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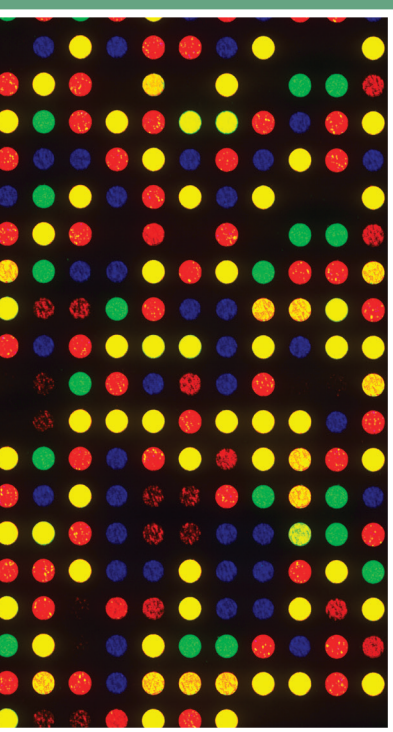
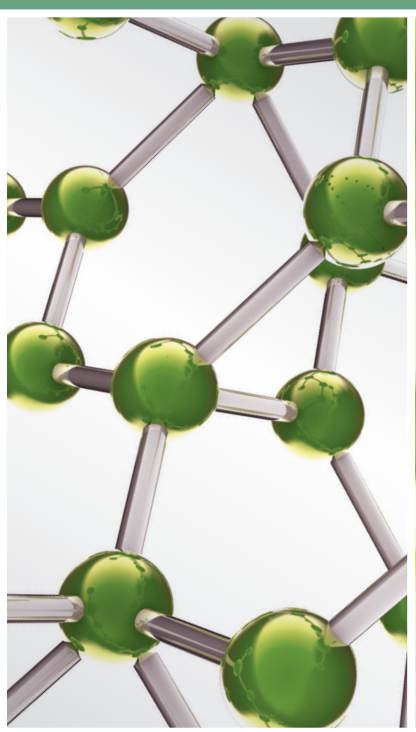
Medical Benefits of Honeybee Products

Guest Editors: José Maurício Sforcin, Vassya Bankova, and Andrzej K. Kuropatnicki



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Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 2702106, 2 pages
https://doi.org/10.1155/2017/2702106

Editorial
Medical Benefits of Honeybee Products

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Received 24 January 2017; Accepted 24 January 2017; Published 9 May 2017

The present Special Issue on medical benefits of honeybee products is dedicated to the memory of Professor Wojciech Krol (1956–2016) who was its initiator and first Lead Guest Editor. He specialized in flavons and flavonoids and his interests included propolis and other natural products. Through his studies of propolis, Professor Krol proved the anti-oxdative properties and possibilities of free radicals scavenging through ethanol extract of propolis (EEP) as well as the synergic antibacterial properties of streptomycin and coloxacine together with EEP. Professor W. Krol was also the Lead Guest Editor of the Special Issue "Propolis: Properties, Application, and Its Potential" published in 2013.

Honeybee products have a long medicinal history. All cultures have folk medicine traditions that include the use of honeybee products, that is, honey, bee pollen, propolis, royal jelly, beeswax, and bee venom. These products have been found to exhibit anti-inflammatory, anti-bacterial, anti-fungal, anti-viral and antioxidant activities. It has been also shown that natural honeybee products inhibit tumor cell growth and metastasis and induce apoptosis of cancer cells. Hence, these bioactive natural products may prove to be useful in cancer therapy.

For this special issue we invited researchers and scholars to submit original research reports and review articles in which they explore aspects of the biological activity of a wide range of honeybee products and their possible applications. A total of 46 papers were submitted out of which, after a rigorous peer-review process, 18 manuscripts have been selected because they represent rich and comprehensive new knowledge. Most of the articles in this special issue are of research character and they present the results of a variety of studies comprising different honeybee products. The accepted papers come from Malaysia, Korea, Lithuania, Chile, Japan, Bangladesh, Saudi Arabia, Egypt, and Poland.

Propolis seems to be the most popular bee product, however highly diversified and chemically complex. One of the articles compares chemical composition and biological activity of yellow, green, brown and red Brazilian propolis. The authors conclude that it is difficult to establish a uniform quality standard for propolis form diverse geographical regions. Some authors show that propolis exhibits anti-diabetic activity and reduces the oxidative stress caused by diabetes. Propolis also plays a very important role in lipid metabolism since its use has positive effect on oxidative status and improvement of HDL-c, contributing to a reduced risk of cardiovascular disease. Antioxidant properties and cardioprotective mechanism of propolis from Malaysia were confirmed in rats.

Various articles deal with separate honeybee products. Bee pollen ointment may be effectively used in burn wound treatment since bee pollen prevents infection of the newly-formed tissue in the healing process of burn wounds. Manuka honey tested on rats proved to possess anti-oxidant and anti-inflammatory activities and seems to be effective in the treatment of chronic ulcer and prevention of mucosal glycoproteins. Another study conducted on rats showed that the consumption of Malaysian honey reduces excessive weight gain and improves obesity-related parameters in obese

rats. Bee venom, a honeybee product used in traditional folk medicine in Korea and China, finds its application in the treatment of arthritis, cancer and inflammation. Some authors prove that bee venom exerts its anti-inflammatory activity through the IRAK1/TAK1/NF-κB signaling pathway.

Oral medicine is another field in which honeybee products are used and their effectiveness was studied. Honey, the best known bee product, can be used in children suffering from oral mucositis. Topical application of honey is recommended in prophylaxis and treatment of mucositis induced by chemo- and radiotherapy. Brazilian propolis may improve poor oral health in patients with cleft lip and cleft lip and palate treated with orthodontic appliances. Toothpaste containing EEP and tea tree oil has been shown to improve hygiene and periodontium condition in patients treated with acrylic partial dentures.

Finally, one article deals with ethnomedicinal uses of honeybee products. Ethnomedicine has evolved over the millennia of human existence but in the last decade the research interests in ethnomedicine, ethnodentistry as well as ethnopharmacy have increased tremendously. The authors analyzed the archival sources concerning honeybee products used for medicinal purposes over the period of more than 100 years.

Acknowledgments

The editors would like to express appreciation to the authors of this special issue for their contributions. Special thanks go to the reviewers for their evaluation of the manuscripts and all the critical comments which helped to improve the content of the submitted papers.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 4272940, 11 pages
https://doi.org/10.1155/2017/4272940

Research Article

The Role of Propolis in Oxidative Stress and Lipid Metabolism: A Randomized Controlled Trial

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Received 4 October 2016; Accepted 14 November 2016; Published 30 April 2017

Academic Editor: Andrzej K. Kuropatnicki

Although there is evidence of the benefits of propolis on human health, the vast majority of studies have been conducted using animal models. The present study includes the chemical characterization and clinical evaluation of the effects of the oral administration of propolis solution on the oxidative status and modulation of lipids in a human population in Talca, Chile. Chemical characterization of propolis, total phenol, flavonoids, and total antioxidant capacity were determined by ORAC. Identification of phenols and flavonoids in propolis was assessed by HPLC-DAD. A double-blind, placebo-controlled clinical trial was conducted. Subjects provided informed consent form and the Bioethics Committee of the Universidad de Talca approved protocol. Eligible subjects ($n = 67$) were randomized in two groups: propolis ($n = 35$) and placebo ($n = 32$). All subjects were evaluated at 0 (baseline), 45, and 90 days. In the propolis group, we observed that increases in HDL-c went from 53.9 ± 11.9 to 65.8 ± 16.7 mg/dL ($p < 0.001$) from baseline to 90 days. Compared to placebo subjects, consumption of propolis induced a net increase in GSH levels ($p < 0.0001$) and a decrease ($p < 0.001$) in TBARS levels for the propolis group. Our findings indicate potential benefits of propolis use in human health. The use of propolis appears to have positive effects on oxidative status and improvement of HDL-c, both of which contribute to a reduced risk of cardiovascular disease.

1. Introduction

Propolis is a sticky, resinous material that honeybees (*Apis mellifera* L.) collect from various plants and mix with wax and other secretions [1]. Numerous biological properties of propolis have been reported including cytotoxic, antimicrobial, antiviral, free radical scavenging, anti-inflammatory, local anesthetic, hepatoprotective, antitumor, and immune system stimulating [2, 3]. For these reasons, propolis is widely used in popular medicine and apitherapy, with extensive use in food and beverages to improve health and prevent diseases [3]. The medical application of propolis has led to increased interest in its chemical composition and potential clinical use in humans.

The chemical composition of propolis is complex and dependent upon the plant source [4]. Analysis of different propolis samples has identified at least 300 different compounds; biological activities are mainly attributed to the phenolic components such as flavonoids in all their forms (flavonols, flavones, flavonones, dihydroflavonols, and chalcones), terpenes, beta-steroids, aromatic aldehydes, and alcohols [5, 6]. The principal antioxidant mechanism of propolis polyphenols can be summarized in the significant ability of "scavenger" reactive oxygen species (ROS) and radical reactive nitrogen species (RNS) to decrease the xanthine oxidase reaction; chelate ion metals are involved in the process of free radical creation and disrupt the cascade of

reactions, leading to the peroxidation of lipids and synergistic action with other antioxidants [7, 8].

It is well known that lipid peroxides are produced through a free radical chain process of autoxidation of lipids containing polyunsaturated fatty acids; their formation by ROS action has been implicated in the pathogenesis of various diseases [9, 10], such as atherosclerosis, myocardial infarction, diabetes mellitus type 2 (DM2), metabolic syndrome, and renal dysfunction [11]. The underlying mechanisms in disease development are different. In the case of atherosclerosis and cardiovascular complications, the primary risk factor is endothelial dysfunction, which is associated with LDL oxidation. For diabetes, beta-cell dysfunction and susceptibility to oxidative stress, which deplete insulin regulation, are fundamental. In renal disease, at the glomeruli and interstitial level, damage is associated with membrane oxidation and the favouring of albumin excretion and other relevant particles.

Previous studies have evaluated the role of propolis in carbohydrate metabolism. The following functions have been described: the epicatechin-mediated stimulation of insulin synthesis in pancreatic β-cells via increased cAMP [12], β-cell proliferation promoted by genistein, and, using in vivo analysis of epigallocatechin gallate, inhibition of glucose production by the liver [13]. Phenolics compounds can also influence glucose absorption in the gut by inhibiting α-amylase, β-glucosidase, and intestinal maltase [13, 14]. Furthermore, it has been reported that benzyl caffeate, isolated from propolis, inhibits the formation of lipid peroxides and very low doses of propolis ethanol extract exert an antilipid peroxidative action [15]. Also it is interesting to mention that not all the studies have shown successful result related to propolis administration [16], leaving open the opportunity to explore the mechanism and doses necessaries of propolis for human health.

It has also been described that propolis may prevent the rise of triglycerides (TG) as well as low and very low density lipoprotein cholesterol (LDL-c and VLDL-c). In an alloxan-induced rat model of type 2 diabetes mellitus [1], the regulation of lipid metabolism by propolis could be explained by its association with key proteins in lipogenesis and lipolysis, such as HMG-CoA reductase [17]. Recently, studies have indicated that the ethanolic extract of propolis and its subfractions are beneficial in increasing high-density lipoprotein (HDL) [17] and enhancing liver ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1). This protein expression is associated with cholesterol efflux from peripheral tissue, suggesting that propolis may be involved in HDL particle formation and lead to an increase in plasma HDL [18, 19].

In light of the available knowledge about the potential benefits of propolis in human health, given its high phenolic profile it would be interesting to demonstrate the effect of propolis on the overproduction of free radicals (oxidative stress) in human beings. Taking into account the role of the oxidative stress in the genesis of several chronic human diseases (degenerative, cardiovascular, cancer, and any other pathologies), this research may contribute to their prevention.

Although there is evidence from traditional medicine about the benefits of propolis, there are few scientific works in human research that support it. Considering the previous information submitted and taking note of the differences observed between the chemical compositions among types of propolis from different geographical areas which depended on the surrounding flora, the present study reports on its chemical composition and provides a clinical evaluation of the effects of a propolis solution (administered orally) on the oxidative status and modulation of serum lipids in human subjects in Talca, Chile.

2. Materials and Methods

2.1. Propolis. Propolis (Beepolis®) is a 3% solution preparation in propylene glycol (PG), manufactured by a bee products company (Laboratorio Rotterdam Ltda) in the Maule Region, Chile (Health Authorization n° 639-18/08/2009, granted by Ministry of Health Regional Office, Maule Region).

2.2. Chemical Analysis of Propolis

2.2.1. Total Phenolic Content (TPC). The TPC of the propolis solution (propolis dissolved in propylene glycol) was determined according to the Folin-Ciocalteu method [20]. Briefly, 20 μL of sample or standard (gallic acid, GAE) was mixed with 1.58 mL of distilled water and 100 μL of Folin-Ciocalteu reagent. The reaction mixture was preincubated for 8 min and then 300 μL of sodium carbonate 20% was added. Finally, each tube was incubated for 2 h at room temperature and the absorbance was obtained in a spectrophotometer (Thermo Spectronic Genesys 10 UV) at a wavelength of 765 nm. The TPC was expressed as GAE in grams per 1000 ml of sample.

2.2.2. Total Flavonoid Content (TFC). The TFC propolis was determined spectrophotometrically using the method reported by Zhishen et al. 1999 [21], based on the formation of a flavonoid-aluminum complex. Briefly, 0.5 mL of propolis solution (3% propolis dissolved in propylene glycol) or standard (quercetin) was mixed with 2 mL of distilled water and 0.15 mL of sodium nitrate ($NaNO_3$, 5%). After 6 min of incubation, 0.15 mL of aluminum chloride ($AlCl_3$, 10%) was added and allowed to incubate for another 6 min, after which 2.0 mL of sodium hydroxide (NaOH, 4%) was added to the mixture. Water was added to achieve a final volume of 5 mL, and the solution was incubated for another 15 min. Absorbance was obtained in a spectrophotometer (Thermo Spectronic Genesys 10 UV) at a wavelength of 510 nm. The results were reported as quercetin equivalents (QE) in milligrams per 1000 ml of sample.

2.2.3. Antioxidant Capacity by Oxygen Radical Absorption Capacity (ORAC) Assay. The antioxidant capacity method was adapted from Dávalos et al. [22]. Briefly, different dilutions of propolis solution (3% propolis dissolved in propylene glycol) or Trolox (standard) were placed in a microplate containing 21 μM fluorescein in 75 mM phosphate buffer, pH 7.4. The mixture was preincubated for 20 min at 37°C, and then

19 mM of 2,2′-azobis(2-aminopropane) (ABAP) was added. Fluorescence intensity (λexc = 485 nm, λem = 512 nm) was registered in a Varioskan Flash microplate reader (Thermo Electron Corp.). The Trolox equivalent concentration for propolis solution was obtained from the calibration curve (the standard curve was obtained by plotting the net area under the curve (AUC) of different Trolox concentrations). ORAC values were calculated using the difference between the area under the fluorescein decay curve and the blank (net AUC). Regression equation between net AUC and antioxidant concentration was calculated for the sample. ORAC values were expressed as μmol of Trolox equivalents per gram of propolis solution.

2.2.4. Compound Identification by HPLC-DAD. Chromatography was assayed according to Pellati et al. [23]. Determination was performed using an Agilent Technologies (Waldbronn, Germany) modular model 1100 system with a diode array detector (DAD). The chromatograms were recorded using Agilent ChemStation for LC and LC-MS systems. The analyses were carried out using Ascentis C18 column (250 mm × 4.6 mm ID, 5 μm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of (A) 0.1% formic acid in H_2O and (B) ACN. The postrunning time was 5 min. The flow rate was 1.2 mL/min. The column temperature was set at 30°C. The sample injection volume was 5 μL. The DAD acquisitions were performed in the range 190–450 nm. The sample preparation for HPLC analysis consisted of 500 μL of propolis and was diluted with MeOH, filtered through a 0.45 μm PTFE (polytetrafluoroethylene) filter into an HPLC vial, and injected into the HPLC system. All sample preparations were carried out in duplicate. The standard solution of each compound (aldehyde benzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, pinobanksin, cinnamic acid, apigenin, veratric acid, and vanillin, among others) was prepared as pure compound (2–6 mg) in MeOH. The external standard calibration curve was generated using five data points. Five μl aliquots (in triplicate) of each standard solution were used for HPLC analysis.

2.3. Subjects. This clinical trial was a randomized, double-blind, and placebo-controlled. Subjects were invited to participate in the study via an institutional email (Universidad de Talca, Talca, Maule, Chile) and 85 subjects were interested in this clinical trial. The subject flowchart is shown in Figure 1. The first subjects were enrolled between March 19 and May 26, 2014. Follow-up was from May 29 to September 11, 2014.

The inclusion criteria were as follows: (i) an age range of 18–69 years; (ii) having at least one of the following altered parameters: fasting glycemia, lipids profile, blood pressure, or diabetes mellitus, cardiovascular disease, and/or overweight. The exclusion criteria were as follows: (i) history of significant alcohol consumption; (ii) reported acute or chronic pathological conditions (liver and/or renal failure, uncontrolled diabetes mellitus, or immunodeficiency and immunological disorders, among others); (iii) being unlikely to cooperate with the study regime. During the study, 8 subjects withdrew voluntarily and 3 additional subjects were excluded for having insulin above the normal range (>100 μU/mL).

2.4. Ethics Statement. The study was performed in compliance with ethical principles and good clinical practice. All subjects provided a written informed consent prior to participation in the study, approved by the Bioethics Committee of the Universidad de Talca (Page Number 2013-064, November 2013).

2.5. Treatment Groups. Eligible subjects were randomized in two groups (A or B) using a Microsoft Excel spreadsheet. Only Rotterdam Laboratory knew the meaning of the codes. They sent two sets of bottles with identical physical characteristics (shape, size, and color), marked only with a single letter code (A or B). One group consumed propolis (n = 35) and the other (n = 32) a placebo with similar flavours (mixture of peppermint, fernet, and synthetic). The propolis and placebo were administered orally twice daily in the same dose and formulation (15 drops each time) for 90 days. At the beginning and during the course of the study the subjects were evaluated for allergic reactions, epigastric discomfort, and any other adverse reaction at 0 (baseline), 45, and 90 days. They were also monitored by phone, focus group, and an in-person interview. All assays were performed according to international standards used in clinical laboratories, which include a calibration curve for each analyte (r = 0,999) and internal quality controls protocols. Blood pressure was taken twice on every measure day (0, 45, and 90), after sitting 5 minutes, and then again after 10 minutes, and the average was used in the analysis. All analyses were conducted comparing baseline to day 90. At the end of the study, the results were unblinded.

2.6. Anthropometric and Blood Pressure Measurements. Height was measured to the nearest 0.5 cm and weight to the nearest 0.1 kg using a mechanical column scale with eye-level beam (Seca 220®, Ca, USA). BMI was classified based on age and sex norms for underweight, normal, overweight, or obese cases (<18.5, 18.5 to 24.9, 25 to 29.9, and >30, resp.). Waist circumference (cm) was measured at the midpoint between the lower ribs and the iliac crest. Hip circumference was measured horizontal at the largest circumference of the hip. Blood pressure (mmHg) was measured in an Omron® digital sphygmomanometer (Osaka, Japan).

2.7. Fasting Blood Samples. These were used to measure the levels of glucose by colorimetric enzymatic hexokinase reagent kit (Glucose-Custom Biotech), insulin (by electrochemiluminescence immunoassay, Insulin ECLIA), and glycosylated haemoglobin (HbA1c, by a turbidimetric inhibition immunoassay, Tina-quant haemoglobin A1c Gen.2®). The lipid profile was determined: (i) total cholesterol by using the CHOD-PAP enzymatic colorimetric test (Cholesterol Gen.2®); (ii) triglycerides (TG) by GPO-PAP enzymatic colorimetric test (Triglycerides GPO/PAP®); (iii) HDL-c by enzymatic colorimetric test (HDL-Cholesterol plus 3rd generation®). The liver enzymatic profile was determined: (i) γ-glutamyltransferase by enzymatic colorimetric test (γ-Glutamyltransferase ver.2®); (ii) alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by quantitative determination of the catalytic activity by colorimetric enzymatic assay (ALT or AST acc. to IFCC without pyridoxal

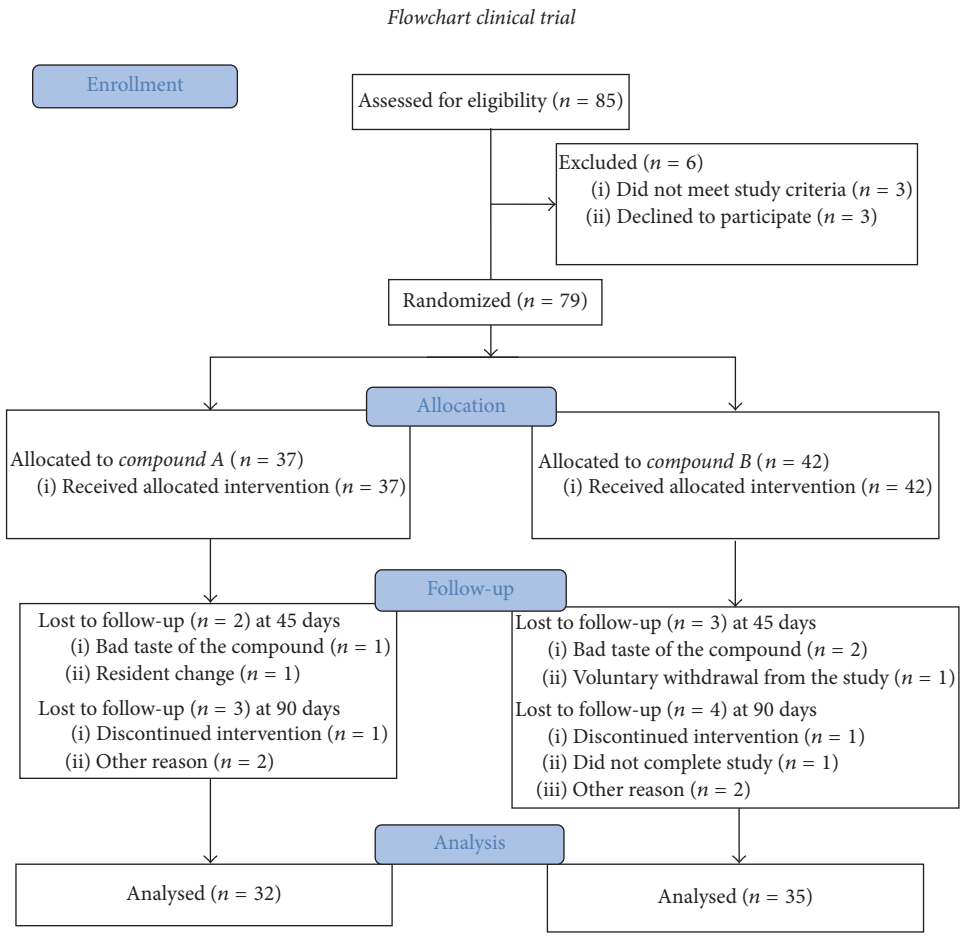


Figure 1: Eligibility, randomization, and patient follow-up. *Compound A* is placebo and *compound B* is propolis.

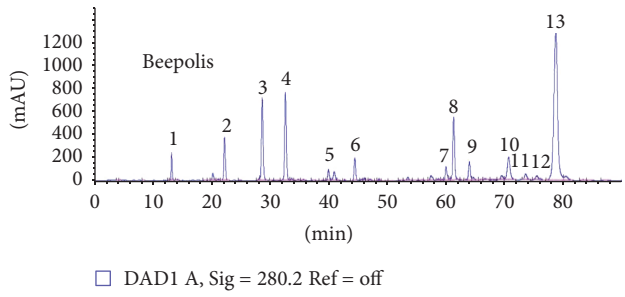
phosphate activation®); and C-reactive protein (CRP), by highly sensitive turbidimetric immunoassay (Cardiac C-Reactive Protein [Latex] High Sensitive®). All analyses were measured in a Cobas c311 autoanalyser Roche (Zurich, Switzerland). LDL-c (mg/dL) was calculated according to Friedewald's protocol [24]: [total cholesterol (mg/dL) − (HDL-c (mg/dL) + TG (mg/dL)/5)]. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according to Blümel et al. [25]: HOMA-IR = [(glycemic mg/dl) × (insulinemic μU/mL)/405].

Clinical Parameters of Oxidation. To determine oxidative damage, TBARS were measured during an acid-heated reaction as previously described [26]. Serum (0.3 mL) was mixed with 180 μL of trichloroacetic acid 50% and 600 μL of thiobarbituric acid 0.67% and then heated in a boiling water bath (90°C) for 30 min. Malondialdehyde (MDA) equivalents were determined by calibration curve (1–10 nmol MDA/mL of sample). TBARS were determined spectrophotometrically (Multiskan Go, Thermo Scientific, Massachusetts, USA) at 532 nm and expressed as nmol MDA/mL of sample. Total levels of GSH were determined according to Beutler et al. [27]; 40 μL of total blood plus 760 μL of water was added to 1200 μL of protein precipitant reactive (1.67 g metaphosphoric acid, 0.2 g EDTA, and 30 g NaCl in a final volume of 100 mL of distilled water); then the mixture was centrifuged at 3500 rpm for 10 min. The supernatant was collected and mixed with 125 μL of DTNB 5,5′-dithiobis(2-nitrobenzoic acid) in 0.4% buffer sodium phosphate 0.1 M, pH 7.5. After 5 minutes the samples were measured spectrophotometrically at 412 nm.

2.8. Statistical Analysis. All the data were evaluated using the by Shapiro-Wilk test for normality of the variable. Values correspond to the mean ± standard deviation (SD). The statistical analysis included intragroup t-test analysis and one-way ANOVA followed by Tukey's posttest. A p value of <0.05 was considered statistically significant. The data were evaluated with GraphPad Prism 6® software (La Jolla, CA, USA).

3. Results

3.1. Total Phenolic and Flavonoid Compounds, Antioxidant Capacity, and Chemical Identification of Propolis Compounds by HPLC-DAD. Table 1 shows the TPC, TFC (flavonols plus OH-flavonols), and ORAC values for the propolis



(a)

Peak	Beepolis	Conc. (mg/L)
1	Aldehyde benzoic acid	12.7
2	Caffeic acid	43.4
3	p-Vanillin	63.3
4	p-Coumaric acid	57.0
5	cis-Ferulic acid	11.1
6	trans-Ferulic acid	21.8
7	Veratric acid	15.9
8	Caffeic acid ester (SI)	74.3
9	Apigenin	51.6
10	Cinnamic acid	42.9
11	Quercetin-7-methyl ester	23.1
12	Pinobanksin-5-methyl ether	24.3
13	Quercetin	838.1

(b)

Figure 2: *Chemical characterization of propolis*. Chromatogram obtained by HPLC-DAD analysis of a propolis sample at 280 nm (a). Peak information of the chromatogram (b).

Table 1: Total phenolic and flavonoids content and antioxidant capacity of propolis.

	Beepolis
TPC (g GAE/L)	22.82
ORAC (μmol equiv. Trolox/g)	42.73
TFC (mg quercetin/L)	937.1

assayed. The HPLC-DAD analysis of the commercial sample of propolis measured at 280 nm denoted a complex composition, as shown in Figure 2(a). The chromatogram of each characterized compound is presented by a number over each chromatographic peak. The corresponding peak identification is described in Figure 2(b). Thirteen different main compounds were identified and ordered from highest to lowest concentration: quercetin > caffeic acid ester > *p*-vanillin > *p*-coumaric acid > apigenin > caffeic acid > cinnamic acid > pinobanksin-5-methyl-ether > quercetin-7-methyl-ester > *trans*-ferulic acid > vetranic acid > aldehyde benzoic acid > cis-ferulic acid.

3.2. Description of General Characteristics. A total of 85 subjects were eligible for this study, 79 of whom provided informed consent and were randomized. Table 2 summarizes the demographics characteristics of the study population. The placebo group was comprised of 7 men and 25 women with an average age of 44.5 ± 13.7 years, a weight of 74.5 ± 14.4 kg, and a height of 162 ± 8 cm. Average BMI was 28.2 kg/m^2. The propolis group was made up of 9 men and 26 women with an average age of 48 ± 12.1 years, a weight of 69.6 ± 12.5, a height of 162 ± 8 cm, and a BMI of 27.9 ± 4.8. Both groups were, on average, overweight. In terms of weight, BMI, and waist circumference there were no significant differences between the groups at baseline or 90 days. All the anthropometric variables analysed are shown in Table 3. In the propolis group systolic and diastolic blood pressure saw a significant reduction: SBP from 126.1 ± 9.5 to 121.9 ± 9.3 mmHg and DBP from 79.4 ± 10.2 to 76.2 ± 6.9 (*t* test; $p < 0.018$).

3.3. Carbohydrate Metabolism and Liver Profile in the Study Group. Fasting glycaemia, HbA1c, and insulin were measured at day 0 and 90 (Table 4), with no significant changes within the groups: blood sugar levels and insulin were stable over time and within normal limits. We calculated the HOMA index and considered the HOMA-IR > 2.5 as a cutoff to determine insulin resistance; HOMA values decreased in the propolis group, from 2.54 ± 1.91 (baseline) to 2.43 ± 1.28 (day 90), but differences were not statistically significant. Analysis of liver enzymatic activity (GGT, GOT, and GPT) did not show any variations within or between groups (Table 5). No signs of allergy or other adverse reactions to propolis consumption were observed among the study participants.

3.4. Lipids Profile in Study Group. The effects of propolis on blood lipids in human subject are given in Figure 3. The propolis group had a 17% increase in total cholesterol (Figure 3(a)) at day 90 from 175.3 ± 29.2 to 206.6 ± 21.6 mg/dL (one-way ANOVA, Tukey posttest: $p < 0.0001$) and a 22% increase in HDL-c (Figure 3(b)) from 53.9 ± 11.9 to 65.8 ± 16.7 mg/dL (one-way ANOVA, Tukey posttest: $p < 0.001$), compared with day 0 of propolis administration and with the placebo subjects. There were no statistically significant differences in LDL-c (intragroup *t* Test: $p > 0.559$) and TG (Intragroup *t* Test: $p > 0.535$) in the propolis group; additionally the placebo group did not show any variation in the lipid parameters measured (see Figure 3(c)).

3.5. Oxidative Parameters in Study Group. In the propolis group, TBARS decreased by 67% (one-way ANOVA, Tukey's posttest: $p < 0.0001$) and GSH levels increased by 175% (one-way ANOVA, Tukey's posttest: $p > 0.0002$), with both changes being observed at day 90 compared with day 0 of intake (Figures 4(a) and 4(c), resp.). Among subjects

Table 2: Demographic characteristics.

Demographics	Total ($n = 67$)	Placebo ($n = 32$)	Propolis ($n = 35$)
Age (mean ± SD)	46.4 (±12.9)	44.5 (±13.7)	48.0 (±12.1)
Gender			
Female	51 (76%)	25 (78%)	26 (74%)
Male	16 (24%)	7 (22%)	9 (26%)

Table 3: Effects of propolis over anthropometric and blood pressure measures.

	Placebo		p value	Propolis		p value
	Day 0	Day 90		Day 0	Day 90	
Weight (kg)	74.5 (±14.4)	74.8 (±14.4)	0.174	69.6 (±12.5)	68.7 (±11.6)	0.488
BMI (kg/m^2)	28.2 (±4.7)	28.4 (±4.7)	0.182	27.9 (±4.8)	27.9 (±4.7)	0.409
Waist circumference (cm)	89.8 (±8.9)	91.1 (±9.2)	0.152	87.6 (±9.8)	87.9 (±9.2)	0.353
SBP (mmHg)	122.2 (±11.1)	121.6 (±11.6)	0.401	126.1 (±9.5)	121.9 (±9.3)	0.018*
DBP (mmHg)	77.5 (±8.2)	74.6 (±9.3)	0.006*	79.4 (±10.2)	76.2 (±6.9)	0.036*

Values represent the mean ± SD for 32 placebo and 35 propolis subjects. Significant differences between the groups are indicated by a single asterisk (intragroup t test: $p < 0.05$). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure.

Table 4: Carbohydrate metabolism in study group.

	Placebo		p value	Propolis		p value
	Day 0	Day 90		Day 0	Day 90	
Glycemia (mg/dL)	92.3 (±8.1)	95.4 (±7.6)	0.174	94.8 (±11.8)	97.9 (±10.8)	0.488
HbA1c (%)	5.49 (±0.35)	5.42 (±0.39)	0.487	5.50 (±0.35)	5.46 (±0.33)	0.194
HOMA	2.54 (±1.21)	2.58 (±1.12)	0.551	2.54 (±1.91)	2.43 (±1.28)	0.512
hs-CRP (mg/L)	2.05 (±1.30)	2.17 (±1.33)	0.465	2.02 (±1.09)	1.81 (±1.11)	0.676

Values represent mean (±SD) for 32 placebo and 35 propolis subjects. Nonsignificant differences between the groups were observed (intragroup t Test: $p < 0.05$); HOMA: homeostatic model assessment.

Table 5: Liver enzyme profile in the study group.

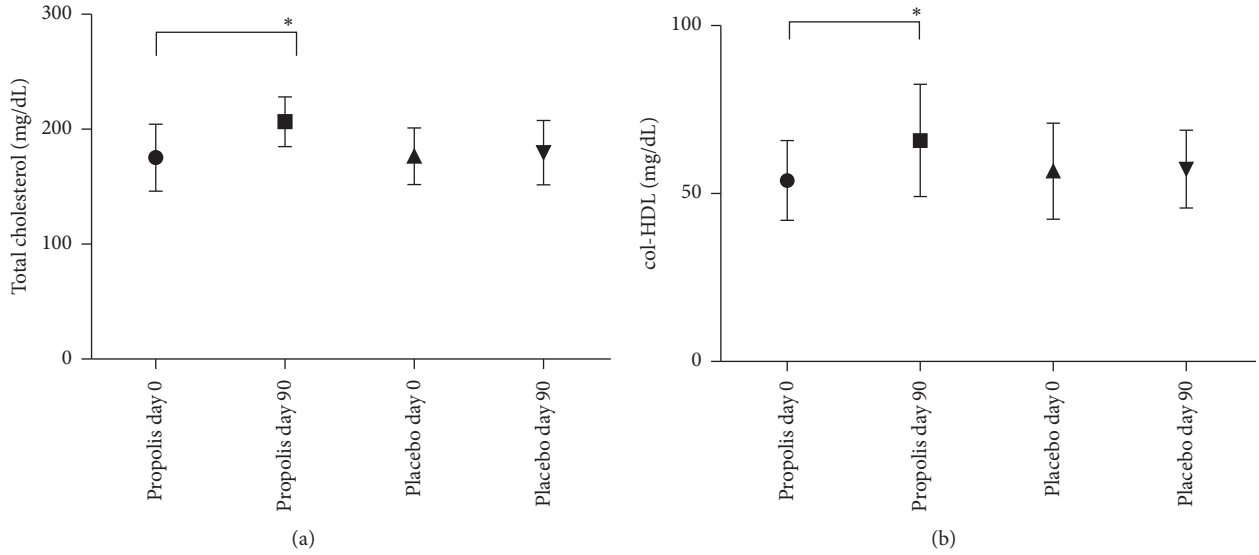
	Placebo		p value	Propolis		p value
	Day 0	Day 90		Day 0	Day 90	
GGT (U/L)	21.3 (±5.7)	18.9 (±7.0)	0.178	20.3 (±9.1)	18.4 (±6.7)	0.372
GOT (U/L)	19.0 (±2.8)	19.7 (±3.0)	0.383	20.6 (±2.9)	20.1 (±3.5)	0.554
GPT (U/L)	23.9 (±8.8)	22.0 (±6.3)	0.406	22.9 (±7.8)	20.3 (±6.7)	0.167

Values represent mean ± SD for 32 placebo subjects and 35 propolis subjects. Nonsignificant differences were found between the groups (intragroup t Test: $p > 0.05$). GGT: gamma glutamyl transferase; GOT: glutamic oxaloacetic transaminase; GPT: glutamate-pyruvate transaminase.

who received the placebo, plasma levels of TBARS and GSH were comparable throughout the study (0 to 90 days), with nonstatistically significant changes. TBARS had a net decrease in subjects in the propolis group, which was higher than that observed in the placebo group (t Test: $p < 0.0001$; see Figure 4(b)). There was a net increase of GSH levels (t Test: $p < 0.0001$), in propolis versus placebo subjects (Figure 4(d)).

4. Discussion

In the last years the interest in functional foods from natural origin for quality of life improvement and disease prevention has increased. Some of these functional foods derived from the hives industry (e.g., honey and propolis). Traditional knowledge has shown benefits when consuming these products, and there is an abundance of scientific work



(a) (b)

	Placebo		p value	Propolis		p value
	Day 0	Day 90		Day 0	Day 90	
Total cholesterol	176.5 (±24.67)	179.6 (±27.95)	0.6361	175.3 (±29.22)	206.6 (±21.61)	0.0001
LDL cholesterol	105.0 (±36.28)	104.0 (±38.65)	0.9089	109.1 (±32.98)	113.8 (±32.26)	0.5590
HDL cholesterol	56.70 (±14.29)	57.28 (±11.60)	0.8628	53.91 (±11.90)	65.81 (±16.69)	0.0010
Triglycerides	128.4 (±69.11)	132.4 (±72.09)	0.8188	116.7 (±58.46)	126.5 (±71.64)	0.5351

(c)

Figure 3: *Lipids profile in study group*. Plasma total cholesterol graph (a), HDL-c graph (b), and table of total cholesterol, LDL-c, HDL-c, and triglycerides (c), all determined by enzymatic methods. Values correspond to mean ± SD for 32 placebo subjects and 35 propolis subjects (one-way ANOVA, Tukey's posttest $^{*}p < 0.05$).

characterizing propolis from different parts of the world and its effects in vitro on cells or rats. There are very few clinical studies, however, that demonstrate conclusively the health effects in human beings. Propolis has traditionally been used to treat infections, but scientific evidence of its value as an antioxidant and/or in the management of chronic diseases such as diabetes, atherosclerosis, and cancer is insufficient. Propolis has a large number of bioactive compounds: a variety of polyphenols and flavonoids, related to the flora surrounding the hives [4]. Nina et al. (2015) described a large variation in in vitro antimicrobial effects, antioxidant activity, and composition in four different geographic areas of propolis from the Maule Region of Chile. Researchers found that propolis sample from the central valley was more effective as an antibacterial than those from the coastal range or Andean slopes [28]. Bankova et al. (2014) analysed the chemical composition and antiviral activity of commercial propolis Extract ACF® (PPE) (ethanolic extract at a concentration of 3%). They showed that PPE had a high antiviral activity against herpes simplex virus type 1 and type 2 which may partly be due to interference in the viral adsorption to the cells [29]. Miyazaki et al. (2015) worked with Brazilian ethanol extracts of propolis, evaluating their action in oxidative stress in both in vivo and in vitro studies, related to the cognitive dysfunction associated with hyperhomocysteinemia. This study found that propolis improved cognitive function, decreasing the accumulation of proteins in the brain, mediated by an increase in homocysteine [30]. On the other hand, Chilean studies with propolis from the Araucanía region showed a modulation of the angiogenesis in both in vivo and in vitro models. Cuevas et al. (2015) showed that ethanolic extracts of Chilean propolis, specifically pinocembrin, one of its main constituents, were able to modulate in vitro angiogenesis, in part by modulating HIF1α stabilization and ERK1/2 phosphorylation, two important factors involved in this process. Other studies have evaluated in vitro the inhibitory activity of 22 propolis extracts from different Chilean regions on 10 strains of *Helicobacter pylori* isolated from gastric mucosa in vitro. The results show that Chilean propolis has an effective anti-*Helicobacter pylori* activity [31, 32].

The principal aim of this trial was to evaluate the most relevant effects associated with propolis, like oxidative status, lipid content, and carbohydrate level normalization in a placebo-controlled human study. First, we evaluated the total phenolic and flavonoid content. Compared to tropical zone propolis, the ORAC antioxidant capacity was higher [33].

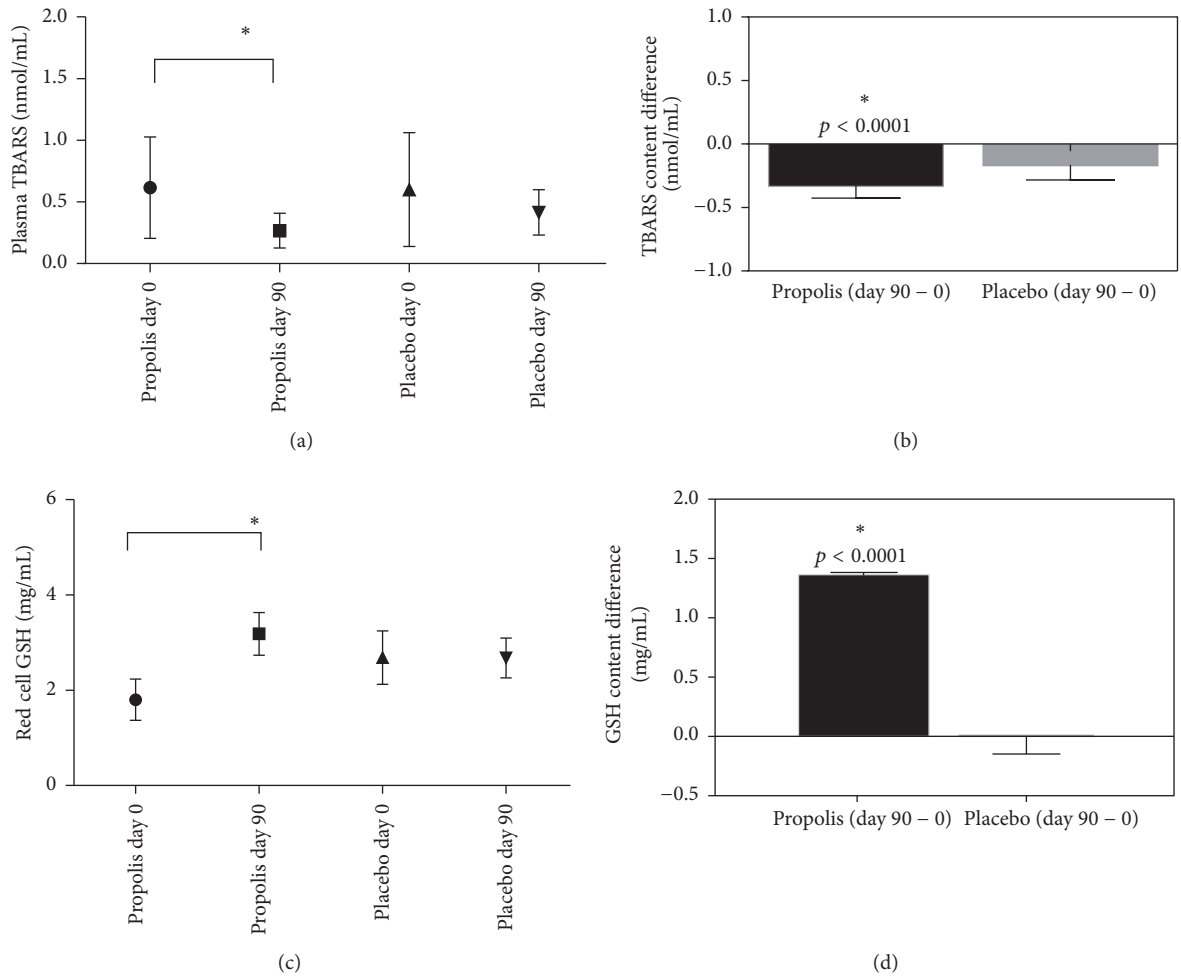


Figure 4: *Oxidative parameters in study group*. Oxidative stress was assayed by TBARS (a), net changes (day 90 − 0) in TBARS (b), reduced glutathione GSH (c), and net changes (day 90 − 0) in GSH (d). Values correspond to mean ± SD for 32 placebo subjects and 35 propolis subjects. Significance was evaluated by one-way ANOVA and Tukey's posttest ((a) and (c) $^{*}p < 0.05$) or t Test ((b) and (d) $^{*}p < 0.05$).

This may be related to the particular characteristics of bioactive compounds, as the variety of polyphenols and flavonoids related to the flora surrounding the hives as a function of botanical and geographical origin [4]. Propolis types found in tropical areas contain a wide variety of phenolic compounds, such as *p*-coumaric acid, flavan-3-ol-flavonols, chalcones, isoflavonoids, pterocarpans, and triterpenoids, among others [33, 34]. In addition, Brazilian propolis has a high content of formononetin, isoliquiritigenin, pinocembrin, biochanin A, and quercetin [33, 35], a phenolic pattern that has many differences with the Chilean propolis assayed in this study, explaining the phenolic and flavonoids differences found among propolis.

The antioxidant capacity was evaluated by ORAC and the flavonoid and phenolic content are directly related with that observed in this clinical study, showing decreases in TBARS and GSH enhancement. Propolis has the capacity to reduce ROS, which could be related to two different mechanisms. According to the literature, the first is the capacity of caffeic acid phenethyl ester (CAPE) to activate the transcription factor NrF2 [36]. NrF2 is a regulatory protein associated with antioxidant protection and with the enhancement of antioxidant enzymes like heme oxygenase-1, phase II detoxification enzymes, and enzymes involved in GSH metabolism [36, 37]. Thus, through the phenolic compound propolis could activate NrF2 and improve cellular antioxidant capacity. The second mechanism could be triggered by the ability of the phenolic and flavonoid compounds like quercetin, CAPE, *p*-vanillin, *p*-coumaric acid, apigenin, and cinnamic acid, all of which are present in Chilean propolis, to neutralize oxidative species [38]. CAPE not only has been shown to inhibit activation of the nuclear transcription factor- (NF-) κB signaling pathway [39], but also has strong ROS scavenging ability and activates NrF2 [40], thereby increasing an antioxidant stress response, which could in part explain the antioxidant effects observed in our study related to the increase in GSH and decrease of TBARS.

Other propolis components have been studied. Pinocembrin (5,7-dihydroxyflavanone), abundant flavonoid in propolis, has been shown to have antioxidant activity related to the nuclear translocation of NrF2, activation of the NrF2/ARE pathway, and induction of HO-1 and ϒ-GCS

expression, which is related to the biosynthetic pathways of GSH formation [41]. The work of Ishige et al. (2001) [42] shows that flavonoids can deplete intracellular ROS indirectly by increasing intracellular GSH. Additionally, propolis can enhance glutamate-cysteine ligase, a rate-limiting enzyme in GSH synthesis [43], and it is therefore associated with strong free radical scavenging activities and improvement of the endogenous antioxidant defense system observed by propolis consumption.

In relation to the effects observed in HDL-c, it is important to highlight that this lipoparticle helps protect against cardiovascular disease [44–46], avoiding LDL oxidation or neutralizing the atherogenic effects of the oxidized-LDL in artery walls [5]. Currently, there are no approved drugs in therapeutics protocols to improve HDL-c levels or such drugs are less controversial. Recent studies have indicated that the ethanolic extract of propolis and its subfractions are beneficial for increasing plasma HDL-c while reducing LDL-c in a model of hypercholesterolemic rabbits [18]. According to the blood plasma analysis Brazilian propolis reduced total cholesterol and elevated HDL-c in LDLr−/− in mice with an initial atherosclerotic lesion [47]. Propolis enhances liver ATP-binding cassette transporters A1 and G1 (ABCA1 and ABCG1) protein expression, which is associated with cholesterol efflux from peripheral tissue. This suggests that propolis may be involved in HDL particle formation and may lead to an increase in HDL [18, 19]. Together with an increase of ABCA1 cassette, Brazilian red propolis upregulated ApoA-1-mediated cholesterol efflux by macrophages, an action related to ABCA1 via induction of PPARY/LXR, an important transcription factor related to lipid metabolism. The relationship of propolis with the lipid metabolism is a good indicator of its potential as a cardiovascular protector [48].

In relation to blood pressure, our findings showed a significant decrease in DBP in both the propolis and placebo groups, and we therefore estimate that these findings are likely only placebo effect. SBP decreased significantly but only in propolis group. Nevertheless, some evidence reported in the literature has described dietary antioxidants as possibly having beneficial effects on hypertension, although this has not been proven with antioxidant supplementation [49]. On the other hand, Teles et al. (2015) [50] demonstrated in an animal model that the antioxidant and anti-inflammatory effects of propolis were able to attenuate hypertension and structural renal damage in Wistar rats models. The reduction in PAS in the study group that took propolis was modest and therefore of uncertain clinical significance, but under the above background it would be interesting to reevaluate future effect on blood pressure in a study designed for hypertension patients. We found no differences in glucose, $HbA_{1\text{-}c}$, or insulin levels. This may be due to the short intervention period, which underscores the importance of future studies over a more prolonged period to detect clinically relevant changes related to propolis consumption. Previously, Babatunde et al. (2015) [51] noted a significant decrease in blood glucose level in alloxan-induced hyperglycemia Wistar rats when given Nigerian propolis, suggesting that long-term administration/intake of this extract may have hypoglycemic effect. Blood glucose reduction may relate to the bioactive compounds of propolis on β-cells, which could enhance the production of insulin or enhance cellular sensitivity response to insulin.

5. Conclusion

Data reported here support the role of propolis in diverse chronic disease, through different mechanisms such as the increase in HDL-c, and the antioxidant effect due to enhanced GSH and decreased TBARS levels, both markers of oxidative stress in humans. Therefore, our findings provide highlighted scientific information in using propolis as an antioxidant agent.

According to the results, the use of propolis may improve the prognosis of several chronic diseases and potentially contribute to decreasing the risk of cardiovascular disease.

Conflicts of Interest

The authors declared that there are no conflicts of interest regarding the publication this paper.

Acknowledgments

This study was supported by Fondos para la Innovación de la Competitividad (FIC), Maule, no. BIP.30.136.439-0 and Rotterdam Laboratory, for the Beepolis.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 5439645, 14 pages
https://doi.org/10.1155/2017/5439645

Research Article

The Antidiabetic Activity of *Nigella sativa* and Propolis on Streptozotocin-Induced Diabetes and Diabetic Nephropathy in Male Rats

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Received 3 July 2016; Accepted 19 January 2017; Published 16 February 2017

Academic Editor: Andrzej K. Kuropatnicki

This study was conducted to compare the ameliorative effect of *Nigella sativa* and propolis methanol extract on *streptozotocin*-induced diabetic male rats and treating diabetic nephropathy. Forty male Albino rats were divided into four groups; the first group was the negative control fed standard diet. The other 30 rats were injected with *streptozotocin to induce* diabetes by a single intravenous injection and then divided equally into three groups; the second group was the positive diabetic control; the third and the fourth groups were treated orally with 20% w/w *Nigella sativa* seeds methanol extract and propolis methanol extract (20% w/w), respectively. The rats of the second group showed increased glucose levels and lipid peroxide accompanied with reduction in superoxide dismutase, catalase, and glutathione-S-transferase enzyme activities compared with the negative control. Carboxymethyl lysine, interleukin-6, and immunoglobulins were also increased as a result of diabetes. Kidney function parameters were also elevated, while potassium and sodium levels were decreased. Moreover, tissues of kidney and pancreas showed severe histopathological changes. Treating the diabetic rats with *Nigella sativa* and propolis methanol extract in the third and fourth groups, respectively, ameliorated all altered biochemical and pathological examinations approaching the negative control. Propolis was more effective than *Nigella sativa*.

1. Introduction

Diabetes mellitus (DM) is a heterogeneous disease, characterized by chronic hyperglycaemia caused by defects in insulin secretion, insulin action, or both, resulting in impaired function in carbohydrate, lipid, and protein metabolism [1]. Moreover, hyperglycemia is considered a major factor responsible for the intense oxidative stress in diabetes through the overproduction of reactive oxygen species [2, 3], which results in an imbalance between excess formation of reactive oxygen species (ROS) and ability of a biological system to readily detoxify the reactive intermediates or to repair the resulting damage. ROS interact with the free amino and sulfhydryl groups of proteins forming Amadori products which further modified to form advanced glycation end products (AGEs) especially carboxymethyl lysine (CML) [4], contributing to development of diabetic complication [5]. The formed AGEs bind to their receptors (AGE-receptors) on the cell membrane resulting in the activation of the nuclear factor kappa B (NF-κB), which plays an important role in inducing genes involved in the control of the immune system as well as in the response to injury and infection such as IL-6 and immunoglobulins [4].

Effective control of hyperglycemia in diabetic patients is critical for reducing the risk of micro- and macrovascular complications [6]. Natural sources play an important role in the management of diabetes mellitus, especially in developing countries, delaying the development of diabetic complications and correcting the metabolic abnormalities [7]. *Nigella sativa* (black seed) and propolis are among the natural sources reported to have beneficial effects in the treatment of many diseases [8]. *N. sativa* has many beneficiary effects such

as an anticancer, anti-inflammatory, cardiovascular, renal, immunomodulatory, and antidiabetic effects as well as many other effects like antiasthmatic, antimicrobial, antiparasitic, and antihypertensive effects. Moreover, the seeds of *N. sativa* are widely used in the treatment of various diseases like bronchitis, diarrhea, rheumatism, and skin disorders [9]. The efficacy of *N. sativa* is related to numerous active components which have been isolated from seeds and its oil including thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine-N-oxide, nigellicine, nigellidine, and alpha-hederin [10], as well as flavonoids [11].

Propolis is a natural resinous mixture produced by honeybees from substances collected from parts of plants, buds, and exudates which is widely used in folk medicine in various parts of the world for several applications as anti-inflammatory [12], antioxidative [13], antiproliferative [14], antidiabetic [15], and antimicrobial [16] agent. More than three hundred organic compounds of different groups, mainly phenolic, such as flavonoids and phenolic acids, have been identified in propolis [17]. Furthermore, caffeic acid of propolis is known to play an important role in reducing the inflammatory response and also aids the immune system by promoting phagocytic activities and stimulates cellular immunity [18].

This study aimed to evaluate the protective effect of *Nigella sativa* and propolis methanol extracts on streptozotocin-induced diabetes and treating diabetic nephropathy in male rats.

2. Materials and Methods

N. sativa and propolis were purchased from a local herbal medicine shop in Jeddah, Saudi Arabia.

2.1. Diet. The animal diet was obtained from a grain mill in Jeddah, Saudi Arabia. A 100 g of the conventional animal basal diet consists of 4 g corn oil (4% fat), 4 g minerals (4% minerals), 12% protein (17.14 g of 70% casein), 0.2 g choline chloride (0.2%), 0.3 g methionine (0.3%), 4 g cellulose (4% fiber), 1 g vitamin mixture (1% vitamin), and 69.36 g of corn starch (69.36%). The diet was stored in a dark dry place.

Total carotenoids were extracted with acetone-hexane mixture and determined with a spectrophotometer at wavelength of 440 nm as described by Dubois et al. [19].

2.2. Preparation of Methanol Extract. Methanol extracts were prepared by soaking 200 g of dry *N. sativa* seeds or propolis in 1 liter of 90% methyl alcohol under shaking for 5 days and then kept in a refrigerator. Methanol was evaporated using a rotatory evaporator apparatus. 20 g of the semisolid extract of both *N. sativa* and propolis was suspended in 100 mL distilled water with 2 mL of tween 80 (suspending agent) to prepare a 20% solution [20].

2.3. Phytochemical Analysis. The total flavonoid content of each extract was determined by a colorimetric method as described by Zhishen et al. [21]. 0.5 mL of each sample was mixed with 2 mL of distilled water, and then 0.15 mL of $NaNO_2$ solution (15%) was added. 0.15 mL of aluminum chloride ($AlCl_3$) solution (10%) was added after 5 m and allowed to stand for 6 minutes, and then 2 mL of 4% NaOH solution was added to the mixture. Water was added to bring the final volume to 5 mL immediately. The mixture was thoroughly mixed and allowed to stand for another 15 minutes. The absorbance of the mixture was measured at 510 nm.

2.4. Animals and Housing Conditions. Forty male Albino rats (180–200 g) were obtained from the animal experimental unit of King Fahd Center for Medical Research, King Abdulaziz University. The animal experiments were carried out according to protocols approved by the Institutional Animal House of the University of King Abdulaziz at Jeddah, Saudi Arabia. Rats were kept for two weeks before the start of the experiment for acclimatization. The animals were then housed 5/cage and received normal basal diet and tap water ad libitum at a room temperature of about 28 ± 2°C, a room humidity of 60 ± 5%, and a 12 h light and 12 h dark cycle.

2.5. Experiment Design. The animals were divided into 4 groups, each consisting of 10 rats. The first group (G1) received only a single tail vein injection of 0.1 mol/L citrate buffer. The other 30 rats were subjected to fasting for 12 h and were then intravenously injected with freshly prepared streptozotocin (65 mg/kg body weight) in a 0.1 mol/L citrate buffer (pH 4.5). After 5 days of injection, rats with blood glucose higher than 200 mg/dL in the fasting state were considered diabetic. The other rats with blood glucose lower than 200 mg/dL were discarded from the study. The experiments were started one week after STZ injection. The 30 diabetic rats were then randomly divided into 3 groups: the second group (G2) received only STZ and fed normal basal diet. The third group (G3) was treated with (20% w/w) *Nigella sativa* seeds methanol extract using stomach tube. The fourth group (G4) was treated with (20% w/w) propolis methanol extract using stomach tube. Treatment was continued for 4 weeks.

2.6. Urine Sample. Urine samples were collected before induction of diabetes and one day before the end of the experiment by placing the rats in individual metabolic cages for 24 h. Albumin and creatinine levels were determined in the urine samples.

2.7. Blood Sampling and Analysis. At the end of the experiment, rats were fasted 14–16 hours after their last feeding, and then blood samples were collected from the heart of each rat under anesthesia with diethyl ether. Blood samples were centrifuged at 2,000*g* for 10 minutes at 4°C and serum was removed and stored at −80°C until analysis.

2.8. Dissection. The abdomen of ether anaesthetic rats was dissected and the kidneys and pancreas were dissected out. One kidney was kept in ice for kidney homogenate preparation and the other kidney and the pancreas were saved in saline buffer (0.9% NaCl) for histopathological investigations.

2.9. Kidney Tissue Homogenate. Kidney tissues were cut into small pieces, washed with phosphate-buffered saline,

and then ground in a homogenization buffer consisting of 0.05 M Tris-HCl pH 7.9, 25% glycerol, 0.1 Mm EDTA, and 0.32 M $(NH_4)_2SO_4$ containing a protease inhibitor tablet (Roche, Germany). The lysates were homogenized on ice using a Polytron homogenizer and then sonicated in an ice bath to prevent overheating for 15 seconds followed by 5-minute centrifugation at 12000 rpm and 4°C. The supernatant was aliquoted and stored at −80°C until use. The kidney homogenate was used for estimating the activity of antioxidant enzymes and lipid peroxidation as well as level of IL-6.

2.10. Determination of Fasting Blood Sugar (FBS). Fasting blood sugar was estimated using glucose kit from HUMAN (Germany) according to Barham and Trinder [22].

2.11. Determination of Lipid Peroxide. Lipid peroxide was estimated by determination of malondialdehyde (MDA) in the serum and in the kidney tissue homogenate according to the method described by Ohkawa et al. [23] using commercially available kits from Biodiagnostic Chemical Company (Egypt).

2.12. Estimation of Antioxidant Enzymes Activity. Superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) activities were estimated in the serum and in the kidney tissue homogenate according to the method described by Nishikimi et al. [24], Aebi [25], and Habig et al. [26], respectively, using commercially available kits from Biodiagnostic Chemical Company (Egypt).

2.13. Determination of Interleukin-6 (IL-6). IL-6 levels in serum and kidney tissue homogenate were determined using Rat IL-6 Immunoassay kit from R&D Systems Inc. (USA) according to the method of Hibi et al. [27].

2.14. Determination of Immunoglobulins (Ig). Immunoglobulins (IgA, IgM, and IgG) were estimated in the serum according to Fahey and Mckelvey [28] and Berne [29] using commercially available kits from GenWay Biotech (USA).

2.15. Determination of Carboxymethyl Lysine (CML). Carboxymethyl lysine (CML) was estimated in the serum using commercially available kit according to the method described by Seigel et al. [30] from MyBioSource (Canada). This kit employs Double Antibody Sandwich Technique.

2.16. Determination of Kidney Functions. Urea, creatinine, and uric acid were estimated in the serum according to the method described by Fawcett and Scott [31], Bartels et al. [32], and Fossati et al. [33], respectively, using enzymatic colorimetric kit from HUMAN (Germany), while albumin was estimated in urine by ELISA according to Sayed [34] using a Nephrat II Albumin Kit (USA) and the concentration of creatinine urine samples was determined by the commercial HUMAN kit (Germany) according to the method of Bartels et al. [32].

2.17. Determination of Electrolytes. Sodium (Na^+) and potassium (K^+) were estimated in the serum according to Trinder [35] and Terri and Sesin [36], respectively, using HUMAN kits (Germany).

2.18. Physiological Evaluations

(i) Food intake and water consumption were calculated daily.

(ii) Food efficiency ratio (FER), food efficiency ratio percentage (FER%), body weight gain (BWG), and body weight gain percentage (BWG%) were calculated according to the method of Davies and Morris [37].

2.19. Histopathological Examination. Kidney and pancreas tissues were kept in saline after animal sacrifice, fixed in 10% formalin, processed routinely, and then embedded in paraffin. 5 μm thick sections were prepared and stained with hematoxylin and eosin (H&E) dye for microscopic investigation (Drury et al. [38]). The stained sections were examined and photographed using Olympus light microscope equipped with a digital camera.

2.20. Statistical Analysis. The scored values were analyzed using SPSS program to calculate t-test and the mean ± SD and then analyzed using one-way analysis of variance (ANOVA, $P < 0.05$) using a protected least significant difference (LSD) test of SAS [39].

3. Results

3.1. Phytochemical Analysis. The spectrophotometric evaluation of the antioxidants (flavonoids and carotenoids) showed that *N. sativa* seed contains 993.6 mg/100 g dry weight flavonoids and 80.6 mg/100 g dry weight carotenoids, whereas propolis contains 4630 mg/100 g dry weight flavonoids and 1.92 mg/100 g dry weight carotenoids.

3.2. Fasting Blood Sugar (FBS). Effect of treating STZ-induced diabetic rats with *N. sativa* and propolis for 4 weeks is illustrated in Table 1. The mean values of serum fasting blood sugar (FBS) were significantly ($P < 0.001$) increased in the positive control group, when compared with those of the negative control. However, treating these rats with methanolic extract of *N. sativa* and propolis for 4 weeks significantly ($P < 0.001$) reduced the fasting blood sugar in the serum of both G3 and G4 groups, respectively, although being higher than that of the negative control values. Methanolic extract of propolis in G4 was more effective in reducing fasting blood sugar than that of *N. sativa* in G3.

3.3. Lipid Peroxide. Table 1 also shows the effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on lipid peroxidation (MDA) in the serum and kidney tissue homogenate. The mean values of MDA in the diabetic positive control (STZ treated) group were significantly ($P < 0.001$) increased compared with those of the negative control group in both serum and kidney tissue homogenate. In G3 and G4, the mean values of MDA in both

Table 1: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on fasting blood sugar, lipid peroxide, and antioxidants enzymes.

Parameters	Statistics	G1 N. control	G2 P. control	G3 *Nigella sativa* extract	G4 Propolis extract
Serum FBS (mL/dL)	Mean ± SE	92.66 ± 1.14^{d}	283.33 ± 2.47^{a}	203.16 ± 3.71^{b}	139.00 ± 1.18^{c}
	LSD 0.05 = 7.230				
	t-test	—	−63.63***	15.32***	59.03***
Serum MDA (nmol/mL)	Mean ± SE	0.93 ± 0.03^{d}	4.50 ± 0.05^{a}	2.82 ± 0.03^{b}	1.94 ± 0.03^{c}
	LSD 0.05 = 0.149				
	t-test	—	−52.66***	20.35***	27.91***
MDA (nmol/g) Kidney tissue	Mean ± SE	2.58 ± 0.06^{d}	16.08 ± 0.18^{a}	4.76 ± 0.05^{b}	3.54 ± 0.11^{c}
	LSD 0.05 = 0.306				
	t-test	—	−70.12***	67.64***	62.91***
Serum CAT (U/mL)	Mean ± SE	2.40 ± 0.19^{a}	0.15 ± 0.01^{d}	1.20 ± 0.01^{c}	1.89 ± 0.02^{b}
	LSD 0.05 = 0.301				
	t-test	—	11.49***	−71.53***	−58.72***
Serum SOD (U/mL)	Mean ± SE	638.68 ± 1.56^{a}	120.83 ± 2.41^{d}	276.45 ± 2.37^{c}	520.18 ± 1.85^{b}
	LSD 0.05 = 25.419				
	t-test	—	178.65***	−48.94***	−116.79***
Serum GST (U/mL)	Mean ± SE	813.20 ± 2.32^{a}	120.93 ± 2.38^{d}	421.56 ± 3.20^{c}	762.65 ± 1.74^{b}
	LSD 0.05 = 6.450				
	t-test	—	228.70***	−136.08***	−275.19***
CAT (U/g) Kidney tissue	Mean ± SE	5.02 ± 0.08^{a}	0.385 ± 0.02^{c}	2.86 ± 0.03^{d}	3.97 ± 0.06^{b}
	LSD 0.05 = 0.144				
	t-test	—	51.41***	−74.97***	−56.95***
SOD (U/g) Kidney tissue	Mean ± SE	917.18 ± 2.59^{a}	175.58 ± 4.53^{d}	675.98 ± 3.94^{c}	818.73 ± 4.78^{b}
	LSD 0.05 = 12.818				
	t-test	—	117.11***	−134.00***	−88.10***
GST (U/g) Kidney tissue	Mean ± SE	826.20 ± 2.75^{a}	315.68 ± 3.56^{c}	684.33 ± 1.99^{b}	771.88 ± 2.69^{ab}
	LSD 0.05 = 181.965				
	t-test	—	109.14***	−71.07***	−103.53***

Data are represented as mean ± SE. t-test values: $***$: significant at $P < 0.001$. ANOVA analysis: within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

serum and kidney tissue homogenate were significantly ($P < 0.001$) decreased compared to those of the positive control group as a result of treating diabetic rats with *N. sativa* and propolis methanolic extract, respectively.

Treating diabetic rats with the methanolic extract of propolis in G4 was more effective on lipid peroxidation compared to that of *N. sativa* in G3.

3.4. Antioxidant Enzymes. The results of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on antioxidant enzymes in the serum and kidney tissue are given in Table 1. The mean values of catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in the positive control group were significantly ($P < 0.001$) decreased compared to those of the negative control.

Table 2: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on immunoglobulins and IL-6.

Parameters	Statistics	G1 N. control	G2 P. control	G3 *Nigella sativa* extract	G4 Propolis extract
Serum IgG (mg/dL)	Mean ± SE	530.66 ± 1.05[b]	754.33 ± 3.46[a]	595.00 ± 100.64[b]	572.33 ± 2.40[b]
	LSD 0.05 = 152.870				
	t-test	—	−63.91***	1.55[NS]	45.12***
Serum IgA (mg/dL)	Mean ± SE	99.16 ± 1.88[d]	359.83 ± 1.74[a]	257.00 ± 1.73[b]	126.00 ± 1.31[c]
	LSD 0.05 = 5.492				
	t-test	—	−85.42***	52.10***	92.71***
Serum IgM (mg/dL)	Mean ± SE	129.83 ± 1.07[d]	357.16 ± 2.24[a]	220.00 ± 2.22[b]	141.50 ± 1.78[c]
	LSD 0.05 = 5.614				
	t-test	—	−138.06***	45.31***	65.29***
Serum IL-6 (pg/mL)	Mean ± SE	5.60 ± 0.26[d]	24.48 ± 0.89[a]	11.90 ± 0.34[b]	8.78 ± 0.19[c]
	LSD 0.05 = 1.630				
	t-test	—	−17.24***	11.26***	20.71***
IL6 (pg/g) Kidney tissue	Mean ± SE	48.80 ± 2.01[d]	90.43 ± 1.55[a]	67.01 ± 0.69[b]	55.43 ± 0.89[c]
	LSD 0.05 = 4.285				
	t-test	—	−14.14***	16.67***	19.38***
Carboxymethyl lysine (CML) (nmol/mL)	Mean ± SE	188.16 ± 2.38[d]	276.00 ± 2.58[a]	234.33 ± 1.85[b]	212.16 ± 2.35[c]
	LSD 0.05 = 6.574				
	t-test	—	−24.84***	10.93***	23.52***

Data are represented as mean ± SE. *t*-test values superscripts (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

In G3 and G4, the mean values of CAT, SOD, and GST in the serum were significantly ($P < 0.001$) increased compared to those of the positive control as a result of treating diabetic rats with *N. sativa* and propolis methanolic extract, respectively. In G4, the mean values of the three antioxidant enzymes were higher than those of G3.

3.5. Interleukin-6 (IL-6). Table 2 shows the effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on interleukin-6 (IL-6) in the serum and kidney tissue homogenate. The mean values of IL-6 in the diabetic positive control group (G2) were significantly ($P < 0.001$) increased. After treating these diabetic rats with methanolic extracts of *N. sativa* and propolis in G3 and G4, respectively, a significant ($P < 0.001$) decrease in IL-6 values compared with the positive control group was observed. Treating diabetic rats with propolis in G4 was more effective than treating them with *N. sativa* in G3.

3.6. Immunoglobulins (Igs). The effect of methanolic extracts of *N. sativa* and propolis on IgG, IgA, and IgM immunoglobulins in the serum with induced diabetic rats is given in Table 2. The mean values of IgG, IgA, and IgM immunoglobulins were significantly ($P < 0.001$) increased in the diabetic positive control (STZ treated) compared with those of the negative control group. In G3 and G4, the mean values of IgG, IgA, and IgM immunoglobulins were significantly ($P < 0.001$) decreased as a result of treatment with *N. sativa* and propolis methanolic extract, respectively. The immunoglobulins result revealed that treating STZ-induced diabetic rats with the propolis methanol extract in G4 was more efficient than treating them with the methanolic extract of *N. sativa* in G3.

3.7. Carboxymethyl Lysine (CML). The percentage of CML in the diabetic positive control group (G2) was significantly increased compared with negative control as shown in Table 2. In G3 and G4, the percentage of CML was very highly significantly ($P < 0.001$) decreased as a result of treating diabetic rats with *N. sativa* and propolis, respectively, compared with the positive control group. In G4, treating diabetic rats with methanolic extract of propolis was more effective on CML compared to treating them with *N. sativa* in G3.

3.8. Kidney Functions. The mean values of urea, creatinine, and uric acid in the serum of the positive control group

Table 3: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on kidney functions and electrolytes.

Parameters	Statistics	G1 N. control	G2 P. control	G3 *N. sativa* extract	G4 Propolis extract
Serum urea (mg/dL)	Mean ± SE	24.50 ± 1.11[d]	74.83 ± 0.87[a]	47.33 ± 0.88[b]	33.33 ± 0.98[c]
	LSD 0.05 = 2.705				
	t-test	—	−29.16***	25.28***	27.26***
Serum creatinine (mg/dL)	Mean ± SE	0.68 ± 0.03[d]	3.63 ± 0.18[a]	2.60 ± 0.09[b]	1.21 ± 0.04[c]
	LSD 0.05 = 0.318				
	t-test	—	−14.90***	6.70***	11.66***
Serum uric acid (mg/dL)	Mean ± SE	3.33 ± 0.08[d]	6.68 ± 0.04[a]	5.15 ± 0.07[b]	4.20 ± 0.05[c]
	LSD 0.05 = 0.179				
	t-test	—	−59.53***	31.01***	28.46***
Urinary albumin (mg/dL)	Mean ± SE	22.16 ± 1.70[d]	411.50 ± 7.74[a]	216.66 ± 3.71[d]	122.66 ± 3.27[b]
	LSD 0.05 = 14.333				
	t-test	—	−47.24***	20.85***	29.03***
Urinary creatinine (mg/dL)	Mean ± SE	85.00 ± 0.85[a]	27.00 ± 0.36[c]	35.16 ± 1.16[b]	73.16 ± 0.60[c]
	LSD 0.05 = 2.405				
	t-test	—	84.90***	−7.57***	−70.58***
Serum Na^+ (mmol/L)	Mean ± SE	143.83 ± 0.94[a]	118.33 ± 0.88[d]	127.83 ± 0.60[c]	138.66 ± 0.42[b]
	LSD 0.05 = 1.996				
	t-test	—	19.85***	−8.99***	−24.11***
Serum K^+ (mmol/L)	Mean ± SE	4.86 ± 0.03[a]	3.03 ± 0.08[d]	3.76 ± 0.04[c]	4.25 ± 0.04[b]
	LSD 0.05 = 0.169				
	t-test	—	17.39***	−10.25***	−13.37***

Data are represented as mean ± SE. *t*-test values: ∗ ∗ ∗: significant at $P < 0.001$. ANOVA analysis: within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

(G2) were significantly ($P < 0.001$) increased compared with those of the negative control group (G1) as a result of induced diabetes shown in Table 3. Treating these diabetic rats with methanolic extracts of *N. sativa* and propolis in G3 and G4, respectively, significantly ($P < 0.001$) decreased urea, creatinine, and uric acid levels compared with those of the positive control group (G2). The methanolic extract of propolis in G4 was more effective than that of *N. sativa* in G3.

Also, Table 3 shows that the mean values of urinary albumin of the positive control group were significantly ($P < 0.001$) increased compared with those of the negative control group (G1). Meanwhile, the mean values of urinary creatinine in G2 were significantly ($P < 0.001$) decreased compared with those of the negative control group (G1). Treating these diabetic rats in G3 and G4 with methanolic extract of *N. sativa* and propolis, respectively, significantly ($P < 0.001$) decreased urinary albumin and increased creatinine in urine when compared with those of the positive control (G2).

3.9. Serum Electrolytes. Table 3 also shows the effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on serum electrolytes. The mean values of serum sodium and potassium ions of the positive group were significantly ($P < 0.001$) decreased compared with those of the negative control group. Treating these diabetic rats with methanolic extracts of *N. sativa* and propolis in G3 and G4, respectively, significantly ($P < 0.001$) increased serum electrolytes levels (Na^+ and K^+) compared with those of the positive control group.

3.10. Food Intake. Table 4 shows that there were nonsignificant differences in food intake (FI) in all groups in the first and the second week, whereas the mean values of FI in all

Table 4: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on food intake.

Food intake (g/day)	Statistics	G1 N. control	G2 P. control	G3 *N. sativa* extract	G4 Propolis extract
1st week	Mean ± SE	15.50 ± 0.22^{a}	15.50 ± 0.22^{a}	15.50 ± 0.22^{a}	15.50 ± 0.22^{a}
	LSD 0.05 = 0.674				
	t-test	—	0.00^{NS}	0.00^{NS}	0.00^{NS}
2nd week	Mean ± SE	16.50 ± 0.22^{a}	16.33 ± 0.21^{a}	16.66 ± 0.21^{a}	16.50 ± 0.22^{a}
	LSD 0.05 = 0.648				
	t-test	—	0.54^{NS}	-1.58^{NS}	-0.54^{NS}
3rd week	Mean ± SE	19.16 ± 0.54^{a}	18.16 ± 0.40^{b}	17.00 ± 0.44^{c}	15.50 ± 0.22^{c}
	LSD 0.05 = 0.933				
	t-test	—	3.87^{**}	2.90^{**}	6.32^{***}
4th week	Mean ± SE	16.41 ± 0.39^{a}	16.12 ± 0.30^{ab}	15.95 ± 0.23^{b}	15.91 ± 0.24^{b}
	LSD 0.05 = 0.426	—	1.77^{*}	0.81^{NS}	1.15^{NS}
	t-test				

Data are represented as mean ± SE. *t*-test values: ∗: significant at $P < 0.05$, ∗∗: significant at $P < 0.01$, and ∗ ∗ ∗: significant at $P < 0.001$. ANOVA analysis: within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

Table 5: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on water consumption.

Water consumed (mL/day)	Statistics	G1 N. control	G2 P. control	G3 *N. sativa* extract	G4 Propolis extract
1st week	Mean ± SE	33.33 ± 1.05^{b}	42.50 ± 1.11^{a}	36.33 ± 0.88^{b}	36.33 ± 0.88^{b}
	LSD 0.05 = 3.257				
	t-test	—	-4.56^{***}	7.40^{***}	4.01^{**}
2nd week	Mean ± SE	35.33 ± 1.17^{b}	42.50 ± 1.11^{a}	34.83 ± 0.90^{b}	37.16 ± 0.79^{b}
	LSD 0.05 = 2.943				
	t-test	—	-4.73^{***}	5.54^{***}	4.54^{***}
3rd week	Mean ± SE	29.16 ± 1.53^{b}	42.50 ± 1.11^{a}	26.66 ± 1.66^{b}	26.66 ± 1.05^{b}
	LSD 0.05 = 3.725				
	t-test	—	-8.00^{***}	7.88^{***}	19.00^{***}
4th week	Mean ± SE	27.50 ± 1.11^{a}	29.16 ± 1.53^{a}	28.00 ± 1.00^{a}	27.50 ± 1.11^{a}
	LSD 0.05 = 4.453				
	t-test	—	-1.58^{NS}	0.63^{NS}	1.00^{NS}

Data are represented as mean ± SE. *t*-test values: ∗∗: significant at $P < 0.01$ and ∗ ∗ ∗: significant at $P < 0.001$. ANOVA analysis: within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

groups in the 3rd week were significantly ($P < 0.01$) lower than those of the negative control. In the fourth, FI of the positive control group was significantly ($P < 0.05$) lower than that of the negative control group. In G3 and G4, FI was nonsignificantly lower than that of the positive control group.

3.11. Water Consumption. Data in Table 5 illustrate the effect of supplementation of methanolic extract of *N. sativa* and propolis for 4 weeks to diabetic rats on water consumption. The positive control group showed significant ($P < 0.001$) increase in water consumption in the first three weeks as a

Table 6: Effect of treating diabetic rats with methanolic extracts of *N. sativa* and propolis for 4 weeks on body weight gain (BWG) and food efficiency ratio (FER).

Biological evaluation	Statistics	G1 N. control	G2 P. control	G3 *N. sativa* extract	G4 Propolis extract
BWG (g/day)	Mean ± SE	0.494 ± 0.040[a]	0.466 ± 0.024[a]	0.316 ± 0.044[a]	0.444 ± 0.126[a]
	LSD 0.05 = 0.191	—	0.57[NS]	3.08**	0.17[NS]
	t-test				
BWG (g/4 week)	Mean ± SE	14.833 ± 1.222[a]	14.000 ± 0.730[a]	9.500 ± 1.335[a]	13.333 ± 3.783[a]
	LSD 0.05 = 5.735				
	t-test	—	0.57[NS]	3.09**	0.17[NS]
BWG%	Mean ± SE	8.305 ± 0.685[a]	7.059 ± 0.436[ab]	5.012 ± 0.889[b]	6.818 ± 1.988[a]
	LSD 0.05 = 2.178				
	t-test	—	1.52[NS]	2.15**	0.12[NS]
FER (g/day)	Mean ± SE	0.030 ± 0.002[a]	0.029 ± 0.001[a]	0.020 ± 0.002[a]	0.028 ± 0.007[a]
	LSD 0.05 = 0.011				
	t-test	—	0.32[NS]	3.09**	0.12[NS]
FER%	Mean ± SE	3.013 ± 0.248[a]	2.894 ± 0.151[a]	1.985 ± 0.279[a]	2.793 ± 0.792[a]
	LSD 0.05 = 1.199				
	t-test	—	0.40[NS]	2.99**	0.12[NS]

Data are represented as mean ± SE. *t*-test values: **: significant at $P < 0.01$. ANOVA analysis: within each row, means with different superscript (a, b, c, or d) are significantly different at $P < 0.05$, whereas means with the same superscript letters mean that there is no significant difference at $P < 0.05$. LSD: least significant difference; NS: nonsignificant.

result of STZ-induced diabetes, compared with that of the negative control group, whereas the 4th week showed no significant difference in water consumption in all groups. Treating diabetic rats with *N. sativa* and propolis significantly ($P < 0.001$) decreased water consumption, compared with that of positive group.

3.12. Physiological Evaluation. Table 6 shows the effect of treating STZ-induced diabetic rats with *N. sativa* and propolis for 4 weeks on physiological evaluation. The mean values of body weight gain (BWG), body weight gain percentage (BWG%), food efficiency ratio (FER), and food efficiency ratio percentage (FER%) in the positive control group were nonsignificantly lower than those of the negative control. Treating these diabetic rats with methanolic extract of *N. sativa* in G3 significantly ($P < 0.01$) decreased the mean values of these parameters when compared with the positive group, whereas the mean values of these parameters in G4 were nonsignificantly lower than those of the positive control group.

3.13. Pathology of Kidney. Microscopically, the histopathological examination of the kidney of the negative control group showed normal histological structure of normal kidney tissues and normal blood vessels with no histopathological changes (Figure 1(a)). Examination of kidney tissues of rats in the positive group, which suffer from diabetes, showed pathological changes in kidney structure compared to that of the negative control group. They showed a collapsed glomerular tuft with marked tubular atrophy associated with interstitial inflammation and interstitial hemorrhage (Figure 1(b)). Meanwhile, the kidney sections of diabetic rats in G3 treated with the *N. sativa* methanol extract for 4 weeks seemed to be restoring the normal appearance of glomeruli and regenerated tubules with interstitial hemorrhage (Figure 1(c)). On the other hand, after treatment with the propolis methanol extract in group (4) for 4 weeks, the kidney nearly restored the normal cortical tissue (Figure 1(d)).

3.14. Pathology of Pancreas. Figure 2 shows the histology of pancreas of rats under study. Figure 2(a) shows the normal pancreatic tissues of the negative control group (G1) with normal pancreatic acini, Langerhans cells, and interductal glands. Figure 2(b) shows the pancreatic tissues of the streptozotocin-induced diabetic rats of the positive control (G2) with degenerated pancreatic acini cells, periductal inflammation, and mild congested edema. Figure 2(c) shows the pancreas of diabetic rats treated with *Nigella sativa* (G3) with improvement in the degenerated pancreatic acini cells, mild inflammation, and congestion. Figure 2(d) shows the

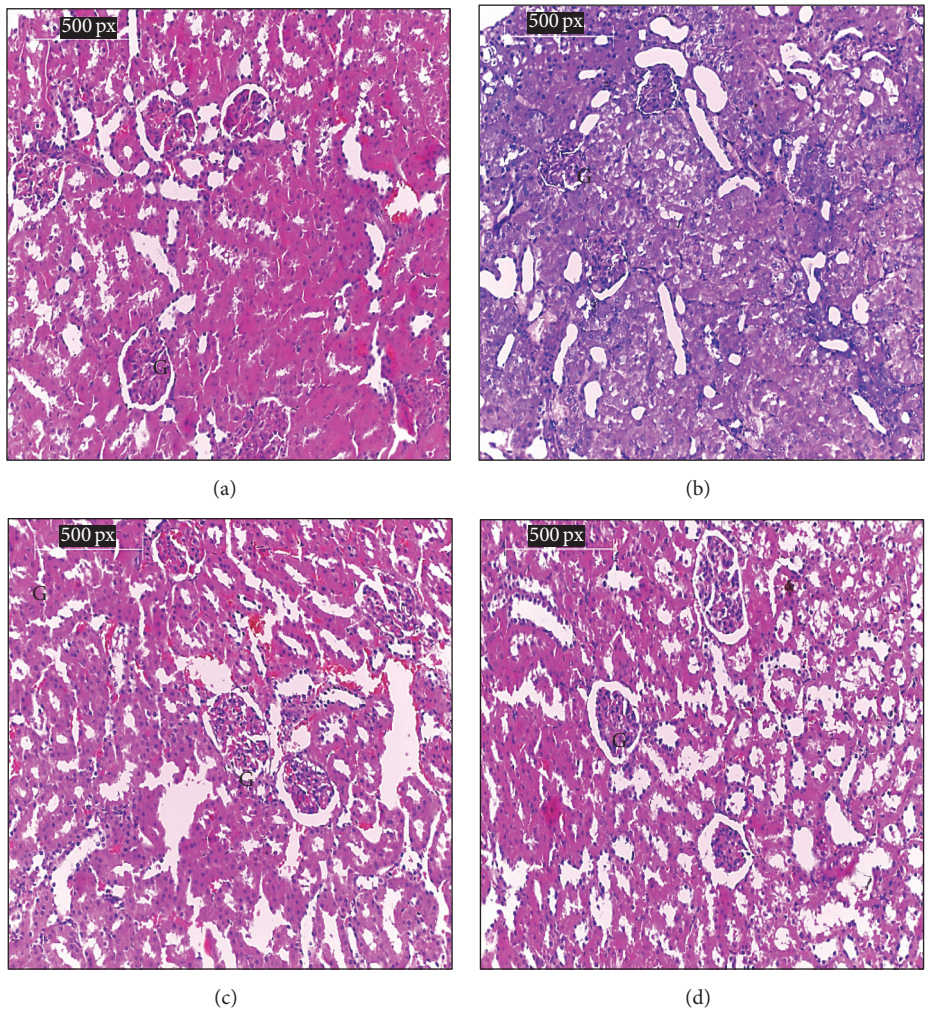

Figure 1: (a) Kidney of rats of the negative control group reveals the normal histological structure of renal tissue with normal parenchyma, normal blood vessels, and being interstitial with no histopathological changes, (b) kidney of rat from the positive control group showing collapsed glomerular tuft with marked tubular atrophy, interstitial inflammation, and interstitial hemorrhage, (c) kidney of diabetic rat treated with *N. sativa* methanol extract showing normal glomeruli and regenerated tubules with interstitial hemorrhage, and (d) kidney of diabetic rat treated with propolis methanol extract (G4) showing near normal renal cortical tissue. G: glomerulus (H&E stain ×200).

pancreas of diabetic rats of the propolis treated group (G4) with no evidence of inflammation in islets or around the large ducts with normal pancreatic tissues.

4. Discussion

Diabetes mellitus is metabolic disorder leading to hyperglycemia, which later develops to micro- and macrovascular complications. The induction of experimental diabetes in the rats using chemicals which selectively destroy pancreatic β-cells is very convenient and simple to use as streptozotocin (STZ) that acts as diabetogenic agent mediated by reactive oxygen species [40]. In the present study, induction of diabetes using streptozotocin (STZ) at a dose of 65 mg/kg in rats of the positive control group showed significant increase in serum glucose level compared with the control group [3, 41]. The concurrent oral administration of 20% of methanolic extract of *N. sativa* or propolis to the diabetic rats of G3 and G4, respectively, for 4 weeks significantly decreased glucose levels most probably due to their antioxidant chemical contents [42, 43].

STZ diabetic rats in G2 also showed an increase in lipid peroxidation level accompanied by decreased CAT, SOD, and GST activity in the serum and the kidney tissue homogenate compared with that of the negative control group after 4 weeks. This result is in agreement with previous investigations [3, 4, 44]. This result may be attributed to the fact that the elevated generation of free radicals resulting in the consumption of antioxidant defense components may lead to disruption of cellular functions and oxidative damage to membranes and may enhance susceptibility to lipid peroxidation [45]. The concurrent treatment with methanolic extract of *N. sativa* and propolis ameliorated these parameters and nearly restored them to their normal levels as a result of

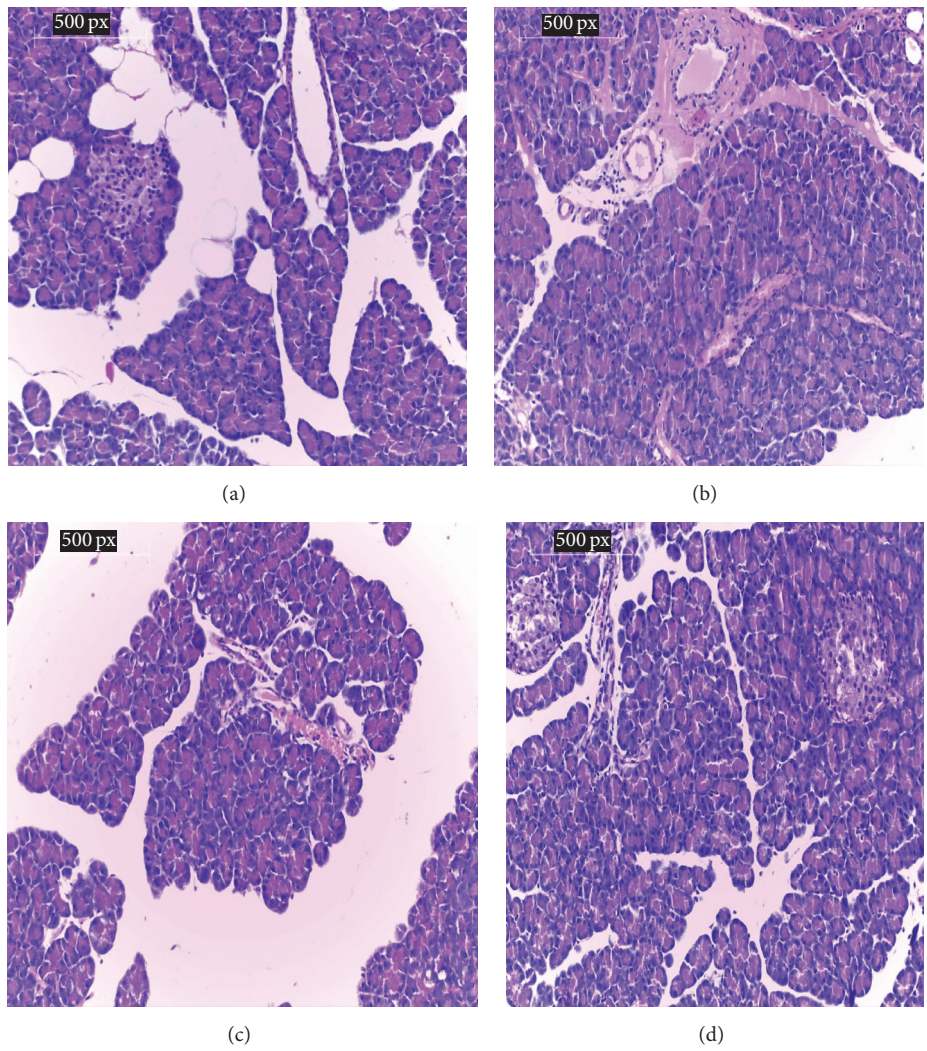

Figure 2: (a) Pancreas of control negative group showing normal pancreatic acini, Langerhans cells, and interductal glands. (b) Pancreas of control positive group showing mild degeneration of pancreatic acini cells with periductal inflammation, edema, and congestion. (c) Pancreas of *Nigella sativa* treated group showing improvement and degeneration of pancreatic tissues with nearly normal tissues. (d) Pancreas of propolis treated group showing restored pancreatic tissues to the normal with no evidence of inflammation in islets or around the large ducts. (H&E, ×200).

their antioxidant activity due to their contents of phenolics and flavonoids that have scavenging effect on the free radicals [11, 46].

Advanced glycation end products (AGEs) increase reactive oxygen species formation and impair antioxidant systems that activate NF-κB signaling pathway which enhanced the production of the cytokine interleukin-6 (IL-6) involved not only in inflammation and infection responses but also in the regulation of metabolic, regenerative, and neural processes [47]. In the current study, IL-6 was increased in the serum and kidney tissue in positive group as a result of STZ-induced diabetes. A similar result was reported by Sayed [34]. However, IL-6 was decreased with the concurrent treatment with methanolic extract of *N. sativa* and propolis. This result is consistent with Bashandy et al. [48] and Al Ghamdi et al. [49].

The increase of immunoglobulins (IgG, IgA, and IgM) as a result of diabetes in the current study is consistent with the increase in IL-6 and other findings revealed a positive correlation between these parameters [3]. This may be attributed to the fact that production of proinflammatory cytokines is increased in patients with diabetes. These include adipocytokines such as interleukin-6 (IL-6), which is a cofactor for immunoglobulin synthesis and a common marker of inflammation [50]. Treating diabetic rats with *N. sativa* and propolis has significantly reduced immunoglobulins (IgG, IgA, and IgM). These results are consistent with previous studies [51, 52].

Diabetes is associated with severe acute and chronic complications that negatively influence both the quality of life and survival of affected individuals [53]. Therefore, protein glycation and formation of AGEs play an important role in

the pathogenesis of diabetic complications like retinopathy, cataract, neuropathy, nephropathy, and cardiomyopathy [54]. Nε-(Carboxymethyl) lysine (CML) was selected as a marker of AGEs in laboratory studies. In our study, CML showed significant increase in the untreated diabetic group after 4 weeks compared with the negative control. This result agrees with that of Van Eupen et al. [5]. On the other hand, our result showed that carboxymethyl lysine (CML) was significantly decreased in the treated diabetic group with *N. sativa* and propolis in G3 and G4, respectively, compared with the untreated diabetic group G2. It is worthy to mention that the efficacy of propolis on lowering carboxymethyl lysine (CML) exceeded that of *N. sativa*.

STZ administration increased serum renal markers in rats, for example, creatinine, urea, and uric acid [3, 55], as a result of diabetic nephropathy which is considered a major complication of diabetes [34]. The current investigation is consistent with the previous studies. Furthermore, overtime diabetic nephropathy will be developed which is characterized by proteinuria, a loss of renal function, and a rapid progression to end stage renal failure [7]. Urine analysis of the STZ-induced diabetes rats showed significant increase in albumin level in the positive control group and decreased urinary creatinine level in G2 after 4 weeks. These results are consistent with other studies [3, 56]. Treatment with *N. sativa* and propolis as natural resources showed significant decrease in the levels of urea, creatinine, and uric acid compared with the positive control. The present results were in conformity with the results of Abdelaziz and Kandeel [57] and Saleh [58]. On other hand, oral administration of propolis revealed a significant reduction of urinary albumin and restoring urinary creatinine approaching the negative control level more than treatment with *N. sativa*. The effect of treatment with 20% of methanolic extract of propolis may be attributed to a strong antioxidant effect of propolis, which can ameliorate oxidative stress and delay the occurrence of diabetic nephropathy in diabetes mellitus [59, 60]. The combination of intracellular and extracellular electrolyte disturbances may be implicated in the pathogenesis of neuropathy, nephropathy, and vascular complications in diabetic patients [61]. Our experiment recorded a significant decrease in serum sodium and potassium in the untreated diabetic group. This result agrees with previous investigation of Al-Rubeaan et al. [62] and Liamis et al. [63]. Diabetic rats treated with propolis restored serum electrolyte (Na^+ and K^+) levels to normal compared to *N. sativa*.

The renal and pancreatic tissues were severely affected in hyperglycemic rats of G2 as a result of STZ supplementation. This result which is consistent with other studies showed a correlation between hyperglycemia and pathological alteration of vital organs [64, 65]. The concurrent supplementation with *N. sativa* and propolis in G3 and G4, respectively, has significantly improved renal and pancreatic tissues and nearly restored them to their normal state [66, 67].

However, administration of 20% (w/w) methanolic extract of either *N. sativa* or propolis to STZ-induced diabetic rats significantly reduced hyperglycemic and oxidative stress resulting from hyperglycemia. Also, they improved all adverse biochemical and histopathological changes resulting from diabetes. These natural resources revealed safe and excellent antidiabetic activity attributed to their antioxidant activity. As well as overcoming most of the histopathology changes in kidney and pancreas tissues, the majority of the cells restored the normal conditions. In addition, methanolic extract of propolis appeared to be more efficient than *N. sativa* as revealed by the different biochemical and histological investigations. Therefore, it is recommended that dietary *N. sativa* and propolis could be excellent adjuvant support in the therapy of diabetes mellitus and preventing its complications.

Abbreviations

AGEs: Advanced glycation end products
BUN: Blood urea nitrogen
BWG: Body weight gain
BWG%: Body weight gain percentage
CAT: Catalase
CML: Nε-(Carboxymethyl) lysine
Cr: Creatinine
CVD: Cardiovascular disease
FBG: Fasting blood glucose
FER: Food efficiency ratio
FER%: Food efficiency ratio percentage
G1: The first negative control group receiving a single tail vein injection of 0.1 mol/L citrate buffer
G2: The second positive control diabetic group intravenously injected with freshly prepared streptozotocin (60 mg/kg body weight) in a 0.1 mol/L citrate buffer (pH 4.5)
G3: The third group, diabetic rats as in G2 treated with 20% w/w *Nigella sativa* methanolic extract for 4 weeks
G4: The fourth group, diabetic rats as in G2 treated with 20% w/w propolis methanolic extract for 4 weeks
GST: Glutathione-S-transferase
IDDM: Insulin-dependent diabetes mellitus
Igs: Immunoglobulins
IL-6: Interleukin-6
MDA: Malondialdehyde
N. sativa: *Nigella sativa*
ROS: Reactive oxygen species
SOD: Superoxide dismutase
STZ: Streptozotocin.

Competing Interests

The authors of this paper declare that they have no competing interests.

Acknowledgments

The authors thank King Abdulaziz City for Science and Technology (KACST) for supporting this project (Grant no. 418-35-ط ا). Thanks are also due to DSR, KAU, for their help and technical support.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 5861024, 7 pages
https://doi.org/10.1155/2017/5861024

Research Article

Effects of Honey on Oral Mucositis among Pediatric Cancer Patients Undergoing Chemo/Radiotherapy Treatment at King Abdulaziz University Hospital in Jeddah, Kingdom of Saudi Arabia

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Received 8 July 2016; Accepted 3 October 2016; Published 7 February 2017

Academic Editor: Andrzej K. Kuropatnicki

One of the most common complications of cancer chemotherapy is oral mucositis. This study evaluates the therapeutic effects of honey with the focus on grade III and IV oral mucositis, reduction of bacterial and fungal infections, duration of episodes of oral mucositis, and body weight in pediatric leukemic patients undergoing chemo/radiotherapy. This is an open labeled randomized controlled study conducted at our hospital on 40 pediatric cancer patients undergoing chemo/radiotherapy. All the 40 patients included in this study experienced a sum total of 390 episodes of fever and neutropenia associated with oral mucositis. A significant reduction of oral mucositis, associated *Candida*, and aerobic pathogenic bacterial infections was noted in patients in the honey treatment group. Also, there is a significant decrease in the duration of hospitalization for all those in the treatment group combined with a significant increase of body weight, delayed onset, and decreased severity of pain related to oral mucositis. Complications of oral mucositis can be tremendously reduced by the topical application of local Saudi honey and honey should be used as an integrative approach in prophylaxis and treatment of chemo/radiotherapy-induced oral mucositis in pediatric cancer patients. Further research is needed to elucidate and better understand the underlying mechanism.

1. Introduction

Mucositis is considered as one of the most common oral problems associated with cancer therapy [1]. Mucositis causes inflammation and ulceration of the oral cavity mucosa and be more susceptible to infection which may result in the demise of the patient due to infections and compromising the cancer treatment. Around 40%–76% of cancer patients

undergoing high dose of chemotherapy and radiotherapy develop mucositis which manifests itself as intense erythema in the treated areas and patients suffer from difficulties with swallowing [1–3]. In general, the incidence rate of mucositis is two to three times higher in patients with blood malignancies associated with bone marrow suppression like lymphoma [4]. Younger cancer patients undergoing chemotherapy are more at risk of developing mucositis and may reach 90% in children under 12 years of age [5]. Some degree of mucositis manifests itself in almost all (nearly 90% to 97%) cancer patients undergoing radiotherapy [6, 7]. Among those patients around 34% to 43% showed severe mucositis [5]. As a consequence to that, patients will suffer from infections caused by both Gram positive and negative bacteria as well as fungi like *Candida* [7]. In addition, the patient's quality of life will be affected, hospital admittance rates will be higher, the use of total parenteral nutrition will be increased, and interruption of treatment will be more frequent, all of which compromise the treatment of cancer [5]. Cancer treatment will be much more effective if it is not associated with short and long term side effects as those associated with oral mucositis. Oral mucositis has also a major impact on the quality of life and nutritional status, prolonged hospital stays, and severe infections. Management essentially consists of pain management, with topical and oral analgesics/anesthetics and anti-inflammatory agents, and systemic use of antifungal medications [8]. In spite of the fact that there are many positive trials, none of those showed overwhelming data to strongly support the use of a certain agent for the treatment of oral mucositis [9].

Currently, the only standard oral hygiene consists of an oral rinse of warm water, salt, and baking soda 4 times a day. Basic oral care (brushing and flossing as tolerated) is recommended to maintain general mucosal health and to reduce the impact of oral microbial flora [10]. Some recent published data showed that honey has a positive effect against oral mucositis [11]. This study was undertaken to evaluate the efficacy of using local Saudi honey as integrative approach in prophylaxis and treatment of chemo/radiotherapy-induced oral mucositis (grades III and IV) among pediatric cancer patients in the cancer ward at King Abdulaziz University Hospital (KAUH). Other parameters were also monitored which are indicative of the success of the integration of honey with ongoing treatment of those patients and they include bacterial and fungal infections, duration of episodes of oral mucositis (as evaluated by the length of hospital stay per episode), and body weight.

2. Materials and Methods

2.1. Design and Setting. This was an open labeled randomized controlled study carried out on 40 patients in the pediatric cancer ward at KAUH, Jeddah, KSA, for a period of one year comparing the efficacy of the consumption of local Saudi commercial honey on chemo/radiotherapy-induced oral mucositis among various pediatric cancer patients who have hematological (acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), Burkett's lymphoma, and langerhans cell histiocytosis) and nonhematological (Wilms' tumour, neuroblastoma, and medulloblastoma) cancer.

2.2. Participants. Sixty patients were admitted to oncology ward diagnosed for both hematological and nonhematological cancer, forty of whom fulfilled the inclusion criteria and as such were included in this clinical trial. The duration of the study was one year (Figure 1).

Patients were assessed for oral mucositis prior to chemotherapy courses and daily during episodes of admission for supportive care of febrile neutropenia. All patients were encouraged to apply hospital provided honey to all areas of oral mucosa, gingiva, and tongue followed by mouth rinsing with alkaline saline, four to six times daily. Clinical assessment was done by attending physicians, nurses, and dentists whenever needed. Normal oral mucosa was defined by pink, moist appearance with no lesions, crusts, or debris. Normal gingiva was recognized by being pink and firm. Patients with healthy oral cavity were still counseled and encouraged to keep up their oral hygiene regimen including local mouth application of hospital honey. The following grading system was used to assess severity of oral mucositis [2]. Grade I oral mucositis was defined with shiny red oral mucosa and/or gingiva with possible swelling and white patches with possibly red coated swollen tongue; patients with grade I oral mucositis may complain of a burning sensation or gingival discomfort. Grade II oral mucositis was defined by same mucosal and gingival findings previously described in grade 1 with added painful ulcers; patients can still tolerate solids and liquids. Grade III oral mucositis was defined with severe erythema, ulceration, or white patches over oral mucosa with severe pain; patient cannot tolerate solid diet but can tolerate fluids only. Grade IV oral mucositis was defined with severe erythema, ulcerations, and white plaques that affect oral intake for both solid and fluid diets even drooling of saliva. Various indicators which are involved in bacterial and fungal infections were monitored. For bacterial infections, aerobic cultures were checked and *Candida* colonization was monitored as an indicator of fungal infections. Inclusion criteria: Pediatric cancer patients at KAUH above 1 year of age treated with chemo/radiotherapy whose parents or their assigned care takers approved them to participate in this study signed a consent form. Exclusion criteria: They include pediatric cancer patients at KAUH who were less than one year old and also those patients who refused to participate in this study or had allergy to honey. Those who were eligible and agreed to participate were invited to our clinic with their parents to get information about them. Such information included the following: sex, age, body weight, educational level, occupation, and their records checked for the presence of any other systemic diseases. They all underwent a physical examination of the mouth and throat for any abnormalities. Routine laboratory tests were conducted. Those patients with other systemic disease were not included in this study.

2.3. Aerobic Bacterial Test and Candida Assay. This was done by taking oral and oropharyngeal swabs. Swabs were sent to the KAUH clinical laboratory for assay. Oral swabs were

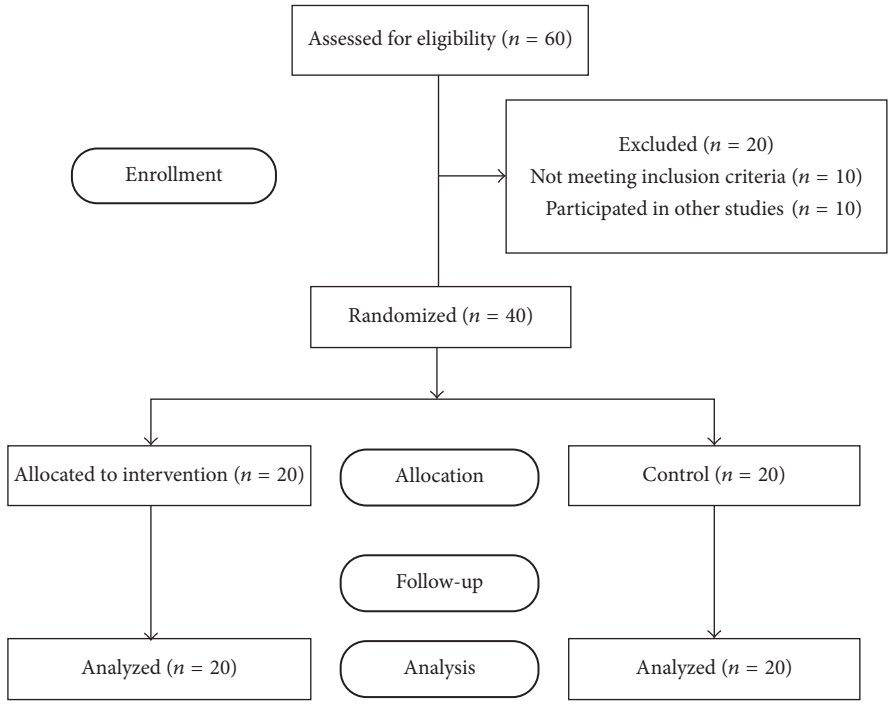

Figure 1: Patient enrollment.

collected by gently rubbing a sterile cotton swab over the labial mucosa, tongue and cancerous lesion [12]. After the swabs were collected, they were inoculated onto sheep blood agar, Sabouraud dextrose agar, MacConkey agar, nutrient agar, and other selective media and then incubated under aerobic conditions for 24–48 hours at 37°C temperature for bacterial pathogens isolation and for 24–72 hours at 30°C in BOD incubator for fungal species isolation [13].

2.4. Intervention. All of the 40 patients were randomly distributed into two groups, each containing 20 patients of both sexes. Patients in both groups then received chemo/radiotherapy in addition to the routine oral hygiene (Lidocaine, Mycostatin, Daktarin mouth gel, and mouth wash).

The experimental group received topical application of pure natural honey as prophylaxis before the development of oral mucositis or during the episodes of fever and neutropenia associated with oral mucositis. Local commercial Saudi honey bought from the supermarkets was used.

2.5. Evaluation of Outcomes. The severity of oral mucositis was described according to the World Health Organization's oral toxicity scale. Grade I: soreness ± erythema, grade II: erythema, ulcers, and patients' ability to swallow solid foods, grade III: ulcers with extensive erythema and patients not being able to swallow solid foods, and grade IV: mucositis to the extent that alimentation is not possible [2]. Oral mucositis was evaluated before and after treatment and also a week after commencing treatment [14]. The duration of stay in the hospital per episode was reported, in addition, to monitor the fluctuations of the body weight in those patients.

2.6. Statistical Analyses. All data was entered using SPSS 17 software (SPSS Inc., Chicago, IL) and analyzed. The data were double checked and cleaned and analyzed in terms of frequencies. Continuous variables were presented as mean and standard deviation (STD) and categorical variables were presented as absolute and relative frequencies. Independent test and Chi-square test were used to investigate whether there was significant difference between the treatment and experimental groups.

Absolute Risk Reduction (ARR) and Number Needed to Treat (NNT) together with the 95% Confidence Interval (95% CI) were presented. P values <0.05 were considered to be significant.

2.7. Ethical Approval. The patients and those who were in charge of them were informed about the objective of this study and the resulting possible benefits, the prescribed ways, and their own role. An informed consent form was signed just before enrolling patients in the study. All personal data was kept confidential. This study design was approved by the Ethical Committee at King Abdulaziz University.

3. Results

The 40 patients experienced episodes of fever and neutropenia associated with oral mucositis. Most of those included were hematological patients (90% in the honey treated group versus 75% in the control group). The characteristics of the patients involved are shown in Table 1.

Both sexes were included in the treatment and control groups with an average age of about 8 years (SD ± 4.2) in

Table 1: Characteristics of study participants.

	Treatment (honey)	Control	*P* value
Gender			
Boys	11 (55)	10 (50)	0.7
Girls	9 (45)	10 (50)	
Age	7.9 (4.1)	8.1 (4.9)	0.8
Diagnosis			
Hematological	18	15	0.4
Nonhematological	2	5	

both groups (Table 1). There was no significant difference between the control and the honey treated group in relation to gender or age. Table 2 shows the Absolute Risk Reduction and Number Needed to Treat for developing grade III and IV oral mucositis, *Candida*, and aerobic bacterial infections. The results show a significant difference between the experimental (honey) and the control group ($P < 0.05$).

The results showed a significant ARR in grade III and IV oral mucositis of 35% in the treatment group ($P = 0.02$) with an NNT of 2. The same was also true in the case of *Candida* colonization with an ARR of 50% in the treatment group ($P = 0.003$) with an NNT of 2. In the case of the aerobic plate count, there was also a significant ARR of bacteria of 50% in the treated group versus the control with an NNT of 2 ($P = 0.003$) (Table 2).

The study, also, showed significant reduction in the duration of hospitalization for oral mucositis patients in the treatment group as compared to the control group. The mean days for hospitalization were 7 ± 3 days/episodes and 13 ± 5 days/episodes for the treatment group and the control group, respectively.

Patients in the treatment group had significant increase in body weight, delayed onset, and decreased severity of pain related to oral mucositis in comparison to those in the control group. The treatment group showed better improvement in all of the outcome variables.

4. Discussion

The results of this study indicated that there was a statistically significant reduction in the number of episodes of oral mucositis, bacterial and fungal infections, and hospital stay among pediatric cancer patients undergoing chemo/radiotherapy who are taking honey in conjunction with their regular therapy. Such an overall improvement was also accompanied by body weight gain in patients in the experimental group in comparison to the control group (Table 3).

Honey is an age-old remedy from the time of Egyptian civilization; mentioned in the holy Qur'an and more recently it has found a place in modern medicine [3, 15, 16].

The results of this study showed that honey had very positive results against oral mucositis among pediatric cancer patients undergoing chemo/radiotherapy. The results showed a significant reduction in grade III and IV oral mucositis in the experimental group (20%) incidence rate versus 55% in the control. Thus yielding a 35% ARR in the treatment group ($P = 0.02$) with an NNT of 2. Those results are in agreement with what was reported by others on the use of honey inside the mouth of cancer patients undergoing chemo/radiotherapy. A higher reduction of 80% in radiation-induced oral mucositis was noted when honey was applied inside the mouth of patient's treatment, directly after and few hours after treatment [17]. Similar results of 20% of participants in experimental group developed grade III or grade IV mucositis, in a study which evaluated the effects of application of honey in management of radiation-induced mucositis, as compared to 75% of participants in control group [18, 19]. In another study regarding the application of honey to prevent radio chemotherapy induced oral mucositis. It was reported that none of the patients in the experimental group developed grade IV mucositis. However, only three patients in the experimental group developed grade III mucositis. This is in contrast to 13 patients in control group who developed grade III or grade IV mucositis [20]. In a different study, one subject in the experimental group developed grade III oral mucositis in comparison to 8 subjects in control group who developed grade III oral mucositis. None in the experimental group developed grade IV oral mucositis [21].

In a single blinded experimental study aimed at evaluating the effect of honey on irradiation induced mucositis, it was noted that there was significant reduction in the degree of oral mucositis in experimental group as compared with control group [22]. In current study, there was a significant reduction in the number of episodes of oral mucositis between the experimental versus the control group. 20% of the patients in the experimental group had developed grade III and IV oral mucositis during the one year of the study in comparison to 55% in the control group.

Honey seemed to enhance the efficacy of therapy in the treatment of oral mucositis as compared to the use of either honey alone or steroids [11, 23–25]. In a randomized controlled study on the effects of honey on oral mucositis, it was noted that there was a statistically significant difference between the experimental and the control group in weeks 4, 5, and 6. For instance, only 7.14% of the participants in the experimental group developed mucositis in comparison to 64.28% in the control group who did not take honey [21].

Honey has long been known to have a soothing action on mucus membranes and recommended for the management of oral mucositis. Honey is the by-product of flower nectar. Because of its high viscosity, acidic PH, hydrogen peroxide, high osmolarity, and rich nutritional properties, honey can inhibit bacterial and fungal growth [7, 26, 27] and enhance healing and is thereby a justified approach in the management of oral mucositis [16].

Infection damaged mucosal tissues are more susceptible to developing a wide variety of bacterial (due to loss of normal tissue response), viral (herpes), and fungal infections. In addition to the impaired effect of the normal immune response caused by decreased saliva volume, alterations in saliva quality and decreased levels of immunity. Such changes result in an increase in the dominance of opportunistic pathogenic organisms at the cost of the normal oral microbiota which are the good bacteria that offer protection [28].

Table 2: Absolute Risk Reduction (ARR) and Number Needed to Treat (NNT) for developing grade III and IV oral mucositis, *Candida*, and aerobic bacterial infections with 95% CI between honey and control group.

	Honey ($n = 20$)	Control ($n = 20$)	ARR* (95% CI)	NNT** (95% CI)	P value
Grade III and IV mucositis	4 (20)	11 (55)	35 (9.6–61.7)	2 (2–10)	0.02
Candida	2 (10)	12 (60)	50 (20.7–69.5)	2 (1–5)	0.003
Aerobic plate count	2 (10)	12 (60)	50 (20.7–69.5)	2 (1–5)	0.003

*Absolute Risk Reduction; **Number Needed to Treat.

Table 3: Effect of honey on the duration of hospitalization per episode of oral mucositis and on the % body weight gain.

	Honey ($n = 20$) mean (SD)	Control ($n = 20$) mean (SD)	Mean difference	P value*
Hospitalization (days/episode)	7 (3)	13 (5)	−4.6	<0.001
Percentage increase in body weight (%)	35.1 (6.5)	15 (4.2)	19.9	<0.001

*t-test.

Septicemia may develop among those patients and may be life threatening infection [29]. Numerous studies have reported that Gram negative bacterial flora of the oropharynx dominates in patients during myelosuppression and in those who are receiving head and neck irradiation [30]. As a result, those colonizing Gram negative bacteria oral microflora may release endotoxins, known to be potent inflammation inducers, thus leading to a cascade of inflammation processes and further intensify the patients' local mucosal injury [15]. Accordingly, it has been hypothesized that oral mucositis may be reduced by using specific antimicrobial therapy against those dominating bacteria [3].

Fungal infections are also common among those patients; particularly Candidiasis, caused by *Candida albicans*, is a common fungal infection present among such patients and is known as oral thrush. Such a symptom is painful and associated with erythema or discrete white plaques and may be easily confused with mucositis. The taste buds in the mouth are affected by radiation and as such they may become impaired as a result and cause changes in taste sensations [28, 31]. Such taste changes may be related to saliva which may modulate some of the tastes (sour, bitter, salt, and sweet) through biochemical interactions [31]. Alteration in the taste will affect the appetite which will be compromised as a result, thus affecting the nutritional status and quality of life of the patient. Nausea and vomiting are a common occurrence among those patients receiving chemo/radiotherapy. Nausea, vomiting, and taste changes affect the ability to eat, tolerate certain foods, and eat less and do not harvest full energy from the entire food consumed and as such lead to a reduction in the patient's body weight.

Our data showed a significant weight gain in the honey treated group versus the control group. The results of a comparative study on the evaluation of honey versus sucralfate against oral mucositis indicated the mean weight loss was more in sucralfate group as compared to honey and it was concluded that honey was more effective in increasing the weight as compared to sucralfate group [32]. Also, the results of another randomized single blind study showed that there was more weight loss in those in the control as compared to the honey treated group [22, 32, 33]. It was reported that patients treated with topical honey showed that 71% of the treated group showed no weight loss as compared to 22% in the control group [19, 32, 34–37]. Oral mucositis is normally associated with pain which results from the loss of the epithelial lining, ulceration and the associated edema. Also pain results from the neurotransmitters related to the inflammatory response associated with oral mucositis [28]. The pain becomes more intense when the pharyngeal mucosa is affected and results in burning sensations experienced by the patient upon swallowing.

Pediatric cancer patients have poor nutritional status before starting chemo/radiotherapy treatment and it decreases with a number of mucositis related side effects such as dysphagia and the loss of taste and saliva. Also, feeling down affects the appetite [38]. Inadequate nutrition leads to weight loss and such patients may require other means of nutrition. Salivary secretions are also reduced [39–41]. Such a decrease will result in dryness of the mouth and causes oral discomfort, altered taste, nutritional impairment (difficulty in mastication and swallowing), and dental decay.

The data obtained in this study revealed that there was a significant reduction in the days of hospitalization during an oral mucositis episode in the experimental group in comparison to the control group throughout the one-year duration of the study. In a study which took place for 4 weeks and indicated that 21.42% of patients in control group were hospitalized due to severe mucositis. This is in contrast to none in the experimental group were hospitalized due to severe mucositis. Due to the development of severe oral mucositis in five patients in the control group were treated, while none in experimental group had treatment interruptions. The results of another study revealed that 16% of patients who received radiotherapy were hospitalized due to severe mucositis. In addition to having unplanned break in the treatment protocol was also reported in 11% of patients in the same study [42].

In spite of the fact that the underlying mechanism of action of honey is not well elucidated, it is likely that factors like osmolality, phenol content, flavanoid levels, acidity, and the release of hydrogen peroxide are thought to be the most important factors for its activity [43]. Honey is known for its

antioxidant and anti-inflammatory activities and the increase of nitric oxide (NO) in the lesions [25, 43, 44]. Being sweet, honey may per se stimulate the salivation reflex due to their hyperosmolarity. As such its efficacy may be related to its hyperosmolarity, anti-inflammatory, and antioxidant properties [34, 45–48]. As a consequence, to all those, honey may accelerate the repair and healing of mucosal damage and reduce associated irritations [11, 49, 50].

Limitations. A larger sample size is recommended for future studies for further validation of the results.

5. Conclusions/Recommendations

This study showed that the topical honey treatment is effective in reducing and minimizing oral mucositis among pediatric cancer patients treated with chemo/radiotherapy and is cost-effective treatment. It also showed a reduction in hospitalization duration, reducing painful mucositis, and increasing body weight. Honey is a natural product, is cheap, has less side effects, is tolerated well by most of the patients, and has a delicious taste. We recommend using topical honey as a part of the standard supportive care for chemo/radiotherapy-induced oral mucositis in pediatric cancer patients. The results warrant further investigation.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 8025752, 10 pages
https://doi.org/10.1155/2017/8025752

Review Article

External Use of Propolis for Oral, Skin, and Genital Diseases: A Systematic Review and Meta-Analysis

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Received 21 September 2016; Accepted 8 November 2016; Published 6 February 2017

Academic Editor: Andrzej K. Kuropatnicki

Objective. The aim of this review is to provide the available evidence on the external use of propolis (EUP) for oral, skin, and genital diseases. *Method.* We searched twelve electronic databases for relevant studies up to June 2016. Randomized clinical trials (RCTs) were included and analysed. *Results.* Of the 286 articles identified, twelve potentially relevant studies met our inclusion criteria. A meta-analysis of two studies on recurrent oral aphthae (ROA) indicated that there were no significant differences in total effective rate (TER) for pain disappearance between EUP and placebo groups (RR = 1.96, 95% CI = 0.97–3.98, and $P = 0.06$). In two studies on skin diseases, the combined treatment of EUP with other interventions revealed significant effects on the duration of treatment or TER. In one study on genital diseases, EUP showed significant differences in genital herpes outcome measures compared to placebo. *Conclusions.* Our results on the effectiveness of EUP for treating oral, skin, and genital diseases are not conclusive because of the low methodological qualities and small sample sizes. Further well-designed randomized controlled trials, with high quality and large samples for specific disorders, must be conducted to obtain firm conclusions.

1. Introduction

Propolis, also known as "bee glue," is a wax-like substance that is collected from local flora by honeybees to protect and repair their hives [1, 2]. Humans have been using propolis since ancient times, from at least 300 BC, and there are records suggesting that propolis has been used as a medicine in many parts of the world, both internally and externally [3, 4]. In general, propolis contains phenol acids, flavonoid, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, β-steroids, and various other substances [5, 6].

Many researchers have studied propolis in recent decades. The major components extracted from propolis have shown antimicrobial activity [7], and the treatment of cells with ethanolic extract of propolis (EEP) has exhibited anti-inflammatory activity [8]. EEP has also been reported to exhibit antitumour effects in cancer cells [9, 10].

The external use of propolis is defined by the application of pharmaceutical or natural products on the surface or point of illness [11]. External uses of propolis (EUP) include the use of pharmaceutical, cosmetic, and oral products such as ointment [12], gel [13], lipstick [14], and mouthwash [15].

A recently published systematic review on propolis for oral health reported that it can reduce oral infection and dental plaque and treat stomatitis [16]. However, no published studies to date have evaluated the effectiveness of propolis for external use. In addition, numerous published randomized controlled trials (RCTs) on Complementary and Alternative Medicine (CAM) in Korea could be omitted if the database searches are restricted to English- and Chinese-language databases [17]. Korean CAM RCTs are typically missed in systematic reviews, which can increase the risk of language bias [17].

Therefore, we conducted a systematic review and meta-analysis following the PRISMA recommendations [18]. The aim of this systematic review is to explore the evidence on the effectiveness of the external use of propolis for oral, skin, and genital diseases.

2. Methods

2.1. Data Sources and Searches. We searched the following electronic databases up to June 2016 without a language restriction: MEDLINE (OvidSP), EMBASE (OvidSP), the Cochrane Central Register of Controlled Trials (CENTRAL), and CINAHL Plus (EBSCOhost). We also searched six Korean medical databases (Korea Institute of Science and Technology Information, Korean traditional knowledge portal, KoreaMed, OASIS, RISS, and the National Library of Korea) and two Chinese databases (CNKI and Wanfang). Furthermore, we conducted nonelectronic searches of conference proceedings, our own article files and nine traditional Korean medical journals (Journal of Korean Medicine, the Journal of Korean Acupuncture and Moxibustion Society, Korean Journal of Acupuncture, Journal of Acupuncture and Meridian Studies, Journal of Pharmacopuncture, Journal of Oriental Rehabilitation Medicine, the Journal of Korean Chuna Manual Medicine for Spine and Nerves, Korean Journal of Oriental Physiology and Pathology, and the Journal of Korean Oriental Internal Medicine).

The following search terms were used in each database's language: "propolis" AND "external use OR external application OR external treatment OR topical application OR ointment OR gel OR dressing OR oral OR skin OR genital" AND "randomized controlled trial OR randomized clinical trial".

2.2. Study Selection

2.2.1. Inclusion Criteria. We defined EUP interventions as any type of intervention in which propolis ingredients were applied to illness points as a treatment. All RCTs evaluating EUP for various diseases were included. Patients diagnosed with any disease were also included. We classified each disease according to the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (ICD-10) [31].

Studies on the combined effects of EUP and other interventions (e.g., EUP plus rinsing therapy) were considered for inclusion when the same intervention was applied to both the EUP group and the control group.

Clinical trials comparing EUP with placebo or other active controls were included. Other active control interventions included rinsing therapy, miconazole, oral antiseptics, silver sulfadiazine, honey, Vaseline, pine pollen packs, and metronidazole gel.

2.2.2. Exclusion Criteria. Non-RCTs, animal or cell studies, and quasi-RCTs were excluded. Trials including healthy participants were also excluded. We did not include studies on the internal use of propolis (e.g., propolis capsules, tablets, or suspensions) or mouthwash interventions (e.g., mouth rinsing, teeth brushing). Unqualified control interventions (e.g., herbal medicine, acupuncture, and bee venom therapy) were excluded because their efficacy was unable to be investigated.

2.3. Data Extraction. Three authors (S. H. Sung, G. H. Choi, and N. W. Lee) independently selected the included studies and extracted data using a predefined data extraction form. N. W. Lee, who is a Traditional Chinese Medicine (TCM) practitioner, searched the Chinese databases and screened the Chinese-language trials. For studies with insufficient information, we contacted the corresponding authors to request additional data. Disagreements were resolved by discussion between two authors (G. H. Choi and B. C. Shin) to reach consensus.

2.4. Assessment of Risk of Bias (ROB). We used the Cochrane risk of bias tool [32]. This tool includes 7 domains, but we assessed random sequence generation, allocation concealment, blinding of participants or personnel, blinding of assessors, incomplete outcome data, and selective outcome reporting. The risk of bias in each study was assessed by two independent authors (S. H. Sung and G. H. Choi) using the Cochrane risk of bias tool; disagreements were resolved by discussion.

2.5. Data Analyses. For meta-analyses, we extracted dichotomous data using risk ratios (RR) for the total effective rate (TER) for pain disappearance. We applied a random-effects model using Review Manager (Revman) software (version 5.3 for windows; the Nordic Cochrane Centre, Copenhagen, Denmark). I^2 tests were used to analyse the heterogeneity between the included studies. I^2 values above 50% were considered to indicate possible heterogeneity [32]. As statistical pooling was not feasible due to the variability of diseases, types of EUP form, control interventions, and outcome measures, a summary of the findings is presented in the results.

3. Results

3.1. Study Selection and Description. Of the 286 potentially relevant records, 221 studies were screened after duplicate trials were removed. Of these 221 studies, 139 were excluded because they were nonclinical trials (reviews, qualitative studies, and animal or in vitro studies) or were not related to propolis. Of the remaining 82 trials, 12 RCTs (English: $n = 8$; Chinese: $n = 2$; Korean: $n = 1$; Persian: $n = 1$) met our inclusion criteria (Figure 1).

Twelve studies were conducted in various countries, including two trials in Brazil and China and one trial in the Democratic Republic of Congo, Iran, Korea, Italy, Macedonia, Poland, Sudan and Ukraine each. We grouped the 12 trials into those addressing three diseases: five trials applied EUP for oral diseases (Table 1), five for skin diseases (Table 2), and two for genital diseases (Table 3).

3.2. Participants

3.2.1. Number of Participants. The 12 studies included 862 participants. The sample size per group ranged from 10 to 52 participants. One study reported on 23 patients with two burn areas, one of which received EUP and the other a control intervention [24].

3.2.2. Types of Disease. We classified the 12 RCTs into those addressing oral, skin, and genital diseases because the types

Table 1: Characteristics of the included RCTs for oral diseases.

First author, year	Location of propolis production, chemical composition of propolis	Used form of propolis, amount used	Patient's disease, sample size (randomized/analysed)	Experimental group (intervention, regimen)	Control group (intervention, regimen)	Outcome measures	Main results	AE
Ali, 2011 [19]	UAE, n.r.	Paste, n.r.	Recurrent oral aphthae, 120/114	(A) PP (containing olive oil), $n = 39$, 2 times per day until healing	(B) PP (containing sesame oil), $n = 35$, 2 times per day until healing (C) Placebo paste, $n = 40$, 2 times per day until healing	(1) TER for pain disappearance (2) Duration of treatment for ulcer (3) Lesion size (4) Duration of drug adherence to mucous membrane	(1) (A)[a], (B)[a] significantly better than (C) (2) (A)[b], (B)[b] significantly better than (C) (3) (A) better than (B), (C) but NS (4) Most patients were approximately 20–30 minutes	n.r.
Atanasovska, 2014 [20]	Macedonia, I 62.5% + B 55% + TP 24.2% + TFF 8% + TFD 49%	Spray, n.r.	Recurrent oral aphthae, 20/20	(A) PS (Proaftol), $n = 10$, 24–32 sessions (3-4 times per day for 8 days)	(B) Placebo spray, $n = 10$, 24–32 sessions (3-4 times per day for 8 days)	(1) Lesion size (2) Severity of pain	(1) On day 3, Positive[c]; on day 5, Positive[c]; on day 8, Positive[b] (2) On day 3, Positive[c]; on day 5,Positive[b]; on day 8, Positive[b]	n.r.
Capistrano, 2013 [21]	n.r., n.r.	Gel, 1 session 5 mL	Candidal stomatitis, 45/45	(A) PG, $n = 15$, 64 sessions (4 times per day for 14 days)	(B) Mouthrinse (containing propolis), $n = 15$, 64 sessions (4 times per day for 14 days) (C) Miconazole, $n = 15$, 64 sessions (4 times per day for 14 days)	(1) CFU (2) Newton's classification	(1) Significant difference in (A)[c], (B)[c], (C)[c] but NS in each group (2) Significant difference in (A)[b], (B)[c], (C)[b] but NS in each group	n.r.
Chen, 2009 [22]	n.r., n.r.	Extract, n.r.	Recurrent oral aphthae, 76/76	(A) PE, $n = 38$, 14 sessions (2 times per day for 7 days)	(B) Placebo (oral antiseptics), $n = 38$, 14 sessions (2 times per day for 7 days)	(1) TER for ROA (2) TER for pain disappearance	(1) Positive[b] (2) Positive[b]	n.r.
Piredda, 2015 [23]	n.r., n.r.	Extract, 1 session 8–10 mg	Oral mucositis, 60/60	(A) PE + mouthrinse, $n = 30$, 15 sessions (1 time per day for 15 days)	(B) Mouthrinse, $n = 30$, 15 sessions (1 time per day for 15 days)	(1) NCI-CTCAE version 4.0	(1) Positive[a]	Manifested suspected skin reaction (2 in group (A))

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

AE: adverse events; B: balm (extract with 70% ethanol); CFU: colony forming units; I: inhibitor against *Staphylococcus aureus*; NCI-CTCAE: National Cancer Institute-Common Terminology Criteria for Adverse Events; n.r.: not reported; NS: no significant difference between groups; PE: propolis extract; PG: propolis gel; positive: (A) significantly better than (B); PP: propolis paste; PS: propolis spray; ROA: recurrent oral aphthae; TER: total effective rate; TFD: total flavonones and dihydroflavonols; TFF: total flavones and flavonol; TP: total phenols.

Table 2: Characteristics of the included RCTs for skin diseases.

First author, year	Location of propolis production, chemical composition of propolis	Used form of propolis, amount used	Patient's disease, sample size (randomized/analysed)	Experimental group (intervention, regimen)	Control group (intervention, regimen)	Outcome measures	Main results	AE
Gregroy, 2002 [24]	n.r., n.r.	Cream, n.r.	Second-degree burns, 33/23[d]	(A) PC, $n = 23$[d], 1 session	(B) SSD, $n = 23$[d], 1 session	(1) CFU (2) Duration of treatment for burns	(1) NS (2) (A) better than (B) but NS	n.r.
Kucharzewski, 2013 [25]	Poland, n.r.	Ointment, n.r.	Varicose veins of lower extremities with ulcer, 56/56	(A) PO + rinsing the ulcer with PSCS + compression treatment, $n = 28$, PO: 7 to 42 sessions (1 time per day until healing); the others: 7 to 42 sessions (1 time per week until healing)	(B) Rinsing the ulcer with PSCS + compression treatment, $n = 28$, 28 to 102 sessions (1 time per week until healing)	(1) Duration of treatment for ulcer	(1) Positive[b]	n.r.
Ngatu, 2011 [26]	Japan, n.r.	Extract, n.r.	Tinea capitis and tinea versicolour, 242/188	(A) PE (50 mg/mL), $n = 26$, 28 sessions (1 time per day for 28 days)	(B) PE (100 mg/mL), $n = 29$, 28 sessions (1 time per day for 28 days) (C) Acacia honey, $n = 31$, 28 sessions (1 time per day for 28 days) (D) Miconazole, $n = 50$, 28 sessions (1 time per day for 28 days) (E) Vaseline, $n = 52$, 28 sessions (1 time per day for 28 days)	(1) Pruritus (2) Papule, pustule (3) Erythema (4) Desquamation (5) WBC (6) Leukocytes	(1) (A)[b], (B)[b], (C)[a], (D)[b] significantly better than (E) (2) Significant difference in (A)[b], (B)[b], (C)[b], (D)[c] (3) (A)[c], (B)[c], (C)[c], (D)[c] significantly better than (E) (4) (A)[c], (B)[c], (C)[c], (D)[c] significantly better than (E) (5) (A)[b], (B)[a], (D)[a], significantly better than (E), but NS between (C) and (E) (6) NS	Itch (1 in group (D))
Park, 2013 [27]	Korea, n.r.	Mask pack, 2 g	Acne, 30/30	(A) PMP, $n = 15$, 10 sessions (1 time per week for 4 weeks)	(B) PPMP, $n = 15$, 10 sessions (1 time per week for 4 weeks)	(1) Skin conditions ① Moisture ② Sebum ③ Elasticity (2) Number of acne lesions ① Stuff ② Papule ③ Pustule	(1) ①Negative[b] ②, ③Negative[a] (2) ①, ② NS ③Negative[a]	n.r.
Yin, 2013 [28]	China, n.r.	Ointment, n.r.	Other specified diabetes mellitus with foot ulcer, 60/60	(A) PO + VT, $n = 30$, PO: 4 times per day for 17 months, VT: 24 months	(B) VT, $n = 30$, 24 months	(1) TER for ulcer	(1) Positive[a]	n.r.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$; [d]same patients with two burn areas received PC and SSD.

AE: adverse events; CFU: colony forming units; negative: (B) significantly better than (A); n.r.: not reported; NS: no significant difference between groups; PC: propolis cream; PE: propolis extract; PMP: propolis mask pack; PO: propolis ointment; positive: (A) significantly better than (B); PPMP: pine pollen mask pack; PSCS: physiological sodium chloride solution; SSD: silver sulfadiazine; TER: total effective rate; VT: vasodilator therapy; WBC: white blood cell.

Table 3: Characteristics of the included RCTs for genital diseases.

First author, year	Location of propolis production, chemical composition of propolis	Used form of propolis, amount used	Patient's disease, sample size (randomized/analysed)	Experimental group (intervention, regimen)	Control group (intervention, regimen)	Outcome measures	Main results	AE
Mousavi, 2016 [29]	Iran, n.r.	Cream, 1 session 3 g	Acute vaginitis, 100/100	(A) PVC, $n = 50$, 7 sessions (1 time per day for 7 days)	(B) MVG, $n = 50$, 7 sessions (1 time per day for 7 days)	(1) Amsel's criteria (2) Gram stain	(1) Positive[a] (2) Positive[a]	n.r.
Vynograd, 2000 [30]	Canada, n.r.	Ointment, n.r.	Herpes viral infection of genitalia and urogenital tract, 90/90	(A) PO, $n = 30$, 40 sessions (4 times per day for 10 days)	(B) AO, $n = 30$, 40 sessions (4 times per day for 10 days) (C) Placebo ointment, $n = 30$, 40 sessions (4 times per day for 10 days)	(1) Number of patients who were healed (2) Lesions ① Crusted lesions ② Ulcer lesions ③ Vesicular lesions (3) Infection of herpetic-bacterial (only for 28 infected women)	(1) Positive[b] (2) ① On day 3, Positive[c] ② (A) better than (B) but NS; (A)[a] significantly better than (C) ③ Positive[a] (3) Positive[b]	None

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

AE: adverse events; AO: acyclovir ointment; MVG: metronidazole vaginal gel; n.r.: not reported; PO: propolis ointment; positive: (A) significantly better than (B); PVC: propolis vaginal cream.

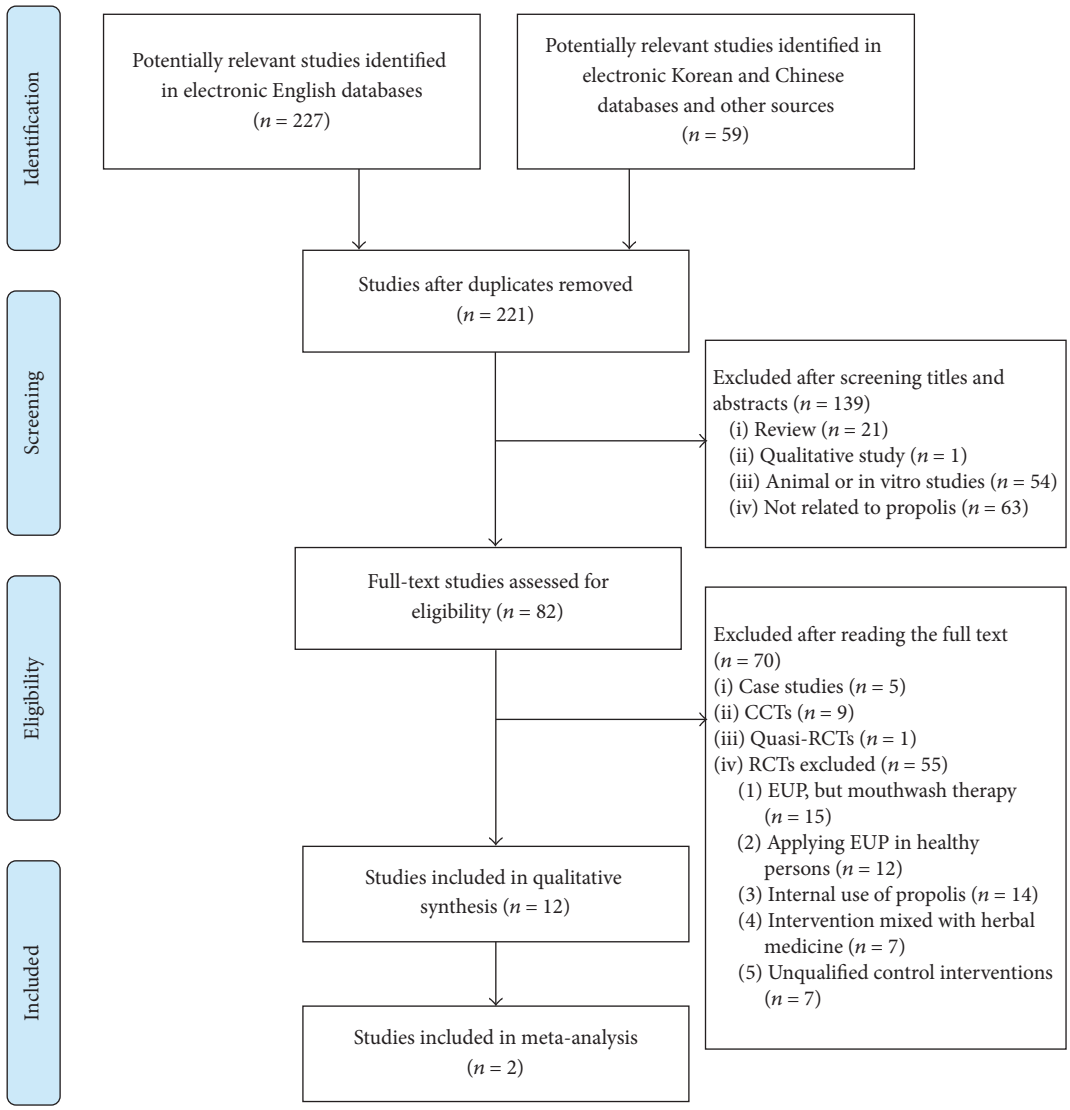

Figure 1: Flowchart of the RCT selection process. CCTs: controlled clinical trials; RCTs: randomized controlled trials; EUP: external use of propolis.

of disease were heterogeneous. The five studies included in the oral diseases group consisted of three studies on recurrent oral aphthae (ROA) [20, 21, 23], one study on candidal stomatitis [21], and one on mucositis [23]. Five trials addressed skin diseases; second-degree burns [24], leg ulcers [25], tinea capitis and tinea versicolour [26], acne [27], and diabetes mellitus with foot ulcer [28] were each assessed by one study. Two studies were included in the genital diseases group; one was on acute vaginitis [29], and the other was on genital herpes [30].

3.3. Interventions. Four studies compared EUP with a placebo intervention that was the same form of EUP [19, 20, 30], and two studies compared EUP with EUP that had a different ingredient [19] and concentration [26]. A combination of EUP and other interventions was compared with a control of the same additional interventions in three trials [23, 25, 28]. Other studies compared EUP with mouthrinse [21], miconazole [21, 26], silver sulfadiazine [24], acacia honey [26], Vaseline [26], pine pollen mask packs [27], and metronidazole vaginal gel [28].

3.3.1. Locations of Propolis Collected. A total of eight studies described the countries where propolis had been collected, including the UAE [19], Macedonia [20], Poland [25], Japan [26], Korea [27], China [28], Iran [29], and Canada [30].

3.3.2. Chemical Composition of Propolis. The chemical composition of propolis was reported in only one study [20]. In this trial, the component of propolis was inhibitor against *Staphylococcus aureus*, minimum 62.5%, balm 55.0%, total phenols 24.2%, total flavones and flavonol 8.0%, and total flavonones and dihydroflavonols 49%.

Table 4: Risk of bias assessment.

First author, year	Selection bias		Performance bias	Detection bias	Attrition bias	Reporting bias
	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Ali, 2011 [19]	U	U	H	U	U	U
Atanasovska, 2014 [20]	U	U	L	U	L	U
Capistrano, 2013 [21]	U	U	H	L	L	L
Chen, 2009 [22]	U	U	L	U	L	U
Piredda, 2015 [23]	U	L	H	U	L	L
Gregroy, 2002 [24]	U	U	H	U	U	U
Kucharzewski, 2013 [25]	H	U	H	U	L	U
Ngatu, 2011 [26]	U	U	H	U	U	L
Park, 2013 [27]	U	U	L	U	L	U
Yin, 2013 [28]	U	U	H	U	L	U
Mousavi, 2016 [29]	U	U	H	U	L	L
Vynograd, 2000 [30]	U	U	H	U	L	U

H: high risk; L: low risk; U: unclear risk.

3.3.3. Types of EUP Form. Seven forms of EUP were used in 12 RCTs; extract and ointment were utilized in three trials each, and other types of EUP included paste [19], spray [20], gel [21], cream [24, 29], and mask pack [27].

3.3.4. Amount of EUP Used. The amount of EUP used was reported in only four studies: the amount of EUP for 1 session ranged from 8 mg to 3 g in three trials [23, 27, 29], and one trial used 5 mL of EUP for 1 session [21].

3.4. Outcome Measures. Twelve studies reported on very diverse outcome measures due to the various types of diseases. The duration of treatment for each disease was investigated in three studies [19, 24, 25]. Two trials utilized Colony Forming Units (CFU), lesion size, and TER. Outcome measures related to pain were applied in three studies on oral diseases [19, 20, 22]. Studies on skin diseases used the measure of skin reactions such as stuff, papules, pustules, and pruritus [26, 27].

3.5. ROB Assessment. The included RCTs had a generally low methodological quality (Table 4). Although the 12 RCTs reported randomization, one study reported an inadequate method of random sequence generation (generated by even and odd numbers) [25], and the other 11 studies did not describe the method of randomization. Allocation was adequately concealed in only one study, in which it was managed by an external centre [23]. The participants and personnel were blinded in three trials (same form of intervention was used in the EUP and control groups) [20, 22, 27]. One study compared EUP with a placebo that was the same form of EUP, but it described a single-blinded method [19]. Blinding of the participants and outcome assessor was employed in one trial [21]. Nine studies properly addressed incomplete outcome data (dropout did not occur) [20–23, 25, 27–30]. The other three studies did not report the reasons for dropout [19, 24, 26]. Finally, for reporting bias, four trials reported their protocol before conducting the RCTs [21, 23, 26, 29].

3.6. Clinical Efficacy of EUP. A meta-analysis was not possible because of the heterogeneity in the diseases or outcome measures, with the exception of two trials on ROA [19, 22] that used a placebo for comparison. We summarized the results of the other studies because statistical pooling was not performed [20, 21, 23–30].

3.6.1. Clinical Efficacy of EUP for Oral Diseases. Of the five studies on oral diseases, a meta-analysis of two studies [19, 22] on ROA reported that there were no significant differences in TER for pain disappearance between EUP and placebo groups (RR = 1.96, 95% CI = 0.97–3.98, and $P = 0.06$) (Figure 2). For ROA, the effect of propolis spray was significantly better than placebo in measures of lesion size ($P < 0.01$ on the 8th day) and severity of pain ($P < 0.01$ on the 8th day) [20]. One trial [23] that compared propolis extract plus mouthrinse with mouthrinse alone showed a significant difference between groups using the National Cancer Institute-Common Terminology Criteria for Adverse Events (NCI-CTCAE) ($P < 0.05$). No significant difference between groups was identified in one trial [21].

3.6.2. Clinical Efficacy of EUP for Skin Diseases. Of the five studies on skin disease, one on burns [24] showed no significant difference between groups in CFU or duration of treatment. For leg ulcers, the combination of propolis ointment with cointerventions was significantly more effective in reducing the duration of treatment ($P < 0.01$) than cointerventions alone in the control group [25]. For tinea capitis

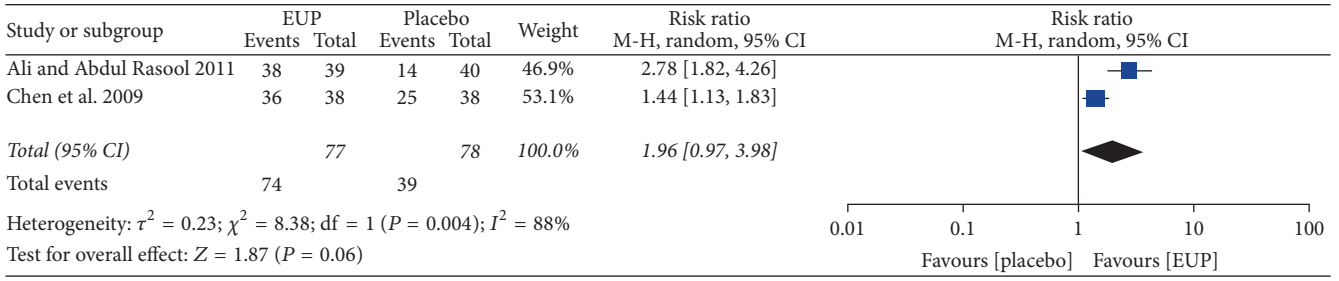

Figure 2: Meta-analysis of the total effective rate (TER) of EUP versus placebo. EUP: external use of propolis; CI: confidence intervals.

and tinea versicolour, propolis extract (50 mg/mL) showed a significant effect on pruritus, erythema, desquamation, and white blood cell count compared with Vaseline [26]. For acne, a pine pollen mask pack improved skin conditions and pustules better than a propolis mask pack [27]. One study [28] reported that propolis plus vasodilator therapy significantly improved the TER ($P < 0.05$) when compared with vasodilator therapy.

3.6.3. Clinical Efficacy of EUP for Genital Diseases. Of the two studies on genital diseases, one study comparing propolis vaginal cream with metronidazole vaginal gel reported a significant improvement in Amsel's criteria and gram stain [29]. Another study [30] compared propolis ointment with acyclovir ointment and placebo and showed a significant effect of propolis on the number of healing patients ($P < 0.01$), crusted lesions ($P < 0.001$ on day 3), ulcer lesions ($P < 0.05$), vesicular lesions ($P < 0.05$), and herpetic-bacterial infections ($P < 0.01$); propolis ointment was significantly more effective at reducing ulcer lesions ($P < 0.05$) than placebo, but no significant difference was found between propolis and acyclovir in reducing ulcer lesions.

3.7. Adverse Events. Three studies described adverse events. One trial reported a skin reaction in 2 cases in the EUP group [23]. One patient suffered from itch in the control group [26]. No adverse events occurred in one study [30].

4. Discussion

The objective of this systematic review and meta-analysis was to provide evidence on EUP for any disease. A total of 12 studies were included in our review. Three studies on ROA [19, 20, 22] showed a significant effect of propolis treatment compared to the placebo groups. Two studies on skin diseases [25, 28] reported a significant effect of combined treatment of EUP with other interventions (e.g., rinsing therapy, compression treatment, or vasodilator therapy) compared to the other interventions alone. The results of one study on genital herpes [30] indicated a significant effect of 10 days of propolis ointment compared with a placebo ointment. We found in this systematic review that EUP has a more beneficial effect on ROA, skin diseases, and genital herpes than controls. However, because most of the RCTs had a small sample size, low methodological quality and groups receiving different forms of propolis, these analyses were not conclusive regarding the effectiveness of EUP for the studied diseases. Piredda et al. [23] reported that adverse events did not occur, and another trial [30] did not mention severe side effects with EUP. However, the evidence was insufficient to draw conclusions about the safety of EUP because only three studies [23, 26, 30] described adverse effects.

Most of the included trials had a low methodological quality based on Cochrane's risk of bias tool. Although the 12 RCTs stated that the participants were randomly assigned, an adequate method of randomization was not described, and only one [23] study had a low risk of bias for allocation concealment. Random sequence generation and allocation concealment are necessary to prevent selection bias. One out of 12 studies reported proper blinding in the outcomes assessment [21]. Low-quality blinding of outcome assessors is more likely to be influenced by placebo effects [33]. Therefore, the included trials have the potential for overestimation.

The propolis used in the 12 included RCTs was collected from diverse countries. Eight out of 12 studies [19, 20, 25–30] reported the locations of where the propolis was collected. Of the included twelve RCTs, only one study [20] mentioned chemical composition of propolis. Huang et al. [34] reported that propolis collected from many countries has similar chemical components but that there is a difference in concentration. Additionally, propolis collected from various regions in the same country has been identified as having a few distinct components [2]. The chemical composition of propolis collected from plants of certain countries can cause adverse effects [35]. Therefore, the efficacy and safety of propolis, considering geographical location, should be investigated in future studies.

Four studies used placebo interventions that used the same form of propolis for the control group [19, 20, 22, 30]; consideration of the smell of the placebo intervention was not mentioned. The use of an indistinguishable placebo compared to the experimental treatment is crucial for appropriate blinding of participants. Because propolis has a specific aromatic smell [36], patients who are familiar with propolis products may have been able to recognize whether the product was propolis. Thus, future studies should assess a proper placebo, considering the scent of propolis.

In this review, trials using propolis as a mouthwash were excluded because we focused on assessing the evidence of the

efficacy of EUP when its ingredients were applied at the point of illness. There was some evidence in a recent dentistry study that propolis mouthwash protects against oral disease due to its antimicrobial properties [37]. One trial [21] compared propolis gel with propolis mouthwash and showed significant effects before and after treatment but no significant difference between groups. It is necessary to conduct comparative studies to identify the different efficacies of EUP between washing and applying forms of propolis.

Although our review indicated the applicability of propolis for external use, few studies on the standardization of EUP have been examined. Therefore, the following factors should be standardized to reduce the heterogeneity of future trials on EUP: (1) type of EUP form based on chemical composition of propolis; (2) effects and safety of EUP considering geographical locations; (3) amount used and number of treatment sessions; and (4) placebo model for EUP.

The strength of our review is that there was no restriction of language or publication status; hence, English, Chinese, Korean, and Persian papers were included in the review. However, there are some limitations to this systematic review. First, although 12 studies on EUP were included in this review, the heterogeneity in the diseases, the types of EUP form, control groups, and outcome measures was high; thus, a statistic pooling of 10 studies could not be assessed. In addition, it is difficult to propose any definitive conclusions regarding the safety of EUP because of the insufficient information on adverse effects. Therefore, researchers should conduct RCTs on EUP considering these limitations. Moreover, the side effects and amount used must be described in future studies to establish clinical practice guidelines for EUP.

As previous publications only investigated propolis for oral diseases [16], our review showed that propolis may be used externally for various diseases. The results could help determine the types of disease and forms of propolis for future research on EUP.

5. Conclusion

Our systematic review and meta-analysis suggested that the effectiveness of EUP for the treatment of oral, skin, and genital diseases was inconclusive because of the low methodological qualities and the small sample sizes. Further RCTs, with a high quality and large samples for specific disorders, must be conducted to provide additional clinical evidence on EUP treatment. Furthermore, the standardization of EUP to ensure clinical efficacy and safety is needed.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Soo-Hyun Sung, Byung-Cheul Shin, Nam-Woo Lee, and Gwang-Ho Choi were responsible for the study concept and design. Soo-Hyun Sung, Gwang-Ho Choi, and Nam-Woo Lee searched and selected studies for inclusion. Nam-Woo Lee, trained in Traditional Chinese Medicine, searched and selected studies of Chinese literature. Soo-Hyun Sung and Gwang-Ho Choi evaluated the risk of bias of the included studies. Soo-Hyun Sung and Byung-Cheul Shin performed the data analysis and interpretation. Soo-Hyun Sung, Gwang-Ho Choi, and Lam-Woo Lee wrote the paper. Byung-Cheul Shin revised the manuscript. All authors were involved in the whole process of the study.

Acknowledgments

This work was supported by a grant to Korean Medical Science Research Centre for Healthy Aging from the National Research Foundation of Korean Government (2014R1A5A2009936).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 4034179, 12 pages
https://doi.org/10.1155/2017/4034179

Research Article

Oral Health of Patients Treated with Acrylic Partial Dentures Using a Toothpaste Containing Bee Product

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Received 21 October 2016; Accepted 23 November 2016; Published 6 February 2017

Academic Editor: Vassya Bankova

This study was carried out to investigate the influence of a propolis and tee tree oil-containing hygienic agent on selected oral health parameters, oral microflora, and the condition of periodontal health. Thirty-seven patients who underwent oral rehabilitation with a removable acrylic denture were selected and randomly assigned into two groups: study group (A) which received a newly formulated propolis and tee tree oil-containing toothpaste or a control group (C) without an active ingredient. API, S-OHI, and mSBI were assessed in three subsequent stages. During each examination swabs were employed for microbiological inoculation: in the study group after 4 weeks use of the active toothpaste showed a decrease in the number of isolated microorganisms. In the control group, after 4 weeks use of the toothpaste without active ingredients resulted in increase in the number of the isolated microorganisms. Improvements in hygiene and the condition of periodontium were observed in patients using active toothpastes. In the study group the oral flora diversity was reduced by the decrease in the number of cultured microorganism species, while in the control group an increase in the number of cultured microorganisms and their species was observed.

1. Introduction

Oral health and condition reflect body's general health. Condition of the oral cavity, level of hygiene, and residing pathogens affect the whole human organism. There are numerous reports on oral bacteria being the cause of bacterial endocarditis, pneumonia, gastric infections, chronic obstructive pneumonia, and other diseases [1]. The risk of contracting the above-mentioned diseases increases with age. Moreover, the amount of patients who use dentures, which are yet

another place of accumulation of microorganisms associated with these infections, rises with age as well. Absences of teeth are closely related to both oral and general health [2]. Loss of a single tooth may cause rotation, shifting, and tipping of neighbouring teeth towards the empty space, which will in turn lead to adverse loads on these teeth, not along the long axis of the tooth [3–5]. Partial lack of teeth, especially in lateral teeth, may cause the loss of mandibular occlusal support, which leads to changes in its position in relation to the maxilla [6]. Such a shift in mandibular position may result in muscle fatigue and other perpetuated occlusal relations in temporomandibular joints [7, 8].

Prosthetic treatment is not only designed to restore partial lacks of teeth. It also serves a prophylactic purpose by preventing the formation of pathological changes in the stomatognathic system and removing already existing changes [9]. It is common to employ acrylic partial dentures. Such dentures shift the loads through mucous membrane and periosteum to the bone, restoring masticatory function to a limited extent, do not protect the residual dentition, cause periodontitis and mucositis, create favourable conditions for the accumulation of bacterial plaque, and accelerate the loss of alveolar process. Due to the adverse impact of partial dentures on the denture medium and residual dentition it is necessary to maintain proper oral hygiene, remove plaque carefully, and take preventive measures against caries and periodontopathy [10, 11]. Regardless of the type of prosthetic restoration chosen, the maintenance of proper denture and oral hygiene is indispensable to achieve a successful treatment outcome [12].

The oral cavity constitutes a natural environment for microflora, whose composition is an individual feature. It is influenced by, inter alia, age, topography of the oral cavity, condition of teeth, nutritional and breathing habits, tobacco smoking, oral hygiene, and loss of teeth or usage of prosthetic restorations [13]. The extent of such changes depends on the type of dentures: fixed dentures cause local changes in the microflora, while removable dentures cause more complex changes. It is associated with the formation of new reservoirs of microorganisms such as the intramucosal surface of the denture base or clasps on retaining teeth. Acrylic base of partial dentures covers a substantial surface of the mucosa, creating favourable conditions for the buildup of bacteria and fungi in the form of denture plaque. It is a place characterised by high humidity, elevated temperature, reduced oxygen supply, and impaired conditions for salivary self-cleaning. Moreover, the acrylic material, due to its heterogeneous and porous structure, absorbs water and swells in the oral cavity, further facilitating the aggregation of microorganisms [14–16]. Perfect oral hygiene is also the basic method for prevention of caries and diseases of the periodontium and oral mucosa. Caries and periodontal diseases are the most common cause of teeth loss. Therefore, it is crucial to protect residual dentition in patients with partial dentures from these diseases. The basic hygienic treatment is mechanical removal of plaque, supported by, for example, antimicrobial chemical agents [17, 18]. The positive effect of ethanolic extract of propolis and tea tree oil on the maintenance of proper oral hygiene has been known for a long time. They are naturally occurring compounds with a broad-spectrum antimicrobial, antiviral, antifungal, and antiprotozoal activity. Additionally, they exhibit antiphlogistic and antioxidant properties [19, 20].

Propolis, so-called bee glue, is made by bees mostly from resinous substances gathered from buds of some trees. Its properties depend on the species of bees which produce it as well as on the plants used for the production [21]. Propolis is a very complex substance with high contents of active ingredients like flavonoids, phenols, aromatic acids, esters, wax substances, aldehydes, coumarin, sterols, enzymes, or fatty acids, as well as proteins, microelements, and vitamins [22, 23]. Propolis is well-soluble in ethyl alcohol; thus medical extracts can easily be prepared [23]. In dentistry propolis is used in the treatment of caries [24] and periodontal diseases [20, 25]. It also exhibits analgesic activity and accelerates wound healing, which is used in dental surgery [26]. There are numerous scientific reports on its anticancer, antiphlogistic, and immunomodulating properties [19, 27]. Tea tree oil is obtained from leaves of a tree growing in Australia and New Zealand, *Melaleuca alternifolia*, using cold water extraction [28].

Tea tree oil is a mixture of terpene compounds, such as terpinen-4-ol, γ-terpinene, p-cymene, α-terpinene, and 1,8-cineole, which are largely responsible for tea tree oil's properties [29, 30]. TTO exhibits strong antiseptic activity, selective for pathogenic microorganisms, and is highly effective against oral cavity pathogens [31]. It has also been demonstrated that TTO has inhibitory properties against *Candida albicans* [32].

The study aims to assess the influence of toothpastes containing ethanolic extract of propolis and tea tree oil on oral hygiene in patients with partial dentures. The number of scientific reports on synergistic activity of these substances in the improvement of oral hygiene is insufficient [33, 34]. The aim of this study was to evaluate the influence of toothpaste with active substances of plant origin, such as ethanolic extract of propolis and tea tree oil, on microflora of the oral cavity and to evaluate the following indices: OHI-S, denture plaque index, mSBI, and API in patients with partial dentures.

2. Materials and Methods

This study was conducted between September 1, 2015, and March 1, 2016, in the Department of Prosthetic Dentistry, Akademickie Centrum Stomatologii i Medycyny Specjalistycznej (Bytom, Poland), and in Specialist Dental Clinic (Katowice, Poland), which provide comprehensive dental care for patients with removable dentures.

2.1. Toothpaste Preparations with Propolis and Tea Tree Oil. Two samples of toothpaste covered anonymously with blank white tag and marked only with letter A or C were compared: the studied toothpaste with the active ingredient (study group, A), 1,5% content of ethanol extract of Polish propolis, and with no active ingredients, placebo (control group, C). The second natural product valued for its properties was tea tree oil (TTO). It is obtained from the *Melaleuca alternifolia* plant. Two other species (*Melaleuca linariifolia* and *Melaleuca*

dissitiflora) are also used to obtain essential oil, only if they contain more than 30% terpinen-4-ol in the essential oil. The major component of tea tree oil is terpinen-4-ol. Its content is 29–45%. The composition of active and placebo toothpaste had the following:

Active Toothpaste

aqua (up to 100% of weight),
glycerine (5–12%),
silica (10–14%),
sorbitol (10–20%),
hydroxyethyl cellulose (0.1–1%),
titanium dioxide (0.5–2%),
xanthan gum (0.3–1%),
ethanolic extract of propolis (EEP) (1.0%),
tea tree oil (TTO) (1.0%),
menthol oil (0.2%),
rosemary oil (0.1%).

Placebo Toothpaste

aqua,
glycerine,
silica,
sorbitol,
hydroxyethyl cellulose,
titanium dioxide,
xanthan gum.

2.2. Study Groups Inclusion and Extrusion Criteria. Subject qualification for the study was based on medical and dental history, interview, and review of clinical records. The exclusion and inclusion criteria from the investigation were as follows:

Inclusion Criteria

(i) written participation consent,
(ii) age (40–85 years),
(iii) patients lacking 5–8 teeth in the maxilla and mandible under prosthetic treatment,
(iv) patients with remaining posterior occluding pairs with a minimum of two supporting zones according to Eichner index.

Extrusion Criteria

(i) lack of a written participation consent, painful masticatory dysfunction,
(ii) edentulous patients or patients with residual dentition (Eichner subgroups C1–C3), patients with full dental arches or lacking 1-2 teeth in an arch,
(iii) patients with retaining teeth mobility over +20 PTV tested with the Periotest instrument,
(iv) patients with class V fillings or acrylic/ceramic crowns around the retaining teeth,
(v) patients with cancer,
(vi) psychosomatic disorders,
(vii) patients after trauma within the craniofacial region,
(viii) pregnant and lactating females,
(ix) patients suffering from asthma or atopic dermatitis, allergic to foods, drugs, or honey and its products, or having other allergy-related ailments.

The study was conducted with the prior approval from the Bioethics Committee of the Silesian Medical Chamber in Katowice, Poland, Resolution number 8/2015 of March 23, 2015.

2.3. Patients. 37 patients aged 41–82 (57.0 ± 10.49) were qualified for the study, including 20 females (54%) and 17 males (46%). Patients were randomly divided into two subgroups. The study group consisted of 18 persons aged 41–79 (55.05 ± 11.09), who received the active toothpaste with propolis and tea tree oil, whereas the control group consisted of 19 patients aged 41–82 (66.79 ± 11.19), who were given the negative control toothpaste.

2.4. Clinical Examination Protocol. The studies were performed in two stages. The first stage consisted of a general medical and dental interview based on a survey and a form written particularly for this purpose. The study was extended by a survey concerning medical history and current diseases, allergies, taken medication, and environmental risk. Mobility of the retaining tooth was also measured with Periotest during the first visit, as a criterion qualifying the patient for the study. The values may be in the following ranges: −08 to +09, physiological teeth mobility; +10 to +19, perceptible teeth mobility; +20 to +29, moderate teeth mobility under investigator's pressure; +30 to +50, severe teeth mobility under pressure from the lips or tongue [35, 36]. Patients qualified for the study were evaluated to determine the value of selected oral hygiene indices included in the form, that is, OHI-S, denture plaque index, mSBI, and API. The measurement procedure according to the above-mentioned indices was carried out with use of a standard WHO periodontal probe. The measurement was based on the assessment of presence and size of plaque on selected tooth surfaces and bleeding from the interdental papilla.

Approximal Plaque Index (API), Lange and Ainamo, 1988: Presence of plaque is assessed only on occlusal surfaces. The presence of bacterial plaque is examined in quadrants 1 and 3 on occlusal surfaces on the side of the oral cavity proper and in quadrants 2 and 4 on occlusal surfaces on the side of the vestibule. The formula used to calculate the value of API [%] = the sum of interdental spaces with plaque/the sum of all examined surfaces × 100%. Index values were interpreted as follows: API 100–70%, bad oral hygiene; API 70–40%, average hygiene, improvement recommended; API 39–25%, quite good oral hygiene; API < 25%, optimal oral hygiene.

Simplified Oral Hygiene Index determines the amount of soft debris or calculus on the four buccal surfaces of the selected teeth: upper right first molar, upper right central incisor, upper left first molar, and lower central incisor, and on the lingual surfaces of the lower left first molar and lower right first molar.

Denture plaque index (Budtz-Jörgensen, 1978) provides the following description for the amount of denture plaque on the fitting surface: 0 = nonvisible, 1 = less than one-third covered, 2 = one-third to two-thirds covered, and 3 = more than two-thirds covered.

The periodontal status (gingival health) was evaluated with use of the modified Sulcus Bleeding Index (mSBI, Muhlemann-Son, 1971) and by recording only "bleeding presence" or "bleeding absence" for all existing teeth. The index describes the presence of localised bleeding in interdental spaces and interdental papilla. In quadrants 1 and 3 the vestibular side is examined, while in quadrants 2 and 4 the lingual one is examined.

After clinical examination acrylic partial dentures were made for all 37 patients. Both groups (study and control) were subjected to hygienisation of the oral cavity and instructed on how to properly brush their teeth with use of Fones circular technique. Fones circular technique consists of putting the toothbrush perpendicularly to slightly opened dental arches and performing circular movements. In the roll technique the toothbrush is placed on attached gingiva at the angle of 45° with the bristles pointed towards the root. Patients were also shown the proper denture hygiene. They were instructed to wash their dentures after every meal with use of a soft denture brush or with hands and hard soap. After brushing the dentures were to be carefully rinsed under running water and put in the oral cavity. Patients were advised not to use dentures on a 24/7 basis. Dentures were supposed to be removed for the night and kept in a dry place after prior washing. All patients were examined in three subsequent stages: preliminary qualification at baseline before hygiene procedure (1st assessment), a follow-up after 7 days (2nd assessment), and a follow-up 28 days after the initial examination (3rd assessment). Patients were examined for regression of lesions, frame of mind, and possible side effects, which was recorded on the control form. During each examination swabs were used for microbiological examination of oral cavity microflora. The microbial material for smear tests was collected from the floor of the mouth.

2.5. Bacterial Strains Isolation and Microbiological Investigation. Microbiological examinations were performed with use of classic methods employed in microbiological diagnostics. Material collected from the patients was cultured on proper growth media for proliferation and subsequent isolation of pure cultures. Aerobic bacteria were proliferated on Columbia agar solid medium with 5% sheep blood at 37°C. Anaerobic bacteria were proliferated on Schaedler K3 solid medium with 5% sheep blood at 37°C in anaerobic conditions obtained with Biomerieux GENbag anaer generators (Marcy-l'Étoile, France). Species identification was performed after isolation and proliferation of cultured microorganism strains with use of the following reagent sets: ENTEROtest 24N, NEFERMtest 24N, STREPTOtest 24, STAPHYtest, ANAEROtest 23, OXItest, PYRAtest, and TNW_lite 6.5 computer program for species identification of microorganisms (Erba-Lachema, Brno, Czech Republic). Also the following Biomerieux (Marcyl'Etoile, France) biochemical tests were used: Katalaza, Slidex Staph Kit, and API Candida. Performance and interpretation of results of the tests was carried out according to the manufacturer's recommendations with diagnostic reagent sets.

2.6. Statistical Analysis. The first stage of statistical analysis consisted of verification of the compatibility of the obtained index values and the number of bacteria with normal distribution with use of the Shapiro-Wilk test. Variables with normal distribution were presented with arithmetic mean and standard deviation, whereas nonparametric variables were presented with median and interquartile range. One-way analysis of variance (ANOVA) and Levene's test were used to compare the results of the study group with the control group for OHI, denture plaque index, and the number of bacteria. The comparison of results between groups was performed with the Tukey-Kramer method. Results obtained for OHI and denture plaque index were compared with Student's t-test for dependent and independent samples. For unrelated variables of API and SBI the results of study and control groups were compared with Mann–Whitney U test, Wilcoxon signed-rank test, and Friedman's ANOVA with Kendall's coefficient of concordance. The results were deemed to be statistically significant if $p < 0.05$.

3. Results

3.1. Oral Health Conditions. The general distribution range for API was presented in Table 1. Statistically significant differences between groups were reported 7 and 28 days after the initial examination ($p < 0.05$ and $p < 0.001$). Improvement of hygiene was reported both in the study group (A) and in the control group (C). It may be associated with the reduction of interproximal spaces in the lateral segments of maxilla and/or mandible. A significant improvement was only observed in the study group (A) (Table 1).

Another assessed index was the simplified Greene-Vermilion hygiene index. Statistically significant improvement of hygiene in the study group (A) was observed (Table 2). In the study group (A) the index was determined after 7 and 28 days of usage ($p = 0.013221$, $p = 0.000468$). No statistical significance was observed in the control group (C) (Table 2). Significant reduction was observed by comparing OHI 7 and 28.

Based on the conducted analysis of denture plaque index values no significant reduction in the values was observed after the period of use of toothpastes in study (A) and control (C) groups compared to the output value (Figure 1).

Statistically significant reduction of gingival bleeding index after 7 and 28 days of the use of toothpastes compared to the gingival bleeding index at the beginning of the treatment was observed both in study (A) and in the control group (C) (Table 3). The study and control group did not differ in terms

Table 1: API ranges: assessment for study and control groups.

	Oral hygiene assessment (interproximal spaces)					
	The assessment criteria	T1	T2	T3	Friedman's ANOVA test (p)/Kendall's coefficient of concordance	Wilcoxon signed-rank test p
Study group (A)	Optimal	11%	39%	56%	$p = 0.0000/0.95894$	(1) : (2) = 0.000438 (2) : (3) = 0.000293 (1) : (3) = 0.000196
	Quite good	15%	28%	44%		
	Average	53%	33%	0%		
	Bad	21%	0%	0%		
Control group (C)	Optimal	17%	21%	42%	$p = 0.00228/0.32012$	(1) : (2) = 0.231060 (2) : (3) = 0.064186 (1) : (3) = 0.000327
	Quite good	11%	37%	37%		
	Average	72%	42%	21%		
	Bad	0%	0%	0%		
Mann–Whitney "U" test (p)		0.784483	0.046554	0.001032	—	—

T1 (1): preliminary examination before hygiene procedure; T2 (2): examination after 7 days; T3 (3): 28 days after the initial examination.

Table 2: OHI ranges: assessment for study and control groups.

	Oral hygiene assessment (interproximal spaces)										
	T1		Mean ± standard deviation	T2		Mean ± standard deviation	p	T3		Mean ± standard deviation	p
Study group (A)	0–0.5	55.5%	0.41 ± **0.25**	0–0.5	66.6%	0.33 ± **0.23**	0.013221	0–0.5	88.8%	0.20 ± **0.17**	0.000468
	0.6–1	44.5%		0.6–1	34.4%			0.6–1	11.1%		
	1.1–2	0%		1.1–2	0%			1.1–2	0%		
	2.1–3	0%		2.1–3	0%			2.1–3	0%		
Control group (C)	0–0.5	58%	0.64 ± **0.50**	0–0.5	63%	0.58 ± **0.30**	0.352356	0–0.5	68.4%	0.57 ± **0.23**	0.382331
	0.6–1	21%		0.6–1	21%			0.6–1	31.6%		
	1.1–2	16%		1.1–2	16%			1.1–2	0%		
	2.1–3	5%		0.006370	0.000002			2.1–3	0%		
Study (A) versus control (C) (p)	0.097301			0,006370				0,000002			

T1 (1): preliminary examination before hygiene procedure; T2 (2): examination after 7 days; T3 (3): 28 days after the initial examination.

of the assessment of the SBI value upon initial examination. After 7 and 28 days of use the control group exhibited a significantly higher level of SBI in comparison with the study group (Table 3).

3.2. Microbiological Investigation. The following observations in study group (A) constituted by 18 patients were made: 77 microorganisms of 31 species were isolated in the first microbiological test. The second test (after 7 days of use of the toothpaste with EEP and TTO) resulted in the isolation of 78 microorganisms of 27 species. The third test after 4 weeks of use of the active toothpaste showed a decrease in the number of isolated microorganisms to 65, of 24 species (Table 4). The oral microflora diversity was reduced by the decrease in the number of cultured microorganism species. The following bacterial species were eliminated: *Actinomces viscocus, Actinomyces israelii, Atopobium parvulum, Bifidobacterium ovatus, Clostridium botulinum, Eubacterium saburreum, Lactobacillus acidophilus, Lactobacillus fermentum,* and *Propionibacterium granulosum,* classified as Gram(+) anaerobes. They are all part of the oral flora and, more specifically, dental biofilm bacteria. *Lactobacillus acidophilus* is a known pathogen responsible for the development of caries. *Actinomyces* spp. belong to pioneer bacteria responsible for the formation of plaque, while *A. israeli* may be an etiological factor for actinomycosis. The following species were also eliminated: *Bacteroides uniformis* and *Parabacteroides distasonis* classified as Gram(−) anaerobes; *Acinetobacter freundii*, *Burkholderia cepacia,* and *Neisseria sicca* (Gram(−) aerobe), as well as *Staphylococcus epidermidis* MSCNS (Gram(+) aerobe), which can cause opportunistic infections, including sepsis. The microflora gained the following species, Gram(+) anaerobes: *Atopobium minutum* and *Clostridium butyricum*, Gram(−) anaerobes: *Capnocytophaga ochracea* and *Prevotella melaninogenica*, Gram(+) aerobes: *Micrococcus* spp. and *Streptococcus oralis*, and Gram(−) aerobes: *Providencia rustigiani* and *Stenotrophomonas maltophilia*. *Micrococcus* spp. and *S. oralis* are classified as microorganisms which participate in plaque formation as one of the first. *Capnocytophaga ochracea* belongs to the green bacterial complex, associated

Table 3: SBI ranges: assessment for study and control groups.

		Sulcus Bleeding Index assessment				
	The assessment criteria	T1	T2	T3	Friedman's ANOVA test (p)/Kendall's coefficient of concordance	Wilcoxon signed-rank test p
Study group (A)	Normal gingiva SBI < 10%	50%	72.5%	100%	p = 0.00000/0.71010	(1) : (2): 0.003283 (2) : (3): 0.001474 (1) : (3): 0.000982
	Bleeding on probing	50%	27.5%	0%		
Control group (C)	Normal gingiva SBI < 10%	53%	65.5%	65.5%	p = 0.09760/0.12247	(1) : (2): 0.726768 (2) : (3): 0.286004 (1) : (3): 0.572082
	Bleeding on probing	47%	34.5%	34.5%		
Mann–Whitney U test (p)		0.346197	0.012713	0.000001		—

T1 (1): preliminary examination before hygiene procedure; T2 (2): examination after 7 days; T3 (3): 28 days after the initial examination.

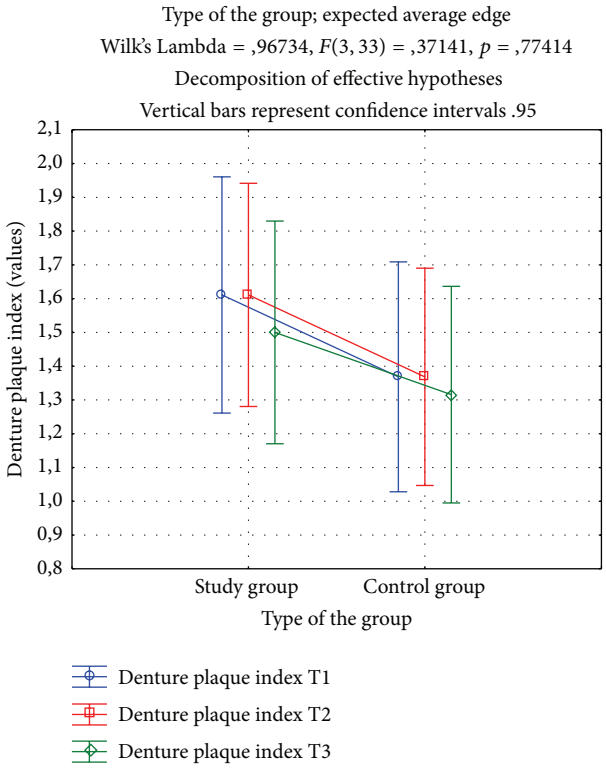

Figure 1: ANOVA Estimated Marginal Means for denture plaque index study and control group.

with advanced periodontitis according to Socransky. In our study the number of the following bacteria declined: *Bifidobacterium adolescentis, Veillonella parvula, Streptococcus salivarius,* and *Streptococcus sanguinis*. They all belong to physiological bacterial flora and participate in the formation of dental biofilms. A reduction of *Candida albicans* was also observed (Table 5). *C. albicans* is often isolated from the oral cavity, although most frequently in patients using dentures, and does not always cause infection symptoms.

In the control group (C) constituted by 19 patients, 79 microorganisms of 25 species were isolated in the first microbiological test. The second test resulted in the isolation of 83 microorganisms of 28 species, while the third test after 4 weeks of use of the toothpaste without active ingredients resulted in the culture of 92 microorganisms of 26 species (Table 4). An increase of the number of cultured microorganisms and a slight increase in the number of species were observed. The following species were eliminated: Gram(+) anaerobes: *Clostridium sporogenes, Eubacterium saburreum,* and *Lactobacillus fermentum,* and Gram(−) anaerobes: *Bacteroides ovatus, Bacteroides uniformis, Fusobacterium mortiferum,* and *Prevotella oralis*, as well as Gram(+) aerobic bacterium *Staphylococcus aureus* MSSA. The microflora gained the following Gram(+) anaerobes: *Bifidobacterium dentium, Bifidobacterium ovatus, Clostridium chauvoei, Gemella morbillorum,* and *Propionibacterium propionicum. Bifidobacterium* spp. are probiotic bacteria dwelling in human intestine. *Gemella morbilorum* is a pathogen isolated from cysts which were not resolved after the second attempt of endodontic treatment. The flora also gained Gram(−) anaerobic bacterium *Veillonella parvula*, Gram(+) aerobic bacterium *Streptococcus mutans*, and Gram(−) aerobic bacterium *Neisseria sicca*. *Streptococcus* spp. participate in plaque formation, while *S. mutans* is a known etiological factor for caries. *V. parvula*, which belongs to the purple complex according to Socransky, is isolated in patients with healthy periodontium. The number of cultured *Candida albicans* also increased. As mentioned before, this fungus is often isolated in patients with removable dentures. The number of the following bacteria declined in the control group: *Blautia producta, Clostridium clostridiforme, Lactobacillus acidophilus, Capnocytophaga ochracea,* and *Streptococcus sanguinis*, and the number of the following bacteria increased: *Actinomyces israelii, Actinomyces naeslundii, Campylobacter gracilis, Streptococcus mitis, Streptococcus salivarium, Escherichia coli,* and *Neisseria subflava*, as well as fungi *Candida albicans* (Table 5).

3.3. Side Effects. No serious side effects have been reported. Some patients reported discoloration of their toothbrush.

4. Discussion

Natural extracts used in the tested toothpastes were intended to provide chemical assistance in the mechanical removal of plaque and thus improve oral hygiene. The first one was

Table 4: The microorganisms species isolated from patients, who used toothpaste with EEP and TTO (study group, A) or without EEP and TTO (control group, C).

Isolated microorganisms	Number of patients from study group (A)			Number of patients from control group (C)		
	Initial	After 7 days	After 28 days	Initial	After 7 days	After 28 days
Gram(+) anaerobic bacteria						
Actinomyces viscosus	1	1	0	0	0	0
Actinomyces israelii	1	1	0	2	3	3
Actinomyces naeslundii	1	2	1	3	7	7
Anaerococcus prevotti	0	0	0	0	1	0
Atopobium minutum	0	0	1	0	1	0
Atopobium parvulum	1	0	0	0	0	0
Bifidobacterium adolesccentis	2	1	1	1	0	1
Bifidobacterium dentium	1	0	1	0	1	3
Bifidobacterium longum	4	5	4	0	1	0
Bifidobacterium breve	0	0	0	5	2	5
Bifidobacterium ovatus	3	2	0	0	0	1
Blautia producta	1	0	2	2	0	1
Clostridium barati	1	1	1	0	2	0
Clostridium botulinum biovar A	1	1	0	0	0	0
Clostridium butyricum	0	0	1	0	0	0
Clostridium chauvoei	0	1	0	0	0	1
Clostridium clostridiforme	0	0	0	2	1	1
Clostridium histolyticum	0	1	0	0	0	0
Clostridium perfringens	0	1	0	1	1	1
Clostridium sporogenes	0	0	0	1	0	0
Eubacterium saburreum	1	1	0	1	0	0
Gemella morbillorum	0	0	0	0	0	1
Lactobacillus acidophilus	1	0	0	3	3	1
Lactobacillus fermentum	1	1	0	1	1	0
Propionibacterium granulosum	1	1	0	0	1	0
Propionibacterium propionicum	0	0	0	0	1	1
Gram(−) anaerobic bacteria						
Bacteroides ovatus	0	0	0	1	0	0
Bacteroides uniformis	1	0	0	1	0	0
Campylobacter gracilis	0	0	0	1	1	2
Capnocytophaga ochracea	0	1	1	3	2	1
Fusobacterium nucleatum	1	1	1	0	1	0
Fusobacterium mortiferum	0	0	0	1	0	0
Mitsuokella multacida	3	3	4	5	4	5
Parabacteroides distasonis	1	1	0	0	0	0
Prevotella melaninogenica	0	0	1	0	0	0
Prevotella oralis	0	0	0	1	0	0
Veillonella parvula	2	3	1	0	1	1
Gram(+) aerobic bacteria						
Micrococcus spp.	0	0	1	0	0	0
Staphylococcus aureus MSSA	1	0	2	1	0	0
Staphylococcus epidermidis MSCNS	1	0	0	0	0	0
Streptococcus mitis	7	10	7	6	7	9
Streptococcus oralis	0	1	1	0	0	0

Table 4: Continued.

Isolated microorganisms	Number of patients from study group (A)			Number of patients from control group (C)		
	Initial	After 7 days	After 28 days	Initial	After 7 days	After 28 days
Streptococcus mutans	0	0	0	0	0	2
Streptococcus salivarius	9	5	8	7	4	10
Streptococcus sanguinis	4	5	2	5	7	3
Gram(−) aerobic bacteria						
Acinetobacter freundii	1	0	0	0	0	0
Burkholderia cepacia	1	0	0	1	0	1
Enterobacter amniogenus	0	1	0	0	0	0
Enterobacter cloacae	0	1	0	0	0	0
Enterobacter spp.	0	0	0	0	1	0
Escherichia coli	0	0	0	1	1	2
Neisseria sicca	1	0	0	0	2	1
Neisseria subflava	15	18	16	18	16	19
Providencia rustigiani	0	0	1	0	0	0
Pseudomonas spp.	0	0	0	0	1	0
Stenotrophomonas maltophilia	0	0	1	0	0	0
Fungi						
Candida albicans	8	8	6	5	9	9
Number of microorganisms strains	**77**	**78**	**65**	**79**	**83**	**92**
Altogether:		**220**			**254**	

ethanolic extract of propolis. There are numerous studies confirming its strong antimicrobial, antiviral, antifungal, and antiphlogistic activity [37–39]. Propolis exhibits a wide spectrum of antimicrobial activities. Research conducted by Koru et al. demonstrated that propolis is more effective against Gram(+) aerobes than against Gram(−) aerobes [40]. In their study Feres et al. compared antimicrobial activity of 11% propolis and the popular 0.12% chlorhexidine against bacteria in saliva in healthy patients and in patients with periodontal diseases. Both substances exhibited statistically significant antimicrobial activity [41]. Antimicrobial activity of 10% propolis and 0.12% chlorhexidine against *S. mutans* and *L. acidofilus*, known pathogens responsible for the development of caries, was compared in 2011. Both proved to be effective [42]. Another study from 2013 compared 0.12% chlorhexidine with propolis at a much lower concentration, namely, 2%. Its antimicrobial activity against *S. mutans* and *L. acidofilus* was confirmed [43]. These studies proved the cariostatic properties of propolis, antiperiodontal pathogen activity, and efficiency at lower concentrations. In the present study we employed 1.5% ethanolic extract of propolis. Hitherto, there have been no studies confirming the antimicrobial effectiveness at the concentration of 1.5%. However, in the toothpaste tested it was combined with tea tree oil, which has similar properties. Thus, the concentration of EEP could be lowered. At the same time, lower concentration of EEP minimises the risk of side effects such as allergic reactions and burning sensation in the oral cavity [34]. An important aspect is the antiphlogistic properties of ethanolic extract of propolis [44]. Skaba et al. conducted an analysis of ethanolic extract of Brazilian propolis, in which they confirmed its antiphlogistic activity using three recognised methods. The same study included an assessment of the influence of toothpaste with 3% ethanolic extract of Brazilian propolis on oral hygiene with use of oral cavity indices: API, OHI, and SBI. Researchers collected also smears from patients in order to assess antimicrobial activity. Patients were divided into two groups. The study group (I) was using an active toothpaste (T) with propolis, while the control group (II) was using a toothpaste with no active substance (G). Patients were examined on the first, initial visit, second visit after a week, and third one after 4 weeks. After 4 weeks of using the T toothpaste there was a statistically significant decrease in OHI in both groups I and II, which may indicate that daily mechanical plaque removal, even without active substances, can be effective in healthy patients. API in group II did not change and patients were qualified for the group with average hygiene, whereas API in group I improved after 7 days and returned to baseline upon the last examination, patient with quite good hygiene. SBI improved in group I, but in a statistically insignificant way. It did not change in group II [45]. Study of toothpastes with 3% ethanolic extract of propolis in patients undergoing orthodontic treatment was conducted in 2013 and in 2016. Apart from the smears, it comprised also API according to Lange, Gingival Index (GI), and Orthodontic Plaque Index (OPI) upon first examination and after 35 days of using a toothpaste with propolis and CT gel. The control group consisted of patients using a toothpaste without propolis and CC gel. The first examination did not demonstrate any statistically significant differences in the assessed indices. Upon examination after 35

Table 5: The changes of oral microflora from patients, who used toothpaste with EEP and TTO (study group, A) or without EEP and TTO (control group, C).

Changes of microorganisms species after 28 days of study	Study group (A)	Control group (C)
Eliminated species	Gram(+) anaerobic bacteria	
	Actinomyces viscosus *Actinomyces israelii* *Atopobium parvulum* *Bifidobacterium ovatus* *Clostridium botulinum* *Eubacterium saburreum* *Lactobacillus acidophilus* *Lactobacillus fermentum* *Propionibacterium granulosum*	*Clostridium sporogenes* *Eubacterium saburreum* *Lactobacillus fermentum*
	Gram(−) anaerobic bacteria	
	Bacteroides uniformis *Parabacteroides dystasonis*	*Bacteroides ovatus* *Fusobacterium mortiferum* *Prevotella oralis*
	Gram(+) aerobic bacteria	
	Staphylococcus epidermidis MSCNS	*Staphylococcus aureus MSSA*
	Gram(−) aerobic bacteria	
	Acinetobacter freundii *Burkholderia cepacia* *Neisseria sicca*	—
Declined species	Gram(+) anaerobic bacteria	
	Bifidobacterium adolescentis	*Blautia producta* *Clostridium clostridiforme* *Lactobacillus acidophilus*
	Gram(−) anaerobic bacteria	
	Veillonella parvula	*Capnocytophaga ochracea*
	Gram(+) aerobic bacteria	
	Streptococcus salivarius *Streptococcus sanguinis*	*Streptococcus sanguinis*
	Fungi	
	Candida albicans	—
Gained species	Gram(+) anaerobic bacteria	
	Atopobium minutum *Clostridium butyricum*	*Bifidobacterium dentium* *Bifidobacterium ovatus* *Clostridium chauvoei* *Gemella morbillorum* *Propionibacterium propionicum*
	Gram(−) anaerobic bacteria	
	Capnocytophaga ochracea *Prevotella melaninogenica*	*Veillonella parvula*
	Gram(+) aerobic bacteria	
	Micrococcus spp. *Streptococcus oralis*	*Streptococcus mutans*
	Gram(−) aerobic bacteria	
	Providiencia rustigiani *Stenotrophomonas maltophilia*	*Neisseria sicca*
Increased species	Gram(+) anaerobic bacteria	
	Blautia producta	*Actinomyces israeli* *Actinomyces naeslundii*
	Gram(−) anaerobic bacteria	
	Mitsuokella multiacida	*Campylobacter gracilis*
	Gram(+) aerobic bacteria	
	Staphylococcus aureus MSSA	*Streptococcus mitis* *Streptococcus salivarius*

Table 5: Continued.

Changes of microorganisms species after 28 days of study	Study group (A)	Control group (C)
	Gram(−) aerobic bacteria	
	Neisseria subflava	*Neisseria subflava*
		Escherichia coli
	Fungi	
	—	*Candida albicans*

days there was a statistically significant decrease in the values of GI and OPI compared to the control group. No significant difference was shown in the decrease of API [46, 47].

Melaleuca alternifolia tea tree oil (TTO) is also a known antiseptic preparation with antimicrobial and antifungal activity. It has been successfully used for many centuries in dermatology, particularly in the treatment of acne vulgaris [48], accelerates wound healing, and has anticancer properties [49]. The basic composition of the oil is terpinen-4-ol, γ-terpinene, α-terpinene, 1,8-cineole, terpinolene, ρ-cymene, α-pinene, α-terpineol, aromadendrene, δ-cadinene, limonene, sabinene, globulol, and viridiflorol. They are terpene compounds with antiseptic and antiphlogistic activities which apart from dermatology can also be employed in dentistry, particularly in the maintenance of proper oral hygiene [50]. In 2003 Hammer et al. examined in vitro the antimicrobial activity of tea tree oil against 161 isolated oral pathogens and detected MIC and MBC for 15 of them. MIC and MBC values were lowest for *Porphyromonas* spp., *Prevotella* spp., and *Veillonella* spp. and highest for isolated *Streptococcus* spp., *Fusobacterium* spp., and *Lactobacillus* spp. Time necessary for the death of *Streptococcus mutans* and *Lactobacillus rhamnosus* in ≥0.5% concentration was 5 minutes. It was thus demonstrated that oral pathogens are susceptible to the activity of TTO [51].

Studies on toothpastes with natural extracts against three species of bacteria *S. mutans, P. aeruginosa,* and *E. faecalis* compared to a toothpaste without antimicrobial ingredients were conducted in 2015. *S. mutans* is a known etiological factor for caries, while *P. aeruginosa* and *E. faecalis* are pathogens isolated in periodontopathies and endodontic infections. 8 toothpastes with the following composition were used: sorbitol (I), tocopherol (II), mentha (III), cinnamon/mentha (IV), propolis/tea tree oil (V), mentha/açaí berry (VI), mentha/guarana (VII), and propolis (VIII). The control group was a toothpaste with no antimicrobial properties (group (IX), negative control), while group (X) (positive control) was a dental gel with triclosan and formaldehyde. The results demonstrated that *E. faecalis* exhibited resistance to the toothpaste with propolis and tea tree oil and susceptibility to the toothpaste with propolis only. The authors explain it with a different concentration of propolis in the toothpastes. Propolis/tea tree oil toothpaste exhibited the strongest antimicrobial activity against *S. mutans* compared to other toothpastes. *P. aeruginosa* proved to be resistant to all tested natural substances [52]. Some other researchers demonstrate alternative methods to reduce pathological oral microflora, by addition to resin composites on element ions with nanogold and nanosilver [53, 54].

In the present study there was an increase in the number of cultured *C. albicans* colonies in patients using both the active toothpaste and the placebo. Both active substances employed in the active toothpaste exhibit antifungal activity. However, it should be remembered that the group of examined patients was using acrylic partial dentures, which are an iatrogenic factor for *C. albicans* infection [55]. The use of acrylic partial dentures caused changes in the oral microflora. The acrylic material is a reservoir for *C. albicans*, whose amount depends on denture hygiene and its quality, particularly roughness [56, 57]. The 2011 study of bee products in comparison with fluconazole confirmed the antifungal activity of propolis and the possibility of its alternative use in antifungal therapy. It exhibits activity against *C. albicans, C. glabrata, C. krusei,* and *Trichosporon* spp. [58]. There are many reports on the antifungal activity of tea tree oil. The 2003 in vitro study examined the antifungal activity of particular components of the oil in concentrations between 0.5% and 2%. The results demonstrated that all components of the oil, apart from β-myrcene, exhibit antifungal activity [59]. The study from 2004 confirmed the hypothesis of the mechanism of action of tea tree oil against *C. albicans*, *C. glabrata,* and *S. cerevisiae*. The oil influences the properties and disrupts the functions of fungal cell membrane [60]. Study conducted by Sudjana et al. demonstrated that TTO inhibits adhesion of *C. albicans* to human cells and polystyrene and inhibits the formation of biofilm through reduction of surface tension [61].

5. Conclusions

(1) Improvements in hygiene and the condition of periodontium were observed in patients using toothpastes with ethanolic extract of propolis and tea tree oil.

(2) The toothpaste with ethanolic extract of propolis and tea tree oil had no influence on the reduction of denture plaque.

(3) The studied toothpaste with active ingredients (ethanolic extract of propolis and tea tree oil) demonstrates the beneficial influence on microorganisms composition in oral microflora.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 5370545, 11 pages
https://doi.org/10.1155/2017/5370545

Research Article

Antioxidant Properties and Cardioprotective Mechanism of Malaysian Propolis in Rats

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Received 8 July 2016; Accepted 20 October 2016; Published 2 February 2017

Academic Editor: Andrzej K. Kuropatnicki

Propolis contains high concentrations of polyphenols, flavonoids, tannins, ascorbic acid, and reducing sugars and proteins. Malaysian Propolis (MP) has been reported to exhibit high 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity and ferric reducing antioxidant power (FRAP) values. Herein, we report the antioxidant properties and cardioprotective properties of MP in isoproterenol- (ISO-) induced myocardial infarction in rats. Male Wistar rats ($n = 32$) were pretreated orally with an ethanol extract of MP (100 mg/kg/day) for 30 consecutive days. Subcutaneous injection of ISO (85 mg/kg in saline) for two consecutive days caused a significant increase in serum cardiac marker enzymes and cardiac troponin I levels and altered serum lipid profiles. In addition significantly increased lipid peroxides and decreased activities of cellular antioxidant defense enzymes were observed in the myocardium. However, pretreatment of ischemic rats with MP ameliorated the biochemical parameters, indicating the protective effect of MP against ISO-induced ischemia in rats. Histopathological findings obtained for the myocardium further confirmed the biochemical findings. It is concluded that MP exhibits cardioprotective activity against ISO-induced oxidative stress through its direct cytotoxic radical-scavenging activities. It is also plausible that MP contributed to endogenous antioxidant enzyme activity via inhibition of lipid peroxidation.

1. Introduction

Propolis, or bee glue, is a resinous product collected by bees from various plant sources, such as buds and exudates. The substance is sticky at and above room temperature (20°C), but at lower temperature it becomes hard and brittle [1–3]. Propolis is used as a sealant by bees to seal cracks in hives, encapsulate invader carcasses, repair combs, and strengthen thin borders [1, 2, 4]. Depending on its source, geographical climate, and age, propolis varies greatly; it possesses a pleasant aromatic odor and occurs in different colors, such as yellow, cream, green, and light or dark brown [5].

Propolis is mainly composed of resins, polyphenols, flavonoids, fatty acids, essential oils, wax, pollen, and other organics and minerals. Propolis contains at least 38 flavonoids [6]. It also contains vitamin B complexes; vitamins C and E; important minerals such as Zn, Mg, Cu, Fe, Mn, Ni, and Ca; and some trace elements [7]. Due to the presence of compounds such as flavonoids, phenolic acids, and their esters, propolis exhibits anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant, and antiproliferative properties [8]. Reactive oxygen species (ROS) such as superoxide anions and hydroxyl radicals are scavenged by antioxidants present in propolis [9, 10]. In addition, the extreme reactivity

of ROS toward lipids and proteins contributes to their rapid damaging capacity [11].

Myocardial infarction (MI), commonly known as "heart attack," occurs due to an interruption in the supply of blood to heart tissue. As a result of coronary artery occlusion, necrosis of part of the myocardium occurs [12]. In fact, an imbalance between coronary blood supply and myocardial demand is the main cause of myocardium necrosis resulting from MI [13]. MI is a common presentation of ischemic heart disease (IHD) and is followed by numerous pathophysiological and biochemical changes, including lipid peroxidation (LPO), hyperglycemia, and hyperlipidemia [14]. In the developed world and most developing countries, MI is one of the main causes of mortality and morbidity [12, 15].

At low concentration, catecholamines exert a positive ionotropic effect and are beneficial in regulating heart function. However, when present in high doses (administered or released in excess from the endogenous stores), catecholamines can deplete the reserved energy of cardiomyocytes, resulting in structural and biochemical changes leading to irreversible damage. Isoproterenol 4-[1-hydroxy-2-(isopropylamino)ethyl] benzene-1,2-diol hydrochloride (ISO) is a synthetic catecholamine and β-adrenergic agonist that causes severe stress to the myocardium, resulting in an infarct-like necrosis of the heart muscle [16]. Free radicals thus produced may attack polyunsaturated fatty acids (PUFAs) within membranes, forming peroxyl radicals. These radicals then attack adjacent fatty acids, causing a chain reaction of LPO. Lipid hydroperoxide end products are harmful and may be responsible for the disruption of the integrity of the myocardial membrane [17, 18].

Recently, a number of Malaysian agro-food-based research institutes and local beekeepers have collected the bee propolis of different species to analyze its composition as well as its health benefits [19, 20]. Propolis from the *Trigona itama* species of Malaysia has been reported to contain a number of bioactive compounds with biological activities. The major phytochemicals identified in MP are hexadecanoic acid, which acts as an antioxidant; phenylethyl alcohol and dodecanoic acid, which act as antimicrobial agents; and norolean-12-ene, ethyl octadecanoate, 8-octadecanoic acid, and 9-octadecanoic acid, which serve as anti-inflammatory agents [19]. A previous study demonstrated that propolis contains remarkable antioxidant properties, mainly attributed to its phenolic and flavonoid contents [21]. The aim of this study was to investigate the antioxidant properties of Malaysian Propolis (MP) and its cardioprotective effect, elucidating the mechanism that occurs in ISO-induced MI in rats.

2. Materials and Methods

2.1. Chemicals and Drugs. Gallic acid, catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ) standards were purchased from Sigma-Aldrich (St. Louis, MO). Tannic acid, L-ascorbic acid, and Folin-Ciocalteu's phenol reagent were purchased from Merck Co. (Darmstadt, Germany). ISO and 1,1,3,3-tetraethoxypropane were purchased from Nacalai Tesque, Inc. Kyoto, Japan. All chemicals and reagents used in this study were of analytical grade.

2.2. Propolis Sample. Stingless bee propolis of *Tetratrigona* subgenus (genus: *Trigona*) was collected from the Min House Camp, Kubang Kerian, Kelantan, Malaysia in June 2013.

2.3. Extract Preparation. Propolis extract (20%) was prepared according to the method described by Laskar et al. [22]. Solid propolis samples (200 g) were first cut into small pieces using a sterile, smooth steel knife. The samples were soaked with ethanol (70%) for 48 hours and then shaken (150 rpm) at 30°C for 72 hours. The extract solution was filtered with Whatman number 1 filter paper and dried in a rotary evaporator (Buchi, Tokyo, Japan) under a reduced pressure (100 psi) and at a controlled temperature (40°C). The dried extract was collected and finally preserved at −20°C for subsequent *in vitro* and *in vivo* studies.

2.4. Phytochemical Analysis

2.5. Estimation of Total Polyphenols. The total polyphenol content of the propolis extract was estimated by spectrometric determination based on Folin-Ciocalteu's method [23] using a PD-303S spectrophotometer (APEL, Japan). Briefly, 0.4 mL of sample extract (10 mg/mL) was mixed with 1.6 mL of 7.5% sodium carbonate solution. Then, 2 mL of 10-fold diluted Folin-Ciocalteu's reagent was added, and the final reaction mixture was incubated for 1 hour in the dark. Total polyphenol content was determined as gallic acid equivalent (GAE) (6.25–100.00 μg/mL) and expressed as mg of GAEs/g of propolis. The color intensity of the blue complex was measured at 765 nm.

2.6. Estimation of Total Flavonoids. The total flavonoid content was estimated using an aluminum chloride colorimetric assay [24]. First, 1 mL of sample (10 mg/mL) was mixed with 0.3 mL of 5% sodium nitrite and added to the reaction mixture; after approximately 5 min, 0.3 mL of 10% aluminum chloride was added. Subsequently, another 2 mL of 1 M sodium hydroxide (NaOH) was added after 6 min, followed by the immediate addition of 2.4 mL of distilled water to produce a total volume of 10 mL. The color intensity of the flavonoid-aluminum complex was measured at 510 nm. Total flavonoid content was determined as catechin equivalent (CE) (6.25–100.00 μg/mL) and expressed as mg of CEs/g of propolis.

2.7. Determination of Ascorbic Acid Content. The ascorbic acid content in the propolis was estimated by a method established by Omaye et al. [25], with slight modifications. Briefly, 1 mL of extract (10 mg/mL) was mixed with 1 mL of a 5% trichloroacetic acid (TCA) solution and centrifuged for 15 min at 3500 rpm. Then, 0.5 mL of the supernatant was mixed with 0.1 mL of DTC (2,4-dinitrophenylhydrazine/thiourea/copper) solution and incubated for 3 hours at 37°C. To the mixture was added 0.75 mL of ice-cold 65% H_2SO_4. The solution was allowed to stand for an

additional 30 min at room temperature. The colored complex that developed was monitored at 520 nm. The ascorbic acid concentration was determined as ascorbate equivalent (AEs) (1–10 μg/mL) and expressed as mg of ascorbate equivalents (AEs) per g of sample.

2.8. Estimation of Reducing Sugar Content. The content of reducing sugars in the propolis was estimated according to the Nelson-Somogyi method [26]. Briefly, 2 mL of propolis extract (10 μg/mL) and standards (made in 0.2% of benzoic acid) were transferred into two different test tubes, followed by the addition of 2 mL of copper reagent to each tube. The tubes were heated for 15 min in a 100°C water bath and then cooled. Finally, 1 mL of arsenomolybdate color reagent was added to the reaction mixture. The absorbance was measured at 520 nm. Dextrose was used as a standard for the preparation of the calibration curve (10–100 μg/mL), and the reducing sugar content was expressed as mg of D-glucose per g of propolis.

2.9. Estimation of Total Protein Content. The total protein content in the propolis was estimated using Lowry's method [27]. Bovine serum albumin (BSA) (12.5–100.0 μg/mL) was used as a standard to prepare a calibration curve. The final results are expressed as mg of BSA equivalent per g of propolis.

2.10. Analysis of Antioxidant Properties. To investigate the antioxidant potential of the propolis extracts, DPPH radical-scavenging and FRAP assays were performed.

2.11. DPPH Free Radical-Scavenging Activity. The antioxidant potential of the propolis was determined according to the DPPH radical-scavenging activity based on a method established by Braca et al. [28]. Briefly, 1 mL of the extract was mixed with 1.2 mL of 0.003% DPPH in methanol at varying concentrations (2.5–80.0 μg/mL). The percentage of DPPH inhibition was calculated using the following equation:

$$\%\ \text{of DPPH inhibition} = \left[\left(\frac{A_{\text{DPPH}} - A_S}{A_{\text{DPPH}}}\right)\right] \times 100, \quad (1)$$

where A_{DPPH} is the absorbance of DPPH in the absence of a sample and A_S is the absorbance of DPPH in the presence of either the sample or the standard.

DPPH scavenging activity is expressed as the concentration of sample that is required to decrease DPPH absorbance by 50% (IC_{50}). The value can be graphically determined by plotting the absorbance (the percentage of inhibition of DPPH radicals) against the log concentration of DPPH and determining the slope of the nonlinear regression.

2.12. Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was performed according to a method established by Benzie and Strain [29]. The reduction of a ferric tripyridyltriazine complex into its ferrous form produces an intense blue color at low pH that can be monitored by measuring absorbance at 593 nm. Briefly, 200 μL of the solution at different concentrations (62.5–1000.0 μg/mL) was mixed with 1.5 mL of the FRAP reagent, and the reaction mixture was incubated at 37°C for 4 min. The change in absorbance was monitored at 593 nm against a distilled water blank. The FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6) with 1 volume of 10 mM TPTZ solution in 40 mM hydrochloric acid and 1 volume of 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$). The FRAP reagent was prewarmed to 37°C and was always freshly prepared. A standard curve was plotted using an aqueous solution of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) (100–1000 μmol), with FRAP values expressed as micromoles of ferrous equivalent (μM Fe [II] per kg of sample).

2.13. Experimental Animals. Adult male Wistar Albino rats ranging from 140 to 160 g were used. The animals were maintained under standard conditions of ventilation, temperature (23 ± 2°C), humidity (40–70%), and light/dark condition (12/12 h) in the animal house facility of the Department of Biochemistry and Molecular Biology, Jahangirnagar University, Savar, Dhaka. The rats were housed in polypropylene cages with soft wood-chip bedding. They were provided with a standard laboratory pellet diet and water *ad libitum*. The experiments were conducted according to the ethical guidelines as approved by Bangladesh Association for Laboratory Animal Science. The experiment protocol was approved by the Biosafety, Biosecurity, and Ethical Committee of Jahangirnagar University [Approval number BBEC, JU/M2014 (3)].

2.14. Induction of Experimental MI. MI was induced by subcutaneous injection of ISO (85 mg/kg). ISO was dissolved in normal saline and administered twice at an interval of 24 h for two consecutive days. The ISO dose was established based on a pilot study for ISO dose fixation and the results of previous studies [12, 18]. Animals were sacrificed (as below) 48 h after first ISO administration.

2.15. Experimental Design. Following one week of acclimation, the animals were randomly divided into four groups (8 rats in each group) and were treated as follows.

Group 1 (Control). Animals were given standard laboratory diet and water *ad libitum*.

Group 2 (MP). Animals were given propolis (100 mg/kg) for four weeks but were not given ISO.

Group 3 (ISO). Animals were injected with ISO (85 mg/kg) on the 29th and 30th days.

Group 4 (MP + ISO). Animals received MP (100 mg/kg) for 4 weeks prior to ISO (85 mg/kg) administration on the 29th and 30th days.

During the experimental period, the rats' body weights were recorded regularly and the doses modulated accordingly. Propolis dosage administered at 100 mg/kg was based on that reported in a previous study [30]. One day after the second ISO injection, the animals were anesthetized with

ketamine hydrochloride injection (100 mg/kg) for sacrifice prior to dissection. Blood samples (5 mL) were collected from the inferior vena cava of the rats. In addition, heart tissue was excised immediately from the surrounding tissues and was washed twice with ice-cold phosphate buffer saline (PBS), followed by storage at −20°C prior to analysis. Some of the heart samples were stored in 10% formalin for histopathological examination.

2.16. Serum Collection for Further Study. Blood samples (3 mL) were transferred to clean dry centrifuge tubes to allow for coagulation at an ambient temperature. The serum was separated by centrifugation at 2000 rpm for 10 min. The serum was kept at −20°C for subsequent biochemical analyses. The heart samples were homogenized in phosphate buffer (25 mM, pH 7.4) using a tissue homogenizer (F 12520121, Omni International, Kennesaw, USA) to make an approximately 10% w/v homogenate. This homogenate was centrifuged at 1700 rpm for 10 min. The supernatant was collected and stored at −20°C for biochemical analyses.

2.17. Estimation of Cardiac Troponin I (cTn I). The heart-specific troponin I (cTn I) level in the sera was estimated via enzyme immunoassay kits (JAJ International Inc., USA) using an ELISA reader (Digital and Analog System RS232, Das, Italy).

2.18. Assay of Cardiac Marker Enzymes. The serum activities of creatinine kinase-MB (CK-MB), aspartate transaminase (AST), lactate dehydrogenase (LDH), and alanine transaminase (ALT) were estimated via commercially available standard assay kits (Stanbio Laboratory, USA) using a PD-303S spectrophotometer (APEL, Japan).

2.19. Analyses of Lipid Profiles. The serum levels of total cholesterol (TC), triglycerides (TGs), and high-density lipoprotein-cholesterol (HDL-C) were estimated using commercially available standard assay kits (Stanbio Laboratory, USA). Serum VLDL-C levels were calculated by a formula provided by Friedewald et al. [31]:

$$\text{VLDL-C} = \frac{\text{TG}}{5}. \quad (2)$$

2.20. Estimation of LPO Products. Malondialdehyde (MDA) levels were assayed for LPO products in heart tissues according to the method described by Ohkawa et al. [32]. Briefly, tissue homogenate (0.2 mL) was mixed with 8.1% sodium dodecyl sulfate (0.2 mL), 20% acetic acid (1.5 mL), and 8% thiobarbituric acid (1.5 mL). The mixture was supplemented up to 4 mL with distilled water and was heated at 95°C in a water bath for 60 min. After incubation, the tubes were cooled to room temperature, and the final volume was increased to 5 mL. A butanol : pyridine (15 : 1) mixture (5 mL) was added, and the contents were vortexed thoroughly for 2 min. After centrifugation at 3,000 rpm for 10 min, the upper organic layer was aspirated and its absorbance was read at 532 nm against a blank. The levels of MDA were expressed as nmol of thiobarbituric acid reactive substances (TBARS) per mg of protein.

2.21. Estimation of Antioxidant Enzymes. The levels of endogenous antioxidant or antiperoxidative enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx), and glutathione-S-transferase (GST), in the rats' heart tissues were estimated using standard assay kits (Abnova Corporation, Taiwan). To this end, the heart tissue homogenates were recentrifuged at 12,000 rpm for 10 min at 4°C using Eppendorf 5415D centrifuges (Hamburg, Germany). The resulting clean supernatants of tissue homogenates were fed into the assays. The SOD activity was expressed as units/mg of protein, GPx and GRx activities were expressed as nmol NADPH oxidized/min/mg of protein, and GST activity was expressed as nmol CDNB conjugated/min/mg of protein.

The total protein content in the recentrifuged tissue homogenates was estimated by the method described by Lowry et al. [27]. Briefly, 0.2 mL of sample (digested with 0.1 N NaOH) was mixed with 2 mL of working reagent (a mixture of 2% sodium carbonate, 0.1 N NaOH, 1.56% copper sulfate, and 2.37% sodium-potassium tartrate), and the reaction mixture was incubated for 10 min at room temperature. The addition of 1 N Folin-Ciocalteu's phenol reagent (0.2 mL) was followed by 30 min of incubation at room temperature. Finally, the absorbance was measured at 660 nm using a spectrophotometer (APEL, Japan). BSA was used as the standard.

2.22. Histopathological Examination. The heart was rapidly dissected out and washed immediately with saline after sacrifice. It was then fixed in 10% formalin. The fixed tissues were then embedded in paraffin. The tissues were sectioned into 5 μm slices using a rotary microtome and then stained with hematoxylin and eosin dye for observation under a light microscope (MZ3000 Micros, St Veit/Glan, Austria) at 40x magnification. The pathologist performing the histopathological evaluation was blinded to the treatment assignment of the different study groups.

2.23. Statistical Analysis. All analyses were conducted in triplicate and the data are expressed as the mean ± standard deviation (SD). The data were analyzed using GraphPad PRISM (version 6.05; GraphPad software Inc., San Diego, CA, USA), Microsoft Excel 2007 (Redmond, Washington, USA), and SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, USA). The mean values of different groups were compared using one-way analysis of variance (ANOVA). Statistical analyses of biochemical data were conducted using Tukey's test. The minimum level of significance was set to <0.05.

3. Results

3.1. Antioxidant Constituents. The bioactive polyphenols, flavonoids, tannin and ascorbate, protein, and reducing sugar contents in MP are presented in Table 1.

Table 1: Total polyphenols, flavonoids, tannins, protein, ascorbic acid, and reducing sugar contents in MP.

Phytochemicals	Amount present in propolis (mg/g)
Total polyphenols (GAEs)	15.93 ± 0.18
Total flavonoids (CEs)	1.65 ± 0.10
Total tannins (TEs)	5.81 ± 1.65
Ascorbic acid (AEs)	0.91 ± 0.02
Total protein (BSA)	24.54 ± 0.26
Reducing sugar (D-glucose)	38.22 ± 3.22

Data are presented as means ± SD.
GAE: gallic acid equivalents; CE: catechin equivalents; TE: tannic acid equivalent; AE: ascorbate equivalent; BSA: bovine serum albumin.

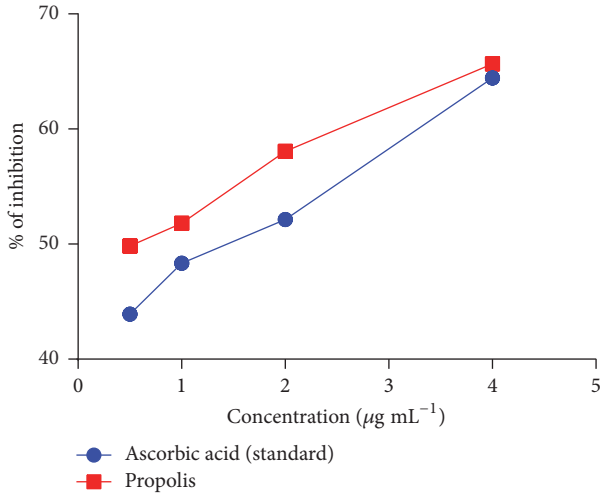

Figure 1: Percentage of DPPH inhibition of propolis against an ascorbic acid standard showing a dose-dependent inhibition potential.

3.2. Antioxidant Activity Assay. The antioxidant potential of MP was determined by both DPPH scavenging activity and the FRAP assay. The estimated IC_{50} value of DPPH scavenging activity was 1.08 μg/mL (Figure 1), and the FRAP value was 954.29 ± 3.40 [μM Fe(II)]/kg.

3.3. The Effects of Propolis on Biochemical Parameters. During the treatment period (4 weeks), no death was observed in any of the experimental groups, and no significant difference in body weight was observed between different groups (Table 2). The heart weight increased significantly in ISO-administered rats compared with that of normal control rats. However, a significant reduction in heart weight was observed in the propolis treatment (MP + ISO) group compared with that of the rats treated with ISO alone.

Serum cTn I levels were significantly increased in ISO-administered rats compared with those of the normal controls (Figure 2). However, a significant reduction in serum cTn I levels was observed in animals receiving prior treatment with propolis compared with those of ISO-treated rats.

A marked increase in serum cardiac enzyme activities was observed in ISO-induced myocardial ischemic rats. Again,

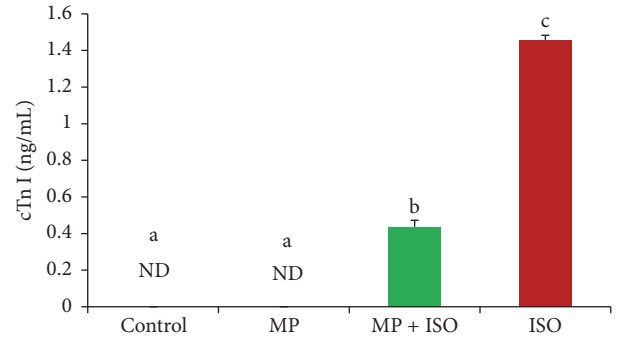

Figure 2: Effect of MP on serum cTn I in normal and treated rats. The bars represent means ± SD ($n = 8$); bars with different letters (a, b, c) indicate significantly different mean values at $p < 0.05$. ND: not detected.

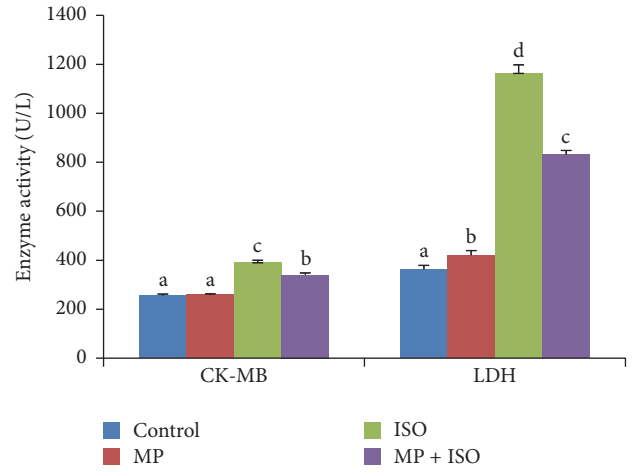

Figure 3: Effects of MP and ISO on serum CK-MB and LDH levels in normal and different treated rats. The bars represent means ± SD ($n = 8$); bars with different letters (a, b, c, d) indicate significantly different mean values at $p < 0.05$. MP: Malaysian Propolis. ISO: isoproterenol.

pretreatment with propolis significantly decreased the levels of CK-MB, LDH, AST, and ALT in rats challenged with ISO (Figures 3 and 4).

A marked increase in circulating levels of TC, TGs, and VLDL-C and a corresponding decrease in HDL-C level were observed in ISO-treated rats (Figure 5). Again, pretreatment with propolis was observed to significantly reduce the levels of TC, TGs, and VLDL-C while increasing the HDL-C level compared with the levels measured for the ISO-challenged group. Administration of MP alone did not significantly affect the lipid profile compared with that of normal control group.

ISO-induced rats exhibited a significant increase in MDA levels compared with the levels measured for the normal control group. Propolis pretreatment, however, significantly ameliorated the increase (Figure 6).

Table 3 presents the effects of propolis on the activities of antioxidant enzymes such as SOD, GRx, GPx, and GST in the heart tissue. Rats induced with ISO exhibited a significant decrease in the level of antioxidant enzymes compared with

Table 2: Effect of MP on body weights (BW) and absolute and relative heart weights.

Parameters	Treatment			
	Control	MP	ISO	MP + ISO
Initial BW (g)	133.25 ± 9.66[a]	148.62 ± 10.07[a]	139.75 ± 13.76[a]	135.12 ± 7.86[a]
Final BW (g)	162.37 ± 4.62[a]	176.25 ± 16.61[a]	169.25 ± 16.61[a]	167.87 ± 7.69[a]
BW gain (%)	17.98 ± 4.49[a]	16.76 ± 2.32[a]	17.39 ± 2.28[a]	19.54 ± 1.24[a]
Absolute heart weight (g)	0.67 ± 0.08[a]	0.68 ± 0.04[a]	0.92 ± 0.05[c]	0.75 ± 0.02[b]
Relative heart weight (g/100 g)	0.41 ± 0.05[a]	0.41 ± 0.02[a]	0.54 ± 0.02[b]	0.45 ± 0.02[a]

Data are presented as means ± SD, $n = 8$.
[a,b,c]Values in the same row that do not share superscript letters (a, b, and c) differ significantly at $p < 0.05$; % of body weight (BW) gain = [(final BW − initial BW)/ final BW] × 100.
MP: Malaysian Propolis. ISO: Isoproterenol.

Table 3: Effects of MP and ISO on the activities of superoxide dismutase (SOD), glutathione reductase (GRx), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) in the heart tissue of normal and treated rats.

Parameters	Treatment			
	Control	MP	ISO	MP + ISO
SOD (units/mg of protein)	1.53 ± 0.15[a]	1.96 ± 0.04[a]	0.13 ± 0.02[c]	0.31 ± 0.05[b]
GRx (nmol NADPH oxidized/min/mg of protein)	96.23 ± 6.70[a]	94.32 ± 1.74[a]	71.07 ± 4.78[b]	89.13 ± 9.28[a]
GPx (nmol NADPH oxidized/min/mg of protein)	2.95 ± 0.57[a]	2.27 ± 0.52[a]	0.90 ± 0.02[b]	1.98 ± 0.51[a]
GST (nmol CDNB conjugated/min/mg of protein)	2.08 ± 0.20[a]	2.55 ± 0.43[a]	1.29 ± 0.18[b]	2.13 ± 0.63[a]

Data are presented as means ± SD, $n = 8$.
[a,b,c]Values in the same row that do not share superscript letters (a, b, and c) indicate significant difference at $p < 0.05$.
MP: Malaysian Propolis. ISO: isoproterenol.

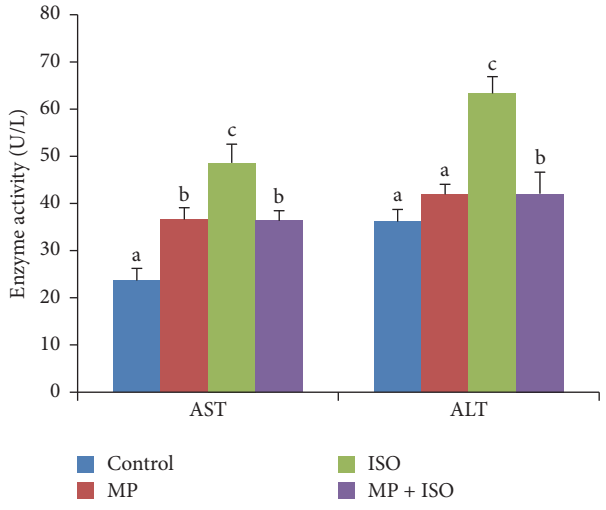

Figure 4: Effects of MP and ISO on serum AST and ALT levels in normal and different treated rats. The bars represent means ± SD ($n = 8$); bars with different letters (a, b, c) indicate significantly different mean values at $p < 0.05$. MP: Malaysian Propolis. ISO: isoproterenol.

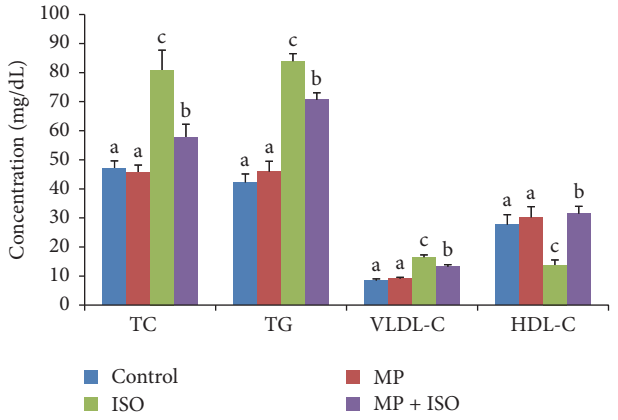

Figure 5: Effects of MP and ISO on serum lipid profile in normal and different treated rats. The bars represent means ± SD ($n = 8$); bars with different letters (a, b, c) indicate significantly different mean values at $p < 0.05$. MP: Malaysian Propolis. ISO: isoproterenol.

the level measured for the control group. However, pretreatment with propolis significantly ameliorated the levels of SOD, GRx, GPx, and GST enzymes compared with the levels measured for the rats treated with ISO alone.

Normal cardiac muscle fibers without any infarction were observed in normal control rats (Figure 7(a)). Rats treated with propolis alone also showed normal cardiac muscle bundles without any infarction or tissue damage (Figure 7(b)). ISO-treated rats showed myocardial structural changes, including coagulative necrosis, separation of cardiac muscle fibers, and infiltration of inflammatory cells (Figure 7(c)). Pretreatment with propolis decreased the degree of infiltration of inflammatory cells (Figure 7(d)).

Histological changes in the heart in the various groups are presented in Table 4.

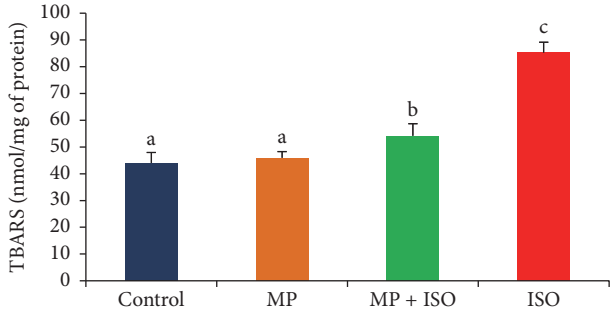

Figure 6: Effects of MP and ISO on cardiac LPO levels in normal and different treated rats. The bars represent means ± SD ($n = 8$); bars with different letters (a, b, c) indicate significantly different mean values at $p < 0.05$.

Table 4: Semiquantitative scoring of the architectural changes evidenced by histopathological examination of rat myocardial tissues.

Parameters	Treatment			
	Control	MP	ISO	MP + ISO
Muscle separation	−	−	+++	+
Cellular necrosis	−	−	+++	+
Inflammatory cells infiltration	−	−	+++	+
Edematous intramuscular space	−	−	+++	++

Scoring was performed as follows: none (−), mild (+), moderate (++), and severe (+++).

4. Discussion

To the best of our knowledge, our study is the first to determine the antioxidant potentials of Malaysian bee propolis and to investigate the effects of a propolis extract on myocardial ischemia based on the findings of several biochemical markers in addition to histopathological findings in a rat model.

Polyphenols and flavonoids are important constituents that contribute to the functional quality, color, and flavor of bee products and serve as powerful antioxidants due to the hydrogen-donating ability of their hydroxyl groups as well as their ability to donate electrons to arrest the production of free radicals as a result of oxidative stress [33]. Tannins are another important subclass of water-soluble phenolic compounds. They have been reported to exhibit significant astringent, antimicrobial, and antioxidant properties [34, 35]. Our findings indicate that MP serves as a reserve source of phenolics, flavonoids, and tannins and may therefore play a significant role as a singlet oxygen quencher and free radical scavenger to minimize molecular damage of the cell.

Ascorbic acid is a small antioxidant molecule that directly interacts with a broad spectrum of ROS, terminating the chain reaction initiated by free radicals via electron transfer as well as participating in the regeneration of vitamin E [33]. To the best of our knowledge, our study is the first to report on the ascorbic acid content of MP, which, when coupled with the presence of other antioxidants, can be used as a natural source of antioxidants. Our study also confirms that MP is a rich source of reducing sugars with a significant amount of protein.

The antioxidant activities of MP were evaluated by FRAP and DPPH assays. FRAP assays primarily measure the abilities of antioxidants to reduce ferric tripyridyltriazine (Fe^{3+}) to their ferrous form (Fe^{2+}), whereas DPPH assays measure percentages of radical-scavenging activity [35]. The FRAP and DPPH assays confirmed the high antioxidant potential of MP, as previously reported [9].

MI is a clinical syndrome arising from sudden and persistent curtailment of myocardial blood supply, resulting in the necrosis of myocardium [36]. Administration of ISO contributes to the release of cellular enzymes in the circulation due to irreversible cardiac damage, which leads to an alteration in the integrity of the plasma membrane as a response to β-adrenergic stimulation. The stimulation generates ROS and downregulates copper-zinc superoxide dismutase activity, reduces glutathione levels (which leads to the loss of membrane integrity), induces heart contractile dysfunction and myocyte toxicity, and finally produces myocardial necrosis [37]. In the present study, the heart weights increased significantly with relatively no change in body weight following ISO administration, which contributed to the increased heart weight to body weight ratio. The increased heart weights may be attributed to increased water content and edematous intramuscular space [38], which was confirmed by our histopathological findings. Pretreatment with propolis, however, helped to maintain near-normal heart weights, indicating the therapeutic benefits of the substance.

Cardiac troponin I (cTn I) is a contractile protein that is a highly sensitive and specific marker of myocardial cell injury; it is normally absent in serum and released only following myocardial necrosis [39]. Our study confirmed that serum cTn I levels were significantly higher in ISO-treated rats than in the normal control group, which may be attributed to ISO-induced cardiac damage. The results are consistent with those reported by Afroz et al. [12]. Animals challenged with ISO after pretreatment with propolis, however, showed significantly lower cTn I levels compared with those of rats treated with ISO alone. This phenomenon may be attributed to the protective effect of phenolics in propolis on the myocardium by preserving the structural and functional integrity of the contractile apparatus and thus preventing oxidative injury of cardiac muscles.

An insufficient supply of oxygen or nutrients to cardiac tissues or chemically induced cardiac damage may increase the permeability or even rupture the cardiac membrane, resulting in leakage of cytosolic enzymes, including CK-MB, AST, LDH, and ALT (diagnostic markers of MI), into the bloodstream and a subsequent increase in their serum concentrations [12, 40]. The CK-MB activity assay is an important and reliable diagnostic index for MI because of the marked abundance of CK-MB in the myocardium and its virtual absence in most other tissues and consequent sensitivity [41]. In the present study, ISO administration in rats caused a marked elevation in the activities of all the cardiac marker enzymes in the serum, in accord with previously reported studies [12, 18], an important indication of ISO-induced necrotic damage of the myocardium and leakiness of the plasma membrane. Pretreatment with MP,

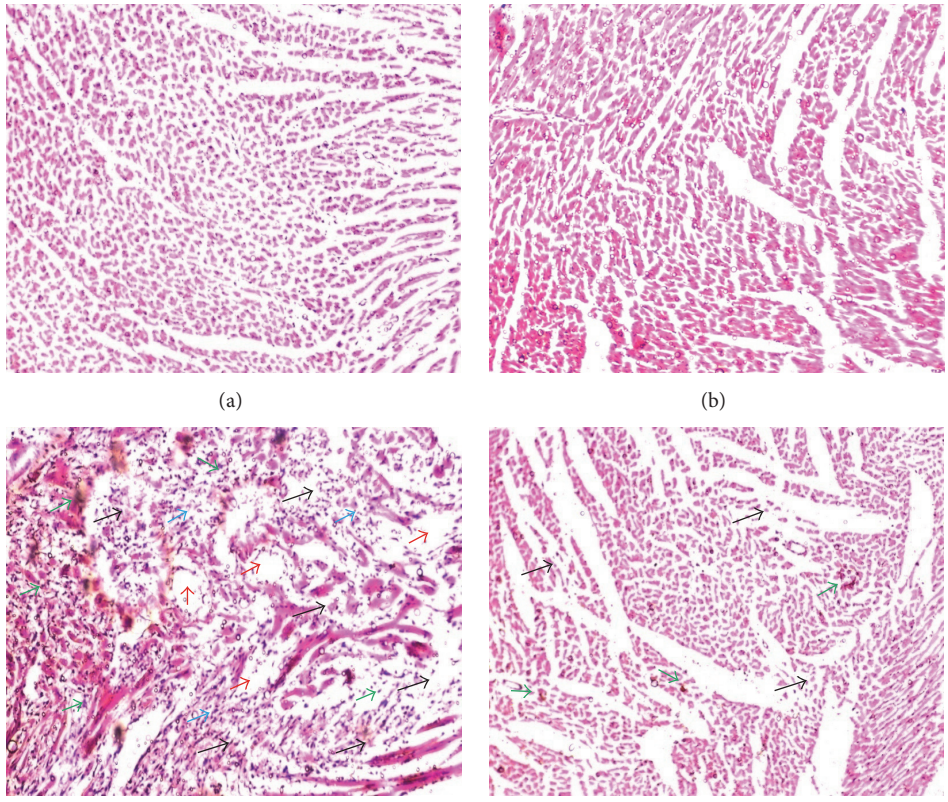

Figure 7: (a) Group I: H & E stained myocardial tissue section from normal control heart showing normal cardiac muscle fibers. (b) Group II: pretreatment with MP (100 mg/kg) showing normal muscle fibers without any pathological changes. (c) Group III: ISO (85 mg/kg) treated heart showing cardiac muscle fibers with muscle separation (black arrows), edematous intramuscular space (red arrows), cellular necrosis (blue arrows), and infiltration of inflammatory cells (green arrows). (d) MP (100 mg/kg) + ISO (85 mg/kg) treated heart showing lowered inflammatory cells (green arrows) and reduced muscle fibrous separation with edematous intramuscular space.

however, significantly ameliorated the activities of all marker enzymes in the serum, indicating that propolis helps maintain membrane integrity, thereby limiting the leakage of the enzymes. Polyphenols such as tannic acid, ascorbic acid, flavonoids, and reducing sugars confirmed to be present in MP are important constitutive antioxidants that may confer a protective effect against oxidative cardiac injury, thus restricting the leakage of these enzymes from the myocardium.

Plasma cholesterols and triglycerides play a key role in the pathogenesis of cardiovascular disease not only due to the development of atherosclerosis but also due to the modification of the composition, structure, and stability of cellular membranes [42]. A significant increase in the levels of TC, TG, and VLDL-C along with a decrease in HDL-C levels was observed in ISO-treated rats, as previously reported [40]. There are a positive correlation between high levels of LDL-C and VLDL-C with MI and a negative correlation between high levels of HDL-C and MI. HDL-C inhibits the uptake of LDL-C by the arterial walls and facilitates the transport of cholesterol from peripheral tissues to the liver and is catabolized and excreted from the body [43]. Administration of MP significantly restored these alterations, thereby maintaining the normal fluidity and function of the myocardial membrane. The improvement in lipoprotein status has been due to the polyphenols in MP, which are responsible for the upregulation of proinflammatory cytokines, chemokines, and angiogenic factors and consequent inhibition of progression of atherosclerosis [44].

Propolis is also reported to downregulate the mRNA expression of key genes, including monocyte chemoattractant protein (MCP)-1, interferon gamma, interleukin-6, cluster of differentiation (CD) 36, and transforming growth factor beta (TGF-β), which have been associated with the atherosclerotic process [44, 45]. Moreover, MP lowered TC levels and elevated HDL-C levels, as also reported by Daleprane et al. [45], which may contribute to the upregulation of ABCA 1 gene expression associated with increased HDL-C levels and restoration of lipid profiles in animals [44].

LPO, a type of oxidative deterioration of PUFAs, has been linked to pathogenic events in MI [14, 40]. The myocardial necrosis observed in the rats receiving ISO can be attributed to peroxidative damage because it has been previously reported that ISO generates lipid peroxides [46]. Increased LPO appears to be the initial stage of the pathogenesis, making heart tissue more susceptible to oxidative damage [18]. ISO administration caused a marked elevation in LPO, which

was expressed as MDA content, in line with previous reports [14, 40]. Oral pretreatment with MP led to a significant reduction in myocardial MDA content. This result can be attributed to the presence of flavonoids in MP, which can scavenge LPO products generated excessively by ISO, thus conferring protection to the cardiac tissue [47]. It has been reported that the synergistic scavenging effects of bee products may be due to both enzymatic and nonenzymatic antioxidants involved in cardiovascular defense mechanisms [48, 49].

The oxidative stress may be exerted through quinone metabolites of ISO that react with molecular oxygen to produce superoxide anions and other ROS, which may interfere with cellular antioxidant enzymes [18]. Endogenous antioxidant enzymatic defense plays a key role in neutralizing oxygen free radical-mediated tissue injury [50]. SOD, catalase, and GPx are the primary free radical-scavenging enzymes involved in the first line of cellular defense against oxidative injury, removing both oxygen (O_2) and hydrogen peroxide (H_2O_2) before they can interact to form more reactive hydroxyl radicals [51, 52].

In our study, significantly reduced activities of SOD and GPx and GRx and GST were observed in the heart tissues of ISO-treated rats compared with the activities observed in control rats. The observed decreases in the activities of these enzymes are in accord with findings reported in previous studies [12, 53]; such decreases may be due to the increased utilization of these enzymes for scavenging ROS and their inactivation by excessive ISO oxidants [52]. Pretreatment with MP, however, improved the activities of cellular antiperoxidative enzymes. The findings could be due to the direct free radical-scavenging potency of phenolics in propolis [47]. It is also plausible that MP restores antioxidant enzyme function via upregulation of the activity or expression of Nrf2, an important intracellular transcription factor, released from its repressor (Keap1) under oxidative or xenobiotic stress [46]. The released Nrf2 binds to the antioxidant response element (ARE) in the promoter region of cytoprotective genes and induces their expression. The transcribed genes subsequently induce the expression of free radical-scavenging enzymes to neutralize, detoxify, and eliminate the cytotoxic oxidants [46, 54, 55].

The biochemical improvements reported in the current study are consistent with the histopathological findings, in which the heart tissues of normal control rats and rats treated with MP alone clearly illustrated the integrity of the myocardial cell membrane. The tissue of rats treated with ISO alone showed widespread myofibrillar disorder, necrosis of myocytes, inflammatory cell infiltration, and cardiac muscle fiber separation. However, MP-pretreated hearts showed a near-normal morphology of cardiac muscle with the absence of necrosis and reduced inflammatory cells compared with the hearts of rats treated with ISO alone. Similar histopathological findings were obtained in ISO-treated rats with respect to gallic acid, a phenolic acid also present in honey [56], which also showed remarkable antioxidant properties, further supporting the antioxidant potential of MP as a cardioprotectant. Nevertheless, further research must be undertaken to investigate the active constituents present in MP to confirm the cardioprotective mechanism.

5. Conclusion

MP is a promising source of natural antioxidants, as confirmed by its high polyphenols, flavonoids, tannins, ascorbic acid, and reducing sugar contents, as well as its considerably high DPPH free radical-scavenging activities and FRAP values. Our *in vivo* study confirmed that MP significantly altered nearly all biochemical parameters associated with ISO-induced myocardial injury, as further supported by histopathological findings. MP may have antilipoperoxidative and antioxidant effects that confer these cardioprotective effects.

Disclosure

Romana Ahmed and E. M. Tanvir are joint first authors.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research is supported by the TWAS research grant (no. 14-385 RG/PH/AS_C-UNESCO FR: 3240283438) and the Research and Development (R&D) project 2015-2016 research grant (39.012.002.02.01.018.2015-R&D-59).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 1342150, 9 pages
https://doi.org/10.1155/2017/1342150

Research Article

Four-Week Consumption of Malaysian Honey Reduces Excess Weight Gain and Improves Obesity-Related Parameters in High Fat Diet Induced Obese Rats

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Received 22 July 2016; Accepted 9 October 2016; Published 26 January 2017

Academic Editor: Andrzej K. Kuropatnicki

Many studies revealed the potential of honey consumption in controlling obesity. However, no study has been conducted using Malaysian honey. In this study, we investigated the efficacy of two local Malaysian honey types: Gelam and Acacia honey in reducing excess weight gain and other parameters related to obesity. The quality of both honey types was determined through physicochemical analysis and contents of phenolic and flavonoid. Male Sprague-Dawley rats were induced to become obese using high fat diet (HFD) prior to introduction with/without honey or orlistat for four weeks. Significant reductions in excess weight gain and adiposity index were observed in rats fed with Gelam honey compared to HFD rats. Moreover, levels of plasma glucose, triglycerides, and cholesterol, plasma leptin and resistin, liver enzymes, renal function test, and relative organ weight in Gelam and Acacia honey treated groups were reduced significantly when compared to rats fed with HFD only. Similar results were also displayed in rats treated with orlistat, but with hepatotoxicity effects. In conclusion, consumption of honey can be used to control obesity by regulating lipid metabolism and appears to be more effective than orlistat.

1. Introduction

2.1 billion people worldwide are overweight and obese, regardless of their socioeconomic status reported in 2013 [1]. Thus, both overweight and obesity are identified as a major contributor to other chronic diseases such as diabetes, cardiovascular diseases, and even certain cancers [2]. Various factors are known to lead to this health problem, but ease of accessibility to unhealthy foods has been identified as the major culprit [3].

Honey can be described as one of the functional foods. Besides its natural sweet taste, honey has a low glycemic index and other medicinal properties. Its benefit to health and its usage have been well known to mankind since ancient times and were recorded in medical texts from various civilizations [4, 5]. Findings from many studies also showed the ability of honey in controlling overweight and obesity when consumed orally, thus making it a potential antiobesity agent [6–10]. However, the effect using the local honey is still unknown and to be investigated in this study.

A variety of honey found in Malaysia are diverse, which include both blossom and honeydew honey, due to their tropical climate and being rich in floral sources [11]. Biochemical and pharmacological activities of honey vary, depending on its location, weather and humidity, nectar source, and handling during harvesting and storage [11]. In this study, two types of Malaysian honey were used to observe its effects on high fat diet-induced Sprague-Dawley male rats. Gelam honey, categorised as a blossom honey, was produced by *Apis dorsata*, a wild and native honeybee, from nectars of *Melaleuca cajuputi* trees and harvested from the forest. Meanwhile, Acacia honey is honeydew honey produced by

A. mellifera, also known as European honeybee, from sugary fluids produced by *Acacia mangium* trees and reared by beekeepers in wooden hives [11].

2. Methods

2.1. Materials. Catechin, Folin-Ciocalteu's reagent, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate (Na_2CO_3), aluminum chloride ($AlCl_3$), sodium nitrite ($NaNO_2$), and sodium hydroxide (NaOH) were received from Merck (Darmstadt, Germany). All the chemicals used were of analytical grade. The gastrointestinal lipase inhibitor for obesity, orlistat (XENICAL), was obtained from a local drugstore.

2.2. Honey Sample Preparation. Gelam honey was harvested from wild honeybees found in a Gelam forest in Terengganu, Malaysia. The Acacia honey was collected from a bee farm under the Department of Agriculture, Johor, Malaysia. The samples were irradiated with 25 kGy of gamma radiation using the radioactive source cobalt 60 (model JS8900), at the Malaysian Nuclear Agency (MINT), Selangor, Malaysia [12]. The irradiated honey was then kept at 4°C away from direct sunlight and in amber bottles.

2.3. Physicochemical Analysis of Honey. The pH of Gelam and Acacia honey was determined according to the method described by the International Honey Commission (IHC) [13]. It was measured using a digital pH meter (HI 98127, Hanna Instruments, Mauritius) which was calibrated at room temperature using buffer solutions [14]. Meanwhile, the acidity of both honey types was determined using the titrimetric method (with 0.1 M sodium hydroxide solution) as described in the IHC method [13].

Moisture content was determined using a refractometer. In general, the refractive index indicates the solid content in a sample of honey. The refractive index of both honey types was measured at ambient temperature using an Atago handheld refractometer (Master Refractometer, Japan) and measurements were further corrected at the standard temperature of 20°C by adding a correction factor of 0.00023/°C. The moisture content was measured in triplicate, and the percentage of moisture content that corresponds to the corrected refractive index was calculated [14].

Ash content was measured according to the Association of Official Analytical Chemists (AOAC, 1990) official method 942.05 [15]. Briefly, two grams of each honey type was placed in a porcelain crucible and weighed. After that, samples were dried in an oven at 110°C for four hours, to remove moisture that would cause foaming during the early stages of ashing. The materials were then ashed in an electrical furnace at 600°C for six hours, followed by cooling in a desiccator and then weighed. The ash content on dry basis was calculated according to the following equation:

$$\text{Percentage ash content on dry basis} = \left[\frac{(C - A)}{(B - A)}\right] \times 100, \quad (1)$$

where A is weight of the crucible, B is weight of crucible and sample after evaporation, and C is weight of crucible and sample after ashing.

2.4. Determination of Total Phenolic Contents. The concentration of total phenolic contents in the Gelam and Acacia honey samples was estimated using Folin-Ciocalteu method with slight modifications. Gallic acid was used to obtain a standard curve (20, 40, 60, 80, and 100 μg/mL; $r^2 = 0.9336$) [16]. The concentration of the phenolic compounds from both honey types was measured in triplicate. The results were reported as the mean ± standard deviation and are expressed as mg of gallic acid equivalents (GAEs) per kg of honey.

2.5. Determination of Total Flavonoid Content. The total flavonoid content in Gelam and Acacia honey samples was measured using the colorimetric assay as described in Zhishen et al. (1999) [17]. A calibration curve was obtained using standard solutions of catechin (20, 40, 60, 80, and 100 μg/mL; $r^2 = 0.998$). The results were expressed as mg catechin equivalents (CE) per kg of honey.

2.6. Animal Husbandry. All animal experiments were carried out under protocols approved by the Research Committee on the Ethical Use of Animals (UITM Care), Reference no. 5/2012. Seven-week-old male Sprague-Dawley rats, with body weight ranging from 200 to 220 g, were obtained from Laboratory Facilities of Animal Management (LAFAM), University Technology MARA (UiTM), Puncak Alam, Selangor, Malaysia. Animals were housed as one rat per cage with a 12-hour light/dark cycle [18].

2.7. Obesity Induction and Treatment. Control rats were fed with a normal diet, while obese induced rats were fed with a high fat diet (HFD) to elicit diet-induced obesity for four weeks. Then, the animals were subdivided into five groups consisting of six rats per group for another 4 weeks with or without treatment. The groups with high fat diet fed with Gelam honey (HFDGH) and the group receiving high fat diet fed with Acacia honey (HFDAH) served as treatment groups. Rats fed with high fat diet treated with orlistat (HFDO) served as a positive control group.

2.8. Body Weight and Meal Pattern Analysis. The body weight of each rat was recorded once a week and the differences in body weight were recorded. In the meal pattern analysis, the amount of food consumed from each rat was measured weekly by subtracting from the quantity of food supplied initially. Energy efficiency of each rat was calculated at the end of the study [18].

2.9. Specimen Collection. Blood was collected from the abdominal aorta of the rat, anesthetised using diethyl ether after fasting for 12 hours on the last day of the 8-week feeding period. The collected blood was processed using a microcentrifuge method, and the serum was stored in a freezer at −80°C. The retroperitoneal, epididymal, and visceral fat pads were removed and weighed [18].

Table 1: Physiochemical analysis and total phenolic and total flavonoid contents of Gelam and Acacia honey.

Samples	pH	Free acidity (meq/kg)	Moisture content (%)	Ash (%)	Total phenolic content (GAE mg/L)	Total flavonoid content (CEmg/L)
Gelam honey	3.40 ± 0.02	59.00 ± 1.02	24.90 ± 0.05	0.14 ± 0.01	55.28 ± 0.40	79.57 ± 2.73
Acacia honey	3.55 ± 0.03	85.00 ± 1.71[a]	22.30 ± 1.03	0.18 ± 0.01	33.72 ± 0.45[a]	27.84 ± 0.08[a]

Results are presented as means ± SEM, $n = 3$.
[a]Values are statistically significant at $p < 0.05$ compared to Gelam honey.

2.10. Anthropometrical and Adiposity Index Determinations. The body weight and body length were used to determine the following anthropometrical parameters:

(i) Body mass index (BMI) = body weight (g)/length^2 (cm^2).

(ii) Lee's index = cube root of body weight (g)/nose to anus length (cm).

Adiposity index was determined by the total weight of epididymal, visceral, and retroperitoneal fat divided by body weight × 100 and expressed as adiposity percentage (% AI).

2.11. Biochemical Analysis. The blood samples were centrifuged at 4 000 revolutions per minute (rpm) for 15 minutes at 4°C. The clear serum obtained was separated, labelled, and subjected to hepatic function tests, including alkaline phosphate (ALP), aspartate aminotransferase (AST) and alanine transaminase (ALT), renal function test (urea and creatinine), and serum lipid profile (glucose, triglycerides, and total cholesterol). These parameters were determined using an Autoanalyser (ILAB 300 Plus Clinical Chemistry Analyser, Milano, Italy). Serum levels of leptin and resistin were assessed using ELISA kits (USCN Life Science and Technology, Wuhan, China). Rat total adiponectin level was determined using Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the instructions provided by the manufacturer.

2.12. Relative Organ Weight. The relative organ weight (ROW) of each organ was then calculated according to the following equation:

$$\text{ROW} = \frac{(\text{absolute organ weight (g)} \times 100)}{\text{body weight of rat on sacrifice day (g)}}. \quad (2)$$

2.13. Histological Evaluation. A comprehensive gross observation was carried out on the internal organs such as the liver, spleen, lung, kidneys, and heart. They were observed for any signs of abnormality and for the presence of lesions owing to any effects of the administration of the treatment and high fat diet intake. The organs were then carefully dissected, cleaned from any fat, and weighed.

Each organ was then preserved in 10% buffered formalin for subsequent histopathological examination. The tissues were embedded in paraffin and then sectioned; the sections were cut at 4-5 microns with the rotary microtone, stained with hematoxylin, and examined microscopically.

2.14. Statistical Analysis. Results were expressed as mean ± standard error mean (SEM). The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's test *post hoc* using SPSS software version 18.0 (SPSS, Chicago, IL, USA). Values with a confidence level of $p < 0.05$ were considered significant.

3. Results

pH values and ash contents of Gelam and Acacia honey were almost similar (Table 1). However, free acidity of Gelam honey was lower than Acacia honey. The moisture content of Gelam and Acacia honey was over 20%. Gelam honey appeared significantly higher in total phenolic content and flavonoid content compared to Acacia honey (Table 1).

Weight gain and adiposity index in rats fed with high fat diet were significantly increased compared to control group (Table 2), indicating obesity model was successfully established for four weeks in this study. Similar pattern was observed for rats in other groups prior to initiating the treatment for another four weeks. Rats fed with Gelam honey and treated with orlistat, respectively, but not Acacia honey, exhibited significant reduction in both parameters. However, rats fed with Gelam honey showed significant increase in total food intake when compared to other groups. In addition, the rats consistently demonstrated high energy efficiency compared to the other groups. BMI and Lee's index in the treatment groups were not significantly different compared to HFD group. Interestingly, BMI for rats fed with Gelam honey and orlistat were in between control and HFD groups.

Glucose, triglyceride, and cholesterol of rats in HFD group were significantly higher compared to the control rats (Table 3). Similar patterns were seen in rats fed with Acacia honey, but significantly lower compared to HFD group. Both rats in HFDGH groups were significantly reduced in their glucose, triglyceride, and cholesterol levels compared to rats in HFD group.

Similar observation was recorded in HFD group in the level of adipocytokines (leptin, resistin, and adiponectin) once compared with control group (Table 4). Mixed results were found in all treatment groups, whereby the values fall between control and HFD groups. But generally, the treatment reduced all the adipocytokines level compared to the HFD group.

Most parameters from liver enzymes and renal function test in HFD group increased significantly when compared to the control group except for levels of urea and total protein (Table 5). Rats in the treatment groups showed all

Table 2: Effect of four-week treatment after four-week obesity inducement in male Sprague-Dawley rats on percentage weight gain, energy efficiency, adiposity index, BMI, and Lee's index.

Test	Group				
	NC	HFD	HFDGH	HFDAH	HFDO
Weight gain (%)	20.32 ± 2.09	44.34 ± 2.15[a]	38.39 ± 2.55[a,b]	40.06 ± 2.40[a]	33.37 ± 1.48[a,b]
Total food intake (kg)	1.23 ± 0.08	1.27 ± 0.11	1.46 ± 0.07[a,b]	1.27 ± 0.10	1.27 ± 0.10
Energy efficiency	0.17 ± 0.03	0.22 ± 0.02[a]	0.26 ± 0.02[a,b]	0.24 ± 0.02[a]	0.18 ± 0.01[b]
Adiposity index (%)	0.60 ± 0.08	1.13 ± 0.04[a]	0.85 ± 0.03[b]	0.96 ± 0.02[b]	0.80 ± 0.12[b]
BMI	0.70 ± 0.01	0.77 ± 0.03[a]	0.74 ± 0.04	0.75 ± 0.06	0.72 ± 0.06
Lee's index	0.30 ± 0.01	0.35 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.30 ± 0.01

Results are presented as means ± SEM, $n = 6$.
Values are statistically significant at $p < 0.05$.
[a]Significantly different compared to control (NC) group; [b]significantly different from high fat diet (HFD) group.
NC: normal control, HFD: high fat diet, HFDGH: high fat diet rats fed with Gelam honey, HFDAH: high fat diet rats fed with Acacia honey, HFDO: high fat diet rats treated with orlistat.

Table 3: Effects of four-week treatment after four-week obesity inducement in male Sprague-Dawley rats on levels of glucose, triglyceride, and cholesterol.

Test	Group				
	NC	HFD	HFDGH	HFDAH	HFDO
Glucose (mmol/L)	4.03 ± 0.40	13.00 ± 1.21[a]	5.72 ± 0.44[b]	7.55 ± 0.70[a,b]	5.53 ± 0.51[b]
Triglyceride (mmol/L)	11.10 ± 0.57	17.45 ± 1.10[a]	11.75 ± 1.19[b]	13.61 ± 1.06[b]	14.02 ± 1.01[a,b]
Cholesterol (mmol/L)	5.57 ± 0.37	14.13 ± 0.76[a]	6.60 ± 0.64[b]	8.73 ± 0.82[a,b]	8.53 ± 0.66[a,b]

Results are presented as means ± SEM, $n = 6$.
Values are statistically significant at $p < 0.05$.
[a]Significantly different compared to control (NC) group; [b]significantly different from high fat diet (HFD) group.
NC: normal control, HFD: high fat diet, HFDGH: high fat diet rats fed with Gelam honey, HFDAH: high fat diet rats fed with Acacia honey, HFDO: high fat diet rats treated with orlistat.

Table 4: Effects of four-week treatment after four-week obesity inducement in male Sprague-Dawley rats on adipocytokines levels of leptin, resistin, and adiponectin.

Test	Group				
	NC	HFD	HFDGH	HFDAH	HFDO
Leptin (ng/mL)	8.95 ± 0.76	21.70 ± 1.52[a]	13.66 ± 0.95[a,b]	12.13 ± 1.23[a,b]	13.32 ± 1.04[a,b]
Resistin (ng/mL)	39.21 ± 1.09	48.39 ± 1.7[a]	41.87 ± 1.13[b]	42.18 ± 1.28[b]	41.57 ± 1.03
Adiponectin (ng/mL)	33.67 ± 1.09	30.51 ± 0.78[a]	34.94 ± 1.12[b]	32.78 ± 1.20	41.57 ± 0.91[a,b]

Results are presented as means ± SEM, $n = 6$.
Values are statistically significant at $p < 0.05$.
[a]Significantly different compared to control (NC) group; [b]significantly different from high fat diet (HFD) group.
NC: normal control, HFD: high fat diet, HFDGH: high fat diet rats fed with Gelam honey, HFDAH: high fat diet rats fed with Acacia honey, HFDO: high fat diet rats treated with orlistat.

Table 5: Effects of four weeks of Gelam honey and Acacia honey consumption on levels of liver enzymes and renal function test in rats fed high fat diet for four weeks.

Test	Group				
	NC	HFD	HFDGH	HFDAH	HFDO
ALP (UI)	118.33 ± 5.16	181.50 ± 6.42[a]	135.17 ± 5.35[a,b]	147.00 ± 4.56[a,b]	136.83 ± 3.48[a,b]
AST (U/L)	119.90 ± 1.91	160.40 ± 3.01[a]	120.23 ± 3.05[b]	138.57 ± 2.56[a,b]	130.90 ± 1.08[a,b]
ALT (U/L)	67.53 ± 1.55	81.13 ± 1.60[a]	69.31 ± 1.31[b]	73.13 ± 1.96[b]	71.97 ± 0.62[b]
Urea (mmol/L)	29.75 ± 0.92	16.55 ± 0.84[a]	18.28 ± 0.78[a]	18.41 ± 1.26[a]	17.45 ± 0.95[a]
Creatinine (μmol/L)	20.48 ± 5.12	31.15 ± 6.32[a]	24.15 ± 2.18[a,b]	28.98 ± 4.17[a]	34.82 ± 7.02[a]
Total protein (g/L)	25.35 ± 1.40	23.66 ± 2.38	16.43 ± 1.37[a,b]	17.32 ± 2.82[a,b]	18.49 ± 1.38[a,b]

Results are presented as means ± SEM, $n = 6$.
Values are statistically significant at $p < 0.05$.
[a]Significantly different compared to control (NC) group; [b]significantly different from high fat diet (HFD) group.
NC: control rats, NC: normal control, HFD: high fat diet, HFDGH: high fat diet rats fed with Gelam honey, HFDAH: high fat diet rats fed with Acacia honey, HFDO: high fat diet rats treated with orlistat, ALP: alkaline phosphate, AST: aspartate aminotransferase, and ALT: alanine aminotransferase.

Table 6: Relative organ weights of rats' organs treated for four weeks after fed four-week high fat diet.

Test	Group				
	NC	HFD	HFDGH	HFDAH	HFDO
Liver	2.47 ± 0.43	3.26 ± 0.54[a]	2.75 ± 0.14[b]	2.56 ± 0.22[b]	2.77 ± 0.34[b]
Kidney	0.57 ± 0.06	0.61 ± 0.04	0.56 ± 0.05	0.57 ± 0.04	0.60 ± 0.06
Spleen	0.20 ± 0.03	0.17 ± 0.01	0.16 ± 0.03	0.15 ± 0.03	0.17 ± 0.02
Heart	0.29 ± 0.02	0.33 ± 0.04[a]	0.30 ± 0.01	0.29 ± 0.03[b]	0.30 ± 0.04
Lung	0.35 ± 0.05	0.39 ± 0.02[a]	0.35 ± 0.02[b]	0.34 ± 0.04[b]	0.35 ± 0.05[b]

Results are presented as means ± SEM, $n = 6$.
Values are statistically significant at $p < 0.05$.
[a]Significantly different compared to control (NC) group; [b]significantly different from high fat diet (HFD) group.
NC: normal control, HFD: high fat diet, HFDGH: high fat diet rats fed with Gelam honey, HFDAH: high fat diet rats fed with Acacia honey, HFDO: high fat diet rats treated with orlistat.

the measured parameters, except for total protein, were in between levels of control and HFD groups. Interestingly, all treatment groups showed significant decrease in total protein compared to both control and HFD groups.

Relative organ weight (ROW) of liver, heart, and lungs for rats in HFD group showed a significant increase compared to control rats (Table 6). In this parameter, rats fed with Acacia honey showed more prominent effect, where its ROW showed significant increase compared to rats in HFD group, particularly the liver, heart, and lung.

Histopathological evaluations revealed that liver section from rats fed with normal diet (control group) had shown normal morphological appearance (Figure 1(a)). However, livers in the rats that were fed with HFD, fat accumulation, and high number of ballooned hepatocytes were observed (Figure 1(b)). Meanwhile, hepatic tissue of rats fed with both honey types showed normal hepatic tissues with normal hepatic strands; hepatocytes were well arranged from the central vein and were separated by sinusoids (Figures 1(c) and 1(d)). However, the histological architecture of liver sections in rats in HFDO group (Figure 1(e)) showed abnormal patterns, with a mild degree of necrosis and slight lymphocyte infiltration, almost comparable to those of the control group (Figure 1(a)).

4. Discussion

Prior to commencing the effect of honey consumption in an obese animal model, physicochemical of Gelam and Acacia honey was measured. The analysis was conducted to standardise the honey condition and to measure the quality of honey used in the study [19, 20]. pH for both honey types was almost similar and within the acceptable range for fresh honey (pH 3.4 to 6.1) set by Codex Standard for honey and the IHC [12, 21, 22]. The pH value was also in parallel to other Malaysian honey reported by Moniruzzaman et al. (2013) [11]. However, Gelam honey was found more acidic compared to Acacia honey. It is because Gelam honey is blossom or floral honey, which contains more antioxidant properties, compared to honeydew honey such as Acacia honey [11].

Antioxidant properties such as total phenolic and flavonoid contents measured in Gelam honey were significantly higher compared to Acacia honey. It contributes to more free acidity in Gelam honey than in Acacia honey as mentioned above. The presence of phenolic acids and flavonoids in honey samples might act as a proton donator leading to the formation of acidified honey with low pH value [23]. This means that phenolic acids and flavonoids are one of the main components responsible for the antioxidant activity of honey that may give a health-protective and therapeutic impact of chronic diseases [24]. Both honey types contain more than 20% of moisture content, slightly higher than level set by the Codex. It is predictable since Malaysia has a tropical climate, which is high in humidity, hot, and rainy all year round [12]. Ash content for both honey types was almost similar. The content depends on the plant nectar and is influenced by several factors including environmental, botanical, and geographical factors [25].

Obesity induction using high fat diet in animal model was used in the study as the approach has high relevancy of mimicking the usual route of obesity occurrence in human [26]. The consumption of high fat diet led to obesity because it facilitates the development of a positive energy balance, leading to an increase in visceral fat deposition, and thus led to abdominal obesity [27]. Seven-week-old male Sprague-Dawley rats, with body weight ranging between 200 and 220 g, were selected for this study, which is a preferred animal model for studying obesity in humans [27, 28]. The rats were induced to become obese for four weeks by monitoring their body weight to increase by more than 125% compared to the rats fed with normal diet [26, 28].

Results showed that, after the first four weeks, followed by the second four-week feeding with high fat diet, rats in HFD group were successfully induced to become obese compared to control rats. Meanwhile, obese induced rats (four weeks) and then fed with honey (four weeks) have shown significant weight reduction compared to HFD group (Table 2). Interesting, rats fed with Gelam honey but not Acacia honey displayed significantly higher total food intake, but low in weight gain compared to HFD group and higher in energy efficiency than displayed by the HFDO group (Table 2). Rationally, a higher kilojoule intake will lead to more weight gain but this was not seen in the rats fed with the honey. The consumption of honey may induce the conversion of excess food into energy instead of being converted into fat for storage.

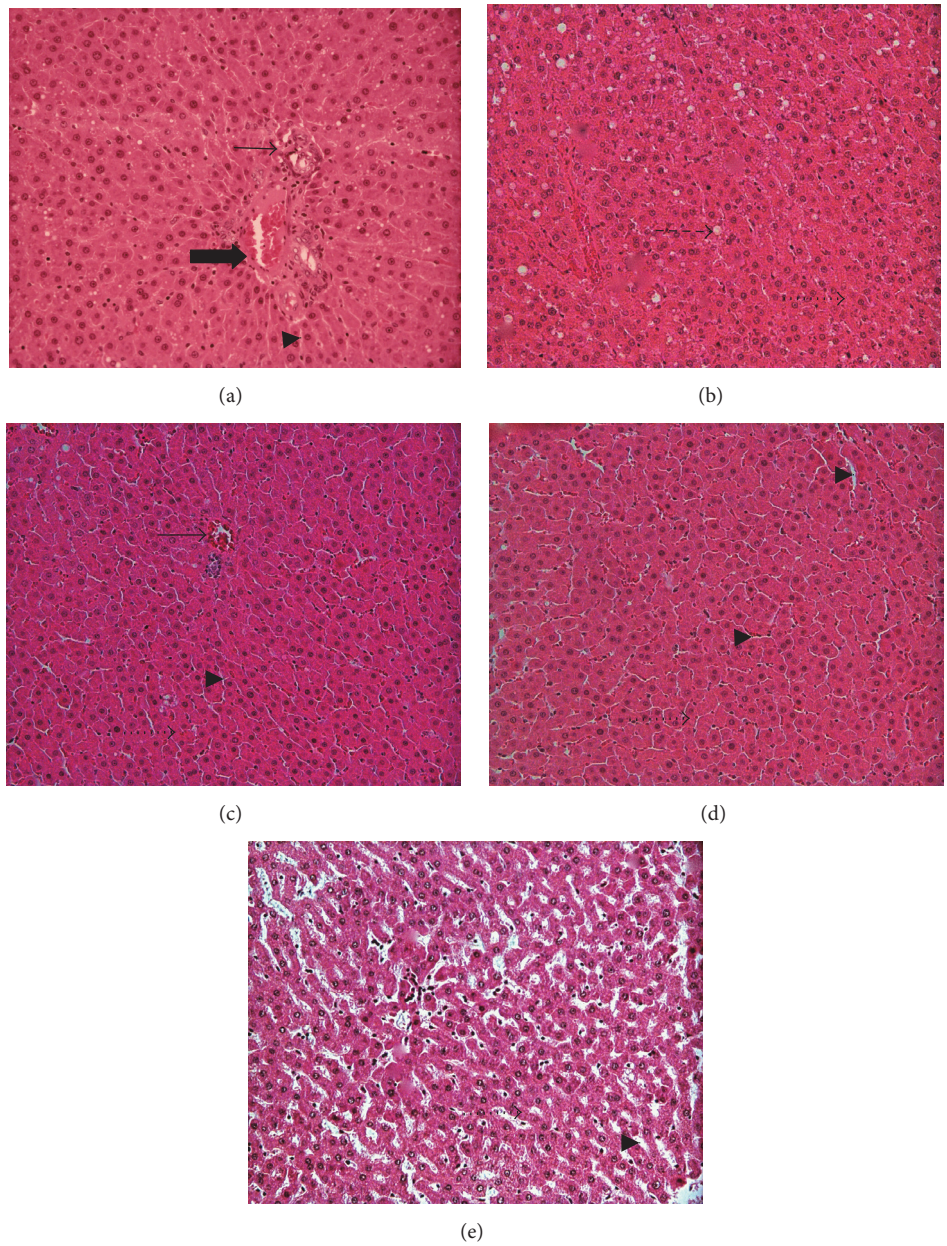

Figure 1: Photomicrographs of rat's liver histology (HE staining ×200) fed with high fat diet for four weeks prior to treatment with Gelam honey, Acacia honey, or orlistat, respectively, for another four weeks. (a) Control (NC); (b) high fat diet (HFD); (c) high fat diet with Gelam honey (HFDGH); (d) high fat diet with Acacia honey (HFDAH); and (e) high fat diet with orlistat (HFDO). Bold arrow: portal vein, normal arrow: central vein, arrow head: sinusoid, dotted arrow: hepatocytes, and dashed arrow: ballooned hepatocytes.

In addition, rats group fed Gelam honey showed a decrease in adiposity percentage index compared to HFD group (Table 2). Both honey groups (Gelam and Acacia) appeared lower in BMI and Lee's index compared to HFD group. The findings demonstrated that Gelam and Acacia honey are capable of preventing body weight gain, concomitantly helping in maintaining the current body weight. The present study investigated the effects of honey on obese rats and sought to determine whether honey is able to reverse the increased body weight gain caused by high fat diet. Nevertheless, the similarities of these findings strengthen the postulation that honey possesses weight-reducing properties. Result showed that Gelam honey might prevent excessive body weight gain and reduce the body weight increase caused by high fat diet intake.

Similar results had been reported by Chepulis (2009), where rats supplemented with 10% honey *ad libitum* showed a significant reduction in their body weight after 6 weeks of treatment [29]. Clover honey from the United States of America was also reported able to reduce excess weight gain and exhibited antihyperlipidemia *in vivo* [7]. Meanwhile, Romero-Silva et al. (2011) revealed that rats fed with diet

containing honey showed significantly reduced adipocyte size after two months of regimen when compared to rats fed sugar [8].

In addition to the results obtained from animal models, data from interventional studies in human subjects supplemented with honey also showed a reduction in body weight, body fat, and a significant reduction in total cholesterol [10]. Another research on overweight subjects done by Yaghoobi et al. (2008) demonstrated that consumption of honey reduced the low-density lipoprotein cholesterol (LDL-C) and triglyceride level and a slight increase in high-density lipoprotein cholesterol (HDL-C) level [10].

Rats in all treatment groups showed significantly lower glucose, triglyceride, and cholesterol levels compared to HFD group. Major composition of honey is mono- and disaccharide sugars, which is glucose, fructose, maltose, and sucrose. However, more than 90% are simple sugars, which are readily absorbed and metabolised after consumption. Usually, not more than 5% sucrose is found in natural blossom honey. Furthermore, hundreds of micronutrients in honey change the way the substance reacts in our digestive system [6]. It is possible that ingestion of honey will reduce plasma glucose level as well, thereby reducing fat deposition [29, 30]. This makes honey have low glycemic index compared to other sweeteners [4].

Moreover, the oral administration of honey significantly decreased the weights of epididymal and retroperitoneal adipose tissues and, ultimately, the total adiposity index of the high fat diet induced obese rats fed with honey. It has been suggested that lower glycemic index of honey may be a good source of quick energy as the calories can be burnt quickly [29, 31]. This may lead to an efficiency in metabolism and decreases amino acid catabolism and fat deposition [29]. Simultaneously, it lowers glucose, triglycerides, and cholesterol level in the bloodstream. Many other studies showed similar findings, as reported by Chepulis (2007); honey fed diet reduced weight gain compared to sucrose [32]. This finding is further strengthened by the previous research done by Nemoseck et al. (2011) and Mushtaq et al. (2011) where they found that honey could reduce weight gain, adiposity, and related biomarkers (leptin, insulin, and adiponectin) and also better blood profiles [7, 31–34]. Another factor that may contribute to a decrease in weight gain is the presence of hydrogen peroxide in honey. Hydrogen peroxide can be a strong insulin mimetic agent [35]. Hydrogen peroxide in honey is produced from the oxidation of glucose by glucose oxidase enzyme.

Adiponectin, leptin, and resistin are adipocytokines that are related to obesity. Leptin is a fat-derived key regulator of appetite and energy expenditure, where its concentration in the plasma is associated with general adiposity [36]. The reductions of leptin and resistin levels in serum level of rats fed with Gelam honey and orlistat reflect a decrease in fat mass but not in HFD (Table 4). This is because hypothalamus receives direct input from hormones, specifically, leptin which crosses the blood-brain barrier and provides information on the levels of peripheral adipose mass [37]. There were predictable changes in neural activity, in brain areas known to be involved in the regulatory, emotional, and cognitive control of food intake, where many of them were reversed by leptin [37]. Following adipocytes loss, leptin decreases in response to visual food cues to hypothalamus [38]. The results from the present study suggested that honey causes significant adipocytes loss (Table 2), indicated by the reduced leptin and resistin level, but an increase in adiponectin level reduced the excess weight gain in the rats (Table 4).

Meanwhile, the biochemical indices such as enzymes found in the liver and kidneys are useful markers for assessment of tissue damage [39]. Tissue enzymes can also indicate tissue cellular damage caused by chemical compounds before structural damage that can be detected by conventional histological techniques [40]. The observation showed that liver enzymes such as ALP, AST, and ALT were significantly higher in HFD and HFDO groups (Table 4). However, for kidney, reductions in urea and total protein level were observed. The abnormality observed indicated liver and kidney problems originated from feeding high fat diet and administration of the chemical compound (orlistat) [41]. There was a significantly higher acid phosphatase activity in the liver of rats fed with HFD and treated with orlistat in HFDO group (Table 6). It may be a result of damage to the lysosomal membrane and consequent leakage of the enzymes from the lysosome into the extracellular fluid [42]. This is an indication of diminished synthetic function of the liver which may consequently lead to enhanced retention of fluid in the tissues spaces and causes organs damage [41].

Above-mentioned results were proven by histological evaluation, where it showed slightly damaged liver tissues in high fat diet (Figure 1(b)) and orlistat (Figure 1(e)). As previously reported by Rao and Hua (2014), fat diet caused mobilization of free fatty acid from adipose tissue and the transportation into hepatocytes [39]. However, in Gelam honey and Acacia honey group it was shown that the levels of liver enzymes and kidney enzymes were within the range [43–45]. The decrease in the level of creatinine, ALP, and ALT activities was a clear indication that honey showed hepatoprotective and renal-protective properties and this is in accordance with the fact that consumption of honey conferred the aforementioned effects [46, 47].

Furthermore, histopathological evaluation for the control rat's liver exhibited normal morphological appearance. It was observed that the hepatic parenchyma consisted of several hepatic lobules separated from each other by very delicate connective tissue septa housing the portal triad (Figure 1(a)). Each hepatic lobule contained a thin walled central vein surrounded by hepatic cords radiating towards the periphery. The hepatic cords were separated from each other by the hepatic sinusoids. The latter appeared wide irregular blood spaces lined by endothelial cells and Kupffer cells. Rats fed with Gelam or Acacia honey displayed similar results with the control rats with no abnormalities in hepatocytes. The hepatocytes were arranged in a trabecula running radiantly from the central vein and were separated by sinusoids containing Kupffer cells (Figures 1(c) and 1(d)). These results were in agreement with previous studies showing that daily consumption of honey gave positive effects on liver function enzymes [46, 47]. However, liver from experimental HFD groups showed lipid accumulation in hepatocytes, abundance

of cytoplasm vacuolization, and ballooned hepatocytes while HFDO group showed severe lipid accumulation in hepatocytes (Figure 1(b)). Cytoplasm vacuolization in parenchymatous cells of the liver and hypertrophied hepatocytes were seen in both high fat diet (Figure 1(b)) and orlistat groups (Figure 1(e)). Nevertheless, liver from HFDO groups showed the degenerated hepatocytes and necrosis (Figure 1(e)). The results showed the protective effect of honey compared to toxicity effects of orlistat, in addition to their potential in reducing excess weight gain and obesity.

5. Conclusion

Honey is a great natural sweetener and medicinal food, which is rich in nutrients and health benefits. This study recapitulates the effects of honey on obese induced rats. The onset of hepatic steatosis in this model resulted from an increased dietary intake of fat, in addition to excess caloric intake. Moreover, we demonstrated that the daily supplementation of honey might reverse the formation of hepatic steatosis. Furthermore, the present investigation proved that Gelam honey possesses lipid lowering and antioxidative effects in obese induced rats, as well as its weight-reducing ability compared to Acacia honey. However, both honey types showed better effects compared to orlistat, a drug used to control obesity. Due to the promising effects of honey in diet-induced obesity, further investigation is important in order to determine the active compounds in honey followed by identifying the probable mechanisms of action of honey in reducing plasma lipid and its weight maintenance property.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to acknowledge UiTM Fellowship and the Research Management Institute (RMI), Universiti Teknologi MARA (UiTM), for the financial and administrative supports. They would like to express their sincere gratitude to UiTM Puncak Alam for the facilities provided throughout the research project. This research was supported by the Research Entity Initiative (REI) research grant under the RMI, UiTM, for the Project 600-RMI/DANA 5/3/REI (9/2013).

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 5413917, 12 pages
https://doi.org/10.1155/2017/5413917

Research Article

Manuka Honey Exerts Antioxidant and Anti-Inflammatory Activities That Promote Healing of Acetic Acid-Induced Gastric Ulcer in Rats

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Received 4 July 2016; Revised 30 October 2016; Accepted 3 November 2016; Published 19 January 2017

Academic Editor: José Maurício Sforcin

Gastric ulcers are a major problem worldwide with no effective treatment. The objective of this study was to evaluate the use of manuka honey in the treatment of acetic acid-induced chronic gastric ulcers in rats. Different groups of rats were treated with three different concentrations of honey. Stomachs were checked macroscopically for ulcerative lesions in the glandular mucosa and microscopically for histopathological alterations. Treatment with manuka honey significantly reduced the ulcer index and maintained the glycoprotein content. It also reduced the mucosal myeloperoxidase activity, lipid peroxidation (MDA), and the inflammatory cytokines (TNF-α, IL-1β, and IL-6) as compared to untreated control group. In addition, honey-treated groups showed significant increase in enzymatic (GPx and SOD) and nonenzymatic (GSH) antioxidants besides levels of the anti-inflammatory cytokine IL-10. Flow cytometry studies showed that treatment of animals with manuka honey has normalized cell cycle distribution and significantly lowered apoptosis in gastric mucosa. In conclusion, the results indicated that manuka honey is effective in the treatment of chronic ulcer and preservation of mucosal glycoproteins. Its effects are due to its antioxidant and anti-inflammatory properties that resulted in a significant reduction of the gastric mucosal MDA, TNF-α, IL-1β, and IL-6 and caused an elevation in IL-10 levels.

1. Introduction

Gastric ulcers develop inside the stomach, affect many people worldwide, and represent discontinuity in the gastric mucosal penetration through the muscularis mucosa [1]. This type of ulcer results from the imbalance between two known factors: aggressive factors (physical, chemical, or psychological) in the lumen and protective ones. The aggressive factors include acid, pepsin, *Helicobacter pylori*, stress, alcohol, administration of nonsteroidal anti-inflammatory drugs, and protective mechanisms which are present in the duodenal mucosa and include mucus, bicarbonate, prostaglandin, blood flow, the antioxidant system, sulfhydryl compounds, nitric oxide, and cell proliferation [1–3]. During the ulcer healing process, the eradication of *Helicobacter pylori* or control of gastric acid secretion was not sufficient to prevent recurrence of gastric ulcer [4–6]. A lot of research has been conducted and acquired knowledge over the years regarding the development of gastric ulcer. This resulted in the development of a wide spectrum of drugs for its treatment, like proton pump inhibitors, antacids, anticholinergics, and histamine receptor antagonists [7]. However, all the current therapies are not always effective, have adverse side effects, and are expensive. For this reason, identifying new potentially agents through natural sources is still essential for more effective and safe antiulcer therapy [8, 9].

Bees belonging to the species *Apis mellifera* collect the nectar from different flowers and convert it to honey. It has a density of around 1.36 g/mL (about 36% more dense than water) [10]. The medicinal application of honey in the treatment of ulcers was originally documented by the Sumerians and goes, as far, back as 2,100–2,000 BC [11]. Honey is effective in the treatment of a broad range of wound types including, but not limited to, burns, scratches, diabetic, malignant, leprosy, fistulas, leg ulcers, traumatic, boils cervical varicose ulcers, amputation burst abdominal wound septic and surgical wounds, cracked nipples, and wounds of the abdominal wall and perineum [12]. Natural honey is composed of around 82% carbohydrates, water, phytochemicals, proteins, minerals, and antioxidants. It is likely thought that the minor ingredients are likely to be responsible for differentiating among the various types of honey and for their medicinal and biological potential [13]. The sugars in honey include in a descending order the following: fructose (38.2%) and glucose (31.2%), sucrose (0.7%–1%), and disaccharides (approximately 9%) some trisaccharides and higher saccharides [14, 15]. Two important innovative commercial types of honey available on the market include manuka honey [16] and Surgihoney [17] and have been known for their effectiveness in wound management. In a previous study, we have demonstrated a gastroprotective effect of manuka honey against gastric lesions induced by ethanol [18]. In this study, we evaluate for the first time the gastric curative effects of manuka honey in rat model with acetic acid-induced chronic gastric ulcer. The underlying mechanism of such an effect is also elucidated.

2. Material and Methods

2.1. Animals. Sprague-Dawley male eight-week-old rats, weighing between 220 and 240 g, were used. The animals were housed for 1 week at a temperature of 24 ± 1°C and a 55 ± 5% relative humidity. They were reared on a standard laboratory diet and tap water ad libitum. The rats were deprived from food 24 hours prior to the experiment; during this period, animals were kept in cages with raised floors of wide mesh to prevent coprophagy while being allowed access to water ad libitum. Rats were handled following the animal care guideline set by our university. The experimental protocol was approved by Research Ethics Committee at King Fahd Medical Research Center.

2.2. Acetic Acid-Induced Gastric Ulcer and Treatment. Ulcer induction was achieved as described elsewhere [19]. Under anesthesia, laparotomy was performed on all animals through a midline-epigastric incision. The stomach was firstly exposed and then was injected with 0.05 mL (v/v) of a 30% acetic acid solution into the subserosal layer in the glandular part of the anterior wall. After that, the stomach was soaked in a bath of saline in order to prevent any adherence to the external surface of the ulcerated region. The abdomen then was closed afterwards to allow the rats to feed normally. Two days after surgery, all rats were randomly divided into six groups each consisting of 6 animals: (1) a SHAM control group which underwent the surgical procedure of ulcer induction with the application of saline instead of acetic acid; (2) control group: acetic acid ulcer induced group; (3) positive control group: acetic acid + ranitidine treated group (ranitidine at the dose of 30 mg/kg) [20]; (4) control group + low dose group of manuka honey (0.625 g/kg); (5) control group + medium dose group of manuka honey (1.25 g/kg); (6) control group + high dose group of manuka (2.5 g/kg). Treatment of rats was carried out two days after the induction of ulcer by gavage once a day for a period of 10 consecutive days. One day after the last treatment, the animals were sacrificed by cervical dislocation carried out under humane conditions and after the animals being anesthetized, the stomachs were removed and the mucosal damage was assessed according to the following: edema (1 point), hyperemia (1 point), petechiae (light, moderate, and intense with 1, 2, and 3 points, resp.), hemorrhagic lesion (3 points), ulcers (not perforated and perforated, 1 point/mm^2 and 2 points/mm^2, resp.), and thickening of the ulcer (1 point/mm^2) [21].

$$\text{Ulcer inhibition rate} = \frac{\text{Control (ulcer index)} - \text{Test (ulcer index)}}{\text{Control (ulcer index)}} \times 100\%. \quad (1)$$

Ulcer inhibition rate was expressed as previously described [22].

2.3. Pathological Effects on Gastric Tissue. Paraformaldehyde (4%) solution was used in order to fix the gastric tissues. This was followed by dehydrating the tissue samples with alcohol and xylene and later embedding them in paraffin for sectioning. 5 μm thick sections were affixed onto slides, deparaffinized, and stained using hematoxylin and

eosin (H&E). Light microscopy was used for the general histopathology examination.

2.4. Periodic Acid Schiff Staining for Determination of Mucin Content. The histochemical assay for the determination of mucin was performed as described earlier [19]. Samples were sectioned and placed on slide. These slides were then deparaffinized, rehydrated, oxidized (0.5% periodic acid for 5 min), and washed with distilled water. Then slides were stained with Schiff's reagent for 20 min, followed by washing the sections with sulfurous water (three times for 2 min) and in tap water for 10 min. Finally, the sections were counterstained with hematoxylin for 20 seconds and dehydrated.

2.5. Assessment of Gastric Mucosa Myeloperoxidase (MPO) Enzyme Activity. MPO was determined in gastric homogenates according to the method of Grisham et al. [20]. A portion of the stomach (100 mg) was homogenized in 10 volumes of ice cold potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 20,000 ×g for 20 min at 4°C. The pellets were then collected and were homogenized in 10 volumes of ice cold 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyl-trimethyl ammonium bromide (HTAB) and 10 mM EDTA. An aliquot of the homogenate (100 μL) was removed and added to a 1 mL reaction volume containing 80 mM potassium phosphate buffer (pH 5.4), 0.5% HETAB, and 1.6 mM tetramethyl benzidine. The mixture was warmed to 37°C and then 100 μL of 0.3 mM H_2O_2 added. The rate of change in absorbance was then measured at 655 nm. The MPO activity was expressed as U/mg tissue.

2.6. Sample Preparation for Antioxidant Analysis. Another portion of the stomach samples were homogenized in a solution of 2% Triton X-100 containing 0.32 M sucrose solution for SOD determination. Additional portions of the stomach were homogenized in 50 Mm potassium phosphate pH 7.5 and 1 Mm EDTA for determination of MDA, GSH, GPx, and CAT. The resulting homogenates were sonicated twice for 30 s intervals at 4°C and then centrifuged at 1800g for 10 min at 4°C [23].

2.7. Determination of Reduced Glutathione (GSH). GSH was determined according to the method of Ellman [24] in stomach homogenates using kit from Biodiagnostic, Egypt. The GSH content of the tissue GSH was expressed as nmol/g tissue.

2.8. Determination of Lipid Peroxide (Measured as MDA). The concentration of MDA was assayed in the stomach homogenates using kits from Biodiagnostic, Egypt. Based on the method of Uchiyama and Mihara [25], tissue MDA content was measured by taking two optical density measurements at two wavelengths (535 nm and 525 nm) and determining the difference between them. The distinct tissue MDA content was expressed as nmol/g tissue.

2.9. Determination of Glutathione Peroxidase (GPx). This was done also on stomach homogenates using kits marketed by Biodiagnostic, Egypt, and based on what has been reported in the literature [26]. GPx activity was expressed in mU/g tissue.

2.10. Determination of Superoxide Dismutase (SOD). The activity of SOD was done on stomach homogenates using kits provided by Biodiagnostic, Egypt; as described by others [27], SOD activity was expressed in U/g tissue.

2.11. Determination of Catalase (CAT). The activity of CAT was assayed on stomach homogenates according to Aebi [28] using kit from Biodiagnostic, Egypt. CAT activity was expressed in U/g tissue.

2.12. Measurement of Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), IL-6, and IL-10. ELISA kits (Assaypro, USA) were used for measurement of TNF-α, IL-1β, and IL-6 concentrations in stomach homogenate, while an ELISA kit (Novex, USA) was used for measurement of IL-10 concentrations in stomach homogenate. Cytokine concentrations were calculated using standard purified recombinant cytokines.

2.13. Analysis of Apoptosis and DNA-Cell Cycle by Flow Cytometry. Cell suspensions from specimens were collected from the stomach mucosa of rats treated with honey and from the control were treated with 0.1% Triton X-100 to create pores in cell membrane to allow the penetration of Propidium Iodide stain into the cell. The cells were washed with PBS at 37°C for 30 min in the dark and stained with a Propidium Iodide solution (50 μg/mL Propidium Iodide and 50 μg/mL RNase) (Sigma-Aldrich, Munich, Germany). Cell clumps of stained cells were removed by passing them through a nylon mesh sieve. Analysis was done using flow cytometry (BD FACSCalibur, San Jose, USA). Data collection and analysis was done using the CellQuest (BD, San Jose, USA) and ModFit Lt (Verity Software House Inc., Topsham, ME, USA) software.

Doublet discrimination was used to analyze the samples. This will allow the distinction between the signals originating from one nucleus versus two or more aggregated nuclei. Single nuclei will only be considered for the computer analysis. For each sample, data for 20,000 events were collected. The analysis of apoptosis was performed by determining hypodiploidy (sub-G1 peak) as previously described [29].

2.14. Statistical Analysis. All results were shown as mean ± SD. Data was entered using Statistics software SPSS 22. One-way analysis of variance (ANOVA) test was used to analyze the data. Statistical differences of P of <0.05 were considered to be significant.

3. Results

3.1. Effect of Manuka Honey on Ulcer Index. In the ulcer control group, the subserosal layer of the glandular part of the anterior stomach wall showed a significant increase in the gastric lesion index as compared to the SHAM value (P = 0.000). The administration of low concentrations of honey did not produce a significant reduction of the ulcer index and

Table 1: Effect of different doses of manuka honey on gastric mucosal lesion index.

Treatment regimen	Gastric lesion index (mm^2/stomach)	Ulcer inhibition rate (%)
SHAM	1.0 ± 0.17	—
Ulcer control group	15 ± 0.43[a]	—
Positive control (ranitidine)	8.0 ± 0.21[b]	47
Ulcer control group + manuka honey (0.625 gm/kg)	13 ± 0.60	13
Ulcer control group + manuka honey (1.25 gm/kg)	12 ± 0.63	20
Ulcer control group + manuka honey (2.5 gm/kg)	5.0 ± 0.31[b]	67

Data are mean ± SEM ($n = 6$).
[a]Significant versus SHAM ($P \leq 0.05$).
[b]Significant versus acetic acid (ulcer) ($P \leq 0.05$).

in the ulcer inhibition rate. However, a significant decrease in the gastric lesion index as well as in the ulcer inhibition rate was noted when rats were treated with 2.5 gm/kg or ranitidine (Table 1).

3.1.1. Macroscopic Examination. In the ulcer control group, the subserosal layer of the glandular part of the anterior stomach wall showed rounded gastric mucosal lesions (Figure 1(b)). The treatment of rats with low concentrations of manuka honey did not produce a significant healing ability (Figures 1(d) and 1(e)). However, when manuka honey was used at a high concentration of 2.5 gm/kg, it resulted in a significant healing effect of ulcer as compared to the SHAM group. The same was true in the case of the positive control ranitidine group (Figures 1(c) and 1(f)).

3.1.2. Histopathological Changes of the Stomach Fundic Mucosa. As demonstrated in Figure 2, the SHAM group showed normal surface mucous columnar cells (black arrows) and intact glandular cells (star). This is in contrast to the ulcer control group (b and c) which showed disruption and desquamation of surface mucous epithelium (black arrows) with inflammatory cell infiltration (star). The positive control ranitidine (d) showed nearly normal surface cells. Using manuka honey (0.6 gm/kg) showed focal surface desquamation (black arrows), capillary congestion (white arrows), and necrosis (star) (e). Manuka honey at concentration of 1.25 gm/kg (f) demonstrated focal loss of mucous surface epithelium (black arrows) and mucosal inflammatory cell infiltrate (white arrows). In the case of manuka honey (2.5 gm/kg), marked proliferation and elongation of surface mucous cells extending deeper into mucosa (black arrows) (g) are shown.

3.1.3. Effect of Manuka Honey on Glycoproteins. The subserosal layer of the glandular part of the anterior stomach wall showed either marked decrease or complete loss in gastric mucosal glycoprotein content of surface cells in the ulcer control group (Figures 3(b) and 3(c)). Treatment with manuka honey at 0.65 gm/kg or 1.25 gm/kg moderately increased PAS reacted glycoprotein (Figures 3(e) and 3(f)). Treatment of rats with either manuka honey (2.5 gm/kg) or ranitidine (30 mg/kg) showed marked preservation of the glycoprotein content of surface epithelium which may extend down along gastric glands (Figures 3(d) and 3(g)).

3.2. Assessment of Oxidative Stress Biomarkers

3.2.1. Myeloperoxidase (MPO). There was a significant increase in the gastric mucosa MPO activity (170%) in the ulcer control group as compared to the SHAM group ($P = 0.007$). A significant decrease in the gastric mucosa MPO activity was noted in both the ranitidine and the honey (2.5 mg/kg) treated groups (50% and 35%, resp.) as compared to ulcer control group ($P = 0.007$ and $P = 0.026$, resp.) (Figure 4).

3.2.2. Glutathione (GSH). There was a significant reduction of the levels of GSH in the ulcer control group (74%) as compared to the SHAM value ($P = 0.000$). This significant decline was reversed upon treating the rats with either manuka honey (2.5 gm/kg) or ranitidine (30 mg/kg) resulting in a significant increase of gastric mucosal GSH content (~250%) as compared to ulcer control group ($P = 0.000$) (Figure 5).

3.2.3. Malondialdehyde (MDA). There was a significant increase in the gastric mucosa MDA levels (31%) in the ulcer control group in comparison to the SHAM group ($P = 0.005$). The treatment of rats with either manuka honey (2.5 gm/kg) or ranitidine (30 mg/kg) resulted in a significant decrease in the gastric mucosal MDA levels (~30%) as compared to ulcer control group ($P = 0.001$ and $P = 0.002$, resp.) (Figure 6).

3.2.4. Glutathione Peroxidase (GPX), Superoxide Dismutase (SOD), and Catalase (CAT) Enzyme Activities. In the ulcer control group, there was a significant decrease in the gastric mucosal GPx, SOD, and CAT activities (50%, 60%, and 28%, resp.) as compared to the SHAM value ($P = 0.001$, 0.003, and 0.000, resp.). Upon treating the rats with manuka honey (2.5 gm/kg), there was a significant increase in the gastric mucosa GPx, SOD, and CAT enzyme activities (78%, 109%, and 29%, resp.) as compared to ulcer control group ($P = 0.001$, 0.000, and 0.007, resp.). The treatment of rats with ranitidine (30 mg/kg) significantly increased gastric mucosa SOD and CAT enzyme activities (87% and 33%, resp.) as compared to ulcer control group ($P = 0.000$ and $P = 0.023$, resp.). However, ranitidine had no significant increase in GPx

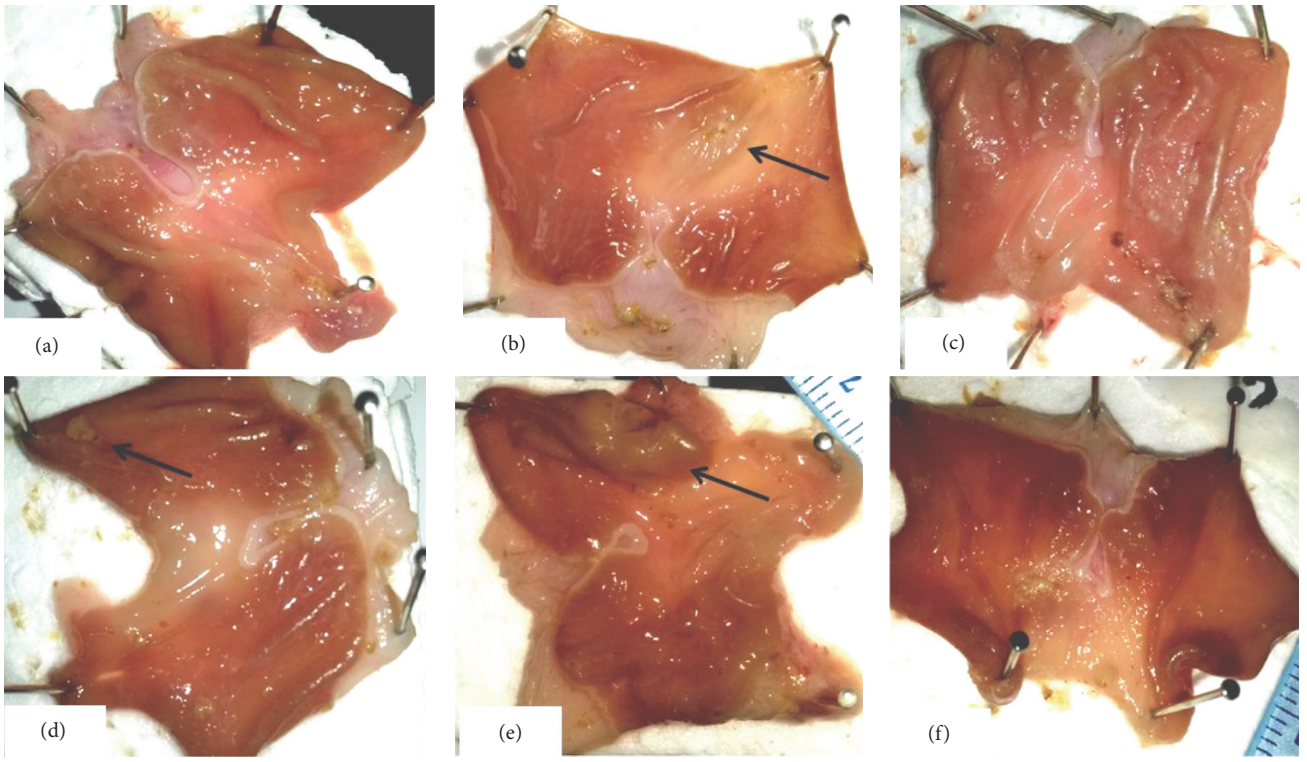

Figure 1: Effect of manuka honey on the regeneration of gastric mucosa examined in acetic acid-induced gastric ulceration in rats (gross examination). The images represent macroscopic photograph of the (a) SHAM, (b) ulcer control group, (c) positive control: ranitidine (30 mg/kg), (d) manuka honey (0.625 gm/kg), (e) manuka honey (1.25 gm/kg), and (f) manuka honey (2.5 gm/kg).

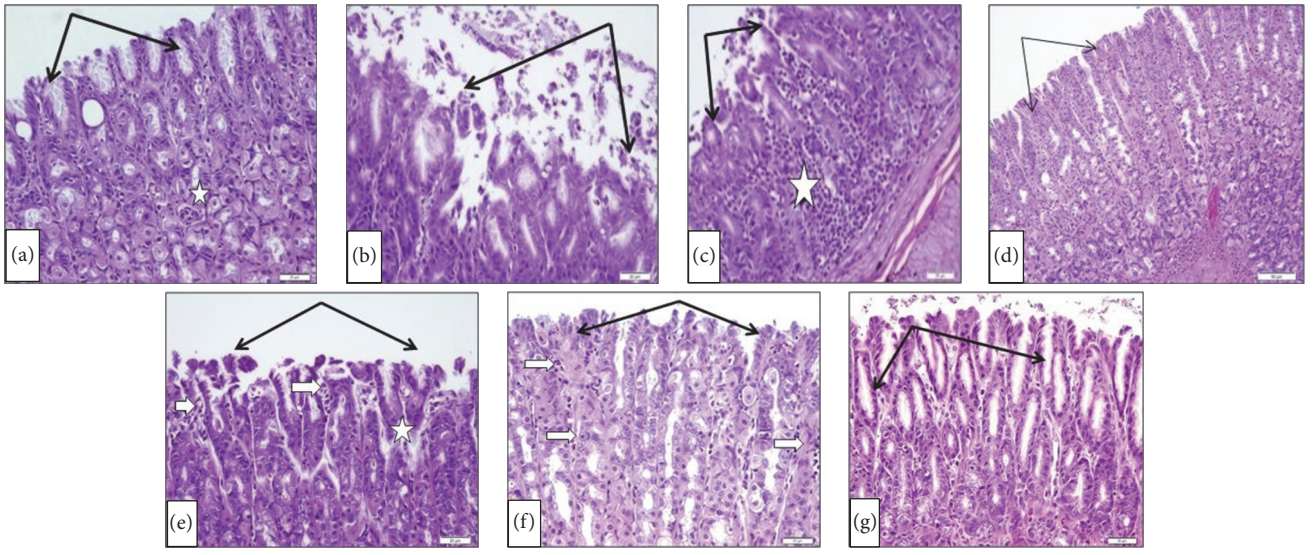

Figure 2: Effect of manuka honey on the histopathological changes of stomach fundic mucosa examined in acetic acid-induced gastric ulceration in rats (H&E ×20). (a) SHAM. (b and c) Ulcer control group. (d) The positive control (ranitidine). (e) Manuka honey (0.6 gm/kg); (f) manuka honey (1.25 gm/kg); (g) manuka honey (2.5 gm/kg).

enzyme activity (19%) as compared to the ulcer control group ($P = 0.238$) (Table 2).

3.3. Effect of Manuka Honey on Gastric Mucosa Proinflammatory Cytokines: Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-1 Beta (IL1-β), and IL-6. There was a significant increase in the gastric mucosal levels of TNF-α, IL1-β, and IL-6 (132%, 800%, and 53%, resp.) as compared to the SHAM group ($P = 0.000$, 0.000, and 0.005, resp.). The treatment of rats with manuka honey (2.5 gm/kg) significantly decreased

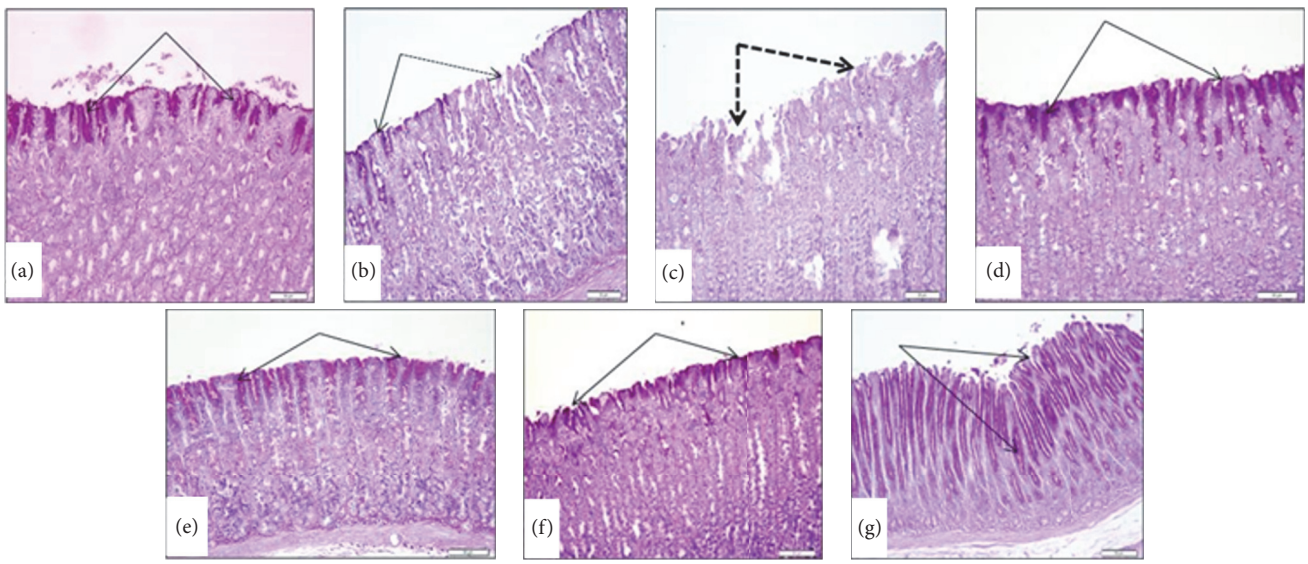

Figure 3: Effect of manuka honey on the gastric mucosal glycoprotein formation detected by PAS staining (PAS ×20). (a) SHAM showed high positive reaction in the surface mucous cells (black arrows). (b and c) Ulcer control group showed marked decrease (black arrows) or loss (dotted arrows) in PAS mucosal glycoprotein content of surface cells. (d) Positive control (ranitidine) showed marked preservation of mucopolysaccharides content of surface epithelium (arrows). (e) Manuka honey (0.6 gm/kg) showed moderate increase in PAS reacted glycoprotein. (f) Manuka honey (1.25 gm/kg) showed moderate increase in PAS reacted glycoprotein. (g) Manuka honey (2.5 gm/kg) showed marked increase in PAS reactive substance extending down along gastric glands (black arrows).

Table 2: Effect of manuka honey on gastric mucosa glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) enzyme activities measured in acetic acid-induced gastric ulceration in rats.

Treatment regimen	GPX (U/g tissue)	SOD (U/mg tissue)	CAT (U/g tissue)
SHAM	1534 ± 113	257 ± 40	31 ± 0.9
Ulcer control group	767 ± 109[a]	101 ± 7[a]	23 ± 1.3[a]
Ulcer control group + ranitidine (30 mg/kg)	915 ± 46	188 ± 13[b]	31 ± 2.5[b]
Ulcer control group + manuka honey (2.5 gm/kg)	1368 ± 74[b]	210 ± 11[b]	30 ± 1.5[b]

Data are mean ± SEM ($n = 6$).
[a]Significant versus SHAM ($P \leq 0.05$).
[b]Significant versus acetic acid (ulcer) ($P \leq 0.05$).

gastric mucosal TNF-α, IL1-β, and IL-6 content (59%, 40%, and 20%, resp.) as compared to the ulcer control group (P = 0.000, 0.013, and 0.001, resp.). The same was true in the case of treatment with ranitidine where a significant decrease in gastric mucosal TNF-α, IL1-β, and IL-6 levels was noted (42%, 33%, and 23%, resp.) as compared to the ulcer control group (P = 0.000, 0.027, and 0.005, resp.) (Table 3).

Effect of Manuka Honey on Gastric Mucosal Interleukin-10 (IL-10) Levels. As was the case with other cytokines, the gastric mucosal levels of IL-10 were significant (45%) as compared to the SHAM group (P = 0.002). The treatment of rats with either manuka honey (2.5 gm/kg) or ranitidine (30 mg/kg) significantly increased gastric mucosal IL-10 levels (292% and 138%, resp.) as compared to the ulcer control group (P = 0.000 and 0.014, resp.) (Figure 7).

3.4. Effect of Manuka Honey on Cell Cycle Progression. Significant increases in gastric mucosal apoptotic cell population (Sub-G1) and proliferation (S-phase) were seen in the ulcer control group as compared to the SHAM group (Figure 8). There was no change in the cell populations in the G_2M phase in the ulcer control group as compared to the SHAM group (P = 0.585, resp.) (Table 4). Acetic acid in the ulcer control group significantly decreased G_1 cell accumulation as compared with the SHAM group (P = 0.003) (Table 4). Treatments of rats with manuka honey (2.5 gm/kg) significantly decreased apoptotic cell population (Figure 8) and decreased sub-G_1, S-phase, and G_2M cell accumulation as compared to the control ulcer group (P = 0.004, 0.05, and 0.04, resp.) (Table 4). Also, manuka honey (2.5 gm/kg) significantly increased G_1 cell accumulation as compared to the ulcer control group (P = 0.001) (Table 4). Treatments of rats with ranitidine (30 mg/kg) significantly decreased apoptotic cell population and G_2M cell accumulation (Figure 8), as compared to the ulcer control group (P = 0.003) (Table 4). Ranitidine did not induce significant changes in cell accumulation in sub-G_1, G_1, and S-phase as compared to the ulcer control group (P = 0.572, 0.511, and 0.644, resp.) (Table 4).

Table 3: Effect of manuka honey (2.5 gm/kg) and ranitidine (30 mg/kg) on gastric mucosa tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL1-β), and IL-6 content measured in acetic acid-induced gastric ulceration in rats.

Treatment regimen	TNF-γ (pg/mg tissue)	IL-1β (pg/mg tissue)	IL-6 (pg/mg tissue)
SHAM	274 ± 2.0	41 ± 0.5	113 ± 8
Ulcer control group	636 ± 30[a]	326 ± 41[a]	173 ± 6[a]
Ulcer control group + ranitidine (30 mg/kg)	367 ± 11[b]	217 ± 8[b]	132 ± 10[b]
Ulcer control group + manuka honey (2.5 gm/kg)	258 ± 13[b]	195 ± 13[b]	139 ± 5[b]

Data are mean ± SEM ($n = 6$).
[a]Significant versus SHAM ($P \leq 0.05$).
[b]Significant versus acetic acid (ulcer) ($P \leq 0.05$).

Table 4: Effect of manuka honey on cell cycle progression of the gastric mucosal cells.

Treatment regimen	Sub-G1%	G1%	S-phase%	G2M%
SHAM	4.39 ± 0.93	81.48 ± 2.46	6.70 ± 1.16	6.89 ± 1.04
Ulcer control group	24.39 ± 5.13[a]	57.82 ± 5.42[a]	10.40 ± 1.49[a]	7.93 ± 1.52[a]
Ulcer group + ranitidine (30 mg/kg)	20.36 ± 4.75[b]	64.01 ± 7.33[b]	8.69 ± 2.26[b]	1.95 ± 0.29[b]
Ulcer group + manuka honey (2.5 gm/kg)	4.91 ± 1.12[b]	84.83 ± 1.87[b]	3.78 ± 0.70[b]	4.13 ± 0.61[b]

Data are mean ± SEM ($n = 6$).
[a]Significant versus SHAM ($P \leq 0.05$).
[b]Significant versus acetic acid (ulcer) ($P \leq 0.05$).

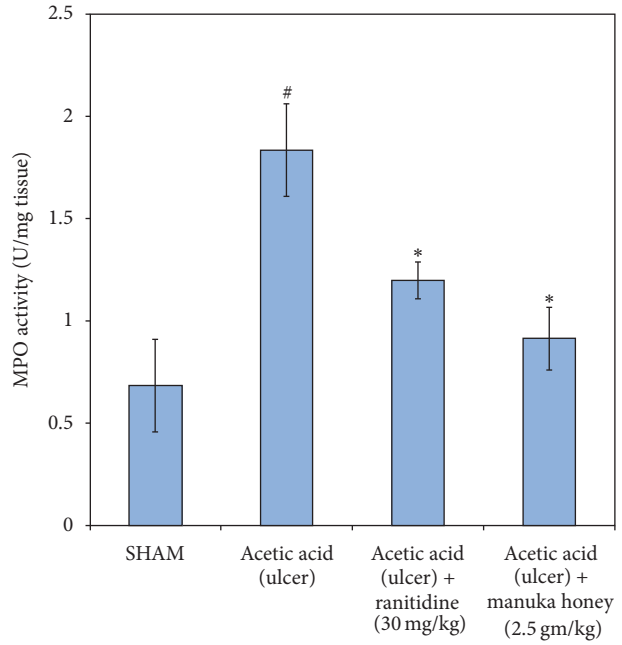

Figure 4: Effect of manuka honey (2.5 gm/kg) and ranitidine (30 mg/kg) on gastric mucosa myeloperoxidase (MPO) enzyme activity measured in acetic acid-induced gastric ulceration in rats. Each value is the mean ± SEM ($n = 6$). #Significant versus SHAM ($P \leq 0.05$). *Significant versus acetic acid (ulcer) ($P \leq 0.05$).

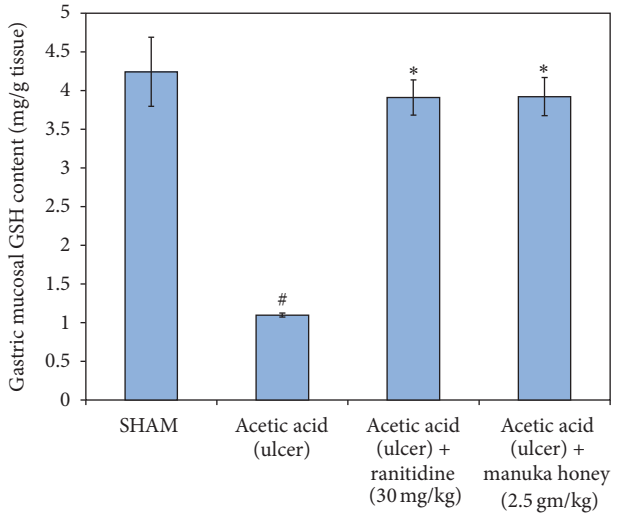

Figure 5: Effect of manuka honey (2.5 gm/kg) and ranitidine (30 mg/kg) on gastric mucosa reduced glutathione (GSH) content as compared to the ulcer control group and SHAM. Each value is the mean ± SEM ($n = 6$). #Significant versus SHAM ($P \leq 0.05$). *Significant versus acetic acid (ulcer) ($P \leq 0.05$).

4. Discussion

In the current study a significant increase was noted in the ulcer index and mean score in the acetic acid-induced ulcer group in comparison to the SHAM control group. The data obtained showed that treatment with manuka honey was safe to the animals used and resulted in zero mortalities. Manuka honey reversed the effects of acetic acid-induced oxidative injury and inflammation in the gastric mucosa and facilitated chronic ulcer healing. Such an effect is likely to be due to its constituents with biological activities including polyphenols such as flavonoids and phenolic acids and total water-soluble vitamins (vitamin B1, B2, B3, B9, and B12 and vitamin C) [30].

The acetic acid produces round, deep ulcers in the stomach and duodenum, resembling to a great extent human ulcer in terms of both pathological features and healing

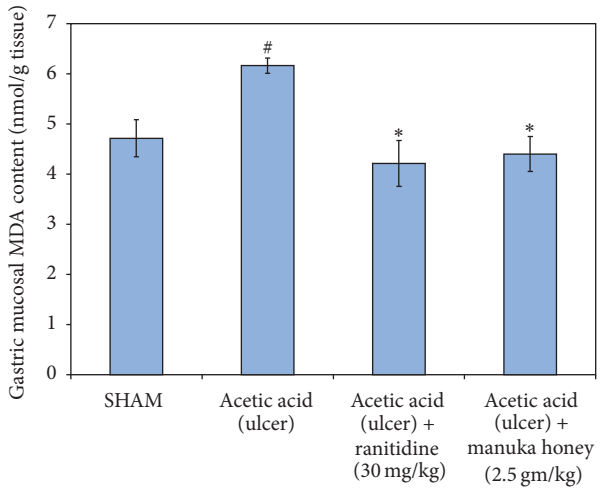

Figure 6: Effect of manuka honey (2.5 gm/kg) and ranitidine (30 mg/kg) on gastric mucosa malondialdehyde (MDA) content as compared to the ulcer control group and SHAM. Each value is the mean ± SEM ($n = 6$). #Significant versus SHAM ($P \leq 0.05$). *Significant versus acetic acid (ulcer) ($P \leq 0.05$).

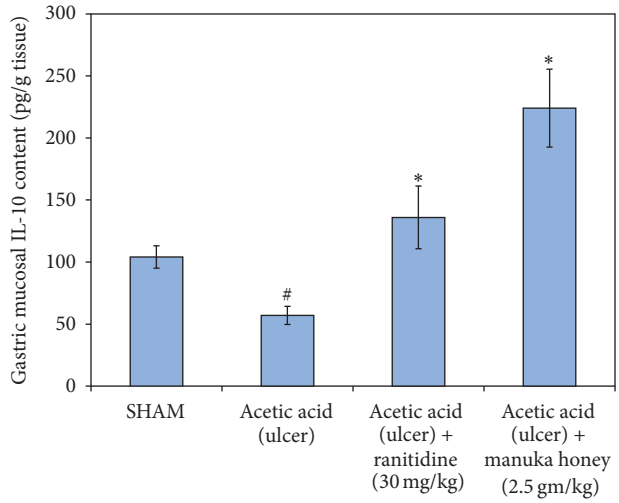

Figure 7: Effect of manuka honey (2.5 gm/kg) and ranitidine (30 mg/kg) on gastric mucosa interleukin-10 (IL-10) content as compared to the ulcer control group and SHAM. Each value is the mean ± SEM ($n = 6$). #Significant versus SHAM ($P \leq 0.05$). *Significant versus acetic acid (ulcer) ($P \leq 0.05$).

process. Such a model has been excessively used to study the pathophysiology and treatment of gastric ulcers and the underlying mechanisms involved in ulcer healing [31, 32].

It has been previously reported that acetic acid induces ulcer by penetrating the gastric mucosa and both the mucous membrane and submucous layers as well as the muscular layer. The ulcers produced by acetic acid become chronic within 2-3 days after ulcer initiation and may be completely treated within 2-3 weeks without the need for perforation or penetration to the surrounding organs [33, 34]. In addition, those ulcers can be treated with various antiulcer drugs [35]. We have previously demonstrated that manuka honey provided significant gastroprotective effects in acute gastric ulcer animal model [18]. The current data showed that manuka honey (2.5 mg/kg) had a healing potential comparable to ranitidine, which is a drug approved by the FDA and prescribed for the treatment of ulcers. Such a conclusion was based on macroscopic, histopathological, and flow cytometric data. These findings corroborate previous clinical published data on the use of manuka honey for chronic wounds healing [36–38]. One of these studies was observational and showed that manuka honey had positive antiulcer effects on 20 patients with spinal cord injuries and who suffered from chronic pressure ulcers (15 with grade III ulcers and 5 with grade IV ulcers) [36]. In the current study, manuka honey was able to reverse the decrease of the mucin-like glycoproteins, as observed by staining with PAS that are critical cytoprotective glycoproteins due to their mucus secretion activities. Such an action may be caused by phenols that are one of the main constituents of manuka honey [39]. Phenols stimulate the production of prostaglandin E2 (PGE2), which in turn produces mucus and, thus, results in providing protection of the gastrointestinal tract against injury [40].

The genesis of acetic acid-induced gastric lesions is a multifactorial process which starts mainly with the depletion of gastric wall mucous content [41]. Such a depletion is often associated with significant production of free radicals, causing damage to the cell and cellular membrane due to excessive oxidative stress [42]. The generation of reactive oxygen species (ROS), for example, superoxide anion, hydrogen peroxide, and hydroxyl radicals, may cause lipid peroxidation, especially in membranes, and results in tissue injury [9]. High levels of lipid peroxidation have been noticed in animals with induced ulcer. For this reason, the presence of MDA levels indicates more tissue damage due to the impairment of the antioxidants activities to deal with oxidative stress and with the handling of free radicals [43]. However, it has been reported that the first line of defense against oxidative damage caused by injury like ulcers involves the migration of free radical scavenging enzymes such as SOD, CAT, and GPx, to eliminate first O_2 and H_2O_2 before forming harmful hydroxyl ($OH^{\bullet}$) radical [44]. The present study revealed that there were significant increases in lipid peroxidation (MDA) and a reduction in the levels of GSH, GPX, SOD, and catalase in the untreated ulcer group compared with normal control group. This observation may emphasize the role of oxidative damage and ulcer induction, development, and/or maintenance. The data revealed that the oral administration of manuka honey as well as ranitidine interfered with the oxidative process through reduction of free radical level (MDA) and increased the levels of GSH, GPX, SOD, and catalase. These data suggest that manuka honey increases the activity of GPx to form GSH and augments the removal of reactive metabolites together with GSH. These data are in agreement with Henriques and colleagues [45] who reported that manuka honey possesses the most powerful antioxidant activities among all the different types of honey they tested and it was able to quench the introduced free hydroxyl radicals within 5 minutes after addition. Such a powerful

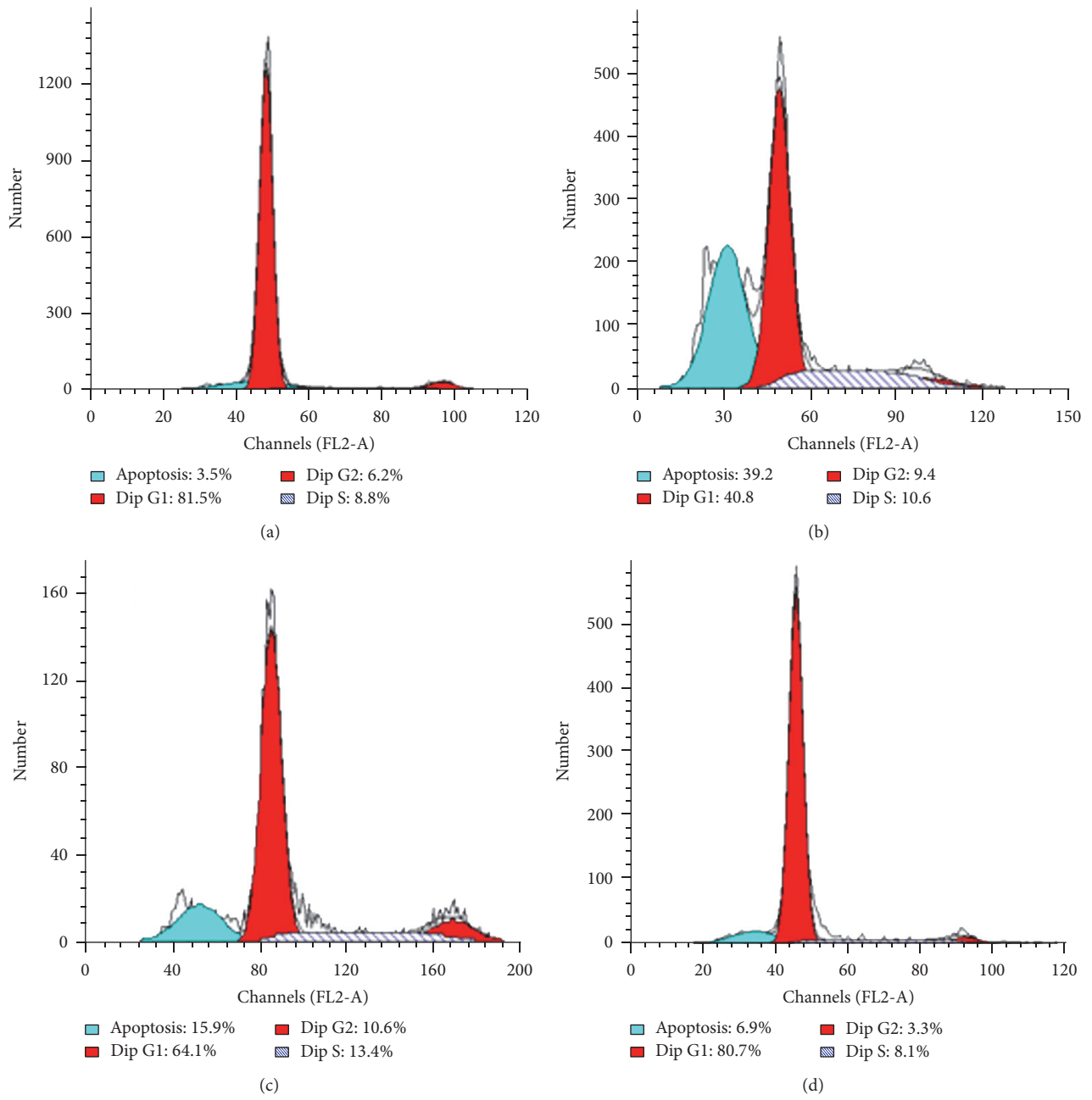

Figure 8: Schematic histograms of DNA-cell cycle analysis using flow cytometry. (a) represents stomach mucosa cells from SHAM group. (b) represents stomach mucosal cells from ulcer control group. (c) represents stomach mucosal cells from ulcer control group + ranitidine (30 mg/kg) group. (d) represents stomach mucosal cells from ulcer control group + manuka honey (2.5 gm/kg) group.

antioxidant ability of manuka honey may be behind its potential to treat chronic inflammations, including ulcers.

Another possible mechanism by which manuka honey treats gastric ulcer may be due to inhibition of the proinflammatory cytokines: TNF-α, IL-1β, and IL-6. These cytokines are involved in production of acute inflammation [46], accompanied with neutrophil infiltration to the gastric mucosa [47], leading to gastric mucosal injury [48, 49]. It has been reported that manuka honey decreased the inflammatory response associated with ulcerative colitis, an inflammatory bowel disease characterized by an overexpression of inflammatory cells [50, 51]. The specific components that give manuka honey its activity are not yet determined [52]. However, it may be due to the presence of specific polyphenols, flavonoids, and caffeic acid phenethyl ester [53, 54]. The data obtained in this study revealed that IL-10 levels were significantly decreased in the ulcer control group. IL-10 is an anti-inflammatory cytokine and can limit tissue damage caused by inflammation [55]. Those results are similar to those reported by Eamlamnam et al. [55]. Such an

effect may be due to the fact that when acetic acid induces gastric mucosal damage, T and B lymphocytes present in the submucosa beneath the damaged area and typically produce basal level of IL-10, become compromised, and fail to yield adequate levels of IL-10 [55]. Manuka honey elevated the IL-10 levels in the honey-treated group in comparison to the ulcer control group.

Inflammatory gastric diseases including gastric ulcer are commonly associated with increased epithelial proliferation [56]. However, chronic gastric ulcer is usually not associated with mucosal thickening, suggesting that the process of epithelial hyperproliferation is counterbalanced with cell losses, mainly through apoptosis [57]. In the current study, applying acetic acid to rats' gastric mucosa resulted in ulceration of the gastric mucosa that was associated with increased proliferation and apoptosis as indicated by DNA-flow cytometry analysis. These observations are consistent with previous reports on the induction of apoptosis in ulcerative gastric mucosa [58, 59]. On the other hand, administration of manuka honey significantly decreased percentage of apoptosis of gastric mucosa compared with that of untreated group. In addition, ulcer group treated with manuka honey showed significantly ($P < 0.05$) decreased proliferation as detected by DNA S-phase using flow cytometry. These data suggest that manuka honey counteracted the inflammatory effect of acetic acid on the gastric mucosa that resulted in the increase in proliferation and apoptosis.

5. Conclusion

This study demonstrated that manuka honey possesses a potent antiulcer activity, which may be due to its antioxidants abilities which result in reducing lipid peroxidation and interfering with the inflammatory process. The current study, therefore, adds to the long list of health benefits that are associated with consumption of honey and thus document its potency as a "functional food" that promotes better health.

Competing Interests

All authors have no competing interests whatsoever.

Acknowledgments

This work was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under Grant no. 503-130-1434. The authors therefore acknowledge, with thanks, DSR's technical and financial support.

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Hindawi
Evidence-Based Complementary and Alternative Medicine
Volume 2017, Article ID 6793456, 10 pages
https://doi.org/10.1155/2017/6793456

Research Article

Induction of Cell Cycle Arrest and Apoptotic Response of Head and Neck Squamous Carcinoma Cells (Detroit 562) by Caffeic Acid and Caffeic Acid Phenethyl Ester Derivative

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Received 3 October 2016; Accepted 19 December 2016; Published 12 January 2017

Academic Editor: Vassya Bankova

Natural polyphenols have been observed to possess antiproliferative properties. The effects, including apoptotic potential of bioactive phenolic compounds, caffeic acid (CA) and its derivative caffeic acid phenethyl ester (CAPE), on cell proliferation and apoptosis in human head and neck squamous carcinoma cells (HNSCC) line (Detroit 562) were investigated and compared. Cancer cells apoptosis rates and cell cycle arrests were analysed by flow cytometry. Exposure to CA and CAPE was found to result in a dose-dependent decrease in the viability of Detroit 562 cells at different levels. CA/CAPE treatment did significantly affect the viability of Detroit 562 cells (MTT results). CAPE-mediated loss of viability occurred at lower doses and was more pronounced, with the concentrations which inhibit the growth of cells by 50% estimated at 201.43 μM (CA) and 83.25 μM (CAPE). Dead Cell Assay with Annexin V labelling demonstrated that CA and CAPE treatment of Detroit 562 cells resulted in an induction of apoptosis at 50 μM and 100 μM doses. The rise of mainly late apoptosis was observed for 100 μM dose and CA/CAPE treatment did affect the distribution of cells in G0/G1 phase. A combination of different phenolic compounds, potentially with chemotherapeutics, could be considered as an anticancer drug.

1. Introduction

Polyphenols, the main constituents of honey bee hive product propolis, are well known to inhibit cell proliferation and induce cell death in human cancer cells [1–3]. The biological activities of propolis are mainly attributed to caffeic acid, cinnamic acid, phenethyl esters, p-coumaric acid, artepillin C, galangin, cardanol, baccarin, chrysin, and other ingredients which possess oxyradical scavenging properties [2–4]. Recent evidence indicates that polyphenols and flavonoids are responsible for an induction of apoptosis and cell cycle inhibition, antiangiogenesis, suppression of matrix metalloproteinases, prevention of metastasis, and augmentation of the effects caused by chemotherapy [3–7]. These compounds are intracellularly metabolized via multiple pathways targeting distinct molecules and exhibiting wide spectrum of cellular cytotoxicity in different cancer types. More specifically, propolis constituents, including phenolic acids effect tumor cells through apoptosis, cell cycle arrest, and cytostatic activity, induced endoplasmic reticulum stress, and caspase activity also reduced mitochondrial membrane potential [8–11]. However, the precise mechanisms by which propolis constituents, caffeic acid and its caffeic acid phenethyl ester, activate apoptosis in human cancer cells still remain uncertain and inconsistent.

Organic phenolic compounds including caffeic acid (CA) and its derivative caffeic acid phenethyl ester (CAPE) are known to be highly bioactive components extracted from honeybee hive propolis [12, 13]. Recent studies indicate that they exhibit cytotoxic, antiproliferative [14, 15],

anti-inflammatory, immunomodulatory [16, 17], antioxidative [17–20], and antibacterial properties [17, 21]. CA and particularly CAPE treatment suppresses proliferation, survival, and invasion of human malignant metaplastic cells, including oral cancer cells [14, 15, 22–25]. Our recent study demonstrated that caffeic acid is able to attenuate the viability and migration rate of oral cancer SCC-25 cells [26]. To the best of our knowledge, there are limited previous studies comparing the growth inhibition of human head and neck squamous carcinoma cells by different polyphenols and/or flavonoids. According to available data, tea catechins are the only flavonoids used in clinical studies on oral cancer [27].

Above 90% of oral and head and neck malignancies are classified histologically as squamous cell carcinoma (SCC) [28, 29]. Squamous cell carcinoma is the most lethal head and neck cancer and, according to epidemiological data, it belongs to the sixth most common epithelial malignancies worldwide. Low survival rate of patients is linked to regional lymph node metastases, poor response to current therapeutic drugs, and local relapse [30]. Although research development in oral and cancer therapy over the recent decades is undoubtedly significant, treatment outcome of HNSCC may not be successful for a significant group of patients, resulting in cancer recurrence and progression, with a decreased overall survival rate. Bioactivity of propolis and plants phytochemicals constituents, including CA and CAPE compounds, is directly attributed to their chemopreventive potential in oral squamous cell carcinoma and generally in human oral carcinogenesis [31–33]. The synergistic and/or additive effects of common components, identifiable in propolis, plants, and vegetable/fruits, are responsible for the chemoprotective action of "healthy organic food" and may play important role in oral and pharyngeal cancer prevention [34, 35].

The current in vitro study has been arranged to investigate the cytotoxic effects of two bioactive phenolic constituents of propolis: caffeic acid and caffeic acid phenethyl ester on the viability, apoptosis, and cell cycle arrest of head and neck (HNSCC) squamous carcinoma cells Detroit 562 line.

2. Material and Methods

2.1. Cell Line Culture Conditions and Reagents. Detroit 562 human squamous carcinoma cell line originating from pharynx primary location was used in the present study and purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK). Detroit 562 HNSCC cells were seeded on 6-well microplates and were cultured in standard culture medium (EMEM; Eagle's Minimum Essential Medium) containing 10% fetal bovine serum (FBS; Pasching, Austria) and 1% penicillin-streptomycin (PAA Laboratories GmbH, Pasching, Austria) at 37°C in 5% CO_2 in air (CO_2 incubator, Heraeus Instruments, Hanau, Germany). Additionally, cells were cultured with 100 μg/mL streptomycin, 100 IU/mL penicillin, and 0.25 μL/mL amphotericin B at 37°C in a 5% CO_2 atmosphere. Reagents were purchased from PAA Laboratories GmbH (Pasching, Austria); caffeic acid and caffeic acid phenethyl ester were purchased from Sigma (St. Louis, MO, USA). Muse™ Annexin V and Dead Cell kit were purchased from Millipore (Billerica, MA, USA).

2.2. Cell Viability/Proliferation Assay. Detroit 562 HNSCC cells proliferation was measured by the (4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cells were seeded on 96-well microplates at 5×10^3 cells/well and left for 48 h in to enable them to attach to the culture medium. Culture medium was decanted and to each well a culture medium-containing CA or CAPE with concentration from 100 to 5 μM was added and left for 24 or 48 h. Next, cell medium was decanted and 10 μL of MTT solution (5 mg/mL MTT in phosphate-buffered saline (PBS)) was added and left for 3 h. Formed formazan crystals were dissolved in DMSO.

Live cells appeared purple in colour in response to MTT. The investigated substances CA and CAPE were applied to monolayer cultures of Detroit 562 human head and neck cancer cells at the final concentrations from 5 to 100 μM, except for the control cells, to which nutrient medium was applied. One hundred microliters of supernatant was transferred to a 96-well plate and cell viability was determined using Elx800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA), by measuring a spectrometric absorbance at 570 nm. The half and quarter maximal Inhibitory Concentration (IC_{50}, IC_{25}) value of the CA and CAPE was determined for monolayer cells. The chemical structure of CA and CAPE is presented in Figures 1(a) and 1(b), respectively.

2.3. Cell Apoptosis Assay: Analysis of Viability and Cell Death Using Flow Cytometry. Detroit 562 cell apoptosis and dead cells, including the percentage of apoptotic cells, were assayed using the multifunctional Muse Annexin V and Dead Cell kit (Millipore, Billerica, MA, USA) according to the user's guide and the manufacturer's instructions. Briefly, after treatment with CA and CAPE, Detroit 562 cancer cells were harvested with trypsin-EDTA and washed twice in PBS. Fresh medium-containing serum was added to each well so final concentration was 1×10^5 cells/mL. Staining protocol included warming the Muse Annexin V and Dead Cell Reagent to room temperature, addition of 100 μL of cells in suspension to each tube, addition of 100 μL of the Muse Annexin V and Dead Cell Reagent to each tube, and mixing thoroughly by vortexing at a medium speed for 5 seconds. Cells were resuspended in PBS with 1% FBS, mixed with the Muse Annexin V and Dead Cell reagent. Samples were incubated for 20 minutes at room temperature in the dark. The percentage of apoptotic cells was analyzed by flow cytometry using Muse Cell Analyzer (Millipore, Billerica, MA, USA) system and were expressed as percentage of apoptotic cells and standard deviation bars represent SD. As a negative control we used pure medium with FBS serum and as a positive control the medium with paclitaxel addition at concentration 100 nM.

2.4. Flow Cytometry Analysis of Cell Cycle Detroit 562 Arrest. Detroit 562 cells were seeded in 4-well plates and incubated with medium containing 10% FBS at 37°C. After treatment with CA and CAPE cell samples were transferred to 15 mL conical tube and the minimum number of cells for fixation

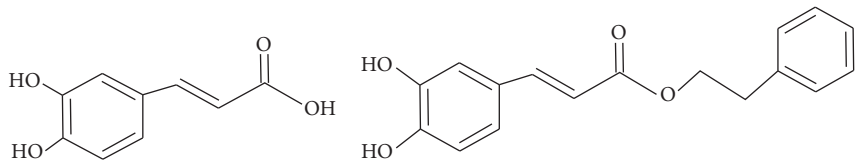

Figure 1: Chemical formulas of bioactive phenolic compounds: caffeic acid (CA) classified as hydroxycinnamic acid (a) and CAPE (b). Caffeic acid comprises both phenolic and acrylic functional groups.

in a tube was amounted at 1×10^6 cells. Samples collected after 24 h and 48 h were gently centrifuged for 5 min at 1500 rpm and washed in PBS. Obtained pellets were fixed in chilled 70% ethanol. Detroit 562 cells were kept in −20°C for 7 days until cell cycle was assayed. After ethanol removal cells were suspended in 0.25 mL PBS per 5×10^5 cells and warmed up to 37°C. Cell pellet was resuspended in 200 μL of Muse Cell Cycle Reagent, incubated for 30 minutes at room temperature, protected from light, and cell suspension was transferred to a 1.5 mL microcentrifuge tube prior to analysis on Muse Cell Analyzer. Cell cycle was assayed by fluorescence-activated cell sorting analysis using a Muse Cell Analyzer (Merck, Millipore, Billerica, MA, USA) with the configuration of 532 nm green laser line, three detection channels, and microcapillary 100 μL round bore.

2.5. Statistical Analysis. Data are presented as means ± standard deviation (SD) and were analyzed by nonparametric methods using the Statistica 9.0v (StatSoft, Tulsa, OK, USA) computer-based statistics programs. Statistical differences between means were evaluated by Friedman ANOVA variance analysis followed by post hoc Dunn's test and Wilcoxon test. The value of $p < 0.05$ was considered to be significant ($*$), $p < 0.01$ and $p < 0.001$ as highly significant ($**$ and $***$, resp.). The results were obtained from three separate experiments performed in quadruplicates ($n = 12$) for cytotoxicity. The experimental means were compared to the means of untreated cells harvested in a parallel manner. IC_{25} and IC_{50} values were calculated from the corresponding concentration inhibition curves according to plotted data presentation based on representative graphs.

3. Results

The study was aimed at comparison of the influence of two common phenolic compounds, constituents of propolis: caffeic acid and caffeic acid phenethyl ester on inhibition of the proliferation, viability and growth of squamous carcinoma cells, as recent reports have confirmed the beneficial effect of propolis-induced cellular stress on selected tumor cells [23–26]. The cellular effect on the HNSCC cell line Detroit 562 was investigated in vitro with the use of MTT assay in a microculture system using various incubation concentrations. Cytotoxic efficacy of CA and CAPE was expressed as the percentage of viable HNSCC Detroit 562 carcinoma cells at different concentrations of CA/CAPE with regard to the unexposed cells. The half maximal Inhibitory Concentration (IC_{50}) was defined as the CA/CAPE concentration value which inhibits the viability of Detroit 562 HNSCC cells in culture by 50% compared to the untreated cells (control). The quarter maximal Inhibitory Concentration (IC_{25}) was defined as the CA/CAPE concentration value which inhibits the viability of Detroit 562 HNSCC cells in culture by 25% compared to the untreated cells (control). IC values were extrapolated from cell viability-CA/CAPE concentration curves. To establish the concentration required to cause effects of 50% growth inhibition in Detroit 562 cells after 24 h and 48 h, a log viability-log dose curve was plotted.

3.1. High Concentrations of CA and CAPE Decrease of Head and Neck Detroit 562 Cell Line Viability and Mitochondrial Function. Results of our experiment revealed that the investigated propolis-derived substances at concentrations up to 25 μM exhibit relatively low cytotoxic activity against Detroit 562 cells. As shown in Figure 2, after 24 h/48 h exposure of Detroit 562 cells to 10 μM of CA/CAPE, the cell viability decreased slightly, except for CA/24 h. However, the absorbance value significantly increased and cytotoxicity increased significantly for CA/CAPE concentrations above 25 μM ($p < 0.05$, $p < 0.01$, and $p < 0.001$, depending on time and substance). The overall viability of Detroit 562 cells significantly decreased for CA and CAPE concentrations of 50 μM and 100 μM ($p < 0.01$, $p < 0.001$), with the cell viability reduction between 16% (CA 24 h 50 μM) and 60% (CAPE 48 h 100 μM). For the concentrations of 25 μM and 50 μM of CA and CAPE the cell viability decrease was similar after 48 hours (Figure 2). These findings were enhanced by validating the dose required to inhibit growth of 50% of HNSCC cells (IC_{50}) which exhibited a value range 201.43 μM–83.25 μM after 48 h of incubation time. The minimum CA and CAPE concentrations required to cause 25% and 50% cell growth inhibition after 48 h were 31.30 μM (IC_{25}, CA), 201.43 μM (IC_{50}, CA) and 18.84 μM (IC_{25}, CAPE), and 83.25 (IC_{50}, CAPE), respectively, while the IC_{25} and IC_{50} values for 24 h of incubation time were much higher: 93.01 μM (IC_{25}, CA), 1061.61 μM (IC_{50}, CA) and 45.03 μM (IC_{25}, CAPE), and 340.95 (IC_{50}, CAPE).

3.2. Exposure to CA/CAPE Stimulates Cell Apoptosis of Detroit 562 Cells. To investigate the apoptotic effect of CA and CAPE, Detroit 562 cells were treated with both substances for 24 h and 48 h, and apoptotic cells were assessed by staining

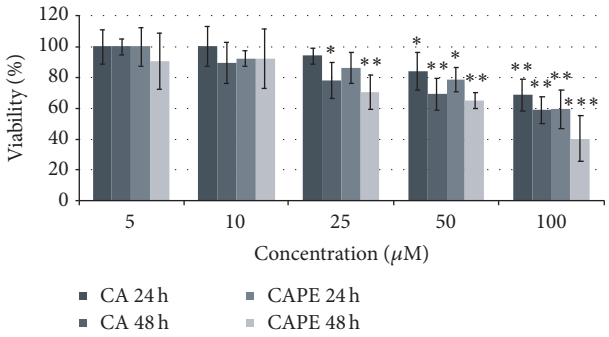

Figure 2: Cytotoxic effects of CA and CAPE at concentrations 5–100 μM on Detroit 562 cancer cells. These effects are highly concentration-dependent. The percentage of cell death measured by MTT cytotoxicity assay. MTT values represent mean ± SD of three independent cytotoxicity experiments performed in quadruplicate ($n = 12$). The lower concentration of CAPE (25 μM) produced similar killing effect on Detroit 562 cells as 50 μM concentration of CA. Mean cytotoxicity between different concentrations alone was statistically significant above the concentration of 25 μM ($^{*}p < 0.05$ and $^{**}p < 0.01$, ANOVA Friedman ANOVA test, Wilcoxon test). CA and CAPE at concentrations range of 25–100 μM induce cytotoxic effects on HNSCC carcinoma cells in a dose-dependent manner and displayed a time-dependent influence during 24 and 48 h of experiment. On the contrary, CA/CAPE concentrations within the range 5–10 μM did not alter markedly the Detroit 562 cells' viability and indirect proliferation during 24 h and 48 hours of exposure, reflected by only a slight increase of absorbance. $^{***}p$ value < 0.001.

with Annexin V. To determine whether CA/CAPE treatment results in apoptosis in Detroit 562 HNSCC cells, we used a Muse Annexin V and Dead Cell kit to measure the changes in cell apoptosis after 24 h and 48 h. We observed that both investigated substances induced cell death through apoptosis in Detroit 562 HNSCC cells (Figures 3 and 4). Comparative and similar results were obtained for 24 h and 48 hours. As shown in Figure 4 total apoptotic Detroit 562 cells following exposure to 100 μM CAPE for 24 h and 48 h were significantly increased (31 ± 2.0% and 55 ± 6.71%, resp.) compared with nontreated control (12 ± 0.6%, $p < 0.05$). In particular, the difference between exposure of Detroit 562 cells to 50 and 100 CAPE in the percentage of early apoptotic cells was minimal (1.47% versus 3.49% and 1.12% versus 1.71%, $p > 0.05$), whereas the variation between the cell groups in the percentage of late apoptotic cells was more pronounced for different concentrations and time laps of both CA and CAPE. These data suggest that phenolic compounds such as CA/CAPE suppress cell viability in Detroit 562 cells via apoptotic pathway.

For the highest CA concentration 100 μM, total apoptosis of Detroit 562 cells increased: 36 ± 6.0% (24 h) and 41 ± 7.0% (48 h) compared to 16 ± 2.1% and 19 ± 1.2%, respectively, in controls. For the highest CAPE concentration 100 μM, total apoptosis of Detroit 562 cells increased: 31 ± 2.0% (24 h) and 55 ± 6.7% (48 h) compared, respectively, to 12 ± 0.6% and 13 ± 0.7% in controls (Figure 4). CA-induced and CAPE-induced total apoptosis of Detroit 562 cells for the concentration 50 μM was determined at 22% (24 h) and 30% (48 h) versus 16% (24 h) and 21% (48 h), respectively, for CA and CAPE (Figure 4). The results suggest that the relative apoptosis efficacy (late and total apoptosis) of 100 μM CAPE in Detroit 562 cells after 48 hours is substantially higher compared to 100 μM CA. The apoptotic spectrum of Detroit 562 cells after 24 h of 100 μM CA treatment seems to be roughly an equivalent of 100 μM CAPE exposure. The difference between two time laps, 24 h and 48 h for both concentrations 50 μM and 100 μM, in the percentage of late and total apoptotic cells, was significant for both substances CA and CAPE ($p < 0.05$), whereas the difference between these two time laps in the percentage of early apoptotic cells was slight. Generally, CAPE induced more apoptosis in Detroit 562 cells than did CA after 48 hours and in opposite, CA induced more apoptosis in Detroit 562 cells than did CAPE after 24 hours. The weakest effect was observed in the cells treated with 50 μM of CA for 24 hours.

3.3. Effect of Two Concentrations of CA and CAPE on Detroit 562 Cell Cycle Phase Distribution: CA/CAPE Arrests HNSCC Cells at the G0/G1 Phase. Due to the fact that previous studies demonstrated modulation of the HNSCC cell cycle by propolis compounds [14, 22], the CA and CAPE effect on Detroit 562 cell cycle status was examined. The effect of CA and CAPE on the cellular cycle distribution was quantified using flow cytometric analysis and cell cycle progression was examined after treatment with 50 and 100 μM of CA and the same concentrations of CAPE for 24 h and 48 h. As shown in Figure 5, treatment of Detroit 562 cells with CA dose of 50 μM and 100 μM for 48 h h resulted in a significantly higher percentage (80 ± 3.2% and 75 ± 1.4%) of cells in the G0/G1 phase than in the control group (56 ± 4.8%, $p < 0.05$ and $p < 0.01$), with a corresponding reduction in the percentage of cells in the S phase (13 ± 5.6% and 21 ± 1.0%, resp., $p < 0.05$ and $p < 0.01$). More pronounced arrest of G0/G1 phase was observed for 100 μM CAPE (50 ± 6.5%) when cells were treated for 48 h compared to the control (19 ± 2.1%, $p < 0.001$) (Figure 5). These data suggest that inhibition of cell proliferation or induction of cell death in Detroit 562 cancer cells by CA/CAPE is associated mainly with the induction of G0/G1 arrest considering the time laps of 48 hours. The different proliferation rates of Detroit 562 cells exposed to CA/CAPE versus control, untreated cells were partially due to the differences in cell cycle regulation.

As shown in Figure 5, no significant difference between the untreated cells and cells exposed to 50/100 μM CA after 24 hours was observed. However, the percentage of Detroit 562 cells in G0/G1 phase slightly increased up to 74% (CA 50 μM), compared to control (64 ± 4.2%, $p < 0.05$), and the S phase cells decreased (21/24% CA 50/100 μM versus 31 ± 3.5% control, $p > 0.05$). The difference between percentage of untreated cells in S phase and G2/M phase and cells treated with 100 μM CAPE for 48 hours was also significant (49±4.7% versus 31±3.8% and 32±2.9% versus 18±2.6%; $p < 0.05$). The data indicated that CA and CAPE arrested Detroit 562 cells cycle after 48 hours at the G0/G1 phase in a dose- and time-dependent manner through disruption G0/G1 checkpoint, which also contributed to the growth inhibition of Detroit 562 cancer cells. This finding suggests an antiviability activity of

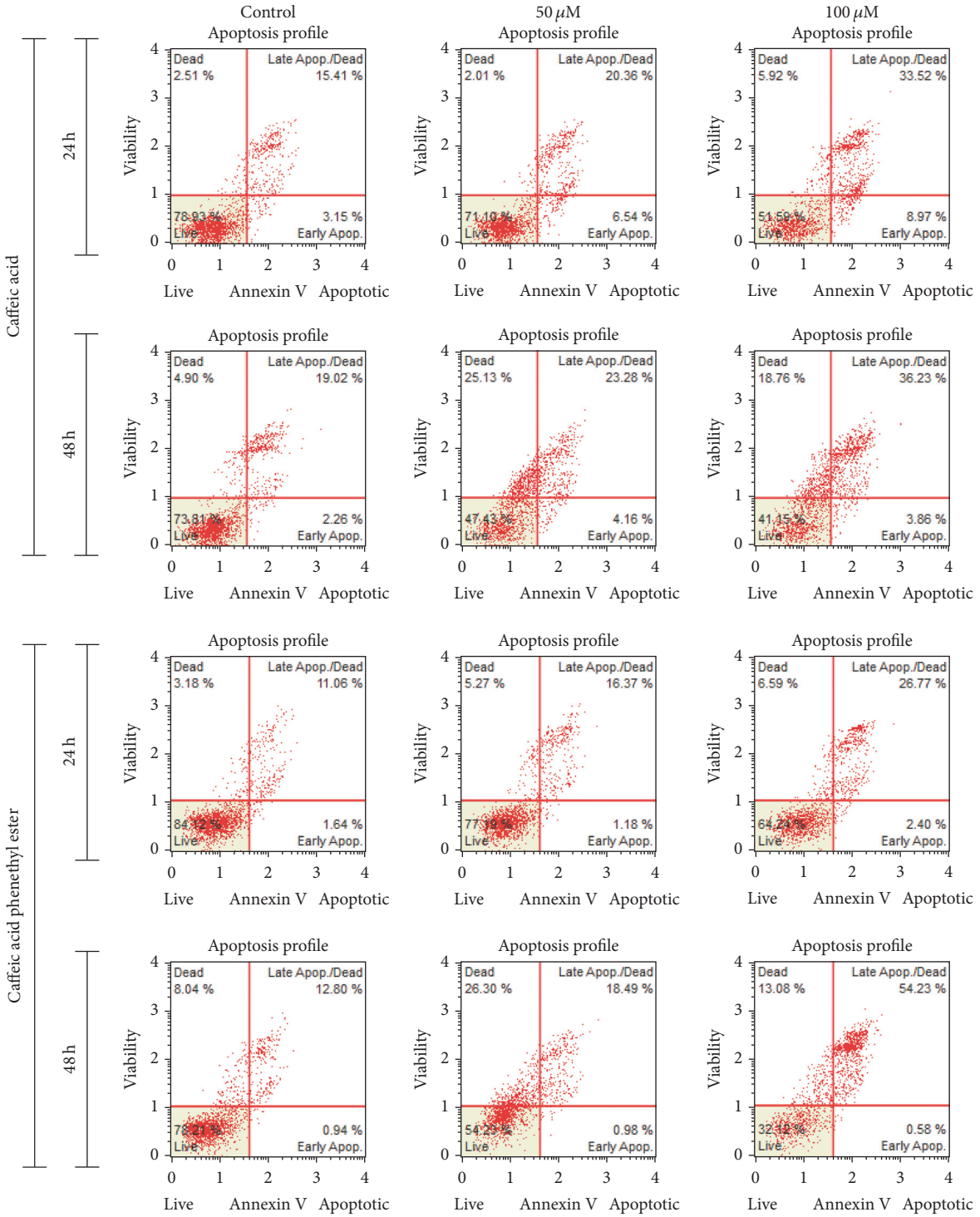

FIGURE 3: Effect of CA and CAPE substances on Detroit 562 cell apoptosis (representative plots). Early apoptotic cells are shown in the lower-right quadrant of the scatter plot, and live cells are in the lower-left quadrant. Both phenolic compounds CA and CAPE induced apoptosis in a dose-dependent manner as measured by the Muse Annexin V and Dead Cell assay. Flow cytometry was shown to induce apoptotic cell death in the epithelial tumor cells Detroit 562 by mainly early and late apoptosis, which was apparent when the percentage of live cells markedly decreased.

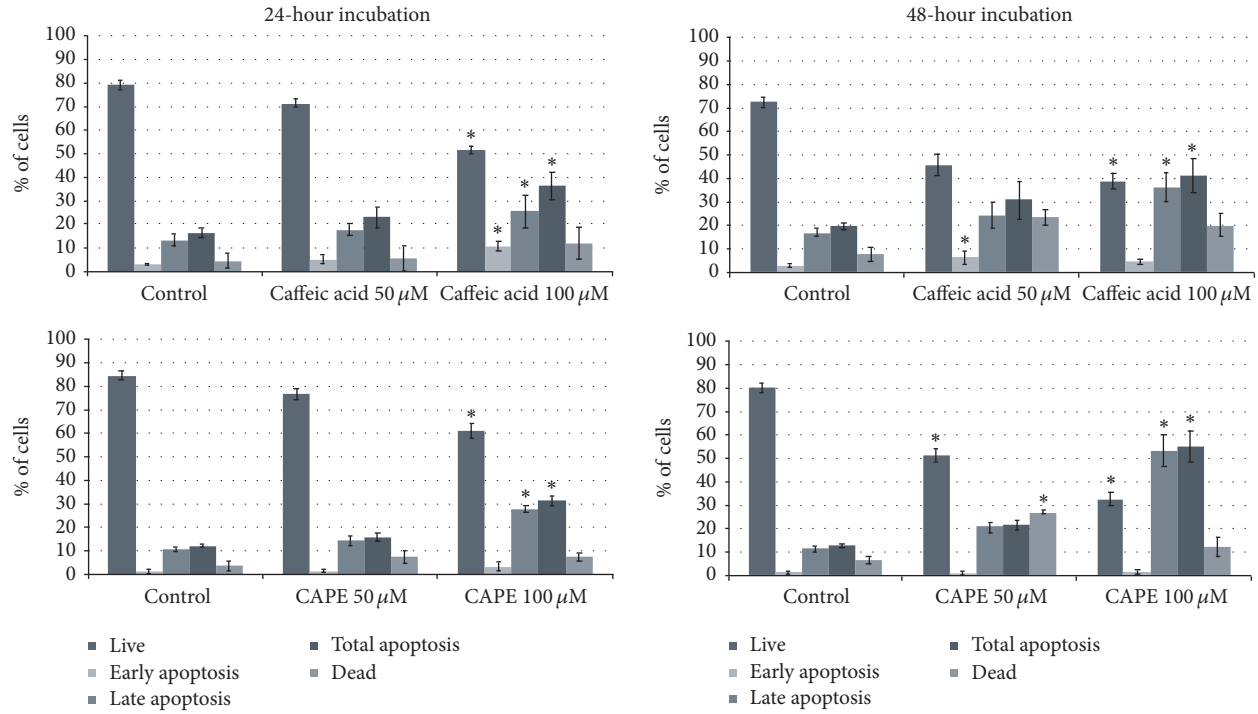

Figure 4: Flow cytometric analysis demonstrated a significant increase in proportion of total apoptotic cells in the NHSCC cells following exposure to mainly CAPE 100 μM and CA 100 μM, also with increased percentage of dead cells. Total apoptotic cells significantly increased following exposure to 100 μM CAPE, compared with 100 μM CA after 48 hours ($p < 0.05$). Specifically, the difference between CA and CAPE for both concentrations 50 μM and 100 μM in the percentage of early apoptotic cells was slight for the time laps 24 h, whereas the difference between them in the percentage of late apoptotic cells was significant for concentration 100 μM ($p < 0.05$). Vertical bars represent the standard deviation of means (SD) ($n = 3$ experiments). $^*p$ value < 0.05.

relatively low concentrations of CAPE in malignant epithelial cells and is consistent with previous reports regarding in vitro squamous cell carcinoma cells studies.

4. Discussion

Head and neck cancers primarily occur in the larynx and pharynx; however, they can be also localized in oral cavity, with a predominant location on the ventral/lateral lingual site or on the floor of the mouth. Considering a relatively high risk of recurrence (20%–30%) and a low five-year survival rate (50–60%), the oncological management of these cancers has to be effective and predictable [36, 37]. Propolis and its constituents have been found to possess a cytotoxic effect on various cancer cells [38], but studies on human head and neck cancer Detroit 562 cells treated with CA/CAPE have not been reported.

The purpose of our study was to investigate the cellular response of Detroit 562 cells to two selected propolis components. Here, we demonstrated and compared the biological effects of phenolic constituents of propolis: caffeic acid and its derivative caffeic acid phenethyl ester in head and neck cancer Detroit 562 cells for the first time. Some natural substances popular in complementary medicine appear to be well suited as a potential novel agent for the adjunct treatment of certain forms of epithelial head and neck malignancy, with supportive clinical trials [39]. The results obtained from flow cytometric assay clearly revealed that CA and particularly CAPE induced dose-dependent growth inhibition and apoptosis in HNSCC, with evident alterations of Detroit 562 cell cycle. This method also identified exclusively dead cells, and CA/CAPE treatment resulted in diminishment of life of HNSCC cells. This presented study is one of the first, to the best of our knowledge, to compare the cytotoxic effects of CA and CAPE in HNSCC Detroit 562 cell line, with the conclusion that CA and CAPE moderately inhibited the proliferation and reduced the viability of HNSCC cells. These results suggest that these phenolic compounds may be potentially considered as supportive chemotherapeutic agent for certain conditions of head and neck (pre)malignancy [40]. Phenolic compounds also have been shown to alleviate the effect of chemotherapeutics in cancer cells and sequential treatment of caffeic acid and paclitaxel induces potent synergistic effect, antiproliferation, and apoptosis of lung cancer cells, which involves NF-kappa B pathway [41].

The inhibitory effect of CA/CAPE on HNSCC cells was due to its ability to induce cell cycle arrest. This is the first step to demonstrate the possibility of cell cycle perturbation by CA and CAPE on this cell line. Whereas G0/G1 arrest was induced with both CA and CAPE treatment after 48 hours of incubation, slight arrest in the S phase was induced when Detroit 562 cells were treated with CA for 24 h. Interestingly, only the concentration of 100 μM of CAPE arrested mildly a Detroit 562 cell cycle in S and G2/M phase. Collectively, our

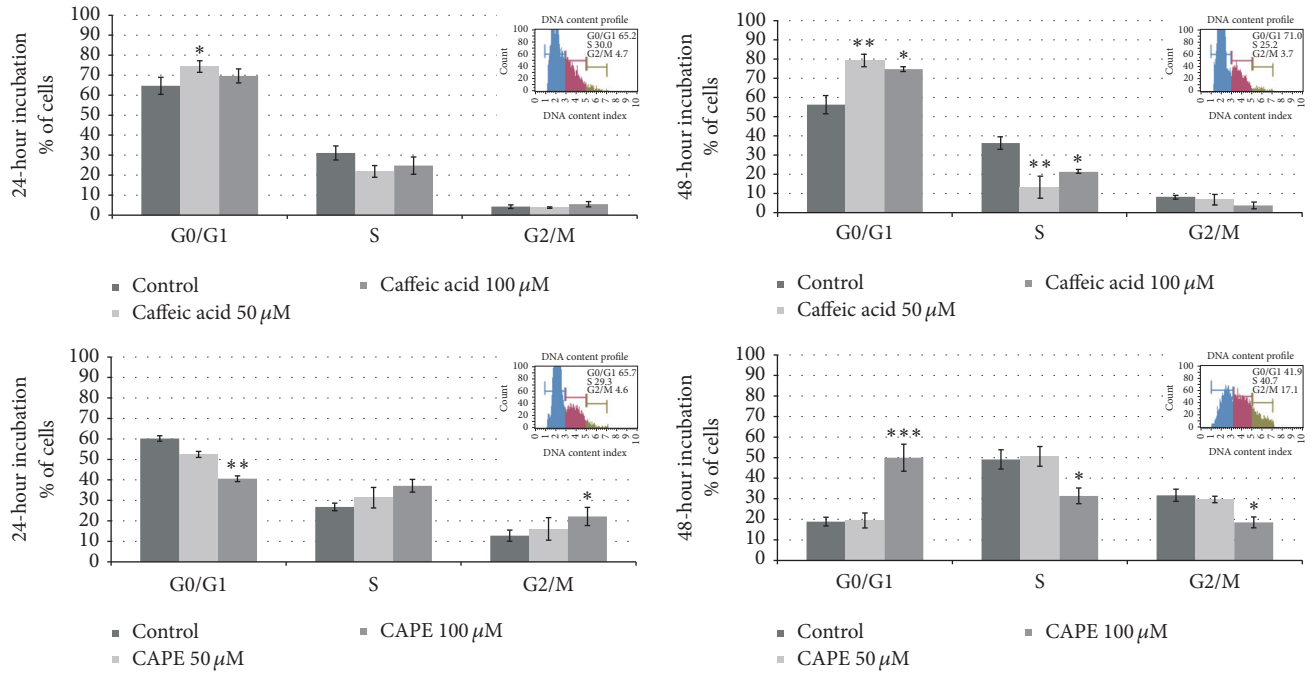

Figure 5: Alterations in the percentage of Detroit 562 cells in G0/G1, S, and G2/M phases of cell cycle are presented as the % of Detroit 562 of three independent experiments (bar graphs). The results show that CAPE at concentration of 100 μM has a mild effect on cell cycle arrest, which is contributing to its anticancer features. Detroit 562 cells exposure to CA and CAPE concentration for 48 h resulted in a cell cycle checkpoint arrest within the G0/G1 phase ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ independent experiments). Four representative flow cytometric plots (right upper) showing the cell cycle distribution following the Detroit 562 cells treatment with CA and CAPE at 50 and 100 μM for 24 h and 48 h. Cells were stained with Muse Annexin V and Dead Cell kit and were subjected to flow cytometric analysis that collected 10,000 events. The cell cycle distribution within 24 h following exposure of Detroit 562 cells to 50 and 100 μM of CA shows that CA treatment did not markedly affect the distribution of cells among the different phases of the HNSCC cell cycle. However, there was a slight increase in cell numbers in the S phase and G2/M phase when treated with 100 μM CAPE for 24 h ($p > 0.05$ and $p < 0.01$). Treatment with 100 μM CAPE for 48 h resulted in a significant accumulation of cells in the G0/G1 phase for Detroit 562 cell line ($p < 0.001$). Subsequently, cell number in the S phases and G2/M phase was decreased to 31% and 18%, respectively, when exposed to 100 μM CAPE for 48 h ($p < 0.05$ versus control).

results suggest that CA/CAPE inhibit head and neck cancer cell proliferation by inducing G0/G1 phase cell cycle arrest and are in agreement with other studies of contained treatment of other cancer cell lines. The G0/G1 phase can allow cells to trigger repair mechanisms or apoptotic pathways. Thus, the effects of CA and CAPE on apoptosis induction of Detroit 562 HNSCC cells were determined, and the results indicated that treatment of head and neck cancer cells with these two phenolic acids effectively induced apoptosis. Chemotherapeutic agents, including propolis constituents, are expected to inhibit the growth of some cancer cells. Apoptotic, antiproliferative and cytotoxic effects of propolis constituents have been reported previously in myeloid leukemia cells [42, 43], malignant melanoma cell line [44–46], human breast cancer cells [46–48], cervical cancer [49], and colon cancer cells [46]. A recent study reported that CAPE efficiently suppresses breast cancer stem cells from MDA-231 cells, a model of human triple-negative breast cancer [50]. Additionally, CAPE induced TRAIL-mediated cell death in Hep3B carcinoma cells [51] and stimulated the expression of death receptor 5 (DR5) and CAPE/TRAIL promoted apoptosis through the binding of TRAIL to DR5. It has been reported that CAPE inhibits proliferation [14, 15, 23, 24], COX-2 activity [52, 53], phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) signaling, and Skp2-F-box protein family, responsible for downregulation of $p27^{Kip1}$ protein [14] in human oral cancer cells. CAPE also induces apoptosis and inhibits cell growth by causing cell cycle G1 or G2/M phase arrest in different types of cancer cells [14]. Additionally, CAPE-treated human cancer cells inhibit cancer cell movement and migration [14, 54].

In particular, the current results indicate that CAPE had a greater apoptotic effect in Detroit 562 cells than did caffeic acid, which are considered the common constituents of propolis. Our findings suggest that certain doses of CA and CAPE (up to 25 μM) acting for 24 hours may not affect Detroit 562 cancer cells' viability and cell cycle. Low doses of biologically active natural substances can be attributed to so-called a "hormesis effect" by even promoting cell proliferation/cell viability and this phenomenon is believed to be an adaptive response of the carcinoma cells [55]. We demonstrated that Detroit 562 HNSCC cells display variable susceptibility to CA and CAPE under different sub- and cytotoxic conditions, considering the incubation time. Kuo et al. assumed that CAPE selectively suppress human oral cancer cells due to the fact that normal human oral fibroblasts

and buccal mucosal fibroblast (BF) cells were more resistant to CAPE treatment, with higher IC_{50} values [14, 56, 57]. In our study the IC_{50} for CA and CAPE treatment of Detroit 562 cells after 48 hours were 201.43 μM and 83.25 μM, respectively, which is coherent with the results of other studies. The IC_{50} of CAPE in cancerous human oral cell lines, to suppress proliferation of oropharyngeal squamous cell carcinoma cell line TW2.6 72.1, neck metastasis of gingiva carcinoma, tongue squamous cell carcinoma, oral squamous cell carcinoma, and oral epidermoid carcinoma-Meng 1 (OEC-M1) were 72.1 μM, 101.0 μM, 120.9 μM, 129.7 μM, and 159.2 μM, respectively [14].

The anticancer activity of natural polyphenols, also present in numerous plants, fruits, and vegetables, has been extensively reported as described in preclinical studies and with regard to oral cancer, many phenolic compounds have been investigated in vitro and in vivo. Ciftci-Yilmaz et al. demonstrated that certain range of concentrations of CAPE reduces the viability of UT-SCC-74A head and neck squamous cancer stem cells [58]. According to recent study carried out by Czyżewska et al. [22] the caffeic acid induced apoptosis in 24% of the human tongue squamous cell carcinoma cell line (CAL-27) and ethanol extract of propolis, polyphenols, and mixture of polyphenolic compounds were cytotoxic for CAL-27 cells in a dose-dependent manner. EEP inhibited cell viability and induced apoptosis by upregulation of caspase-3, caspase-8, and caspase-9 in human tongue squamous cell carcinoma cell line [22]. Quercetin (flavonol, propolis ingredient) suppressed oral squamous cell proliferation by arresting G1 cell cycle phase via mitochondria-mediated apoptosis and inhibiting cell migration [40]. An inhibition of SCC-25 OSCC cells migration induced by caffeic acid was also demonstrated in oral cancer cells [26]. Some polyphenols may reverse epithelial-to-mesenchymal transition and suppress cancer invasion and in human oral cancer SCC-4 cell line [59]. To sum up, these findings indicate that caffeic acid and caffeic acid phenethyl ester could play a potential adjunct role in the therapeutic management of oral and/or head and neck cancer.

The mechanisms of activity of polyphenols comprise induction of apoptosis and cell cycle arrest, scavenging of free radicals, regulation of gene expression, and stimulation of the immune system [6–10, 60, 61]. Apoptosis plays a crucial role during oncological treatment of malignant conditions. The apoptotic range in a cell culture is a crucial parameter of cell health/viability and it can be referred to specific morphological changes. The Muse Cell Analyzer designed for a quantification of cellular apoptosis enables multidimensional cell assessment using a simplified method and does not require complicated protocols. In this study, we used the Muse Cell Analyzer for apoptosis detection using the Muse Annexin V and Dead Cell Assay. The results of available studies [62] indicate that Muse Annexin V and Dead Cell Assay allows the highly accurate assay of cellular apoptosis for both suspension and adherent cell lines using multiple treatment conditions.

Caffeic acid, CAPE, and the broad range of propolis-originated compounds are currently under scientific research and clinical investigation as a novel antitumor agents with a view at the treatment outcomes for certain types of malignancies [63]. Potentially, synergistic effects of polyphenols in propolis are responsible for their potential anticancer activities [22]. In conclusion, a combination of propolis constituents could be considered as a chemopreventive measure in a human squamous cell carcinoma originated from oral cavity or head and neck region. Due to highly individual dietary habits, populations are exposed to huge variation of bioactive natural substances present in foods. Moreover, the synergistic or additive effects of ingredients and natural compounds are responsible for the health-promoting properties of propolis-based products [30]. What is more promising, the novel technologies may enhance the therapeutic and chemopreventive potential of propolis-originated constituents, such as functionalization with nanoparticles, enhancing the efficacy of biologically active natural substances [30]. Studies explaining and clarifying the mechanisms involved in anticancer efficacy can bring invaluable data to this area of chemotherapy.

5. Conclusions

With limitations of in vitro study, we summarize that the current evidence of human head and neck and oral cancer adjuvant therapy and/or chemoprevention with the use of caffeic acid and/or CAPE is positive but still inconclusive. Promising results have been obtained for selected biologically active substances isolated from bee products and propolis, though the definite conclusions are still incoherent. Further advanced studies are required, following an evidence-based approach, in particular clinical trials, to confirm the clinical effectiveness of polyphenols on oral cancer treatment and prevention.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The study was supported by grants of Ministry of Science and High Education/Medical University of Silesia: KNW-1-206/N/5/0 and KNW-2-005/N/6/K, Katowice, Poland.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 3816460, 8 pages
http://dx.doi.org/10.1155/2016/3816460

Research Article

Evaluation of Beeswax Influence on Physical Properties of Lipstick Using Instrumental and Sensory Methods

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Received 21 September 2016; Revised 26 October 2016; Accepted 27 October 2016

Academic Editor: José Maurício Sforcin

The aim of this study was to optimize the lipsticks formulation according to the physical properties and sensory attributes and investigate the relationship between instrumental and sensory analyses and evaluate the influence of the main ingredients, beeswax and oil, with analysis of lipsticks properties. Central composite design was used to optimize the mixture of oils and beeswax and cocoa butter for formulation of lipsticks. Antioxidant activity was evaluated by DPPH free radical scavenging method spectrophotometrically. Physical properties of lipsticks melting point were determined in a glass tube; the hardness was investigated with texture analyzer. Sensory analysis was performed with untrained volunteers. The optimized mixture of sea buckthorn oil and grapeseed oil mixture ratio 13.96 : 6.18 showed the highest antioxidative activity (70±0.84%) and was chosen for lipstick formulation. According to the sensory and instrumental analysis results, optimal ingredients amounts for the lipstick were calculated: 57.67% mixture of oils, 19.58% beeswax, and 22.75% cocoa butter. Experimentally designed and optimized lipstick formulation had good physical properties and high scored sensory evaluation. Correlation analysis showed a significant relationship between sensory and instrumental evaluations.

1. Introduction

Safety of various cosmetic products has become a major trend in recent years. The abundant composition of cosmetics is of increasing concern for consumer's health and environmentally friendly products for the earth. Consumers are searching for natural-based cosmetic products to avoid allergic reactions and any side effects and for the safety of their health and the important criteria are natural raw materials [1].

Acceptable lipstick for the consumers should have a suitable texture and spreadability. Descriptive sensory profiling is an essential tool in this process as it allows an experienced panel to assess the qualitative and quantitative characteristics of a product [2]. Hardness and melting point are the main physical properties important for the stability of lipstick in all usage period and transportation. These characteristics can vary according to the composition of ingredients [3]. Therefore, the optimization of mixture composition is important and experimental design is very useful. Statistical mixture design is more satisfactory and effective than other methods such as classical one-at-a-time or mathematical methods because it can study many variables simultaneously with a low number of observations, saving time and costs [4]. In previous works, for the optimization of mixtures, D-optimal cross and mixed designs were used and were quite effective [4]. In this work, response surface central composite design was applied in order to investigate the relationship between composition, physical properties, and sensory analysis of consumers, which can be the most important factor [5].

The common ingredients of lipstick are wax, butter, and oil [6]. Waxes are very useful cosmetic ingredients based on their various advantageous properties. Beeswax is a natural compound secreted by bees and is widely used for dermatological products due to its countless benefits. Beeswax is mainly composed of a mixture of hydrocarbons, free fatty

acids, monoesters, diesters, triesters, hydroxy monoesters, hydroxy polyesters, fatty acid polyesters, and some unidentified compounds [7]. This substance contains natural ingredients, which help retain moisture in the skin, especially helpful for dry and chapped lips. Various researches have also discovered that beeswax contains small amounts of natural antibacterial agents and can help prevent a painful inflammation that comes with an infection [8]. Beeswax is vitamin-rich, containing plenty of vitamin A, which helps to improve wound healing, reduces wrinkles, protects the skin against UV radiation, and stimulates skin cells turnover [9]. Natural oils are used in a wide variety of cosmetic products and influence the sensory characteristics of the products [10]. The contents of bioactive lipophilic compounds promote elasticity and healing and moisturize the skin and help it maintain a proper moisture balance and protect the skin from free radical damage. Biologically active compounds of natural oils ensure the beneficial properties of lipsticks: moisturizing and protection from damage of free radicals. There are many scientific lines of evidence on antioxidant, anti-inflammatory, immunomodulatory, regenerative, and other valuable activities of biocompounds of sea buckthorn and grapeseed [11–13]. In this present study, the mixture of buckthorn and grapeseed oils was evaluated for antioxidant activity and used for the lipstick formulation to achieve the highest benefit for the consumers. It was important to evaluate the influence of the main ingredients, beeswax and oil, with analysis of lipsticks properties.

2. Materials and Methods

2.1. Materials. Yellow beeswax was purchased from Bitute, Lithuania; cocoa butter was purchased from Henry Lamotte GmbH, Germany; jojoba oil, grapeseed (*Vitis vinifera* L.) oil, and sea buckthorn (*Hippophae rhamnoides* L.) oil were from Biokosmetikos Akademija, Lithuania; olive (*Olea europaea* L.) oil was manufactured by Anira, Spain; castor (*Ricinus communis* L.) oil was manufactured by Henry Lamotte Oils, Germany; cedar (*Pinus sibirica*) balm oil was manufactured by Alsu, Russia; essential oil of thyme (*Thymus vulgaris* L.) was manufactured by Aromatika, Ukraine, and was purchased from a local market. Ethanol 96% (Stumbras, Lithuania) and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) reagent (Sigma-Aldrich, Germany) were used for the study.

2.2. Preparation of Lipsticks. Lipsticks were formulated using a mixture of natural ingredients according to the composition suggested by the central composite response surface design (Table 2). Lipsticks were produced in the laboratory by the moulding method. Beeswax was added to the preheated mixture of oils and heated to 60–67°C until melting. The mixture was homogenized together and cocoa butter was added and melted. The preservative, essential oil of thyme, was added to lipstick base mixture and cooled till 50°C. All of the ingredients were homogenized and poured into clean and lubricated moulds.

2.3. Experimental Design. The preliminary formulation of the lipstick was prepared for the suitable lower and upper limits of solid and liquid parts. The results showed that the amount of beeswax 70–30 in percentages and the amount of oil 40–70 in percentages formulate appropriate lipstick. The experimental design is conducted by using Design-Expert® 6 (version 6.0.8, Stat-Ease Inc., Minneapolis, USA). A set of mixtures was formed using response surface design central composite criterion. The experimental mixture design was applied to study the effect of two-component system, amount of oil and amount of beeswax, on the response variables: melting point and the results of sensory analysis. The set of mixtures is shown in Table 2.

2.4. Antioxidant Activity. Antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical inactivation method with modifications [14]. For the test, DPPH powders were dissolved in a small volume of ethyl acetate and diluted with ethyl acetate by adjusting the absorbance to 0.700 ± 0.020 at 520 nm. A 20 mg oil sample was weighed in a test tube, and 80 μL ethyl acetate as well as 2.9 mL DPPH$^{\bullet}$ free radical solution was added. The samples were agitated and incubated for 30 min in darkness. Absorbance was measured at 520 nm against ethyl acetate. The percent inhibition of the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical was calculated according to the formula

$$\text{DPPH}^{\bullet}\ (\%\ \text{inhibition}) = \frac{\left(A_{\text{blank}} - A_{\text{sample}}\right)}{A_{\text{blank}}} \times 100, \quad (1)$$

where A_{blank} is the absorbance of the blank solution and A_{sample} is the absorbance sample and 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) free radical solution after 30 min.

2.5. Melting Point. Lipstick sample of 2 g was placed into a glass tube. This tube was dipped into a plate full of water, which was heated on the water bath. The temperature at which the material forms a liquid drop was considered its melting point.

2.6. Sensory Analysis. Sixty female participants aged 18–25 years who used lip products were selected for sensory evaluation. Filler questions were included so that the applicants could know what the tested lipstick was and their answers would be as honest as possible. Sensory analysis was performed in a room that was well naturally lit with the temperature (25 ± 2°C), humidity (≈55%), and noise controlled. All samples were stored at room temperature (25 ± 2°C) and kept away from direct sunlight. The participants were instructed in the sensory analysis procedure [5]. The bioethics committee of the Lithuanian University of Health Sciences permitted the sensory analysis.

Sensory analysis was performed in three stages before application: initial appearance, during application, and after application ("after texture"). The initial attributes were appearance, colour, and smell. During the application, consistency (hardness), spreadability, and greasiness were evaluated. Ten minutes after the application, the degree of absorption, amount of residue, and moisturizing were evaluated. Thirteen designed lipsticks formulations (Table 2) were evaluated by a

panel of female participants. All samples were evaluated in randomized order and lipstick tubes were coded. The panelists filled specific protocols based on 0–10 scale for evaluation of each attribute. Appearance, colour, and smell were scored as follows: 0 means unacceptable and 10 means acceptable. Consistency evaluation scale was as follows: 0 means very soft and 10 means very hard. Spreadability evaluation scale was follows: 0 means very bad and 10 means very good. Greasiness was evaluated as follows: 0 means no feeling and 10 means very greasy. Degree of absorption was scored as follows: 0 means very slow and 10 means very fast. Moisturizing evaluation was as follows: 0 means no feeling and 10 means very good. Amount of residue was scored as follows: 0 means no feeling and 10 means too much. Filled questionnaires were analyzed and profiles of sensory analysis were created for lipsticks comparison.

2.7. Texture Profile Analysis. The lipstick hardness is a very important physical characteristic. The texture profile analysis on the formulated products was conducted using a TA.XT.plus (Stable Micro Systems Ltd., Godalming, Surrey, UK) texture analyzer. The penetration depth of a standard 2 mm needle (P/2N) at a constant 5 kg load force was measured to represent the hardness of the lipstick. The sample cuts were placed centrally under the needle probe, which penetrate the sample at 1 mm/s until force of 50 g was achieved. All tests were conducted at room temperature (25 ± 2°C) and repeated three times.

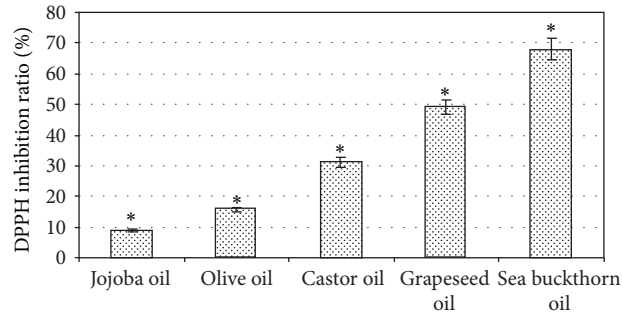
2.8. Statistical Analysis. The results are presented as mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test with the software package Prism v. 5.04 (GraphPad Software Inc., La Jolla, CA, USA). A value of $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Optimization of Mixture of Natural Oils. Natural oils of olive, jojoba seed, castor seed, cedar balsam, grapeseed, and sea buckthorn fruits were chosen as potent active constituents for lipstick production. For comparison of their possible antioxidant activities, DPPH free radical scavenging activity was evaluated (Figure 1). The results are expressed as inhibition percentage of free radicals and ranged from 3.92% to 68%. Grapeseed oil and sea buckthorn oil were selected for the lipstick formulation due to their highest inhibition percentage.

Mixture of grapeseed oil and sea buckthorn oil was optimized by central composite design to obtain the highest free radical scavenging effect. The experimental mixture design of the amount of oils and the responses with DPPH inhibition percentages are shown in Table 1.

Response of experimental design with DPPH inhibition percentage ranged from 47.37% to 59.91%. According to the results, it can be concluded that the highest scavenging activity shows mixtures with higher amount of sea buckthorn oil. The target of mixture optimization was the highest DPPH inhibition percentage. To achieve the highest desirability, the amount of oils was changed (from 6 till 14 g). The program designed experimental model and determinations of radical scavenging activity were performed. DPPH scavenging activity ranged from 52.08% to 65.76%. The highest activity was observed in mixture: 14 g of sea buckthorn oil and 6.0 g of grapeseed oil. The lack of fit (F value = 0.0352) implies that the model is significant and the optimum mixture was suggested to consist of 13.96 g sea buckthorn oil and 6.18 g grapeseed oil, with the highest desirability of 0.96.



* $p < 0.05$ versus all different oils

Figure 1: DPPH inhibition percentage of jojoba, olive, castor, grapeseed, and sea buckthorn oils, $n = 5$.

Table 1: Experimental data of sea buckthorn and grapeseed amounts obtained from mixture design and response, DPPH inhibition percentages of oils mixtures.

Number	Sea buckthorn oil (g)	Grapeseed oil (g)	Response, DPPH inhibition (%)
1	6.00	6.00	53.94 ± 1.10
2	10.00	10.00	59.91 ± 1.15*
3	6.00	6.00	54.98 ± 1.10
4	6.00	11.66	57.72 ± 1.12
5	6.00	6.00	52.60 ± 1.10
6	11.66	6.00	59.86 ± 1.15*
7	2.00	10.00	47.37 ± 1.06
8	10.00	2.00	54.62 ± 1.11
9	2.00	2.00	51.72 ± 1.10
10	6.00	6.00	56.28 ± 1.12
11	6.00	0.34	52.24 ± 1.10
12	6.00	6.00	55.63 ± 1.10
13	0.34	6.00	48.88 ± 1.10

* $p > 0.05$ *versus* formulation number 6.

3.2. Evaluation of Lipstick Formulations. The experimental design for the lipstick formulations is given in Table 2. Sensorial and physical evaluation was performed as response. Sensory properties of the produced lipsticks were assessed in three stages: before, during, and after application; results are presented in Figures 2(a), 2(b), and 2(c). All tested attributes evaluated of lipsticks are not significantly different: the smell in the acceptability assessment ranged from 6.7 to 8.6 points,

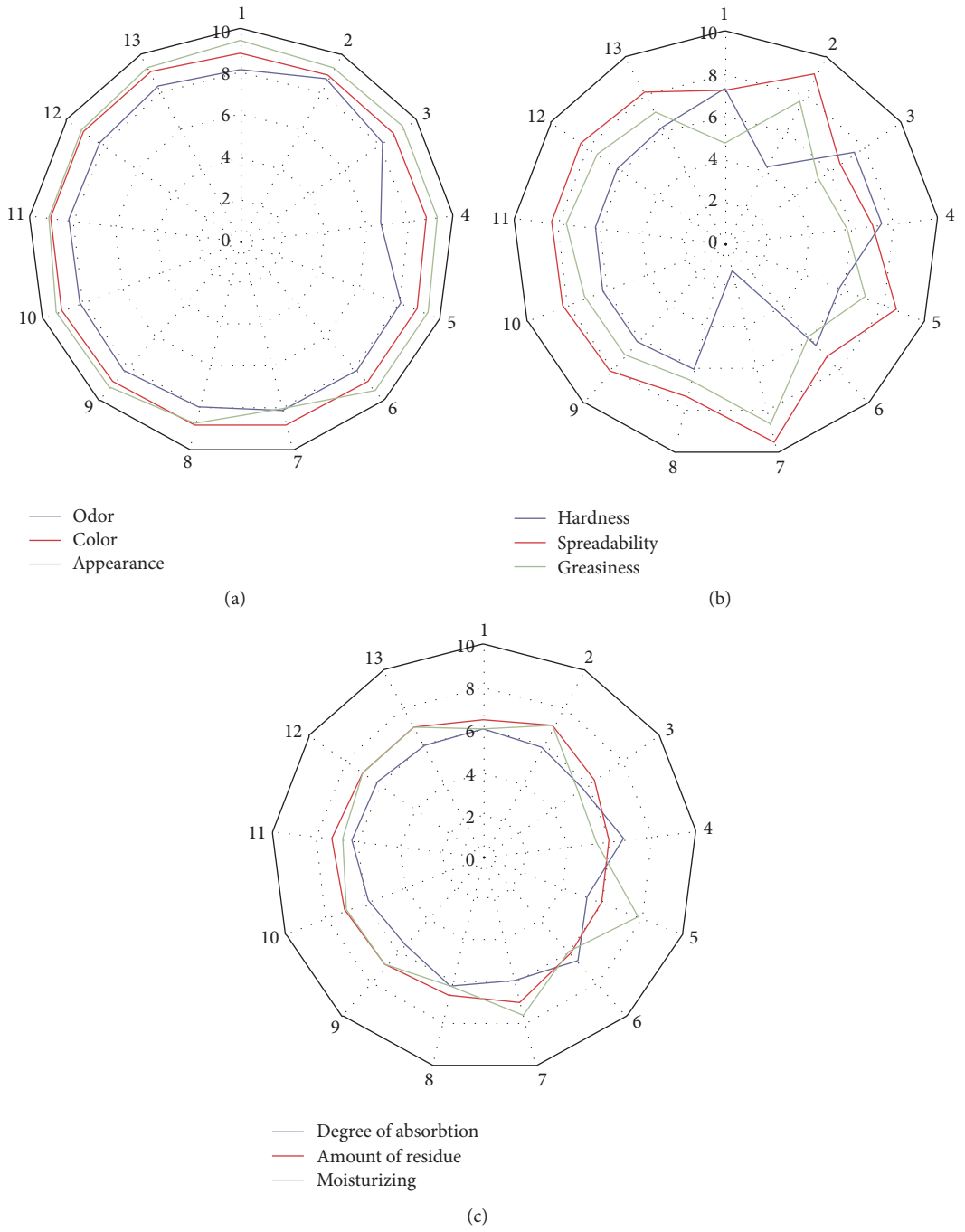


Figure 2: (a) The profile of sensory assessment of the produced lipsticks before application. (b) The profile of sensory assessment of the produced lipsticks during application. (c) The profile of sensory assessment of the produced lipsticks after application.

while appearance and colour were assessed quite well and ranged from 8.1 to 9.4 points. By analyzing the sensory attributes during application, results showed a more noticeable difference. The consistency varied between 1.3 and 7.6 points, while the desirable parameter was medium at −5 points. The lipstick assessed as the softest consists of 11.89% of beeswax; this was the lowest amount of all formulations. The hardest lipsticks were assessed at −7.4 and 7.4 points and beeswax amount was 30%. All remaining samples were evaluated approximately the same as the average of hardness. It can be concluded that different hardness assessment of lipsticks depends on beeswax amount. Greasiness ranged from 4.7 points to 8.7 points. Medium greasiness was assessed for 5.3 points, which was the desirable assessment, and the amount of oils was medium. The spreadability was assigned to a group of attributes, which should be assessed maximally for 10 points. The highest points were 9.6 points for lipstick consisting of the lowest amount of beeswax and the highest

Table 2: The set of component mixtures for the lipstick formulations and the response, melting point.

Number	Oil mixture, %	Beeswax, %	Cocoa butter*, %	Melting point, ˚C
1	40.00	15.00	45.00	56.0
2	70.00	15.00	15.00	55.0
3	40.00	30.00	30.00	64.0
4	70.00	30.00	0	64.0
5	33.79	22.50	43.71	62.0
6	76.21	22.50	1.29	62.0
7	55.00	11.89	33.11	51.0
8	55.00	33.11	11.89	69.0
9	55.00	22.50	22.50	63.0
10	55.00	22.50	22.50	63.0
11	55.00	22.50	22.50	63.0
12	55.00	22.50	22.50	62.0
13	55.00	22.50	22.50	62.0

*Amount of cocoa butter is calculated till 100%.

amount of cocoa butter. The lowest assessment was 6.6 points for the lipstick consisting of a medium amount of beeswax, oils, and butter. After application, the amount of residue was the highest for lipstick containing a high amount of butter and a low amount of beeswax. The moisturizing sensory attribute was evaluated very similarly: the highest evaluation for lipsticks containing a high amount of oils and low amounts of beeswax and the lowest evaluation without cocoa butter. The highest degree of absorption was evaluated for lipsticks containing a high amount of oils (70% and 76.21%) and minimum amount of cocoa butter (1.29% and 0%). The highest amount of cocoa butter (43.71%) was associated with the lowest absorption of lipstick. It can be concluded that absorption of lipsticks depends on cocoa butter amount.

Desirable points of sensory evaluation were divided into two groups. Maximum 10 points were for appearance, smell, colour, spreadability, and moistening attributes. Medium 5 points were for hardness, greasiness, amount of residue, and absorption. An average of two groups of assessment points were calculated and used as responses for the mathematical design program.

Another response was the melting points of designed lipsticks (Table 2), which varied from 51.0˚C to 69.0˚C. The melting point must be high to avoid technical deterioration during preparation and use. Previous studies and researches state the melting point of lipsticks to be in the 60.6–64.0˚C acceptable limit by the consumer [4], or else in the 40–56˚C range [1]. In this study, the desirable temperature of melting point was set at 60˚C. *Design-Expert 6* program calculated the optimal mixture for the lipstick formulation oil (57.67%), beeswax (19.58%), and cocoa butter (22.75%). The lack of fit (F value < 0.0001) implies that the model is significant and the optimum mixture was suggested with the highest desirability of 1.0.

Optimal lipstick formulation of the stated composition was produced and physical parameters were determined: melting point at 59.1˚C, when the predicted value was 60˚C.

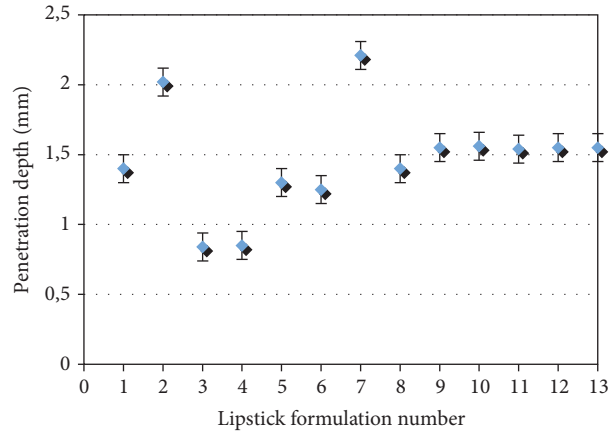


Figure 3: Texture profile penetration depth (mm) for all samples*, $n = 5$.

The essential oil of thyme as a natural preservative was added to the final lipstick formulation. It improved smell significantly ($p < 0.05$) while other sensory attributes remained similar (data not shown). Stability studies at one year confirmed that physical parameters remained stable.

Texture profile analysis was performed to compare sensory evaluation by volunteers and instrumental analysis. Hardness evaluation is an important physical characteristic of lipstick and is a useful tool in objective determination. Assessment points of consistency stated as "hardness" (Figure 2(b)) were compared with penetration depth test. The results of the thirteen designed lipsticks are presented in Figure 3. The penetration depth increases with the increase of oil mixture and decreases with the increase of beeswax. This dependency was observed by other researches also [4, 15].

3.3. Correlation Analysis. Correlation analysis was performed applying Spearman's rank coefficient. Relationship

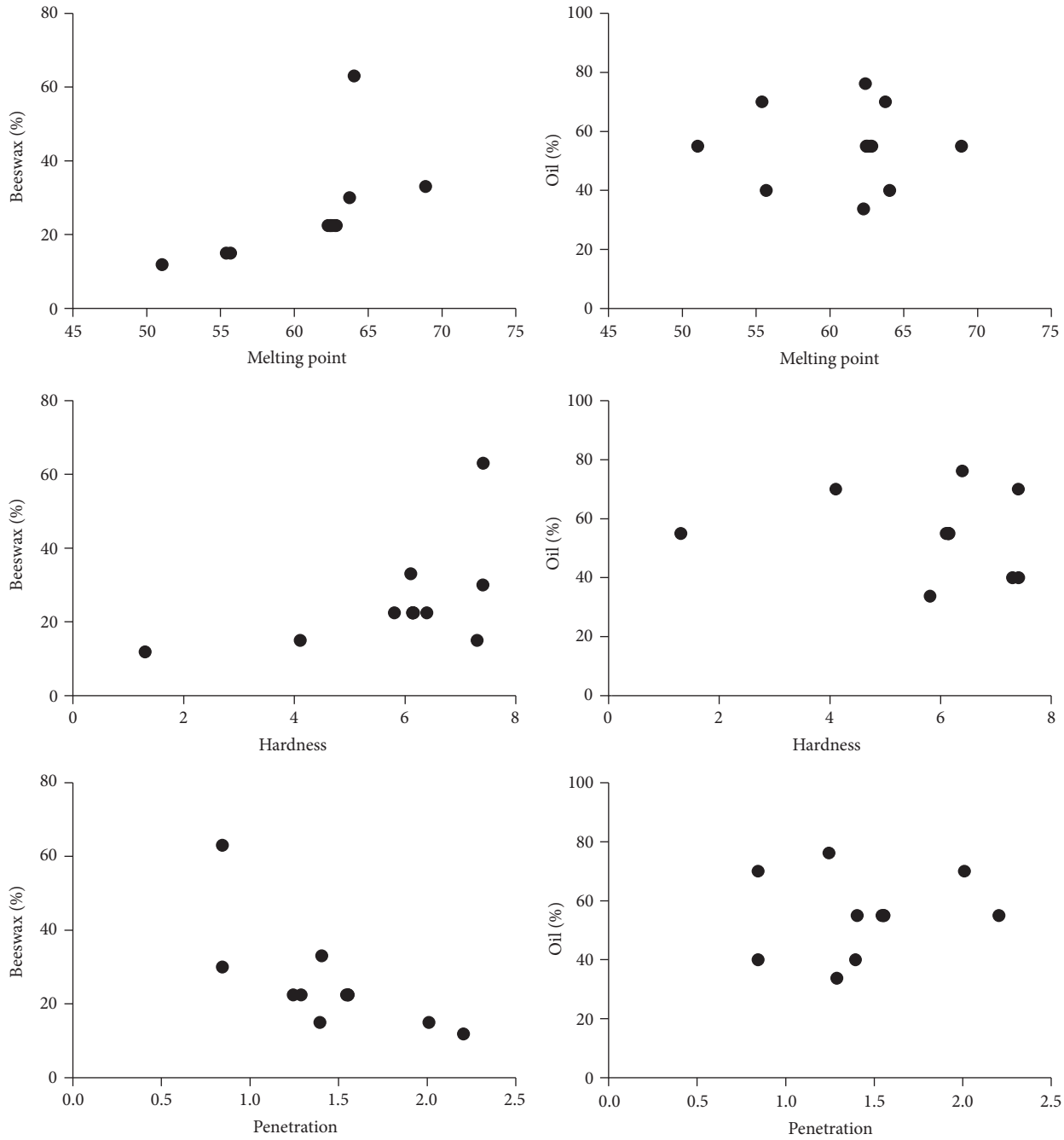


Figure 4: Graphical presentation of correlations of melting point, hardness, and penetration with the amount of beeswax and of oils (mixture of sea buckthorn oil and grapeseed oil in ratio 13.96 : 6.18).

between hardness, penetration, melting point, and components of lipsticks formulations (beeswax and mixture of oils amounts) was evaluated. Graphical data of correlations is presented in Figure 4. Negative corelation was determined between hardness and penetration depth, $R = -0.729$. High correlation was determined in another study, which showed that the mechanical test (objective method) was in good agreement with the sensory analysis (subjective method) [16]. It can be concluded that sensory analysis and instrumental analysis data are confirmed. Another important characteristic, melting point, correlates with the amount of beeswax ($R = 0.912$), while the amount of oils mixture produces a minimal effect. Amount of beeswax had higher influence on hardness and penetration depth than amount of oils mixture also. Hardness and penetration depth showed medium dependance on the melting point, $R = 0.484$ and $R = -0.441$, respectively. Results of this study correspond to the results of other researches [1].

4. Discussion

There are a lot of researches on the health benefits of various vegetable oils depending on their composition and biological activities. In this research, we evaluated the natural oils with the goal of providing the biological activity for the lipstick. Lips are very sensitive to the negative environmental factors and to the damage of free radicals; therefore, it was important to choose natural oil with high antiradical activity. We evaluated six natural oils and the highest results showed grapeseed oil 49,03±0,53% and sea buckthorn oil 68±0,72% of inhibited DPPH radicals. High antioxidant capacity of various oils of grapeseed was determined by other researches and such activity is due to powerful polyphenols constituents like flavan-3-ols and procyanidins [17]. Previous studies revealed that sea buckthorn oil is a potent antioxidant with 73,5±3,39% of DPPH free radicals inactivation and this can be due to the amount of carotenoids and other antioxidants [11, 18].

A good lipstick should have acceptable characteristics for the consumers; therefore, the sensory evaluation was performed. After initial evaluation before use, the mean point was 8.05, which showed that appearance, smell, and colour were well approved. During application of the lipstick, hardness, spreadability, and greasiness were evaluated. Sensory evaluation of consistency varied mostly, from 1.3 to 9.6 points, and the main outcomes are the hardness depending on the amount of beeswax and spreadability depending on the amount of cocoa butter. The feeling after use was divided into three attributes: degree of absorption, moisturizing, and amount of residue. Sensory evaluation results showed that all ingredients were important, but the most influencing ingredient was cocoa butter amount. For comparison with other researches, there are missing published studies, but the main consumers' demand is a natural-based product with good feeling after use.

Instrumental analysis consisted of determination of physical characteristics: melting point and penetration depth. Results of this study show that increasing the amount of beeswax gives a higher value of melting point and smaller value of penetration depth. Previous studies and researches demonstrated that the melting point of lipsticks and the hardness depend on the amount variations of waxes [1, 4, 15]. The influence of beeswax was confirmed as high by correlation analysis.

5. Conclusion

Experimental design was used to optimize mixture of grapeseed and buckthorn oils with the highest antioxidative activity and the final lipstick formulation. The optimal ratio of ingredients was assessed to evaluate the sensory and physical properties. Sensory acceptance and instrumental analysis showed that beeswax had higher influence on the lipstick properties, followed by other ingredients. The penetration depth increases with the increase of oil mixture and decreases with the increase of beeswax. Correlation analysis showed a significant relationship between sensory and instrumental evaluations.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

The authors would like to thank the Open Access Centre for the Advanced Pharmaceutical and Health Technologies (Lithuanian University of Health Sciences) for giving them the opportunity to use modern infrastructure and perform this research.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 8175265, 7 pages
http://dx.doi.org/10.1155/2016/8175265

Research Article

Formulation of Propolis Phenolic Acids Containing Microemulsions and Their Biopharmaceutical Characterization

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Received 20 May 2016; Accepted 10 July 2016

Academic Editor: Vassya Bankova

Microemulsions (MEs) were formulated using PEG-8 caprylic/capric glycerides and ethanolic propolis extracts. Characterization of MEs was performed by determining mean droplet size, polydispersity index, stability under varying external factors, and formulation effect on delivery of phenolic compounds into the skin *ex vivo*. Essential oils were included into the formulations of MEs and their influence on physical characteristics of the nanostructured systems as well as penetration into epidermis and dermis were evaluated. The droplet size, their distribution, and stability of the formulated MEs were not affected. Presence of essential oils in the formulation increased penetration of phenolic compounds in general, but only the amount of ferulic acid increased significantly. Mean droplet size increased with increase of oily phase amount, suggesting that phenolic compounds and components of essential oils were not modifying the formation of the interphase film composition and/or structure. Phenolic compounds were predominantly located in the lipid phase of the MEs thus minimizing their availability at the surface of the skin.

1. Introduction

Increasing understanding of risks affecting human skin and threats of resultant pathology is supporting search for new approaches and formulating innovative products to protect the outmost cells of human organism. External stress factors and ultraviolet radiation in particular can cause damage of the exposed skin cells resulting in oxidative stress, premature aging, and skin cancers [1]. The association between skin ageing and increased oxidative stress is clarified by the fact that skin-ageing changes could cause elevated levels of oxidative stress. It should be emphasized that increased oxidative stress may cause skin ageing, and skin ageing and elevated oxidative stress levels may both indicate existence of some underlying general cause [2]. Topically applied sunscreen products can protect the skin from the harmful effects of UV radiation. The possibility to incorporate naturally occurring substances in sunscreen formulations is becoming an actual topic as those secondary metabolites typically demonstrate antioxidant and UV-absorbing ability. It is determined that phenolic acids, nonflavonoids, and flavonoids can act as UV blockers reducing inflammation, oxidative stress, and DNA damage [3].

Scientific evaluation of propolis and its products demonstrates antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, antitumoral, and wound healing properties of propolis; it stimulates tissue regeneration, wound healing, suppresses pain, and tumefaction [4–6]. Propolis is commonly used as a liquid extract in cosmetic and medicinal preparations because of its antiseptic, anti-inflammatory, and anesthetic properties. Complex varying qualitative and quantitative composition of propolis extracts is a recognized challenge in standardizing propolis products. Phenolic acids and their esters, flavonoids, aromatic alcohols and aldehydes, and terpenes are basic components of different origin propolis and their presence defines quality of propolis products as well as biological activity and possible application [7, 8]. Efficacy of topically applied products is resulting from ability of active components to diffuse into the skin from the applied carrier system. The intensity and the rate of skin penetration by propolis extract components depend on the physicochemical properties of individual compounds and on the structure and composition of the carrier system. The limited ability of propolis polyphenolic compounds (vanillic, caffeic, *p*-coumaric, ferulic acids, and vanillin) to penetrate into the skin has been determined; thus the application of could

be considered as potential solution for achieving improved delivery of propolis antioxidant components into skin [9].

Cosmeceuticals are developed to enhance the health and beauty of skin and are classified as a category of products positioned between cosmetics and pharmaceuticals [10]. Protective cosmeceutical formulations should be both efficient and acceptable for consumer. The protective potential of biologically active components of propolis products could be enhanced by development of efficient delivery systems to transport required quantity of protecting compounds to the right site in the skin. Microemulsions are considered as promising carrier system for cosmetic active ingredients as they perform as efficient solubilizers for hydrophilic and lipophilic ingredients with high encapsulation capacity, improving product efficiency, stability, and appearance. They offer good cosmetic qualities and high hydration properties thus possibly enhancing skin penetration which may emphasize their importance in topical products [11]. Microemulsions are optically isotropic systems containing hydrophilic, lipophilic phases, and a mixture of surfactants. Thermodynamically stabile and transparent microemulsions are easily formulated systems with high diffusion and absorption rates. The low interfacial tensions of microemulsions provide excellent wetting properties ensuring good contact between formulations and the skin. The ingredients of microemulsions can effectively overcome the diffusion barrier and permeate the stratum corneum of the skin, offering efficient dermal and transdermal drug delivery [12]. The average droplet diameter in microemulsions could be in the range from 10 to 140 nm thus exhibiting properties of nanostructured systems. Appropriate selection of components for formulation of microemulsions can guarantee its biocompatibility, nontoxicity, and clinical acceptability.

The objective of this study was to formulate microemulsions containing propolis extracts and evaluate their physicochemical characteristics, stability, and ability to deliver propolis phenolic compounds into the skin *ex vivo*.

2. Materials and Methods

2.1. Chemicals and Reagents. Raw propolis was obtained from UAB Medicata Filia (Vilnius, Lithuania). Acetonitrile (Chromasolv) and acetic acid (glacial) were gradient grade for HPLC, ≥99.8% pure quality (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Ultrapure water was produced filtering purified water through the Millipore Simplicity HPLC grade water preparation cartridge (Bedford, USA). PEG-8 caprylic/capric glycerides (Labrasol) were purchased from Gattefosse (Saint-Priest, France); ethanol from AB "Vilniaus degtinė", Vilnius, Lithuania; isopropyl myristate, synthesis grade from Scharlab SL, Barcelona, Spain; sodium chloride from Carl Roth GmbH, Karlsruhe, Germany; and sodium azide from POCh, Gliwice, Poland.

2.2. Ethanolic and Aqueous Propolis Extract. Phenolic compounds from raw propolis were extracted using 96% ethanol or purified water at a material-to-solvent ratio of 1 : 10 (w/v), stirring on a hotplate magnetic stirrer WiseStir MSH-20D (Wertheim, Germany) for 1 hour and at 70°C temperature (aqueous propolis extract). Produced ethanolic or aqueous propolis extract was filtered using Buchner vacuum filtration system.

2.3. Propolis Samples Analysis by High-Performance Liquid Chromatography. Phenolic acids (*p*-coumaric, ferulic, caffeic, and vanillic acids) and vanillin were quantified in propolis extract and samples using Agilent 1260 Infinity capillary LC (Agilent Technologies, Inc., Santa Clara, CA, USA) with Agilent diode array detector (DAD) and applying validated HPLC method: C18 column (150 × 0.5 mm, 5 μm particle size); the linear elution gradient from 1 to 21% of solvent A (acetonitrile) in B (0.5% (v/v) acetic acid in ultrapure water) 25 min; the injection volume was 0.2 μL, the flow rate was 20 μL/min, and the column temperature was 25°C. The integration of phenolic compounds peaks was performed at 290 nm [9].

2.4. The Construction of Pseudoternary Phase Diagrams. Labrasol, 96% ethanol, isopropyl myristate, and purified water were selected as surfactant, cosurfactant, and oily and aqueous phase, respectively, for the construction of pseudoternary phase diagrams. The oil titration method was used for the production of the oil-in-water (o/w) microemulsions [13, 14]. The ratios of surfactant and cosurfactant in the microemulsions were 2 : 1, 3 : 1, 4 : 1, 5 : 1, 6 : 1, 7 : 1, 8 : 1, and 9 : 1. First, purified water (15–70%), surfactant, and cosurfactant (30–85% of mixture) were mixed stirring on a hotplate magnetic stirrer WiseStir MSH-20D at room temperature. Then oil was added by drops under stirring when the samples appeared as cloudy liquids (the limit of the o/w microemulsion). The regions of oil-in-water microemulsions were plotted at the pseudoternary phase diagrams (Figure 1).

2.5. Formulation of the Microemulsions Containing Ethanolic Propolis Extract. The same percentage composition of the o/w microemulsion was selected from the pseudoternary phase diagrams: 5% of oil phase, 25% of aqueous phase, and 70% of a mixture of surfactant and cosurfactant in different ratios (2 : 1, 3 : 1, 4 : 1, 5 : 1, 6 : 1, 7 : 1, 8 : 1, and 9 : 1). Purified water, labrasol (surfactant), ethanolic propolis extract (cosurfactant), and isopropyl myristate were mixed when microemulsions appeared as clear liquids (Table 1).

2.6. Thermodynamic Stability Studies of Propolis Microemulsions. Thermodynamic stability of o/w propolis microemulsions was evaluated performing centrifugation test and heating-cooling and freeze-thaw cycles test [15]. The microemulsions were centrifuged at 3500 rpm for 30 min. The microemulsions were stored at 4°C, 20°C, 32°C, and 45°C temperature for not less than 48 hours during heating-cooling cycle and at −21°C, 4°C, and 25°C temperature for not less than 48 hours during freeze-thaw cycle.

2.7. Physical Characterization of O/W Microemulsions. The droplet size, standard deviation, and polydispersity index (PDI) of o/w microemulsions were measured using Zetasizer Nano ZS particle size analyzer (Malvern, UK) [16]. The microemulsions pH was determined using pH-meter 766

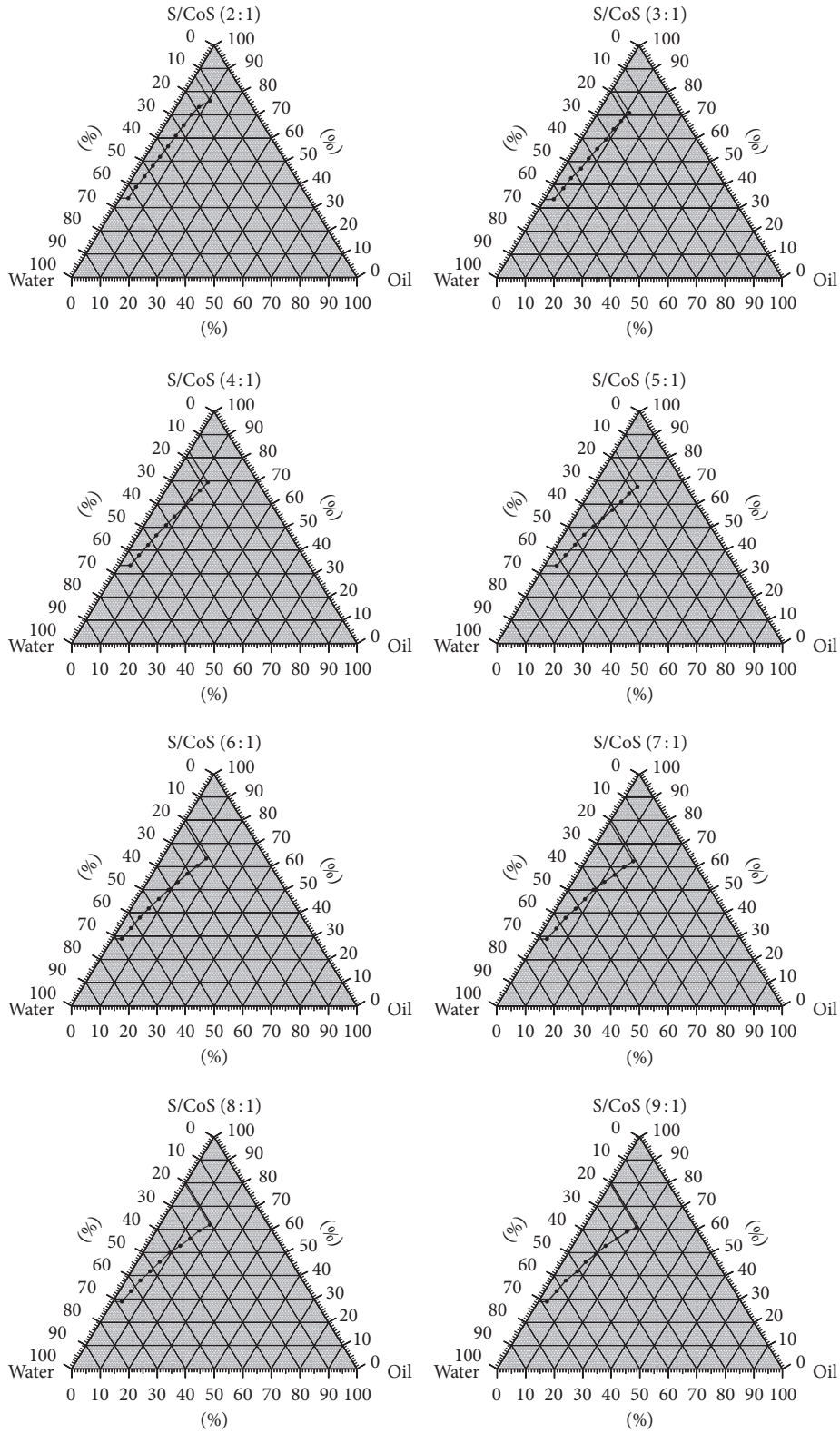

Figure 1: Pseudoternary phase diagrams of oil-in-water microemulsions.

Table 1: Compositions of oil-in-water propolis microemulsions.

Ratio of S : CoS	2 : 1	3 : 1	4 : 1	5 : 1	6 : 1	7 : 1	8 : 1	9 : 1
Isopropyl myristate (%)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Purified water (%)	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Labrasol (%)	46.7	52.5	56.0	58.3	60.0	61.3	62.2	63.0
Ethanolic propolis extract (%)	23.3	17.5	14.0	11.7	10.0	8.8	7.8	7.0

(Knick, Germany); conductivity was determined using conductivity meter (Cond 3110 SET 1, Germany); viscosity was determined using Vibro-Viscometer SV-10 (I&D Company, Limited, Japan).

2.8. Ex Vivo Skin Penetration Study. Caucasian women (age range of 25–40 years) abdominal skin was obtained from Department of Plastic and Reconstructive Surgery, Hospital of Lithuanian, University of Health Sciences, after cosmetic surgery. Kaunas Region Bioethical Committee has approved the use of human skin for transdermal penetration studies. *Ex vivo* skin penetration studies ($n = 6$) were performed using Bronaugh type flow-through diffusion cells with full-thickness human skin. The efficient diffusion area in the cells was 0.64 cm^2. The diffusion cells were placed on the metallic heating block maintaining 37°C temperature by a Grant TC120 thermostated circulating water bath (Grant Instruments Ltd., Cambridge, Great Britain). The acceptor medium (0.9% NaCl solution with 0.005% NaN3) was circulated underneath the skin samples maintaining 0.6 mL/min of circulation rate by Masterflex L/S peristaltic pump with multichannel pump head (Cole-Parmer Instrument Co., IL, USA). The infinite dose of the o/w propolis microemulsion was applied on the outer human skin side surface, and the diffusion cells were covered with aluminum foil. After 24 hours, microemulsions were removed from the human skin surface. The skin samples (0.64 cm^2) were trimmed off removing the outer residuals. Epidermis was separated from dermis applying dry heat separation method [9, 17] and they were separately extracted with a mixture of methanol and deionized water (1 : 1) under sonication.

2.9. Statistical Analysis. Statistical analysis of experimental data was performed using SPSS software (version 19.0). Mann-Whitney *U* test and one-way ANOVA (Tukey's honestly significant difference criterion) were used for data analysis. Correlation analysis was performed applying Spearman's rank coefficient.

3. Results and Discussion

The concentrations of phenolic acids and vanillin were determined in the ethanolic and aqueous propolis extract (Table 2). The total concentration of phenolic acids and vanillin (2472.7 ± 24.6 μg/mL) in ethanolic propolis extract was up to 7.5-fold higher comparing with that in aqueous propolis extract (330.5 ± 8.9 μg/mL). Ethanolic propolis extract was used as an active component and cosurfactant for the formulation of microemulsions (MEs).

As presented in the constructed pseudoternary phase diagrams (Figure 1), the composition of MEs containing 5% of oily phase, 25% of aqueous phase, and 70% of a mixture of surfactant and cosurfactant in ratios 2 : 1, 3 : 1, 4 : 1, 5 : 1, 6 : 1, 7 : 1, 8 : 1, and 9 : 1 resulted in stable MEs and was selected for further development. Ethanol was used as cosurfactant in formulating o/w MEs during initial testing and obtained data were used for evaluation of phenolic compound effect on MEs characteristics.

Thermodynamic stability studies demonstrated that o/w MEs were stable in centrifugation and heating-cooling tests although minor instability was determined in freeze-thaw testing.

The measurements of pH, conductivity, and viscosity were performed 24 hours after formulating o/w MEs [18] to detect possible effects of ethanolic propolis extract (Table 3). The results confirmed absence of critical effects of propolis phenolic compounds on the physical characteristics of MEs.

The pH and viscosity of the MEs were not significantly ($P > 0.05$) affected by changing ethanol to ethanolic propolis extract. The conductivity of the MEs containing ethanolic propolis extract was similar to the MEs containing ethanol thus confirming the same type of o/w dispersion.

All o/w microemulsions were characterized measuring their droplets size and polydispersity index (PDI) (Table 4) and determining number of particle fractions in MEs. The measurements of droplet size in MEs after 24 hours and 1 week were performed to evaluate possible changes of nanostructured systems.

Mean droplet size of MEs containing ethanol or ethanolic propolis extract after 24 hours was similar to the values determined after 1 week. The increase of the surfactant-cosurfactant ratio in MEs resulted in increased mean droplet size but had no effect on the PDI of the MEs. Two-peak particle distribution pattern was determined for MEs formulated with S-CoS ratio 2 : 1; thus it was not considered for further development of ME formulation. Propolis microemulsion with S-CoS weight ratio 3 : 1 and containing 5% isopropyl myristate as oily phase was selected as offered maximum quantity of ethanolic propolis extract to be incorporated. The evaluation of oily phase amount effect on the physical parameters and stability of MEs was performed variating isopropyl myristate content from 3 to 7% (Table 5).

The values of mean droplet size of o/w propolis MEs containing 3%, 5%, or 7% of isopropyl myristate 1 week after formulation did not differ from values determined 24

Table 2: Quantitative profile of phenolic compounds in Lithuanian propolis extracts.

Propolis extract type	Phenolic compound ± SD (μg/mL)				
	Vanillic acid	Caffeic acid	Vanillin	*p*-Coumaric acid	Ferulic acid
Ethanolic	267.7 ± 23.5	59.0 ± 4.4	507.3 ± 11.2	983.7 ± 8.0	654.9 ± 4.1
Aqueous	56.1 ± 3.8	10.2 ± 2.0	80.6 ± 3.0	119.6 ± 2.8	64.1 ± 2.0

Table 3: Characteristics of o/w microemulsions containing ethanolic propolis extract and controls.

Ratio of S : CoS	pH		Conductivity (μS/cm)		Viscosity (mPa·s)	
	ME with EtOH	ME with propolis extract	ME with EtOH	ME with propolis extract	ME with EtOH	ME with propolis extract
2 : 1	4.79	4.60	10.4	10.8	11.2	13.2
3 : 1	4.72	4.57	9.7	9.9	14.7	17.8
4 : 1	4.68	4.56	9.3	9.4	18.5	22.1
5 : 1	4.64	4.56	9.1	9.0	22.1	24.4
6 : 1	4.59	4.55	8.8	8.8	26.9	27.7
7 : 1	4.58	4.54	8.6	8.6	29.1	29.6
8 : 1	4.57	4.52	8.5	8.5	31.0	32.3
9 : 1	4.56	4.52	8.4	8.3	31.2	36.4

Table 4: Mean droplet size and PDI of o/w microemulsions.

Ratio of S : CoS	Mean droplet size ± SD (nm)/PDI			
	24 hours		1 week	
	ME with EtOH	ME with ethanolic propolis extract	ME with EtOH	ME with ethanolic propolis extract
2 : 1	34.95 ± 0.19 0.299 ± 0.003	43.69 ± 0.41 0.274 ± 0.020	34.97 ± 0.17 0.300 ± 0.005	43.49 ± 0.46 0.268 ± 0.017
3 : 1	40.67 ± 0.32 0.310 ± 0.004	48.83 ± 0.31 0.274 ± 0.006	40.45 ± 0.35 0.304 ± 0.003	48.62 ± 0.42 0.285 ± 0.020
4 : 1	46.80 ± 0.86 0.312 ± 0.005	55.30 ± 0.45 0.285 ± 0.022	46.23 ± 0.29 0.312 ± 0.005	55.42 ± 0.36 0.275 ± 0.008
5 : 1	51.53 ± 0.56 0.312 ± 0.002	62.10 ± 0.39 0.271 ± 0.009	51.34 ± 0.35 0.305 ± 0.008	62.17 ± 0.54 0.272 ± 0.007
6 : 1	57.10 ± 0.43 0.309 ± 0.004	66.24 ± 0.41 0.271 ± 0.007	57.32 ± 0.17 0.307 ± 0.009	66.45 ± 0.50 0.271 ± 0.005
7 : 1	61.85 ± 0.41 0.299 ± 0.004	72.02 ± 0.62 0.266 ± 0.006	62.07 ± 0.10 0.299 ± 0.009	72.15 ± 0.53 0.269 ± 0.009
8 : 1	65.88 ± 0.63 0.300 ± 0.003	79.58 ± 1.07 0.288 ± 0.021	66.78 ± 0.62 0.295 ± 0.002	77.50 ± 0.15 0.268 ± 0.003
9 : 1	69.56 ± 0.68 0.292 ± 0.003	80.24 ± 1.12 0.274 ± 0.011	70.26 ± 0.85 0.289 ± 0.005	80.68 ± 0.13 0.267 ± 0.002

Table 5: Characteristics of propolis microemulsions containing different concentrations of oily phase.

Isopropyl myristate (%)	Mean droplet size (nm)		PDI	
	24 hours	1 week	24 hours	1 week
3	41.69 ± 0.26	41.29 ± 0.30	0.291 ± 0.023	0.299 ± 0.017
5	48.83 ± 0.31	48.62 ± 0.42	0.274 ± 0.006	0.285 ± 0.020
7	60.53 ± 0.32	60.84 ± 0.58	0.248 ± 0.007	0.254 ± 0.008

Table 6: Characteristics of propolis microemulsions containing 1% of essential oil.

Essential oil	Mean droplet size (nm)		PDI	
	24 hours	1 week	24 hours	1 week
Pine needle	57.08 ± 0.43	57.28 ± 0.44	0.256 ± 0.009	0.253 ± 0.006
Spruce needle	57.19 ± 0.36	57.11 ± 0.28	0.246 ± 0.008	0.251 ± 0.005

Table 7: The fluxes of phenolic compounds from propolis microemulsions into skin layers after 24 hours.

Phenolic compounds	Flux ($\mu g/cm^2$)		
	PME (3 : 1) containing 7% IMP	PME (3 : 1) containing 6% IMP and 1% pine needle essential oil	PME (3 : 1) containing 6% IMP and 1% spruce needle essential oil
Epidermis			
Vanillic acid	BLD	BLD	BLD
Caffeic acid	BLD	BLD	BLD
Vanillin	BLD	BLD	BLD
p-Coumaric acid	BLD	ALD	ALD
Ferulic acid	BLD	BLD	BLD
Dermis			
Vanillic acid	ALD	BLD	ALD
Caffeic acid	BLD	BLD	ALD
Vanillin	0.43 ± 0.10	0.49 ± 0.13	0.62 ± 0.14
p-Coumaric acid	0.28 ± 0.10	0.34 ± 0.11	0.36 ± 0.10
Ferulic acid	ALD	ALD	0.35 ± 0.12

ALD: above limit of detection.
BLD: below limit of detection.

hours after formulation. Single peak droplet distribution was identified for the MEs containing 7% of isopropyl myristate while MEs with 3% and 5% concentration of isopropyl myristate contained droplets of two size fractions.

Biopharmaceutical characterization of MEs containing 7% of isopropyl myristate was performed evaluating penetration of *p*-coumaric, ferulic, caffeic, vanillic acids, and vanillin into skin layers *ex vivo*. No propolis polyphenolic compounds were determined in epidermis after 24 hours of application of ME, and only vanillin and *p*-coumaric acid were determined in dermis. Essential oils of pine needle and spruce needle were added to o/w propolis microemulsions to improve the penetration of phenolic compounds into the skin [19]. The MEs contained 1% of the essential oil decreasing the concentration of isopropyl myristate, respectively. The propolis MEs were characterized measuring their droplets size and PDI and number of peaks after 24 hours and 1 week (Table 6). The mean droplet size and PDI were not affected by the added essential oil and did not change after 1 week.

The determined fluxes of phenolic compounds into human epidermis and dermis from propolis MEs containing 7% of isopropyl myristate and chemical penetration enhancers are presented in Table 7. Inclusion of pine needle essential oil or spruce needle essential oil resulted in no significant increase of vanillic, caffeic, *p*-coumaric, ferulic acids, and vanillin in epidermis. Only *p*-coumaric acid was identified but the quantity was below limit of quantification. The increased amounts of *p*-coumaric, ferulic acids, and vanillin were determined in dermis with the addition of essential oils, and their increase was higher when ME contained 1% spruce needle essential oil. The presence of vanillic and caffeic acids was determined in dermis but amounts their quantities were below limit of quantification.

Low penetration of propolis phenolic compounds from MEs could be explained by the possible distribution pattern of vanillic, caffeic, *p*-coumaric, ferulic acids, and vanillin in the MEs as due to their solubility they could be concentrated in the interfacial film, formed by surfactant and cosurfactant as well as in the oily phase of MEs [9, 13]. Thus the availability of phenolic compounds in the external phase of the MEs for absorption into the skin can be a limiting factor for their penetration into skin. The addition of essential oils to the MEs increased the amounts of phenolic compounds penetrating into skin, but the increase was not statistically significant. Only in case of ferulic acid did the quantified amount in dermis after application of ME with 1% spruce needle essential oil increase significantly.

4. Conclusion

Propolis phenolic compounds are considered as potent antioxidants that could be applied for minimization of deleterious effects of oxidative stress on biological systems of the living organisms. The important prerequisite for achieving desirable biological effect is availability of propolis phenolic compounds at the site of possible oxidative damage. Therefore

the techniques to increase intradermal penetration of phenolic compounds are attracting much interest. Application of MEs could result in increased quantities of phenolic compounds in skin layers due to presence of relatively high amounts of surface active agents and their ability to disturb the lipid matrix structure and resultant increased permeability of the skin. The results of demonstrated limited ability of MEs formulated using PEG-8 caprylic/capric glycerides and ethanolic propolis extracts to improve delivery phenolic compounds into skin *ex vivo*. The inclusion of 1% of pine needle or spruce needle essential oils into MEs produced no effect on the droplet size and polydispersity index, and this may indicate that the components of essential oils were concentrated in oily phase; hence their potential effect on the skin barrier was limited. Considering limited penetration of propolis phenolic compounds into the skin in the *ex vivo* testing, the efforts should be made to formulate MEs able to incorporate higher quantities of propolis extract. The compositions of MEs had to be optimized to achieve higher concentrations of propolis phenolic compounds in the external phase thus ensuring the availability of biologically active compounds for penetration into biological membranes.

Competing Interests

The authors declare they have no conflict of interests.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 5040528, 9 pages
http://dx.doi.org/10.1155/2016/5040528

Research Article

Quantitative Analysis of Apisin, a Major Protein Unique to Royal Jelly

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Received 6 May 2016; Revised 11 July 2016; Accepted 27 July 2016

Academic Editor: Vassya Bankova

Apisin, a protein that is unique to royal jelly (RJ), is known to compose the greater part of the RJ proteins and to exist as a heterooligomer containing major royal jelly protein 1 and apisimin. However, few reports on the methods for quantifying apisin have been published. Thus, we attempted to quantify apisin using HPLC, a widely used analytical technique, as described below. Isoelectric precipitation and size-exclusion chromatography were used to obtain the purified protein, which was identified as apisin by SDS-PAGE and LC-MS analyses. The purified apisin was lyophilized and then used to generate a calibration curve to quantify apisin in RJ. The apisin content was fairly constant (i.e., 3.93 to 4.67 w/w%) in natural RJ. This study is the first to describe a simple, standardized method for quantifying apisin using HPLC and suggests that apisin can be used as a benchmark for future evaluations of RJ quality.

1. Introduction

Queen honeybees have an approximately 2-fold larger body size and 10-fold longer lifespan than worker bees. Royal jelly (RJ) is a special food for queen bees, and royalactin, an RJ protein, has been reported to play an important role in the differentiation of queen bees [1]. In humans, RJ has been utilized in medicines, health-promoting foods, and cosmetics for many years. RJ primarily consists of water (60–70%), proteins (9–18%), lipids (3–8%), carbohydrates (7–18%), and other components [2]. Proteins, the second most abundant component of RJ, include the major royal jelly protein family (MRJP1 to MRJP9), apisimin, royalisin, and jelleins [3–7], among others, and MRJP1 is the major protein in RJ [8]. Royalactin is thought to be the same protein as MRJP1.

A 350 kDa glycoprotein from RJ exhibiting high-mannose-type N-glycosylation was first reported in 1995 [9]. In the next year, this glycoprotein was found to include two types of proteins with the following N-terminal sequences, as determined by N-terminal amino acid sequence analysis of its structural components: Asn-Ile-Leu-Arg-Gly- and Lys-Thr-Ser-Ile-Ser- [10]. This 350 kDa RJ glycoprotein was named "apisin" after *Apis mellifera* L. [11, 12]. Subsequently, another research group identified an oligomer consisting of MRJP1 and apisimin using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-mass spectrometry (LC-MS) and suggested that the MRJP1 oligomer is the same as the 350 kDa RJ glycoprotein apisin [13]. In fact, the N-terminal Asn-Ile-Leu-Arg-Gly- and Lys-Thr-Ser-Ile-Ser- sequences, which were identified in the 350 kDa glycoprotein in 1996 [10], coincided with the N-terminal sequences of MRJP1 and apisimin, respectively. Thus, apisin is considered to be a unique, major protein complex in RJ that consists of MRJP1 and apisimin. Furthermore, recent research revealed that most MRJP1 exist as an oligomer in apisin [14]. Therefore, we predict that the apisin content can be used as an RJ quality standard. Several researchers have reported an enzyme-linked immunosorbent assay (ELISA) method for quantifying apisin [14–16]. However, the ELISA method directly measures only the amount of MRJP1, not apisin itself. In addition, few reports have addressed the direct quantification of apisin by high performance liquid chromatography (HPLC). One possible reason is the difficulty of preparing a pure, stable standard of

apisin to construct a calibration curve for quantification. In previous reports, apisin was fractionated by anion-exchange gel chromatography [17] or ultracentrifugation [18] which is time consuming and requires a dedicated apparatus. Furthermore, the resulting apisin fraction was crude and produced multiple HPLC peaks.

In the present study, we attempted to establish a simple method for quantifying apisin by HPLC based on a standard curve. Then, we quantified the apisin content in various RJ products using the developed HPLC method.

2. Materials and Methods

2.1. RJ. Natural and lyophilized RJ, which originated from *Apis mellifera* L. in China, were obtained from API Co., Ltd. (Gifu, Japan), and stored at −20°C and 4°C, respectively, until further use. Fresh RJ was also collected from the Kawashima Apiary of API Co., Ltd. Briefly, female honeybee larvae (approximately 1 mm in length) were gently placed into individual artificial queen cells (diameter 10 mm × height 12 mm). After 72 h, the larvae were removed from the cells, and then fresh RJ was collected with a soft spoon. The fresh RJ was aliquoted into tubes containing 150 mg each and used immediately or stored at −20°C, 4°C, 25°C, and 40°C.

2.2. Isolation and Purification of Apisin. Natural RJ samples (3 g) were suspended in 100 mL of ultrapure water, and then the pH of the RJ suspension was increased from 4.0 to 6.0 by 0.2 by adding 10% (w/v) sodium hydroxide solution. Each pH-controlled suspension was centrifuged at 1,880 ×g for 20 min at 4°C to separate the supernatant and pellet. The supernatants were diluted to 100 mL with 0.1 M phosphate buffer containing 0.3 M NaCl (pH 7.2) by reference to a previous study [19], and the pellets were resuspended and diluted to 100 mL with the same solution. These samples were filtered and analyzed using size-exclusion columns (Protein KW-803, 8.0 mm I.D. × 300 mm; Protein KW-G, 6.0 mm I.D. × 50 mm, Showa Denko K.K., Tokyo, Japan) connected to an HPLC (Alliance, Waters, Massachusetts, USA) under the following conditions: a mobile phase of 0.1 M phosphate buffer containing 0.3 M NaCl (pH 7.2), an injection volume of 30 μL, a flow rate of 0.3 mL/min, a column temperature of 30°C, and a wavelength of 280 nm. The column was calibrated using Gel Filtration Calibration Kit High Molecular Weight Proteins (GE Healthcare, Pittsburgh, USA). The degree (%) of purity of each sample was assessed by the ratio of the apisin peak area to the total peak area in the chromatograms.

The apisin-rich fraction was prepared by suspending the pellet in the buffer solution and was purified by size-exclusion column chromatography using Sephacryl S-300HR (25 mm I.D. × 460 mm, 225 mL, GE Healthcare, Tokyo, Japan) with a mobile phase of 0.1 M phosphate buffer containing 0.3 M NaCl (pH 7.2). The purity of the apisin fraction was further analyzed on an anion-exchange chromatography using a TSK gel DEAE-5PW column (TOSOH Corporation, Tokyo, Japan). The apisin fraction was collected, desalted by ultrafiltration with an Amicon Ultra-15 unit (NMWL 50,000, 15 mL, UFC910008, Merck Millipore, Darmstadt, Germany), added to ampoules with a 9 : 1 ratio of trehalose (D-(+)-trehalose anhydrous Tokyo Chemical Ind., Co., Ltd., Tokyo, Japan) to apisin, and then lyophilized. The net amount of apisin was calculated by subtracting the trehalose weight from the total weight, including the weight of the container.

In the stability test, the lyophilized apisin was preserved at −20°C or 4°C for 1, 3, 6, 9, and 12 months and was quantified using an internal standardization method at each point. The purified apisin and ovalbumin (Sigma-Aldrich Co., LLC., Missouri, USA), which was used as an internal standard, were dissolved in the mobile phase solution consisting of 0.1 M phosphate buffer and 0.3 M NaCl (pH 7.2) and analyzed by size-exclusion HPLC. The residual amounts of apisin are expressed as a percent of the initial (day 0) value.

2.3. Apisin Identification. The fractionated, purified protein was identified as apisin using SDS-PAGE, followed by LC-MS analysis. The test samples were dissolved in SDS sample buffer (SDS-SB; 62.5 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 2% (w/v) SDS, and 50 mM dithiothreitol), vortexed, and then boiled for 3 min at 95°C. After measuring the protein concentrations using a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) and adding the tracking dye bromophenol blue, 5 μg of the protein was loaded onto 10–20% acrylamide SDS-PAGE gels (80 mm × 80 mm × 1 mm, BIO-CLAFT Co., Ltd., Tokyo, Japan) and electrophoresed with a constant current of 25 mA/gel for 70 min. A buffer consisting of 0.025 M Tris, 0.192 M glycine, and 0.2% (w/v) SDS was used as the running buffer. The proteins were visualized with Oriole Fluorescent Gel Stain (Bio-Rad Laboratories Inc., Hercules, CA, USA). The gel was calibrated by measuring the migration distances for standard proteins of known molecular masses (Precision Plus Protein™ Dual Xtra Standards [Bio-Rad; Hercules, CA, USA]).

Each protein band on the gel was excised, washed with 100 mM ammonium bicarbonate (NH_4HCO_3) in 50% acetonitrile (ACN), dehydrated with 100% ACN, reduced with 50 μL of 10 mM dithiothreitol/100 mM NH_4HCO_3 at 56°C for 30 min, and then alkylated with 50 μL of 55 mM iodoacetamide/100 mM NH_4HCO_3 for 45 min at room temperature in the dark. The gel slices were then washed with 100 mM NH_4HCO_3 and dehydrated with 100% ACN, and the proteins were digested with 50 μL of 0.01 g/L lysyl endopeptidase/100 mM NH_4HCO_3 (Lys-C, #125-05061, Wako Pure Chemical Industries, Ltd., Osaka, Japan) or with 50 μL of 0.005 g/L aspartic protease/100 mM NH_4HCO_3 (Asp-N, #V162A, Promega K.K., Tokyo, Japan) at 37°C for 16 h (overnight). The peptides were extracted by sonication for 3 min at ambient temperature, followed by the addition of the extraction buffer (50% ACN containing 0.5% formic acid), and vortexed twice for 10 min each. The extracted peptides were dried in a speed vacuum, dissolved in 20 μL of 0.1% formic acid by sonication, and then subjected to LC-MS analysis.

The peptides were separated on a Protein Separation Technology BEH300 C18 column (1.7 μm I.D. × 150 mm, Waters Corporation, MA, USA) by binary gradient elution (from 0.1% formic acid/water to 0.1% formic acid/ACN in

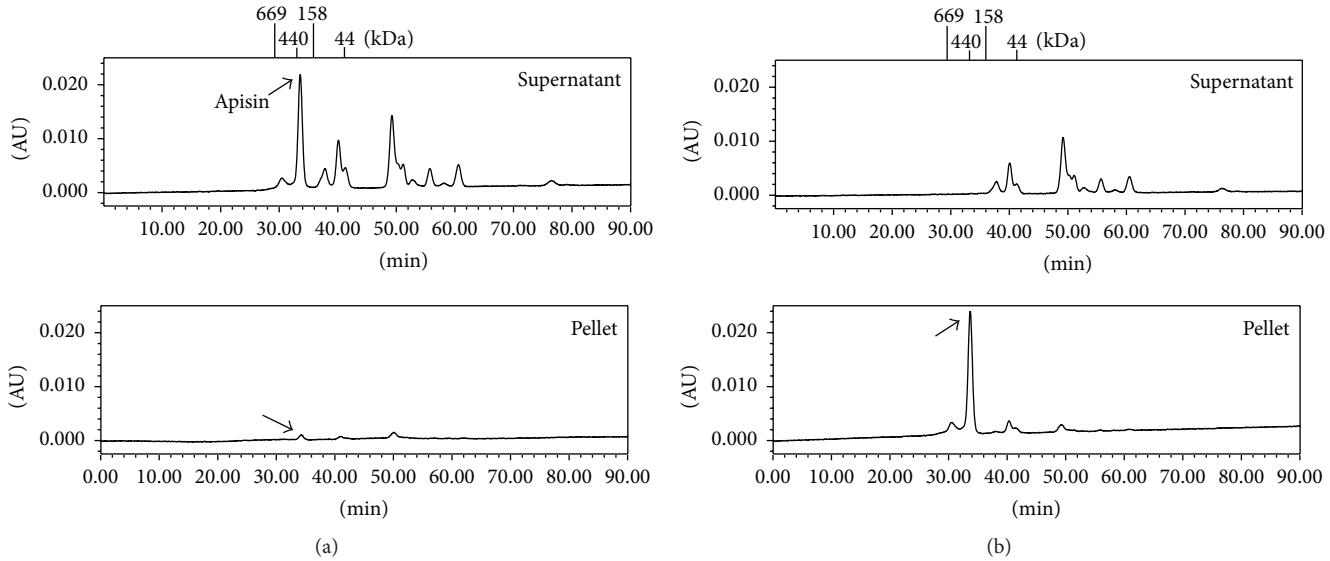

Figure 1: Fractionation of an apisin-rich protein by isoelectric precipitation. Elution profiles of apisin in the supernatant and pellet of the RJ solution at pH 3.9 (a) and pH 4.6 to 4.8 (b) using size-exclusion gel chromatography. The methodological details are provided in Section 2. The changes in the purity (%) of apisin in the supernatant and pellet at pH 3.9 to 6.0 are shown in Table 1.

80 min) at a flow rate of 0.2 mL/min via an ultraperformance liquid chromatograph (UPLC) connected to a liquid chromatograph-electrospray ionization quadrupole/time-of-flight mass spectrometer (LC-ESI-QTof MS) (ACQUITY UPLC coupled to Xevo G2 QTof, Waters). The LC-MS was operated in MS^E (positive/sensitivity) mode. The MS^E spectra were searched against the NCBI Taxonomy database, in which 64,296 proteins were registered with "Apis" (ID: 7459), using the ProteinLynx Global SERVER software (version 5.2, Waters). The parameters used in this analysis were as follows: (1) Lys-C or Asp-N as the protease, (2) one missed cleavage site, (3) carbamidomethylation of cysteine as a fixed modification, and (4) oxidation of methionine and tryptophan and phosphorylation of serine, threonine, and tyrosine as variable modifications. To identify the protein, at least 3 different theoretical y or b ions were assigned to one unique peptide, and a total of 7 different unambiguous y or b ions were necessary for the successful manual verification of the protein. The MS and MS^E ion intensity thresholds were 150 counts and 50 counts, respectively.

The protein bands on the SDS-PAGE gel were extracted using the alkaline extraction method [20]. Briefly, the gel was excised, washed with ultrapure water, and minced in 100 μL of 100 mM NH_4HCO_3/50% ACN. After removing the NH_4HCO_3 solution, the gel was soaked in 50 μL of 0.1 M NaOH for 10 min at 25°C to extract the protein and then was subjected to SDS-PAGE analysis or mass analysis of the intact protein. The intact protein was analyzed using LC-ESI-QTof MS as described below. The protein solution was diluted 2-fold with 0.1% formic acid, filtered, and separated on a Protein BEH C4 column (300 Å, 1.7 μm I.D. × 100 mm, Waters) using a gradient elution with a water/ACN mobile phase (80 : 20 to 80 : 20 in 10 min) containing 0.1% formic acid at a flow rate of 0.2 mL/min. The MS results were analyzed using MAXENT I software (Waters).

2.4. Quantitation of Apisin in RJ. Natural RJ (150 mg) or lyophilized RJ (50 mg) was dissolved in 50 mL of 0.1 M phosphate buffer containing 0.3 M NaCl at pH 7.2 and centrifuged at 12,500 ×g for 5 min at 4°C. The supernatant was filtered (0.2 μm), and apisin was quantified by size-exclusion HPLC analysis according to the methods described in Section 2.2, except for the wavelength 210 nm at which the peak intensity of apisin was higher, using a standard curve generated with known concentrations of the lyophilized apisin as the standard reference material. The experiments were performed in quadruplicate.

2.5. Stability of Apisin in RJ. As described above, fresh RJ was prepared from our bee (*Apis mellifera* L.) keeping farm. The fresh RJ was immediately used to test the stability of apisin at −20°C, 4°C, 25°C, and 40°C. The residual quantity of apisin at each temperature was measured by the standard curve method using size-exclusion HPLC after 1, 4, 7, 14, and 32 days. The experiments were performed in triplicate.

3. Results

3.1. Isolation and Purification of Apisin. The natural RJ was suspended in ultrapure water, and then the pH of the RJ suspension was increased from 4.0 to 6.0 by adding sodium hydroxide solution. As shown in Figure 1, the peaks indicated with arrows were attributed to apisin. At the natural pH of 3.9 (Figure 1(a)), apisin was detected in the supernatant fluids, whereas it was detected in the pellets at pH 4.8 (Figure 1(b)).

Table 1: Changes in the apisin purity (%) in RJ suspensions at various pH values.

pH	3.9	4.0	4.2	4.4	4.6	4.8	5.0	5.2	5.4	5.6	5.8	6.0
Supernatant	27.6	27.7	27.9	16.9	0.0	0.0	0.7	2.1	13.0	24.6	27.0	26.6
Pellet	28.8	33.6	49.2	66.0	70.3	66.5	67.7	67.4	60.5	32.2	44.5	15.1

Table 2: List of identified peptides corresponding to the 5 kDa protein.

Treated enzyme	Peak R.T. (min)	Sequences	Matched proteins
Lys-C	11.04	TSISVK	Apisimin (5,540 Da)
		TSLSVK	A0A088AFZ8_APIME Uncharacterized protein (26,380 Da)
Asp-N	12.60	KTSISVKGSNV	Apisimin (5,540 Da)
		KTSLSVKGESNV	Not applicable
Asp-N	15.25	DANVFA	Apisimin (5,540 Da)

These sequences were searched against the NCBI Taxonomy database (the classification "Apis", ID: 7459).

The apisin-rich (>60%) protein fraction was obtained at pH values from 4.4 to 5.4 (Table 1).

The apisin-rich fraction, which was obtained by isoelectric precipitation at pH 4.8, was purified by size-exclusion column chromatography, and a single peak for apisin was observed in the HPLC chromatogram (Figure 2). Additionally, apisin was detected as a single peak on anion-exchange chromatography (data not shown).

The purified apisin fraction was desalted and lyophilized with trehalose, which almost completely protected the apisin from lyophilization-induced denaturation (Figure 3). The lyophilized apisin was stable for at least 12 months when stored at 4°C or −20°C (Figure 4). The calibration curve constructed from the lyophilized apisin showed good linearity (Figure 5). These findings suggested that lyophilized apisin could be used as a standard reference material for quantification.

3.2. Identification of the Purified Protein as Apisin. SDS-PAGE and LC-MS analyses were performed to identify the purified protein as apisin. The SDS-PAGE results for natural RJ, the isoelectric point (4.8) precipitated proteins, and the purified proteins are shown in Figure 6(a). The purified protein (lane 3) migrated as approximately 55 kDa and 5 kDa species on SDS-PAGE gels. The 55 kDa protein was identified as MRJP1 by a proteomics approach involving Lys-C treatment. However, the 5 kDa protein could not be identified by this approach and was instead subjected to de novo sequencing. The 5 kDa protein was digested with Lys-C or Asp-N, and the following three peptides were detected: "KTSISV or KTSLSV," "KTSISVKGESNVD or KTSLSVKGESNVD," and "DANVFA" (Table 2). After searching these sequences against the Taxonomy NCBI database, only "KTSISV," "KTSISVKGESNVD," and "DANVFA" shared an identical amino acid sequence with apisimin, which has a theoretical mass of 5,540 Da (all the amino acid sequences of mature apisimin are shown in Figure 7). The KTSLSV peptide was assigned to A0A088AFZ8_APIME uncharacterized protein, with a theoretical mass of 26,380 Da; KTSLSVKGESNVD did not match any homologous protein families. Next, the 5 kDa protein was extracted from the SDS-PAGE gel and used for intact protein mass analysis (Figure 6). The accurate mass of the 5 kDa protein was estimated to be 5,540.0 Da (Figure 6(e)). The theoretical mass of apisimin is believed to be 5,540 Da, and it has been reported to have a mass of 5,540.4 or 5,540.9 [5, 13]. Taken together, these findings confirm that the purified protein is a two-protein complex consisting of MRJP1 and apisimin.

Table 3: The apisin content in natural and lyophilized RJ.

Samples	Lot number	Apisin (%)
Natural RJ	I	4.62 ± 0.11
	II	4.43 ± 0.10
	III	4.67 ± 0.11
	IV	4.39 ± 0.10
	V	3.93 ± 0.09
	VI	4.41 ± 0.10
	VII	4.62 ± 0.11
Lyophilized RJ	A	11.63 ± 0.29
	B	11.27 ± 0.27
	C	11.39 ± 0.27
	D	10.93 ± 0.24
	E	11.03 ± 0.24
	F	11.86 ± 0.28

Apisin was quantitated using a standard curve generated with known concentrations of lyophilized apisin. The methodological details are provided in Section 2. All measurements were performed in triplicate, and the data represent the mean ± S.D.

3.3. Quantitation of Apisin in RJ. We demonstrated that apisin could be accurately quantified with a standard curve using HPLC. The apisin content in natural and lyophilized RJ was 3.93 to 4.67% (w/w) and 10.93 to 11.86% (w/w), respectively (Table 3), indicating that the apisin content is fairly constant in RJ. Incidentally, the water content in natural and lyophilized RJ was 63.5 to 65.8% (w/w) and 1.4 to 2.4% (w/w), respectively, when measured by a conventional vacuum-drying method (70°C for 4 h).

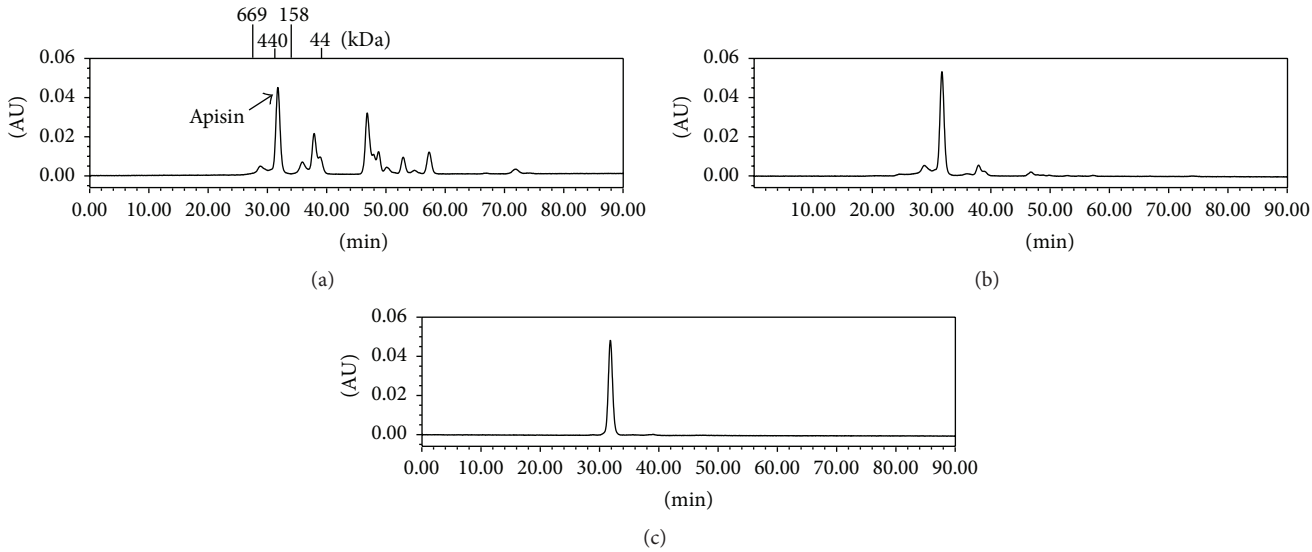

Figure 2: Purification of apisin by size-exclusion HPLC. Elution profiles of the RJ proteins obtained by size-exclusion gel chromatography: (a) natural RJ proteins, (b) proteins after isoelectric precipitation at pH 4.8, and (c) proteins after purification by size-exclusion HPLC.

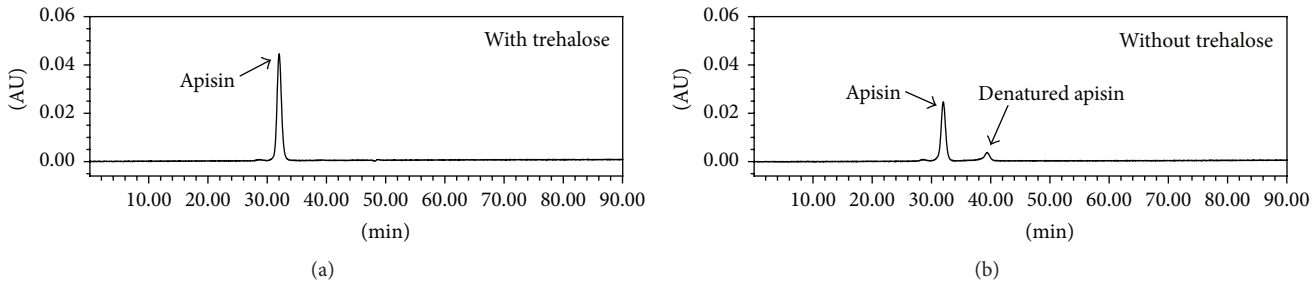

Figure 3: Elution profiles of apisin lyophilized with trehalose (a) and without trehalose (b) by size-exclusion gel chromatography using HPLC.

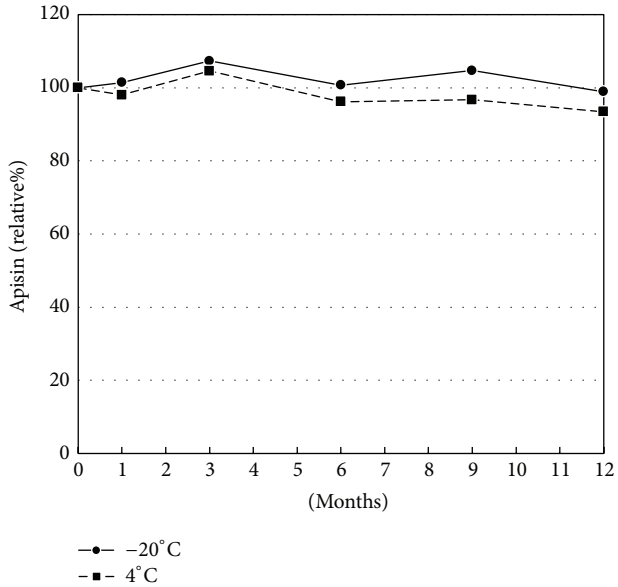

Figure 4: The stability of the lyophilized apisin powder. The lyophilized apisin was preserved at −20°C or 4°C for 1, 3, 6, 9, and 12 months, and the residual amounts of apisin are expressed as percentages of the initial (day 0) value. The methodological details are provided in Section 2.

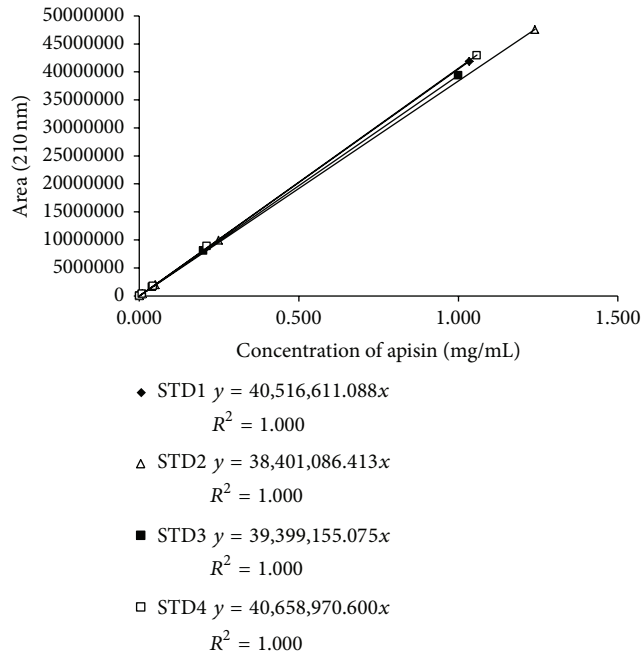

Figure 5: Calibration curves were determined using the lyophilized apisin powder. All of the calibration curves were linear ($R^2 = 1.000$) from 0.01 to 1.00 mg/mL. The experiments were performed in quadruplicate.

3.4. Stability of Apisin in RJ. We assessed the residual quantity of apisin in fresh RJ after preservation at various temperatures. The quantity of apisin was unchanged at −20°C and 4°C for at least 32 days but decreased by approximately 10% and 80% at 25°C and 40°C, respectively (Figure 8).

4. Discussion

Here, we successfully demonstrated the first HPLC-based method for the accurate quantitation of the RJ protein apisin. As shown in Figure 1, we identified the chromatographic peak indicated by an arrow as apisin because apisin exhibited the highest peak on supercritical fluid chromatography at 280 nm, and its molecular weight was estimated to be 280 to 420 kDa in previous studies [9, 13, 21, 22].

Initially, we attempted to fractionate crude apisin by isoelectric precipitation at the optimum pH values. Apisin is a heterooligomer containing MRJP1 and apisimin, and their theoretical isoelectric points were computed to be 5.03 and 4.55, respectively, using the ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/) based on their amino acid sequences. Based on these isoelectric points, the RJ proteins were precipitated at pH values ranging from 4.0 to 6.0, whereas the apisin-rich protein fractions were obtained at pH values from 4.4 to 5.2; thus, as expected, apisin has an isoelectric point of approximately 4.6. The apisin-rich fraction was also purified by size-exclusion gel chromatography, which is frequently used to separate various biomaterials, such as proteins and polysaccharides, according to their molecular sizes. Ultimately, we were able to derive apisin, which was detected as a single peak in the HPLC chromatogram, from the RJ proteins. To the best of our knowledge, this is the first report of the isolation of apisin using isoelectric precipitation followed by size-exclusion gel chromatography. Isoelectric precipitation is a commonly used, rapid, and simple method for fractionating individual proteins. In addition, this relatively easy method provides a large amount of the apisin-enriched fraction, which could be used for additional functional, physicochemical, and analytical studies of RJ.

We then identified the purified protein as apisin using SDS-PAGE and LC-MS analyses. The protein migrated as approximately 55 and 5 kDa species on SDS-PAGE gels. Using a proteomics approach and automatic software-based analysis, the 55 kDa protein was identified as MRJP1, but we failed to identify the 5 kDa band as apisimin. The reasons may include the following: (1) the sequences of the mature protein and the protein in the database, which includes a signal peptide sequence, differ. (2) Only one or two detectable peptides can be generated by the LC-MS analysis because two cleavage sites—Lys-C and Asp-N—exist in the apisimin sequence. (3) The detectable peptides include Ile, which can be distinguished from Leu by LC-MS analysis (Table 2). Thus, we ultimately identified the 5 kDa protein as apisimin via de novo analysis and intact protein mass measurements. Therefore, we determined that the purified protein is indeed apisin, a two-protein complex consisting of MRJP1 and apisimin. Interestingly, the MRJP1 oligomer has been reported to consist of the 55 kDa protein MRJP1 and the 5 kDa protein apisimin [13], suggesting that our purified protein is the same protein as the MRJP1 oligomer, which is also called apisin (350KRJGP).

Third, we tried to measure the apisin content in various RJ products. The apisin fraction that was purified by gel

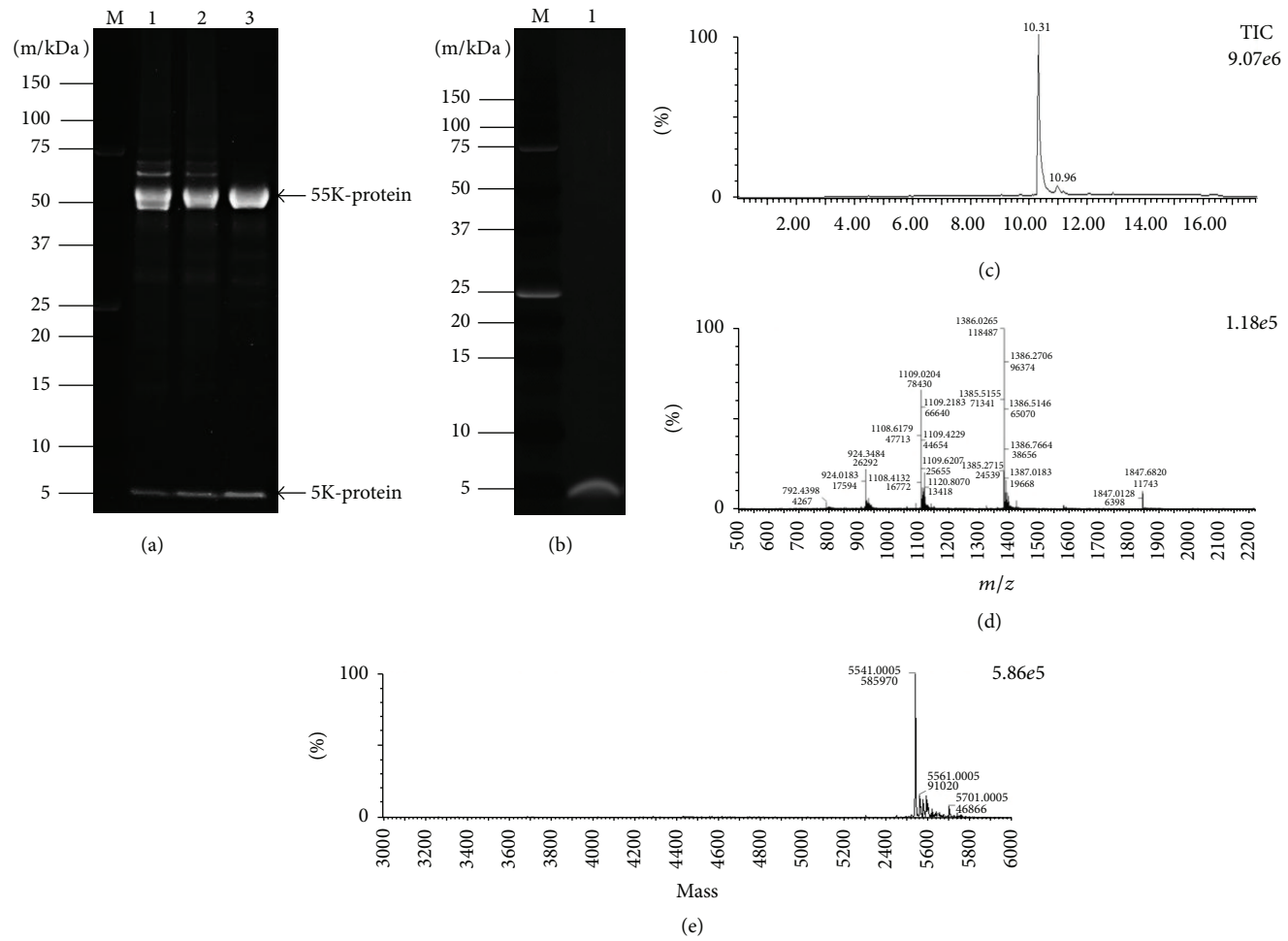

Figure 6: Identification of apisin by SDS-PAGE and LC-MS. (a) SDS-PAGE profiles of natural RJ (lane 1), the isoelectric point-precipitated protein (pH 4.8) (lane 2), and the protein purified by size-exclusion HPLC (lane 3). (b) SDS-PAGE profile of the protein extracted from the 5 kDa band (lane 1). (c) Total ion chromatogram of the 5 kDa protein. (d) Mass spectra of the 5 kDa protein. (e) Calculated molecular weight of the 5 kDa protein according to MAXENT I analysis. The methodological details are provided in Section 2.

```
1          11         21         31
MSKIVAVVVL AAFCVAMLVS DVSAKTSISV KGESNVDVVS
        Signal peptide

41         51         61         71
QINSLVSSIV SGANVSAVLL AQTLVNILQI LIDANVFA
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Figure 7: Amino acid sequence of apisimin [*Apis mellifera*, NP_001011582.1].

chromatography was desalted and lyophilized with trehalose to obtain a dried apisin powder, which was used as a standard reference material. Trehalose is typically used to prevent the lyophilization-induced denaturation of proteins [23]. Indeed, trehalose prevented the denaturation of apisin (Figure 3). The lyophilized apisin powder was stable for at least 12 months at 4°C or −20°C and allowed us to generate a linear calibration curve for the HPLC analysis, suggesting that the prepared apisin powder can be used as a reference standard for the quantitative analysis of apisin by HPLC. Here, we found that the apisin content in RJ was fairly constant, in the ranges of 3.93 to 4.67% (w/w) for natural RJ and 10.93 to 11.86% (w/w) for lyophilized RJ. We also found that the apisin content in RJ did not change for at least 32 days when it was stored at −20°C, 4°C, or 25°C but decreased by approximately 80% at 40°C after 32 days. Previous studies revealed that RJ lost the ability to enhance the proliferation of rat hepatocytes when stored at 40°C for 7 days and suggested that the thermal denaturation of royalactin, which is assumed to be the same protein as MRJP1, is responsible for this phenomenon [1, 19].

Currently, the content of 10-hydroxy-2-decenoic acid (10-HDA), which is a chemically stable fatty acid unique to RJ, is required for the specification of RJ products. However, a potential disadvantage of using 10-HDA is that the amount of 10-HDA in RJ decreases during the production process and can be manipulated by adding an extraneous 10-HDA-rich RJ fraction. In contrast, the content of apisin was fairly constant among RJ products, for example, the fresh RJ from our bee farm, the imported RJ from China, and the lyophilized RJ. Thus, there is a possibility that the MRJP1 oligomer apisin can be used as a benchmark for future evaluations of RJ quality in

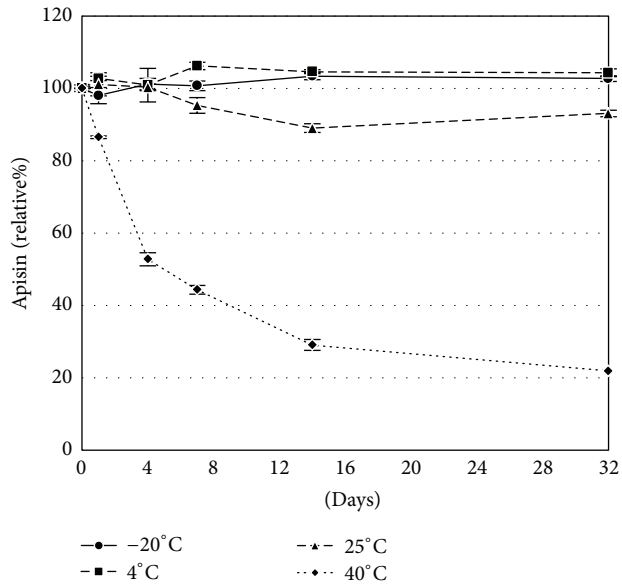

Figure 8: The stability of apisin in RJ. Fresh RJ was preserved at −20°C, 4°C, 25°C, or 40°C for 1, 4, 7, 14, and 32 days, and the residual amounts of apisin were quantified with a standard curve using size-exclusion HPLC and expressed as percentages of the initial (day 0) value. The methodological details are provided in Section 2. All measurements were performed in triplicate, and the data represent the mean ± S.D.

addition to 10-HDA, although there are a few critical issues that must be overcome relating to achieving a secure supply of the apisin reference standard for quantitative analysis.

In conclusion, we developed the first method for the rapid isolation and purification of the RJ protein apisin and then quantified this protein by HPLC using a standard curve. This analysis revealed that the apisin content is fairly constant among natural RJ samples.

Competing Interests

There is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are grateful to the Kawashima Apiary of API Co., Ltd., for supplying the fresh RJ samples. They also thank all of the staff members of their laboratory for providing technical advice.

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Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 2038407, 10 pages
http://dx.doi.org/10.1155/2016/2038407

Research Article

Effects of Brazilian Propolis on Dental Plaque and Gingiva in Patients with Oral Cleft Malformation Treated with Multibracket and Removable Appliances: A Comparative Study

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Received 28 May 2016; Accepted 15 August 2016

Academic Editor: Andrzej K. Kuropatnicki

Orthodontic appliances modify the local environment of the oral cavity, increase the accumulation of dental plaque, and affect the condition of the gingiva. The aim of this study is assessment of Brazilian propolis toothpaste's effect on plaque index (PLI) and gingival index (GI) in patients with CL/CLP treated using orthodontic appliances in the 35-day study period. The study population included 96 patients of an Orthodontic Outpatient Clinic, ACSiMS in Bytom. All the patients participated in the active phase of orthodontic treatment using buccal multibracket appliances or removable appliances. During the first examination, each patient was randomly qualified to the propolis group or control group. A statistically significant decrease in GI and PLI in the entire propolis group ($P < 0.01$) was shown during repeated examination. Insignificant change in GI was in the entire control group during the repeated examination compared to the baseline. Similar result was obtained in patients treated with multibracket and removable appliances. The orthodontic appliance type did not affect the final dental plaque amount and gingival condition in patients using the propolis toothpaste. These results may be clinically useful to improve prevention and control oral infectious diseases during orthodontic treatment patients with oral cleft.

1. Introduction

Orthodontic appliances modify the local environment of the oral cavity, affecting the quantity, flow, composition, and biochemical properties of saliva, as well as microflora of the oral cavity [1–3]. Moreover, these appliances increase the accumulation of dental plaque and the quantity of exfoliated epithelial cells and obstruct teeth self-cleaning [4, 5]. All these factors affect the condition of the gingiva and hard tissues of the tooth and may contribute to the development of caries and periodontium diseases. In patients with cleft lip (CL) and cleft lip and palate (CLP), apart from the risk related to wearing orthodontic appliances, there are additional factors that may be responsible for poor oral health when compared to individuals with no cleft. These include oral and nasal cavity communication, contributing to mixing the microfloras of these environments and pain and fear after surgical procedures which is responsible for avoiding toothbrushing. Loss of lip elasticity in the cleft scar obstructing the access to oral cavity [6], drying mucosa,

and teeth in the cleft area due to nonclosing lips and prolonged oral clearance time favouring aciduric bacteria growth [2, 7] also do not remain insignificant. Furthermore, more frequent hypoplasia and hypomineralisation of tooth enamel, presenting other locations of plaque retention, are observed in this developmental malformation compared with the general population [8].

The results of studies indicating toothpaste-containing chemical agents side effects [9, 10] have convinced the investigators to look for natural ingredients which may effectively remove dental plaque and simultaneously present a favourable effect on entire oral cavity health. One such substance includes propolis, the beneficial properties of which were already known and used in ancient times [11]. Propolis is acquired by honey bees from plant buds and tree bark splits and then enzymatically modified and used to seal the beehive entrance, line its walls, and protect against microbes [11–13]. Propolis is a thick, highly adhesive wax-resin substance composed of plant balms, essential oils, and biologically active compounds such as phenolic acid and its esters, flavones, flavonols and flavanones, aromatic aldehydes and esters, terpenes, fatty acids, β-steroids, mineral salts, terpenes, and vitamins (A, B1, B2, B3, and B7) [11–14]. Propolis presents strong antibacterial, antiviral, antiparasitic, antifungal, and antioxidative properties. *In vivo* and *in vitro* studies have confirmed propolis' anti-inflammatory properties and proved its strong immunomodulating effect [3, 15, 16]. Numerous scientific reports indicate propolis's beneficial effect on oral cavity health condition, yet there remain few clinical trials evaluating propolis's effect on gum hygiene and condition in patients with CL/CLP wearing different types of orthodontic appliances.

The aim of this study is the clinical assessment of ethanol extract of Brazilian propolis (EEP) toothpaste's effect on plaque index (PLI) and gingival index (GI) in patients with CL and CLP treated using buccal multibracket appliances (fixed appliances) or removable orthodontic appliances.

2. Materials and Methods

2.1. Study Population. The study material included patients of an Orthodontic Outpatient Clinic, Academic Center of Dentistry and Specialist Medicine in Bytom, Poland. All the patients participated in the active phase of orthodontic treatment using buccal multibracket appliances or removable appliances. The following inclusion criteria were used: (1) presence cleft lip and alveolus (CL) or cleft lip, alveolus and palate (CLP), (2) lack of coexisting additional developmental malformations, (3) 9–16 years of age, (4) presence of at least 10 own permanent teeth, (5) treatment using fixed buccal multibracket appliances or removable orthodontic appliance for at least 6 months, (6) no antibiotic therapy for at least 2 months prior to the study inclusion, and (7) good general health condition. Exclusions included (1) patients with bone grafting in the dental ridge of the maxilla sooner than 3 months from the study commencement and those planning these procedures in the following 2 months after qualification tests and (2) individuals with confirmed adverse reactions to bee products.

A multibracket buccal appliance (Biomim, 0.22 Roth, manufactured by Ortho Classic) was placed on at least 6 permanent teeth. A removable appliance was an acrylic palatal plate with screws for the patient to unscrew, with a labial bar, arrowhead clasps, and/or Adams clasps. Removable appliances were worn by patients for about 14 hours per day.

2.2. Clinical Protocol. During the first examination (baseline), every patient was provided with detailed instructions on oral cavity hygiene. Every patient was shown the method of cleaning teeth, using a set of teeth model. What is more, the patients and their parents were given spoken information regarding oral cavity hygiene and the teeth cleaning method. Each patient was recommended to clean their teeth with the Fones method, using an ordinary and an interdental toothbrush 3 times a day, and the toothpaste provided on the first visit.

During the first examination, each patient was randomly assigned, by a postgraduate students not participating in the study, to the propolis group (PG) or control group (CG). All recruits with buccal multibracket appliances and removable orthodontic appliance were qualified in a 1 : 1 ratio to one of two groups (PG, CG), so that there was a similar proportion of patients with fixed and removable appliances in both groups. PG patients were told to use toothpaste with 3% ethanol extract of Brazilian propolis, and CG patients were told to use toothpaste without an active substance, EEP (placebo). The patients were not informed which toothpaste they were given; they also could not identify it through the packaging.

2.2.1. Preparation of Brazilian Green Propolis Extract. Propolis used in the study was collected from the beekeeping section of the Seiri Alimentos Naturales, Brazil. Propolis was acquired from honey bees' beehives (*Apis mellifera*) in Minas Gerais State, Southeast Brazil, from the plant *Baccharis dracunculifolia*, which is the source of resin for green propolis (propolis G12) [17]. The toothpastes with 3% of EEP and without EEP (placebo) were prepared in Nippon Zettoc Co., Ltd., Tokyo, Japan. The toothpastes used in the study were of legal origin and contained ingredients commonly used in oral cavity hygiene.

2.2.2. Oral Clinical Assessment. Oral cavity hygiene and gingival condition were assessed in every patient twice, in the starting period (baseline) and after 35 days (final study). The study was conducted by a single investigator with the same illumination every time, using a mirror, a probe.

The amount of dental plaque was marked using plaque index [PLI] according to Silness and Löe [18]. Permanent superior incisors and first molars of the maxilla and the mandible were included in the study, assessing the thickness of dental plaque in the perigingival area of the tooth, using a 0–3 scale. Buccal and lingual surfaces of all examined teeth were taken into consideration. The study was performed without staining, after thorough tooth surface and its adjacent gingival margin drying using delicate air stream.

The clinical condition of the gingival margin was assessed using gingival index [GI] according to Löe and Silness [19],

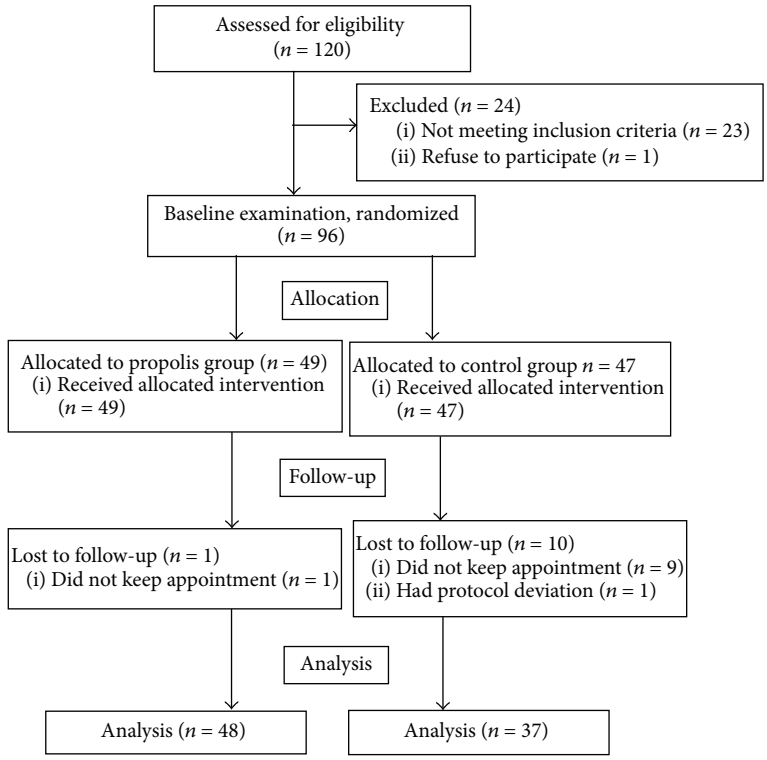

Figure 1: Flowchart of the progress of the phases of clinical trial.

using the same teeth and examined surface selection criteria as with PLI. The study used a 3-step scale taking into account qualitative signs of inflammation, from 0, lack of inflammation signs, to 3, severe inflammation.

Moreover, in the case of every patient DMFT index was calculated for permanent teeth: the Decayed (D), Missing (M), and Filled (F) Teeth (T).

The study was approved by the Bioethics Committee of the Silesian Chamber of Medicine (Resolution number 6/2010). All participants were informed about the type of tests and provided written consent. The data of every patient was collected as confidential, while maintaining personal data confidentiality.

2.3. Statistical Analysis. Data was presented as means, SD, medians (interquartile ranges 25th, 75th percentile [IQR]). All assessed variables did not present normal distribution; therefore, nonparametric tests were used. Categorical variables were reported as proportions. Dichotomous variables were compared using chi-square tests. Comparison of scores between PG and CG was performed using Mann-Whitney U test. A comparison of the GI and PLI scores between the multibrackets appliance group and removable appliance group was performed using Mann-Whitney U test. Wilcoxon matched pairs singed-rank test was used for an intragroup comparison. The Spearman rank correlation coefficient was used to calculate the relations of gingival index and plaque index final study with initial score of gingival index, plaque index, D, M, F, T, DMFT, and type of orthodontic appliance. Statistically significant differences were considered at the level of $P < 0.05$. Statistical analysis was performed using StatSoft, Inc. (2014), STATISTICA (data analysis software system), version 12. http://www.statsoft.com/.

3. Results

3.1. Baseline. The study involved 96 patients and was completed by 85 patients, including 33 girls and 52 boys (Figure 1). The patients from the propolis group and the control group did not differ significantly in terms of age and cleft distribution, nor orthodontic appliance type (Table 1). The median age was 12.3 in the propolis group and 11.9 in the control group. The majority of patients in both the control group and the propolis group included patients with unilateral cleft lip and palate (UCLP), 56.7% and 50.0%, respectively. Bilateral cleft lip and palate (BCLP) was present in 16.6% of patients in the propolis group and 27.0% of patients in the control group. The majority of individuals in the propolis group and in the control group were treated with buccal multibracket appliances, 64.6% and 54%, respectively. Removable appliances were present in 35.4% of the propolis group and 46% of the control group (Table 1). Mean DMFT was similar in the propolis group and the control group, being 4.45 and 4.51, respectively (Table 1). Clinical mean gingival condition expressed in GI during the first examination did not differ between both groups and was 1.5 (Table 1). Mean dental plaque amount was insignificantly higher in the propolis group compared to the control group, being 1.47 and 1.22 ($P = 0.06$), respectively (Table 1).

During the first examination, worse clinical condition of the gingiva and the periodontium in patients wearing buccal multibracket appliances was found in both groups,

Table 1: Characteristic of propolis group and control group; intergroup differences.

	Propolis group			Control group			Intergroup difference
	Female	Male	Total	Female	Male	Total	
	$n = 18$	$n = 30$	$n = 48$	$n = 15$	$n = 22$	$n = 37$	P
Age in years							
Median	12.6	12.3	12.3	12,43	11,62	11,90	
25th–75th percentile (IQR)	11.2–13.6 (2.4)	10.9–14.7 (3.8)	11.14–14.1 (2.9)	10.7–13.8 (3.1)	9.8–12.8 (3.0)	10.3–13.1 (2.8)	*0.375*[a]
UCLP *n* (%)	9 (18.7%)	15 (31.3%)	24 (50%)	8 (21.6%)	13 (35.1%)	21 (56.7%)	*0.536*[b]
BCLP *n* (%)	3 (6.2%)	5 (10.4%)	8 (16.6%)	3 (8.1%)	7 (18.9%)	10 (27%)	*0.955*[b]
UCL *n* (%)	6 (12.5%)	10 (20.9%)	16 (33.4%)	4 (10.8%)	2 (5.4%)	6 (16.2%)	*0.246*[b]
Multibracket appliances *n* (%)	10 (20.9%)	21 (43.7%)	31 (64.6%)	8 (21,6%)	12 (32.4%)	20 (54.0%)	*0.074*[b]
Removable appliances *n* (%)	8 (16.7%)	9 (18.7%)	17 (35.4%)	7 (19.0%)	10 (27.0%)	17 (46.0%)	*0.325*[b]
DMFT							
Mean (SD)			4.45 (3.55)			4.51 (4.48)	
Median (IQR)			5.0 (6.0)			5.0 (5.0)	*0.22*[a]
GI							
Mean (SD)			1.58 (0.85)			1.52 (0.87)	
Median (IQR)			1.5 (1.2)			1.3 (1.3)	*0.67*[a]
PLI							
Mean (SD)			1.47 (0.62)			1.22 (0.83)	
Median (IQR)			1.5 (1.0)			0.96 (1.0)	*0.06*[a]

IQR, interquartile range; [a]*U* Mann-Whitney test; [b]chi-square test, *significance if $P < 0.05$.

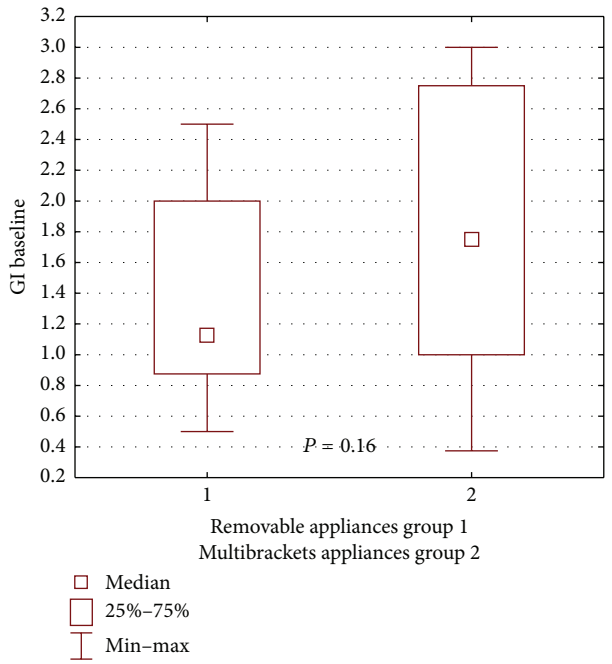

Figure 2: Statistical comparison of GI baseline value between removable appliances and multibrackets appliances at propolis group. Median, IQR, min, max, and significant if $P < 0.05$.

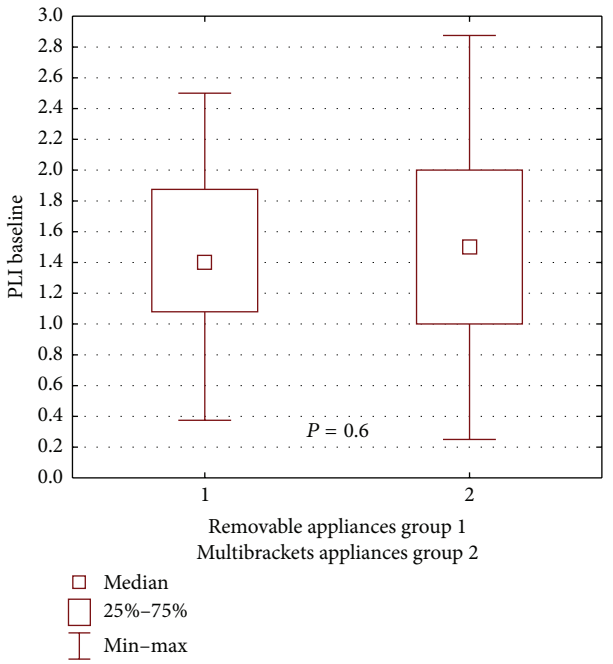

Figure 3: Statistical comparison of PLI baseline value between removable appliances and multibrackets appliances at propolis group. Median, IQR, min, max, and significant if $P < 0.05$.

compared with patients wearing removable appliances, yet the difference was not statistically significant for the propolis group: GI $P = 0.16$, (Figure 2); PLI, $P = 0.60$ (Figure 3); for the control group: GI, $P = 0.72$, (Figure 4); PLI, $P = 0.72$ (Figure 5).

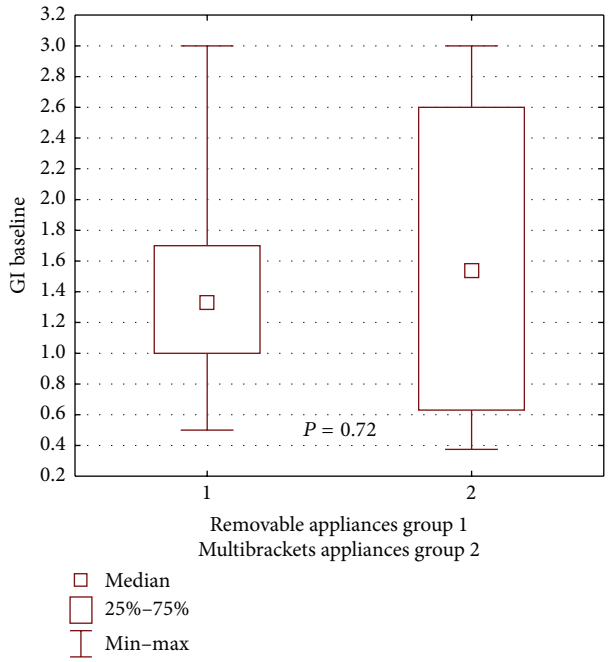

Figure 4: Statistical comparison of GI baseline value between removable appliances and multibrackets appliances at control group. Median, IQR, min, max, and significant if $P < 0.05$.

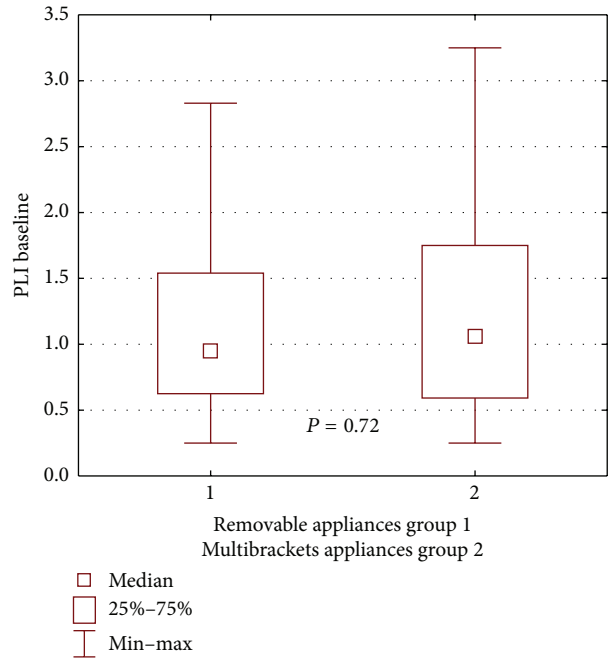

Figure 5: Statistical comparison of PLI baseline value between removable appliances and multibrackets appliances at control group. Median, IQR, min, max, and significant if $P < 0.05$.

3.2. Final Study

3.2.1. Propolis Group. A statistically significant decrease in GI in the entire propolis group ($P < 0.01$) was shown during repeated examination performed after five weeks of using the propolis toothpaste (Table 2). When analysing the gingival index depending on the orthodontic appliance type, a statistically significant improvement of the gingival condition, expressed by a decrease in GI, occurred in both the buccal multibracket appliances patients group ($P = 0.01$) and the removable appliance group ($P = 0.02$) (Table 2).

During repeated examination, a statistically significant decrease in PLI and thus the amount of dental plaque were also demonstrated both in the entire propolis group ($P < 0.01$) and in patients wearing multibracket ($P < 0.01$) and removable appliances ($P = 0.03$) (Table 2).

A worse periodontal condition was demonstrated in patients wearing buccal multibracket appliances during the repeated examination, that is, a higher mean GI compared with patients treated with removable appliances, yet the difference was not statistically significant ($P = 0.48$) (Figure 6). A similar result was received when assessing the amount of dental plaque during repeated examination. PLI was higher in patients treated with buccal multibracket appliances, compared with patients treated with removable appliances. These differences were not statistically significant ($P = 0.34$) (Figure 7).

3.2.2. Control Group. A statistically insignificant change in GI gingival index ($P = 0.25$) was found during the repeated examination in the entire control group compared to the baseline (Table 2). Similar result was obtained in patients treated with buccal multibracket appliances ($P = 0.24$) and removable ($P = 0.64$) appliances (Table 2).

A statistically significant decrease in dental plaque amount was also demonstrated between the baseline and final study in the entire control group ($P = 0.03$) and in patients treated with buccal multibracket appliances ($P = 0.02$) (Table 2). A statistically significant change in median PLI between the baseline and final study was not found in patients treated with removable appliances ($P = 0.87$) (Table 2).

In the control group, the patients with buccal multibracket appliances had a higher median GI at the end of the study, compared with the removable appliance patients, but the difference was not statistically significant ($P = 0.46$) (Figure 8).

A statistically significant difference in dental plaque amount between the buccal multibracket appliances patient group and the removable appliance group was also not found on repeated examination ($P = 0.71$) (Figure 9).

3.2.3. Generally. The analysis of final GI value correlations in the propolis group demonstrated a high statistically significant correlation with the initial GI value (Figure 10). Lack of significant correlation between final study GI and initial value of PLI, DMF and its elements, and orthodontic appliance type was also found.

It was determined that in the control group patients there are high statistically significant correlations of final GI and PLI values with their initial values and DMFT (Figure 11).

Both toothpastes were well accepted by the patients and no adverse effect was recorded. Some patients reported taste altering and too small degree of foaming of toothpaste

Table 2: Plaque index (PLI) and gingival index (GI) scores of patients propolis group and control group treated with multibrackets or removable appliances; intragroup differences between baseline and final study.

		Propolis group			Control group		
		Median (IQR)		Intragroup difference	Median (IQR)		Intragroup difference
		Baseline	Final study	P^*	Baseline	Final study	P^*
GI	Multibracket appliances	1.75 (1.75)	1.5 (1.62)	0.01*	1.54 (1.97)	1.79 (1.15)	0.24
	Removable appliances	1.12 (1.25)	1.12 (1.23)	0.02*	1.33 (0.70)	1.75 (0.93)	0.64
	Total	1.50 (1.25)	1.25 (1.37)	<0.01*	1.33 (1.37)	1.75 (1.02)	0.25
PLI	Multibracket appliances	1.50 (1.0)	1.50 (1.04)	<0.01*	1.06 (1.15)	0.96 (0.52)	0.02*
	Removable appliances	1.40 (0.79)	1.08 (0.79)	0.03*	0.95 (0.91)	0.96 (0.64)	0.87
	Total	1.50 (1.0)	1.25 (1.0)	<0.01*	0.96 (1.0)	0.96 (0.42)	0.03*

IQR, interquartile range; * significance if $P < 0.05$.

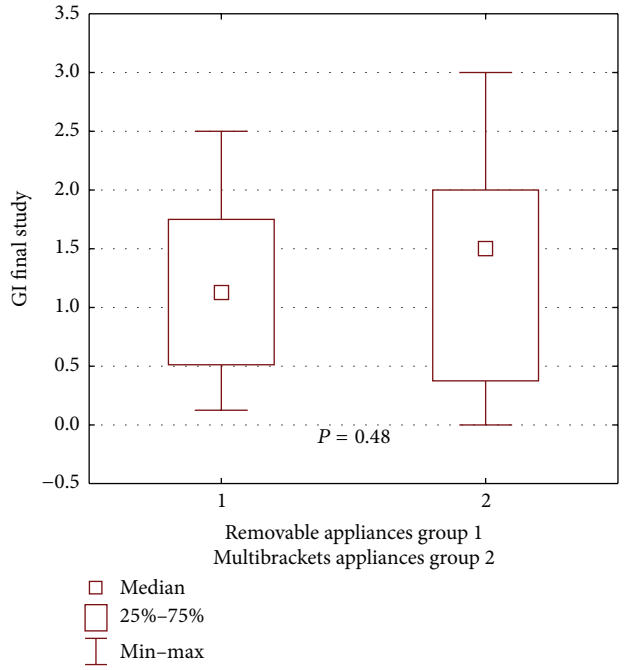

Figure 6: Statistical comparison of GI final value between removable appliances and multibrackets appliances at propolis group. Median, IQR, min, max, and significant if $P < 0.05$.

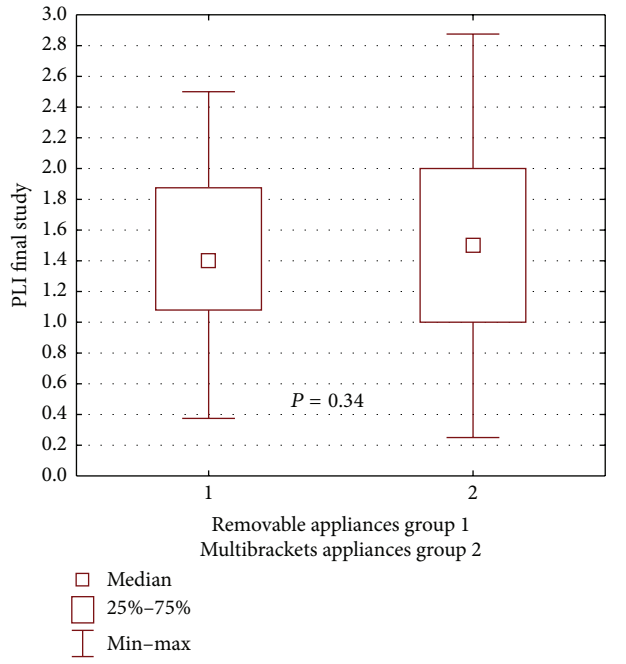

Figure 7: Statistical comparison of PLI final value between removable appliances and multibrackets appliances at propolis group. Median, IQR, min, max, and significant if $P < 0.05$.

with propolis. The main complaint was discoloration of the toothbrush, experienced by 6 patients using toothpaste with propolis.

4. Discussion

Oral cavity malformation, numerous surgical procedures, and long-term orthodontic treatment are factors that may have an effect on oral cavity health in children with oral cleft. These patients are particularly at risk of developing teeth decay and inflammation of the periodontium [5, 20–22]. In the original study it was shown that the mean DMFT in children with CL/CLP is 4.45. Similar DMFT was obtained by Stec-Slonicz et al. when examining children with CL/P during active phase of orthodontic treatment [21] and Zhu et al. in patients aged 6–12 years [22]. Lower mean DMFT of 1.18 in children with cleft lip and palate was obtained by Lucas et al. [23], yet the mean age of the studied children was lower than in the original work. DMFT value applies to permanent teeth and its evaluation in younger children, with the majority of deciduous teeth, may have an effect on its lower value. Higher DMF than in the original study was demonstrated by Al-Wahadni et al. in children with CLP aged 10–15 years [24].

The gingival condition on initial examination, evaluated in the entire material through median GI, was 1.5. A higher GI value in patients with cleft lip and palate aged 10–15 years was obtained by Al-Wahadni et al. [24]. Medium and low gingivitis in patients with unilateral complete cleft were reported by Costa et al. [25]. However, it is important to emphasize that the original study included the condition of marginal gingiva in the area of superior incisors and molars.

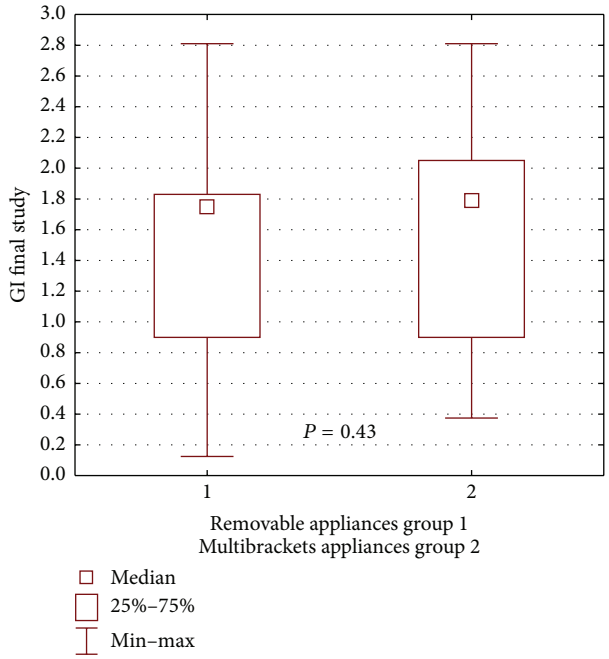

Figure 8: Statistical comparison of GI final value between removable appliances and multibrackets appliances at control group. Median, IQR, min, max, and significant if $P < 0.05$.

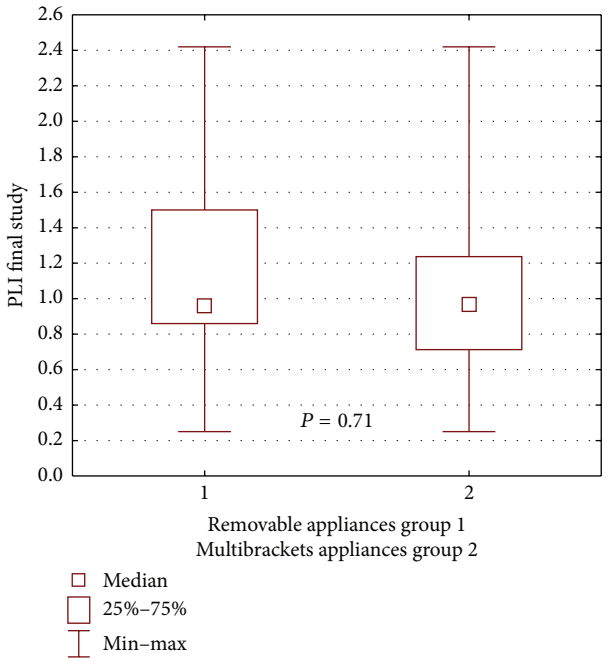

Figure 9: Statistical comparison of PLI final value between removable appliances and multibrackets appliances at control group. Median, IQR, min, max, and significant if $P < 0.05$.

In patients with CL/CLP, the anterior segment of the maxilla is the location of cleft fissure course, scars from primary surgical procedures, and alveolar bone grafting. Furthermore, in patients with CL/CLP, the structure malformation of maxilla's alveolar processes, and vestibular fornix, fistulas to the nasal cavity may coexist with lip structure malformation. This anomaly often prevents correct lip contraction, resulting in nonphysiological drying of the maxilla alveolar processes' mucosa. The described structural and functional changes in the oral cavity in CL/CLP, as well as the methodology of the conducted research, taking into consideration only superior permanent incisors and all first molars, may have an effect on relatively high median GI obtained in this study compared with the results of other authors [20]. This view is confirmed by research conducted by Dahllof et al. who proved a statistically significant higher percentage of units exhibiting bleeding upon probing the upper anterior region, including the cleft area, in children with CL/P, compared with anterior maxillary segment in children without cleft [6].

The authors of this study examined patients with CL/CLP only, during the active phase of orthodontic treatment performed using fixed or removable appliances. Patients treated with fixed appliances presented worse oral cavity hygiene on initial examination, expressed by a higher mean PLI, and worse gingival condition expressed by a higher GI, compared with patients treated with removable appliances. The obtained results matched the research of Karkhanechi et al. who also demonstrated significantly higher PLI and GI in patients without oral cleft, treated with fixed buccal orthodontic appliances versus removable orthodontic aligners during the period between the 6th and 12th therapy month [26].

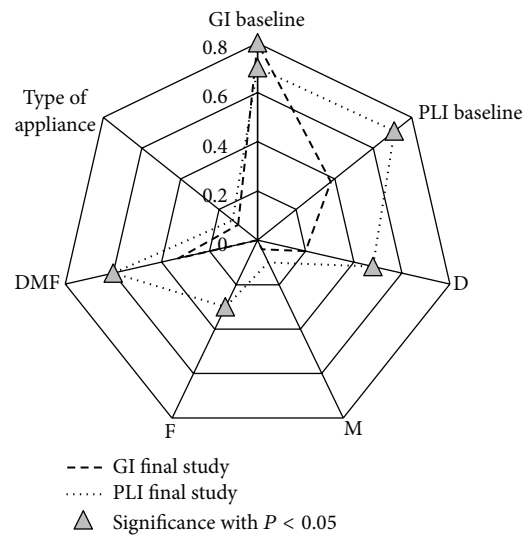

Figure 10: Correlation between GI baseline, PL baseline value and GI final, PLI final, Decayed, Missing, Filled Teeth, DMFT, and type of appliance in propolis group. Statistical significant correlation marked as triangle.

Both an improvement in gingival condition and an improvement in oral cavity hygiene were demonstrated during repeated examination in both patients using the propolis toothpaste, as well as those using the placebo. In the propolis group patients, both those treated with fixed and removable appliances, there was a statistically significant decrease in GI and PLI on final examination, compared with the initial examination. Similarly, a statistically significant PLI and GI

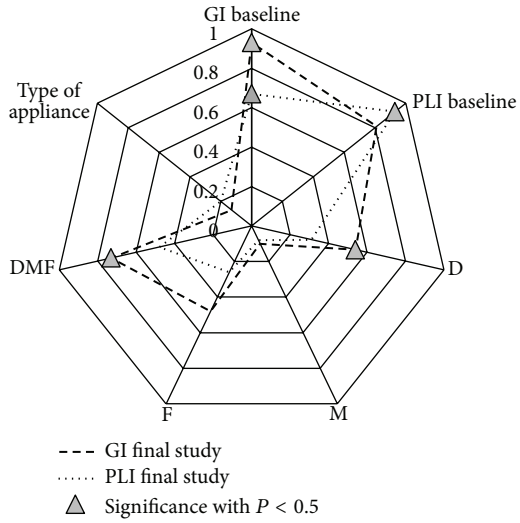

Figure 11: Correlation between GI baseline, PL baseline value and GI final, PLI final, Decayed, Missing, Filled Teeth, DMFT, and type of appliance in control group. Statistical significant correlation marked as triangle.

decrease in patients using alcohol-free mouthwash containing 5.0% Brazilian green propolis over 45 and 90 days was reported by Pereira et al. [27]. Tanasiewicz et al. presented a beneficial propolis effect on oral cavity condition in patients with gingivitis and those with periodontitis [28], whereas Morawiec et al. presented a similar beneficial propolis effect in patients with implants [29].

The results of this study showed a lack of correlation between gingival condition in the final examination and initial oral cavity hygiene and DMFT intensity in propolis group. Therefore, the original study found that propolis masks the effect of initial plaque accumulation and DMFT on gingival condition in the final examination. This effect may indicate a beneficial propolis effect on gingival condition both in children with satisfactory and poor oral cavity hygiene and in those with high and low tooth decay intensity.

Brazilian propolis was used in the study as the active toothpaste component. The compounds present in Brazilian propolis include cinnamic acid and derivatives, coumaric acid, prenylated compounds of chroman and chromen, substituted phenolic esters, flavonoids (flavones, flavanones, and flavonols), benzoic acid, benzopirans, dihydrobenzofurans, and benzofurans [30–32]. Among the cinnamic acid derivatives of Brazilian propolis, artepillin C is the best known [30–33]. According to the reviewed specialist references, artepillin C is one of the components responsible for the antibacterial, antineoplastic, anti-inflammatory, and immunomodulating properties of propolis [34].

The research performed on an animal model showed that the anti-inflammatory action of artepillin C consists of, for example, inhibiting tissue swelling and decreasing total neutrophil count, as well as decreasing prostaglandin E2 level in peritoneal fluid [34]. The mechanism of artepillin C anti-inflammatory action was also confirmed through *in vitro* tests in which the concentration of nitrite in the supernatant in macrophage cell culture decreased as an effect of artepillin C [34]. The permeability of blood vessel capillaries increases during the inflammatory process in the gingiva which promotes plasma protein penetration and causes tissue swelling. Apart from the colour change, it is the second symptom grading the gingivae inflammatory process intensity, assessed in this study using GI index. A statistically significant decrease in the mean value of this index confirms propolis's effect on periodontal inflammation suppression and its effectiveness as an active toothpaste component in patients with CL/CLP.

Detailed studies of propolis's properties have shown that ethanol extract of propolis (EEP) is able to regulate gene expression of bacteria and can cause changes in the bacterial protease activity [35]. The statistically significant PLI and GI decrease during the final study in patients using propolis toothpaste, obtained in the original research, may be related to modifying the maturation and differentiation of bacterial biofilms. Propolis's effect of modifying the dental plaque composition may explain the lack of correlation between final gingivae condition and initial residue amount. This is why it is also effective in patients with high initial plaque accumulation. Propolis's antibacterial action in patients with CL/CLP was also confirmed in the study author's works regarding microflora of the oral cavity [36]. In the control group, the gingival condition in the final study worsens with the increase of initial dental plaque amount; therefore, GI increases.

The ingredients of commonly used toothpastes mostly include chemical compounds such as chlorhexidine [37], triclosan [38], fluorine compounds [39–41], peroxides and hydrogen peroxide [42], cetylpyridinium chloride [38], sodium lauryl sulphate [43, 44], and the salts of metals [45]. Some of these substances may penetrate into the organism through the mucosa, and also as a result of swallowing [46], and disrupt the metabolism of the oral cavity's epithelium [9, 10]. Propolis as a natural substance has very few side effects, which can be minimized by removing the constituents responsible for allergy. However, the incidence of beekeeper's allergic contact dermatitis due to topical application of propolis and adverse reactions due to propolis ingestion are described [47].

Propolis is relatively low-cost, nontoxic and has mucoprotective properties for oral and gastric mucosa [48]. There are no reports dealing with bacterial resistance to propolis [49]; moreover, synergism between propolis and antibacterial agents has been observed [50]. These findings support view that propolis is a promising therapeutic agent in prevention of oral diseases caused by microorganisms.

According to the results of the study, it should be stressed that toothpaste containing propolis is suitable for improvement gingival condition and elimination of the dental plaque and might be used as a potential anti-inflammatory preparation for infectious oral diseases. However, it needs to be emphasized that further step should be given to remove discoloration properties and to improve organoleptic features of studied toothpaste, including more accepted colour, smell, and taste.

5. Conclusions

The conducted study showed that propolis, as an active toothpaste component, may be an effective agent used in oral cavity hygiene in patients with CL/CLP treated with both fixed and removable appliances. The orthodontic appliance type did not affect the final dental plaque amount and gingival condition in patients using the propolis toothpaste. Propolis's action was visible through an improvement in gingivae condition, as well as oral cavity hygiene. The final study decrease in gingival inflammation symptoms in patients using the propolis toothpaste was not related to the initial amount of dental plaque and dental caries intensity. In fact it was quite the opposite: in patients using the placebo toothpaste, the final gingival condition was highly correlated with the initial amount of dental plaque. Instruction on oral cavity hygiene, used on its own, is an effective method of improving oral cavity hygiene in patients with fixed appliances, yet it had no significant effect on gingival condition in the 35-day study period.

Competing Interests

The authors declare no conflict of interests.

Acknowledgments

The authors would like to thank Mr. Rindai Yamamoto, the President of Nihon Natural Food Co. Ltd. (Tokyo, Japan), for the samples of toothpaste-containing ethanol extract of Brazilian green propolis. The paper is original, and the authors take the responsibility for the submission. The authors declare no conflict of interests.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 9272635, 7 pages
http://dx.doi.org/10.1155/2016/9272635

Research Article

Ethnomedicinal Uses of Honeybee Products in Lithuania: The First Analysis of Archival Sources

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Received 17 May 2016; Revised 20 July 2016; Accepted 1 August 2016

Academic Editor: José Maurício Sforcin

Lithuania has old ethnomedicine traditions, consisting of many recipes with herbal, animal, and mineral original ingredients. All these findings were mostly collected in Lithuanian language, often in local community's dialects, and stored only in archives. We analyzed archival sources about honeybee and its products used for medicinal purposes dated from 1886 till 1992 in different parts of Lithuania. We systematized and presented the most important information about bees and their products: indication for usage, ingredients used in the recipe, their preparation techniques, and application for therapeutic purposes. Researchers in Lithuania are now looking for new evidence based indications and preparation and standardization methods of bee products. Archival sources are a foundation for studies in Lithuania. The results can be integrated into scientifically approved folk medicine practices into today's healthcare.

1. Introduction

The first knowledge about Lithuanian ethnomedicine is mentioned in the chronicles of the Teutonic Knights about spells, prejudice, and traditions, since they have close connection to the folk medicine. There are documents about ethnomedicine in the 16th-17th centuries' law books of Grand Duchy of Lithuania. Cases of witches courts with witchcraft methods were mentioned. Lithuania became Christian country latest in Europe, while pagan traditions still existed after christening for a long time. These traditions had close connection with ethnomedicine [1, 2].

The biggest part of all Lithuanian ethnomedicine consists of the usage of traditional medicinal plants. Ethnobotanical expeditions were organized in various historical periods with the aim of preserving local knowledge about traditional uses of medicinal plants for medicinal purposes. All these findings were mostly collected in Lithuanian language, often in local community's dialects, and stored only in archives [3].

Many countries have recently engaged into studies of traditional medicine [4, 5] and Lithuanian researchers begin to collect, systematize, analyze, and publish ethnomedicinal studies as well [6–8]. According to ethnomedicinal global trend studies, it is important to find analysis of food on one hand and medications on the other. These categorizations offered possibility of generating information that could be ordered in a similar way in studies from different regions of the world and thus helped to make comparisons between them [9].

Ethnomedicinal preparations of animal origin make up smaller part of preparations; however, usage of preparations of animal origin is very important part of Lithuanian ethnomedicine. Bee products were important not only for nutritional purposes but also for their healing features and wide medicinal application [10–12]. Bee products can be used alone or in combination with medicinal plants, substances of animal origin, or strongly acting materials for synergistic effect, cumulative impact, or just better taste

and administration form. In addition to bee products as food, active substances used for medicinal purposes, and inactive substances used as bases (e.g., as ointment base), they show close relationship between animal and herbal origin substances used in homemade medicine. This is a part of traditional ecological knowledge, which represents a close relationship between people and places [13].

To find parallels within ethnopharmaceutical (including bee products) research, we look to countries with similar history, nature, and ethnomedical traditions. For this reason, in most cases, Lithuanian researches compare their studies with studies from Poland. Unfortunately, such works are limited [3, 14]. Information about medicinal plants and animals traditionally used for therapeutic purposes is mainly deficient, since archive material is not systematized and mostly presented in small ethnographic papers published in native languages [15, 16]. This problem, also with scarce information on bee products, highlights Polish researches [3, 6, 17]. Also researches organize studies with migrant communities and investigate situation; then people moved to urbanized areas and despite the availability of primary healthcare they often bring with them the traditional medical knowledge and actively use it. Systematic archival material can facilitate comparative analysis in such kind of investigations [18, 19].

Purpose of our study was to systematize unpublished archival material which concerns the use of bee products from ethnographic expeditions in Lithuania. This is the first analysis of unpublished material not only for scientific audience but also in local literature too. This study is important for complementing Lithuanian ethnomedicine data base and also can be useful for researches from other countries to find parallels between studies. Recently, researchers in Lithuania are looking for new evidence based indications and preparation and standardization methods of bee products. Certainly, ideas come from Lithuanian ethnomedicine archives and nowadays public opinion research.

2. Methods

We analyzed archival sources, that is, the material from ethnographic expeditions, dated from 1886 till 1992 in different parts of Lithuania. Among plenty of ethnobotanical information, it also includes preparations from animal origin. We systematized and presented the most important information about bee and its products: indication to use, ingredients used in the recipe, their preparation techniques, and application for therapeutic purposes.

3. Results and Discussion

In Lithuanian ethnomedicine, we can find recipes with bee products, and even whole bee, for the treatment of various ailments. We registered 65 reports regarding bee products used for medicinal purposes (Table 1). Only 39% of all usage was for internal use and the most popular preparation methods were ointments and compresses (22% and 19% from all reports, resp.). The most popular indications were abscesses, wounds, and contagious diseases, such as measles and smallpox. These explain bee products as antimicrobic substances. Honey is the only one of our registered bee products used internally (despite some cases of whole bee, honeycomb, and propolis) and it is the most popular from registered products.

Scientifically inexplicable usage of bee products is explained by archaic traditions and observation of the natural world and an understanding based on the theory of signatures. Only in this way can we explain bee usage as an antidote from viper's bite and fumigation with honeycombs for the same indication.

We registered cases when bee products are used alone or in combination with plants, animals, or additional material: 11 plant species and 8 animal products, such as powders of dried toad, swine bile, goose, chicken fats, dog's and pig's lard, cow's milk, and chicken's eggs. As additional products, cream, butter, vodka, and soap material were used in household.

3.1. Bee, Apes. Until the 18th century bee was described as diuretic and hair loss inhibitive remedy in pharmaceutical literature [20]. It was used in homeopathy for the treatment of allergy and swellings. In Lithuanian folk medicine, according to archival sources, bee's poison was used as antidote to viper's poison. "For epilepsy healing, the drinking the water with boiled dead bees (dead after the winter time) were used" (Table 1). Numerous practices used whole bee for medicinal purposes: "bee glue was used to put on ulcers and boils"; "bee glue was used to put on purulent gatherings for faster removing of purulence" [archival source: LLTI, 1081, pp. 55].

3.2. Honey, Mel. In Old Egypt, in the Ebers Papyrus, honey was the only active ingredient in an ointment described for application to the surgical wound of circumcision [5]. Honey could provide some kind of protection from various kinds of bacteria. It was used on the infected wounds to encourage the healing processes. The ancient Egyptians were not the only people who used honey as medicine. The Chinese, Indians, Ancient Greeks, Romans, and Arabs used honey in combination with other herbs or on its own to treat wounds and various other diseases [21].

Honey is one of the oldest and most famous materials of animal origin. There was a big amount of external drug forms containing honey in Lithuania. According to archival sources, honey (for external usage, alone or in combination) was usually used to treat wounds, abscess (Table 1), and even pain in the body: "from the pain in the body I have mix honey, butter, fat and rue leaves and used a teaspoon daily. After one week my health improved and pain disappeared" [archival source: LLTI. B4575.733/117]. In the Polish traditional medicine, honey has been used for respiratory diseases, gastrointestinal disorders, and dermatological problems. According to our study, the most popular indication for internal use of honey was respiratory disorders and no reports for gastrointestinal disorders were mentioned. But today's study with Polish settlements in Argentina demonstrates that most popular indication is also respiratory disorders [9]. Honey is the most popular material of bee products used up to now according to ethnomedicine studies in other countries and researches mainly focus on these studies [22, 23].

Table 1: Honeybee and its products used for medicinal purposes in Lithuania.

Bee and its products	Usage	Indication	Ingredients	Preparation method	Usage method	Archival source number
Bee, *Apes*	External	Viper's bite	Whole bee	Compress	Application to poisoned part	MT T3, P. 118–120
	External	Toothache	Whole dead bee	Decoction	Rinse the painful part	LLTI BK III-1167
	Internal	Viper's bite	Whole bee	Live bee	Oral	MT T3, P. 118–120; MMNB F127-87, P. 2–4
	Internal	Swollen throat	Whole bee	Dried bee	Oral	MT T5, P. 19–25
	Internal	Epilepsy	Whole bee	Decoction of dried bees	Oral	LNMA F.NM B75, P. 12; VUBRS F81-115, P. 54-55
Honeycomb	External	Viper's bite	Whole honeycomb		Fumigation	MT T3, P. 118–120
	Internal	Children's startle	Whole honeycomb	Decoction	Oral	LKDA B6160, P. 19–81
Honey, *Mel communis*	External	Tetter	Whole	Ointment	Applied to damaged part	LLTI BK III-653
	External	Neck pain	Whole	Ointment	Applied to damaged part	MT T5, P. 198
	External	Tonsillitis	Whole	Ointment	Applied to damaged part	LMDA I 211 a./20
	External	Joint pain	Whole	Ointment	Applied to damaged part and heated in the sun	IIES 208, P. 872
	External	Abscess	Whole	Ointment	Applied to damaged part	LLTI BK III-901
	External	Tonsillitis	Honey, water	Decoction	Rinse the painful part	MMNB F 25-188, P. 45
	External	Abscess	Honey, backfat	Compress	Application to damaged part	VUBRS F81-92, P. 30; LKDA B642/5, P. 58
	External	Pneumonia	Honey, *Nicotiana tabacum* L.	Compress	Application to the breast	VUBRS F81-92, P. 21
	External	Abscess	Honey, powder of dried toad	Compress	Application to damaged part	LLTI B2574, P. 18
	External	Abscess	Honey, tuber of *Allium sativum* L.	Compress	Application to damaged part	LLTI KN.605, P. 193–195
	External	Wound	Honey, *Arctostaphylos uva-ursi* (L.) Spreng.	Compress	Application to damaged part	LMDA I 283/44921
	External	Slag	Honey, milk	Compress	Application to damaged part	VUBRS F81-92, P. 33
	External	Tonsillitis	Honey, dried toad powders	Compress	Application to damaged part	LNMA KN 270, P. 477
	External	Bone pain	Honey, *Nicotiana tabacum* L.	Compress	Application to damaged part	LKDA B642/5, P. 58
	External	Hernia	Honey, swine bile	Compress	Application to damaged part	MMNB F25-188
	External	Abscess	Honey, goose fat	Compress	Application to damaged part	IIES 208/192, P. 882
	External	Wounds	Honey, tuber of *Allium cepa* L., cream, butter	Ointment	Application to damaged part	LMM, P. 171
	External	Wounds	Honey, beeswax, chicken fat, butter	Ointment	Application to damaged part	LMM, P. 155
	External	Wounds, abscess	Honey, decoction of *Matricaria recutita* L. flowers, juice of *Aloe vera* (L.) Burm.f., melted butter	Ointment	Application to damaged part	MMA A424, P. 36-37
	External	Infected wound	Honey, beeswax, fir or pine resin, tuber of *Allium sativum* L., melted butter or suet, juice of *Aloe vera* (L.) Burm.f.	Compress with linen cloth	Application to damaged part	MMNB F 127-87, P. 35–37
	External	Eyestrain	Wild bee's honey	Raw	Application to damaged part	LMDA I 211 a./26
	Internal	Measles	Honey	Raw	Oral	MMNB F 127-87, P. 35–37
	Internal	Wounds	Honey	Raw	Oral	LLTI B 2750, P. 326
	Internal	Eyestrain	Wild bee's honey	Raw		LMM, P. 155
	Internal	Cough	Honey, leaves of *Aloe vera* (L.) Burm.f.	Maceration	Oral	LMM, P. 10
	Internal	Phthisis, tonsillitis	Honey, juice of *Aloe vera* (L.) Burm.f.	Maceration	Oral	MMNB PR.957, P. 2

Table 1: Continued.

Bee and its products	Usage	Indication	Ingredients	Preparation method	Usage method	Archival source number
	Internal	High blood pressure	Honey, dried herb of *Tanacetum vulgare* L.	Maceration	Oral	MMA A418, P. 2
	Internal	Smallpox	Honey, vodka	Extraction	Oral	MMNB 544
	Internal	Tonsillitis	Honey, *Zingiber officinale* Roscoe	Extraction	Oral	MMNB F127-87, P. 5
	Internal	High blood pressure	Honey, *Anethum graveolens* L.	Maceration	Oral	MMA A418, P. 2
	Internal	Bronchitis	Honey, juice of *Viburnum opulus* L. berries, juice of *Beta vulgaris* L.	Maceration	Oral	IIES 208/191, P. 876
	Internal	Cough, phthisis, sore throat, dyspnea	Honey, hot milk		Oral	LLTI BK III-451; LLTI B4260, P. 63/210
	Internal	Scarlatina	Honey, dried toad powders	Decoction	Oral	LLTI B4258, P. 3; LLTI B2574, P. 6;. 63.
	Internal	Tonsillitis	Honey, ashes of toad		Oral	MMA A418, P. 1
	Internal	Cough	Honey, tuber of *Allium sativum* L.		Oral	LNMA F NM B75, P. 4
	Internal	Phthisis	Honey, dog's lard, butter		Oral	LLTI B4258, P. 22-23
	Internal	Rheumatism	Honey, ants	Decoction	Oral	MMA A418, P. 1
Bee stings	External	Rheumatism, arthritis	Bee stings	Raw	Application to damaged part	LLTI B4258, P. 18
Propolis	External	Abscess	Propolis	Raw	Application to damaged part	LMDA I 1060/412; LLTI B1081, P. 55
Propolis	External	Joints pain	Propolis, ethanol	Extraction	Application to damaged part	MMNB F117-168, P. 2
Propolis	External	Toothache	Propolis, ethanol	Extraction	Application to damaged part	VUBRS F81-921, P. 23–25
Propolis	External	Wounds	Propolis, ethanol	Extraction	Application to damaged part	LLTI BK III-343
Propolis	External	Joints pain	Propolis, sunflower oil	Solution in oil	Application to damaged part	LNMA FNM B172, P. 18
Propolis	Internal	Inside wounds	Propolis, ethanol	Extraction	Oral, drops in water	LNMA FNM B155, P. 8–10
Beeswax	External	Ear pain	Beeswax, linen cloth		Burning of rolled waxy linen cloth	LMDA I 1060 4/239
Beeswax	External	Abscess	Beeswax, sheep fat	Ointment	Application to damaged part	LLTI BK III-1154
Beeswax	External	Burned skin	Beeswax, egg		Application to damaged part	LLTI BK III-343
Beeswax	External	Abscess	Beeswax, butter, tuber of *Allium sativum* L.	Ointment	Application to damaged part	LKDA B6160, P. 19/74
Beeswax	External	Wounds	Beeswax, chicken fat, honey	Ointment	Application to damaged part	LMM, P. 155
Beeswax	External	"Rose" disease	Beeswax, fir resin, *Tilia cordata* Mill. flowers, *Matricaria recutita* L. flowers, butter	Ointment	Application to damaged part	MMA A418, P. 2
Beeswax	External	Infected wounds	Beeswax, tuber of *Allium sativum* L., lard, fir resin, soap	Ointment	Application to damaged part	MMNB, F127-87, P. 24
Beeswax	External	"Rose" disease	Beeswax, fir resin, lard	Ointment	Application to damaged part	LLTI BK III-1088

Nowadays, scientific studies indicate that honey contains major amounts of carbohydrates, lipids, amino acids, proteins, vitamins, and minerals that have important roles in wound healing with minimum trauma during redressing. Laboratory studies and clinical trials have shown that honey promotes autolytic debridement, stimulates growth of wound tissues, and stimulates anti-inflammatory activities, thus accelerating the wound healing processes [24]. Internally, it was used for various respiratory tract disorders. When ingested, honey also promotes healing and shows antibacterial action by decreasing prostaglandin levels, elevating nitric oxide levels, and exerting prebiotic effects. The use of honey leads to improved wound healing in acute cases, pain relief in burned patients, and decreased inflammatory response in such patients [25, 26].

According to the scientific studies, honey has antiseptic, curative properties and acts as effective broad-spectrum antibacterial agent [26]. The antimicrobial qualities of honey explain the external and internal uses of honey in Lithuania.

3.3. Bee Stings, Venenum Apium. Bee stings are some kind of injections and were used for treating rheumatism (Table 1). According to archival material, "bee stings were used for arthritis treatment"; "bee stings were used for rheumatizm healing" [archival source: LLTI, 2574, pp. 13].

Other researches highlight the therapeutic application of bee venom which has been used in traditional medicine to treat diseases, such as arthritis and rheumatism, and to relieve pain [27] and clinical trials also reveal bee sting therapy for rheumatoid arthritis and get positive results [28]; also bee venom acupuncture for rheumatoid arthritis is one of the opportunities for the treatment [29].

After viper's bite, according to archival sources, "just let bee to sting or put bee into bread and give it to eat" [archival source: LLTI, 3503, pp. 84].

3.4. Propolis, Propolim. Dioskorid described propolis as sliver extractor and also for fumigation from chronic cough. This is a pharmacopoeial remedy from the 16th century to the 18th century [20]. In Lithuania, it was used as oily or alcohol extract. According to our archival sources, propolis was used to treat wounds and joint pain. It is prepared with ethanol or with oily solution (Table 1).

Despite recent advances in wound care products, traditional therapies based on natural origin compounds, such as plant extracts, honey, and propolis, are interesting alternatives. These therapies offer new possibilities for the treatment of skin diseases and allow overcoming some limitations such as the increase in the bacterial resistance. Current trends move to the development of innovative wound care treatments, combining the use of traditional healing agents (such as propolis and honey) and modern products, such as dressing films and hydrogel sheets containing honey [30]. Also studies show that propolis is a potent antioxidant and a free radical scavenger [31, 32].

Lithuanian scientists have been focusing on investigation of propolis qualities and propolis preparations development. They identified that propolis therapeutic application does not induce germ resistance and does not destroy useful microflora [33]; study explains our findings using propolis for wound care not only with ethanolic solution, but also in a form of oily solution (Table 1). Nowadays, bee products, particularly honey and propolis and its preparations (tablets, suppositories, ointments, mouth sprays, and others), are available in most of the Lithuanian community pharmacies usually positioned as dietary supplements [34].

Figure 1: Ear pain relief by burning of rolled waxy linen cloth (demonstration).

3.5. Beeswax and Honeycomb, Cera Alba, Flava, and Favum Mellis. Fumigation with Ibio shaped piece of wax was described in Ebers Papyrus. It was used when the uterus went down. Dioscorides described the pills of beeswax as a remedy that stops diarrhea [20].

Fumigation with wild honeycombs is very old method of cure, used to cure viper's bite (Table 1). Also, "if children got a fright, the honeycomb tea was used for treating" [LLTI number 6160.81].

White beeswax and yellow beeswax were an important part of ointments and plasters. Other researches investigating ethnopharmaceutical formulations in other countries also find beeswax as material for formulation of ointments [35]. In a study done by Kacániová et al. [36], it was found that the extracts of beeswax were effective against pathogenic bacteria, so this material can be used as antimicrobial agent too. It explains our findings for beeswax used in ointments form to treat wounds, abscesses, and burned skin.

In Lithuanian ethnomedicine, ear pain relief by burning of rolled waxy linen cloth is very interesting (Figure 1). The same method is known in Chinese medicine but instead of linen silk is used. Also beeswax still is used as a component in cosmetic preparations (ointments, lip pencils, etc.), for its acting as protective film on the skin and mucous membranes [37].

4. Conclusions

Treatment techniques with bee and its products in Lithuanian ethnomedicine have survived since the times when qualified medical assistance was hardly accessible. It is a unique fact that in modern times of developed medical assistance even young people in Lithuania actively use traditional bee products and combine them with modern medicine. These unpublished archival materials demonstrated that bee products not only were a part of plant or animal origin homemade medicines but also were among main ingredients in the recipes for the treatment and prevention of common diseases in the studied area. Archival sources are a foundation for studies in Lithuania.

The results can be integrated into scientifically approved folk medicine practices into today's healthcare.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 3704764, 11 pages
http://dx.doi.org/10.1155/2016/3704764

Research Article

Anti-Inflammatory Activity of Bee Venom in BV2 Microglial Cells: Mediation of MyD88-Dependent NF-κB Signaling Pathway

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Received 26 February 2016; Accepted 30 June 2016

Academic Editor: José Maurício Sforcin

Bee venom has long been used as a traditional folk medicine in Korea. It has been reportedly used for the treatment of arthritis, cancer, and inflammation. Although its anti-inflammatory activity in lipopolysaccharide- (LPS-) stimulated inflammatory cells has been reported, the exact mechanism of its anti-inflammatory action has not been fully elucidated. Therefore, the aim of this study was to investigate the anti-inflammatory mechanism of bee venom in BV2 microglial cells. We first investigated whether NO production in LPS-activated BV2 cells was inhibited by bee venom, and further iNOS mRNA and protein expressions were determined. The mRNA and protein levels of proinflammatory cytokines were examined using semiquantitative RT-PCR and immunoblotting, respectively. Moreover, modulation of the transcription factor NF-κB by bee venom was also investigated using a luciferase assay. LPS-induced NO production in BV2 microglial cells was significantly inhibited in a concentration-dependent manner upon pretreatment with bee venom. Bee venom markedly reduced the mRNA expression of COX-2, TNF-α, IL-1β, and IL-6 and suppressed LPS-induced activation of MyD88 and IRAK1 and phosphorylation of TAK1. Moreover, NF-κB translocation by IKKα/β phosphorylation and subsequent IκB-α degradation were also attenuated. Thus, collectively, these results indicate that bee venom exerts its anti-inflammatory activity via the IRAK1/TAK1/NF-κB signaling pathway.

1. Introduction

Toll-like receptors (TLRs) are highly expressed in microglial cells, and they are involved in the functioning of the innate inflammatory response to a wide range of invading microorganisms by releasing proinflammatory cytokines and chemokines [1]. One of these TLRs, that is, TLR4 which can be rapidly activated by lipopolysaccharide (LPS), acts as a potent activator and initiates the inflammatory cascade in cells [2]. Consequently, activation of TLR4 triggers its association with downstream adopter molecules within the cytoplasm, such as myeloid differentiation primary response gene 88 (MyD88), and subsequent association with interleukin-1 receptor-associated kinase 1 (IRAK1). This leads to activation of the transcription factor, that is, nuclear factor-kappa B (NF-κB) [3].

In the central nervous system (CNS), microglia, which are macrophage-like innate immune cells, play a crucial role in host defense mechanisms and tissue repair [4]. Under pathological conditions such as brain tissue injury or in the presence of immunological stimuli, microglia are rapidly activated and produce inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), and IL-1β [5]. Expression of these inflammation-related cytokines is regulated at the transcriptional level. NF-κB is a key transcriptional regulator of inflammatory cytokine expression in the immune cells. Accumulation of inflammatory cytokines can cause severe neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and trauma [6–8]. Therefore, suppression of these inflammation-related mediators is particularly important for the prevention of neurodegenerative diseases in the CNS.

Bee venom, which is extracted from honeybees, has long been used in alternative medicine and as a traditional Korean folk medicine [9]. A number of studies have shown that bee venom has diverse physiological activities such as antiarthritic [10], anticancer [11], and anti-inflammatory action [9]. Bee venom contains melittin, apamin, adolpin, and mast cell-degranulating peptide [12]. Recent reports have shown that two of the main constituents of bee venom, melittin and apamin, have anti-inflammatory effects on LPS-stimulated BV2 microglial cells through p38 mitogen-activated protein kinase (MAPK) and NF-κB-dependent signal transduction [9]. Although several studies on the bioactivities of bee venom have been conducted, the actual molecular mechanism by which bee venom regulates inflammation and the signaling pathway has not yet been completely elucidated.

In the present study, in order to determine the mechanism underlying the anti-inflammatory effects of bee venom in BV2 microglia, we investigated its inhibitory effect on the expression of proinflammatory cytokines and the associated molecular signaling pathways. We established that bee venom could attenuate the expression of proinflammatory mediators through a MyD88-dependent signaling pathway.

2. Materials and Methods

2.1. Materials. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from Welgene (Daegu, South Korea). Streptomycin and penicillin were obtained from Lonza (MD, USA). TRI Reagent® solution (AM9738) was obtained from Applied Biosystems/Ambion (Warrington, UK); Oligo(dT) primers were obtained from Bioneer oligo synthesis (Daejeon, Korea). SYBER® green master mix was obtained from Applied Biosystems (Warrington, UK). iNOS, COX-2, TNF-α, and IL-1β primers were obtained from Bioneer (Daejeon, Korea). Total protein lysis buffer (PRO-PREP) and the PRO-MEASURE protein assay kit were obtained from iNtRON Biotechnology (Seoul, Korea). LPS (*Escherichia coli* 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Specific antibodies against phosphor and/or total forms of ERK, JNK, p38, IKKα/β, IκB, NF-κB p65, PI3K, Akt, PARP, iNOS, COX-2, HO-1, and β-actin, as well as a rabbit HRP-conjugated antibody, were purchased from Cell Signaling Technology (Danvers, MA, USA). All other reagents and chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2. Bee Venom Preparation. The crude honeybee venom was obtained using Large Quantity Bee Venom Collector (P10-1003672, Wissen Co., Ltd., Daejeon, Korea). The bee venom (2.5 g) was solubilized with 250 mL ultrafiltered water and filtered through a 0.45 μm nylon membrane filter (Millipore, Billerica, MA, USA) under vacuum. The filtrates were dried using a freeze dryer (Han IL Sci., Clean Vac 8). The dried powder (10 mg) was dissolved in 1 mL of ultrafiltered water and analyzed by HPLC (Figure 1). The contents of melittin and phospholipase-2 were found to be 59.62 ± 4.25% and 12.03 ± 0.66%, respectively.

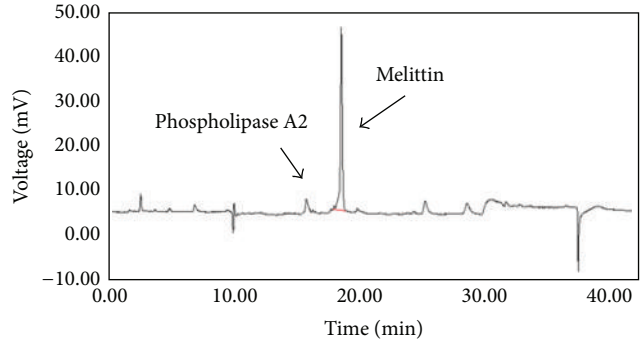

Figure 1: HPLC analysis of bee venom extract used in this experiment.

2.3. Cell Culture. BV2 microglia were maintained in DMEM enriched with 10% heat-inactivated FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin and incubated in a humidified atmosphere of 5% CO_2 at 37°C [13].

2.4. Nitric Oxide Determination. Nitric oxide (NO) in the culture supernatant was measured as previously described [14]. Briefly, BV2 microglia cultured in 24-well plates were incubated with or without LPS (0.1 μg/mL) in the absence or presence of bee venom at the indicated concentrations for 18 h. The cell culture supernatants (100 μL) were mixed with Griess reagent (1% sulfanilamide in 5% phosphoric acid [H_3PO_4] and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride [NEDHC]) and incubated for 5 min at room temperature. The absorbance was measured at 540 nm in a microplate reader.

2.5. Cell Viability Assay. BV2 microglial cells were seeded in 24-well plates and treated with or without LPS (0.1 μg/mL) in the absence or presence of various concentrations of bee venom in culture medium. After 18 h, the BV2 microglial cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent for 4 h, following which the absorbance was measured at 560 nm in an ELISA reader.

2.6. RNA Extraction and Quantitative PCR. BV2 microglial cells were pretreated with various concentrations of bee venom for 30 min and then stimulated with LPS (0.1 μg/mL) for 18 h. Total RNA was isolated using TRI Reagent solution according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using reverse transcriptase premix and oligo(dT) primers. Quantitative PCR was performed with the CFX96™ Real-Time System (Bio-Rad) by using power SYBR® Green Master Mix. The relative quantity of target mRNA was calculated using the comparative threshold (Ct) method by normalizing to GAPDH Ct values. The quantitative PCR program used was as follows: predenaturation (95°C, 5 min), denaturation (95°C, 20 sec), annealing (55°C,

20 sec), and extension (72°C, 45 sec), using primers specific for *iNOS*, *COX-2*, *IL-6*, and *TNF-α*.

2.7. Western Blotting. BV2 microglial cells were pretreated with various concentrations of bee venom for 30 min in DMEM. After incubation with LPS (0.1 μg/mL), BV2 microglial cells were washed and scraped in ice-cold phosphate-buffered saline (PBS), and the cell pellets were resuspended in lysis buffer (PRO-PREP) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 2 mM sodium fluoride, and 1 mM sodium orthovanadate. The cell lysate was centrifuged at 9,500 ×g for 10 min following which a protein extract was obtained from the supernatant. Protein concentration was measured using the PRO-MEASURE assay kit. Extracts containing equal amounts of protein (40 μg) were mixed with 1x SDS-PAGE loading buffer (Biosesang Inc., Korea) and boiled for 5 min, separated by using 10% SDS-PAGE, and further transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with blocking buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk) for 2 h at room temperature and then incubated with primary antibodies for 18 h at 4°C. After washing with TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20), the membranes were incubated with the secondary antibody for 2 h at 4°C. Immunoreactive protein bands were visualized using enhanced chemiluminescence (ECL). β-actin was used as a loading control.

2.8. Nuclear and Cytoplasm Protein Extraction. Nuclear and cytosolic protein extracts were prepared as previously described [15]. Cells were treated with different concentrations of bee venom prior to LPS stimulation. After incubation, the cells were washed three times with ice-cold PBS, resuspended in lysis buffer A (10 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 2 μg/mL pepstatin), centrifuged, and transferred to cytosolic proteins. After isolating the cytosolic proteins, the pellet was resuspended in lysis buffer B (10 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 10% glycerol) and centrifuged again. The supernatant was transferred to new tubes and was used as the nuclear protein extract.

2.9. Immunoprecipitation. BV2 microglial cells were pretreated with bee venom and then stimulated with LPS for 30 min. After 15 and 30 min, stimulated BV2 microglial cells were resuspended in IP buffer (50 mM Tris-HCl (pH 7.5), 20 mM NaF, 25 mM beta-glycerophosphate (pH 7.5), 120 mM NaCl, 2% NP-40, and various protease inhibitors). This was followed by addition of primary antibody and incubation with gentle rocking overnight at 4°C. After the overnight incubation, protein A agarose beads were added, and the pelleted beads were washed five times with 500 μL of 1x IP buffer. The pellet was resuspended in Laemmli sample buffer (Bio-Rad) and loaded on an SDS-PAGE gel. After electrophoresis, the proteins were transferred onto a PVDF membrane. The membrane was blocked with 5% defatted milk and then incubated with the primary and secondary antibodies. Immunoreactive bands were visualized using enhanced ECL.

2.10. Luciferase Assay. BV2 microglial cells were cultured in 24-well plates for 24 h and then transfected in triplicate with TK-renilla (pRL-TK) and NF-κB firefly luciferase (pNF-κB-Luc) constructs (Stratagene, La Jolla, CA, USA) by using Lipofectamine™ 2000 according to the manufacturer's instructions. Briefly, transfected cells were pretreated with bee venom for 30 min and then stimulated with LPS for 6 h. Next, the cells were washed twice with ice-cold PBS and then 150 μL of 1x passive lysis buffer was added. After centrifugation at 12,000 ×g for 5 min at 4°C, a 10 μL aliquot of the supernatant was analyzed using a Glomax luminometer (Promega, Madison, WI, USA). NF-κB luciferase activity was measured using a luciferase assay system according to the manufacturer's instructions. Luciferase activity was normalized to renilla luciferase activity.

2.11. Statistical Analysis. One-way ANOVA with a post hoc Dunnett's multiple comparison and Student's *t*-test was used to determine the statistical significance of differences between the experimental and control groups. *p* values of 0.05 or less were considered statistically significant. Data represent the means ± SEM of three experiments conducted in triplicate.

3. Results

3.1. Inhibitory Effect of Bee Venom on Nitric Oxide Production in LPS-Stimulated BV2 Microglial Cells. Nitric oxide (NO) not only acts as an inflammatory mediator and a regulator of inflammatory action, but also has detrimental effects on host tissues [16]. Activated BV2 microglial cells induce iNOS expression and NO *production* in neuronal inflammation. Therefore, we initially examined whether bee venom extract affected NO production in LPS-activated BV2 cells. It was observed that LPS treatment prominently increased NO production (17.3 ± 1.4 μM) in BV2 microglial cells compared to untreated cells (Figure 2(a)), and this increase was markedly reduced by bee venom pretreatment in a concentration-dependent manner. Next, we evaluated the cytotoxicity of bee venom and found that it did not display any cytotoxicity even at 2.5 μg/mL in the presence of LPS stimulation (Figure 2(b)). Therefore, it was concluded that the inhibitory effect of bee venom was not due to cytotoxicity.

3.2. Inhibitory Effect of Bee Venom on the mRNA and Protein Expression of iNOS and COX-2 in BV2 Microglial Cells. NO, which has a crucial role in the initiation of inflammation, is produced in high amounts by iNOS [17]. To determine whether the inhibitory effect of bee venom on NO production was due to decreased iNOS expression, we measured iNOS mRNA and protein expression by real-time PCR and immunoblotting, respectively. iNOS was highly expressed

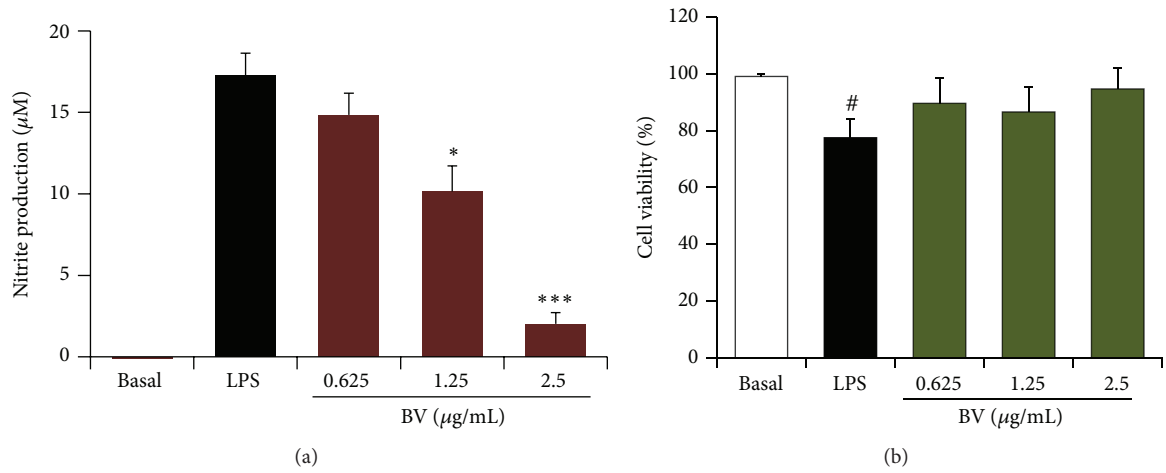

Figure 2: Bee venom inhibits lipopolysaccharide- (LPS-) induced nitric oxide (NO) production in BV2 microglia. (a) The effect of bee venom on the LPS-induced NO production in BV2 cells. (b) The effect of bee venom on the cell viability of LPS-stimulated BV2 cells. BV2 microglial cells were pretreated with different concentrations of bee venom extract (0.625 μg/mL–2.5 μg/mL) for 30 min and then incubated with LPS (0.1 μg/mL) for 24 h. NO production was determined in the cell supernatants as described in Section 2. After determination of NO production, the viability was immediately examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in Section 2. The data are presented as mean ± standard error (SEM), and the experiments were repeated three to five times. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus LPS alone. $^{\#}p < 0.05$ versus basal.

following LPS stimulation (Figures 3(a) and 3(c)). However, this enhanced mRNA and protein expression was greatly suppressed by bee venom pretreatment in a concentration-dependent manner. We then investigated whether bee venom also had an effect on COX-2 mRNA and protein expression in BV2 microglial cells. It was observed that bee venom treatment inhibited the expression of COX-2 mRNA and protein in a dose-dependent manner (Figures 3(b) and 3(c)).

3.3. Inhibitory Effect of Bee Venom on LPS-Induced mRNA Expression of Proinflammatory Cytokines in BV2 Microglial Cells. Microglia cell activation upregulates proinflammatory cytokines such as TNF-α and IL-6, and these can be toxic to neurons and other glial cells. In addition, activated microglial cells contribute to the development of neurodegenerative diseases in the CNS. Therefore, these cytokines merit interest as potential targets in the treatment of neurodegenerative disorders [18]. Following LPS stimulation, TNF-α and IL-6 were highly expressed (Figures 4(a) and 4(b)). When BV2 microglial cells were pretreated with bee venom (0.625, 1.25, and 2.5 μg/mL) and then stimulated with LPS (0.1 μg/mL) for 24 h, a significant inhibition of proinflammatory cytokine expression was detected. These findings suggested that bee venom could disrupt the expression of IL-6 and TNF-α at the transcriptional level.

3.4. Inhibitory Effect of Bee Venom on LPS-Induced NF-κB Activation, IκB-α Degradation, and IKKα/β Phosphorylation. The transcription factor NF-κB is activated by the degradation of phosphorylated IκB-α, which is phosphorylated by IκB-α kinase (IKK) [19]. To examine the effect of bee venom on the degradation of IκB-α and the phosphorylation of IKKα/β, BV2 microglial cells were stimulated with LPS in the presence or absence of bee venom. LPS treatment alone augmented the phosphorylation of NF-κB at 15 and 30 min (Figure 5). However, bee venom pretreatment decreased NF-κB translocation from the cytoplasm to the nucleus, IκB-α degradation, and IKKα/β phosphorylation in a time-dependent manner. Next, we determined whether bee venom could reduce NF-κB transcriptional activity. It was observed that bee venom extract significantly repressed NF-κB activity in a concentration-dependent manner, suggesting that NF-κB is a critical target in bee venom-mediated anti-inflammatory action (Figure 5(e)).

3.5. Inhibitory Effect of Bee Venom on LPS-Induced Transforming Growth Factor-β- (TGF-β-) Activated Kinase 1 and MAPK Phosphorylation. TGF-beta activated kinase 1 (TAK1) functions as an upstream signaling molecule of NF-κB. An activated TAK1 complex will phosphorylate critical kinases, including p38 MAPK, c-jun N-terminal kinase (JNK), and IKK, which activates NF-κB [20]. Since we learnt that bee venom suppressed NF-κB activation, we hypothesized that bee venom might inhibit the augmentation of NF-κB translocation via TAK1 phosphorylation in the LPS-TLR4 signaling pathway. To determine whether bee venom disrupts MAPKs and TAK1 phosphorylation, we evaluated the levels using immunoblot analysis. Bee venom attenuated LPS-induced ERK1/2 and JNK phosphorylation but not p38 MAPK phosphorylation with statistical significance at 30 min (Figures 6(a) and 6(b)). In addition, bee venom significantly inhibited LPS-induced TAK1 phosphorylation at earlier activation of 5 min (Figure 6(b)).

3.6. Inhibitory Effect of Bee Venom on the Interaction between MyD88 and Its Downstream Signaling Molecules. MyD88 is an important component of signal transduction in TLR4

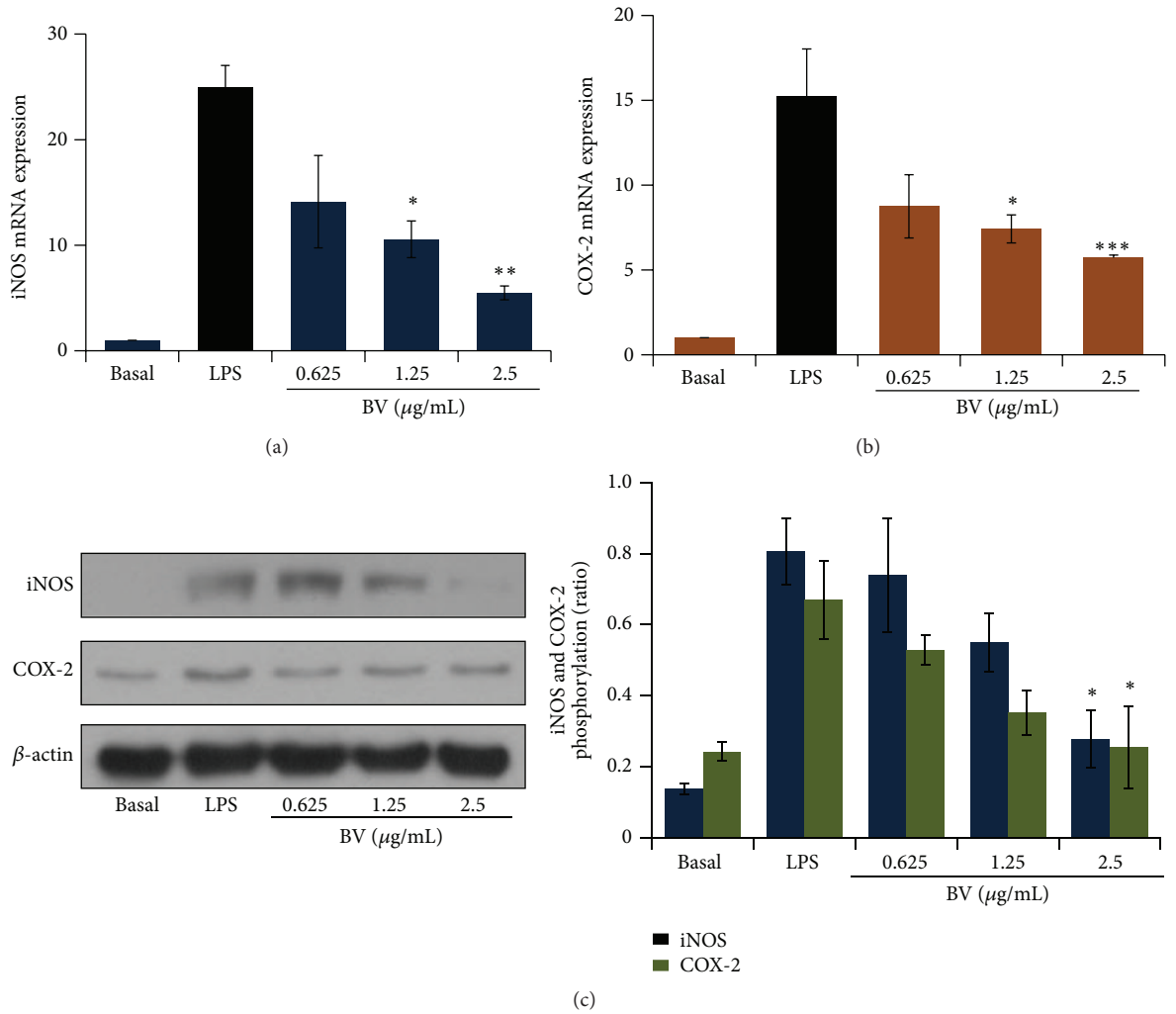

Figure 3: Bee venom inhibits the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein in LPS-activated BV2 microglia. (a) The effect of bee venom on *iNOS* mRNA expression in LPS-stimulated BV2 cells. (b) The effect of bee venom on *COX-2* mRNA expression in LPS-stimulated BV2 cells. (c) The effect of bee venom on iNOS and COX-2 protein expression in LPS-stimulated BV2 cells. BV2 microglial cells were pretreated with bee venom (0.625–2.5 μg/mL) or vehicle for 30 min and then stimulated with LPS (0.1 μg/mL) for 24 h. Then, total RNA was prepared (to assess mRNA expression), or protein was extracted as described in Section 2. ((a) and (b)) The levels of *iNOS* and *COX-2* mRNA expression were determined using quantitative real-time polymerase chain reaction (PCR). The protein concentration of the cell extracts was determined with PRO-MEASURE (iNtRON Biotechnology, Korea). The protein separation and immunoblot procedures are described in Section 2. The data are presented as mean ± SEM, and experiments were performed three to five times. Representative images of experiments performed at least in triplicate are shown. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus LPS alone.

activation. Upon binding of LPS to TLR4, an adapter molecule MyD88 was recruited into the receptor and then subsequently recruits IL-1 receptor-associated kinase (IRAK1) into the TLR-MyD88 complex. Interaction between MyD88 and IRAK1 leads to TRAF6 phosphorylation, which activates the downstream signaling pathway [21]. It has recently been reported that the MyD88-dependent pathway is involved in activation of LPS and expression of inflammatory cytokines in BV2 microglial cells [22, 23]. Therefore, we hypothesized that the anti-inflammatory effect of bee venom might be via inhibition of the MyD88-dependent pathway. In the present study, we examined the interaction between MyD88 and other signaling molecules, TRAF6, IKKα/β, MKK4, and TAK1, *using protein complex immunoprecipitation technique*. The association of the tested signaling molecules, including TRAF6, IKKα/β, MKK4, and TAK1, with MyD88 was greatly diminished after 30 min of bee venom treatment. This suggests that the bee venom diminished the signaling pathway at earlier components in the Myd88-dependent pathway of LPS-TLR4 activation (Figure 7).

4. Discussion

Several reports have shown that bee venom exhibits antineuroinflammatory activity [9, 24, 25]. However, the signaling

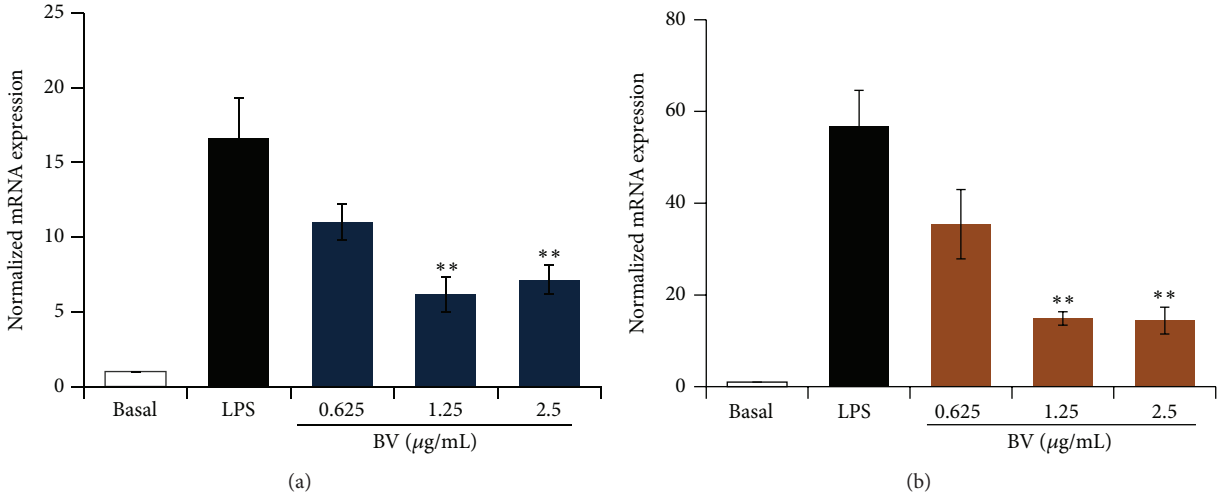

Figure 4: Bee venom inhibits the expression level of tumor necrosis factor-α (*TNF-α*) and interleukin-6 (*IL-6*) mRNA in LPS-stimulated BV2 microglia. Dose-dependent inhibition of *TNF-α* (a) and *IL-6* (b) mRNA expression were assessed using quantitative real-time PCR. BV2 cells were pretreated with bee venom for 30 min, and then 0.1 μg/mL LPS was added and the cells were incubated for an additional 24 h. The total RNA preparation and real-time PCR were performed as described in Section 2. GAPDH was used as an internal control, and relative expression levels of *TNF-α* and *IL-6* mRNA were calculated by normalization to *GAPDH*. The data are presented as mean ± SEM of three to five experiments. $^{**}p < 0.01$ versus LPS alone.

pathways that govern this activity are still not clear. Therefore, we explored the anti-inflammatory activities of bee venom and the actual signaling pathways that inhibit LPS-induced inflammation in BV2 microglial cells. Our data indicated that bee venom could neutralize LPS-induced inflammatory responses in microglial cells through a MyD88-dependent pathway. To be precise, bee venom extract significantly inhibited LPS-induced NO production and expression of the proinflammatory cytokines TNF-α and IL-6 in BV2 cells in a concentration-dependent manner. In addition, bee venom was found to inhibit the transcriptional activity of NF-κB. Analysis of the downstream signaling molecules of LPS-TLR4 showed that the MyD88-IRAK1-TRAF6-TAK1-MKK4 pathway and the MyD88-TAK1-IKKα/β pathway were modulated by bee venom treatment. The major finding of this study was that the MyD88-IRAK1-TRAF6-TAK1-MKK4 and MyD88-TAK1-IKKα/β pathways are the novel inhibitory mechanisms underlying bee venom-mediated inhibition of the LPS-induced inflammatory response in BV2 microglial cells.

Microglial cells are resident immune cell population in the CNS that are intensely responsive to brain injury and neuronal disorders, and when these occur, they become rapidly activated. This reaction is a part of the normal response to maintain brain homeostasis [5]. However, overactivated microglia can cause neuronal death and neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, and trauma. Therefore, regulation of microglial activation by bee venom might be a useful option for the prevention or therapy against neurodegenerative diseases [26].

LPS, a macromolecular complex found in the cell walls of gram-negative bacteria, is an endotoxin that is covalently linked to a lipid A [5]. Several reports have shown that LPS can strongly activate microglia. Activated microglia release a large variety of inflammatory mediators, including proinflammatory cytokines, chemokines, and neurotoxic factors [27] In particular, NO is an interesting target molecule due to its Janus-like ability to mediate the inflammatory response by inhibiting or prompting the inflammatory response through several different pathways. Exposure to LPS or other stimuli induces the expression of iNOS in immune cells, and iNOS can constantly produce a large amount of NO [28, 29]. In the present study, we found that LPS-induced NO production was significantly attenuated by bee venom treatment, and this effect could emanate from its inhibitory effect on iNOS.

TLRs are members of the IL-1R/TLR superfamily that play crucial roles in inflammatory responses to invading pathogens by recognizing specialized microbial components. Among several TLR subtypes, TLR4 is required for LPS stimulation and is involved in host defense against inflammation, apoptosis, and cancer [30]. MyD88 is an adaptor protein that possesses a TIR domain in its C-terminus and a death domain in its N-terminus. The association of its TIR domain with TLRs is responsible for transmitting the intracellular signal from TLR4 after LPS stimulation [31]. Upon TLR4 activation, MyD88 recruits IRAK4 and then induces IRAK1 phosphorylation. Phosphorylated IRAK associates with TRAF6, which leads to activation of JNK and NF-κB [32]. Interaction of TAK1 with ubiquitinated TRAF6 leads to TAK1 activation. TAK1 is an MAP3 K that is activated by TGF-β and plays a critical role in relaying the signal that promotes inflammation-related cytokine expression [33]. Our data clearly showed that bee venom blocked LPS-activated inflammatory responses in a MyD88-dependent manner (Figure 7).

The transcription factor NF-κB has a critical role in the innate inflammatory response and is a key mediator that is responsible for several key biological processes such as

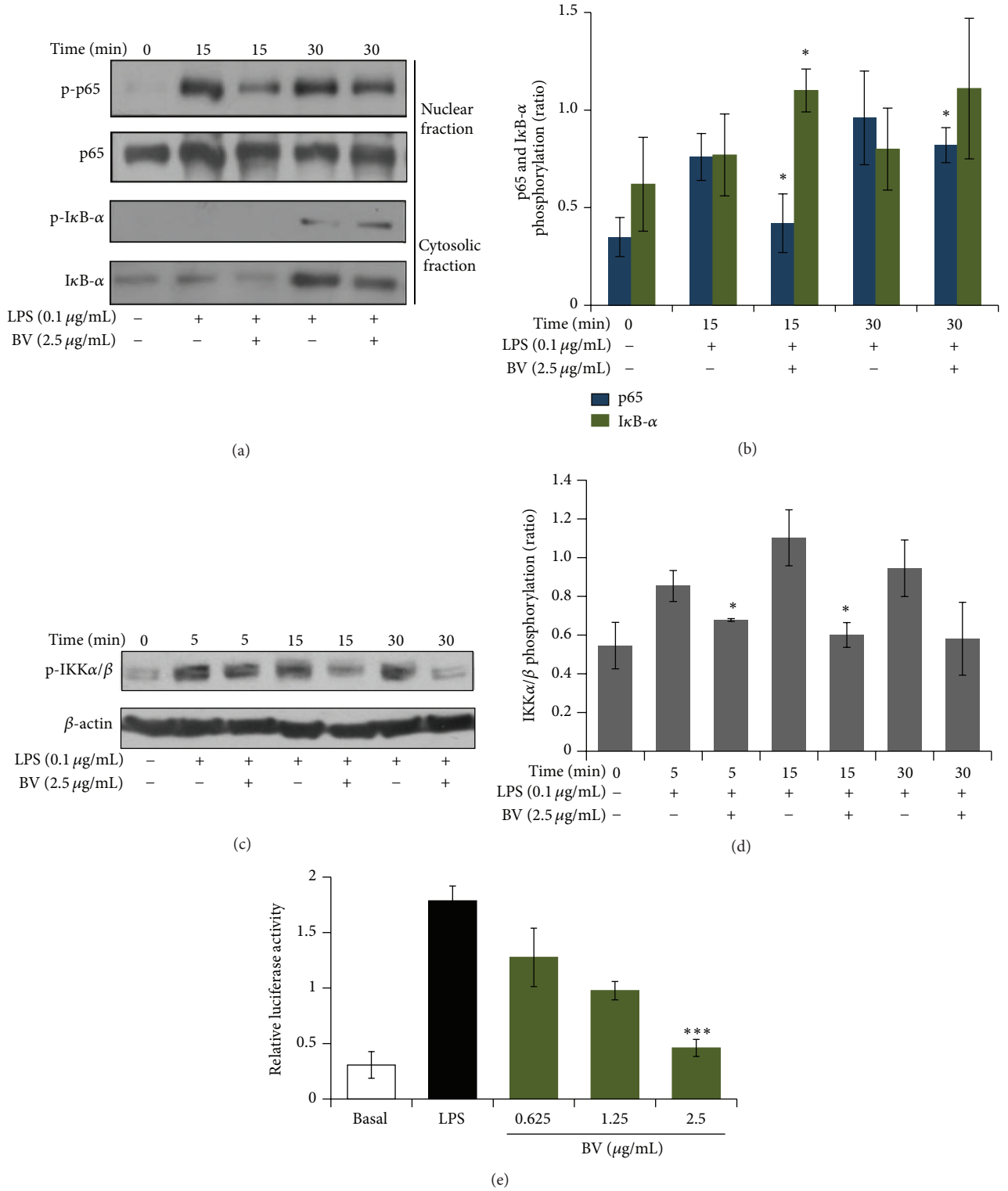

FIGURE 5: Inhibition of degradation of IκB-α, phosphorylation of IKKα/β, nuclear translocation of the p65 subunit of NF-κB/Rel, and NF-κB transcriptional activity by bee venom in LPS-stimulated BV2 microglia. The inhibitory effect of bee venom on the nuclear translocation of the p65 subunit of NF-κB/Rel, degradation of IκB-α (a), phosphorylation of IKKα/β (b), and NF-κB transcriptional activity (c). BV2 cells were pretreated with bee venom or vehicle for 30 min and then stimulated with 0.1 μg/mL LPS for the indicated times. The protein extraction and SDS-PAGE methods are described in Section 2. The β-actin was used as an internal loading control for the immunoblot analysis. The effect of bee venom on NF-κB transcriptional activity was determined by firefly luciferase activity by using a luminometer. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus LPS alone.

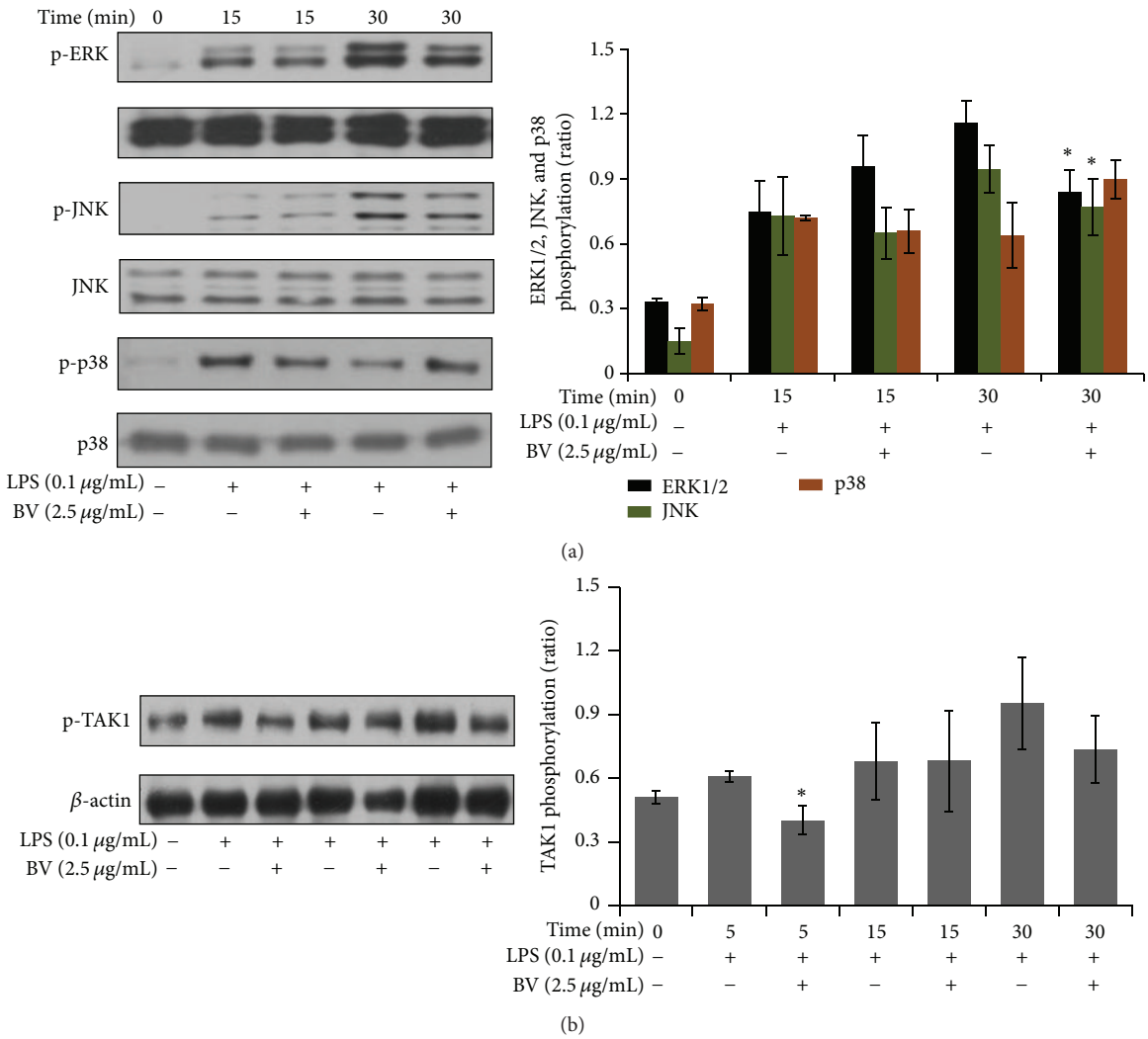

Figure 6: Bee venom modulates MAPK and TAK1 phosphorylation in LPS-stimulated BV2 microglia. The inhibitory effects of bee venom on the phosphorylation of extracellular-signal regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38-MAPK) (a), and TGF-β-activated kinase 1 (TAK1) (b) in LPS-activated microglial cells. BV2 cells were pretreated with bee venom or vehicle for 30 min and then incubated with LPS for the indicated times. The protein extraction and protein separation by SDS-PAGE are described in Section 2. β-actin was used as an internal loading control for the immunoblot analysis. Representative images of experiments performed at least in triplicate are shown. $^{*}p < 0.05$ versus LPS alone.

immune and inflammatory responses. NF-κB is activated in the macrophages upon stimulation with proinflammatory cytokines [34]. Activated IKKα/β causes degradation of IκB-α and translocation of NF-κB to the nucleus [35]. In addition, bee venom abolished the translocation of NF-κB into the nucleus, thereby deactivating the NF-κB transcriptional activity. This may be due to blockade of the interaction between MyD88 and IKKα/β (Figure 6(b)).

In summary, we found that bee venom diminished LPS-induced proinflammatory cytokines, iNOS, and COX-2 expression. In addition, the phosphorylation of the three MAPKs was significantly attenuated following bee venom treatment. Interestingly, the association of MyD88 with IRAK and TRAF6 was disrupted (at 15 min) by bee venom pretreatment. Moreover, the interaction of MyD88 with MKK3/4 and IKKα/β was also significantly inhibited at 30 min. Therefore, bee venom limits LPS-induced neuroinflammation through inhibition of the association of MyD88 with TRAF6 and IRAK1, which inhibits activation of downstream signaling molecules (Figure 8). Bee venom has been shown to prevent LPS-induced IκB-α/β phosphorylation, which, in turn, inhibits the translocation of NF-κB and the MAPK-dependent pathways. These results support our rationale of the novel mechanism underlying bee venom's anti-inflammatory effects in BV2 microglia.

Competing Interests

The authors declare that they have no conflict of interests.

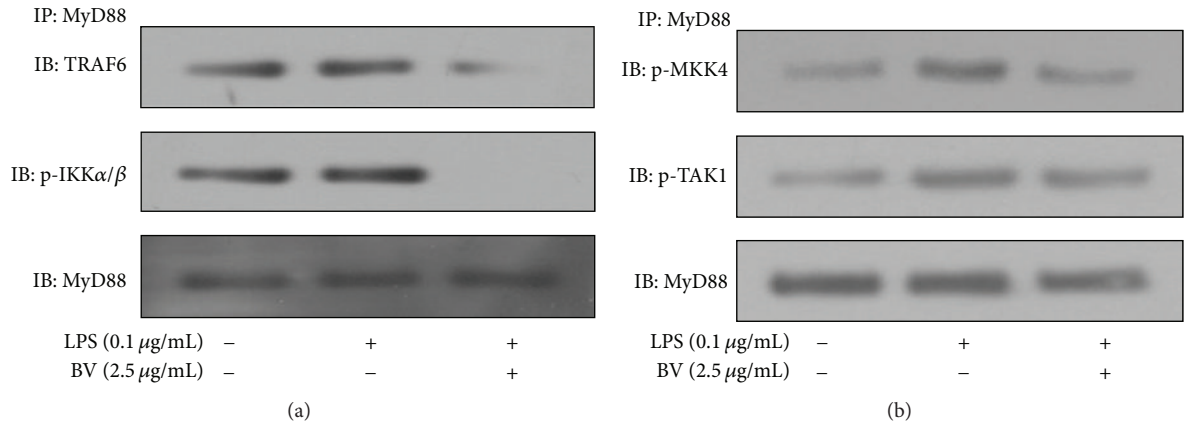

Figure 7: The interaction of MyD88 with either TRAF6 and IKKα/β or MKK4 and TAK1 was diminished by bee venom treatment in LPS-stimulated BV2 microglia. The inhibitory effects of bee venom on the interaction of MyD88 with TRAF6 (a) and IKKα/β or MKK4 and TAK1 (b) in LPS-activated microglial cells are shown. BV2 cells were pretreated with bee venom or vehicle for 30 min and then incubated with LPS for the indicated times. The protein extraction, immunoprecipitation with MyD88 antibody, and protein separation by SDS-PAGE are described in Section 2. Representative images of experiments performed at least in triplicate are shown.

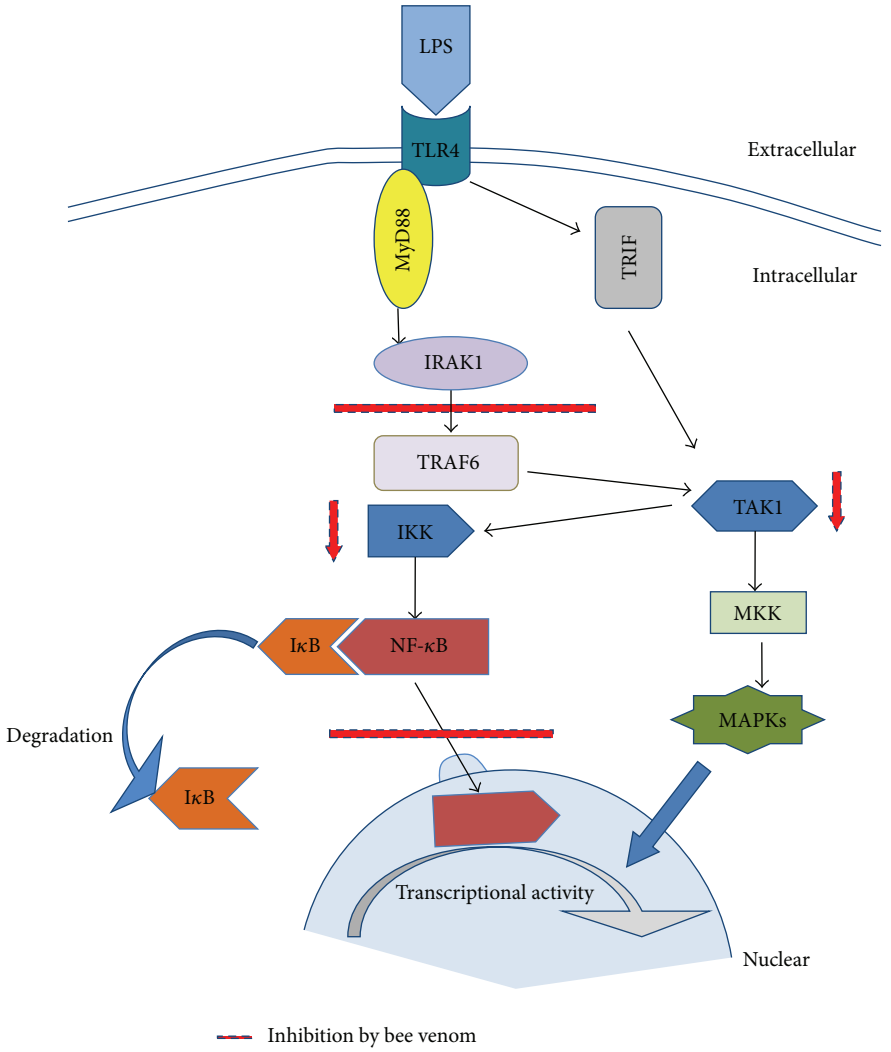

Figure 8: Graphical summary of anti-inflammatory activity of bee venom in LPS-activated BV2 glial cells.

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Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 7292379, 8 pages
http://dx.doi.org/10.1155/2016/7292379

Research Article

Influence of Temperature on Free Radical Generation in Propolis-Containing Ointments

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Received 29 April 2016; Revised 16 June 2016; Accepted 17 June 2016

Academic Editor: José Maurício Sforcin

Free radicals thermally generated in the ointments containing propolis were studied by electron paramagnetic resonance (EPR) spectroscopy. The influence of temperature on the free radical concentration in the propolis ointments was examined. Two ointment samples with different contents of propolis (5 and 7%, resp.) heated at temperatures of 30°C, 40°C, 50°C, and 60°C, for 30 min., were tested. Homogeneously broadened EPR lines and fast spin-lattice interactions characterized all the tested samples. Free radicals concentrations in the propolis samples ranged from 10^{18} to 10^{20} spin/g and were found to grow in both propolis-containing ointments along with the increasing heating temperature. Free radical concentrations in the ointments containing 5% and 7% of propolis, respectively, heated at temperatures of 30°C, 40°C, and 50°C were only slightly different. Thermal treatment at the temperature of 60°C resulted in a considerably higher free radical formation in the sample containing 7% of propolis when related to the sample with 5% of that compound. The EPR examination indicated that the propolis ointments should not be stored at temperatures of 40°C, 50°C, and 60°C. Low free radical formation at the lowest tested temperatures pointed out that both examined propolis ointments may be safely stored up to the temperature of 30°C.

1. Introduction

Propolis is a natural agent produced by *Apis mellifera*, resulting from the addition of salivary enzymes to resins collected from various plant sources, mixed with wax, and used for narrowing nest hive entrances, sealing gaps, and embalming dead organisms inside the hive, thus preventing decomposition and spreading of odors [1–3].

Although the chemical composition and biological activity of propolis are highly changeable due to the variability of plant species occurring around the hive, from which the bees collect the exudates, the mentioned apitherapeutic comprises approximately 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollens, and 5% of impurities [2, 4, 5]. Until the present time, over 300 ingredients, contained in the biologically potent fractions such as flavonoids, phenolics, and aromatic compounds, have been identified in propolis [4, 5]. The mentioned constituents determine the properties of propolis including anti-inflammatory, antimicrobial, antioxidant, antitumor, antiulcer, regenerating, and anti-HIV activities [3, 4, 6–10].

Due to special properties, high activity, and a broad application of propolis-containing ointments in medicine and pharmacy [1–5], they should not contain large amounts of free radicals. The ointments should be stored at specified conditions, among others at a proper temperature, which is safe for the chemical structure of the therapeutic constituents of the ointment. Thermal formation of free radicals should not occur. Too high temperature may lead to undesirable free radicals thermal formation in the ointment environment.

The rupturing of the chemical bonds of the ointment compounds, accompanied by the generation of unpaired electrons, may weaken the therapeutic efficacy of the mentioned medicine. The toxic impact of free radical reactions on the exposed skin must be avoided. The aim of this work was to determine the concentrations and properties of thermally formed free radicals in two ointments different in terms of the amounts of propolis. Moreover, different temperatures of the propolis ointment storage were analyzed.

The performed EPR analysis had the innovatory character since the free radical scavenging activity of 5% and 7% ointment samples was not examined by electron paramagnetic resonance earlier probably because of technical and analytical difficulties. In our work we implemented for the first time electron paramagnetic resonance (EPR) spectroscopy as an experimental tool for the examination of broadened EPR lines and fast spin-lattice interactions present in the used propolis formulations. Moreover, the instrumental method applied by us was nondestructive and demanded only a small amount of sample for the experimental estimation.

The undertaken study is supposed to broaden our earlier knowledge about the usefulness of electron paramagnetic resonance spectroscopy studies in the assessment of propolis antioxidative properties [11–13].

2. Experimental

2.1. Samples Characterization. The subjects of the study were propolis-containing ointments at two different propolis concentrations, 5% and 7%, respectively. The abovementioned topical propolis ointments were obtained from the Apiary "Barć" Galenowa Wytwórnia Farmaceutyczna, Kamianna, Poland, and were authorized under the certificate RK/221957/2008 for 5% propolis ointment and RK/157056/2006 for the ointment containing 7% of propolis.

The applied propolis ointments at concentrations of 5% and 7% were chosen on the basis of the literature knowledge and experimental studies investigating the anti-inflammatory effect of the topical formulation containing propolis at abovementioned concentrations [14, 15]. On the other hand, 5% and 7% propolis ointments are commonly used as topically applied propolis extracts that belong to the natural product available on the polish pharmaceutical market, being also listed in the Journal of Medicinal Products List admitted to trade on Polish territory.

2.2. Thermal Treatment of the Ointment Samples. The ointments containing propolis were heated at temperatures of 30°C, 40°C, 50°C, and 60°C for 30 min. The mentioned temperatures of heating were chosen as the potential storage temperatures of the medicine. These temperatures reflected the possible thermal conditions in the storage environment. A professional hot air oven produced by Memmert Firm (Germany) with temperature programmer with additional air exchange rates controlled by AtmoControl software was used. The mentioned experimental device possesses extended temperature protection ensured by the integrated PT100 sensor for independent temperature monitoring.

2.3. EPR Measurements. Free radicals in thermally treated ointments were examined at room temperature 15 minutes after heating. The individual samples were located in thin-walled glass tubes of high paramagnetic purity with the external diameter of 1 mm. The empty glass tubes were free of EPR signals. The masses of the samples in the tubes were determined as the difference of the mass of the tube with the ointment and the mass of the empty tube by the use of Sartorius CPT weight (Germany). To measure the EPR spectra the samples in glass tubes were placed in the resonance cavity of the EPR spectrometer.

The measurements were done by an X-band (9.3 GHz) electron paramagnetic resonance spectrometer of Radiopan Firm (Poznań, Poland) with a magnetic modulation of 100 kHz and a system of numerical data acquisition, Rapid Scan Unit of Jagmar Firm (Cracow, Poland). The lines were detected as the first-derivative EPR spectra in the range of microwave power from 2.2 mW to 70 mW. The microwave frequency was measured by MCM101 recorder produced by EPRAD Firm (Poznań, Poland).

The following parameters of the EPR spectra were determined: g-factors [±0.0002], amplitudes (A) [±0.01 a.u.], integral intensities (I) [±0.02 a.u.], and linewidths (ΔB_{pp}) [±0.02 mT]. The g-factors were calculated from the resonance condition as [16, 17]: $g = h\nu/\mu_B B_r$, where h is Planck constant, ν is microwave frequency, μ_B is Bohr magneton, and B_r is induction of resonance magnetic field. The influence of microwave power on amplitudes (A) and linewidths (ΔB_{pp}) was examined. Integral intensities (I) were obtained by double integration of the first-derivative EPR curves. Integral intensities (I) were used in calculations of free radical concentrations (N) in the samples.

The references in free radical concentrations (N) studies were ultramarine [18, 19] and a ruby crystal (Al_2O_3:Cr^{3+}). Free radical concentrations (N) in the ointments were calculated according to the following formula [9, 10]: $N = N_u[(W_u A_u)/I_u] \cdot [I/(WAm)]$, where N_u is number of paramagnetic centers in the reference and ultramarine; W, W_u are receiver gains for the tested sample and ultramarine; A, A_u are amplitudes of ruby signal for the tested sample and the ultramarine; I, I_u are integral intensities for the tested samples and ultramarine, and m is mass of the sample. Ultramarine was obtained from Professor Andrzej B. Wieckowski from the Institute of Molecular Physics of Polish Academy of Sciences in Poznan (Poland) and the Institute of Physics of University in Zielona Góra (Poland).

The EPR measurements and analysis were performed by professional spectroscopic programs of Jagmar Firm (Cracow, Poland), LabView (National Instruments, USA), and ORIGIN 2016, OriginLab Corporation (Boston, USA).

3. Results and Discussion

The tested unheated propolis ointments did not contain free radicals. Free radicals were thermally formed in these samples. EPR signals were not observed for the unheated samples, which indicated the fact that they were diamagnetic and free of unpaired electrons. The propolis ointments become paramagnetic after thermal treatment at temperatures of

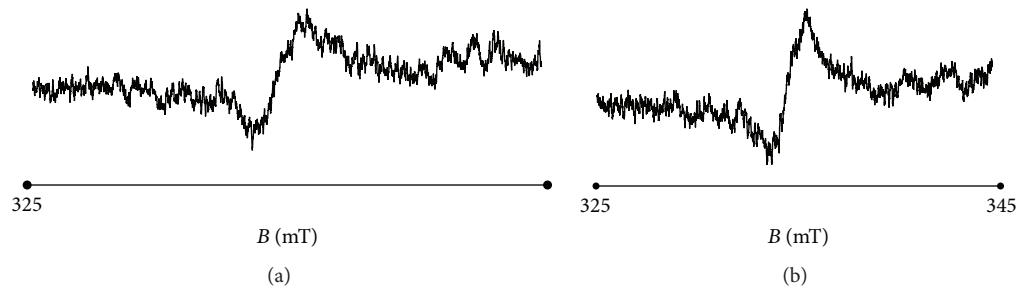

Figure 1: EPR spectra of the ointments containing (a) 5% and (b) 7% of propolis heated at the temperature of 60°C. *B*: magnetic induction. The spectra were measured with low microwave power of 2.2 mW.

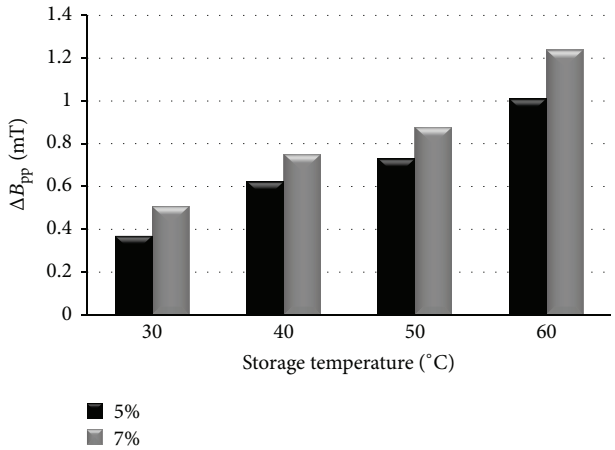

Figure 2: The influence of the heating temperature on linewidths (ΔB_{pp}) of the EPR spectra of the ointments containing 5% and 7% of propolis. The data for the measurements with the microwave power of 2.2 mW.

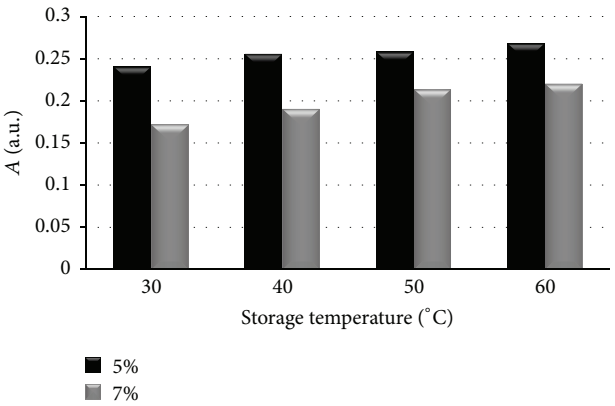

Figure 3: The influence of the heating temperature on amplitudes (A) of the EPR spectra of the ointments containing 5% and 7% of propolis. The data for the measurements with the microwave power of 2.2 mW.

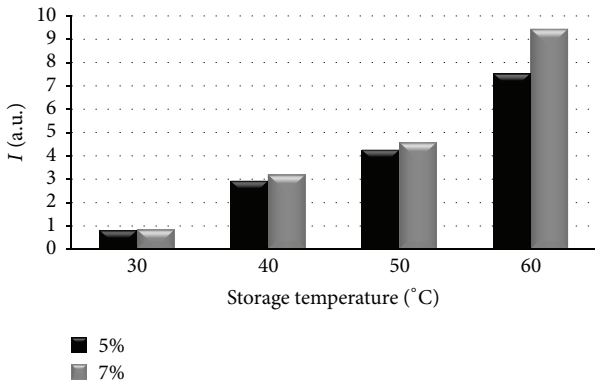

Figure 4: The influence of the heating temperature on integral intensities (I) of the EPR spectra of the ointments containing 5% and 7% of propolis. The data for the measurements with the microwave power of 2.2 mW.

30°C, 40°C, 50°C, and 60°C. For the heated propolis samples, the EPR lines with free radical g factor of 2.00 were measured. The exemplary EPR spectra for the ointments containing 5% and 7% of propolis heated at the temperature of 60°C were shown in Figures 1(a) and 1(b), respectively.

The spectral parameters, linewidths (ΔB_{pp}), amplitudes (A), and integral intensities (I), for the two ointments heated at different temperatures (30–60°C), were compared. The linewidths (ΔB_{pp}) of the EPR lines of the ointments containing 5% and 7% of propolis for the samples heated at temperatures of 30°C, 40°C, 50°C, and 60°C were shown in Figure 2. The influence of the heating temperature on amplitudes (A) and integral intensities (I) of the EPR lines of the tested propolis samples was presented in Figures 3 and 4, respectively. The high values of linewidths (ΔB_{pp}: 0.39–1.22 mT) (Figure 2) were caused by strong dipolar interactions between unpaired electrons of free radicals. The linewidths (ΔB_{pp}) and dipolar interactions were increasing along with rising heating temperature in both tested propolis ointments. The broader EPR lines were observed for the ointment containing 7% of propolis. Dipolar interactions of free radicals were stronger in this ointment. The character of dependence of amplitudes (A) and integral intensities (I) on the heating temperature was visible in Figures 3 and 4. The temperature dependence of the values of amplitudes (A) and integral intensities (I) caused a temperature dependence of free radical concentrations (N) in the examined ointments.

Free radical concentrations (N) in the two examined ointments differing in terms of propolis contents when heated at temperatures of 30°C, 40°C, 50°C, and 60°C were

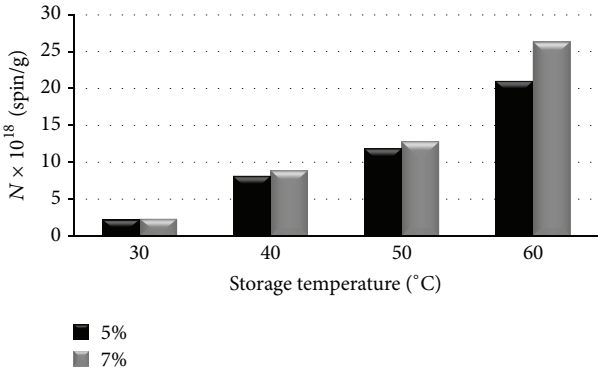

Figure 5: The influence of the heating temperature on free radical concentration (N) in the ointments containing 5% and 7% of propolis. The data for the measurements with the microwave power of 2.2 mW.

compared in the graph in Figure 5. Free radicals in concentrations of ~10^{18}–10^{20} spin/g were detected for the thermally treated propolis samples. Free radical concentrations (N) in both ointments (5% and 7% of propolis contents) strongly depended on the heating temperature. The free radical concentrations (N) increased with increasing heating temperature of the samples. The differences between free radical concentrations (N) in the ointments containing 5% and 7% of propolis treated with temperatures of 30°C, 40°C, and 50°C were not strong. The two tested propolis samples differed in free radical concentrations (N) after heating at 60°C. The significantly higher free radical concentrations (N) were formed in the ointment containing 7% of propolis compared to the sample containing 5% of propolis. Taking into account the results presented in Figure 5, it was visible that temperatures of 40°C, 50°C, and 60°C were not recommended for storage of propolis ointments. Considering the lowest free radical formation the storage at 30°C was recommended for these samples.

The amplitudes (A) and linewidths (ΔB_{pp}) of the analyzed EPR spectra depended on microwave power (M/M_o). The changes of amplitudes (A) of the EPR lines of the ointment containing 5% of propolis heated at temperatures of 30°C, 40°C, 50°C, and 60°C, with increasing microwave power (M/M_o), were presented in Figures 6(a), 6(c), 6(e), and 6(g), respectively. The influence of microwave power (M/M_o) on amplitudes (A) of the EPR lines of the ointment containing 7% of propolis heated at temperatures of 30°C, 40°C, 50°C, and 60°C was shown in Figures 7(a), 7(c), 7(e), and 7(g), respectively. The changes of linewidths (ΔB_{pp}) of the EPR lines of the ointment containing 5% of propolis heated at temperatures of 30°C, 40°C, 50°C, and 60°C, with increasing microwave power (M/M_o), were presented in Figures 6(b), 6(d), 6(f), and 6(h), respectively. The correlations between linewidths (ΔB_{pp}) of the EPR lines of the ointment containing 7% of propolis heated at temperatures of 30°C, 40°C, 50°C, and 60°C and microwave power (M/M_o) were visible in Figures 7(b), 7(d), 7(f), and 7(h), respectively.

The linewidths (ΔB_{pp}) of the EPR spectra of the ointments containing 5% and 7% of propolis increased along with increasing microwave power (M/M_o) (Figures 6(b), 6(d), 6(f), 6(h), 7(b), 7(d), 7(f), and 7(h)). This effect was characteristic for homogeneously broadened EPR lines [20, 21]. The amplitudes (A) for the ointments containing 5% and 7% of propolis heated at temperatures of 30–60°C increased along with increasing microwave power (M/M_o) (Figures 6(a), 6(c), 6(e), 6(g), 7(a), 7(c), 7(e), and 7(g)). The absence of the microwave saturation along with the decrease of amplitudes (A) at the higher microwave powers confirmed fast spin-lattice relaxation processes in the thermally treated propolis samples. The similar times of spin-lattice relaxation processes in the samples heated at different temperatures indicated that magnetic interactions were unchanged. The spin-lattice relaxation of unpaired electrons was retained at temperatures up to 60°C. However, the increase discussed above of free radical concentrations in higher temperatures (Figure 5) did not allow using them during storage of the examined propolis ointments.

The performed X-band (9.3 GHz) electron paramagnetic resonance examination of the ointments containing propolis brings to light the thermal conditions of their storage. The higher temperatures (40–60°C) which produced high concentration of free radicals in the ointments should be rejected in practice. The safe temperatures of storage of the ointments containing both 5% and 7% of propolis were up to 30°C. This work gives information about free radical properties of the tested propolis samples. The obtained EPR results were important for pharmacy and medicine application of propolis.

The apitherapeutic ointments applied in the present study possess numerous medical applications and properties. It is commonly known that the propolis ointment may be successfully applied in case of female patients with cervical erosions, inflammation of the mucous membrane of the uterus, inflammation of the cervix, and nonspecific inflammation of the vulva and vagina [22].

Many studies have been carried out on the antimicrobial effect of topically applied propolis in case of different skin diseases caused by microorganisms. These include suppurative diseases of the skin caused by staphylococci, such as folliculitis, boils, sweat gland infection, and mixed staphylococcal-streptococcal pathologies, including ecthyma, pyoderma or skin tuberculosis, and various fungal and viral diseases [23]. Earlier studies also demonstrated that topical application of propolis extract turned out to be effective in inhibiting carrageenan-induced rat hind paw edema and, additionally, inhibiting chemotaxis of human polymorphonuclear leukocytes (PMNs), the phenomenon of which may also contribute to the anti-inflammatory effect of ointment containing propolis extract [15]. Furthermore, it was also observed that in the course of phonophoresis the propolis ointment caused the decrease in skin sensitivity, due to the anesthetizing effect on the skin receptors [24].

Propolis ointment management was also reported to stimulate the efficacy of the short stretch bandage compression stocking and the combined venous ulcer treatment which was more effective than Unna's boot compression alone [14]. Pessolato et al. reported in their studies that burn treatment with propolis stimulated the process of tissue

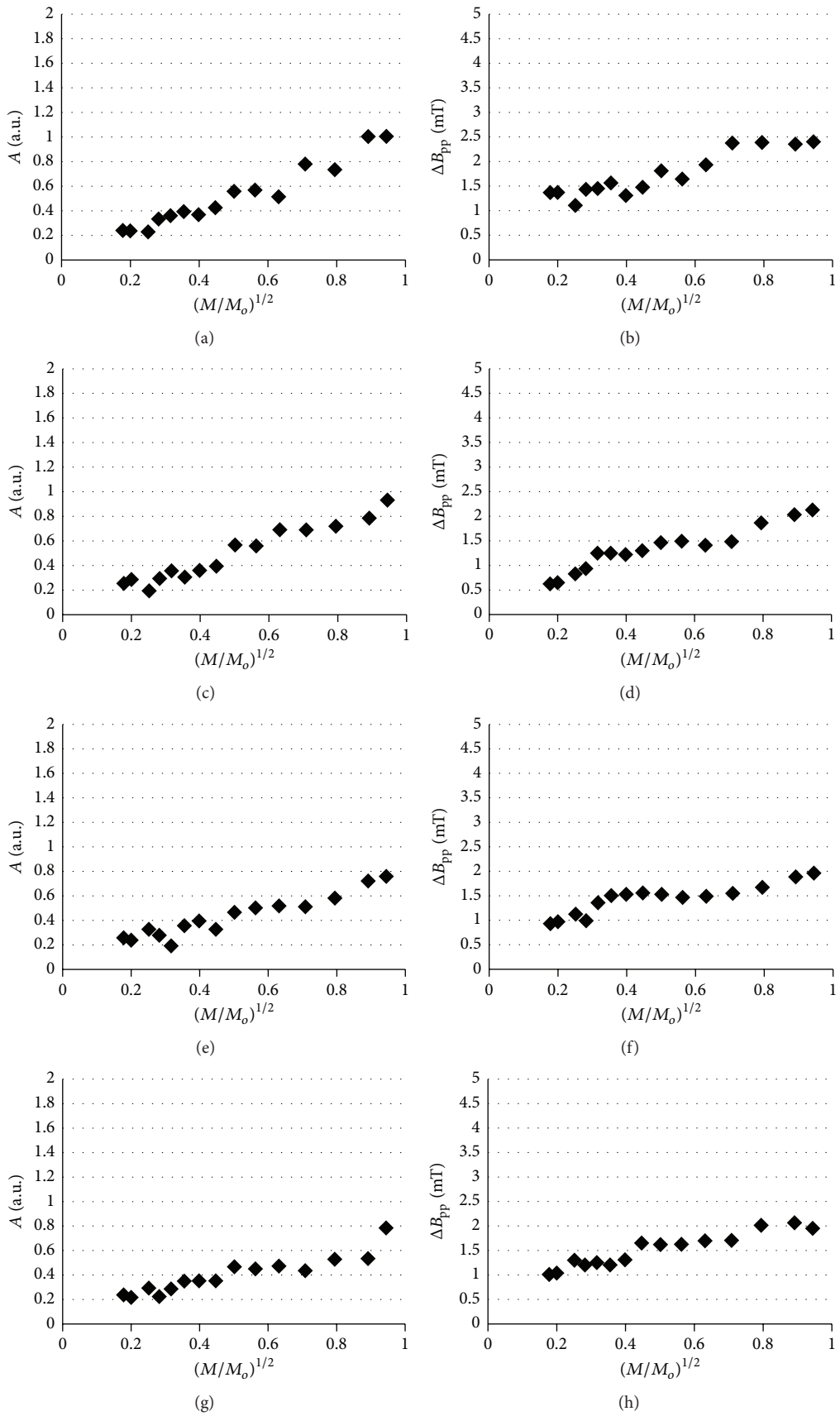

Figure 6: The influence of microwave power (M/M_o) on (a, c, e, and g) amplitudes (A) and (b, d, f, and h) linewidths (ΔB_{pp}) of the EPR spectra of the ointment containing 5% of propolis thermally treated at temperatures 30°C (a, b), 40°C (c, d), 50°C (e, f), and 60°C (g, h). M: microwave power used during the measurement of the EPR spectra; M_o: total microwave power produced by klystron (70 mW).

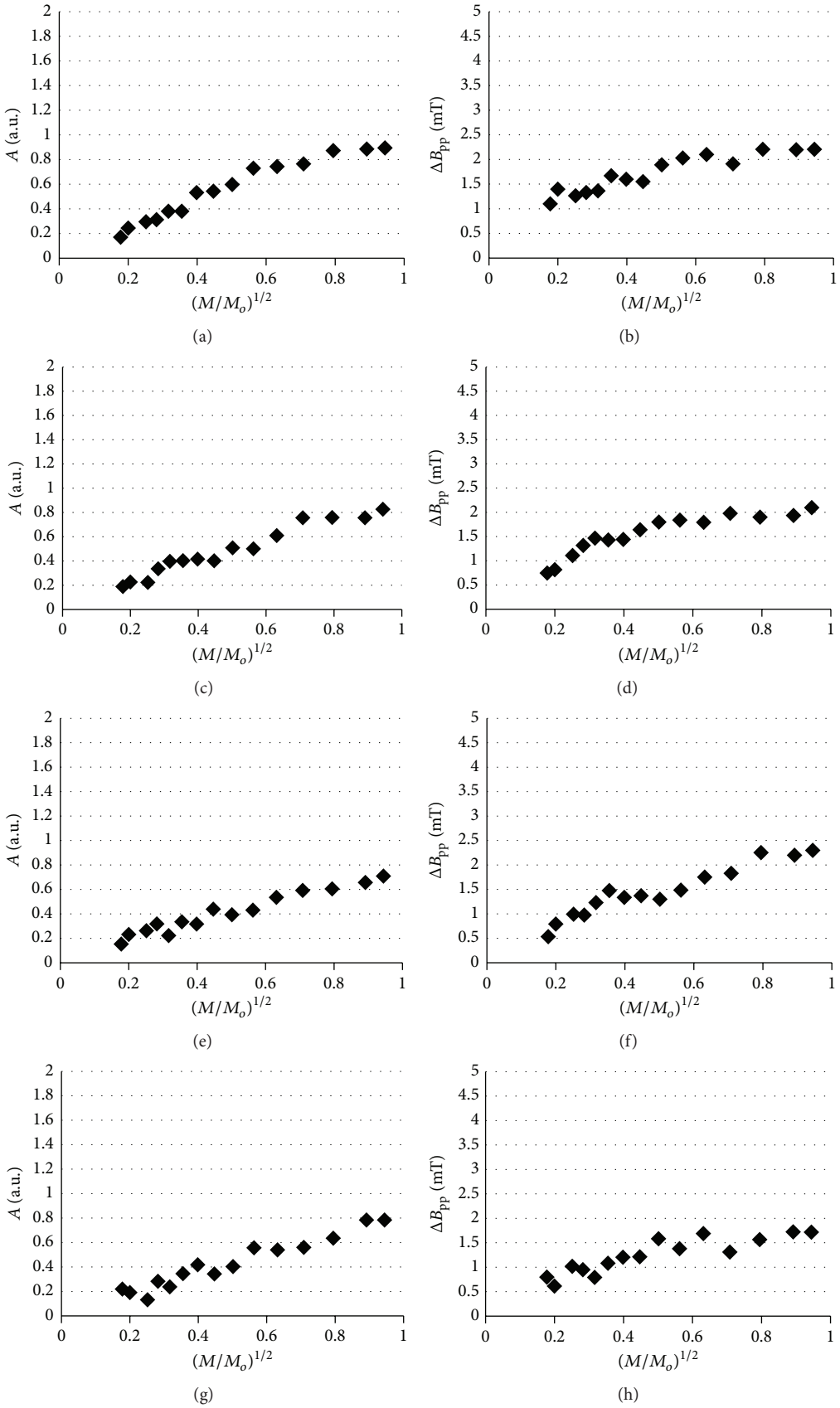

Figure 7: The influence of microwave power (M/M_o) on (a, c, e, and g) amplitudes (A) and (b, d, f, and h) linewidths (ΔB_{pp}) of the EPR spectra of the ointment containing 7% of propolis thermally treated at temperatures 30°C (a, b), 40°C (c, d), 50°C (e, f), and 60°C (g, h). M: microwave power used during the measurement of the EPR spectra; M_o: total microwave power produced by klystron (70 mW).

regeneration and led to inhibition of local inflammation, which indicates that treatment with the mentioned apitherapeutic was successful in the initiation of the burn healing, and accelerated the biosynthesis of collagen fibres (estimated by morphometry) in all the evaluated periods [25].

Anti-inflammatory, antimicrobial, and regenerating properties of the propolis ointment were also examined in our previous studies. We observed that, according to clinical and histopathological evaluation, propolis ointment accelerates regenerative and reconstructive processes and reduces wound healing time [26]. Furthermore, our previous experimental studies revealed that the apitherapeutic ointment accelerates the burnt tissue repair by stimulation of the glycosaminoglycan accumulation in the wound bed, needed for granulation, tissue growth, and wound closure. Moreover, our previously published studies showed that propolis accelerates chondroitin/dermatan sulfates structure modification responsible for binding growth factors that play a crucial role in the tissue repair process [27, 28]. And, last but not least, we also examined antimicrobial properties of propolis ointment. We observed that propolis topical formulation applied in burn wound treatment displayed higher antimicrobial efficacy than commonly used silver sulfadiazine which was demonstrated by significant reduction in microbial colonization as well as bactericidal properties against the isolated strains [29].

4. Conclusions

Free radicals were not found in the tested unheated propolis ointments of diamagnetic character. Electron paramagnetic resonance examination pointed out that all the thermally treated propolis ointments independently of the heating temperature contained free radicals (~10^{18}–10^{20} spin/g). Free radicals were responsible for paramagnetism of these samples and for their EPR spectra. The measured EPR spectra were homogeneously broadened lines. The EPR lines of the heated propolis ointments were not saturated up to the microwave power of 70 mW, which indicated fast spin-lattice interactions in the samples. The increase of free radical concentrations in the ointments containing propolis (5% and 7%) with increasing of the heating temperature was observed. Thermal formation of free radicals at temperature 60°C was considerably higher in the ointment containing 7% of propolis than in the sample containing 5% of propolis. Taking into account the values of free radical concentrations, it was concluded that both propolis ointments may be stored at the temperature of 30°C, but they should not be stored at higher temperatures 40°C, 50°C, and 60°C. The usefulness of EPR spectroscopy in optimizing storage temperature for the propolis ointments was confirmed.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This work was financially supported by Medical University of Silesia in Katowice, Grant no. KNW-1-023/N/6/O.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 6057650, 11 pages
http://dx.doi.org/10.1155/2016/6057650

Research Article

Comparative Study of Chemical Composition and Biological Activity of Yellow, Green, Brown, and Red Brazilian Propolis

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Received 29 March 2016; Accepted 16 June 2016

Academic Editor: Jose M. Prieto

The chemical composition and biological activity of a sample of yellow propolis from Mato Grosso do Sul, Brazil (EEP-Y MS), were investigated for the first time and compared with green, brown, and red types of Brazilian propolis and with a sample of yellow propolis from Cuba. Overall, EEP-Y MS had different qualitative chemical profiles, as well as different cytotoxic and antimicrobial activities when compared to the other types of propolis assessed in this study and it is a different chemotype of Brazilian propolis. Absence of phenolic compounds and the presence of mixtures of aliphatic compounds in yellow propolis were determined by analysing ^{1}H-NMR spectra and fifteen terpenes were identified by GC-MS. EEP-Y MS showed cytotoxic activity against human tumour strain OVCAR-8 but was not active against Gram-negative or Gram-positive bacteria. Our results confirm the difficulty of establishing a uniform quality standard for propolis from diverse geographical origins. The most appropriate pharmacological applications of yellow types of propolis must be further investigated.

1. Introduction

Propolis is a beehive product popularly used to treat or prevent several disorders such as wound infections and respiratory conditions [1]. The regular intake of propolis has been indicated in traditional medicine as a way to promote health and enhance human resistance to infections or malignant affections with no unwanted side effects.

Brazilian propolis was previously classified by Park et al. [2] who described twelve distinct groups of Brazilian propolis. Green propolis (type 12) from south-eastern Brazil is currently the most exported Brazilian propolis. Green propolis is rich in artepillin C and other prenylated phenolic compounds with potent antitumour properties [3]. A brown type of propolis is found in the southern regions of Brazil (states of Paraná and Santa Catarina) [1, 4]. Yellow propolis

samples from northeast and south Brazil were found by Park et al. [5] and classified in group 1 from southern region and groups 9 and 11 from northeast. In addition, yellow propolis from Cuba was described by Cuesta-Rubio et al. [6]. A 13th type of Brazilian propolis was later identified as the red propolis from northeastern Brazil [7]. Trusheva et al. [8] showed that this red propolis was rich in phenolics, triterpenoids, isoflavonoids, and prenylated benzophenones and a naphthoquinone epoxide was isolated for the first time from a natural source. Since then, red Brazilian propolis has been the target of further investigation by several research groups [6, 7, 9–13]. Sawaya et al. [4] have also carried out several studies seeking to classify the Brazilian propolis based on their ESI-MS fingerprints and chemometric multivariate analysis.

Despite the great number of studies about Brazilian propolis, the chemical composition of propolis from the central-western region of Brazil has been scarcely investigated [4, 19]. Neither the chemical composition nor the pharmacological properties of yellow Brazilian propolis have been described so far. In the current study, we investigated the chemical composition and cytotoxic and antimicrobial activities of a sample of yellow Brazilian propolis collected in the Pantanal ecosystem in Mato Grosso do Sul, Brazil. Moreover, four samples of typical Brazilian classes of propolis, green propolis from São Paulo (EEP-G SP), green propolis from Minas Gerais (EEP-G MG), red propolis from Bahia (EEP-R BA), brown propolis from Paraná (EEP-B PR), and a sample of yellow propolis from Cuba (EMP-Y Cuba), were included in the study, permitting the direct comparison with the chemical characteristics and pharmacological potency of yellow propolis.

2. Experimental

2.1. Propolis Samples and Extracts. Propolis was supplied by beekeepers from different states of Brazil: red propolis (Bahia), green propolis (São Paulo), yellow propolis (Mato Grosso do Sul), and brown propolis (Paraná). A sample of green propolis from the state of Minas Gerais was purchased in a local market. A sample of yellow propolis from Cuba was prepared as a methanol extract. All propolis samples were stored at −18°C until extraction. Ethanol extracts of propolis (EEP) were prepared using 10 g of each propolis samples mixed with 100 mL of absolute ethanol. The mixture was stirred for one day under controlled speed (160 rpm) at room temperature and then filtered. The filtrates were kept in freezer overnight (temperature of −18°C) and filtered again to remove waxes. Solvent from the extractive solutions was removed in a rotatory evaporator at 50°C to obtain the dry ethanol extracts of propolis (EEPs).

2.2. ^{1}H-NMR Analysis. Samples of EEPs for ^{1}H-NMR analysis were prepared as follow: a mixture of 100 μL D_2O (buffer phosphate pH 7.04) and 600 μL CD_3OD was added to 30 mg of propolis extracts and sonicated for 15 min. The mixture was centrifuged at 13000 rpm for 20 min at room temperature. The supernatant (600 μL) was transferred into an NMR tube of 5 mm. Sodium-3-trimethylsilyl-2,2,3,3-d_4 propionate (TMSP 0.324 mg/mL) was used as internal reference. All NMR experiments were recorded at 298 K in a Bruker UltraShielding™ Plus 600 MHz spectrometer operating at 14.6 T, equipped with a TCI cryoprobe for H-C/N-D. ^{1}H NMR spectra were acquired using a noesy pulse sequence for presaturation on water resonance and spoil gradient during mixing time (noesygppr1d, Bruker terminology). The parameters set in this sequence were 4.0 s for relaxation delay time, acquisition time of 3.99, data points of 140k, mixing time of 10 ms, and 128 scans with a spectral window of 30 ppm. Spectra were processed by applying an exponential line broadening LB of 0.3 and manually phased trough Topspin 3.0 (Bruker Biospin).

2.3. UPLC-ESI(−)-MS/MS. The EEPs were analysed on a UPLC Acquity Chromatographer (Waters, Milford, USA) coupled with a TQD Mass Spectrometer (Micromass-Waters Manchester, England), with an Electrospray source (ESI). Fingerprints were obtained by direct injection of 5 μL of extracts by flow infusion into the ESI source. Chromatographic separation was carried out in a C_{18} BEH Waters Acquity analytical column (50 mm × 2.1 mm i.d., 1.7 μm particle size) held at 30°C. A linear gradient elution was carried out with mili-Q purified water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.2 mL/min. Elution started with 95% of solvent A and 5% of solvent B. A linear gradient was performed until 100% of solvent B in 9 min and this condition was held for 1 min. Afterwards, the initial elution condition was reestablished in 2 min, totalizing 12 min for the chromatographic run. ESI(−)-MS and tandem ESI(−)-MS/MS were obtained under the following analytical conditions: capillary −3.5 kV, cone −30 V, source, and desolvation temperature were 150 and 350°C, respectively. For ESI(−)MS/MS, the energy for the collision induced dissociations (CID) was 25 V. Data were acquired in the m/z 100–700 range. Diagnostic ions in the different propolis samples were identified by the comparison of their ESI(−)MS/MS dissociation patterns with authentic analytical standards and/or by comparison with fragmentation pattern from the literature.

2.4. GC-EIMS. An aliquot of dry yellow propolis extracts was dissolved in ethyl acetate at a concentration of 500 μg/mL. Volume samples of 1 μL were injected in the splitless mode into an AGILENT gas chromatograph (model 7890A GC System), coupled with a mass spectrometer operating in EI mode at 70 eV. A 5% phenyl 95% dimethylpolysiloxane capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was held at 250°C for 1 min and then heated to 300°C at the rate of 5°C/min. The final temperature was maintained for 10 min. Helium was used as the carrier gas. Injector and detector temperature was 230°C and 150°C, respectively. Compounds were identified by searching against a database of mass spectra (NIST 2011 Mass Spectral Library, Agilent Technologies).

2.5. Cytotoxicity Assays. The cytotoxicity of propolis extracts was evaluated against four human tumour cell lines: OVCAR-8 (ovary carcinoma), HCT-116 (colorectal carcinoma), SF-295 (human glioblastoma), and LH-60 (promyelocytic leukemia) obtained from the National Cancer Institute (Bethesda, MD, USA). The general viability of cultured cells was determined by the reduction of the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product, as previously described by Mosmann [20]. The tumour cells were maintained in RPMI 1640 medium, supplemented with 10% foetal bovine serum, 1% penicillin, and streptomycin at 37°C with 5% CO_2. For all experiments, cells were seeded at 0.1×10^6 cells/mL (LH-60, OVCAR-8, and SF-295) and 0.7×10^5 cells/mL (HCT-116) and incubated during 72 h with propolis extracts at 37°C with 5% CO_2. After centrifugation and solution removing, MTT solution was added and the plates were incubated and centrifuged and the solids dissolved in pure and sterile DMSO. The absorbance was measured in a plate spectrophotometer DTX-800 (Beckman Coulter) at 595 nm. Investigation of the survival viability for nontumour cell line L929 (mouse fibroblast) was also carried out. DMSO (solvent) and doxorubicin (reference standard drug) were used as negative and positive controls, respectively.

2.6. Antibacterial Activity. Antibacterial activity was evaluated against the following standard strains: (i) Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538) and *Enterococcus faecalis* (ATCC 29212) (ii) bacteria Gram-negative: *Pseudomonas aeruginosa* (ATCC 25853) and *Escherichia coli* (ATCC 8739). All samples were obtained from INCQS/FIOCRUZ (National Institute of Quality Control in Health, Brazil). Furthermore, one clinic isolate, methicillin-resistant *Staphylococcus aureus* (MRSA), was also applied as test organism and obtained from cultures of patient samples existing in the Public Hospital (Bacteriological Laboratory) of Belém city, Pará, Brazil.

All bacteria were previously seeded in Petri plates containing Mueller Hinton agar (Merck, Germany) and incubated at 35°C for 24 hours. For bacterial inoculum preparation, strains were grown to exponential phase in Mueller Hinton broth (Merck, Germany) at 37°C for 24 h and adjusted by diluting fresh cultures to turbidity equivalent to 0.5 McFarland scale (approximately 2×10^8 CFU/mL) and then diluted until 1×10^3 CFU/mL, as described by Clinical and Laboratory Standards Institute [21]. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were performed using the broth microdilution method in MHB [21]. MIC is defined as the lowest concentration of extract with no visible growth of the microorganism in the resazurin colorimetric assay. To determine MIC, propolis extracts were dissolved in DMSO at the highest concentration (19000 μg/mL) to be tested. A serial twofold dilution was made in a concentration range from 100 to 19000 μg/mL in 1 mL sterile test tubes containing MHB. For the microdilution test, the inoculum (100 μL) containing 5×10^3 CFU/mL was added to each well and 100 μL was transferred into consecutive wells. After 24 h of incubation, 15 μL of resazurin (1 μg/mL), which was metabolically reduced by active cells to a colour derivative, was added to the wells to allow visual identification of metabolic activity [22]. After incubation, the development of a purple-pink colour was considered as the indicative of bacterial growth. Therefore, MIC was read as the lowest concentration of the extract where the purple-pink colour was not observed. To determine MBC, 10 μL of broth was taken from each well and incubated in Mueller Hinton agar at 37°C for 24 h and for each bacterium. The MBC was defined as the lowest extract concentration that resulted in a colony count lower than three colonies per mL (99.9% killing) or no bacterial growth, as described by de Quadros et al. [23]. Each test was performed in three replicates. Negative control consisted of 100 μL of the bacterial inoculum and 100 μL of DMSO. Chloramphenicol (50 μg/mL) and gentamicin (10 μg/mL) were used as positive controls for Gram-positive and Gram-negative bacteria, respectively.

2.7. Statistical Analysis. For chemometrics analysis of NMR a bucket table was created using AMIX Statistics software (version 3.9.7, Bruker Biospin). A bucket window of 0.04 ppm was choosen for spectral binning and residual water signal (4.90–5.00 ppm) and methanol (3.29–3.33 ppm) were excluded together with noise regions prior bucketing process. Principal component analysis (PCA) was used in order to compare the qualitative chemical composition of the diferent types of propolis extracts from several states of Brazil. Statistical differences between experimental types of propolis were verified by ANOVA followed by Fisher and Tukey test at 95% significance ($p < 0.05$).

3. Results and Discussion

3.1. Qualitative Comparison of the Chemical Composition of Propolis Extracts by ^{1}H NMR, ESI(−)-MS, and LC-ESI(−)-MS/MS. The qualitative profile of green, red, brown, and yellow propolis from different regions of Brazil and one yellow propolis from Cuba was evaluated first by ^{1}H NMR (Figure 1) and by ESI(−)-MS fingerprinting (Figure 2). The samples of Brazilian propolis showed clearly distinct ^{1}H NMR (Figure 1) and ESI(−)-MS (Figure 2) patterns, indicating different chemical composition. The most striking feature in the ^{1}H NMR spectrum of yellow propolis is the absence of signals from aromatic compounds and the most down field hydrogens are vinyl hydrogens at δ 5.0 to 6.0 ppm. This indicates the lack of abundance of phenolics compounds that are typically present in the green, brown, and red types of Brazilian propolis. In Figure 1, solvent signal (D_2O/CD_3OD) at 3.36–3.27 ppm and the signal for the internal reference TMSP were excluded from NMR spectra for better graphic quality.

Through ^{1}H-NMR technique, it is possible to verify the chemical class of only the most abundant compounds in the extracts, once NMR technique is quite susceptible to compound concentration. Chemometric analysis of ^{1}H NMR data clustered propolis samples into three groups (Figure 3): a group comprising the green propolis; a second group for

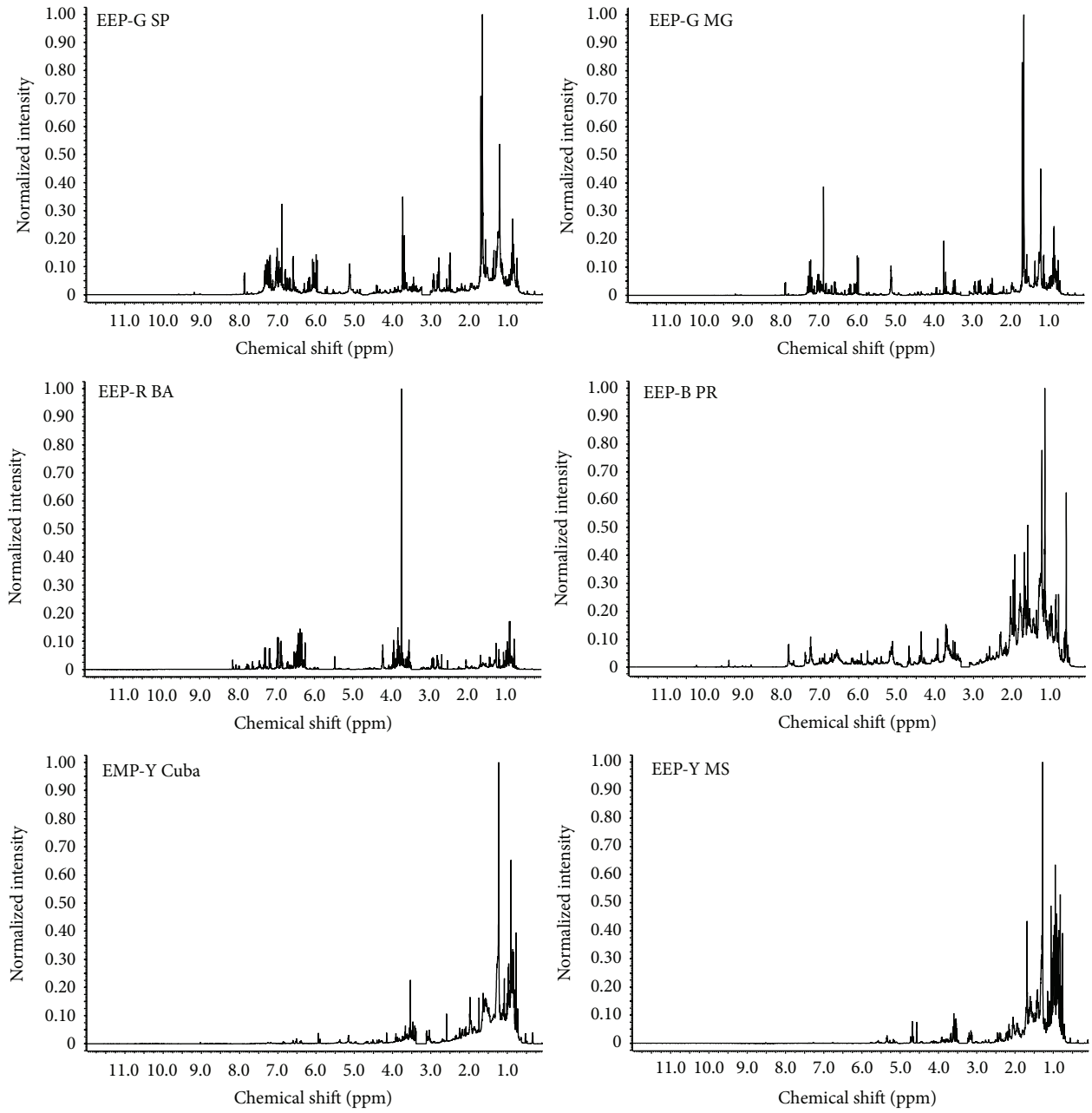

Figure 1: ^{1}H NMR (600 MHz, CD_3OD/D_2O) of propolis extracts (solvent signal at δ 3.36–3.27 and the signal for the internal reference TMSP were excluded from NMR spectra).

the brown and red propolis; and a third group for the yellow propolis. The Cuban and Brazilian yellow propolis were grouped due to the predominance of resonances at δ 0.7–1.1 ppm and δ 1.23–1.32 ppm characteristic of hydrogens bonded to Csp^3 in aliphatic compounds.

The six samples of propolis were also analysed by UPLC-ESI(−)-MS/MS to tentatively identify some of the ions observed by ESI(−)-MS fingerprinting (Figure 2). A total of twenty-nine known compounds were identified in green, red and brown Brazilian propolis and Cuban yellow propolis (Table 1). All these compounds have been previously identified in propolis and correspond to phenolic compounds, such as derivatives of benzoic and cinnamic acids, flavonoids and prenylated phenolic compounds, but labdanic terpenes were also found [3, 4].

3.2. Chemical Composition of Yellow Brazilian Propolis by GC-EIMS. The lipophilic nature of the constituents in yellow propolis made GC-MS the most suitable analytical technique to analyse the chemical composition of this type of propolis.

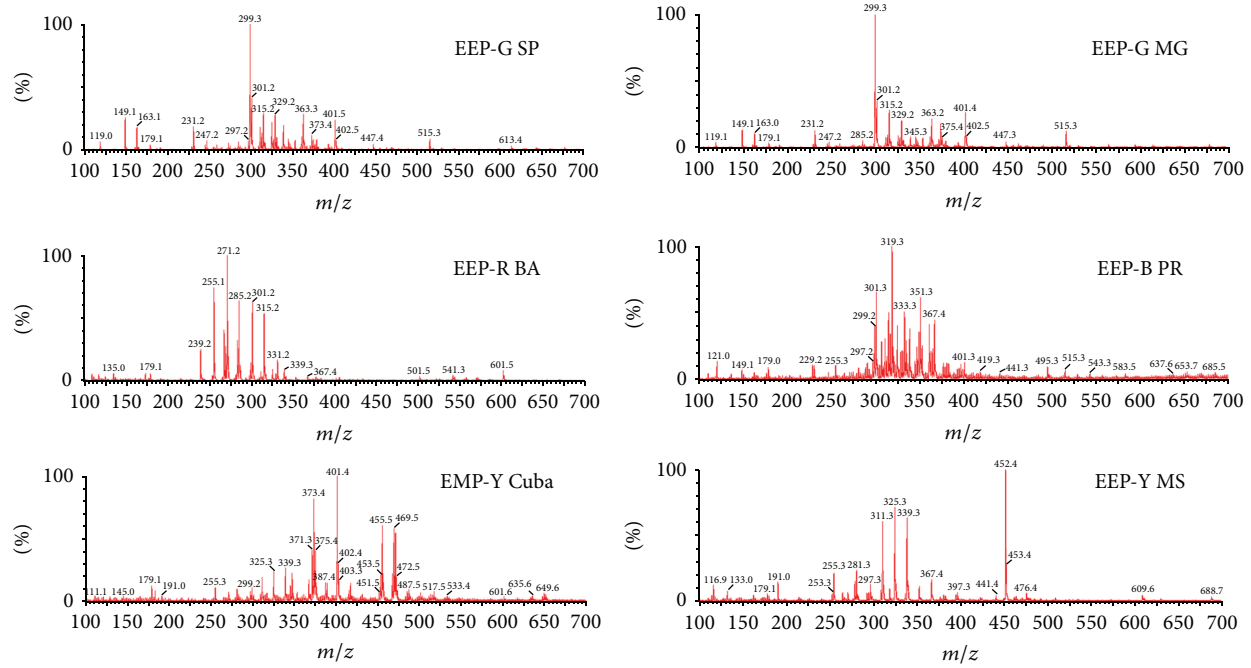

Figure 2: ESI(−)-MS fingerprints of propolis extracts.

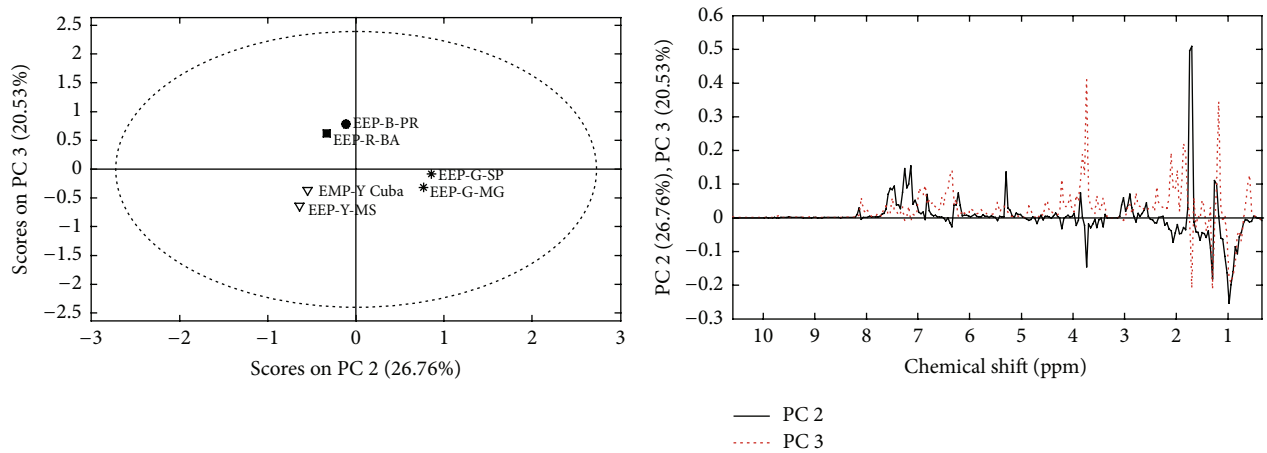

Figure 3: Principal component analysis of ^{1}H-NMR data for extracts of propolis.

A total of fifteen triterpenoids were tentatively identified in the extract of yellow propolis (Figure 4) through comparison of their fragmentation profile with data from the NIST library and the literature data. At least three triterpenoids were already reported for Brazilian type 6 propolis (red colour), such as β-amyrin, lupeol, and olean-12-en-3-one [8, 18, 24].

A study of the chemical composition of propolis produced in the state of Piauí (Brazil) resulted in the identification of six triterpenoids derived from cycloartane: isomangiferolic acid, mangiferolic acid, mangiferonic acid, ambonic acid, ambolic acid, and 24-methylene cycloartane-3β,26-diol. The authors mentioned that these compounds were previously isolated from the stem bark of species of *Mangifera indica* (Anacardiaceae) and suggested that this species could be the source of propolis from Piauí. Pentacyclic triterpenoids, such as lupeol, α-amyrin and β-amyrin, and tetracyclic cycloartenol type, have shown significant anti-inflammatory activity [25].

Hernández et al. [26] reported a quali-quantitative GC-MS study of 19 samples of yellow Cuban propolis. Yellow Cuban propolis comprised two major groups: type A, rich in triterpenic alcohols and with the presence of polymethoxylated flavonoids as minor constituents, and type B, containing acetyl triterpenes as the main constituents [6]. Through GC-MS compounds of low polarity were identified such as lanosterol, β-amyrone, β-amyrin, germanicol, lupeol, cycloartenol,

Table 1: Propolis constituents identified by UPLC-ESI(−)-MS/MS.

m/z $[M\text{-}H]^-$	Compound	Propolis origin	ESI(−)-MS/MS m/z (relative intensity%)	Reference
151		SP	150 (100); 147 (65); 127 (10); 121 (15)	
163	*p*-Coumaric acid	SP, Cuba, MG, PR	163 (20); 119 (100)	a, [4, 14, 15]
179	Caffeic acid	SP, MG, PR	179 (40); 135 (100); 116 (50)	[14]
229	2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopyrane	SP, MG	185 (100); 168.9 (50); 146 (20)	a, [4]
231	4-Hydroxy-3-prenylcinnamic acid	SP, MG, PR	231 (15); 187 (30); 132 (100)	a, [4, 15, 16]
233	Viscidone	SP, MG, PR	233 (25); 188 (100); 133 (50); 132 (85)	a
247	3,4-Dihydroxy-5-prenylcinnamic acid	SP, MG, PR	247 (40); 203 (60); 148 (100)	a, [16]
253	2-Hydroxy-4-methoxychalcone	BA	253 (80); 237 (95); 209 (100); 161 (70); 136 (92)	[17]
253		SP, MG	220 (5); 162 (20), 145 (30); 118 (100)	
255	Liquiritigenin	BA	255 (35); 135 (40); 119 (100)	[7, 9, 17, 18]
255		BA, Cuba	255 (45); 135 (35); 119 (100)	
255		MS	254 (52); 209 (100); 191 (50); 153 (62); 123 (62); 109 (61)	
285	(3*S*)-Vestitone	BA, MG	285 (25); 269 (33); 147 (18); 109 (100)	[18]
285	Pinobanksin-5-methyl-ether	SP, MG	285 (100); 252 (30); 163 (45); 151 (35); 136 (50)	[14]
285	Kaempferol	Cuba, BA	285 (83); 149 (100); 122 (85)	[14]
297		BA	267 (85); 205 (100)	
299		BA	299 (40); 284 (100)	
299	Luteolin-methyl-ether	BA	299 (40); 284 (100); 255 (25); 227 (30)	[14, 15]
299	Kaempferide	SP, Cuba, PR	299 (100); 284 (90); 200 (30); 151 (23)	[15, 16]
299	Artepillin C	SP, MG, PR	299 (90); 255 (100); 200 (97)	a, [4, 16]
301	Dihydrokaempferide	BA, SP, Cuba, MG, PR	301 (100); 283 (5); 152 (30)	a, [16]
315	(3*S*)-Violanone	BA	315 (20); 108 (100)	[18]
315	Quercetin-3-methyl-ether	BA	315 (80); 300 (55); 271 (50); 243 (100); 165 (48)	a, [14]
315	Isorhamnetin	SP, MG, PR	315 (100); 284 (20); 252 (30)	a, [15]
315	(3-4-Hydroxy-3-methyl-2-butenyl-4-hydroxy-5-prenyl) cinnamic acid	SP, MG	315 (100); 253 (90); 241 (88); 198 (98)	a
315	3-Hydroxy-2,2-dimethyl-8-prenylchromane-6-propenoic acid	SP, MG	315 (75); 271 (25); 253 (20); 244 (85); 198 (100); 146 (45)	[15]
317		BA	317 (20); 270 (23); 166 (48); 109 (100)	
317		SP, MG	317 (25); 314 (50); 241 (100); 124 (73)	
317		SP, MG	317 (10); 242 (40); 200 (100)	
317		SP	317 (45); 273 (55); 160 (100)	
317		MG	317 (40); 312 (100); 253 (50); 147 (80)	
319		BA, PR	301 (100); 193 (25)	
319		MS	319 (20); 223 (100);	
329		BA	329 (30); 314 (55); 299 (100); 285 (35); 271 (100); 182 (60)	
329	Quercetin-dimethyl-ether	SP, Cuba, MG, PR	229 (100); 314 (70); 299 (42); 270 (80); 227 (25)	[14]
329	Betuletol	SP, MG	329 (85); 314 (45); 299 (100); 270 (85); 257 (90) 198 (50); 160 (35)	a
333	Agathic acid	SP, MG, PR	333 (45); 314 (40); 257 (30); 245 (35)	a

Table 1: Continued.

m/z [M-H]$^-$	Compound	Propolis origin	ESI(−)-MS/MS m/z (relative intensity%)	Reference
347	Agathic acid 15-methyl ester	SP	247 (70); 259 (20); 187 (100); 163 (60); 146 (80)	a
353		Cuba, MS	353 (40); 335 (45); 151 (40); 112 (50)	
361	15-Acetoxy cupressic acid	MG, SP	361 (100); 317 (70); 242 (75); 159 (45); 126 (72)	a
363	3-Prenyl-4-dihydrocinnamoyloxy cinnamic acid	SP, MG	363 (20); 187 (80); 149 (100)	a, [15]
447	(*E*)-3-{-4-hydroxy-3-[(*E*)-4-(2,3-dihydrocinnamoyl oxy)-3-methyl-2-butenyl]-5-prenylphenyl}-2-propenoic acid	SP, MG, PR	447 (10); 297 (50); 197 (15); 149 (100)	a, [16]
515	3,5-di-*O*-caffeoylquinic acid	SP, MG, PR	515 (25); 353 (100); 173 (30)	a, [4, 15]

[a]Identification based on comparison with authentic analytical standards.

β-amyrin acetate, 24-methylene-9,19-ciclolanostan-3β-ol, α-amyrin acetate, and lupeol acetate [26]. In our current report, all these compounds were found in the sample of yellow propolis from Pantanal (Mato Grosso do Sul, Brazil) demonstrating a similar chemical profile for the Cuban and Brazilian yellow propolis samples, which consequently formed a group in PCA (Figure 3).

3.3. In Vitro Cytotoxicity Assays. In preliminary screening of cytotoxic activity, the percentage of inhibition on three tumour cell lines was measured for each type of propolis (data not shown). Green samples (EEP-G SP and EEP-G MG) had low inhibition of all studied tumour cell lines, presenting low cytotoxic potential. Brown (EEP-B PR) and red (EEP-R BA) propolis had the highest cytotoxic potential with inhibition percentages greater than 75% in at least two tumour cell lines. Yellow propolis from Brazil (EEP-Y MS) showed high cytotoxic activity only against OVCAR-8 tumour cell, thus showing greater specificity against ovarian carcinoma.

Only extracts of red, brown, and yellow propolis, which showed inhibition percentages above 75% in at least one tumour cell, were considered for further experiments. An analytical curve obtained by linear regression, varying the concentration of extract of propolis and measuring inhibition of cell proliferation, enabled the calculation of the concentration required for each extract to inhibit 50% (IC_{50}) of tumour cells OVCAR-8 (ovary carcinoma), HCT-116 (colorectal carcinoma), SF-295 (human glioblastoma), and LH-60 (promyelocytic leukemia) (Table 2). The highest concentration tested for each extract was 100 μg/mL.

According to criteria established by the National Cancer Institute (NCI, USA), the IC_{50} threshold for extracts with promising cytotoxic activity value is 30 μg/mL. The results showed that yellow propolis contains substances with cytotoxic effects. Brown propolis was effective against the four cell lines studied. To evaluate the degree of selectivity against tumour cell lines, IC_{50} was also investigated against the non-tumour cell line L929 (mouse fibroblast) and a selective index (SI) was calculated as IC_{50} (nontumour cell)/IC_{50} (tumour cell). The highest selectivity against all tumour cells was shown by red propolis especially against LH-60 (leukemia promyelocytic). da Silva Frozza et al. [17] also found that red propolis had selective cytotoxic activity for tumour cell lines. Yellow propolis had selective indexes between 0.91 and 1.84; however, only values of SI $\geq$ 2 are considered significant [27]. Therefore, our results suggest that brown and red propolis and, to a lesser extent, yellow propolis may act in a selective way against tumour cells and show potential antitumour activity. We previously demonstrated that propolis extracts showed an *in vivo* antitumour activity in the experimental model Sarcoma 180 tumour cells with moderate toxicity effect at experimental exposure levels when compared to 5-FU [16]. Propolis has been a subject of intensive research, especially in the area of cancer, and its selectivity *in vivo* against tumours must be further investigated.

3.4. Antibacterial Activity. Samples of green (EEP-G MG and EEP-G SP) and red propolis (EEP-R BA) had high antibacterial activity against Gram-positive bacteria, mainly *S. aureus* (Table 3). Particularly, EEP-G SP showed excellent activity against *S. aureus* (MIC = 159 μg/mL), *E. faecalis* (MIC = 310 μg/mL), and MRSA isolate (MIC = 630 μg/mL).

As previously reported, the antimicrobial activity and chemical composition of propolis are directly associated with geographical location, biodiversity, bee species, and method and time of harvest [28, 29]. The high antibacterial activity of propolis against Gram-positive bacteria, mainly strains of *S. aureus* and *Enterococcus* sp., has been widely described [29–31]. However, previous studies have shown a wide variation in MIC and MBC values [29, 31]. Up to now, there are few studies on the antimicrobial activity of green and red propolis. A study of Brazilian red propolis from state of Alagoas reported that red propolis inhibited *Streptococcus pyogenes* and various Gram-negative bacteria, such as *P. aeruginosa* and *E. coli* with MIC values of 256 to 512 μg/mL [18]. Another study about Brazilian red propolis from the state of Alagoas reported MICs values of 50 to 100 μg/mL against *S. aureus* ATCC 25923 and *Streptococcus mutans* UA159 [10]. Regarding green propolis types, a propolis sample collected in São Paulo state inhibited *S. mutans* at concentrations

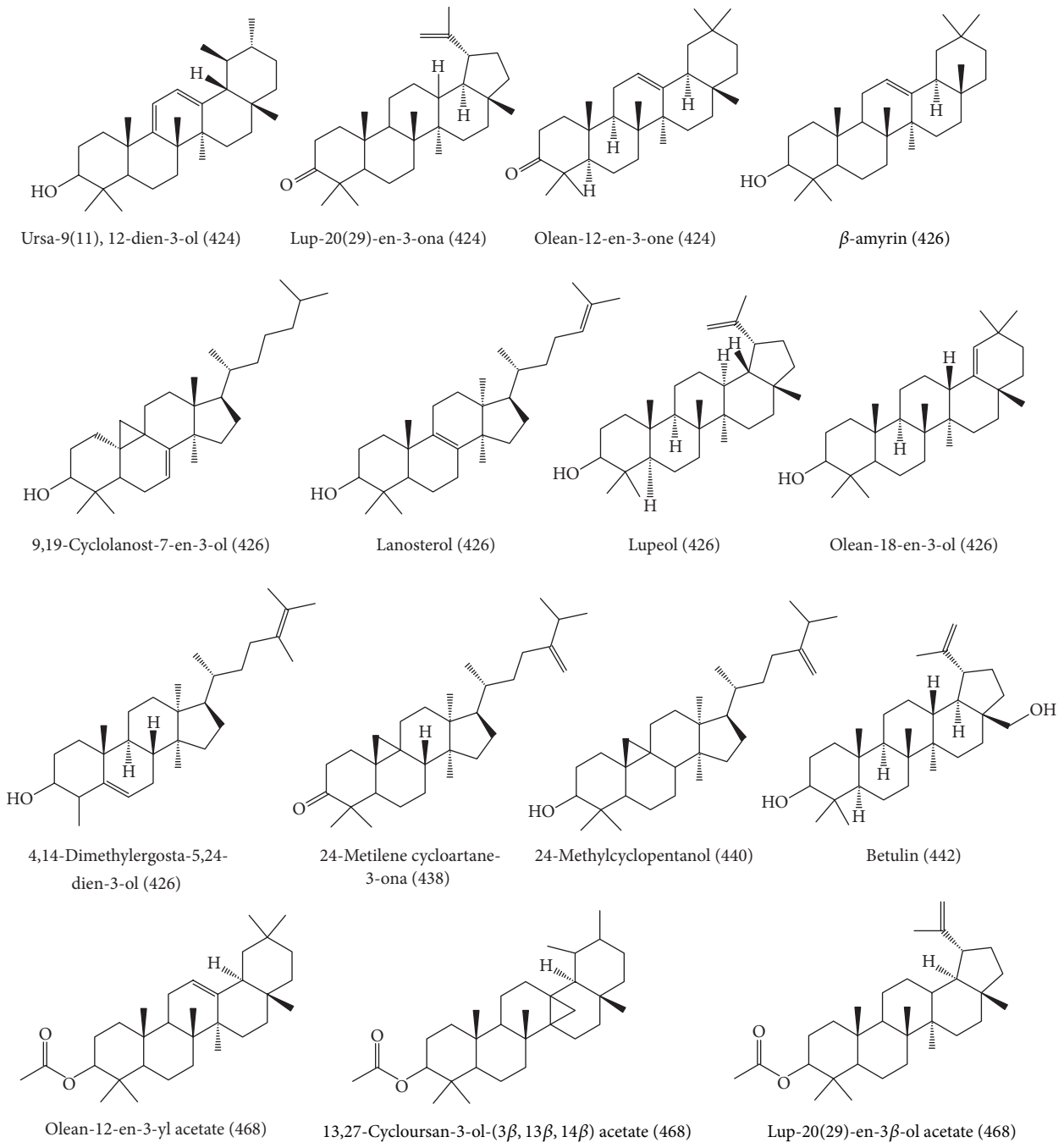

Figure 4: Compounds identified by GC-EIMS in the yellow propolis from Brazil.

of 400 μg/mL [32]. If compared with previous studies, our results showed that green propolis from São Paulo and Minas Gerais states was more effective at inhibiting Gram-positive bacteria, with MIC values of 159 to 310 μg/mL, whereas Brazilian red propolis from Bahia state showed antibacterial action similar to other studies published. The higher antibacterial activity expressed by Brazilian green propolis may be attributed to its different chemical composition and its high concentration in flavonoids and aromatic acids such as galangin, kaempferol, pinostrobin, and pinocembrin which have shown high antibacterial effect, as reported by Grenho et al. [33]. In addition, Scazzocchio et al. also reported that some constituents such as flavonoids (kaempferide, quercetin, galangin, and pinocembrin), caffeic, benzoic, and 4-hydroxy-3,5-diprenylcinnamic acids may probably act by the inhibition of bacterial RNA polymerase but may also act on the local microbial membrane or cell wall, causing structural and functional damage, resulting in antimicrobial action [30].

On the other hand, our data showed that the two yellow propolis samples (EEP-Y MS and EMP-Y Cuba) were not active against Gram-negative bacteria or Gram-positive

Table 2: IC_{50} (μg/mL) and selectivity index (SI) for brown, red, and yellow propolis extracts against a panel of tumour cells and against a normal cell L929.

	Tumour cells								Nontumour cells
	SF-295		HCT-116		OVCAR-8		HL-60		L929
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	
EEP-BPR	27.91 (26.88–28.98)	7.77	19.43 (16.52–22.84)	11.17	26.97 (25.81–28.19)	8.05	9.44 (8.67–10.28)	22.99	217 (101.9–461.8)
EEP-RBA	70.66 (9.06–551.0)	20.20	42.53 (14.93–121.2)	20.20	53.46 (44.30–64.52)	16.07	17.48 (14.66–20.85)	49.15	859.1 (137.5–5368)
EM-YMS*	16.4 (11.6–23.1)	1.84	43.73 (32–59)	0.69	20.67 (18.7–38)	1.46	31.56 (22.4–44.4)	0.95	30.11 (23.45–38.66)
EEP-YMS	34.77 (25–48.3)	0.91	>50	—	17.9 (17.63–52.65)	1.70	>50	—	31.58 (21.29–46.87)
Dox	0.145 (0.127–0.162)	2.71	0.063 (0.046–0.081)	6.03	0.26 (0.16–0.30)	1.46	0.011 (0)	34.54	0.38 (0.28–0.48)

*Only for yellow propolis an extract was also obtained with methanol as extractive solvent.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for different extracts of propolis.

Propolis	Bacteria, MIC and MBC/(μg/mL)									
	Staphylococcus aureus		Methicillin-resistant *S. aureus (MRSA)*		*Enterococcus faecalis*		*Pseudomonas aeruginosa*		*Escherichia coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
EEP-Y MS	>12,800	>12,800	>12,800	>12,800	>12,800	>12,800	6,400	>12,800	>12,800	>12,800
EMP-Y Cuba	>10,500	>10,500	>10,500	>10,500	5,020	10,500	5,020	6,400	>10,500	>10,500
EEP-B PR	>19,900	>19,900	>19,900	>19,900	860	>1,730	>13,900	>13,900	>13,900	>13,900
EEP-G MG	200	400	400	>400	400	800	6,450	12,900	6,450	12,900
EEP-G SP	159	315	630	>630	310	>630	10,110	20,220	10,110	20,220
EEP-R BA	390	780	780	>780	780	1,570	6,300	12,600	6,300	12,600
Chloramphenicol	50	>50	>50	>50	50	>50	—	—	—	—
Gentamicin	—	—	—	—	—	—	4	10	10	10
DMSO	>19,900	>19,900	>19,900	>19,900	>19,900	>19,900	>19,900	>19,900	>19,900	>19,900

bacteria, with MIC values above 5020 μg/mL. So far, there are no previous reports on the chemical composition of yellow propolis from Brazil and reports about their biological activities are also scarce. Park et al. [5] reported that samples of yellow propolis from Brazil were not active (or weakly active) against *S. aureus*. Additionally, yellow propolis samples had low antioxidant and anti-inflammatory activities. On the other hand, a previous report about yellow propolis from Cuba showed that extracts were able to inhibit 50% of *S. aureus* growth at low concentration, but no activity was observed against *E. coli* [34]. These yellow propolis samples from Cuba were abundant in triterpenoids and had a small proportion of phenolics and flavonoids if compared with green and red propolis.

4. Conclusions

The chemical composition of a yellow propolis from Mato Grosso do Sul/Brazil was analysed and its *in vitro* biological activity was assessed for the first time. This sample is rich in triterpenes and presents a different qualitative profile from other well-known types of Brazilian propolis. Yellow propolis showed cytotoxic activity against human ovarian carcinoma but was not active against Gram-negative or Gram-positive bacteria. Our results indicate that the Brazilian brown, red, and, to a lesser extent, yellow propolis inhibited, in a selective way, the growth of tumour cells and therefore show potential for anticancer therapy. Brazilian green propolis showed better antibacterial action, mainly against *S. aureus* and one multidrug-resistant clinical isolate (MRSA).

The results of the present study expand the knowledge about the chemical composition and biological activities of different chemotypes of propolis from Brazil, showing its variability and difficulty of standardization. Additionally, there is a need to investigate the most appropriate pharmacological applications for the yellow type of propolis due to its unique composition when compared to other types of Brazilian propolis.

Competing Interests

The authors declare no conflict of interests regarding the publication of this paper.

Acknowledgments

Financial support was provided by Finep (Fundo Paraná). Christiane Schineider Machado thanks CAPES and João Benhur Mokochinski thanks FAPESP (Grant no. 2013/21306-0) for doctoral fellowships. Alexandra Christine Helena Frankland Sawaya wishes to thank FAPESP and CNPq for the financial support. Authors also thank beekeepers who kindly supplied propolis samples.

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Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2016, Article ID 8473937, 12 pages
http://dx.doi.org/10.1155/2016/8473937

Research Article

Bee Pollen as a Promising Agent in the Burn Wounds Treatment

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Received 10 February 2016; Revised 18 April 2016; Accepted 24 April 2016

Academic Editor: José Maurício Sforcin

The aim of the present study was to visualize the benefits and advantages derived from preparations based on extracts of bee pollen as compared to pharmaceuticals commonly used in the treatment of burns. The bee pollen ointment was applied for the first time in topical burn treatment. Experimental burn wounds were inflicted on two white, domestic pigs. Clinical, histopathological, and microbiological assessment of specimens from burn wounds, inflicted on polish domestic pigs, treated with silver sulfadiazine or bee pollen ointment, was done. The comparative material was constituted by either tissues obtained from wounds treated with physiological saline or tissues obtained from wounds which were untreated. Clinical and histopathological evaluation showed that applied apitherapeutic agent reduces the healing time of burn wounds and positively affects the general condition of the animals. Moreover the used natural preparation proved to be highly effective antimicrobial agent, which was reflected in a reduction of the number of microorganisms in quantitative research and bactericidal activity of isolated strains. On the basis of the obtained bacteriological analysis, it may be concluded that the applied bee pollen ointment may affect the wound healing process of burn wounds, preventing infection of the newly formed tissue.

1. Introduction

Wound healing, being the result of dynamic cooperation between many molecular factors, is a dynamic reaction whose undisturbed course enables restoring the continuity and functionality of damaged skin [1–3]. The process consists of 4 specific phases which smoothly proceed and change from one to the other even coexisting at times. The duration period of particular healing phases may vary depending on the type of the damage and possible coexistence of interfering additional factors, that is, the size and place of the damage, blood supply of the wound edges, cleanness of the wound, the degree of microbiological contamination, presence of necrotic tissue, and properly conducted healing management [1, 2, 4, 5].

The therapy of burn wounds may be properly conducted either by applying surgical methods or by topical application of therapeutic preparations. Besides contemporary, conventional treatment methods of thermal skin damage, apitherapy, which uses the therapeutic effect of standardized, pharmacologically active fractions obtained from bee products, is becoming more and more noticeable. Apitherapeutic agents have a beneficial effect on the skin condition, due to the reduction of water loss, and influence the reconstruction of

the lipid barrier. One of the most frequently used apitherapeutic agents is bee pollen. This is a varied, natural product which is rich in such biologically active substances as amino acids, fatty acids, phytosterols, phospholipids, nucleic acids, carbohydrates, vitamins, mineral substances, enzymes, and coenzymes as well as phenolic compounds including phenolic acids and flavonoids [6–9]. The plethora of biologically active substances gives this natural raw material significant biotic properties such as antimicrobial, anti-inflammatory, immunomodulatory, or antioxidative activity [7, 10].

Such a high efficiency of this natural bee product with a significantly low risk of adverse reactions makes bee pollen a potentially optimal remedial factor in the therapy of local burn wounds [11, 12]. Therefore, the subject of this study was the assessment of efficiency and therapeutic usability of the bee pollen which has not been studied before.

Bee pollen, also flower pollen, is male reproductive organs produced by flowers of entomophilous plants. It is collected by worker bees, transported, and stored in beehives. It constitutes a basic ingredient in bee's nutrition used for current needs or stored for later period [13]. Bee pollen results from agglutination of flower pollen, nectar or honey, and bee's salivary substances [14].

Bee pollen treatment of topical, thermal damage of the skin was compared with the commonly applied pharmaceutical preparation such as silver sulfadiazine (SSD), which has many side effects.

AgSD not only may be responsible for the development of argyria and dysfunctions of liver, spleen, and kidney due to systemic accumulation of silver or determined by sulphadiazine presence, dermatitis, erythema multiforme rashes, and acute hemolytic anemia but also unfortunately could be responsible for cytotoxic effect on fibroblasts and keratinocytes [15, 16].

The clinical assessment of the treatment process of burn wounds was conducted. It concerned wound pathomorphology including the extent and depth of the burn, wound maceration, occurrence, and character of the exudate as well as the process of scar formation. The histopathological assessment of the burn wound epithelialization of the dynamics was done together with qualitative and quantitative assessment of particular microorganisms in tissue samples collected from beds of experimental burn wounds.

2. Material and Methods

2.1. Therapeutic Agents. The following therapeutic preparations were used: 1% silver sulfadiazine (SSD) (Lek, Poland), 0.9% NaCl (Polpharma), and bee pollen formulation. The analyzed bee pollen came from the apiary "Barć" in Kamienna. These are clean and ecological regions of Poland. In this apiary the European Dark Bee also known as Western Honey Bee is bred. The pollen was a composition of many pollens of various plants. Taking into account the location of the apiary, the dominating pollens came from such plants as oilseed rape (lemon-yellow color), shamrock (brown color), coltsfoot (bright yellow), common dandelion (bright orange), linden (bright green), or heather (red-yellow). Macroscopically, it was a multicolor blend of granules which were ground. 50 g of the ground pollen was added to 500 mL of 70% ethanol. The extraction of the solution was conducted for 4 weeks at room temperature. After that period, the solution underwent microfiltration. Next, the ethanol was distilled with vacuum evaporator. The result was dry matter which was used to prepare the bee pollen formulation (ointment containing 5% bee pollen ethanolic extract and 95% of petroleum jelly (according to Polish norm PN-R-78893)). The procedure was performed under general anesthesia according to the dosage regimen: atropine sulfate, 0.05 mg/kg body weight (Polfa Warszawa); ketamine hydrochloride, 3 mg/kg body weight (Biovet, Puławy); xylazine hydrochloride, 1 mg/kg body weight (Sandoz GMBH). Silver sulfadiazine was used in order to prolong the analgesic effect, 5 mg/kg body weight (Polpharma).

2.2. Tissue Material. The study protocol was approved by the Ethics Committee of the Medical University of Silesia. Two, 16-week-old, domestic pigs have been chosen as the useful experimental animals for the evaluation of wound repair because of many similarities of pig skin to human one. The usage of the limited number of experimental animals was consistent with validated animal model developed by Hoekstra et al. [17] in modification of Brans et al. [18]. The last mentioned pig model is based on the application of one experimental animal [18]. Moreover, in accordance with the guidelines of good laboratory practice for animal testing, the established principle is to use the minimum number of animals necessary to arrive at scientifically robust data and to ensure reliable data. Thus, the animals used in our study were bred and selected for the highest degree of genetic purity. This form of breeding purpose prevents genetic contamination and allows minimizing the number of animals necessary for the experiment, with very reliable results to be obtained.

Pigs were housed according to G.L.P. standards of Polish Veterinary Law. Each animal was inflicted with 18 skin burn wounds with equal gaps (9 wounds on each side along the line of the backbone). The size of each wound was identical, 1.5 cm × 3 cm. Totally, the wounds took about 2% of the surface of each animal's body subject to the experiment.

Burns were classified as 2nd-degree deep partial thickness burns. Animals were divided into two groups: control (C) and experimental ones (E). 36 dermal burns were inflicted. The wounds of animals in the control group were either untreated (subgroup C1) or treated with physiologic saline (subgroup C2).

The postburn wounds of the experimental group were also treated with SSD (subgroup E1) and with the bee pollen containing ointment (subgroup E2). The wounds in question were treated with mentioned substances twice a day, starting on the first day of the experiment. Three replications of biopsies were taken from the same wound of each animal, using surgical knife. Occlusive dressings were applied every 12 hours in all animals of all subgroups.

2.3. Clinical Study. Clinical observation was to assess the extent and depth of the burn, its maceration, and presence

of necrotic tissue in it. Macroscopic reading of pathomorphological picture of the wound considered occurrence and intensification of typical symptoms of burn wounds: erythema, swelling, exudate, bleeding, and eschar. The process of granulation tissue formation together with the course of scar formation, ongoing on the burn wound surface, was also assessed.

2.4. Histopathological Study. The process of granulation, the type of the granulation tissue, intensification of swelling around the burn angiogenesis, and possible scarring of the wound were assessed. The microscopic picture of skin preparations included degree of the damage in the area and near the wound as well as the repair processes in next stages of the observation. Histopathological studies concerned the samples which were collected from burn wounds and from the adjacent, unchanged tissue in general anesthesia on 0, 3rd, 5th, 10th, 15th, and 21st day from the moment of inflicting the burn. After consolidation, tissues samples were collected form skin specimen in order to make histopathological preparations. The basic slides with samples were stained to achieve optical differentiation and verification of the elements of cell structure. Two different kinds of dyes were used: hematoxylin and eosin. Two histopathological preparations, which resulted from that process, underwent the microscopic assessment.

2.5. Microbiological Study. Microbiological study was performed from the material collected from the burn wounds on 0, 3rd, 5th, 10th, 15th, and 21st day of the experiment. In the case of quantitative study, the material was collected with a sterile swab stick from the burn wound surface of $1\,cm^2$ and was subsequently put into the $10\,cm^3$ of a sterile solution of 0.9% NaCl. This suspension of microorganisms served as the basis for a series of dilutions. Then, a $1\,cm^3$ of the suspension was collected and spilt on the slide and dissolved in both the Mueller Hinton agar (MH), in order to assess the amount of bacteria, and Sabouraud agar, in order to assess the amount of fungi and mould. The material to microbiological purity test of the skin was simultaneously collected from the places where the burns were not inflicted. In the case of quantitative studies, the material was collected with AMIES transport medium with active carbon (HAGAMED, Poland), which was stored at 5°C up to the moment of performing microbiological tests (max. up to 2 hours). Simultaneously, the samples for microbiological purity test were collected from the skin of animals not taking part in the experiment. Microbiological diagnosis was conducted in accordance with the standards of National Committee for Clinical and Laboratory Standards [19]. The cultures were conducted on the following enrichment and differential media such as liquid media (Carbohydrate broth, an enrichment medium for aerobic bacteria) and solid media (blood agar, to enrich aerobic microorganisms and characterize the type of hemolysis; Mannitol Salt Agar (Chapman), to differentiate *Staphylococcus* spp.; MacConkey Agar, to differentiate *Enterobacteriaceae* species; Sabouraud Agar, to identify fungi; Agar D-Coccosel, to identify *Enterococcus faecalis*; Cetrimide Agar, to identify *Pseudomonas* spp.). The identification of isolated bacteria species was conducted by microscopic tests, culture tests, and commercial biochemical test API (bioMerieux, France). The growth promotion test was carried out with reference strains. The next stage of the test was to assess the amount of bacteria on $1\,cm^2$ of the burn wound surface. Therefore, the material was collected from $1\,cm^2$ of the wound which was then shaken in $10\,cm^3$ of the sterile solution of physiological saline.

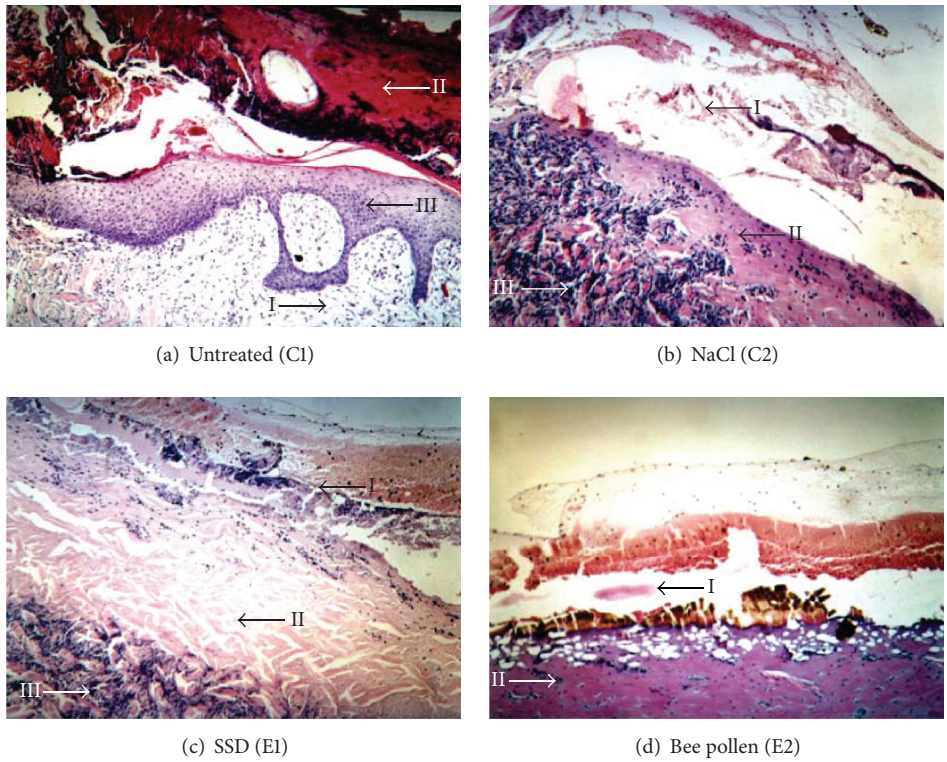
2.6. Data Analysis. In addition to the analytical methods mentioned above, the automatic measurement of the time constants was proposed. They concern the change rate analysis of the number of bacteria, fungi, or moulds in the wound. Therefore, the electrical-analog method, the inertial first-order object with delay, was suggested. For such a proposed model, the time constants for particular groups C1, C2, E1, and E2 were measured.

The microbiological data analysis was performed using Statistica 7.0 package (StatSoft, Cracov, Poland). The normality of distribution was verified with Kolmogorov-Smirnov test. Statistical differences between variables were verified by analysis of variance (ANOVA), followed by post hoc NIR test.

3. Results

3.1. Clinical Test Results. The clinical view of the wounds was compared on 3rd, 5th, 10th, 15th, and 21st day after burn infliction.

Differences in the clinical view of healing wounds were noticed on the 5th day of the observation. In the control subgroups, untreated wounds (C1), wounds treated with 0.9% NaCl solution (C2), and the study subgroup (E1) in which the wounds were treated with silver sulfadiazine, the erythema was observed to exceed the area of the burn wound. The skin surrounding the wound was very swollen with visible exudate. In the case of the wounds treated with the ointment with a 5% bee pollen, the subgroup (E2), the area of the wound was covered with a thin, flexible eschar accompanied by bleeding. On 10th and 15th day of the experiment, the untreated wound, in the control subgroup (C1), was covered with a hard, dry, and cracked eschar strongly adhering in the center. Under the eschar, there was a pink granulation tissue. During the same days, in the control subgroup, treated with 0.9% NaCl solution (C2), the burn wound was covered with a softened eschar with a small amount of serosanguineous exudate. In the experimental subgroup (E1) treated with silver sulfadiazine, the area of the wound was covered with a hard eschar and there was an erythema. The burn wounds of subgroup (E2), treated with the bee pollen ointment on the 10th day, were covered with a thin, flexible eschar with a visible granulation, while, on the 15th day, there was a clear epithelium being formed. The area of the wound decreased. The tissues surrounding the wound were characterized by a weak, atrophic inflammatory condition. On the 21st day of the observation, the clinical view was still significantly differentiated. In subgroup (C1) the untreated wounds were covered with a dry, cracked eschar. In subgroup (C2),



(a) Untreated (C1)

(b) NaCl (C2)

(c) SSD (E1)

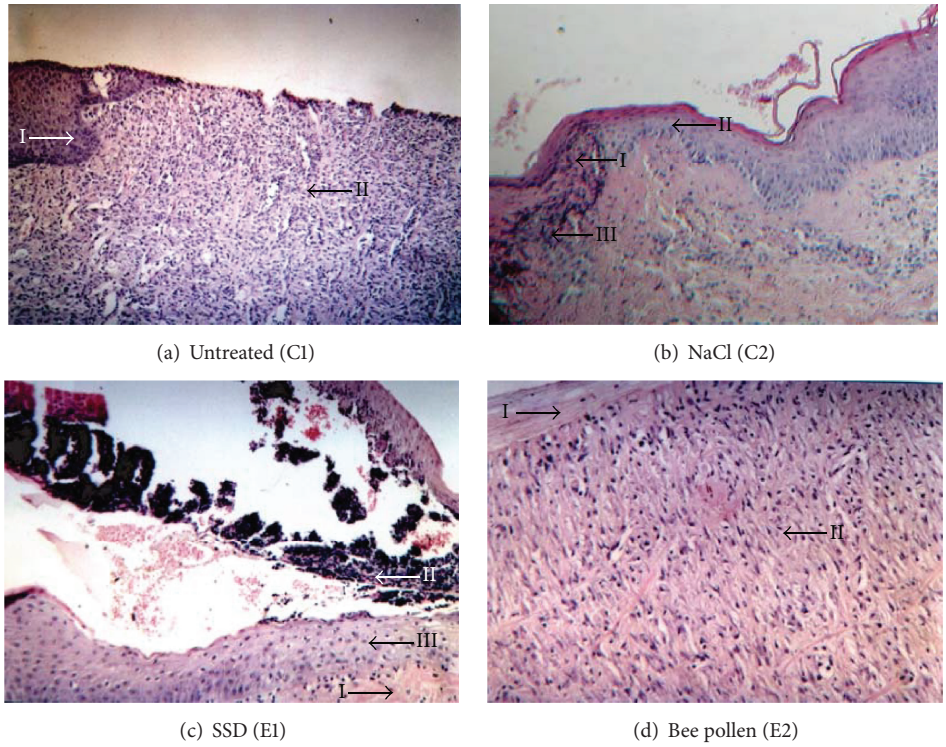
(d) Bee pollen (E2)

Figure 1: The picture of microscopic changes of skin samples collected from burn wounds on the 10th day of the experiment: (a) untreated (I: swollen inflammatory granulation tissue in the area of dermis, II: eschar with a slight bleeding, and III: visible, pink, and swollen granulation tissue); (b) washed with NaCl (I: petechial hemorrhages, loss of stratified squamous epithelium, II: coagulative necrosis, and III: massive lymphocytic infiltration); (c) treated with SSD (I: petechial hemorrhages, II: area of aseptic necrosis with many inflammatory infiltrations, and III: inflammatory infiltrations on the verge of necrosis); (d) treated with bee pollen (I: petechial hemorrhages, II: area of necrosis with many inflammatory infiltrations).

the wounds, being constantly washed with 0.9% NaCl, were covered with an irregular eschar tightly adhering to the wound in its central part. After the eschar was removed mechanically, a pink granulation tissue without the features of epithelialization could be seen. The wound, treated with silver sulfadiazine (subgroup E1), was covered with a pink epithelium. The tissues surrounding the wound had no significant inflammatory features. The wound area did not decrease. The wounds, treated with the bee pollen ointment, in subgroup E2, were covered with a thick epithelium. The features of the healing process were strongly visible. Within the surrounding tissue there were not any signs of erythema or the ongoing inflammatory process.

3.2. Histopathological Test Results. The histological view of wound healing of animals from all groups until the 5th day of the experiment were identical. Figure 1 shows differentiated dynamics of repairing processes which occurred on the 10th day of the experimental healing process for all analyzed groups. Application of the bee pollen (E2) achieved its therapeutic effect on the 10th day of the experiment. The whole wound surface was filled with collagen fibers, which affected scar formation, and the stratified squamous epithelium was being created.

On the 15th day of the observation, other changes in the histopathological view were observed. In the control subgroups C1 and C2 and in the E1 subgroup, a slow wound healing process in the phase of fibroplasia with the sustaining inflammation could be observed. In subgroup (E2), in which the wounds were treated with the ointment with 5% bee pollen extract, fibroplasia was significantly proceeding, while the present granulation tissue was covered with a regenerated epithelium. In the wound area there were no clear signs of inflammatory reaction. The regenerated stratified squamous epithelium was appearing on the wound edges together with existing inflammatory infiltrations in the histopathological view on the 21st day of the experiment in the case of the untreated wounds (subgroup C1). In case of wounds washed with 0.9% of NaCl (subgroup C2) as well as in subgroup (E1), in which the wounds were treated with silver sulfadiazine, the developed stratified squamous epithelium was covered with an eschar, under which there was a visible mature granulation tissue with a lot of fibers. In subgroup (E2), in which the wounds were treated with the bee pollen ointment, the whole wound surface was filled with a scar together with a thick



(a) Untreated (C1) (b) NaCl (C2)

(c) SSD (E1) (d) Bee pollen (E2)

Figure 2: The microscopic changes of skin samples collected from burn wounds on the 21st day of the experiment: (a) untreated (I: regenerated stratified squamous epithelium on the sample edge, II: vessel-rich and cell-rich granulation tissue); (b) washed with NaCl (I: eschar, II: regenerated stratified squamous epithelium, and III: vessel-rich and cell-rich inflammatory granulation tissue); (c) treated with SSD (I: a slightly swollen dermis, II: eschar with petechial hemorrhages, and III: regenerated stratified squamous epithelium); (d) treated with bee pollen (I: connective tissue scar covered with epithelium and II: inflammatory granulation tissue with predominating collagen fibers).

stratified squamous epithelium. There was no granulation tissue. The E2 subgroup showed a correctly healed burn wound. The description of histopathological observations on 21st day of the experiment is shown in Figure 2.

3.3. Microbiological Test Results

3.3.1. Quantitative Study. The Logarithmic CFU (colony forming unit) values of bacteria cultured on particular days of the burn wound healing are summarized in Table 1.

The result of the quantitative study conducted on the 0 day, immediately after burning, showed no microorganisms from none of the experimental groups. The effect of thermal feature made the skin sterilized. On the 3rd day of the study, the bacteria were isolated only from the tissue specimens collected from the untreated wounds. On the 5th day, the microorganisms were present in the tissue material of all studied groups. Further growth of the average number of bacteria in 1 cm^2 of the wound was found on the 10th day of the experiment. However, the number of bacteria decreased in wounds washed with 0.9% of NaCl (C2) and in wound treated with the bee pollen ointment (E2). A further decrease of the number of bacteria in most analyzed groups was observed on the 15th day after burning. However, the wounds treated with the bee pollen ointment were characterized by the smallest number of bacteria in relation to the previous measurement. A systematic decrease of the number of bacteria in the wounds classifying to control and experimental groups was confirmed on the 21st day of the experiment and; what is more, the beds of thermal damage treated with silver sulfadiazine and with the bee pollen ointment were characterized by the biggest decrease of the bacteria number (Figures 3 and 4).

Logarithmic CFU (colony forming unit) values of fungi and mould cultured on particular days of the burn wound healing are summarized in Table 2.

The growth of fungi and mould in the wound area of animals, evaluated on 0 and 3rd day of the C1, C2, E1, and E2 subgroups, resulted in finding no such microorganisms. The experimental studies conducted on 5th and 10th day showed that the number of fungi and moulds increased particularly in the case of untreated wounds as well as those treated with silver sulfadiazine. Next days showed a decreased general number of fungi and mould in untreated wounds and those treated with SSD. The wounds washed with NaCl and those exposed to bee pollen ointment were characterized by the lowest number of fungi and mould on the 21st day of the experiment (Figures 5 and 6).

Variable number of fungi and moulds in time was analytically analyzed. This analysis is to approximate the

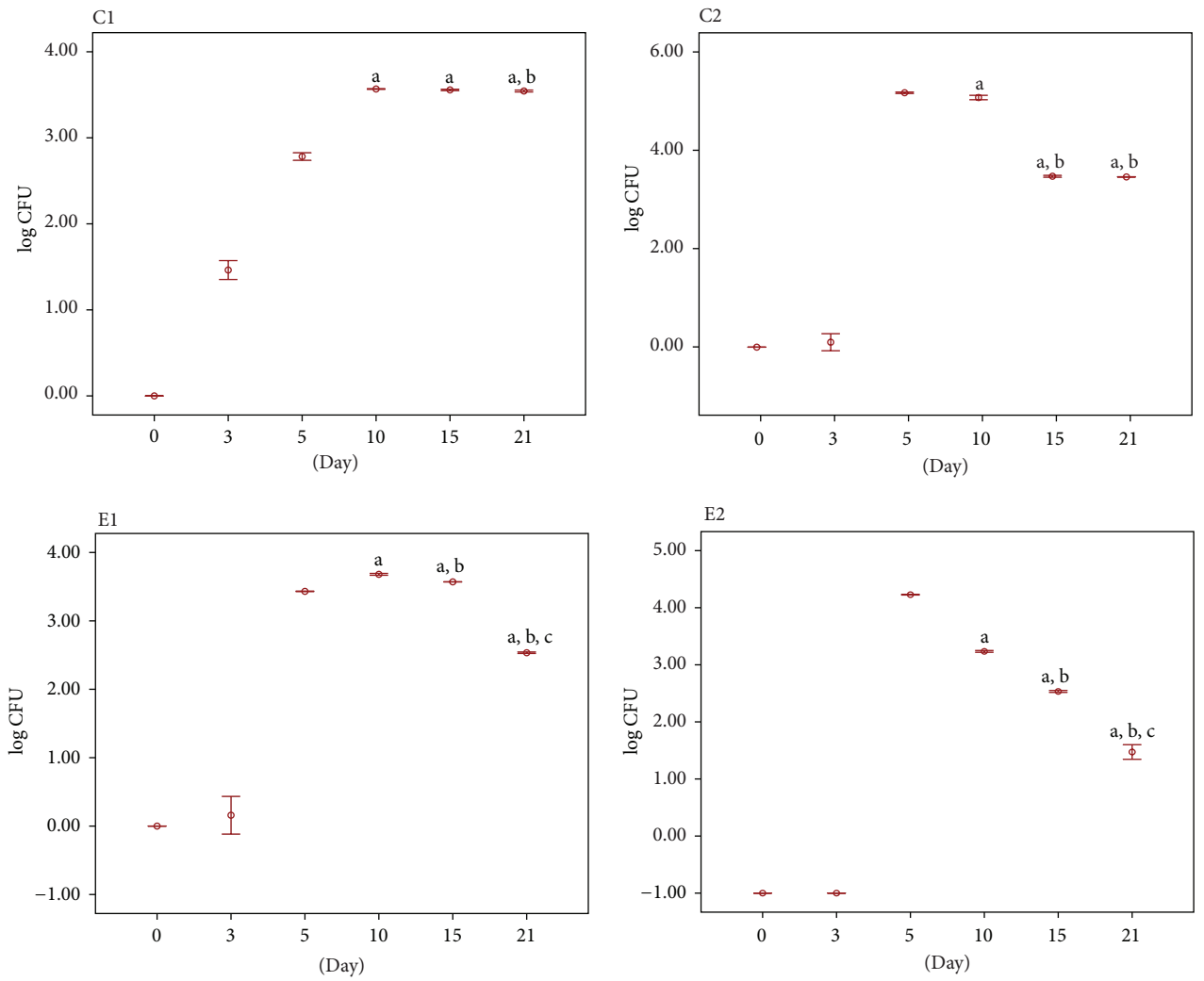


Figure 3: Quantitative study: log CFU value of bacteria cultured on particular days of the burn wound healing: C1: tissue material from untreated wounds; C2: tissue material collected from wounds washed with NaCl; E1: tissue material from wounds treated with silver sulfadiazine; E2: tissue material from wounds treated with bee pollen ointment. Results are expressed as mean ± standard error of the mean (SEM) of the assays performed in triplicate. [a] $p < 0.05$ compared with value determined on 5th day, [b] $p < 0.05$ compared with value determined on 10th day, and [c] $p < 0.05$ compared with value determined on 15th day.

Table 1: log CFU values of bacteria on the following days of the experiment.

	0 day	3rd day	5th day	10th day	15th day	21st day
C1 (untreated)	—	1.47	2.78	3.57	3.56	3.54
C2 (NaCl)	—	—	5.18	5.08	3.48	3.46
E1 (SSD)	—	—	3.43	3.68	3.57	2.53
E2 (bee pollen)	—	—	5.23	4.24	3.53	2.48

Table 2: log CFU values of fungi and mould on the following days of the experiment.

	0 day	3rd day	5th day	10th day	15th day	21st day
C1 (untreated)	—	—	1.59	1.47	1.19	0.99
C2 (NaCl)	—	—	1.01	1.01	1.19	0.18
E1 (SSD)	—	—	1.68	1.60	1.07	1.00
E2 (bee pollen)	—	—	0.99	0.88	1.16	0.75

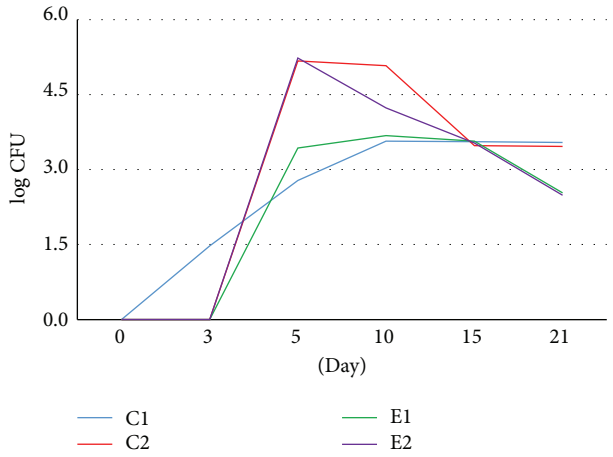


Figure 4: Dynamics of log CFU value of bacteria cultured on particular days of the burn wounds treated with NaCl (C2), silver sulfadiazine (E1), bee pollen ointment (E2), and untreated wounds (C1).

mode of changes in time with a model. A model, being the inertial first-order object with delay, has been chosen. The very choice of the model results from earlier authors' experiences concerning the analysis of dynamic changes (e.g., linked to temperature) occurring in humans and animals. The model enables parameterization of characteristics linked to the change rate of the number of fungi and moulds. These parameters are time constant T_1 and delay. The time constant enables the determination of the change rate of the number of fungi and moulds in time. According to the theory of automatic control (the processes occurring in living organisms) the steady state takes place after third up to fifth time constants (95% and 99% of the steady state). For the cases in question it means that if the obtained results are approximated with this model (the inertial first-order object with delay) it will become possible to determine the time after which the decrease in the number of fungi and moulds to the values close to 0 (zero) will appear. It will be, for example, $3 * T_1$ for which only 5% of fungi and mould will remain when related to the maximum value. Similarly for $5 * T_1$ only 1% of fungi and mould will remain in relation to the maximum value. The approximation of changes of the number of fungi and mould in time with the inertial first-order object with delay enables obtaining one more error parameter of matching δ, which gives the information about the matching compliance of the model with the obtained experimental data.

The time change of the average number of bacteria, fungi, and moulds is a nonlinear relationship. Due to the analogy to the control systems, the response of the system (in this case it is the number of bacteria, moulds, and fungi) may be approximated by the inertial first-order object with delay. It results from the biological and medical rationale concerning the growth rate (development) of the bacteria, fungi, and moulds on the healing wound surface (regardless of the fact if it was C1, C2 or E1, E2). The general transmittance form

Table 3: The error value of matching the inertial first-order object model with the experimental data (bacteria, fungi, and mould) for given time constants T_1.

	C1	C2	E1	E2
$\delta^{(B)}$ [%]	3	44	5	58
T_1 [day]	20	1	18	1
$\delta^{(R)}$ [%]	25	17	18	17
T_1 [day]	7	20	6	20

of the response relationship for the multi-inertial object is as follows:

$$G(s) = \frac{k}{(1 + sT_1)(1 + sT_2)\cdots(1 + sT_n)}, \quad (1)$$

where k is amplification and $T_1, T_2, \ldots, T_n$ is time constant.

This was the basis for formulating the error of matching the model with the source data, for example, for the bacteria (superscript) and tissue material from untreated wounds (subscript) done as follows:

$$\delta_{C1}^{(B)} = \frac{100}{I \cdot \max_i y_{C1}^{(B)}(i)} \sum_{i=1}^{I} \left| y_{C1}^{(B)}(i) - y_{SC1}^{(B)}(i) \right| [\%], \quad (2)$$

where $y_{C1}^{(B)}(i)$ is change in the number of bacteria (superscript B) in the next i-measurements, $y_{SC1}^{(B)}(i)$ is simulation of change in the number of bacteria (superscript B) in the next i-measurements for the model (unit response) described by transmittance, and I is total number of measurements.

Similarly, the error of experimental data match with the standard for fungi and mould (superscript R) and different materials is calculated. For such a formulated error the method of a tuned model was applied in order to match the multi-inertial object with the data and to specify the order of the model. The smallest values of errors, shown in Table 3, were obtained for multi-inertial first-order object.

In Table 3 the calculated error values of the match $\delta^{(B)}$ and $\delta^{(R)}$ for the materials C1, C2, E1, and E2 were shown. The calculations were done for the inertial first-order object (with the time constant T_1) with delay (5 days) for which the value of particular errors is smaller. In the graph in Figure 7 the exemplary obtained results are shown, the behaviors of $y_{C1}^{(R)}(i)$ and $y_{SC1}^{(R)}(i)$ for $T_1 = 8$.

As it can be concluded from Figure 7, the biggest error values (>44%) occur for materials C2 and E2, which results from the specification of changes in the number of bacteria in the wound. Due to individual variation of pigs, this specification depends on many factors. The smallest error values and, simultaneously, the best match of the model with experimental data occur for materials C1 and E1. The time constants for them are 20 and 18 days. Similar error values were obtained for fungi and mould which fluctuate around the value of 18%. The time constants are also different (as in the case of bacteria) for materials C1 and E1 amounting to 6 and 7 days, while for materials C2 and E2 they are equal to 20 days. Summing up the obtained results, the time constant average value of the growth of bacteria, mould, and fungi in the wound is at the level of 18 up to 20 days.

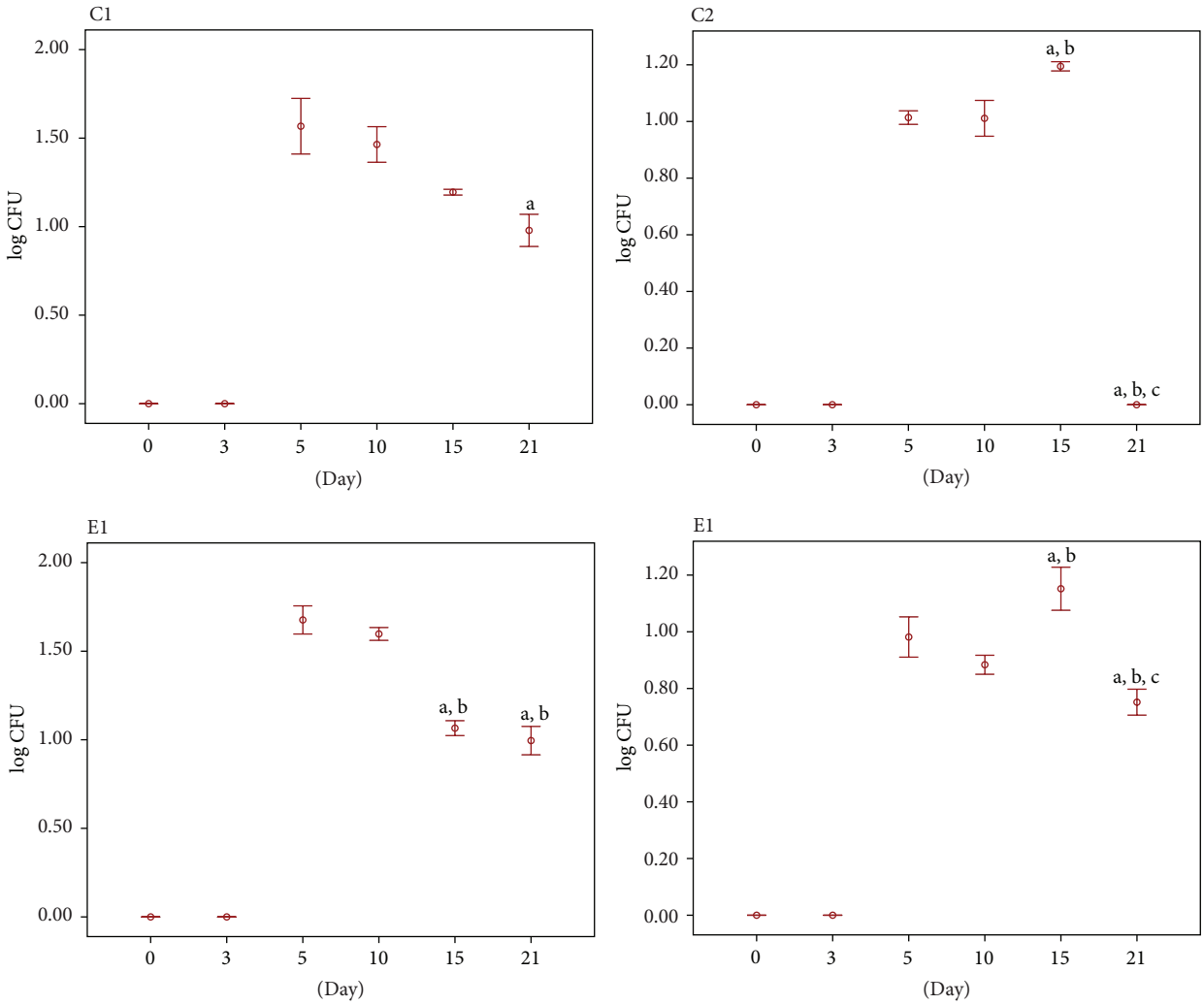


Figure 5: Quantitative study: log CFU value of fungi and mould cultured on particular days of the burn wound healing: C1: tissue material from untreated wounds; C2: tissue material collected from wounds washed with NaCl; E1: tissue material from wounds treated with silver sulfadiazine; E2: tissue material from wounds treated with bee pollen ointment. Results are expressed as mean ± standard error of the mean (SEM) of the assays performed in triplicate. [a] $p < 0.05$ compared with value determined on 5th day, [b] $p < 0.05$ compared with value determined on 10th day, and [c] $p < 0.05$ compared with value determined on 15th day.

3.3.2. Qualitative Study. In the qualitative study, changes of microbial species from the swabs of burn wounds treated with appropriate experimental agents and of the healthy skin surface were evaluated during next days of the experiment. On 0 day, the number of microorganisms, which constitute the physiological flora of the skin and the environment, increased in healthy skin (Table 4). However, no bacteria were cultured from none of the samples collected from the burn wounds immediately after burning.

On the 3rd day of the study, the wounds were colonized with microorganisms from *Micrococcus* species only in the subgroup in which the wounds were untreated (C1). On the 5th day of the study, the number of isolated microorganisms species significantly increased in the animals of all subgroups. Besides typical physiological flora of the skin and the environment (*Micrococcus* spp., *Bacillus* spp.) there were also microorganisms which are characteristic for wound inflammation. In the subsequent days of the experiment, all burn wounds were characterized by a lower number of strains. On the 21st day of the study, in subgroups C2, E1, and E2, the bacterial flora was reduced to only one environmental species, such as *Bacillus* spp., while in the group of untreated wounds (C1), only *Staphylococcus hyicus* was found.

4. Discussion

Wound healing is a dynamic and time-synchronized reaction of the organism connected both with the actions of many cells, such as inflammatory cells, vascular cells, connective tissue cells, and epithelial cells, and with accumulating extracellular matrix (ECM) components, which leads to creation of a new tissue [20]. A significant role in the healing process is played by ECM components: glycosaminoglycans (GAGs), fibronectin, proteoglycans, vitronectin, and collagens [21, 22].

Table 4: Changing of species of microflora in burn wounds in the following days of the experiment; N: tissue material from healthy skin not inflicted with a burn; C1: tissue material from untreated wounds; C2: tissue material from places washed with 0.9% NaCl; E1: tissue material from places treated with silver sulfadiazine salt; E2: tissue material from places treated with the with bee pollen ointment.

	0 day	3rd day	5th day	10th day	15th day	21st day
N	*Micrococcus* spp. *Bacillus* spp. *Staphylococcus lentus*	*Micrococcus* spp. *Bacillus* spp. *Staphylococcus lentus*	*Micrococcus* spp. *Bacillus* spp. *Gemella* spp. *Aerococcus viridans*	*Micrococcus* spp. *Bacillus* spp. *Aerococcus viridans*	*Micrococcus* spp. *Bacillus* spp. *Aerococcus viridans* *Enterococcus faecalis*	*Micrococcus* spp. *Bacillus* spp. *Aerococcus viridans*
C1	—	*Micrococcus* spp.	*Micrococcus* spp.	*Micrococcus* spp. *Staphylococcus hyicus* *Candida* spp.	*Candida* spp.	*Staphylococcus hyicus*
C2	—	—	*Micrococcus* spp.	*Micrococcus* spp.	*Bacillus* spp.	*Bacillus* spp.
E1	—	—	*Bacillus* spp. *Staphylococcus hyicus* *Enterococcus faecalis*	*Bacillus* spp. *Micrococcus* spp.	*Bacillus* spp.	*Bacillus* spp.
E2	—	—	*Bacillus* spp. *Staphylococcus hyicus* *Pseudomonas aeruginosa*	*Bacillus* spp. *Pseudomonas aeruginosa*	*Bacillus* spp.	*Bacillus* spp.

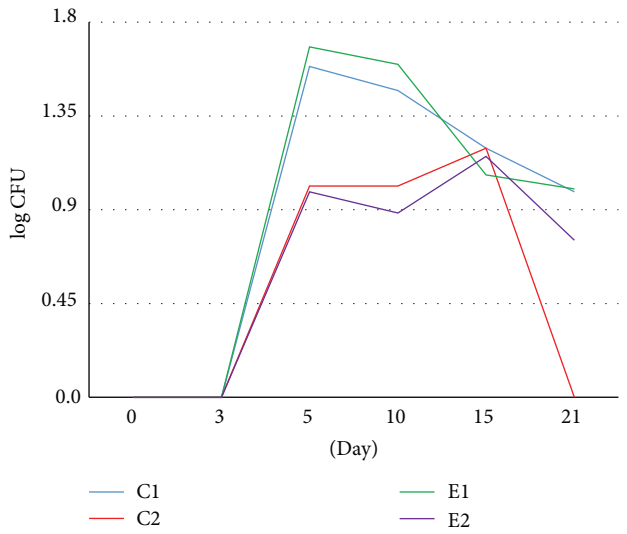


Figure 6: Dynamics of log CFU value of fungi and mould cultured on particular days of the burn wounds treated NaCl (C2), silver sulfadiazine (E1), bee pollen ointment (E2), and untreatedwounds (C1).

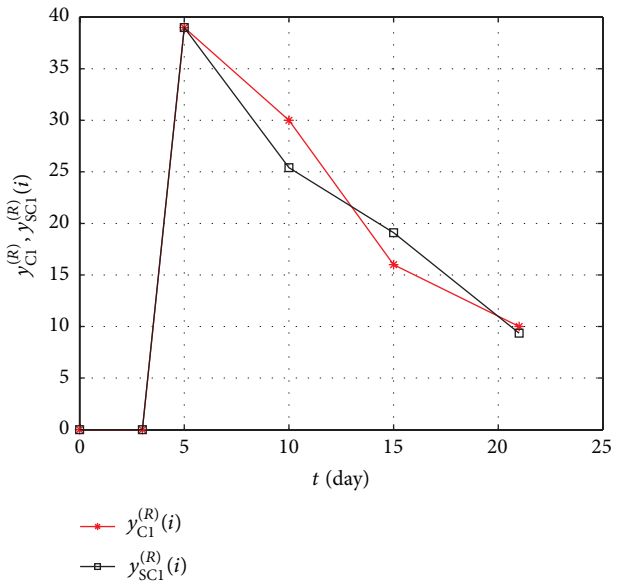


Figure 7: The graph of changes in the number of fungi and moulds in time for experimental and simulation data of the material C1.

The therapeutic effect of a natural bee preparation, propolis, in the treatment of experimental burn wounds was the subject of our previous experimental studies. They showed that application of propolis modulated the expression of glycosaminoglycans, collagens, noncollagenous glycoproteins, and free radicals in the burn wound bed, which favors the intensification of healing process and, therefore, confirmed a positive influence of the mentioned apitherapeutic agent on the metabolism of ECM components [21–24].

The aim of the present study was to compare the therapeutic efficiency of another natural agent based on bee pollen extract with a commonly used pharmaceutical silver sulfadiazine in treatment of thermal burns.

Although silver sulfadiazine is considered as a gold standard in the topical treatment of burn wounds, this

therapeutic agent is characterized by many side effects such as the risk of crystalluria, methaemoglobinaemia, neutropenia, erythema multiforme, and prolonged reepithelialization and the impairment of the mechanical strength of newly created tissue [25, 26]. Such side effect cannot be found in the case of bee pollen. This apitherapeutic agent demonstrates strong immune-modulating properties, which accelerate epithelialization and has bacteriostatic, bactericidal, and anesthetic properties [9, 27]. Moreover, bee pollen has a strong anti-inflammatory activity, decreases the healing period, and reduces the duration and intensity of ailments [9, 28].

The experimental model implemented in the present study was based on the tissue material collected from the domestic pig skin. The choice of the animal was made mainly due to the similarity between pig skin and human one [29].

Clinical and histopathological observation comprising the assessment of the extent and depth of the burn wounds, wound maceration, presence of necrotic tissue, granulation tissue type, and swelling around the burn wound indicated that, on the first days of the experiment, the pathomorphological view of the wounds for every group was the same. It became significantly differentiated on the 5th day of the observation. In the case of the wounds treated with the ointment with bee pollen ointment (E2), the wound area was covered with a thin, flexible eschar with a slight bleeding. In the wound area there were signs of swelling and reddening.

On the next days of the observation of the wounds treated with the apitherapeutic agent, a strong granulation and, subsequently, epithelium formation with clearly visible fully healed characteristics were noted. The wound surface decreased and was the size of 1 cm × 1 cm. In the area of the tissues surrounding the healing wound there were no signs of swelling or the ongoing inflammatory process. The clinical and histopathological assessment led to a conclusion that the applied apitherapeutic agent ointment reduces the time of burn wound treatment. Similar results were obtained in our previous studies where the therapeutic usability of another apitherapeutic agent, propolis, was assessed in the course of regeneration of experimental thermal skin damage. Propolis ointments in comparison with SSD preparation significantly accelerated the regenerative-reparative process of tissue damage not demonstrating any undesirable effects at the same time [30]. The beneficial effect of standardized propolis formulation on the healing process was also proved in Jastrzębska-Stojko et al. experimental studies [31]. The healing process of burn wounds treated with Sepropol was faster as compared to the standard SSD therapy. Moreover, histopathological tests showed that the process of scar formation in wounds treated with propolis formulation started considerably earlier as compared to the control group [31]. The other part of our studies concerning microbiological examinations during experimental burn wound healing proved that bee pollen ointment had an effective antimicrobial activity, reducing both the number of microorganisms and presenting bactericidal activity in isolated strains. The antibacterial properties of another apitherapeutic agent, propolis, were already assessed in the study with animal model of burn wounds. The mentioned study indicated a higher antimicrobial effectiveness of propolis ointment as compared to SSD in the course of burn wounds healing. A more beneficial action of the first from the mentioned preparations was manifested by a significant reduction of microorganisms as well as a more effective bactericidal action of the applied apitherapeutic agent. A similar trend in the effects of SSD action and a bee product in the range of antibacterial action were described by Kabała-Dzik et al. [32].

The therapeutic mechanism of bee natural products is based, among others, on antimicrobial activity and on inducing processes of damaged tissues regeneration. These characteristics proved their usability in wound healing and ulcerations of different etiology [31, 33].

The results in this study confirmed the beneficial effect of the bee pollen ointment on the burn wound healing process which could be seen in the decreased number of bacteria in the burn wounds during subsequent days of the experiment.

Different mechanisms could be responsible for the observed antibacterial effects of bee pollen. The first one results from the presence of active compounds, such as flavonoids and phenolic acids, whose forming complexes with bacterial cell walls lead to the disruption of cell wall integrity, blocking ion channels, and inhibiting electron flow in the electron transport chain [34].

The second mechanism by which bee pollen exerts antibacterial activity might be based on the inhibition of bacterial RNA-polymerase by phenolic compounds such as flavanone pinocembrin, flavonol galangin, and caffeic acid phenethyl ester [35].

Besides high antimicrobial activity, bee pollen ointment was also characterized by a bactericidal effect for isolated strains.

Moreover, the study also proved that thermal damage and bacterial infection of the wound favor yeast multiplication including *Candida albicans.* The yeast of the *Candida* species in proper conditions is saprophytes which live in the natural environment and colonize mucosa and human skin. However, they may induce life-threatening candidiases. Burns and necrotic lesions, which are the gates for fungal infection, may contribute to sepsis. *Bacillus cereus* and *Bacillus subtilis*, which are usually harmless, may induce infections in the condition of decreased immunity.

The clinical and histopathological observations performed in our study led to a conclusion that the bee pollen exerts a beneficial effect on wound healing cellular events providing reepithelization and wound closure. The microbiological studies proved that bee pollen ointment had an effective antimicrobial activity. The benefits and advantages of the bee pollen ointment in burn wound treatment imply the usability of the applied apitherapeutic agent preparation in topical burns therapy.

Competing Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by a grant from the Medical University of Silesia, Poland (KNW-1-018/K/4/0).

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