Nutraceuticals: Recent Advances of Bioactive Food Components
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Nutraceuticals are numerous and have been studied intensely for basic science and applied research. The term is generally used to refer to those chemicals that may have biological significance, for example, antioxidants, disease resistance, and regulating the immune systems, but are not established as essential nutrients. It utilizes traditional and rapidly advancing analytical methods and instrumentation such as differential solvent extraction, gas or liquid chromatography coupled with tandem mass spectrometry, nuclear magnetic resonance and electron-spin resonance, and gene and protein arrays for fractionation, purification, chemical and genetic fingerprinting, derivatization, modification, synthesis, and clinical trials of photochemicals starting from cell-line to animal and patient studies. The goal of this special issue is to provide a platform for scientists all over the world to promote, share, and discuss various new issues and developments in the area of nutraceuticals research.

This issue compiles biological activities of bioactive food components.

Kombucha is described in a review and research paper. Kombucha tea is produced by fermenting sugared black tea with a mixed culture of yeast and bacteria. Kombucha tea has gained immense popularity in recent times due to many associated health benefits. The therapeutic effects of this beverage are thought to be derived from the chemical composition of this beverage, mainly the polyphenols and secondary metabolites which are produced during fermentation. The stability of antioxidant activity and polyphenol contents were also investigated by M. I. Watawana et al.

Calcium compounds of Opuntia ficus-indica are reported by I. Rojas-Molina et al. from Mexico which have a deficiency in calcium intake. They characterize the distribution of calcium compounds in soluble and insoluble dietary fiber extracted from Opuntia ficus-indica.

Enriching pasta with grape marc is studied by V. Marinelli et al. from Italy. Grape marc is an interesting source of natural compounds as polyphenols and fibers. This is the study to enrich fresh and dry pasta with grape marc extracts obtained by means of UAE using only water as solvent extraction.

Antioxidant peptide is obtained from Spirulina maxima by W. S. Kim et al. This paper deals with the antioxidant peptide derived from Spirulina maxima possessing an effective constituent in antitumor progression products based on the mechanism of tumor progression through ROS and the HIF1α signaling pathway.

Tunisian Cupressus sempervirens chemical composition and some biological activities are analyzed by A. B. Nouri et al. Some of the studies already focused on chemistry and biological activities of Cupressus sempervirens; however they are originated from different areas in the world. This study is to ascertain the chemical composition of Tunisian Cupressus sempervirens to evaluate its antioxidant and antibacterial activities.

4-(4-Methylbenzamino)benzoate is observed to suppress adipocyte differentiation by J. T. Hwang et al. 4-(4-Methylbenzamino)benzoate has estimated adipogenesis-suppressing activity compared to resveratrol or genistein.
The biologically active substances of regrowth velvet antler are described in this issue. In this study, regrowth velvet antler is subjected to extraction by DW to allow determination of its constituent biologically active substances, including uronic acid, sulfated GAGs, sialic acid, uracil, hypoxanthine, and uridine. In addition, the antioxidant activities of RVA are determined by assessing DPPH, H$_2$O$_2$, hydroxyl, and ABTS radical scavenging activity as well as FRAP and ORAC.

We thoroughly reviewed these papers and we believe that they contribute to the applications of bioactive food components. Moreover, we believe that the papers in this special issue will be milestones for the advancement for the next generation of nutraceuticals researchers.

Pyo-Jam Park
Thomas Ty Wang
Eun-Kyung Kim
Qian Zhong-Ji
Research Article

Specific Anthocyanin Contents of Whole Blue Maize Second-Generation Snacks: An Evaluation Using Response Surface Methodology and Lime Cooking Extrusion

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Lime cooking extrusion (LCE) is a widely applied technology for producing second-generation snacks, as an alternative to traditional nixtamalization (TN). Pigmented maize has been used to produce snacks with similar organoleptic characteristics to TN products and to obtain a product with additional functional benefits due to the anthocyanic compounds contained in those grains. However, during the process, anthocyanins are degraded, and several chemical modifications occur. Response surface methodology is applied to evaluate extrusion factors and their effects on the response variables of extrudates. The aim of this study was to evaluate the changes in specific anthocyanins after extrusion in second-generation blue maize snacks. Three anthocyanins were identified and quantified by HPLC-UV-DAD: cyanidin 3-glucoside and pelargonidin 3-glucoside, which have been previously reported in blue maize and its products, and cyanidin 3,5-diglucoside. Higher retention values were found in the extrudates making LCE a viable option for producing second-generation blue maize snacks.

1. Introduction

Anthocyanins represent the major group of water-soluble pigments in the plant kingdom and are widely distributed in food crops (e.g., vegetables, roots, tubers, and cereals). Several investigations have reported not only nontoxic and nonmutagenic effects but also therapeutic and antioxidant activities of anthocyanic compounds. The aglycones (basic forms) of anthocyanins are termed anthocyanidins. These structures are based on the flavylium ion or 2-phenyl benzopyrylium, which presents hydroxyl and methoxyl groups in different positions [1]. Anthocyanidins are not accumulated as such but rather in a glycosylated form, that is, linked to sugars, in which case they are known as anthocyanins. The sugars attached to anthocyanidins provide high solubility and stability. Generally, the sugar molecule is linked to the phenolic acid at the 3 position, although it can be linked at the 5 and 7 positions as well [2]. Cyanidin 3-glucoside has been reported as the most abundant anthocyanin contained in pigmented maize (approximate from 48% up to 87%), but pelargonidin 3-glucoside, peonidin 3-glucoside, and malvidin 3-glucoside can also be present [3]. The pigmented maize cultivated in
Table 1: Experimental design of LCE showing different combinations of FM, T, and LC and experimental values of anthocyanin contents of the extrudates.

<table>
<thead>
<tr>
<th>Tx¹</th>
<th>FM</th>
<th>T</th>
<th>LC</th>
<th>C3,5-diG</th>
<th>C3G</th>
<th>Pel3G</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>ºC</td>
<td>%</td>
<td>mg·kg⁻¹</td>
<td></td>
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<tr>
<td>1</td>
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<td>16.98</td>
<td>28.10</td>
<td>1.63</td>
</tr>
<tr>
<td>2</td>
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<td>130 (0)</td>
<td>0.13 (0)</td>
<td>16.47</td>
<td>27.22</td>
<td>1.71</td>
</tr>
<tr>
<td>3</td>
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<td>17.09</td>
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</tr>
<tr>
<td>4</td>
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<td>0.13 (0)</td>
<td>17.06</td>
<td>27.61</td>
<td>1.66</td>
</tr>
<tr>
<td>5</td>
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<td>141.89 (1)</td>
<td>0.2 (1)</td>
<td>16.97</td>
<td>28.07</td>
<td>1.73</td>
</tr>
<tr>
<td>6</td>
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<td>0.13 (0)</td>
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<td>28.21</td>
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</tr>
<tr>
<td>7</td>
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<td>28.36</td>
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<tr>
<td>8</td>
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<td>16.89</td>
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<td>1.59</td>
</tr>
<tr>
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</tr>
<tr>
<td>10</td>
<td>21.38 (1)</td>
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<td>0.05 (−1)</td>
<td>16.92</td>
<td>27.96</td>
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<tr>
<td>11</td>
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</tr>
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<td>27.64</td>
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</tr>
<tr>
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</tr>
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<td>16.59</td>
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<td>16.70</td>
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</tr>
<tr>
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<td>130 (0)</td>
<td>0.13 (0)</td>
<td>16.69</td>
<td>27.26</td>
<td>1.47</td>
</tr>
</tbody>
</table>

¹Numbers in parentheses are the coded levels. Tx: treatment; FM: feed moisture; T: temperature; LC: lime concentration; C3,5-diG: cyanidin 3,5-diglucoside; C3G: cyanidin 3-glucoside; Pel3G: pelargonidin 3-glucoside.

Extrusion is a technology widely used to obtain different maize products, such as expanded snacks. Lime cooking extrusion (LCE) is considered a faster and effective alternative method to traditional nixtamalization (TN) processing, the latter based on the addition of Ca(OH)₂ to maize and water at 100 ºC to form a solution that is cooked for 20–40 min and steeped for at least 16 h [5]. LCE processing involves thermal treatment, low moisture, and high-pressure conditions, among other factors (incorporation of additives and conditioning of flours). Many studies [6, 7] have been conducted on pigmented maize using LCE to retain more anthocyanins, limiting the losses due to processing. However, none of these studies have analyzed the profile of anthocyanins as being affected by multiple process factors using response surface methodology (RSM), a statistical method used to investigate the effects of factors in complex processes, and examined how these factors influence the different anthocyanins contained in the extrudates. The aim of this study was to evaluate the changes in specific anthocyanin contents that occurred in extruded products elaborated with LCE, applying RSM.

2. Materials and Methods

2.1. Raw Material. Blue maize was obtained from a local market in Toluca, Mexico. The grains were cleaned and stored at 5 ºC in black polyethylene bags until use. Commercial lime (Calhidra de Sonora, SA de CV, Hermosillo, SON, Mexico) and distilled water were used.

2.2. Methods

2.2.1. Lime Cooking Extrusion (LCE). The process was performed according to the method described by Escalante-Aburto et al. [4]. Ten kilograms of blue maize was passed through a blade mill (Pulvex SA de CV, Model 200, serial 1030401, Mexico, DF) with a 0.8 mm mesh. Twelve hours before the extrusion experiment, the ground maize was divided into 20 batches of 300 g, and commercial lime and distilled water were added to each batch according to the experimental design shown in Table 1.

To obtain the extrudates, the experiment was performed in a single screw extruder (Brabender Instruments, Model E19/25 D, OHG Duisburg, Germany). The temperatures in the first, second, and third zones were set at 60, 80, and 110 ºC, respectively, and the fourth zone varied from 110 ºC to 150 ºC (Table 1). The screw had a diameter of 19 mm, and the nominal compression ratio was 3 : 1. The other extrusion conditions were a screw speed of 120 rpm, feed hopper of 50 rpm, and die opening diameter of 4 mm. The extrudates were cooled at room temperature and stored in sealed bags until the chemical analyses were performed.
2.2.2. Anthocyanin Profile by HPLC Analysis. The extraction of anthocyanins was based on the methodology of Abdel-Aal and Hucl [8]. Extrudates samples were ground (Moulinex, model 980-18, France) and passed through a number 60 sieve. Two mg of sample was weighed into a 50 mL centrifuge tube (tube 1) with 20 mL of acidified ethanol (ethanol + HCl 1N, 85:15 v/v) and agitated (Wrist Action Shaker, model 75, Burrell Corp., EUA) for 1h. Then, the solution was centrifuged (Thermo Scientific, model Heraeus Biofuge Primo Burrell Corp., EUA) for 1h. The supernatants from both steps were combined, giving an approximate volume of 40 mL, and then concentrated to1.0mg/mL. Moreover, a backward regression analysis was applied, and nonsignificant factors (P > 0.1) were eliminated from the second-order polynomial equation. Then, a new equation was recalculated to achieve the final predictive model for each response variable. RSM was used [9] and contour plots for each determination were obtained using Design Expert Software V.7.0.0.

3. Results and Discussion

HPLC analyses demonstrated that of the five anthocyanins analyzed only three were present in raw blue maize and its extrudates. Cyanidin 3,5-diglucoside, cyanidin 3-glucoside, and pelargonidin 3-glucoside were identified. C3,5-diG was the first anthocyanin eluted. The glucose molecules affected the polarity, degree of glycosylation, and nature of the sugar moiety, and thus the elution order was galactoside > glucoside > arabinoside [10]. Moreover, the presence of multiple glycosidic substituents affected the retention characteristics, provoking a faster elution of the compounds, as in the case of C3,5-diG.

3.1. Effects of FM, T, and LC on C3,5-Diglucoside Concentration. The coefficients of the second-order equations, ANOVA, and determination coefficients of the effects of FM, T, and LC on the C3,5-diG are shown in Table 2.

The results demonstrated that FM in its linear and quadratic (FM)^2 terms exerts highly significant (P < 0.0024) and significant (P < 0.0200) effects, respectively, on the C3,5-diG concentration. In addition, temperature in its quadratic term (T)^2 presented a very significant effect (P < 0.092) on the anthocyanin. The prediction model equation was as follows:

\[
C_{3,5-diG} = -3.92 + 0.81(\text{HA}) - 0.01(\text{HA})^2 - 0.0007(T)^2. \tag{1}
\]

Table 1 displays the average values of the concentration of C3,5-diG, which varied from 16.40 mg·kg\(^{-1}\) to 17.29 mg·kg\(^{-1}\). C3,5-diG was the second most abundant compound after C3G. The latter situation could occur because diglycosylated anthocyanins have higher stability than monoglycosylated ones, which diminishes the susceptibility to degradation by temperature and UV radiation [II]. Moreover, when the
The retention of C3,5-diG in the extrudates was evaluated in comparison to the values obtained in raw blue maize, it was found that between 91.2 and 96.2% of the anthocyanin was retained in the products. The stability of C3,5-diG to comparison to the values obtained in raw blue maize, it was found that between 91.2 and 96.2% of the anthocyanin was retained in the products. The stability of C3,5-diG in the extrudates was evaluated in compounds with glucose or galactose substituents. Moreover, there was a significant production of anthocyanins such as delphinin and malvidin (present in pigmented maize) when their corresponding anthocyanins were submitted to heating at 100°C for 30 minutes in conditions of very low moisture.

Figure 2(a) shows the effects of FM and T on the C3,5-diG concentration of extrudates. It was observed that in a range of 21–23% FM and at T of 120 and 130°C the anthocyanin reached its maximum concentration. When the HA * LC interaction was analyzed (Figure 2(b)), a directly proportional effect of FM and C3,5-diG was observed: as FM increases, the C3,5-diG content of the extrudates also increases, independently of LC.

Salinas Moreno et al. [13] reported the presence of C3G, Pel3G, peonidin 3-glucoside, three acylated anthocyanins, cyanidin-3-(6″malonylglicoside), cyanidin-3-(3″,6″dimalonylglicoside), and four unidentified anthocyanins. This investigation demonstrated that C3,5-diglucoside was present in blue maize and its extrudates.

Figure 2(c) showed the effect of the T * LC interaction on C3,5-diG concentration. Only T significantly affected this response variable, and the maximum average values of C3,5-diG were found at 130°C.

3.2. Effects of FM, T, and LC on the Cyanidin 3-Glucoside (C3G) Concentration. The coefficients of the second-order equations and ANOVA of the effect of FM, T, and LC are shown in Table 2. The FM presented a very significant effect in the linear and quadratic terms, while T in the quadratic term (T²) showed a very significant effect (P < 0.0195) on the C3G values. The prediction model equation was as follows:

\[ C3G = -16.56 + 1.25 (FM) - 0.029 (FM)^2 - 0.001 (T)^2. \] (2)

The average values of C3G were in the range of 26.67 to 28.36 mg·kg⁻¹ (Table 1). There were slight losses of these anthocyanins, as the extrudates retained between 77.1 and 82% of C3G compared with raw blue maize (34.60 mg·kg⁻¹).

Figure 3(a) shows the effects of FM and T on the C3G content. Increased FM and T of 130°C resulted in the highest concentrations of these compounds. The interaction FM * T did not show a significant effect; nevertheless, there were significant effects only of the individual terms, meaning that the results could be related to the findings reported by Yue and Xu [14]. These authors concluded that although anthocyanins degraded at temperatures higher than 100°C, thermal stability increased in compounds with glucose or galactose substituents. Moreover, there was a significant production of anthocyanidins such as delphinin and malvidin (present in pigmented maize) when their corresponding anthocyanins were submitted to heating at 100°C for 30 minutes in conditions of very low moisture.

Figure 3(b) shows the effects of FM and LC. A significant effect of FM can be observed; as these process factors increased, the concentration of C3G grew higher. It has been reported that LC and pH affect the anthocyanin concentration. In an alkaline medium, anthocyanin compounds diminish in content or are completely destroyed. However, there were no significant effects of LC. Figure 3(c) shows the effects of temperature and LC. Between 130°C and 140°C, the C3G content in the extrudates was higher, independently of LC. Sánchez-Madrígal et al. [15] evaluated the effect of LC addition on the concentration of C3G extracted from blue maize nixtamalized flours obtained by extrusion. Those authors concluded that the concentration of C3G increases without significant effects from the calcium source added.

3.3. Effects of FM, T, and LC on Pelargonidin 3-Glucoside (Pel3G) Concentration. The coefficients of the second-order equations, ANOVA, and determination coefficients of the effects of FM, T, and LC on Pel3G concentration in the extrudates are shown in Table 2. The results demonstrated that LC in its quadratic term (LC²) presented a significant effect (P < 0.0273) on Pel3G concentration. The prediction model equation was as follows:

\[ Pel3G = 1.76 - 2.15 (LC) + 8.79 (LC)^2. \] (3)

Table 1 displays the average values of Pel3G in the extrudates, which are in a range of 1.47–1.78 mg·kg⁻¹. Low amounts of these compounds were lost in the extrudates compared with raw blue maize (2.29 mg·kg⁻¹), as between 64.1 and 77.7% of Pel3G was retained in the products. The Pel3G
concentration in the extrudates was higher than that reported by Sánchez-Madrigal et al. [15] in blue maize nixtamalized flours obtained by extrusion processing. This result corroborates the different chemical transformations of anthocyanic compounds during processing, in addition to the differences in the anthocyanin profile for each maize variety, despite the same grain coloration. Even when Pel3G was present at minimum concentrations, it had a high stable chemical structure.

Garzón and Wrolstad [16] reported that Pel3G exerted a very significant effect on the degradation velocity of anthocyanin contained in certain food products with these compounds added, due to the higher stability to thermal processing.

Figure 4(a) shows the effect of the FM * T interaction on Pel3G concentration in the extrudates. Maximum concentrations of these compounds were found at 130°C and low levels of FM (14%), which agreed with the work by Garzón and Wrolstad [16] with respect to anthocyanin stability at higher processing temperatures.

When the effect of the FM * LC interaction was analyzed (Figure 4(b)), it was observed that, at minimum and maximum LC (0–0.25%), Pel3G reached its highest concentration independently of FM. Figure 4(c) shows the effect of the T *
LC interaction, where Pel3G presented higher degradation at LC 0.05–0.16% and T 110–120°C.

Although the interaction of FM and T did not show significant effects and only the individual effects were significant, our results could be related with the findings reported by Yue and Xu [14]. They found that even when anthocyanins were degraded at temperatures higher than 100°C, the thermal stability of these compounds with glucose or galactose substituents was better. Moreover, higher production of anthocyanidins such as cyanidin, delphinin, and malvidin (present in pigmented maize) was observed when their anthocyanins were subjected to temperatures higher than 100°C and very low moisture conditions.

In general, the results indicated higher retention percentages due to the short time of residence of raw material, implying minimum conditions of thermal treatment and hence higher retention of pigments. Anthocyanins showed certain sensitivity to the individual and combined effects of processing, such as temperature, pressure, treatment time, and the mechanisms and kinetics of degradation [17]. As we can see in the models, Pel3G was the only anthocyanin that showed a significant effect of LC, which was correlated with the pH. Andrés-Bello et al. [18] reported that, with increasing pH, anthocyanins become paler in color; however, if the food matrix contains substances capable of acting as copigments, the color may be retained and stabilized to a certain extent.
When anthocyanins are processed in an alkaline medium (pH 7), the pericarp is not completely hydrolyzed by calcium hydroxide (lime), and the release of ferulic acid is lower. It has been reported that ferulic acid can act as a copigment of anthocyanins, increasing their retention during processing.

4. Conclusions

Higher retention percentages of anthocyanins were observed in the extruded snacks, even when an alkaline medium was induced with the lime cooking extrusion and the materials were exposed to high temperatures. Feed moisture, temperature, and their interactions showed significant effects on the anthocyanin content. Cyanidin 3-glucoside was the most abundant anthocyanin in the extruded snacks at the highest values at feed moisture contents (21–23%) and temperatures on the fourth zone of the extruder (130–140°C), with no significant effects of LC. Cyanidin 3,5-diglucoside due to its chemical composition showed higher stability and had the highest values at feed moisture contents of 21–23% and temperature of 130°C, regardless of the LC. On the other hand, pelargonidin 3-glucoside was significantly affected by LC, showing the highest values at temperature of 150°C, and
lime concentration range of 0–0.25%, regardless of the feed moisture content. These results indicated that it is possible to produce snacks with beneficial or nutraceutical compounds using the lime cooking extrusion process.

Conflict of Interests

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

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Escalante-Aburto thanks CONACYT for the postdoctoral scholarship provided.

References


Research Article

Ultrasound-Assisted Extraction of Total Flavonoids from Corn Silk and Their Antioxidant Activity

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Object. Ultrasound-assisted extraction of total flavonoids from corn silk and their antioxidant activities were studied. Methods. Response surface methodology was adopted to optimize the extraction conditions and antioxidant activities of the extracted total flavonoids were detected through ferric reducing antioxidant power (FRAP) assay. Results. Through a three-level, three-variable Box-Behnken design of response surface methodology (RSM) adopting yield as response, the optimal conditions were determined as follows: ultrasonic power 500 W, extraction time 20 min, material-solvent ratio 1:20, and ethanol concentration 30%. Under the optimum conditions, the extraction yield of total flavonoids was 1.13%. FRAP value of total flavonoid extracted from corn silk was 467.59 μmol/L. Conclusion. The total flavonoids of corn silk could be developed as food natural antioxidant reagents.

1. Introduction

Corn silk (Zea mays L.) is one of the Chinese traditional herbals and contains flavonoids, sterol, alkaloids, carbohydrates, inorganic elements, vitamins, and other chemical constituents [1, 2] which show remarkable bioactivities, such as diuresis, hypoglycemic, bacteriostatic, antihypertensive, enhancing immune, and anticancer activity [3]. The extracts of corn silk are approved by FDA as OTC drug [4].

Flavonoids are effective components of many Chinese herbal medicine with the function of antihypertensive, reducing blood lipid, being antibacterial and antitumor, enhancing immune, antioxidant, and eliminating free radicals [5]. Therefore, it is helpful to look for an effective method to extract flavonoids from corn silk.

However, the literature on efficient extraction of corn silk is limited. Traditional methods (distillation or liquid solvent extraction) for the extraction of flavonoids from plant often need long extraction times, use of large amounts of solvent, and low efficiencies. Moreover, flavonoids are thermally unstable and easily degrade during the extraction. In order to increase the extraction yield, ultrasound-assisted extraction [6–9], which is inexpensive, simple, and efficient alternative to conventional extraction technique, was adopted in our study. In addition, ultrasound also shows a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid and liquid phases. Meanwhile, Box-Behnken design of response surface methodology (RSM) was applied in the determination of the optimal extraction conditions with the independent variable of material-solvent ratio, extraction time, and ethanol concentration. The antioxidant activities of extracted total flavonoids were also determined using reliable method [10, 11].

2. Materials and Methods

2.1. Instruments. UV-1100 spectrophotometer, ultrasonic extractor, rotary evaporator, multiuse recycle water vacuum pump, grinder, and electronic balance.

2.2. Reagent. Corn silk was harvested in Sichuan Agricultural University green nursery garden, Rutin standard was obtained from Sichuan Institute for Food and Drug (99% purity, batch number 130910), ethanol, sodium hydroxide,
aluminum nitrate, sodium nitrite, ferrous sulfate, concentrated hydrochloric acid, and sulfuric acid were of analytical grade and purchased from Chengdu Kelong Chemical Reagent Company. TPTZ was purchased from Beijing Solarbio Science and Technology Company for the preparation of the FRAP solution.

2.3. Determination of Standard Curve. A total of Rutin standard solution was prepared by dissolving Rutin reference material in 70% ethanol; Rutin solution (0.0 mL, 1.0 mL, 2.0 mL, 4.0 mL, 6.0 mL, 8.0 mL, and 10.0 mL) was accurately pipetted into 25 mL volumetric flasks, respectively. Then, 12 mL of the 70% ethanol and 2 mL of the sodium nitrate solution were added. The mixture was shaken up and placed for 10 min, followed by the addition of 2 mL 10% nitric acid solution, and was shaken up. After 10 min, 20 mL sodium hydroxide was added to scale. The mixture was deposited for 5 min, and then the absorbance of the solution was measured at 500 nm with the reagent blank as reference. Make the standard curve with the concentration of Rutin standard solution as abscissa and absorbency as vertical; the regression equation was

\[ Y = 8.3968x - 0.0043, \quad R^2 = 0.9998. \]

2.4. Single Factor Experiment. Fixed extraction conditions are as follows: ultrasonic time 20 min, material solvent ratio 1: 20, ethanol concentration 30%, ultrasonic temperature 60°C, ultrasonic power 500 W, and one-time extraction. The supernatant was taken after being centrifuged for 10 min under 3000 r/min, and then the absorbance was measured according to 2.1. Extraction time (10 min, 15 min, 20 min, 25 min, and 30 min), material liquid ratio (1: 5, 1: 10, 1: 15, 1: 20, and 1: 25), and ethanol concentration (20%, 30%, 40%, 50%, and 60%) were selected as the key variables. Their impact on the yield of the total flavonoids was tested separately:

Among them, the flavonoids content \( C \) (mg/mL) = \( X \cdot \text{diluted multiples; flavonoids yield} \%) = \frac{[C \cdot V]}{1000 W} \times 100 \% ; \\
X: \text{the concentration of flavonoids, calculated by standard curve} (\text{mg/mL}); \\
W: \text{sample quality} (g); \\
V: \text{the original volume of extracting solution} (\text{mL}).

2.5. The Response Surface Analysis Factor Levels Design [12]. According to single factor experiments, coded level of the three factors for Box-Behnken design of RSM were settled as follows: ethanol concentration (20%, 30%, and 40%), extraction time (15 min, 20 min, and 25 min), and material liquid ratio (1: 10, 1: 15, and 1: 20).

2.6. FRAP Method to Determine the Antioxidant Activity of Flavonoids [13, 14]

2.6.1. Antioxidant Activity Determination. The total flavonoids were vacuum-concentrated and dried after being extracted under the optimal extraction conditions, and then the crude flavonoids were obtained and certain concentration of flavonoids solution was prepared with distilled water. A volume of 0.3 mL sample solution was pipetted, and 2.7 mL FRAP solution (preheated to 38°C, prepared by 10 mmol/L TPTZ working solution, 20 mmol/L FeCl₃–6H₂O, 0.3 mol/L H₂SO₄, and 0.3 mol/L Hac buffer at 1: 1: 10, pHi = 3.6) was added. The mixture was shaken up and placed for 15 min. We keeping record of the absorbance values at 593 nm. Absolute ethanol was used as blank control for zeroing. FeSO₄ concentration (μmol/L) could be obtained from the standard curve according to the absorbance and defined as FRAP value. Sample with higher FRAP has higher antioxidant ability.

2.6.2. Determination of FeSO₄ Standard Curve. A volume of 6.08 mg FeSO₄ was dissolved with distilled water, 0.25 mL 18 mol/L H₂SO₄ was added, and the mixture was diluted with distilled water to the scale of 50 mL, and then an iron nail was put into it. 5 mL of the solution was pipetted into 50 mL volumetric flask and was diluted with distilled water to the scale to make 800 μmol/L FeSO₄ standard solution. The 200, 400, and 600 μmol/L standard solutions were prepared sequentially. Regression equation of the standard curve was obtained as \( y = 0.0021x + 0.3210, \quad R^2 = 0.9996. \)

3. The Results and Analysis

3.1. Single Factor Results

3.1.1. Effect of Extraction Time. From Figure 1, the extraction yield of total flavonoids increased with the extension of time and reached maximum value at 20 min, but after 20 min, the yield decreased, which might be due to denaturation of the total flavonoids through long period of ultrasonication.

3.1.2. Effect of Solid Liquid Ratio. From Figure 2, as ethanol scaled up, the extraction yield increased with the increase of the dissolved extracts, which reached top at ratio of

![Figure 1: Effects of extraction time on yield of total flavonoids.](image1)

![Figure 2: Effects of material solvent ratio on yield of total flavonoids.](image2)
3.1.3. Effect of Ethanol Concentration. From Figure 3, the extraction yield of total flavonoids increased gradually and reached top at 30%. Then the yield dropped sharply, caused by enhanced volatilization with high ethanol concentration.

3.2. The Response Surface Method to Optimize the Extraction

3.2.1. The Response Surface Analysis Factor Levels of Design Results. Based on single factor experiments, extracting time, material solvent ratio, and ethanol concentration were selected, to study the effects of different combinations of the three factors by Box-Behnken design of RSM adopting Design-Expert V8.0.6 (Stat-Ease, Inc.) (Table 1).

3.2.2. Regression Model of Flavonoids Extraction Yield and the Significance Test. The regression equation was obtained as

\[ R_1 = 1.08 + 8.750e - 003 \times A + 0.058 \times B + 0.044 \times C \\
- 0.048 \times A \times B + 0.080 \times A \times C + 2.500E \times A \times C - 0.01 \times A_2 - 0.073 \times B_2 - 0.020 \times C_2. \]  

Variance analysis results suggested that the effect of the factors in flavonoids extraction yield were B, C, and A in turn. While material solvent ratio and extracting time had significant effects on the total flavonoids extraction rate, ethanol concentration showed little effect. Effect of cross-term AC was significant, indicating the significant interaction of ethanol concentration and material solvent ratio. From Table 2, the relationship of the regression curve line with the actual statistical data was well described and used to determine the optimum conditions.

3.2.3. Response Surface Analysis. Comparison of Figures 4–6 showed that extracting time played a critical role for achieving higher extraction yields, observed as steep curve line with optimum value of 21 min. This may due to the strengthened medium motion caused by ultrasound and increased effect of vibrating homogenization and cavitation, which accelerated the dissolution of the total flavonoids and improved the extraction rate. The flat curve line and nonsignificant response value change of the other two factors (ratio of material solvent ratio and ethanol concentration) indicated their little influence on the flavonoids yield. Their optimum values were about 1:20 and 20 min, respectively.
Table 2: Variance analysis of the regression model.

<table>
<thead>
<tr>
<th>Sources of variance</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>The mean square</th>
<th>F</th>
<th>Pr &gt; F</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.15</td>
<td>9</td>
<td>0.016</td>
<td>3.29</td>
<td>0.0655</td>
<td>*</td>
</tr>
<tr>
<td>A-Ethanol concentration</td>
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<td>1</td>
<td>$6.125 \times 10^{-4}$</td>
<td>0.12</td>
<td>0.7371</td>
<td></td>
</tr>
<tr>
<td>B-extracting time</td>
<td>0.026</td>
<td>1</td>
<td>0.026</td>
<td>5.27</td>
<td>0.0553</td>
<td>*</td>
</tr>
<tr>
<td>C-material solvent ratio</td>
<td>0.015</td>
<td>1</td>
<td>0.015</td>
<td>3.05</td>
<td>0.1242</td>
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</tr>
<tr>
<td>$AB$</td>
<td>$9.025 \times 10^{-3}$</td>
<td>1</td>
<td>$9.025 \times 10^{-3}$</td>
<td>1.80</td>
<td>0.2218</td>
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<tr>
<td>$AC$</td>
<td>0.026</td>
<td>1</td>
<td>0.026</td>
<td>5.10</td>
<td>0.0484</td>
<td>*</td>
</tr>
<tr>
<td>$BC$</td>
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<td>1</td>
<td>$2.500 \times 10^{-5}$</td>
<td>$4.982 \times 10^{-3}$</td>
<td>0.9457</td>
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</tr>
<tr>
<td>$A_B$</td>
<td>0.042</td>
<td>1</td>
<td>0.042</td>
<td>8.39</td>
<td>0.0231</td>
<td>*</td>
</tr>
<tr>
<td>$B_C$</td>
<td>0.022</td>
<td>1</td>
<td>0.022</td>
<td>4.41</td>
<td>0.0739</td>
<td>*</td>
</tr>
<tr>
<td>$C_B$</td>
<td>$1.684 \times 10^{-3}$</td>
<td>1</td>
<td>$1.684 \times 10^{-3}$</td>
<td>0.34</td>
<td>0.5805</td>
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<tr>
<td>The residual error</td>
<td>0.035</td>
<td>7</td>
<td>5.018</td>
<td>$10^{-3}$</td>
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<td>Loss of quasi item</td>
<td>$7.725 \times 10^{-3}$</td>
<td>3</td>
<td>$2.575 \times 10^{-4}$</td>
<td>0.38</td>
<td>0.7762</td>
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<tr>
<td>Pure error</td>
<td>0.027</td>
<td>4</td>
<td>$6.850 \times 10^{-3}$</td>
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<td></td>
</tr>
<tr>
<td>Sum</td>
<td>0.18</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* means the difference is significant at the 0.05 level.

3.2.4. The Determination of the Optimal Process Conditions and Validation. The optimal conditions were determined by Design-Expert as follows: extraction time 21.45 min, material solvent ratio 1:20, and ethanol concentration 33.75%. Under the optimum conditions, the extraction yield of total flavonoids was 1.13%, which allowed higher extraction yields with lower temperature and extraction time when compared with conventional solvent extraction methods. With stable results, this method offered a theoretical basis for industrial and experimental extraction of total flavonoids from corn silk. FRAP value of the total flavonoids extracted from corn silk was determined as 467.59 μmol/L, which indicated good antioxidant activities of the total flavonoids from corn silk.

3.3. To Generate the Determination Results of Flavonoid Antioxidant Capacity. The FRAP value is 467.59 μmol/L, which indicated that the total flavonoids of corn silk have good antioxidant activity.

4. Conclusions

In this study, the optimization of ultrasound-assisted extraction of total flavonoids from corn silk and evaluation of their antioxidant activity were conducted. On the basis of single-factor test and Box-Behnken experimental design, the quadratic regression model was established to fit the experiment data in good effect. Through response surface methodology (RSM) of yield, the optimal conditions were determined as follows: extraction time 20 min, solid-liquid ratio 1:20, and ethanol concentration 30%. Under the optimum conditions, the extraction yield of total flavonoids was 1.13%, which allowed higher extraction yields with lower temperature and extraction time when compared with conventional solvent extraction methods. With stable results, this method offered a theoretical basis for industrial and experimental extraction of total flavonoids from corn silk. FRAP value of the total flavonoids extracted from corn silk was determined as 467.59 μmol/L, which indicated good antioxidant activities of the total flavonoids from corn silk.

Conflict of Interests

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

Authors’ Contribution

Ling-Li Zheng and Guan Wen contributed equally.

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References


Review Article

Health, Wellness, and Safety Aspects of the Consumption of Kombucha

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Functional foods have been identified as whole foods and fortified, enriched, or enhanced products which have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels. As consumer awareness on functional food escalates, the interest towards conducting scientific studies in this field has also proportionately increased. Many of the traditional food products are known to possess bioactive components, thus qualifying as functional food. Kombucha tea is produced by fermenting sugared black tea with a mixed culture of yeast and bacteria. Kombucha tea has gained immense popularity in recent times due to many associated health benefits. The therapeuticeffects of this beverage are thought to be derived from the chemical composition of this beverage, mainly the polyphenols and secondary metabolites which are produced during fermentation. However, the safety aspects of the beverage also need to be taken into account when qualifying the beverage as a functional food. Nevertheless, Kombucha tea could be easily recognized as a beverage which is able to replace the consumption of carbonated beverages due to its possession of health benefits and therapeutic properties.

1. Kombucha: Preparation and Fermentation Process

Kombucha tea is known under different names throughout the world such as red tea fungus, Champignon de longue vie, Ling zhi, kocha kinoko, Chainii grib, and Chainii kvass [1]. It is traditionally prepared by fermenting sugared black tea with a symbiotic culture of yeast and bacteria. This beverage is thought to have originated in China over 2000 years ago [2], while there are many historical reports of this beverage being consumed in countries such as Russia, Germany, and the Middle East as well [3]. In many countries this beverage is produced in large-scale for commercial use as well as in domestic conditions. Despite being a fermented beverage, the flavor of Kombucha tea is considered to be satisfactory and nonacrimonious, though mildly acidic and mildly alcoholic, similar in taste to apple cider [4]. As the fermentation progresses, the taste of Kombucha tea changes to a mild vinegar-like taste, thus increasing the consumer acceptability of the flavor and other sensory aspects of the beverage [4, 5]. The microbial composition of the Kombucha tea culture is known to vary from one culture to another depending on factors such as geographic location, climate, the local species of bacteria and yeast, and the source of the inoculum [6]. In some studies it has been proven that the use of different Kombucha starter cultures can cause a development of different antioxidant activity pathways even though the same substrate has been used [1, 7].

The yeast and bacteria involved in this microbial fermentation form a mat-like pellicle known as a “tea fungus.” The yeast component of this culture commonly consists of osmophilic yeast species, while the bacterial component includes acetic acid bacteria. In many studies it has been found that the dominant acetic acid bacterial species found in the microbial cultures are Acetobacter xylinum, A. xylinoideas, A. aceti, A. pausterianus, and Bacterium gluconicum [5]. The dominant yeast strains were found out to be Kloeckera
Approximately 50 g of white sugar is dissolved in 1 liter of boiling water

Approximately 5 g of tea leaves are added to the mixture and allowed to infuse for 5 min

The tea leaves and filtered and allowed to cool to room temperature

The cooled tea is added to a sterile glass jar and inoculated with the freshly grown Kombucha starter culture

The fermentation is allowed to be carried out

The pellicle which is formed is removed and the liquid portion is strained away for consumption

**Figure 1:** The typical method of preparation of the Kombucha beverages using sugared black tea.

spp., *Schizosaccharomyces pombe*, *Saccharomyces ludwigii*, *S. cerevisiae*, *Torulaspora* spp., *Zygosaccharomyces bailii*, and *Pichia* spp., [5]. The fungal-like structure is formed by the presence of these microbes in a zoogal mat. This mat is produced due to the formation of a thin layer of floating cellulose to which the cell mass of bacteria and yeast is attached [6]. The cellulose is produced by the bacterial component of the microbial consortium. *A. xylinum* is primarily known to be responsible for the cellulose production and this cellulose network enhances the association between the bacteria and the fungi [8]. It has been reported that the caffeine and related xanthines found in tea have the ability to stimulate the synthesis of this cellulose production by the bacteria [8]. This powerful symbiotic association and its byproducts have the ability to inhibit the growth of potential contaminating bacteria [6]. The bacterial cell and yeast cell numbers are generally thought to reach $10^4$–$10^6$ cfu mL$^{-1}$ in a Kombucha culture which has been allowed to ferment for a span of approximately 10 days [9]. Many scientific studies have proven that yeast outnumbers the bacterial count [5, 10]. As the fermentation progresses, the acidity of the broth increases due to the production of organic acids. Due to high acidity-induced oxygen starvation, the number of viable microbial cells present in Kombucha tea decreases as well [10]. The number of yeast and bacterial cells in the broth has been reported to be more than the cell number in the cellulolic pellicle [5].

In terms of the traditional preparation, the starter culture is added to a sugared black tea infusion and the fermentation is allowed to happen for a period of 3 to 10 days under ambient temperature [6]. A flow-chart containing the general preparation method is shown in Figure 1. Nevertheless, under domestic conditions of preparation, the amount of tea used for the fermentation process and the method of preparation differ according to personal preferences. The flavor and concentration of the compounds found in this beverage also differ with the starter culture, amount of starter culture used, type of tea, and type of sugar [6]. Even though traditionally black tea is being used as the substrate, Kombucha prepared by other substrates such as green tea and oolong tea are commonly available. Green tea has shown to have a better stimulation effect on the Kombucha fermentation compared with black tea fermentation and this leads to formation of the product in a shorter period of time [11, 12]. In some rare instances this beverage has been prepared by lemon balm tea, mulberry tea, jasmine tea, and peppermint tea [6, 11, 13]. The resulting broth is filtered out and the liquid portion is consumed. The tea fungus which is initially added to the tea is called the “mother tea fungus,” where during the fermentation the development of a “daughter tea fungus” takes place. The cellulosic pellet rests on top of the tea broth and produces a fresh thin layer of the daughter mat which is available as a new layer above the old Kombucha culture mat. This new daughter mat is formed with each successful fermentation step, and it is used to reinoculate a new batch of tea. At the beginning of the fermentation, a small portion of previously prepared Kombucha broth may be added to the new tea to decrease the pH in order to stop the growth of undesired microorganisms [8]. As the fermentation progresses the appearance of gas bubbles will occur due to carbonic acid produced during the fermentation.

### 2. Composition of Kombucha Tea and How It Differs from Black Tea

The microbes in this culture are able to ferment the sugared black tea and produce a complex cocktail of molecules. At the end of the fermentation process, the resulting beverage would consist of sugars, polyphenols such as catechins, organic food acids, lysine, fiber, ethanol, amino acids, essential elements such as Na, K, Ca, Cu, Fe, Mn, Ni, and Zn, water-soluble vitamins such as vitamin C, vitamin B, and vitamin B$_2$, catalase, carbon dioxide, substances which act as antibiotic substances, and some hydrolytic enzymes [1, 14]. The chemical structures of the commonly produced acids are shown in Figure 2. As the fermentation progresses, the yeast component of this mixed culture is able to break down sucrose to produce glucose, fructose, and carbon dioxide which gives off the effervescence and the sparkling appearance. The acetic acid bacteria have the ability to convert glucose to gluconic acid and fructose in to acetic acid. Also, the yeast component is able to produce ethanol which is then oxidized into acetalde-hyde by the bacterial counterpart of this colony [15]. The yeast prefers fructose as the substrate when producing ethanol [8]. The acetic acid produced by the acetic acid bacteria has the ability to stimulate the production of ethanol by yeast, and ethanol in turn can facilitate the acetic acid bacteria to grow and produce acetic acid. The ethanol and acetic acid present in the Kombucha broth have been reported to be involved in the antimicrobial activity of the broth against pathogenic bacteria, thus providing a protection against contamination of the tea fungus [8]. It has been reported that even if the byproducts of the fermentation process decrease, the pH...
value of this beverage has a buffering capacity. This buffering capacity is due to carbon dioxide dissociation, and when this process happens, production of amphiprotic hydrocarbonate anion (HCO$_3^-$) occurs. This anion has the ability to react with hydrogen ions (H$^+$) from organic acids and inhibit further change of H$^+$ concentration in the broth and thus contribute to formation of buffer in the system [16].

Even though Kombucha is based on the preparation of black tea, other than the fermentation process, it is considered different due to the chemical contents themselves. Tea (Camellia sinensis), in general, is one of the most popular beverages used worldwide and it has been consumed by many, for centuries. There are three major types of teas, namely, black, green, and oolong. Out of these three types, black tea is the most popular and it accounts for nearly 80% of the tea consumed worldwide [15]. This form of tea is prepared by the infusion of dried leaves of Camellia sinensis in hot water. The tea leaves, after picking, are air dried then crushed to release the enzymes and then allowed for enzymatic fermentation. Then, they are dried again to obtain the final product. Some of the main components of tea are purine alkaloids such as caffeine, theaflavins, gallotannins, triterpene, saponins, flavonoids, mineral compounds, carbohydrates, and vitamins. Out of these three types, black tea is the most popular and it accounts for nearly 80% of the tea consumed worldwide [15]. This form of tea is prepared by the infusion of dried leaves of Camellia sinensis in hot water. The tea leaves, after picking, are air dried then crushed to release the enzymes and then allowed for enzymatic fermentation. Then, they are dried again to obtain the final product. Some of the main components of tea are purine alkaloids such as caffeine, theaflavins, gallotannins, triterpene, saponins, flavonoids, mineral compounds, carbohydrates, and vitamins [15, 16]. Black tea is known to possess a higher radical scavenging property and the major contributor of this property is known to be polyphenols [17]. Depending on the geographical location, climate, season, soil fertility, and plantation method, a variation can be seen in this particular property [17]. Kombucha tea differs from its parental food by the antioxidant content, starch hydrolase inhibitory activity, anionic mineral composition, and acid content. Kumar et al. [18] have demonstrated the presence of the anionic minerals such as fluoride, chloride, bromide, iodide, phosphate, sulphate, and nitrate in both black and Kombucha tea. Table I shows a comparison of anionic mineral concentrations of Kombucha and Black tea. They have further revealed that the anionic mineral compositions of Kombucha and black tea are significantly different from each other [18]. Kombucha tea is known to exhibit an increase in the radical scavenging properties during the fermentation process, showing a higher antioxidant capacity than black tea [19].

3. Health Benefits Associated with Consumption of Kombucha

The American Diabetic Association has defined functional food to be products including whole foods and fortified, enriched, or enhanced foods, which have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels [20]. Kombucha beverage is known to possess many prophylactic and therapeutic benefits; it is believed to help in digestion, give relief against arthritis, act as a laxative, prevent microbial infections, combat stress and cancer, provide relief against hemorrhoids, impart a positive influence on the cholesterol levels, and facilitate excretion of toxin as well as blood cleansing [1, 6, 8]. This beverage is also associated with influencing the gastrointestinal microbial flora in humans by acting as a probiotic drink and helping in balancing the intestinal flora, thus facilitating the normalization of intestinal activities to a certain extent [1, 21, 22]. It is also known to have the

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Table 1: Comparison of anionic mineral concentration of Kombucha tea and Black tea [7].

<table>
<thead>
<tr>
<th>Anion</th>
<th>Kombucha tea (mg/g)</th>
<th>Black tea (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F$^-_3$</td>
<td>3.20 ± 0.16</td>
<td>1.20 ± 0.06</td>
</tr>
<tr>
<td>Cl$^-_3$</td>
<td>0.96 ± 0.04</td>
<td>3.12 ± 0.13</td>
</tr>
<tr>
<td>Br$^-_3$</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>NO$_3^-_3$</td>
<td>0.18 ± 0.01</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>HPO$_4^{2-}_2$</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>SO$_4^{2-}_2$</td>
<td>1.02 ± 0.04</td>
<td>4.20 ± 0.17</td>
</tr>
<tr>
<td>I$^-_3$</td>
<td>1.04 ± 0.08</td>
<td>0.44 ± 0.04</td>
</tr>
</tbody>
</table>

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**Figure 2**: The most abundant organic acids found in Kombucha tea as a result of the fermentation process.
ability to improve the health of hair, skin, and nails, reduce stress and nervous disturbances, reduce insomnia, relieve headaches, reduce the craving for alcohol of an alcoholic person, and prevent the formation of bladder infections [6, 8]. Reducing the kidney calcification is also known to be a beneficial effect of this beverage [6]. Reduction of menstrual disorders and menopausal hot flashes, improving eye sight, cellular regeneration, stimulation of glandular systems in the body, relieving bronchitis and asthma and the enhancement of general metabolism are a few more of the health benefits which have been claimed to be associated with consumption of the Kombucha broth [6]. The beneficial effects of this beverage are known to be attributed to the presence of the metabolic products released into the broth during the fermentation, although most of the health benefits are hypothesized to be due to its radical scavenging potential. The microbial community has the ability to enhance the radical scavenging activity of black tea by the fermentation process involved in producing the beverage, the review also summarizes the safety issues and aspects of caution which need to be borne in mind when consuming the beverage.

### 3.1. Antimicrobial Activity.

Kombucha tea is known to show a remarkable antimicrobial activity against a broad range of microorganisms. Many scientific studies have been done on this subject and the Kombucha broth has demonstrated inhibitory activity against many pathogenic microorganisms of both Gram positive and Gram negative origin [8]. Kombucha tea has demonstrated the ability to inhibit the growth of pathogens such as *Helicobacter pylori* (the causative organism of peptic ulcers), *Escherichia coli* (the causative organism of common diarrhea), *Entamoeba coliaceae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Agrobacterium tumefaciens*, *Bacillus cereus*, *Aeromonas hydrophila*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella sonnet*, *Leuconostoc monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, and *Candida albicans* [8, 24, 25]. This antimicrobial activity of the broth is attributed to the low pH value of this beverage, especially owing to the presence of acetic acid in particular and a range of other organic acids and catechins which are shown in Figure 2 as well as many large proteins which are produced during the fermentation [8, 24]. Acetic acid and catechins are specially known to inhibit a range of Gram positive and Gram negative microorganisms [6]. It has also been demonstrated that the broth may contain antibiotic substances which give the antimicrobial property [24, 26]. Some studies have demonstrated that Kombucha tea shows not only antibacterial activity but also antifungal activity [27]. The antifungal activity is attributed to the production and

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### Table 2: Some constituents of Kombucha related to health claims and their recommended values for consumption.

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Amount present in Kombucha green tea (g/L)</th>
<th>Amount present in Kombucha black tea (g/L)</th>
<th>Recommended maximum level as per standard guidelines</th>
<th>LD₅₀ value in rats via oral route (mg/kg of body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>9.51 ± 0.35 (on day 15) [33]</td>
<td>6.17 ± 0.3 (on day 15) [33]</td>
<td>2.1 g per day [62]</td>
<td>3310 [63]</td>
</tr>
<tr>
<td>Glucuronic acid</td>
<td>1.57 ± 0.14 (on day 15) [33]</td>
<td>1.5 ± 0.17 (on day 15) [33]</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.15 ± 0.02 (on day 15) [33]</td>
<td>0.33 ± 0.07 (on day 15) [33]</td>
<td>FDA requirement substance added directly to the human food affirmed as generally recognized as safe (GRAS) [64]</td>
<td>3730 [65]</td>
</tr>
<tr>
<td>Citric acid</td>
<td>Not available</td>
<td>Not available</td>
<td>3000 mg/kg per day [66]</td>
<td>3000 [67]</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>0.03 (on day 3) [33]</td>
<td>0.11 (on day 3) [33]</td>
<td>0.14 mg/kg per day (the scientific basis for this conclusion is not available and therefore no safe dietary dose for oxalic acid can be established) [68]</td>
<td>7500 [69]</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Not available</td>
<td>Not available</td>
<td>0–2.5 mg/kg per day [70]</td>
<td>661 [71]</td>
</tr>
</tbody>
</table>
presence of acetic acid in this beverage. In recent times, the emergence of resistant strains of pathogens associated with human diseases has been widely seen, and the use of Kombucha tea as an antimicrobial product can be used to overcome this problem [27]. In this aspect, it has been demonstrated that the antimicrobial activity of Kombucha tea prepared from green tea shows a higher activity than Kombucha tea prepared traditionally from black tea [27].

3.2. Probiotic Effects. Probiotics are known to be living microorganisms; when administrated in adequate amounts, they are able to result in health benefits. Most often, the bacterial component of a probiotic mixture comes from *Lactobacillus* or *Bifidobacterium* or a cocktail of these two strains. In support of these lines, there can be a few common yeast types such as *Saccharomyces boulardii* and *S. cerevisiae* in this mixture as well [14]. Probiotic microbes are known to play a vital role in the wellness of human health. Probiotic microorganisms provide a balance in intestinal microbiota, normalizing processes in gut and boosting the immune system. In addition, they help in improving digestion, fighting against harmful bacterial overgrowths, and achieving mental clarity and mood stability and against psychological conditions such as anxiety and depression. Many studies have claimed that this beverage not only is a probiotic but also acts as a symbiotic, a combination of prebiotics and probiotics [8, 21]. A prebiotic selectively helps the growth and activity of the consortium of beneficial microbes present in the human gut [21]. The bacteria and yeast present in this beverage act as probiotics and the microcellulose which is present can help in the growth of the beneficial microbes present in the intestine [14]. The popularity of this beverage as a probiotic and a symbiotic has increased in recent times as scientists have found that this beverage can be used to give the required nutrition and help maintain health and wellness in humans who work under unhealthy environments, such as workers in mines and polar expeditors [14]. When the human body is exposed to such conditions for a prolonged period of time, the normal microbial consortium of microbes in the intestine changes due to the unnatural conditions, psychoemotional discomfort, and drastic change in the diet. This may lead to the disappearance of the protective gut microbes and the emergence of harmful secondary infections by opportunistic microbes. This shift in the gut microbiota can lead to many health issues such as allergies, autoimmune diseases, multiple sclerosis, and transplant infectious disease. The change in the gut microbiota can be corrected to some extent with the help of Kombucha tea. In light of these mentioned possibilities, scientists have started to consider this beverage to be used by astronauts as a supplement to their diet in outer space [14].

3.3. Anticancer Properties. Dietary phytochemicals have been identified as effective anticancer agents. Thus, there is a recent trend of consuming food rich in these bioactive compounds. Scientific studies have claimed that Kombucha has anticancer effects as well [8]. The Central Oncological Research Unit in Russia and the Russian Academy of Sciences in Moscow have conducted population studies on this fermented beverage and have found that the daily consumption of Kombucha broth has a correlation with an extremely high resistance to cancer [8]. Scientists have come up with many possible mechanisms for the anticancer ability of this beverage. For instance, it has been reported that the ability of this fermented beverage to act as an anticancer agent is due to the presence of tea polyphenols and the secondary metabolites produced during the fermentation process [6, 28]. Many studies have shown that the ability of the tea polyphenols present in this fermented beverage to inhibit gene mutations, inhibit the proliferation of cancer cells, and induce cancer cell apoptosis and the ability to terminate metastasis have been highlighted.

<table>
<thead>
<tr>
<th>Bioactivities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antihypercholesterolemic effect</td>
<td><em>In vitro</em> studies on rats [33]</td>
</tr>
<tr>
<td>Antioxidant activity against chromate</td>
<td><em>In vivo</em> studies on rats [47]</td>
</tr>
<tr>
<td>Antioxidant activity against lead</td>
<td><em>In vivo</em> studies on rats [3]</td>
</tr>
<tr>
<td>Antistress activity against cold and hypoxia</td>
<td><em>In vivo</em> studies on rats [46]</td>
</tr>
<tr>
<td>Cytogenetic activity</td>
<td><em>In vitro</em> studies on human peripheral blood lymphocytes [72]</td>
</tr>
<tr>
<td>Healing activity against indomethacin-induced acute gastric ulceration</td>
<td><em>In vivo</em> studies on mice [50]</td>
</tr>
<tr>
<td>Hypoglycaemic effect</td>
<td><em>In vivo</em> studies on mice [6, 73]</td>
</tr>
<tr>
<td>Inhibitory activity towards CCl₄ induced hepatic injury</td>
<td><em>In vivo</em> studies on rats [45]</td>
</tr>
<tr>
<td>Longevity</td>
<td><em>In vivo</em> studies on mice [74]</td>
</tr>
<tr>
<td>Paracetamol induced hepatotoxicity</td>
<td><em>In vivo</em> studies on mice [47]</td>
</tr>
<tr>
<td>Prevention of weight loss in diabetes</td>
<td><em>In vivo</em> studies on rats [75]</td>
</tr>
<tr>
<td>Protective effect on chromosomal aberrations induced by gamma radiation</td>
<td><em>In vitro</em> studies on human peripheral lymphocytes [76]</td>
</tr>
<tr>
<td>in human peripheral lymphocytes</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Bioactivities of Kombucha and mode of studies which have been carried out.
Detoxification is the complex process of removal of toxic substances from the body of a living organism. This process can be physiological or medicinal. In the human body, this process is carried out mainly by the liver. Detoxification helps in the maintenance of a healthy liver and is also known to play a part in cancer prevention. The enzymes, bacterial acids, and other secondary metabolites produced by the microbes during the fermentation process done in the preparation of Kombucha tea have displayed the ability to detoxify body [8]. In addition, most of the enzymes and bacterial acids found in Kombucha tea are very similar to the chemicals produced by the body for the purpose of the detoxifying process. Thus, incorporation of Kombucha tea into one's diet may result in the reduction of toxic substances in the body [8].

3.4. Detoxification. Detoxification is the complex process of removal of toxic substances from the body of a living organism. This process can be physiological or medicinal. In the human body, this process is carried out mainly by the liver. Detoxification helps in the maintenance of a healthy liver and is also known to play a part in cancer prevention. The enzymes, bacterial acids, and other secondary metabolites produced by the microbes during the fermentation process done in the preparation of Kombucha tea have displayed the ability to detoxify body [8]. In addition, most of the enzymes and bacterial acids found in Kombucha tea are very similar to the chemicals produced by the body for the purpose of the detoxifying process. Thus, incorporation of Kombucha tea into one's diet may result in the reduction of toxic substances in the body [8].

3.5. Antioxidant Activity. The popular definition of an antioxidant is any substance, when present at low concentrations compared with that of an oxidizable substrate, that significantly delays or inhibits oxidation of the substrate [36]. This bioactivity could be broadly presented in the form of (1) scavenging properties of molecules, (2) binding of prooxidant metals, and (3) inhibition of prooxidant enzymes. Many studies have proven the effect of these antioxidant properties on many human diseases such as cancer and diabetes [7]. The primary mechanism of the action of antioxidants in these disease conditions is to remove free radical intermediates, and these free radicals are generated in oxidation reactions which happen across the human body. Free radicals have the ability to start multiple chain reactions which will eventually lead to cell damage or the death of the affected cell [36]. When an antioxidant comes in contact with free radicals, they have the ability to oxidize themselves and inhibit other oxidation reactions which lead to harmful chain reactions. The oxidative stress caused by free radicals plays an important role in many of the commonly prevalent human diseases such as Parkinson's disease, coronary heart disease, and cancer, the reasons being the lack of appropriate nutrition and exercise, air pollution in the environment, and smoking [2]. In order to counterbalance this oxidative stress caused by free radicals, it is important to incorporate antioxidant containing food stuff to the daily diet [36].

During the Kombucha fermentation, many compounds with radical scavenging properties are released from the tea leaves themselves [1]. Polyphenols and catechins are the main group of compounds which are found in tea belonging to flavanol group [1, 27, 37]. Polyphenols are considered as having high levels of broad antioxidant properties since they have the ability to scavenge free radicals and reactive oxygen species (ROS) [2]. Polyphenols are about 30% of the total dry weight of fresh tea leaves and epigallocatechin, epigallocatechin-3-gallate, epicatechin-3-gallate, and epicatechin are the most prominent types of polyphenols found in tea leaves [19]. Kombucha tea when prepared using green tea, black tea, and tea waste material has been shown to have a high radical scavenging activity [19]. When the complex phenolic compounds are present in an acidic environment or when enzymes liberated by bacteria and yeasts in tea fungus are present, degradation of complex molecules to small molecules happens and this causes an increase in the total phenolic compounds available in the Kombucha tea broth [2]. Therefore, when the fermentation happens, the total phenolic content increases [2]. The production of compounds possessing radical scavenging properties depends on the culture period and starter origins where they decide which metabolites are to be produced [7]. However, prolonged fermentation is not suitable as accumulation of organic acids can cause a harmful effect when Kombucha tea is directly consumed [2].
3.6. Hepatoprotective Effects. Hepatoprotection is the ability to prevent the damage occurring to the liver by toxic substances [22, 38]. Many studies carried out on cell lines and animal models have shown that Kombucha broth shows hepatoprotective activity against various environmental pollutants [6]. Many of the environmental pollutants have the ability to induce hepatotoxicity and damage the liver. Many scientific studies were carried out to assess the ability of this tea broth to effectively attenuate the physiological changes which are caused by many of the hepatotoxicity-causing agents such as aflatoxin B1 [39], cadmium chloride [40], tert-butyl hydroperoxide [41], and acetaminophen [42, 43]. Carbon tetrachloride (CCl₄) is a xenobiotic that induces the lipid peroxidation and it forms a free radical CCl₅⁻, and this involves accumulating lipid derived oxidants which leads to liver injury [44]. Kombucha tea consumption has been demonstrated to inhibit the activity of CCl₄ and prevent liver injury in rats [45]. In vivo studies have suggested that Kombucha tea is capable of preventing paracetamol induced hepatotoxicity [46]. Studies have been carried out to investigate how Kombucha tea can induce oxidative stress in Albino rats by chromate (VI) [47]. Studies have also been carried out to find protective effects of Kombucha tea against thioacetamide-induced hepatotoxicity and the results have shown that the antioxidant activity of polyphenol substances of Kombucha tea is responsible for this function [22]. These studies have further explained that Kombucha tea prevents the apoptotic cell death of the hepatocytes which is triggered from the exposure of the liver to the environmental toxins [22]. Histological analyses of alloxan-induced diabetes rats given a diet containing Kombucha tea have revealed protective liver-kidney functions [48]. This is supported by the reduction in the activity of aspartate transaminase, alanine transaminase, and gamma-glutamyl transpeptidase in the plasma, as well as in the creatinine urea concentrations [48]. Pathophysiological evidences are also available for hepatoprotective effects of Kombucha tea in rat models [41, 43].

3.7. Other Therapeutic Effects. There are many allegations of health benefits and uses related to this beverage, some of which are not discussed in this review in further detail. For instance, the microbial mat produced in the fermentation had been used to produce artificial skin in Nossa Senhora da Conceição Hospital from Lagarto, SE, Brazil [49]. Some researchers have used this skin to accelerate the healing process and also as an antiseptic by adhering it to open injuries, the so-called Bioskin [49]. The bacterial cellulose which is produced during the Kombucha tea fermentation process has many potential applications in fields such as food and biopharmaceuticals. The ability and trend to use bacterial cellulose in these fields are due to the high purity and the unique physicochemical properties which are present in the fermented beverage. In addition, this bacterial cellulose is preferred in instances where plant based cellulose cannot be used [5]. In the food industry bacteria-based cellulose is used as food matrices, thickeners, dietary fibers, stabilizes, and binders [5]. Lactic acid is one of the organic acids produced during the Kombucha tea fermentation. It is able to enhance the blood circulation and helps prevent constipation [8]. Oxalic acid, which is also produced as a byproduct, can be useful in the production of adenosine triphosphate (ATP) [6]. Bacteria found in the Kombucha mat produce gluconic acid by breakdown of caprylic acid which can prevent certain types of yeast-based infections and candidiasis [33]. Butyric acid is a production of yeast available in the Kombucha mat and helps in protecting the human cellular membrane, where it combines with gluconic acid and strengthens the gut walls in conditions such as candidiasis [6]. Moreover, healing of gastric ulcers in rats due to Kombucha tea has been evident as per histopathological and biochemical studies [50]. The usage of this beverage to eliminate the growth of gray hair and the usage in the improvement of eyesight are a few of the health benefits which have been claimed as well [8]. Oral supply of Kombucha tea at a dose of 5 mg/kg of body weight in alloxan-induced diabetes rats has depicted better inhibition on α-amylase and lipase enzyme activity in the plasma and pancreas and also better suppression of increased blood glucose levels [48]. Thus, Kombucha tea has potential hypoglycemic and antilipidemic activity as well.

4. Safety Issues and Controlling Potential Hazards of Kombucha

Since Kombucha is a complex mixture of microorganisms, it is essential to discuss the safety of Kombucha tea for consumption. As mentioned previously, bacteria and fungi in the Kombucha zoogal mat are capable of forming a powerful symbiosis which can inhibit the growth of contaminating bacteria [8, 51]. Nevertheless, pathogenic microorganisms can contaminate the Kombucha tea throughout the preparation. Due to fermentation, the pH reaches ≤ 4.2. However, until this is achieved, there is a high possibility for contaminations to occur [52]. Mold contamination can occur on Kombucha cultures, especially with Penicillium and Aspergillus, when the Kombucha tea is home-made. Aspergillus species are known to cause carcinogenic and toxigenic effects [53]. Therefore, it is important to be cautious when administering infected beverages by immune-compromised individuals. It is essential to concern that that the efficacy of Kombucha tea as a therapeutic beverage is hardly proven by clinical trials involving human subjects [54]. Beside, even though consumption of Kombucha claims to have no adverse side effects, there have been a few exceptions over past years. Few case reports and case series question the safety of Kombucha tea with suspected liver damage, metabolic acidosis, and cutaneous anthrax infections [54]. Allergic reactions and an uncomfortable stomach are a result of consuming Kombucha tea by people with acid sensitivities and renal insufficiencies [53]. Several studies have confirmed that Kombucha can cause nausea, shortness of breath, throat tightness, headache, dizziness, and jaundice [34, 53]. One case revealed that two persons had developed allergic reactions, a third person developed jaundice, and another developed nausea, vomiting, and head and neck pain. The case reports an etiological association of all four patients since all of them
had consumed Kombucha tea in proximity to the onset of symptoms and their subsequent disappearance after cessation of drinking the beverage [55]. Some individuals have reported that they felt dizziness and nausea after consumption of Kombucha tea [56]. However, it was not explained whether these symptoms are a result of unusual toxins developed in a particular batch of Kombucha tea.

Overfermentation can increase the availability of high acetic acid concentrations, and this might lead to the leeching of some chemical contaminants from the fermentation vessel or packaging materials. There is evidence that severe lead poisoning can be caused by regular use of Kombucha tea which was brewed in a ceramic pot [57, 58]. This might have occurred due to Kombucha tea being acidic and the resulting reactions caused by some ceramics. Most of the ceramics contain very low levels of lead which would not be of any danger when brewing Kombucha tea. Nevertheless, if the Kombucha is steeped in them for a long time, then high amounts of lead can dissolve in the tea [6, 57]. It is important to use glass containers for the preparation and storage of Kombucha tea to prevent leaching of toxic elements such as lead into the beverage. In addition to acetic acid, Kombucha tea contains several organic acids [6, 33]. Some of these metabolites have the potential to damage liver and kidney at high concentrations, as evident by few case reports [56, 59, 60]. Kombucha tea is contraindicated in pregnant and lactating women [53]. Studies have also reported the presence of Bacillus anthrax in Kombucha tea fermented under unhygienic conditions [61]. The source of B. anthrax was found to be cows and anthrax was passed to an individual who rubbed it on his skin to alleviate the pain.

Due to the detoxification effects of Kombucha tea, toxic materials are forced to be excreted from the body. However, if the kidneys are not working properly, these individuals may not be able to successfully discharge the toxic materials. Thus, it is recommended to drink plenty of water to facilitate the elimination of toxins to overcome this problem [53]. From this aspect, it is essential to give attention to the abnormal odour or colour development in Kombucha tea to overcome any adverse side effects. Domestic cultivation of Kombucha is one of the most possible ways of contamination by pathogenic bacteria and yeasts. Since this tea fungus is being grown under aseptic conditions and it is propagated from one house to another, the transfer of contaminations is high [13, 53]. A study reported that consumption of Kombucha might also be a health risk for HIV-positive patients [6]. It was discovered that acute renal failure may occur with lactic acidosis and hyperthermia due to consumption of this beverage. The possibility of getting infected by toxins is higher when Kombucha tea is consumed in large quantities. Another report states that one individual died due to perforations of the intestinal tract and severe acidosis, who consumed home-made Kombucha tea 4 oz per day for two months [52]. Another individual who consumed Kombucha tea coming from the same initial zoogleal mat had suffered from cardiac arrest and severe acidosis [52]. Despite these reports, most of these studies are limited to a very small number of individuals. Hence, further studies should be carried out to form a substantial conclusion about the safety aspects in the consumption of Kombucha tea.

Using clean and sanitized utensils during preparation of Kombucha tea helps in preventing any contamination. It is better to keep the preparation and fermentation areas clean to control growth of any microorganisms since tea must be cooled to about 20°C within two hours prior to adding the Kombucha culture into the tea. It is also important to control the pH during the fermentation process since overproduction of acetic acid can be hazardous. According to the British Columbia Center for disease control, the fermentation should be terminated at the pH 4.2 [56]. Overproduction of alcohols and carbon dioxide can also be prevented by pasteurizing the finished product [57]. Adding 0.1% sodium benzoate and 0.1% potassium sorbate to the finished product, followed by refrigeration, can also be carried out for safety purposes [61]. Refrigeration of the final product is the most common method followed by the commercial producers of Kombucha tea [13].

5. Conclusions

The chemical composition of the Kombucha tea depends on type of tea leaf variety, amount of sugar, and fermentation and composition of tea fungus. However, there are a very few studies focused on the safety of consuming Kombucha tea. Most of the health properties of Kombucha are related to the acidic composition of the beverage and acetic acid, which is the major acidic component to inhibit the fungal growth. Overproduction of acids can be controlled during the fermentation duration as well as the packaging of the final product. Yet, the importance of studying the safety of Kombucha tea consumption is important as there are only a very few such studies been carried out throughout the years. Since this a popular beverage around the world, investigating the advantages and disadvantages of consuming this beverage in similar capacities can be extremely meaningful. According to literature, there is no evidence about systematic human trials being done using Kombucha tea. This could be an area in which future research could be focused in establishing this beverage as a functional food. Since studies carried out to assess qualitative and quantitative properties of constituents of Kombucha are scattered, scientific research should be carried out to clarify the health beneficial claims and safety aspects, which might help in promoting this beverage among consumers. Despite the scattered safety issues, production and consumption of this beverage in a safe manner can be used to substantiate the stand of this beverage as a replacement for carbonated beverages. Attention of relevant authorities should be focused on establishing national and international policies and regulations regarding the safety of Kombucha consumption and mass production of this beverage as a readily available commercial beverage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.
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Characterization of Calcium Compounds in Opuntia ficus indica as a Source of Calcium for Human Diet


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Analyses of calcium compounds in cladodes, soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) of Opuntia ficus indica are reported. The characterization of calcium compounds was performed by using Scanning Electron Microscopy, Energy Dispersive Spectrometry, X-ray diffraction, and infrared spectroscopy. Atomic Absorption Spectroscopy and titrimetric methods were used for quantification of total calcium and calcium compounds. Whewellite (CaC$_2$O$_4$·H$_2$O), weddellite (CaC$_2$O$_4$·(H$_2$O)$_{2.375}$), and calcite (CaCO$_3$) were identified in all samples. Significant differences ($P \leq 0.05$) in the total calcium contents were detected between samples. CaC$_2$O$_4$·H$_2$O content in cladodes and IDF was significantly higher ($P \leq 0.05$) in comparison to that observed in SDF, whereas minimum concentration of CaCO$_3$ was detected in IDF with regard to CaCO$_3$ contents observed in cladodes and SDF. Additionally, molar ratio oxalate:Ca$^{2+}$ in all samples changed in a range from 0.03 to 0.23. These results support that calcium bioavailability in O. ficus indica modifies according to calcium compounds distribution.

1. Introduction

In Mexico, 21% of people over 20 years have a deficiency in calcium intake. Calcium content in the Mexican diet covers only 50% of the recommended daily intake (1200 mg/day) [1]. Calcium deficiency causes skeletal system diseases, that is, osteoporosis, which is a public health problem, due to the fact that this disease is among the eight leading causes of hospital morbidity with a prevalence of 8% in the Mexican population [2]. For this reason, there is an amplified interest in monitoring and increasing consumption of dietary calcium. Moreover, recently, health professionals have been taking action on the matter [3].

In addition, it has been demonstrated that a high bioavailability of calcium from the diet improves bone health [4], although calcium is distributed in different foodstuffs such as milk and dairy foods that provide more than 80% of calcium to the human diet. Furthermore, the calcium bioavailability in milk and dairy foods is significant, and, due to this, mineral absorption is associated with absorption promoters such as
lactose among other factors [5]. In developing countries, calcium intake from dairy products is limited by the high costs of these foodstuffs, as well as the problems associated with lactose intolerance [6]. These facts restrict the consumption of animal products causing a reduction of calcium in the diet. Consequently, Mexico and Central American countries depend on nixtamalized products as their primary source of calcium in their diet [7]. Thus, it is necessary to propose alternative sources of calcium to improve daily intake of this mineral. *Opuntia ficus indica* cladodes (pads) represent a potential source of calcium in human diet, due to the fact that calcium content in pads increases with the growing stage [8, 9].

McConn and Nakata [10] observed a reduction in calcium availability in prickly pear cactus by using an *in vitro* assay; this was attributed in part to the presence of calcium oxalate crystals. In addition, Contreras-Padilla et al. [11] reported that the concentration of oxalate in *O. ficus indica* in different phases of maturity appears to have a cyclic tendency that could be determined by the presence of calcium content in the soil, the plant’s needs during active growth, and seasonal and environmental conditions.

On the other hand, several researches have shown the presence of calcium compounds in mucilage cell and cell walls of *Opuntia ficus indica* [12, 13]. However, there is no previous report about the distribution of these compounds extracted from the matrix of *O. ficus indica* with the purpose of increasing the bioavailability of calcium from this Cactaceae.

The goal of the present research was to characterize the distribution of calcium compounds in soluble and insoluble dietary fiber extracted from *O. ficus indica* cladodes at a late maturation stage (400 g of weight), in order to underline the potential of this cactus as a source of calcium to help the formation of bone mass. These findings will promote the utilization of powder of *O. ficus indica*, with no commercial value, due to the fact that this cactus is not consumed as a vegetable, which can be used as a dietary supplement with an affordable price to increase calcium intake in the Mexican population.

### 2. Materials and Methods

#### 2.1. Samples

*Opuntia ficus indica* cladodes were cultivated in an experimental field in Silao, Guanajuato (Rancho Los Lorenz), Mexico, with organic fertilizer and harvested during the spring of 2014. The *O. ficus indica* cladodes of 400 g (100 days of maturation stage) were washed with distilled water and the thorns were manually removed. Then, *O. ficus indica* cladodes were dehydrated by drying cladode slices (2 × 2 cm) in a force air oven (BG model E102). The dehydration process was carried out at 50°C during 70 minutes, each pan containing 5 kg of *O. ficus indica* slices. The dry material was milled using a hammer mill (PULVEX 200, DF, Mexico) equipped with a 0.8 mm screen.

#### 2.2. Chemicals and Reagents

Ethyl alcohol (95% v/v) reactive grade, hydrochloric acid analytical grade, nitric acid ultrapure, and distilled water were obtained from J. T. Baker (DF, Mexico). The Total Fiber Dietary Kit (TDF-1100 A) was purchased from Sigma (St. Louis, MO, USA) as also were oxalic acid and potassium bromide standards.

#### 2.3. Extraction of Soluble and Insoluble Dietary Fiber from *O. ficus indica*

The dried material was mixed with distilled water (4% w/w). This suspension was homogenized using a blender (IKA-WERKE, Mod. Eurostar BSC.S1) (450 rpm for 20 min). Subsequently, the suspension was left to stand for four hours to ensure hydration of the solids. Then, the suspension was placed in the feed tank of a disk centrifuge (DIDACTA Italia, Mod. TAGI/d), which was operated at 450 rpm. The speed of centrifuge was increased gradually until it reached 7000 rpm. Next, the feed valve of the centrifuge was opened to allow the flow of soluble solids through the gravity rings and the upper hopper of equipment, while the insoluble solids (insoluble fiber) were retained in the bowl of the centrifuge. The insoluble dietary fiber was dehydrated in Teflon pans at 80 kPa and 40°C in a vacuum oven (Barnstead International, Mod. 3618) for 35 min, until a humidity content of 4% (w/w). Soluble solids recovered were mixed with ethyl alcohol at 95% (v/v) in a 1:2 v/v ratio. This suspension was subjected to vacuum filtration at 4 kPa to remove excess water and alcohol in order to obtain the soluble dietary fiber. Finally, this precipitate was dehydrated at the same conditions as before.

#### 2.4. Separation of Oxalate Crystals

Suspensions of dried material (cladodes, soluble dietary fiber, and insoluble dietary fiber) and distilled water (4% w/w) were prepared. These suspensions were processed as was reported by Malainine et al. [14].

#### 2.5. Chemical Characterization

##### 2.5.1. Total, Soluble, and Insoluble Dietary Fiber Content in Dehydrated Cladodes of *O. ficus indica*

Total dietary fiber, soluble dietary fiber (SDF), and insoluble dietary fiber (IDF) in samples were analyzed according to methods 991.42 and 993.19 [15], respectively, by using a dietary fiber kit.

##### 2.5.2. Characterization of Calcium Compounds in Cladodes, Soluble, and Insoluble Dietary Fiber of *O. ficus indica* by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectrometry (EDS)

The morphology of calcium compounds was analyzed in a Scanning Electron Microscopy (Jeol JSM 6060LV, Japan). Prior to the analysis, the samples were fixed on an aluminum specimen holder with carbon tape and dried under critical point conditions in a Cryo-SEM preparation system (Quorum Technologies, Mod. PP30105, UK) operated with liquid CO₂. Subsequently, the mounted samples were then sputter coated with gold. The micro-compositional analysis of the samples was carried out using an Energy Dispersive Spectrometer (INCA x-sight) provided with software (Oxford Instrument, UK). Each sample was turned to move the focus position of the microscope. Further, surface views of isolated samples were taken to obtain...
the micrographs. The conditions of the analysis were high vacuum, 20 KV electron acceleration voltage, and secondary electron mode. Additionally, standards of pure compounds were observed with comparative purposes.

2.5.3. Characterization of Calcium Compounds in Cladodes, Soluble, and Insoluble Dietary Fiber of O. ficus indica by X-Ray Diffraction. Before analysis, samples were calcinated in a furnace (Nabherthm, Mod. L-P 330, GER) at 168°C in order to decompose organic matter and to prevent the formation of new mineral compounds or to avoid decay of calcium compounds commonly present in the Opuntioideae subfamily as was previously reported [14, 16–18]. The samples were ground to a fine powder and passed through a 150 μm screen. The powder samples were then densely packed into an aluminum sample holder. The X-ray diffraction patterns of the samples were recorded on a diffractometer (Rigaku Miniflex) operating at 35 kV and 15 mA, with a CuKα radiation wavelength of λ = 1.5406 Å. The measurements were obtained from 10 to 70° on a 20 scale with a step size of 0.05°. Spectrum analysis software (Materials Data Inc. Jade V 5.0) was used for the samples analysis.

2.5.4. Characterization of Calcium Compounds in Cladodes, Soluble, and Insoluble Dietary Fiber of O. ficus indica by Infrared (IR) Analysis. The IR spectra of dehydrated samples of O. ficus indica cladodes, SDF, and IDF were recorded on a Bruker Vector 33 spectrophotometer in the spectral range between 4000 and 400 cm⁻¹, using the KBr pellet technique (4 mg of the powdered sample dispersed in 100 mg of KBr).

2.5.5. Total Calcium and Oxalate Content in Cladodes, Soluble, and Insoluble Dietary Fiber of O. ficus indica Cladodes. Total calcium and oxalate content was determined according to AOAC Official Method 983.27 and 974.24, respectively [15]. The oxalate concentration was measured with a double beam atomic absorption (Analyst 300 Perkin Elmer), equipped with a deuterium lamp, background corrector, and a hollow cathode lamp. The operating parameters for calcium were a hollow cathode lamp with a wavelength of 422.7 nm, 70 psi of acetylene, nitrous oxide as an oxidant, and slit aperture of 0.7 mm and for oxalates 12 psi of dry air at 70 psi of acetylene with a wavelength of 422.7 nm, 10 mA lamp current, and a 0.7 nm slit width.

2.5.6. Calcium Carbonate Content in Cladodes, Soluble, and Insoluble Dietary Fiber of O. ficus indica. Calcium carbonate content in samples was analyzed by volumetric analysis according to AOAC [19].

2.6. Statistical Analysis. Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test with α = 0.05 and using the Statgraphics procedure (Graphics Software System, Manugistics, Inc., USA).

3. Results and Discussion

3.1. Soluble and Insoluble Dietary Fiber Content. The SDF and IDF contents in samples were 2.53 ± 0.90 and 43.44 ± 1.69%, respectively. These results differ from those reported by Hernández-Uribola et al. [9]. These authors found that, in nopal pads with 100 days of age (400 g of weight approximately), the SDF and IDF contents were 8 and 52%, respectively. Nutrient profile of cladodes from different harvests and regions varies due to the fact that this profile depends on environmental factors, that is, edaphic factors at the cultivation site, the season, and the age of the plant [8, 20]. A higher content of IDF with respect to SDF is attributed to the process of lignification, where polyphenolic polymer lignin is formed due to maturation of cladodes. On the contrary, young Opuntia cladodes lack lignin [21]. The increase of fibers in cladodes involves mainly cellulose and hemicelluloses [22]; these compounds in conjunction with lignin constitute IDF [23]. Total dietary fiber (TDF) content in O. ficus indica in the present study is higher than TDF values in cactus pear (fruit) reported by Jiménez-Aguilar et al. [24].

3.2. SEM and EDS Analysis. Figure 1 shows representative images of microscopic examinations of samples. Presence of calcium oxalate crystals in O. ficus indica cladodes is evident in Figure 1(a) (see arrows) in accordance with previous reports [10, 12, 13, 25]. Biominalized calcium oxalate crystallites in Cactaeaceae species were identified either as CaC₂O₄·2H₂O (wedellite) or as CaC₂O₄·3H₂O (whewellite). Whewellite druses differ from wedellite druses principally by their stellate shapes, with individual crystallites having acute sharp points emerging from the center of the druse. On the other hand, wedellite druses are usually made up of individual tetragonal crystals [26]. In this study, also wedellite crystals were detected (see Figure 1(b)) according to Malainine et al. [14] and Saenz et al. [25]. Figure 1(c) shows crystalline calcium oxalate in IDF extracted from O. ficus indica. As it can be seen, the quantity of crystals in IDF is more than that detected in cladodes (see arrows). The size of druses ranged from 150 to 250 μm; these crystals are larger than druses observed by Rodriguez-Garcia et al. [8] and Saenz et al. [25]. These authors reported that the crystal size of whewellite of O. ficus indica cladodes (from 60 to 200 g of weight) ranged from 30 to 70 μm. At this point, it is important to mention that, in the present study, the weight of cladodes was 400 g (100 days of age) and the oxalate crystal size increases as a function of maturation [26]. Figure 1(d) shows a detail of a vessel from the xylem of O. ficus indica with a calcium oxalate crystal adhered to the vascular tissue with a high content of lignin. Calcium oxalate crystals are present in all tissues of O. ficus indica cladodes [13]; nevertheless, SEM analysis shows that the presence of calcium oxalate crystals in IDF is very noticeable. This result is in agreement with those of Ginestra et al. [13]; indeed, these authors found that calcium oxalate crystals are strongly associated with an alcohol-insoluble residue (constituted by vascular bundles, clumps of parenchyma, and skin) obtained from a cell-wall fractionation of powdered lyophilized cladodes. Figure 2(a) shows a prismatic crystal in SDF extracted from O. ficus
Figure 1: SEM images of cladodes and insoluble dietary fiber of *O. ficus indica*, (a) calcium oxalate crystals (whewellite) in *O. ficus indica* cladodes, (b) calcium oxalate crystals (weddelite) in *O. ficus indica* cladodes, (c) calcium oxalate crystals in insoluble dietary fiber extracted from *O. ficus indica*, and (d) vessel from the xylem of *O. ficus indica* with a calcium oxalate crystal (whewellite).

Figure 2: SEM images and EDS analysis of soluble dietary fiber extracted from *O. ficus indica*, (a) prismatic crystal in soluble dietary fiber with elemental analysis by EDS and (b) prismatic crystal emerging from a globular structure located in soluble dietary fiber.

*A. indica*. A qualitative EDS analysis of this material revealed the presence of calcium, oxygen, carbon, potassium, and magnesium (inserted in Figure 2(a)). Contreras-Padilla et al. [18] have reported similar crystalline structures. These authors associate this composition with the presence of calcium carbonate or calcium-magnesium carbonate. Globular structures were detected by microscopic observation of the SDF (Figure 2(b)). Furthermore, a quadratic crystal is emerging from a globular structure (see white arrow). In this regard, Contreras-Padilla et al. [18] found that calcium
carbonate crystals grow into prismatic forms comparable to the crystal observed in SDF extracted from experimental samples. These authors suggest that the growth of these crystal structures can be correlated with the age of the plant.

3.3. X-Ray Diffraction Analysis. Figure 3 corresponds to the X-rays diffraction patterns of *O. ficus indica* samples (cladodes, SDF, and IDF). These diffractions revealed the presence of calcium oxalate monohydrate (whewellite) in cladodes and IDF, which fit with the PDF # 20–0231 of ICDD-JCPDS database. Monje and Baran [17] and Contreras-Padilla et al. [18] reported two characteristic peaks in X-rays diffraction patterns for oxalate monohydrate at 14-15 and 24-25° diffraction angle 2θ in different Cactaceae species belonging to the Opuntioideae subfamily, including *O. ficus indica*; both peaks are evident in cladodes and IDF samples. In contrast, these peaks were not detected in SDF samples. Furthermore, characteristic peaks in X-rays diffraction patterns for calcium carbonate at 29-30, 39-40, and 45-50° diffraction angle 2θ observed by the same authors were identified in SDF only. This indicates the presence of CaCO₃ (PDF # 47-1743) in these samples. These findings are in agreement with the distribution of crystalline compounds observed by the SEM and EDS analyses. Two small Bragg reflections located on 14.36 and 32.2° in the IDF sample are indicative of the presence of weddellite crystals (CaC₂O₄·(H₂O)₂,375) (PDF # 75-1314); nevertheless, they do not appear in the SDF sample. This result is in agreement with Malanine et al. [14]; these authors also reported the presence of weddellite crystals in *Opuntia ficus indica* cladodes. The other two crystalline compounds containing calcium were detected in SDF sample: spurrite (Ca₃(SiO₄)₂CO₃) (PDF # 13-0496) and glauberite (Na₃Ca(SO₄)₂) (PDF # 74-2340). These two compounds reveal the presence of other elements such as sodium, silicon, and sulfur. The analysis performed with the MDI Jade software showed no presence of other crystalline compounds. This could be attributed to the fact that peaks of crystalline compounds different to calcite, whewellite, and weddellite are very weak and the strongest calcite and whewellite reflection peaks are superimposed with other compound peaks or possibly they are amorphous.

3.4. Infrared Analysis. Figure 4 shows the infrared emission spectra of SDF and IDF isolated from *O. ficus indica* and cladodes. The infrared spectra confirm the existence of calcium carbonate in SDF. This is supported by the presence of bands near 1420 cm⁻¹ (vₐs CO₃²⁻) and 875 cm⁻¹ (CO₃²⁻ out-of-plane bending vibration). The intensity of these bands indicates a large amount of calcite. Nevertheless, this spectrum shows no evident absorption bands for oxalate, whereas infrared spectra of cladodes and IDF reveal the presence of whewellite and calcite. It is important to denote that calcite is in trace amounts in IDF. Calcium oxalate is responsible for bands at 1625 cm⁻¹ (vₐs COO⁻), 1312 cm⁻¹ (vₐ s COO⁻), and 750 cm⁻¹ (OCO deformations). These findings are consistent with the X-ray diffraction results. Finally, the presence of an intense band centered at 1070–1080 cm⁻¹ indicates a high content of silicon oxide (SiO₂) in all samples as it was observed in the X-ray diffraction results. Biomineralized silicon in plants has been related with a structural role in the cell wall and defense as a mineral barrier to both the invasion of pathogen and insect attacks, as well as the translocation of water and salts [27].

3.5. Total Calcium, Calcium Oxalate, and Calcium Carbonate in *O. ficus indica*. The average total calcium, oxalate, and calcium carbonate contents in *O. ficus indica* samples are shown in Table 1. It is evident that total calcium content in cladodes is significantly higher (P ≤ 0.05) in comparison to calcium content in SDF and IDF. Nevertheless, it is worth noting that calcium content in SDF is higher than calcium content in IDF. This means that calcium content in SDF is on average 18.24% higher than calcium content in IDF. Calcium oxalate content in SDF significantly decreases (P ≤ 0.05) with respect to that in cladodes and IDF. In addition, the highest concentration of calcium carbonate was detected in cladodes, while the content of this compound is approximately 50% higher in SDF in comparison with that observed in IDF. These results may be explained as follows: for salts (ionic solids) that dissociate into ions in water, such as the compounds contained in *O. ficus indica*, a solubility product (Kₚ) is typically given. In the case of CaCO₃, Kₚ (25°C) = 3.36 × 10⁻⁹, while, for CaC₂O₄, Kₚ (25°C) = 2.32 × 10⁻³ [28]. The smaller the solubility product of a substance, the lower its solubility. This means that CaCO₃ is more soluble in water than CaC₂O₄; consequently, this fact justifies a major concentration of oxalate in IDF compared with that in SDF and a higher content of calcium carbonate in SDF with respect to IDF.

Calcium carbonate in plants has been related to a mechanism to control soluble Ca²⁺ levels within plant tissues [29]. Similarly, calcium oxalate has a role as a calcium regulator and other functions, that is, mechanical support, intracellular pH regulation, ion balance detoxification, and gravity perception between others [30].
Table 1: Total calcium, oxalate, calcium carbonate content, and molar ratio oxalate:calcium in *Opuntia ficus indica* samples (cladodes, soluble, and insoluble dietary fiber).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total calcium content (mg/g) dry matter</th>
<th>Calcium oxalate content (mg/g) dry matter</th>
<th>Oxalate content (mg/g) dry matter</th>
<th>Calcium carbonate content (mg/g) dry matter</th>
<th>Molar ratio oxalate:calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladodes</td>
<td>32.33 ± 2.90a</td>
<td>6.71 ± 0.80a</td>
<td>8.31 ± 0.80a</td>
<td>70.81 ± 3.40a</td>
<td>0.12a</td>
</tr>
<tr>
<td>Soluble dietary fiber</td>
<td>18.99 ± 1.30b</td>
<td>0.27 ± 0.20b</td>
<td>1.25 ± 0.20b</td>
<td>49.67 ± 1.87b</td>
<td>0.03b</td>
</tr>
<tr>
<td>Insoluble dietary fiber</td>
<td>13.08 ± 1.10c</td>
<td>5.39 ± 0.17a</td>
<td>6.61 ± 0.17a</td>
<td>21.95 ± 2.17c</td>
<td>0.23c</td>
</tr>
</tbody>
</table>

*Values ± SD followed by the same letter are not significantly different (P < 0.05).

In a food evaluation, the chelating agent to mineral chelated molar ratio is an important factor for determining potency of mineral bioavailability. The World Health Organization considers this value as a good index as a preliminary criterion for mineral bioavailability [31].

In order to predict the bioavailability of calcium in samples [oxalate]/[Ca$^{2+}$], ratios were calculated (Table 1). The values obtained from this molar ratio (oxalate: Ca$^{2+}$) are below the critical level of 1, known to impair calcium bioavailability. This means that molar oxalate: Ca$^{2+}$ ratios ≥ 1 are indicative of calcium unavailability [32]. These results are in agreement with those reported by Contreras-Padilla et al. [11]. These authors found that the molar ratio between oxalate and calcium in *O. ficus indica* pads at different maturity stages was lower than 1, suggesting that the bioavailability of calcium is not compromised.

4. Conclusions

Calcium carbonates and calcium oxalates were detected in cladodes, IDF, and SDF of *O. ficus indica*. Nevertheless, significant differences in total calcium, calcium carbonate, calcium oxalate contents, and molar oxalate: Ca$^{2+}$ ratio were observed in all samples. This means that calcium bioavailability in *O. ficus indica* varies according to calcium compounds distribution.
Conflict of Interests
The authors declare that they have no conflict of interests.

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References


Research Article

New Approach to Enrich Pasta with Polyphenols from Grape Marc

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Food industry produces significant amount of waste that represents a problem for the sector. However, by-products are also promising sources of compounds which may be reused for their nutritional properties. The aim of this work is to exploit wine-making by-products, obtaining an extract by ultrasound-assisted extraction only using water as solvent. The characteristics of spaghetti enriched with grape marc were assessed and compared to control samples. In particular, total phenolic and flavonoids contents, the antioxidant activity, the cooking quality, and the sensory acceptability were evaluated at various steps of pasta production. The enriched spaghetti showed higher total phenolic and flavonoids contents and higher antioxidant activity than the control pasta. In addition, low cooking losses were found. In terms of sensory properties fortified pasta is acceptable as the traditional product, thus demonstrating that it is possible to exploit food waste to better satisfy consumer demand for healthy food products in a more sustainable perspective.

1. Introduction

Food industry and in particular fruit and vegetable processing generally produce significant wastes that represent the major disposal problem for food sector, from both the environmental and the economic point of view. However, it is worth noting that by-products can be also promising sources of compounds, being rich in carotenoids, polyphenols, tocopherols, vitamins, and other substances [1]. In general, agroindustrial wastes are disposed, utilized as animal feed or as fertilizer, but their use as sources of natural food ingredients recently recorded considerable attention from food research. The interest for by-products is due to the fact that they represent raw materials at low cost and are widely available. In addition, the natural compounds contained in by-products could allow satisfying consumer demand for food products with new functional properties, typical of compounds from plant world, which are natural and health-promoting. It is widely recognized that an adequate consumption of fruit and vegetables plays an important role in the prevention of diseases, for example, reduced risk of heart disease and stroke, as well as certain types of cancer [1]. Due to this consumer demand for healthier foods and due to the environmental consequences of by-products, food industry points towards formulation of products enriched with bioactive compounds that can be also extracted from by-products [2]. In the scientific literature several extraction techniques have been proposed. Some of them are traditional systems based on the extraction power of solvents or heat application and other ones are unconventional techniques of new generation as the ultrasound-assisted extraction (UAE). This extraction technique is preferred compared to the classical extraction methods because it allows to shorten the extraction time, reduces the organic solvent waste, increases the extraction yield, and enhances the quality of extracts and it is possible to use water as solvent [3, 4]. The UAE is based on sound mechanical waves that go beyond human hearing, whose frequencies are superior to 20 kHz. This type of extraction requires a liquid medium previously selected that allows ultrasonic wave to propagate up to the product, also preserving the integrity of the molecules that can be thermostable, thermostable, hydrosoluble, and liposoluble. This is possible due to the cavitation forces resulting from the ultrasound application. This phenomenon could be explained as the formation and the final collapse of microbubbles
into the liquid medium that may cause different mechanical effects, such as turbulent streaming, particle collisions, and cell wall disruption. These mechanical effects could cause a greater penetration of the solvent into the food matrix and, consequently, an increase of the mass transfer rates of the bioactive compounds from the food matrix into the extraction solvent [3]. Under these conditions the transfer of compounds to be extracted is facilitated and the extraction time is reduced.

According to the literature consulted, most of the studies on by-products are focused on optimization conditions for the extraction to obtain potential bioactive compounds, but the applications of the extracted substances to foods are very scarce. Calvo et al. [5] have designed sausages formulation enriched with lycopene by means of direct addition of tomato skins, previously dried and ground. The same group of researchers also studied the influence on the physicochemical and sensorial properties of raw and cooked hamburgers enriched with dry tomato peels, demonstrating that the addition of tomato peel to meat products results in healthier products due to both lycopene and fiber content present in tomato by-product [6]. Özvural and Vural [7] incorporated grape seed flour, rich in polyphenols obtained from wine by-products, into frankfurters.

In this context, grape marc is taken into account, being an interesting source of natural compounds as polyphenols (anthocyanins, catechins, flavonols, and phenolic acids) and fibers [3]. The aim of this work was to enrich fresh and dry pasta with grape marc extracts obtained by means of UAE using only water as solvent, thus demonstrating that it is possible to increase polyphenols amount and antioxidant activity of pasta without compromising the sensory characteristics and valorize wine-making by-products that are generally discarded.

2. Material and Methods

2.1. Raw Materials. Grape marc made up of skins, seeds, and stalks was provided by a local company of Foggia (Southern Italy), during the 2014 harvest. The samples were dried at 30–35°C in a dryer (SG600, Namad, Rome, Italy) for 48 hours. The dried grape marc was reduced in a fine powder by a hammer mill (16/BV-Beccaria s.r.l., Cuneo, Italy) and then stored at 4°C until further utilization.

2.2. Chemicals. Folin-Ciocalteu reagent, gallic acid monohydrate, methanol, hydrochloric acid, ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt), potassium persulfate (K2S2O8), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), aluminum chloride (AlCl3), sodium nitrite (NaNO2), sodium hydroxide solution (NaOH), and quercetin were supplied from Sigma-Aldrich (Milan, Italy). All reagents were of analytical grade.

2.3. Extraction Process. Grape marc extract (GME) was obtained by means of UAE (USR-1500-50WL, Weal s.r.l., Milan, Italy) using only water as solvent. In the reactor grape marc was suspended in water at a ratio of 1:10 (w/v) and ultrasonically treated for 60 minutes at acoustic frequency of 25 kHz and with ultrasonic power density of 50 W/L. The obtained extract was centrifuged at 10000 rpm for 10 minutes at room temperature and then it was filtered by means of 0.45 μm PTFE filters (Teknokroma, Sant Cugat del Vallès, Barcelona, Spain) and stored at −20°C until the analytical determinations.

2.4. Total Phenols, Flavonoids, and Antioxidant Activity Determination. To determine total phenols, flavonoids, and antioxidant activity, the extraction was performed as described by Biney and Beta [8] from all the various samples: dough, extruded fresh spaghetti, pasteurized fresh spaghetti, dry spaghetti, and cooked fresh and cooked dry spaghetti. The samples were dried at 30°C, grounded, and sieved through an 800 μm sieve. For total phenols and antioxidant activity determination, 2 g of powdered sample was mixed with 20 mL of acidified methanol (HCl/H2O:MeOH, 20:80), while, in the case of flavonoids, the same quantity of sample was combined with 10 mL of acidified methanol. The mixtures were included in 50 mL centrifuge tubes and shaken at room temperature in darkness for 2 h at 300 rpm using orbital shaker (HS 260 BASIC, IKA, Staufen, Germany). Next, the samples were centrifuged at 5°C for 15 minutes at 10000 rpm (5804R, Eppendorf, Milan, Italy) and supernatant was collected and filtered (PTFE, 0.45 μm) prior to the analytical determinations.

2.5. Spaghetti Preparation. Commercial durum wheat semolina was purchased from Agostini Mill (Montefiore dell’Aso, Italy). Semolina was mixed with proper amount of water or grape marc extract (GME) in the rotary shaft mixer (Namad, Rome, Italy) at 25°C for 20 min to distribute liquid uniformly throughout the semolina particles. The dough was extruded with a 60VR extruder (Namad) as described by Padalino et al. [9]. After extrusion, pasta was pasteurized with steam for 3 min at 90°C (Namad, Rome, Italy). The pasteurization system uses air at room temperature to cool down the pasta after the thermal treatment. Then, the extruded pasta was dried in a dryer (SG600; Namad). The drying process conditions applied were in accordance with Padalino et al. [9].

2.6. Sensory Analysis. Fresh-extruded and dry spaghetti samples were submitted to a panel of 15 trained tasters (seven men and eight women, aged between 28 and 45 years) in order to evaluate the sensory attributes. The panelists were selected on the basis of their sensory skills (ability to accurately determine and communicate the sensory attributes as appearance, odor, flavor, and texture of a product). The panelists were also trained in sensory vocabulary and identification of
particular attributes by evaluating durum wheat commercial spaghetti [10]. They were asked to indicate color, homogeneity, and resistance to breaking of fresh and dry uncooked spaghetti. In addition, to color and homogeneity, odor was also evaluated for fresh-extruded and pasteurized spaghetti. Elasticity, firmness, bulkiness, adhesiveness, color, odor, and taste were evaluated on fresh and dry cooked spaghetti. To the aim, a nine-point scale, where 1 corresponded to extremely unpleasant, 9 to extremely pleasant, and 5 to the threshold acceptability, was used to quantify each attribute. On the basis of the aforementioned attributes, panelists were also asked to score the overall quality of both cooked and uncooked products using the same nine-point scale [9].

2.7. Cooking Quality. The optimal cooking time (OCT) was evaluated according to the AACC [11] approved method 66–50. The cooking loss, that is, the amount of solid substance lost into the cooking water, was determined according to the AACC [11] approved method 66–50. The swelling index and the water absorption of cooked pasta (grams of water per gram of dry pasta) were determined according to the procedure described by Padalino et al. [12]. Moreover, cooked spaghetti samples were submitted to hardness and adhesiveness analysis by means of a Zwick/Roell model Z10 Texture Analyzer (Zwick Roell Italia s.r.l., Genoa, Italy) equipped with a stainless steel cylinder probe (2 cm diameter). The hardness (mean maximum force, N) and adhesiveness (mean negative area, Nmm) were measured according to the procedure described by Padalino et al. [12]. Six measurements for each spaghetti sample were performed.

2.8. Chemical Analysis

2.8.1. Determination of Total Phenolic Compounds. Total phenolic compounds were determined by UV-vis spectrophotometry according to Folin-Ciocalteu method [13]. In particular, GME was 1:10 diluted with water before analysis, while extracts obtained from samples relative to each production step of spaghetti, previously described, were analyzed without any dilution. Specifically, 0.5 mL of grape marc or pasta extract was mixed with 2 mL of distilled water and 150 μL of 5% sodium nitrite (NaNO₂) solution. After 6 minutes, 150 μL of a 10% aluminum chloride (AlCl₃) solution was added and the mixture was added to 2 mL of ABTS²⁻ dilution solution and after 3 minutes at room temperature for 12–16 h. The ABTS⁺ solution was diluted with 5 mM phosphate buffered saline, pH 7.4 (PBS), and absorbance 0.70 ± 0.02 at 734 nm. Then, 200 μL of sample extract was added to 2 mL of ABTS⁺ diluted solution and after 3 minutes at 30°C the mixture was measured through a spectrophotometer (UV1800, Shimadzu Italia s.r.l., Milan, Italy) at 734 nm. A calibration curve was previously built using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard, at concentrations between 0.98 and 250 μM (R² = 0.9997) and the antioxidant activity was expressed as μmol Trolox equivalents for gram of dry weight (dw). All analyses were carried out in triplicate.

2.8.2. Determination of Antioxidant Activity. The antioxidant activity was assessed using ABTS test, which is based on the ability of antioxidants to interact with the radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS⁺) inhibiting its absorption at 734 nm, according to the method of Re et al. [14]. 7 mM ABTS stock solution and 140 mM potassium persulfate were utilized. The ABTS radical cation (ABTS⁺) was obtained by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h. The ABTS⁺ solution was diluted with 5 mM phosphate buffered saline, pH 7.4 (PBS), and absorbance 0.70 ± 0.02 at 734 nm. Then, 200 μL of sample extract was added to 2 mL of ABTS⁺ diluted solution and after 3 minutes at 30°C the mixture was measured through a spectrophotometer (UV1800, Shimadzu Italia s.r.l., Milan, Italy) at 734 nm. A calibration curve was previously built using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as standard, at concentrations between 0.98 and 250 μM (R² = 0.9997) and the antioxidant activity was expressed as μmol Trolox equivalents for gram of dry weight (dw). All analyses were carried out in triplicate.

2.8.3. Determination of Total Flavonoids. Total flavonoids content both in GME and in all the extracts was determined by aluminum chloride colorimetric method, according to Huang and Ho [15] with modifications, using quercetin as standard. Extracts (0.5 mL), prepared as previously described, were mixed with 2 mL of distilled water and 150 μL of a 5% sodium nitrite (NaNO₂) solution. After 6 minutes, 150 μL of a 10% aluminum chloride (AlCl₃) solution was added and the mixture was allowed to stand for 6 minutes. Finally, 1 mL of 1 M sodium hydroxide (NaOH) was added and total volume was made up to 5 mL with distilled water. Then, the solutions were mixed and for each sample the absorbance was read in triplicate against blank at 415 nm. The standard curve was prepared using quercetin as standard in the range 3,13–500 mg/L (R² = 0.9981) and total amount of flavonoids was expressed in mg of quercetin/100 g of dry weight (dw).

2.9. Statistical Analysis. Experimental data were compared by a one-way analysis of variance (ANOVA). Duncan’s multiple range test, with the option of homogeneous groups (P < 0.05), was carried out to determine significant differences between spaghetti samples. STATISTICA 7.1 for Windows (StatSoft, Inc., Tulsa, OK, USA) was used.

3. Results and Discussion

In this study the total phenolic and flavonoids content together with the antioxidant activity were evaluated on both control and enriched samples: dough, extruded fresh pasta, and fresh-pasteurized/dry pasta (uncooked and cooked). Details on chemical characterization, cooking quality (only for dry pasta), and sensory properties are reported below separately.

3.1. Chemical Quality. Total phenolic (mg gallic acid/100 g dw), flavonoids (mg quercetin/100 g dw), and antioxidant activity (μmol Trolox/g dw), measured by ABTS assay, of GME were shown in Table 1. The obtained results indicate that the extract has a high content of polyphenols, equal
Table 1: Total phenols, total flavonoids, and antioxidant activity of the grape marc extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenols (mg gallic acid/100 g dw) ± SD</th>
<th>Total flavonoids (mg quercetin/100 g dw) ± SD</th>
<th>Antioxidant activity (μmoli Trolox/g dw) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>GME</td>
<td>443 ± 1.7</td>
<td>405 ± 12</td>
<td>518 ± 7.4</td>
</tr>
</tbody>
</table>

GME: grape marc extract.

to 443 mg acid gallic/100 g dw, greater than that reported by González-Centeno et al. [3], who studied the effect of acoustic frequency, ultrasonic power density, and extraction time of grape pomace by UAE. Moreover, the extract was characterized by a high flavonoid content and antioxidant activity, 405 mg quercetin/100 g dw, and 518 μmoli Trolox/g dw, respectively.

The phenolic compounds of the experimental samples were expressed as mg gallic acid for 100 g dw and shown in Figure 1. In every step of the spaghetti production process, the phenolic content of the ACTIVE samples was higher than spaghetti without any extract. In particular, among the experimental samples, the extruded fresh pasta showed the highest quantity of bioactive compounds (737 mg gallic acid/100 g dw). As it can be inferred by the figure, the phenolic content decreased as temperature increased; in fact the cooked dry pasta revealed the lowest phenolic value, probably due to the combination of drying and cooking procedure. The same trend was observed by Pasqualone et al. [2], who studied biscuits enriched with grape marc extract. According to Abdel-Aal and Rabalski [16] the effect of cooking on phenols is not always the same but depends on the type of bioactive compound and type of product.

ABTS assay was used to evaluate the antioxidant activity. The results are shown in Figure 2 and expressed as μmoli Trolox per gram of dry weight. According to obtained data, the fortification of spaghetti with aqueous grape marc extract led to a significant increase in antioxidant capacity. As in the case of total phenols, also in this case, the ACTIVE samples proved an antioxidant activity greater than the corresponding CNT sample. In fact, according to Alonso et al. [17] there is a positive correlation between the antioxidant activity and the total polyphenolic content of samples. In every step of production process a statistically significant difference between CNT and ACTIVE samples was detected. According to our findings, the entire pasta process did not cause any significant change in antioxidant activity; in fact, after extrusion an increase of antioxidant activity was observed, which remains constant in the subsequent steps. Only after cooking a decrease of antioxidant activity was found, probably because heat degraded phenols, thus decreasing their concentration.

The flavonoid content of both uncooked and cooked spaghetti is presented in Figure 3. The addition of grape marc extract to spaghetti significantly increased the flavonoid content in every phase of the production process. The highest flavonoids content was recorded for the extruded fresh pasta, which presented 58 mg quercetin/100 g dw, greater than the dough sample enriched with the extract. According to the obtained results, it is possible to infer that the extrusion phase increased the flavonoids amount, as observed also in the case of total phenols and antioxidant activity, probably because this step frees the bounded compounds. The pasteurization and the drying process exerted little effect on flavonoids content; in fact, these phases recorded 56 and
Cooking Quality is an important parameter for dry pasta evaluation. The cooking performances of the investigated spaghetti samples in terms of optimum cooking time, cooking loss, water absorption, swelling index, hardness, and adhesiveness are shown in Table 2. As can be observed from Table 2 there was no difference in optimum cooking time. In fact, for both samples studied, the optimum cooking time was around 10 min.

The spaghetti sample with grape marc extract showed a lower cooking loss than the CNT sample. One possible explanation could be due to the capacity of the antioxidant compounds from grape marc extract present in the form of complex with proteins around the starch granules, encapsulating them during cooking and restricting excessive swelling and diffusion of the amylose content [18]. Also Rizk et al. [18] found a reduction of cooking loss in sample enriched with antioxidant compounds from tomato peels (carotenoids) with respect to the control sample (100% wheat flour).

Regarding the water absorption and swelling index, ACTIVE sample recorded the lowest values with respect to CNT sample (Table 2). These results are in agreement with Rizk and Tolba [19] who also observed that pasta enriched with carotenoids from tomato peels had similar swelling index value of control sample (100% wheat flour).

As compared to the CNT sample, ACTIVE sample recorded lower adhesiveness value. This could be due to the fact that the antioxidant compounds generally form with the gluten proteins a stronger gluten network that entraps the starch granules, slowing down the amyllose release during cooking. On the contrary, the hardness value of ACTIVE sample was similar to that of the CNT sample.

### 3.3. Sensory Quality

The sensory properties of the investigated samples were evaluated by means of a group of trained panelists and the results are listed in Tables 3 and 4 for fresh-extruded/pasteurized and dry spaghetti samples (uncooked and cooked), respectively. Sensory data of uncooked fresh-extruded/pasteurized spaghetti samples (E-ACTIVE and P-ACTIVE) showed that addition of grape marc extract determined a slight decrease in overall quality as compared to the CNT, even though no statistically significant differences were observed among samples. Specifically, samples E-ACTIVE and P-ACTIVE recorded the smallest color scores with respect to the CNT samples. In fact, the spaghetti samples containing GME showed a light brown color in comparison to the bright yellow color of the CNT sample. Pasta color is essential for assessing pasta quality. Generally, consumers prefer pasta with a bright yellow color [20]. Moreover, the lowest color score has been observed for both pasteurized samples P-CNT and P-ACTIVE with respect to extruded samples. Most probably, the high temperature during the pasteurization temperature promoted development of Maillard reaction, giving to pasta a brownish color that slightly affects panelist judgment. Regarding the extruded and the pasteurized cooked spaghetti samples, the incorporation of GME caused a little rise of overall quality, even though no significant difference was observed between the studied samples. In particular, E-ACTIVE and P-ACTIVE spaghetti samples recorded a decline in adhesiveness and bulkiness (high score) in comparison with the E-CNT and P-CNT. Besides, the addition of GME did not determined significant differences in the other sensorial attributes. Concerning dry spaghetti (uncooked and cooked) the addition of the GME did not cause any significant differences in overall quality with respect to the CNT sample (Table 4).

### 4. Conclusions

The reutilization of wine-making by-products was proposed with success to enrich fresh or dry pasta from durum wheat.
Table 3: Sensory characteristics of fresh-extruded (E) and fresh-pasteurized (P) uncooked and cooked spaghetti samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Uncooked spaghetti</th>
<th>Cooked spaghetti</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Color</td>
<td>Odor</td>
<td>Overall quality</td>
<td>Elasticity</td>
<td>Firmness</td>
<td>Bulkiness</td>
<td>Adhesiveness</td>
<td>Color</td>
<td>Odor</td>
</tr>
<tr>
<td>E-CNT</td>
<td>7.52 ± 0.18</td>
<td>7.92 ± 0.18</td>
<td>7.80 ± 0.17</td>
<td>7.07 ± 0.34</td>
<td>6.98 ± 0.23</td>
<td>6.75 ± 0.24</td>
<td>7.32 ± 0.31</td>
<td>7.21 ± 0.27</td>
<td>7.21 ± 0.24</td>
</tr>
<tr>
<td>E-ACTIVE</td>
<td>7.25 ± 0.23, b</td>
<td>7.80 ± 0.36</td>
<td>7.50 ± 0.33</td>
<td>7.45 ± 0.27</td>
<td>7.21 ± 0.41</td>
<td>7.38 ± 0.27</td>
<td>7.25 ± 0.28</td>
<td>7.25 ± 0.27</td>
<td>7.04 ± 0.37</td>
</tr>
<tr>
<td>P-CNT</td>
<td>7.20 ± 0.18, b</td>
<td>7.92 ± 0.18</td>
<td>7.50 ± 0.24</td>
<td>7.08 ± 0.34</td>
<td>6.95 ± 0.25</td>
<td>7.00 ± 0.24</td>
<td>7.20 ± 0.31</td>
<td>7.20 ± 0.27</td>
<td>7.20 ± 0.24</td>
</tr>
<tr>
<td>P-ACTIVE</td>
<td>7.08 ± 0.23</td>
<td>7.80 ± 0.36</td>
<td>7.20 ± 0.33</td>
<td>7.45 ± 0.27</td>
<td>7.21 ± 0.41</td>
<td>7.50 ± 0.27</td>
<td>7.50 ± 0.28</td>
<td>7.35 ± 0.27</td>
<td>7.04 ± 0.37</td>
</tr>
</tbody>
</table>

\(^a,b\) Data in columns with different superscripts are significantly different (P < 0.05).
E-CNT: fresh-extruded spaghetti samples without the addition of grape marc extract.
E-ACTIVE: fresh-extruded spaghetti samples with the addition of grape marc extract.
P-CNT: fresh-pasteurized spaghetti samples without the addition of grape marc extract.
P-ACTIVE: fresh-pasteurized spaghetti samples with the addition of grape marc extract.
Table 4: Sensory characteristics of dry uncooked and cooked spaghetti samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Color</th>
<th>Uncooked spaghetti</th>
<th>Cooked spaghetti</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Res. to break</td>
<td>Overall quality</td>
<td>Elasticity</td>
</tr>
<tr>
<td>CNT</td>
<td>7.61 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.41 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.24 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACTIVE</td>
<td>7.40 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.30 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.21 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Data in columns with different superscripts are significantly different (<i>P</i> < 0.05).

CNT: samples without the addition of grape marc extract.

ACTIVE: samples with the addition of grape marc extract.
The obtained results show that it is possible to use grape marc aqueous extract, obtained from ultrasound extraction, instead of simple water to produce pasta, without altering its sensory characteristics. In fact, according to sensory analysis no significant differences have been found among the experimental samples. Moreover, enriched spaghetti was characterized by a higher content of phenolic compounds, flavonoids, and consequently antioxidant activity compared to the control sample. Therefore, data demonstrate with a concrete example that it is possible to reuse an agroindustrial waste such as grape marc to design new foods with healthful properties. In this way, it is possible to face environmental problems and satisfy at the same time consumer demand for food products with a recognized premium quality.

Abbreviations

- ABTS: 2,2-Azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt
- Trolox: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
- PBS: Phosphate buffered saline
- GME: Grape marc extract
- CNT: Samples without the addition of grape marc extract
- ACTIVE: Samples with the addition of grape marc extract

Conflict of Interests

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

References


Research Article

Evaluation of the Stability of the Total Antioxidant Capacity, Polyphenol Contents, and Starch Hydrolase Inhibitory Activities of Kombucha Teas Using an In Vitro Model of Digestion

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The objective of this study was to evaluate and compare antioxidant and starch hydrolase inhibitory activity of three different types of Kombucha beverages prepared by three pellicles with different microbial compositions. The fermentation process was carried out for 7 days and the assessments of antioxidant and starch hydrolase inhibitory activities as well as tea phenolic compounds were carried out. These parameters were also evaluated after subjecting the final fermented samples to gastric and duodenal digestion in an in vitro digestion model. The pH had a statistically significant decrease during the period of fermentation. The total phenolics content and antioxidant activities had increased during the fermentation process as well as when subjected to digestion. The starch hydrolase inhibitory activities also increased in a similar manner during the different phases. The $\alpha$-amylase and $\alpha$-glucosidase inhibitory activities showed statistically significant increases ($P < 0.05$) as the fermentation progressed, while an increase was observed after being subjected to pancreatic and duodenal digestion as well. All three types of tea showed a higher $\alpha$-amylase inhibitory activity than $\alpha$-glucosidase inhibitory activity.

1. Introduction

The interest towards conducting systematic studies on traditionally consumed food items for the purpose of maintaining good health and well-being has increased during recent times. Many of the traditional food products are known to possess bioactive components which enable them to be considered as functional foods. Essentially, this category of food products includes whole foods and fortified, enriched, or enhanced foods, which have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels [1]. Kombucha, a fermented tea beverage, has gained immense popularity throughout the world in recent times as a functional food, especially in Western and Mediterranean regions, primarily due to numerous associated health benefits such as anticancer, anti-diabetic, anti-inflammatory, hepatoprotective, and detoxification as well as due to the ability to act as a probiotic and symbiotic beverage [2–4]. This beverage is traditionally prepared by fermenting sugared black tea with a symbiotic mixed culture of yeast and bacteria [5]. It is believed that this beverage originated in China and it is popular for its high antioxidant activity and starch hydrolase inhibitory activities which have been systematically demonstrated by many recent studies [5–8]. The period of fermentation is typically known to be a minimum of 3 days with a maximum of 60 days depending on the native practices, whereby the broth is filtered and consumed after the process [5]. The flavor of this tea varies widely from sparkling flavor to a mild vinegar-like taste [9]. The studies focusing on the antioxidant activity of the beverage have also demonstrated the possession of bioactive ingredients of therapeutic interest, mainly the polyphenols and secondary metabolites developed during the fermentation process itself.

The bacterial component of the Kombucha culture consists of strains such as Acetobacter xylinum, A. xylinoides,
A. aceti, A. pauserianus, and Bacterium gluconicum [9]. The dominant yeast strains are Zygosaccharomyces bailii, Schizosaccharomyces pombe, Saccharomyces ludwigii, S. cerevisiae, Kloeckera spp., Torulaspora spp., and Pichia species [9, 10]. Many scientific studies have proven that yeast outnumber the bacterial count during the fermentation and it has also been discovered that the number of yeast and bacterial cells in the broth is more than the number of cells in the cellulosic pellicle [8, 10, 11]. The organic acids produced during the fermentation decrease the pH in the broth, which leads to acidity-induced oxygen starvation. Due to this process, the number of viable pathogenic microbial cells, if present at all, decreases as well, resulting in a beverage which is safe for consumption despite being of microbial origin [11]. The acetic acid bacteria present in this consortium have the ability to produce cellulose which forms a zoogleal mat, where this network enhances the association between bacteria and yeast [12]. Caffeine and related xanthines found in tea have the ability to stimulate the synthesis of this cellulose by the bacteria [7, 12]. Many factors such as climate, geographic location, local species of bacteria and yeast, and source of inoculum have been known to play a significant role in deciding the microbial composition of the Kombucha culture [12].

One objective of this study was to evaluate the enhancement of the antioxidant activity, the changes in polyphenolic contents, and the starch hydrolase inhibitory activity in sugared black tea fermented with three Kombucha cultures of different microbial composition. The obtained results were compared with the unfermented values of tea prior to the fermentation process. Additionally it was necessary to quantify and study the bioaccessible antioxidant capacity and starch hydrolase inhibitory activity. This is essentially the antioxidant and starch hydrolase inhibitory properties which are released from the fermented beverage and subsequently released for absorption during the intestinal and duodenal digestion processes [13]. An in vitro digestion model was utilized for this purpose, where the fermented tea samples were subjected to gastric and duodenal digestion phases. Measurement of the total antioxidant capacity (TAC), total polyphenolics content, and starch hydrolase inhibitory activity before and after the in vitro digestion phases were quantified of all three different Kombucha teas prepared from the varied strains.

2. Materials and Methods

The bacterial and fungi strains pellicular mats used for the study were verified and authenticated according to the method by Marsh et al., [8] using DNA amplification and high-throughput sequencing. The results are reported in Table 1. Black tea dust was obtained from Watawala Plantations, Sri Lanka. All other reagents, chemicals, and HPLC standards used for the study were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.1. Preparation of Kombucha Teas and Determination of the pH and Titratable Acidity (TA). One gram of black tea (Camellia sinensis) dust was added to 100 mL of boiling water and infused for 5 min after which they were filtered through a sterile sieve. Sucrose (10%) was dissolved in each beverage and the preparation was left to cool to room temperature at 24 ± 3°C. The cooled tea was aseptically inoculated with each of the freshly grown tea fungi for 7 days. The fermentation was carried out at 24 ± 3°C. Sampling was carried out only once per day in order to avoid contamination. The fermented teas were centrifuged at 7240 × g for 10 min prior to the assays and analyses. The pH values were measured with a hand-held pH meter (Testo 206 PHI, Keison, London, UK), while the TA was measured according to the method by Chen and Liu [10].

2.2. Enumeration of Bacteria and Fungi Population in the Broth and Pellicle. Enumerations of the overall population of bacteria and yeast in the broth and pellicle of the fermented beverages were determined according to the method by Chen and Liu [10]. Both figures were expressed as colony-forming units per mL (cfu/mL).

2.3. Determination of the Total Phenolics Content and Antioxidant Activity. The method by Huang et al. [14] was used for determining the total phenolics content and the Oxygen Radical Absorbance Capacity (ORAC) assay was carried out according to Prior et al. [15]. Results for the total phenolics content were expressed as milligrams of gallic acid equivalents (GAE) per milliliters (mg GAE/mL), while the ORAC values were expressed as micromoles of trolox equivalents (TE) per milliliters (μmol TE/mL). The di(phenyl)- (2,4,6-trinitrophenyl) iminoazanium (DPPH) and superoxide radical scavenging activity assays were carried out according to the method described by Lee et al. [5]. All assays were carried out using the Synergy HTX multimode microplate reader and Gen5 software (Biotek, Winooski, VT, USA).

2.4. Determination of the α-Amylase and α-Glucosidase Inhibitory Activities. The α-amylase inhibitory activity was evaluated according to the method by Liu et al. [16] while the α-glucosidase inhibitory activity was carried out according to the method by Lee et al. [17]. Acarbose was used as the positive control for both assays and the data were expressed as IC50 (mg/mL).

2.5. In Vitro Digestion Process. The in vitro digestion model was adapted from Ryan et al. [18]. In brief, the fermented tea samples at day 7 of the process were transferred to clean amber bottles and mixed with saline (balanced salt solution) to create a final volume of 20 mL. The samples were acidified to pH 2.0 with 1 mL of a porcine pepsin preparation (0.04 g pepsin in 1 mL 0.1 M HCl) and incubated at 37°C in a shaking water bath at 3000 × g for 1 h. After gastric digestion, 500 μL of each sample was extracted and stored at −20°C. The pH was then increased to 5.3 with 0.9 M sodium bicarbonate followed by the addition of 200 μL of bile salts glycicyoxyxolcholate (0.04 g in 1 mL saline), taurodeoxycholate (0.025 g in 1 mL saline), taurocholate (0.04 g in 1 mL saline), and 100 μL of pancreatin (0.04 g in 500 μL saline). The pH of each sample was increased to 7.4 with 1 M NaOH. Samples were incubated
<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Bacteria</th>
<th>Fungi</th>
<th>Bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluconacetobacter</td>
<td>85.6</td>
<td>Zygosaccharomyces</td>
<td>84.1</td>
<td>Gluconacetobacter</td>
<td>82.3</td>
<td>Zygosaccharomyces</td>
</tr>
<tr>
<td>Acetobacter</td>
<td>2.92</td>
<td>Dekkera</td>
<td>6.28</td>
<td>Acetobacter</td>
<td>3.49</td>
<td>Dekkera</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>2.85</td>
<td>Pichia</td>
<td>5.24</td>
<td>Lactobacillus</td>
<td>3.59</td>
<td>Pichia</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>0.81</td>
<td>Other</td>
<td>4.38</td>
<td>Leuconostoc</td>
<td>1.65</td>
<td>Other</td>
</tr>
<tr>
<td>Lactococcus</td>
<td>0.78</td>
<td>Other</td>
<td>4.38</td>
<td>Leuconostoc</td>
<td>1.58</td>
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<tr>
<td>Bifidobacterium</td>
<td>0.69</td>
<td>Other</td>
<td>5.85</td>
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</tr>
<tr>
<td>Other</td>
<td>6.35</td>
<td>Other</td>
<td>5.85</td>
<td>Other</td>
<td>4.26</td>
<td></td>
</tr>
</tbody>
</table>
in a shaking water bath at 95 rpm at 37 °C for 2 h to complete the intestinal phase of the in vitro digestion process. After the intestinal phase, 500 μL of each sample was extracted and stored at −20°C.

2.6. High Performance Liquid Chromatography (HPLC) Determination of the Phenolic Compounds. A Shimadzu LC2010 HPLC system (Kyoto, Japan) equipped with an SPD-M10AVP diode array detector (Kyoto, Japan) and a phenomenex Luna C-18(2) column (4.6 mm i.d. × 25 cm, 5 μm) was used for the quantification of (−)-epicatechin (EC), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG), theaflavin (TF), and gallic acid (GA). A gradient profile using two solvents was applied following the method by Jayabalan et al. [19] with a few modifications for the quantification of EC, ECG, EGC, EGCG, and GA. The solvents used were as follows: solvent A: 8% aqueous formic acid; solvent B: acetonitrile/methanol (10:90, v/v). A flow rate of 0.9 mL/min was maintained. The gradient was as follows: 0 min—20% B; 7 min—35% B; 14 min—45% B; 21 min—65% B; 25 min—85% B; 32 min—95% B. The TF content was determined according to the method by Thanaraj and Seshadri [20] using the same HPLC setup which was used to quantify the epicatechin isomers. The wavelengths of the diode array detector were set at 260, 280, and 320 nm for monitoring of the phenolic compounds. The concentrations of all the compounds in the extracts were quantified using standard curves and expressed as micrograms per mL (μg/mL).

2.7. Statistical Analysis. IBM SPSS Statistics version 21.0 released in 2012 (IBM Corp., Armonk, NY, USA) for Windows was used for the statistical analyses. Results were calculated and expressed as mean ± standard error mean (SEM) of ≥3 independent analyses. P values of <0.05 were considered to be significant.

3. Results and Discussion

The initial values of all the analytical parameters prior to the fermentation process are indicated as day 0 in Tables 1–3 and Figures 1–3. Sample results at day 7 are considered as the values prior to the digestion process, which are subsequently subjected to the pancreatic and duodenal digestion phases.

3.1. Changes in Population of Viable Bacteria and Fungi. Table 2 shows the changes of the composition of the overall population of bacteria and yeasts in the pellicle prior to fermentation, on day 1 and day 7, as well as in the broth on days 1 and 7. In all three Kombucha tea types, an increase in the overall bacteria and yeast population was observed. The increase in the bacteria and yeast population can be correlated to the fact that these microbes have the ability to utilize the substrate and successfully and thrive in the given environment. In addition, bacterial and fungal proportions of the fermented teas matched those of the corresponding pellicle, thereby suggesting that the fungal composition of the cellulosic pellicle used to inoculate the tea is the key determinant. *Gluconacetobacter* was generally observed to be the common and most populated bacterial entity in all three types of Kombucha pellicles. In previous studies, it has been identified as the key bacterial strain which enhances the bioaccessibility of polyphenols and antioxidant activities in Kombucha beverage [11]. According to Table 2, the cell concentrations of bacteria and yeast in the broth were higher than the cell concentrations in the cellulose pellicle. The *Lactobacillus* spp. present in all three pellicles may possess potential therapeutic properties including acting as a probiotic organism. Such microorganisms are known to provide a balance in intestinal microbiota, normalizing processes in gut and boosting the immune system [21]. The presence of both *Gluconacetobacter* and *Lactobacillus* spp. may also contribute to the fact that the antioxidant activity of the beverages was primarily imparted due to the metabolic activities of these two microbial genera as shown by previous studies [12, 21, 22].

3.2. pH and TA. Figure 1 shows the pH values of the samples on days 0, 1, and 7 as well as the results when the fermented samples are subjected to pancreatic and duodenal digestion. A statistically significant decrease (P < 0.05) was observed in the pH of all three types of Kombucha teas during the period of fermentation starting from day 1 onwards, as compared with their unfermented counterparts on day 0. Compared with the sample values at day 7, a statistically significant (P < 0.05) change in the pH was observed when the teas were subjected to the gastric and duodenal digestion phases. K1 displays a statistically significantly lower pH (P < 0.05) than its nondigested counterpart, whereas K2 and K3 display statistically significantly higher (P < 0.05) values. Prior to the fermentation, the pH of all three teas remained between 6.4 and 6.5. By day 7 of the fermentation period, the pH of the teas varied between 4.8 and 4.5. K1 was observed to be the least acidic of the three teas at the end of the fermentation process with a pH value of 4.8 while K2 and K3 had a value of 4.5. The decrease in pH during the fermentation process would be due to the production of organic acids by the yeast and bacteria present in the tea fungus. The decrease in pH can be beneficial in terms of maintaining the bioactivity and preventing the chemical degradation of phenolic compounds. Previous studies have proven that the organic acids produced during the fermentation are gluconic acid, glucuronic acid, L-lactic acid, malic acid, tartaric acid, malonic acid, citric acid, and oxalic acid [22, 23]. The TA of the unfermented teas ranged between 0.14 and 0.15, and at the end of the fermentation process, the TA values ranged between 2.5 and 2.7 which are statistically significant increase (P < 0.05) compared with the unfermented samples. After pancreatic digestion the TA had statistically significant increase (P < 0.05) in all 3 samples as compared with the day 7 fermented sample. Following duodenal digestion, the TA values have shown a statistically significant decrease (P < 0.05) compared with the fermented sample at day 7. According to Reiss [24], the optimum consumable acidity level of a beverage is 4.0–4.5 g/L; thus all three tea types are within the range of the acceptable TA level and can be acceptable for consumption.
observed in all 3 samples after intestinal digestion. In addition, statistically significant increases (P < 0.05) in all fermented samples compared with their unfermented counterparts. When the teas were subjected to the in vitro digestion, statistically significant increases (P < 0.05) were observed in all 3 samples after intestinal digestion. In addition, statistically significant increases (P < 0.05) were observed in the total phenolics contents following the duodenal digestion as compared with the undigested sample at day 7 of the fermentation as well. The reason for the increase in total phenolics content during the fermentation process has been explained by Blanc [25], where it was demonstrated that the bacteria and yeast present in the Kombucha microbial consortium have the ability to liberate enzymes, such as phytase which has the capability of breaking down the cellulose backbone of Camellia sinensis to release polyphenol compounds. Partial oxidation of polyphenols can be observed when the tea is fermented for a longer period of 1-2 months, resulting in a decrease in the total phenolics content and the antioxidant potential [26]. Thus, extended periods of fermentation can lead to the accumulation of unwanted

### Table 2: Changes to the composition of the overall population of bacteria and fungi present in the broth and the pellicle prior to fermentation (day 0) and on day 1 and day 7.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Microorganisms</th>
<th>Days (population of microorganisms)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 (cfu/mL)</td>
</tr>
<tr>
<td><strong>Broth</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>Bacteria</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>—</td>
</tr>
<tr>
<td>K2</td>
<td>Bacteria</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>—</td>
</tr>
<tr>
<td>K3</td>
<td>Bacteria</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>—</td>
</tr>
<tr>
<td><strong>Pellicle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>Bacteria</td>
<td>4.7 ± 0.1 × 10^3</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>4.8 ± 0.2 × 10^5</td>
</tr>
<tr>
<td>K2</td>
<td>Bacteria</td>
<td>3.8 ± 0.1 × 10^5</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>3.9 ± 0.1 × 10^3</td>
</tr>
<tr>
<td>K3</td>
<td>Bacteria</td>
<td>1.9 ± 0.2 × 10^5</td>
</tr>
<tr>
<td></td>
<td>Fungi</td>
<td>2.2 ± 0.1 × 10^5</td>
</tr>
</tbody>
</table>

### Table 3: Changes in the polyphenol contents in the three Kombucha cultures throughout the 7-day period of analysis expressed as mean ± SEM. *P < 0.05 versus the value of each tea at day 0. †P < 0.05 versus the value of each tea at day 7.

<table>
<thead>
<tr>
<th>Kombucha</th>
<th>Polyphenol⁴</th>
<th>Days/phase of digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (µg/mL)</td>
<td>1 (µg/mL)</td>
</tr>
<tr>
<td><strong>1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>26.5 ± 1.3</td>
<td>27.5 ± 1.1</td>
</tr>
<tr>
<td>EGC</td>
<td>27.8 ± 1.2</td>
<td>27.9 ± 1.1</td>
</tr>
<tr>
<td>ECG</td>
<td>10.8 ± 1.1</td>
<td>11.2 ± 1.2</td>
</tr>
<tr>
<td>EC</td>
<td>9.8 ± 1.4</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>TF</td>
<td>12.6 ± 1.2</td>
<td>13.9 ± 1.1</td>
</tr>
<tr>
<td>GA</td>
<td>31.5 ± 1.9</td>
<td>36.8 ± 1.1*</td>
</tr>
<tr>
<td><strong>2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>26.5 ± 1.3</td>
<td>28.4 ± 1.2</td>
</tr>
<tr>
<td>EGC</td>
<td>27.8 ± 1.2</td>
<td>28.7 ± 1.2</td>
</tr>
<tr>
<td>ECG</td>
<td>10.8 ± 1.1</td>
<td>12.5 ± 1.1</td>
</tr>
<tr>
<td>EC</td>
<td>9.8 ± 1.4</td>
<td>14.8 ± 1.4</td>
</tr>
<tr>
<td>TF</td>
<td>12.6 ± 1.2</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td>GA</td>
<td>31.5 ± 1.9</td>
<td>37.2 ± 1.2</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>26.5 ± 1.3</td>
<td>27.6 ± 1.5</td>
</tr>
<tr>
<td>EGC</td>
<td>27.8 ± 1.2</td>
<td>28.1 ± 1.5</td>
</tr>
<tr>
<td>ECG</td>
<td>10.8 ± 1.1</td>
<td>13.9 ± 1.0</td>
</tr>
<tr>
<td>EC</td>
<td>9.8 ± 1.4</td>
<td>15.1 ± 1.2</td>
</tr>
<tr>
<td>TF</td>
<td>12.6 ± 1.2</td>
<td>15.1 ± 1.1</td>
</tr>
<tr>
<td>GA</td>
<td>31.5 ± 1.9</td>
<td>38.5 ± 1.5</td>
</tr>
</tbody>
</table>

³The denoted abbreviations are as follows: (−)epicatechin (EC), (−)epicatechin-3-gallate (ECG), (−)epigallocatechin (EGC), and (−)epigallocatechin-3-gallate (EGCG), theaflavin (TF), and gallic acid (GA).

3.3. Total Phenolics Content. The total phenolics contents are shown in Figure 2(a). The values of the unfermented teas varied between 470 and 476 mg GAE/mL. Following the initiation of the fermentation process, the total phenolic content shows a statistically significant increase (P < 0.05) in all fermented samples compared with their unfermented counterparts. When the teas were subjected to the in vitro digestion, statistically significant increases (P < 0.05) were observed in all 3 samples after intestinal digestion. In addition, statistically significant increases (P < 0.05) were observed in the total phenolics contents following the duodenal digestion as compared with the undigested sample at day 7 of the fermentation as well. The reason for the increase in total phenolics content during the fermentation process has been explained by Blanc [25], where it was demonstrated that the bacteria and yeast present in the Kombucha microbial consortium have the ability to liberate enzymes, such as phytase which has the capability of breaking down the cellulose backbone of Camellia sinensis to release polyphenol compounds. Partial oxidation of polyphenols can be observed when the tea is fermented for a longer period of 1-2 months, resulting in a decrease in the total phenolics content and the antioxidant potential [26]. Thus, extended periods of fermentation can lead to the accumulation of unwanted
metabolites, and, therefore, the beverage may be deemed as therapeutically inadequate for imparting the required health benefits.

3.4. ORAC, DPPH EC_{50}, and Superoxide Scavenging Activities. The ORAC, DPPH EC_{50}, and superoxide scavenging activity results are shown in Figures 2(b)–2(d). In observing the ORAC results, statistically significant increases (\( P < 0.05 \)) were observed in all 3 samples as the fermentation progresses, in comparison to the unfermented tea samples. At the unfermented stage all three tea samples had ORAC values in the range of 1840 to 1880 \( \mu \text{mol TE/mL} \). As the fermentation progressed, the ORAC values had increased. By day 7 of the fermentation process, the ORAC value for all teas was within a range of 2460–2640 \( \mu \text{mol TE/mL} \). In comparing the values during the digestion processes, statistically significant increases (\( P < 0.05 \)) were observed at both phases. It was noted that K3 had the highest ORAC value among all 3 fermented samples by the end of the fermentation process. An increase in the ORAC value represents the maintenance of the antioxidant potential throughout the fermentation period and this could be of therapeutic importance. It is also known that the oxidation of the polyphenols can result in the formation of stable intermediates which demonstrates strong antioxidant activity [27]. However, in this sample, the increase in the ORAC value was less than K1 and K2 in the duodenal digestion phases as compared with the undigested counterparts. Nevertheless, the final ORAC values of all three types of teas were within a comparable range at the end of the digestion phases. The DPPH EC_{50} values of the 3 fermented Kombucha teas remained within the range of 56–59 mg/kg by day 7. As for the DPPH EC_{50} values and the superoxide scavenging activities, the trend was not as clear as in the instances of the ORAC assay. A better correlation between the total phenolics content and the ORAC value was observed in comparison to the correlation between DPPH EC_{50} and superoxide scavenging values. This better correlation could be due to the fact that the phenolic compounds present in all three types of tea samples may possess a better scavenging activity of peroxide radicals which are generated during the ORAC assay.

3.5. Starch Hydrolase Inhibitory Activity. The results obtained for starch hydrolyase inhibitory activity are shown in Figure 3. In all three tea samples statistically significant increases (\( P < 0.05 \)) in the \( \alpha \)-amylase inhibitory activity and \( \alpha \)-glucosidase activity were observed following the completion of the fermentation process. Prior to fermentation the \( \alpha \)-amylase inhibitory activity of all three Kombucha samples remained in the range of 55-56 \( \mu \text{g/mL} \) in terms of the IC_{50} value. As the fermentation progressed, the inhibitory activity has increased in a statistically significant (\( P < 0.05 \)) manner. When subjected to pancreatic digestion, the \( \alpha \)-amylase inhibitory activity remains more so the same compared with the undigested counterpart. However, after duodenal digestion the \( \alpha \)-amylase inhibitory activity displayed statistically significant increases (\( P < 0.05 \)) compared with the undigested sample at day 7 of the fermentation process. The \( \alpha \)-glucosidase inhibitory activity in the unfermented teas ranged between 69 and 70 \( \mu \text{g/mL} \) in terms of IC_{50} value. As the fermentation progressed, the inhibitory activity has shown statistically significant increases (\( P < 0.05 \)). When the fully fermented samples were subjected to pancreatic digestion, the \( \alpha \)-glucosidase inhibitory activity was observed to be similar to the undigested counterpart. When it was subjected to duodenal digestion, statistically significant decrease (\( P < 0.05 \)) in the IC_{50} value was observed, as compared with the unfermented sample. When considering the results obtained for the three Kombucha strains used in this study, it can be noted that the \( \alpha \)-glucosidase inhibitory activity of K3 after duodenal digestion was higher than the other two strains. In comparing \( \alpha \)-amylase inhibitory activity and \( \alpha \)-glucosidase inhibitory activities in all three tea samples it can be noted that the increase in \( \alpha \)-amylase inhibitory activity
3.6. Changes in the Tea Polyphenol Quantities. The changes in the tea polyphenol contents are shown in Table 3. It can be observed that all EGCG, EGC, ECG, EC, TF, and GA showed a statistically significant increase ($P < 0.05$) by day 7 compared with day 0 or the unfermented counterpart, in all three types of Kombucha teas. There was also a statistically significant increase ($P < 0.05$) in the polyphenol contents after subjecting the teas to pancreatic and duodenal digestions. Samples subjected to duodenal digestion showed the highest value of polyphenol content for all three tea types. Quantities of these phenolic compounds were reflective of the total phenolics content displayed in Figure 2(a). It was heartening to observe an increase in the tea catechins themselves as a result of the fermentation process as well as the pancreatic and duodenal digestion, given the reported health benefits of this

is higher than the $\alpha$-glucosidase inhibitory activity. Thus, it may be concluded that the tea fungus has the ability to enhance the $\alpha$-amylase inhibitory activity better than the $\alpha$-glucosidase inhibitory activity. $\alpha$-amylase is required for the subsequent reactions of $\alpha$-glucosidase. Therefore, the conclusion above may be considered as an important finding in terms of therapeutic effects of the Kombucha beverages. The observations also throw light on the therapeutically beneficial effects of these teas, since the enhancement of the inhibitors has the ability to retard glucose absorption by inhibition the enzymes $\alpha$-amylase and $\alpha$-glucosidase, which are found in the intestinal brush border [16, 17]. Thus, it has an enhanced ability to prevent the breakdown of starch and the subsequent curbing of glucose release into the physiological system of the body.

![Figure 2: Changes in the (a) total phenolics content, (b) ORAC, (c) DPPH EC$_{50}$, and (d) superoxide scavenging activities of the three Kombucha beverages. Error bars represent the SEM. *$P < 0.05$ versus the value at day 0. **$P < 0.05$ versus the value at day 7 (prior to being subjected to the digestion phases).](image-url)
group of compounds, especially catechin and epicatechin. The structural criteria for compounds to be considered as potent free-radical scavengers are that these should possess either a 3-hydroxy group on unsaturated C ring, or a 2,3-double bond with the 3-OH group and 4-one in the C ring, or an ortho-OH substitution pattern in the B ring where the OH groups are not glycated [28–30]. Being the major polyphenolic components, catechin and epicatechin fulfill the first and third structural criteria for effective antioxidants. In addition to antioxidant and starch hydrolase inhibitory properties, tea polyphenols have displayed antimicrobial and antifungal activities in vitro [31, 32]. Thus, in addition to enhancing the human microbiome with beneficial microbes, the organic compounds produced during the Kombucha fermentation further expand the list of therapeutic effects and benefits gained from the consumption of the fermented beverage.

4. Conclusions

This study was able to demonstrate the ability of the tea fungus to increase the total phenolics, antioxidant activity, and starch hydrolase inhibitory activities, due to the increase in phenolic compounds. Three types of Kombucha pellicles were studied individually as well as in comparison with each other. Also, this study was able to demonstrate the statistically significant increase ($P < 0.05$) in total phenolics, antioxidant activity, and starch hydrolase inhibitory activities after subjecting the fermented beverage to an in vitro digestion model. It was also observed that the $\alpha$-amylase inhibitory activity in particular was increased after the fermentation process, whereby this increase could be associated with the increase in the total phenolics content as well as the increased presence of compounds which have the ability to inhibit $\alpha$-amylase produced during the fermentation process. The increase in starch hydrolase inhibitory activity contributes in enhancing the health and wellness; thus this beverage can be considered as a functional food with notable therapeutic effects. The tea fermented with K3 pellicle was discovered to be the better Kombucha beverage in terms of having the highest antioxidant and starch hydrolase inhibitory activity following fermentation. Since K3 Kombucha sample contains Lactobacillus spp., this beverage can be used as a potentially good probiotic supplement. Finally, the study can be used as a platform to carry out further studies based on different microbial compositions and their effect on changing the chemical composition of Kombucha beverage.

Conflict of Interests

The authors report no conflict of interests, financial or otherwise.

Acknowledgment

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References

Antioxidant Peptide Derived from *Spirulina maxima* Suppresses HIF1α-Induced Invasive Migration of HT1080 Fibrosarcoma Cells

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1. Introduction

Hypoxia causes the malignant progression of tumor cells; hence, it has been considered a central issue that must be addressed for effective cancer therapy. The initiation of tumor metastasis requires invasive cell migration. Here, we show that an antioxidant peptide derived from *Spirulina maxima* suppresses hypoxia-induced invasive migration of HT1080 human fibrosarcoma cells. HT1080 cells treated with a hypoxia-inducing agent, CoCl₂, exhibited an increase in invasive migration and intracellular reactive oxygen species (ROS), which is associated with an increase in the expression of hypoxia-induced factor 1α (HIF1α) accompanied by the activation of PI3K/Akt and ERK1/2. The inhibition of PI3K/Akt and ERK1/2 with specific inhibitors diminished the CoCl₂-induced increase in HIF1α expression and invasive cell migration. Moreover, CoCl₂-induced HIF1α expression was associated with an increase in the expression of molecules downstream of β-integrin, such as N-cadherin, vimentin, and β-catenin. Therefore, the *S. maxima* peptide effectively attenuated the CoCl₂-induced ROS generation and downregulated the HIF1α signaling pathway involving PI3K/Akt, ERK1/2, and β-integrin in cells. These results suggest that the *S. maxima* antioxidant peptide downregulates the HIF1α signaling pathway necessary for hypoxia-induced invasive migration of HT1080 cells by attenuating intracellular ROS. *S. maxima* peptide may be an effective constituent in antitumor progression products.

1. Introduction

The rapid growth and proliferation of tumor cells result in a dramatic surge in oxygen demand. Tumor hypoxia caused by inadequate oxygen supply is strongly associated with tumor propagation, malignant progression, and resistance to therapy [1, 2]. Therefore, tumor hypoxia is an important factor in tumor biochemistry and an important challenge in tumor treatment. Accumulating evidence indicates that the effect of hypoxia on the malignant progression of tumor cells is mediated by a series of hypoxia-induced cellular changes that activate anaerobic metabolism, angiogenesis, and metastasis and enable tumor cells to survive or escape their oxygen-deficient environment [1, 3]. The transcription factor hypoxia-inducible factor 1 (HIF1), containing HIF1α and HIF1β subunits, has been known to be a key regulator in tumor cell adaptation to hypoxic environments. In particular, HIF1α regulates the expression of a number of genes affecting the metastatic progression of tumor cells [4–6]. Tumor metastasis is a complex multistep process by which tumor cells disseminate from the primary site, penetrate into lymphatic and blood vessels, and spread to other sites in the body. HIF1α expression has been implicated in the increased invasive migration of tumor cells during the initiation of metastasis [2, 7].

Reactive oxygen species (ROS) have been recently identified as key mediators involved in tumor propagation and malignant progression. ROS activate downstream PI3K/Akt, ERK1/2, and β-integrin pathways that regulate HIF1α expression [8–10]. ROS-induced upregulation of HIF1α also causes the invasive migration of tumor cells through the regulation of target genes such as N-cadherin, vimentin, and β-catenin.
against the invasive migration of tumor cells. Therefore, there has been a marked increase in the study of the role of ROS and antioxidants in the prevention of tumor progression.

Peptides purified from protein hydrolysates have received much attention because of their anticancer, anti-inflammatory, and antioxidant activities [11, 12]. We recently reported that an antioxidant peptide identified from the enzymatic hydrolysates of *Spirulina maxima* is effective against FcεRI-mediated allergic reactions in mast cells [13]. In the present study, we examined the protective effects of this peptide against the invasive migration of tumor cells in vitro.

2. Materials and Methods

2.1. Cell Culture and Reagents. HT1080 human fibrosarcoma cells, obtained from the American Type Culture Collection (ATCC), were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin, in 5% CO₂ at 37°C. For the cell-culture experiments, cells were passaged at least 3 times and detached with trypsin–EDTA. Matrigel Invasion Chambers were purchased from BD Biosciences (USA). Antibodies against HIF1α, Akt, ERK1/2, N-cadherin, vimentin, β-catenin, and actin were obtained from BD Biosciences (USA), Santa Cruz Biotechnology (USA), Cell Signaling Technology (USA), and Sigma-Aldrich (USA), respectively. DCFH-DA was purchased from Molecular Probes. Chemicals and reagents were purchased from Sigma-Aldrich, unless stated otherwise.

2.2. Preparation of the *Spirulina maxima* Peptide. The *S. maxima* peptide was prepared as reported by Vo et al. [13]. The purity of the peptide was >98% according to RP-HPLC assessment and N-terminal sequence analysis. The amino acid sequence of the final purified peptide was determined to be LDAVNR by electrospray ionization mass spectrometry (ESI/MS).

2.3. Cell Viability (*MTT*) Assay. The cytotoxicity of CoCl₂ and/or peptide was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] formazan assay. HT1080 cells were seeded in 96-well plates at a density of 1 × 10⁵ cells/well in DMEM containing 10% fetal bovine serum. Twenty-four hours after seeding, the medium was changed to DMEM containing 0.1% bovine serum albumin (DMEM-BSA) and the cells were incubated with 100 μM CoCl₂ with or without 100 μM peptide for 24 h. Thereafter, the medium was carefully removed and 160 μL of MTT (0.5 mg/mL final concentration) solution was added to each well prior to incubation for an additional 4 h at 37°C in 5% CO₂. The medium was aspirated without the formazan crystals and 1 mL of DMSO was added to each well. The absorbance was measured on a microplate reader (iMark, Bio-Rad) at 540 nm.

2.4. Invasive Cell Migration Assay. For the invasion assay, the lower surface of the porous membranes in the Matrigel Invasion Chambers (BD Biosciences, USA) was coated with fibronectin (25 μg/mL) at room temperature for 1 h and washed 3 times in DMEM-BSA. DMEM-BSA was added to the lower compartment of the chamber. Cells were starved in DMEM-BSA overnight and treated with 100 μM CoCl₂ or 100 μM peptide as described above, trypsinized, and collected. Thereafter, 200 μL of each cell suspension (2 × 10⁵ cells/well in DMEM-BSA) was added to the upper compartment of the chamber and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 24 h. Cells on the upper surface of the membrane were removed, and cells that had migrated to the lower surface of the membrane were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS), stained with crystal violet (0.4% dissolved in 10% ethanol) for 15 min, washed twice with PBS, and counted under a phase-contrast microscope with a 10x objective lens. Cells in 9 randomly selected fields from triplicate chambers were counted in each experiment.

2.5. Measurement of ROS. Dichlorofluorescein diacetate (DCF-DA) was used to evaluate the generation of ROS by oxidative stress. HT1080 cells (4 × 10⁵ cells/well) in 24-well plates were first incubated with 100 μM CoCl₂ or 100 μM peptide for 24 h. The cells were then washed with PBS and incubated with 10 μM DCFH-DA for 30 min at room temperature. Fluorescence was measured by using a fluorescence plate reader. Furthermore, cellular ROS levels were determined by dihydroethidium (DHE) (Sigma-Aldrich) staining and by using the Muse Oxidative Stress Kit (Millipore) according to the manufacturer’s instructions.

2.6. Western Blotting. After proper treatment, cells were washed 2 times with PBS, harvested, and solubilized in 2x sodium dodecyl sulfate (SDS) protein sample buffer containing 100 mM Tris–HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.4% bromophenol blue, and 20% glycerol. Equal amounts of protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The resolved proteins were transferred to polyvinyl difluoride (PVDF) membranes (Millipore Corp.). The membranes were blocked by incubation with 1% bovine serum albumin (BSA) in TBS-T (10 mM Tris–HCl, 150 mM NaCl [pH 7.5], with 0.1% Tween-20) at room temperature for 1 h and further incubated with the specific primary antibody for 1 h. The membranes were washed 3 times with TBS-T and incubated for 30 min with the appropriate secondary antibody conjugated to alkaline phosphatase (AP). Proteins were detected by colorimetric reactions using the respective BCIP/NBT substrates.

3. Results

3.1. *S. maxima* Peptide Decreases CoCl₂-Induced Invasive Migration of HT1080 Cells. We first examined whether the peptide purified from *S. maxima* affects the invasive migration of highly metastatic HT1080 fibrosarcoma cells. HT1080 cells were precultured in the presence or absence of *S. maxima* peptide for 24 h, transferred onto Matrigel-coated transwell membranes, and further incubated for 16 h under preculture conditions. Cells that had migrated to the lower
surface of the membrane were fixed, stained, and counted under a phase-contrast microscope. The results demonstrated that treatment with the S. maxima peptide inhibited the invasive migration of HT1080 cells (Figure 1(a)). After treatment with the peptide, invasive migration was decreased to 97.4%, 84.7%, and 78.3% (*P < 0.05) at 25, 50, and 100 μM of peptide, respectively. Interestingly, the inhibitory effect of the S. maxima peptide on the invasive migration of HT1080 cells was more dramatic under CoCl$_2$-induced hypoxic condition (Figure 1(b)). HT1080 cells incubated with 100 μM CoCl$_2$ demonstrated an approximately 1.7-fold increase in invasive migration compared to that of blank which were not incubated with CoCl$_2$. Additional treatment with the S. maxima peptide significantly attenuated CoCl$_2$-induced invasive migration of cells. Compared to control cells without peptide treatment, treatment with 25, 50, and 100 μM of the peptide reduced the invasive migration of the cells to 85.2%, 66.2%, and 54.7%, respectively. Since treatment with <100 μM of the S. maxima peptide did not affect cell viability (Figure 2), these results suggest that the S. maxima peptide regulates intracellular signaling involved in the hypoxic invasive migration of HT1080 cells.

3.2. S. maxima Peptide Effectively Decreases CoCl$_2$-Induced ROS Generation in HT1080 Cells. Since many studies have shown that elevated ROS generation under hypoxic conditions is associated with tumor progression and metastasis, and that the S. maxima peptide decreases ROS production in FceRI-mediated mast cell activation [13], we attempted to examine whether the S. maxima peptide regulates CoCl$_2$-induced ROS generation in HT1080 cells. The fluorescent probe DCFH-DA was used to measure the effect of the S. maxima peptide on the intracellular ROS level in HT1080 cells. As shown in Figure 3(a), CoCl$_2$ treatment increased the ROS level in HT1080 cells, whereas the S. maxima peptide effectively attenuated the CoCl$_2$-induced increase in the ROS levels. Treatment with 100 μM CoCl$_2$ increased the intracellular ROS level to approximately 2.57-fold compared to that in blank without CoCl$_2$ stimulation. In contrast, the addition of 100 μM S. maxima peptide attenuated the CoCl$_2$-induced increase in ROS levels to similar level to that in control cells without CoCl$_2$ stimulation (Figure 3(a)). Similarly, microscopic fluorescence image and flow cytometry assays showed that the S. maxima peptide is effective in the suppression of CoCl$_2$-induced ROS generation (Figures 3(b) and 3(c)). Figure 3(c) shows that the addition of 100 μM S. maxima peptide decreased CoCl$_2$-induced ROS generation (67.42%) in HT1080 cells to approximately 37.14%. Therefore, these data suggest that the S. maxima peptide may regulate the invasive migration of HT1080 cells by attenuating the hypoxia-induced increase in intracellular ROS levels.
Figure 3: *S. maxima* peptide attenuates CoCl$_2$-induced ROS level in HT1080 cells. (a) HT1080 cells (4 × 10$^4$ cells/well) were incubated in the presence or absence of 100 μM CoCl$_2$ and 100 μM peptide for 24 h. Cellular ROS levels were assessed by DCFH-DA. All data are presented as mean ± S.D. *P < 0.05 compared with blank; †P < 0.05 compared with CoCl$_2$ control. (b) Representative images of HT1080 cells stained with DCF-DA. Intracellular ROS is observed with fluorescence microscopy (ZEISS, MIC00266). Bar, 10 μm. (c) Cellular ROS levels were determined by dihydroethidium (DHE) staining and flow cytometry assay. Representatives of at least 3 independent experiments are shown in the panel.
24h. Cell lysates were analyzed for HIF1α. HT1080 cells were incubated with 100 μM peptide for 24 h in the presence or absence of PI3K inhibitor, 10 μM LY294002 or ERK inhibitor, and 10 μM PD98059. Cell lysates were prepared, and the expression level of HIF1α and phosphorylation levels of Akt and ERK1/2 were examined by western blot analysis. Representatives of 3 independent experiments are shown. (b) Cells were prepared as in (a). Invasive migration of HT1080 cells was examined. All data are presented as mean ± S.D. †P < 0.05 compared with blank without CoCl2 stimulation; ††P < 0.05 compared with CoCl2 control. (c) HT1080 cells were incubated with or without 100 μM CoCl2 and 100 μM peptide for 24 h. Cell lysates were analyzed for HIF1α, β-integrin, N-cadherin, vimentin, β-catenin, and β-actin.

3.3. S. maxima Peptide Downregulates the HIF1α Signaling Pathway Necessary for CoCl2-Induced Invasive Migration of HT1080 Cells. Hypoxia-induced cellular ROS induces the expression and activation of transcription factor hypoxia-inducible factor 1α (HIF1α), which leads to aggressive cellular changes that are associated with tumor cell invasion through the regulation of target genes [2, 7]. Since PI3K/Akt and ERK1/2, upstream molecular regulators of HIF1α, have been reported to be responsible for invasive migration in response to oxidative stress during tumorigenesis [14, 15], we first examined whether these proteins are further involved in the CoCl2-induced hypoxic condition of HT1080 cells. HT1080 cells were incubated with 100 μM CoCl2 for 24 h in the presence or absence of PI3K inhibitor, LY294002 or ERK inhibitor, and PD98059. Thereafter, the expression level of HIF1α, phosphorylation levels of Akt and ERK1/2, and invasive migration of HT1080 cells were examined (Figures 4(a) and 4(b)). CoCl2 treatment increased the phosphorylation levels of Akt and ERK1/2 as well as expression level of HIF1α in HT1080 cells. However, LY294002 or PD98059 treatment reduced the CoCl2-induced increase in the expression of HIF1α and phosphorylation levels of Akt and ERK1/2 (Figure 4(a)). The CoCl2-induced invasive migration of HT1080 cells was decreased by treatment of these inhibitors (Figure 4(b)), indicating that PI3K/Akt and ERK1/2, which are upstream of HIF1α, are associated with CoCl2-induced invasive migration of HT1080 cells.

We also examined whether the S. maxima peptide regulates the PI3K/Akt and ERK1/2 signaling necessary for CoCl2-induced invasive migration of HT1080 cells (Figure 4(a)). HT1080 cells were incubated with 100 μM CoCl2 for 24 h in the presence or absence of 100 μM S. maxima peptide, and the expression and phosphorylation levels of the related proteins were examined. Treatment with the S. maxima peptide attenuated the CoCl2-induced increase in the phosphorylation levels of Akt and ERK1/2 as well as expression of HIF1α. Furthermore, CoCl2 treatment was found to increase the expression of β-integrin, another regulator of HIF1α, and the S. maxima peptide conversely attenuated CoCl2-induced increases in the expression of β-integrin (Figure 4(c)). Since many studies have previously indicated that the ROS/HIF1α-induced activation of β-integrin is associated with the activation of N-cadherin, vimentin, and β-catenin, the underlying mechanisms were further investigated (Figure 4(c)). The treatment of HT1080 cells with CoCl2 increased the expression levels of N-cadherin, vimentin, and β-catenin, whereas the S. maxima peptide treatment conversely attenuated the CoCl2-induced increase in the expression levels of these proteins.
proteins. Taken together, we confirmed that the antioxidant S. maxima peptide downregulates the HIF1α signaling pathway necessary for hypoxia-induced invasive migration of HT1080 cells by attenuating intracellular ROS.

4. Discussion

Tumor hypoxia has been regarded as a potential therapeutic problem since it can cause a more aggressive malignant progression of tumor cells. Sustained tumor hypoxia increases the potential for invasive growth and metastatic migration and enhances the intrinsic resistance of tumors to cancer treatments [1, 2]. Therefore, an understanding of the effects of hypoxia on tumor physiology is required to counteract tumor progression. Recently, it has been accepted that the ROS-induced upregulation of HIF1α causes the invasive migration of tumor cells through the regulation of genes such as PI3K/Akt, ERK1/2, and β-integrin/catenin [8–10]. Therefore, there has been a marked increase in the study of ROS and antioxidants for the prevention of tumor progression through the HIF1α signaling pathway. In this study, we showed that an antioxidant peptide derived from S. maxima attenuates CoCl2-induced intracellular ROS generation and downregulates the HIF1α signaling pathway, leading to a decrease in the invasive migration of HT1080 fibrosarcoma cells.

Previous studies have reported that hypoxia-induced ROS activate downstream PI3K/Akt and ERK1/2 pathways that regulate HIF1α. The inhibition of ROS production reduces not only the phosphorylation of Akt and ERK1/2 but also the expression of HIF1α, which is associated with a decrease in cell migration and invasion [8–10]. The suppression of PI3K/Akt or ERK1/2 inhibits the migration of tumor cells in response to the extracellular matrix and growth factors under hypoxic conditions [16–18]. Therefore, these results suggest a dependency of ROS on the PI3K/Akt and ERK1/2 signaling pathway and HIF1α expression in invasive tumor cell migration. Our results indicate that these HIF1α upstream molecules are further involved in CoCl2-induced invasive migration of HT1080 cells, and that the antioxidant S. maxima peptide has antitumor effects based on this molecular mechanism. In addition, the β-integrin family, which is heterodimeric receptors involved in cell-cell and cell-extracellular matrix interactions [19], has been reported to promote invasive cell migration [20]. A relationship between the metastatic potential of tumor cells during invasion and quantitative changes in β-integrin expression has been reported in several hypoxic cancers [21, 22]. The upregulation of β-integrin expression is associated with the upregulation of epithelial-mesenchymal transition marker proteins such as N-cadherin and vimentin, and the downregulation of E-cadherin which is a cell-cell adhesion molecular marker in cancer cells [14, 21]. Therefore, it has been suggested that the regulation of molecules downstream of β-integrin such as N/E-cadherin, vimentin, and β-catenin is also important for hypoxia-induced invasive migration of tumor cells. The present results clearly support this hypothesis by demonstrating the relationship between the expression of HIF1α and N-cadherin, vimentin, and β-integrin/β-catenin in hypoxia-induced invasive tumor cell migration. Taken together, our results suggest that the antioxidant peptide derived from S. maxima may be an effective constituent in antitumor progression products based on the mechanism of tumor progression through ROS and the HIF1α signaling pathway.

5. Conclusion

The S. maxima peptide attenuates the CoCl2-induced intracellular ROS generation and downregulates the HIF1α signaling pathway involving PI3K/Akt, ERK1/2, and β-integrin in HT1080 cells, which results in a decrease in the CoCl2-induced invasive migration of HT1080 fibrosarcoma cells.

Conflict of Interests

The authors declare no conflict of interests.

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References


Chemical Composition, Antioxidant Potential, and Antibacterial Activity of Essential Oil Cones of Tunisian Cupressus sempervirens

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The extraction yield of the essential oil (EO) extracted by hydrodistillation from the cones of Tunisian Cupressus sempervirens L. was of 0.518%. The chemical composition was analyzed by GC-MS. Results showed that this essential oil was mainly composed of monoterpene hydrocarbons (65%) with α-pinene as the major constituent (47.51%). Its antioxidant activity was ascertained by evaluating the total antioxidant capacity and also by evaluating its inhibitory effect against DPPH and ABTS radicals. In addition, it showed a strong antioxidant power against the DPPH (IC_{50} = 151 \mu g/mL) and ABTS (IC_{50} = 176.454 \mu g/mL) radicals scavenging. Moreover, its antibacterial activity was tested against different species of pathogenic bacteria (three Gram-positive and eight Gram-negative bacteria). The bacterial strains susceptible to the evaluated oil were Bacillus subtilis, Escherichia coli, Klebsiella oxytoca, Morganella morganii, Shigella, and Vibrio cholerae.

1. Introduction

Cupressus (Cupressaceae), comprising twelve species, is distributed in North America, the Mediterranean region, and subtropical Asia at high altitudes [1]. The geographic area of Cupressus genus is limited to the northern hemisphere and many species have been studied [2, 3]. In Tunisia, only one species of the genus Cupressus, Cupressus sempervirens L. [4], was native.
is used externally for headache, colds, cough, and bronchitis [12].

Studies on phytochemical compounds of Cupressus sempervirens L. revealed that it contains active constituents such as flavonoids (cupressusflavone, amentoflavone, rutin, quercitin, quercetin, and myricitrin), phenolic compounds (anthocyanidin, catechins flavones, flavonols and isoflavones, tannins, and catechol), and essential oils (EO) [13, 14]. It has been demonstrated that principals active from Cupressus sempervirens L. display antiseptic, aromatherapeutic, astringent, balsamic, and anti-inflammatory activities [15]. Cupressus sempervirens L. antimicrobial activity has been reported in several studies [14, 16].

Even studies have focused on chemistry and biological activities of Cupressus sempervirens L. leaves originating from different areas in the world [14, 16, 17]; there is no report concerning this species (cones) in Tunisia. The aim of this study is to ascertain the chemical composition of the EO of Tunisian Cupressus sempervirens L. cones and to evaluate its antioxidant and antibacterial activities.

2. Materials and Methods

2.1. Plant Material. Cones (the aerial parts) of Cupressus sempervirens L. were collected from Sidi Thabet (North of Tunisia) in March 2014. The botanical identification was achieved by Pr. Mohammed Chaieb from the Faculty of Sciences of Sfax-Tunisia. Female cones were dried at room temperature for 7 days and used for analyses. Voucher specimens of the plants were deposited in the Herbarium of this laboratory.

2.2. Extraction of the Essential Oil. Cupressus sempervirens cones were dried at room temperature. After that, 100 g of dry matter was used for essential oil extraction by hydrodistillation in a Clevenger apparatus for four hours. The resulting essential oil recovered is dried by anhydrous sodium sulphate and then stored at 4°C for further analysis.

2.3. Analysis of the Essential Oil. Cupressus sempervirens L. essential oil composition was investigated by GC and GC/MS. The analytical GC was carried out on an HP5890-series II gas chromatograph (Agilent Technologies, California, USA) equipped with Flame Ionization Detectors (FID) under the following conditions: the fused silica capillary column, apolar HP-5, and polar HP Innowax (30 m × 0.25 mm ID, film thickness of 0.25 mm). The oven temperature was held at 50°C for 1 min, then programmed at rate of 5°C/min to 240°C, and held isothermal for 4 min. The carrier gas was nitrogen at a flow rate of 1.2 mL/min; injector temperature: 250°C; detector: 280°C; the volume injected: 0.1 mL of 1% solution (diluted in hexane). The percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction. GC/MS was performed in a Hewlett-Packard 5972 MSD System. An HP-5 MS capillary column (30 m × 0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometry. The carrier gas was helium, with a flow rate of 1.2 mL/min. Oven temperature was programmed (50°C for 1 min, then 50–240°C at 5°C/min) and subsequently held isothermal for 4 min; injector port: 250°C; detector: 280°C; split ratio: 1:50; volume injected: 0.1 mL of 1% solution (diluted in hexane); mass spectrometer: HP5972 recording at 70 ev scan time: 1.5 s; mass range: 40–300 amu. Software adopted to handle mass spectra and chromatograms was ChemStation. The identification of the compounds was based on mass spectra (compared with Wiley 275L, 6th edition mass spectral library). Further confirmation was done from Retention Index data generated from a series of alkanes retention indices (relative to C9–C28 on the HP-5 and HP-20M columns) [18].

2.4. Antioxidant Activities

2.4.1. Evaluation of Total Antioxidant Capacity. The total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the oil and the formation of a phosphate subsequent green/Mo (V) complex at acid pH [19]. In a reaction volume of 1 mL was added to different concentrations of tested oil and standard sulfuric acid (0.6 M), sodium phosphate (28 mM), and ammonium molybdate (4 mM). The solutions were then incubated in a water bath at 95°C for 1 hour. After cooling to room temperature, the optical density is measured at 695 nm. Each fraction was analyzed in triplicate.

2.4.2. DPPH Radical-Scavenging Activity. The ability of our essential oil to reduce the DPPH was measured according to the method described by Tuberoso [20]. For each concentration, one milliliter was added to 0.25 mL of ethanolic solution of DPPH. The mixture was stirred vigorously and then incubated at room temperature for 30 min in the dark. The absorbance illustrating the power of the extract to reduce the free radical DPPH to the yellow-colored diphenylpicrylhydrazine was measured at 520 nm. So, antiradical activity is expressed as IC50 (µg mL−1), the extract dose required to induce a 50% inhibition. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect = [(A0 − A1)/A0] × 100, where A0 and A1 were the absorbance of the control and the sample after 30 min, respectively. Each experiment was analyzed in triplicate.

2.4.3. ABTS Assay. For ABTS assay, we used the method described by Hayouni et al. [21]. The stock solutions included 7 mM ABTS** and 2.45 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS** solution with 50 mL methanol to obtain an absorbance of 0.7 ± 0.02 units at 734 nm using the spectrophotometer 6305 UV-VIS. Fresh ABTS** solution was prepared for each assay. 50 µL of each concentration of the essential oil is added with 950 µL of ABTS** solution, allowed to react in the dark and then we follow the kinetics of this mixture every 5 min for 30 min. Then the absorbance was measured at 734 nm. Antiradical activity is expressed as IC50 (µg mL−1), the extract dose required to induce a 50% inhibition. A low IC50 value corresponds to
a high antioxidant activity of plant extract. Results are expressed in μg Trolox equivalents (TE)/mg dry mass. The percentage inhibition of the ABTS cation radical by the samples was calculated according to the following formula: Inhibition percentage (% inhibition) = \((A_0 - A_t)/A_0 \times 100\), where \(A_0\) is absorbance of control sample \((t = 0 \text{ h})\) and \(A_t\) is absorbance of a tested sample in 5 or 30 min.

2.5. Antimicrobial Activities

2.5.1. Microorganisms. The tested microorganisms include Gram-positive bacteria, Staphylococcus aureus ATCC 29213, Bacillus subtilis ATCC 6633, and Bacillus cereus ATCC 11778, and Gram-negative bacteria: Escherichia coli ATCC 8739, Klebsiella oxytoca CECT 8207, Salmonella salamae ATCC 43972, Salmonella typhi ATCC 25241, Morganella morganii ATCC 25830, Salmonella anatum ATCC 9270, Vibrio cholerae CECT 8265, and Shigella ATCC 29930.

2.5.2. Disk-Diffusion Assay. Antibacterial activity was evaluated using the method described by Choi et al. (2006) [22]. The principle of this method is to use Whatman paper discs of 6 mm in diameter. The discs were impregnated with essential oil dilutted in hexane. A disc soaked in hexane was used as negative control. These discs are then deposited on the surface of a medium swab with a bacterial suspension to an optical density of 0.5 McFarland standard. We used the bacterial strains for the culture medium Muller-Hinton. At the end of the incubation, 24 hours at 37°C, the diameters of the zone of inhibition were measured.

2.5.3. Minimum Inhibitory Concentration. A broth microdilution was used to determine the minimum inhibitory concentration (MIC). The tests were performed in Muller-Hinton Broth. The essential oil was dissolved in Tween 80. A serial dilution of tested oil was prepared in a 96-well microtiter plate over the range 78 μg/mL–1 mg/mL. In each well containing a concentration was added 100 μL of bacterial suspension of 10^6 (0.5 MacFarland standard). We incubated plates overnight at 37°C, and then measured the optical density at 620 nm.

3. Results and Discussion

3.1. Chemical Composition. The extraction yield of the EO from the cones of Tunisian Cupressus sempervirens L. was of 0.518%. The coupling analysis of GC/MS and GC-FID/KI revealed 67 compounds (Figure 1). The major compound was α-pinene (47.51%). It was followed by δ-3-carene, α-terpinyl acetate, β-caryophyllene, and α-cedrol whose proportions were 7.40, 4.11, 4.53, and 4.99%, respectively (Table 1). These results are in accordance with those reported by Boukhri et al. (2012) [17] for Cupressus sempervirens L. collected from the random gardens in Sfax, Tunisia, and characterized by α-pinene (37.14%), δ-3-carene (19.67%), limonene (5.43%), and α-terpinolene (4.69%) as the most abundant volatiles. Furthermore, Riahi et al. (2012) [23] reported a yield of 0.92% for Tunisian Cupressus sempervirens L. It is higher than that obtained for Algerian C. sempervirens (0.26%) [14] and comparable to that of the Cameroon species (1%) [24].

The major components of Tunisian Cupressus sempervirens L. as reported by Riahi et al. (2012) [23] were α-pinene (24.44%), 3-carene (18.60%), α-limonene (11.61%), terpinen-4-ol (10.56%), β-myrcene (4.89%), camphor (4.62%), and β-linalool (4.23%). For essential oils of most Cupressus species, α-pinene and 3-carene were cited as the major compounds [25]. However, in our sample α-pinene component is absent. Results by Taponjout et al. (2005) revealed that the oil from Cupressus sempervirens L. in Cameroon mainly consists of α-pinene (9.9%), terpinen-4-ol (11.2%), and sabine (14.8%).

Moreover, α-pinene and γ-terpinene accounted, respectively, for 39.5 and 11.56% of the whole essential oil of Cupressus sempervirens L. cones originating from Greece [26]. The cone essential oil of Egyptian Cupressus sempervirens L. showed antibacterial activity [27]. It is in contradiction with the results reported by Chéraif et al. (2005) [28]. Indeed, α-pinene is present in the essential oil of Cupressus sempervirens L. leaves at a low rate (20%) compared with that of cone essential oil. In addition, the proportion of δ-3-carene is important (22.9%) in the essential oil branches of Cupressus sempervirens L. by comparison with that of cones (74.0%). Limonene is of the order of 5.1% in the essential oil branches of Cupressus sempervirens L. Its rate is lower in cones essential oil (1.75%). The same results have been reported for α-terpinyl acetate whose rates were of 7.5% and 4.11%, respectively, in branches and cones. Furthermore, β-caryophyllene was detected as trace in twigs whereas its rate was of 4.53% in cones. Among volatiles, α-pineneol was present in the branches essential oil with an amount of 9.4%. However, it was absent in cones essential oil.

Loukis et al. (1991) [26] reported that α-pinene and γ-terpinene accounted, respectively, for 39.5 and 11.56% of the whole essential oil of Cupressus sempervirens L. cones originating from Greece.

Emami et al. (2004) [29] detected 42 compounds in the essential oil of cones Cupressus sempervirens L. originating from Egypt. They reported a yield of 0.26% for leaves essential oil. Tognolini et al. (2006) [30] reported that the α-pinene is the major compound. This is the second monoterpenoid hydrocarbon predominant compound according to the results of
Table 1: Essential oil composition from cones of *Cupressus sempervirens*.

<table>
<thead>
<tr>
<th>Number</th>
<th>Volatile compound</th>
<th>RI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amount (% of whole EO)</th>
<th>Methods of identification</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>Tricyclene</td>
<td>924</td>
<td>1015</td>
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<td>1032</td>
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<tr>
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<td>α-Fenchene</td>
<td>953</td>
<td>1044</td>
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</tr>
<tr>
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<td>Camphene</td>
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<td>1076</td>
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<tr>
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<td>1132</td>
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</tr>
<tr>
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<tr>
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<td>26</td>
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</tr>
<tr>
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<td>1709</td>
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<tr>
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<td>41</td>
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<td>1687</td>
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<tr>
<td>42</td>
<td>Alloaromadendrene</td>
<td>1474</td>
<td>1661</td>
<td>0.54</td>
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<td>43</td>
<td>Germacrene D</td>
<td>1480</td>
<td>1696</td>
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<td>44</td>
<td>α-Muurolene</td>
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<td>46</td>
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<td>1837</td>
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<td>Cadina-1,4-diene</td>
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<td>β-Bisabolene</td>
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</tr>
<tr>
<td>50</td>
<td>α-Cedrol</td>
<td>1597</td>
<td>2021</td>
<td>4.99</td>
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</tr>
</tbody>
</table>
Sacchetti et al. (2005) [31]. Furthermore, the most abundant volatile compounds in the essential oil of Turkish Cupressus sempervirens L. are \( \alpha \)-pinene and \( \Delta ^{3} \)-carene. Obviously, the composition of essential oils is significantly influenced by the organ. It is also influenced by many other factors including environmental factors such as rainfall, sunlight, soil, and climatic conditions and agronomic factors such as the date of harvest and the density of the culture.

3.2. Antioxidant Activities. Considering the many aspects of antioxidants and their reactivity, several tests are applied as antioxidants. Among them, the total antioxidant capacity, DPPH, and ABTS radical scavenging tests are used to determine the antioxidant power of essential oil.

The chemical complexity of essential oils, often a mixture of dozens of compounds with different functional groups, polarity, and antioxidant activity, may lead to scattered results, depending on the test employed [31].

3.2.1. Total Antioxidant Capacity. The total antioxidant activity of Cupressus sempervirens L. is expressed as Trolox equivalent. The phosphomolybdenum method is based on the reduction of Mo (VI) with Mo (V) by the antioxidant compounds and the formation of a green phosphate/Mo complex (V). Our results showed that the antioxidant activity is proportional to the extract concentration (0.0434 \( \mu \)g ET/g DW) (Figure 2). Baykan Erel et al. (2012) [32] studied the antioxidant activity of essential oils of six species of Artemisia. It was noted that only essential oil of Artemisia absinthium and Artemisia arborescens has a total antioxidant activity with values, respectively, in the order of 2.89 mg ET/g DW and 3.39 mg ET/g DW.

3.2.2. DPPH Radical-Scavenging Activity. Free radical-scavenging activities of the tested oil and positive control (Trolox) are presented in Figure 3. In fact, Cupressus sempervirens L. essential oil remarkably reduced the concentration of DPPH free radical and transformed its stable, purple color into the yellow-colored DPPH-H with an efficiency IC\( _{50} \) = 151 \( \mu \)g/mL. The effect of antioxidant on DPPH radical scavenging was thought to be due to its hydrogen-donating ability. DPPH
Table 2: Antibacterial activity from essential oil cones of *Cupressus sempervirens*.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Concentration (mg/mL)</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Bacillus subtilis ATCC 6633</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Escherichia coli ATCC 8739</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca CECT 8207</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Morganella morganii ATCC 25830</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Shigella ATCC 29930</em></td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Vibrio cholerae CECT 8265</em></td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [33].

In two antioxidants screening studies *Cupressus sempervirens* L. was reported to display moderate radical-scavenging effect against DPPH [31, 34]. The leaf methanolic extract of Egyptian *Cupressus sempervirens* L. had strong DPPH radical-scavenging activity as reported by Ibrahim et al. (2009) [35].

Needless to say, potency of antioxidant activity of a substance is correlated with the applied method. In a study on some Iranian conifers the leaf and fruit MeOH extracts of *Cupressus sempervirens* L. were highly effective in ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods [36]. Nevertheless, activity of these extracts was also changeable according to the method.

3.2.3. ABTS Assay. Essential oil of *Cupressus sempervirens* L. had an antioxidant according to the DPPH test. The results relative to ABTS radical cation scavenging confirmed the previous result. In fact, IC$_{50}$ obtained at the end of this test is 176.454 µg/mL (Figure 4). Ghazghazi et al. (2010) [37] obtained, by studying the essential oil samples of *R. canina* of Feija and Ain Draham (North of Tunisia) during the test of the ABTS, IC$_{50}$ values of 159.0 µg/mL and 201.8 µg/mL, respectively. From these results we can conclude that the essential oil of *Cupressus sempervirens* L. has a strong antioxidant against ABTS cation. The antioxidant activity of essential oil could be assigned to the synergistic effects of two or more of its components. In this context, Lu and Foo (2001) [38] reported that most natural antioxidant compounds often work synergistically to produce a broad spectrum of antioxidant properties that create an effective defense system against free radicals. *Cupressus sempervirens* L. essential oil consists of a very complex mixture of various chemical classes (Table 1), which may produce either synergistic or antagonistic effects on the process of lipid oxidation [39].

3.3. Antibacterial Activity. The antibacterial test allowed us to determine the sensitive strains to the essential oil of *Cupressus sempervirens* L. The screening of this activity was firstly determined by the disk-diffusion method on agar for predicting the inhibitory activity of the oil on the growth of a bacterial culture. Our results showed that the evaluated oil had significant antibacterial effect. In fact, among twelve tested strains, six were sensitive to this oil (Table 2). Gram-negative bacteria (*Klebsiella oxytoca, Vibrio cholerae, Shigella*, and *E. coli*) were the most sensitive strains with the MIC values that do not exceed 125 µg/mL of essential oil (Table 2). This finding was in agreement with other findings. For example, the cone EO of Egyptian *Cupressus sempervirens* L. showed...
antibacterial activity [27]. Chéraif et al. (2005) [28] tested the antibacterial activity of essential oil Cupressus sempervirens L. on Gram-positive and Gram-negative bacteria and found that this essential oil has a moderate activity against tested bacteria. It is important to mention that the activity was more pronounced for Gram-positive than Gram-negative bacteria. Similarly, Mazari et al. (2010) [14], when studying the biological activities of essential oils of Cupressus sempervirens L. and Juniperus phoenicea, reported that both oils constitute sources of antimicrobial agents. There are often large variations in the intensity of the antimicrobial activities against Gram-negative and Gram-positive bacteria.

The EO of Cupressus sempervirens L. presented antibacterial activity against Gram-positive and Gram-negative bacteria, showing the biggest inhibition with B. subtilis and E. coli. Additionally, the cypress essential oil was found to have moderate antimicrobial activity when compared to vancomycin (30 mcg) and erythromycin (15 mcg) as antibiotics [40].

The antibacterial activity could be affected by the solubility of the oil, the diffusion range in the agar and the evaporation [41, 42]. In addition, the antibacterial activities of the essential oils suggest their usefulness in the treatment of various infectious diseases caused by the tested bacteria.

It is well known that Gram-negative bacteria are more resistant to essential oil compound antibacterial properties than Gram-positive bacteria because of hydrophobic lipopolysaccharide in the outer membrane which provides protection against different agents [43]; however, the obtained results indicated that the evaluated oil possesses selective antibacterial activity and its effect was pronounced against Gram-negative bacteria compared to Gram-positive ones.

4. Conclusion

According to our results, the essential oil of cypress cones is very rich in α-pinene which is its major component. This compound is followed by Δ-3-carene, α-terpinyl acetate, β-caryophyllene, and cedrol. This composition is different from those found in other studies, and this difference may be due to climatic factors.

Furthermore, the evaluation of the antioxidant activity by chemical tests (total antioxidant capacity, DPPH, and ABTS) revealed a variable behavior of the essential oil against the used radicals. Moreover, IC₅₀ values calculated are very low such that this reflects the high antioxidant power: they are of 176.45 and 151 μg/mL for ABTS and DPPH tests, respectively. The evaluation of the antibacterial activity of the tested essential oil showed that it possesses a significant activity. These promising results allow us to expand our studies to ascertain other activities of this essential oil such as antiparasitic, antifungal, and anticholinesterase activities.

Conflict of Interests

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

Acknowledgment

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References

Inhibitory Effects of 4-(4-Methylbenzamino)benzoate on Adipocyte Differentiation

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The potent suppression of adipocyte differentiation by 4-(4-methylbenzamino)benzoate was discovered during the search for new antiobesity compounds. 4-(4-methylbenzamino)benzoate was observed to suppress adipocyte differentiation in 3T3-L1 cells by 96.8% at 50 𝜇M without cytotoxicity. In addition, 4-(4-methylbenzamino)benzoate reduced the cellular expression of fatty acid synthase in a concentration-dependent manner, as well as suppressing PPAR-gamma activity, which controls fatty acid storage and glucose metabolism. Based on these results, 4-(4-methylbenzamino)benzoate shows potential as an antiobesity material.

1. Introduction

Obesity, a major factor in the development of heart disease, cancer, hypertension, diabetes, and degenerative arthritis, is induced by adipocyte differentiation due to hormonal changes and imbalances in energy metabolism caused by excessive fat intake [1–5]. Adipogenesis in the body is the process of cell differentiation by which preadipocytes become adipocytes during fat accumulation. Mature adipocytes are differentiated from immature adipocytes such as fibroblasts and form lipid droplets inside cells [6, 7].

Studies have shown that natural compounds, resveratrol and genistein, have antiobesity effects [8–11]. Resveratrol and genistein are contained in grapes and beans, respectively. Although some antiobesity drugs are currently available, such as orlistat, sibutramine, or sertraline, several side effects have been reported [12]. Thus, studies are being conducted on compounds with antiobesity effects to replace these drugs.

In this study, the potent adipogenesis-suppressing activity of 4-(4-methylbenzamino)benzoate (MBAB, Figure 1) was observed in 3T3-L1 cells without cytotoxicity, during the search for new antiobesity substances. MBAB exhibited higher adipogenesis-suppressing activity compared to resveratrol or genistein. Therefore, MBAB show great potential as a new antiobesity substance.

2. Materials and Methods

2.1. Materials. MBAB was synthesized using a previously reported method [13]. Resveratrol and genistein, which were used as positive controls, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 3T3-L1 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and the FAS antibodies from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell Culture and Differentiation. 3T3-L1 cells were cultured in DMEM culture medium with 10% FBS, at 37°C, and 5% CO2 conditions. For the differentiation, 3T3-L1 cells were grown in a 48-well plate until confluence, and differentiation was induced by incubation with a hormonal cocktail containing 10 𝜇g/mL insulin, 0.5 𝜇M dexamethasone, and 0.5 𝜇M IBMX for 24 h. The cells were then incubated with a normal medium containing 10 𝜇g/mL insulin in the presence or absence of MBAB, resveratrol, and genistein for 8 days.
2.3. **Cell Viability.** After differentiation was completed, 0.5 mg/mL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added and incubated at 37°C for 4 hours. After eliminating the MTT solution, 200 μL of DMSO was added and absorbance at 540 nm was measured to determine cell viability.

2.4. **Oil Red O Staining and TG Assay.** When differentiation was complete, the cells were washed with PBS twice and fixed with 3.7% formaldehyde. After incubating the cells for 1 hour using Oil Red O dye, isopropanol was added, and the absorbance was measured at 510 nm to determine the amount of triglycerides.

2.5. **Fatty Acid Synthase (FAS) Expression.** The 3T3-L1 cells were washed twice with ice-cold PBS and then lysed with a lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 mM sodium orthovanadate, 1 mM NaF; and 0.2% protease inhibitor cocktail, pH 7.2). The collected protein was centrifuged at 14,000 rpm for 5 minutes, and the supernatant was collected for protein quantification. Then, 30 μg of protein was loaded in 10% SDS-PAGE for electrophoresis, after which it was transferred to a nitrocellulose membrane and reacted with FAS antibodies (1:1000), anti-mouse antibody (1:1000), and detected by ECL.

2.6. **Peroxisome Proliferator-Activated Receptor γ (PPAR-γ) Transcription Activity.** The effect on PPAR-γ activity in 3T3-L1 cells was measured using a PPAR-γ transcription factor assay kit (Cayman Chemical). The 3T3-L1 cells were treated with rosiglitazone and each concentration of MBAB, and then the cell extract was added to a dsDNA sequence-coated plate. The PPAR-γ antibodies and the secondary antibodies were reacted in order, and then a detection reagent was added and absorbance was measured at 450 nm.

2.7. **Statistical Analysis.** All data were presented as mean ± standard deviation (SD). The significance of the differences between groups was tested using one-way analysis of variance (ANOVA).

3. **Results and Discussion**

3.1. **Effects on Cell Viability.** The MTT assay showed that MBAB did not cause significant cell death at a concentration of less than 50 μM, whereas resveratrol and genistein reduced the cell viability in a concentration-dependent manner (Figure 2). Therefore, our results indicate that MBAB has lower cytotoxicity compared to resveratrol and genistein.
3.2. Inhibitory Effects on Adipocyte Differentiation. After confirming the absence of cytotoxicity in 3T3-L1 cells treated with MBAB at concentrations of less than 50 μM, the effect on adipocyte differentiation was measured. As shown in Figure 3, MBAB suppressed the adipocyte differentiation of 3T3-L1 cells in a concentration-dependent manner after they were induced to differentiate after being treated with a hormone mixture. MBAB strongly suppressed adipocyte differentiation at a concentration of 50 μM, which was similar to the control group. In particular, MBAB exhibited a higher adipocyte differentiation suppressing activity at all concentrations compared to the same concentrations of resveratrol or genistein. Figure 4 shows photographs of 3T3-L1 cells treated with MBAB and stained with Oil Red O.

3.3. Inhibitory Effects on FAS Expression. Fatty acid is synthesized by the action of fatty acid synthase (FAS), an enzyme with a molecular weight of 250 kDa, using malonyl-CoA as substrate [14–17]. FAS is known as an important factor in the regulation of fat biosynthesis and obesity. Thus, FAS inhibitors can effectively reduce fat production and suppress obesity.

The effect of MBAB on the intracellular expression of FAS was measured as shown in Figure 5. MBAB inhibited the increase in FAS production in 3T3-L1 cells that were induced by a hormone mixture in a concentration-dependent manner. Therefore, it is considered that MBAB impairs lipid production by suppressing fatty acid biosynthesis through FAS reduction.

3.4. Inhibitory Effects on PPAR-γ Activity. PPAR-γ regulates fatty acid storage and glucose metabolism [18]. Since the PPAR-γ signaling pathway is known to be a major target for the development of antiobesity drugs, the effect of MBAB
Conflicts of Interests

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

References


We investigated the biologically active substances contained in RVA (regrowth velvet antler) by comparing the composition of biologically active substances and antioxidant potential of different antler segments. RVA was subjected to extraction using DW (distilled water). RVA was divided into 3 segments: T-RVA (top RVA), M-RVA (middle RVA), and B-RVA (base RVA). The T-RVA section possessed the greatest amounts of uronic acid (36.251 mg/g), sulfated GAGs (sulfated glycosaminoglycans) (555.76 mg/g), sialic acid (111.276 mg/g), uridine (0.957 mg/g), uracil (1.084 mg/g), and hypoxanthine (1.2631 mg/g). In addition, the T-RVA section possessed the strongest antioxidant capacity as determined by DPPH, \( \text{H}_2\text{O}_2 \) (hydrogen peroxide), hydroxyl, and ABTS (2,2'-azinobis-3-ethylbenzthiazoline-6-sulphonate) radical scavenging activity as well as FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity). The values of those were 53.44, 23.09, 34.12, 60.31, and 35.81 TE/\( \mu \)g/mL and 113.57 TE/\( \mu \)g/mL at 20 \( \mu \)g/mL. These results indicate that the T-RVA section possesses the greatest amount of biologically active substances and highest antioxidant potential. This is the first report on the biologically active substances and antioxidant potential of RVA.

1. Introduction

Velvet antler consists of the cartilaginous, prequalified antlers of moose, elk, and sika deer, which regrow yearly. The growth of deer antlers is one of the fastest types of tissue growth in mammals. Growing antlers contain nerves and blood vessels and are covered with a hairy, skin covering tissue commonly known as “velvet” [1]. Velvet antler is a widely used traditional Asian medicine that has been used clinically in East Asia for millennia to treat various diseases and as a tonic [2]. Velvet antler is generally harvested twice per year. The first velvet antler harvest occurs after 40–45 days of growth, while a second harvest occurs after 50–55 days of regrowth, at which point the harvested velvet antler is known as RVA. Although studies have been conducted on the chemical composition of RVA [3, 4], there have been no comprehensive reports on the composition of biologically active substances and antioxidant potential of RVA.

Numerous studies have demonstrated that free radicals are generated by oxidative damage to biomolecules such as lipids, nucleic acids, proteins, and carbohydrates [5–7]. Overproduction of free radicals and reactive oxygen species is believed to be associated with cellular and tissue pathogenesis, which leads to several chronic diseases such as cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases [8]. Many medical reports and clinical observations convincingly show that disease-resistance can be conferred by enhancing antioxidative processes [9–14].
Therefore, antioxidant supplementation could prevent or inhibit oxidative stress induced by ROS. Antioxidants terminate free radical chain reactions by removing free radical intermediates while inhibiting other oxidation reactions. Because of the clinical potential of antioxidants, significant interest has been focusing on the development of natural antioxidants that are safe and effective.

In this study, RVA was subjected to extraction by DW to allow determination of its constituent biologically active substances, including uronic acid, sulfated GAGs, sialic acid, uracil, hypoxanthine, and uridine. In addition, the antioxidant activities of RVA were determined by assessing DPPH, \( \text{H}_2\text{O}_2 \), hydroxyl, and ABTS radical scavenging activity as well as FRAP and ORAC.

2. Materials and Methods

2.1. Materials. Seven specimens of sika deer (Cervus nippon) RVA were collected at the same farm (Fanrong farm, China). Carbazole, sodium tetraborate, dimethylmethylene blue, glycine, sodium thiosulfate, acetatoacellulose, uracil, hypoxanthine, uridine, DPPH, ABTS, potassium persulfate, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), FL, and AAPH (2,2′-azobis(2-amidinopropane)dihydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of Samples. The RVA specimens were divided into 3 sections, T-RVA, M-RVA, and B-RVA, lyophilized and homogenized with a grinder. Next, 10 g of each segment was added to 100 mL of DW and subjected to extraction in boiling DW for 1 h. The RVA extracts were filtered (0.45 \( \mu \)m pore size) and lyophilized (yields: T-RVA, 3.87%; M-RVA, 3.61%; B-RVA, 2.66%) in a freeze dryer for 5 days.

2.3. Analysis of Bioactive Compounds

2.3.1. Uronic Acid. Uronic acid content was determined by the carbazole reaction [15]. Briefly, a 50 \( \mu \)L serial dilution of the standards or samples was placed in a 96-well plate, after which 200 \( \mu \)L of 25 mM sodium tetraborate in sulfuric acid was added to each well. The plate was heated for 10 min at 100°C in an oven. After cooling at room temperature for 15 min, 50 \( \mu \)L of 0.125% carbazole in absolute ethanol was carefully added. After heating at 100°C for 10 min in an oven and cooling at room temperature for 15 min, the plate was read in a microplate reader at a wavelength of 550 nm.

2.3.2. Sulfated GAGs. GAGs content was determined by the DMB (dimethylmethylene blue) dye binding method [16]. Briefly, the color reagent was prepared by dissolving 0.008 g of DMB in a solution containing 1.185 g NaCl, 1.520 g glycine, 0.47 mL HCl (12 M), and 500 mL DW. Each sample was mixed into 1 mL of color reagent and the absorbance was read immediately at 525 nm.

2.3.3. Sialic Acid. Sialic acid content was determined based on the procedures described by Matsuno and Suzuki [17]. All solutions were precooled in an ice bath. Sodium periodate solution (10 mM, 20 \( \mu \)L) was added to 200 \( \mu \)L of a glycoconjugate sample in a 15 mL polypropylene test tube. The solution was chilled in an ice bath for 45 min. The reaction was terminated by the addition of 100 \( \mu \)L of 50 mM sodium thiosulfate solution. Next, 500 \( \mu \)L of 4.0 M ammonium acetate (pH 7.5) and 400 \( \mu \)L of ethanolic solution of 100 mM acetocetanilide were added to the solution, which was left standing for 10 min at room temperature. The fluorescence intensity of the solution was measured at 471 nm with an excitation wavelength of 388 nm.

2.3.4. Uracil, Hypoxanthine, and Uridine. Uracil, hypoxanthine, and uridine were determined as described previously [18]. 1 mg of the DW extract was dissolved in 1 mL of 3% methanol solution, after which 1 mL of the resulting solution was filtered for HPLC analysis. The analysis was performed on an HPLC system equipped with an isocratic pump (Kyoto, Japan) and RI (refractive index) detector (Lab Alliance, model 500). The separation was conducted on a ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 5 \( \mu \)m, Agilent Technologies, USA). The mobile phase was 0.07% acetic acid/methanol/water (3:97, v/v/v; pH 3.5) at a flow rate of 1.0 mL/min. A series of standards of uracil, hypoxanthine, and uridine in the range of 0.625–40.00 ppm were prepared in the mobile phase. Quantification was carried out by integration of the peak areas using external standard calibration. A linear response with a correlation coefficient of 0.999 (\( r = 6 \) ) was obtained for the standards. For all experiments, the extracts and standards were filtered through a 0.45 \( \mu \)m cellulose ester membrane before injection into the HPLC system. Detection was performed at a wavelength of 254 nm.

2.4. Antioxidant Activity

2.4.1. DPPH Radical Scavenging Activity. The DPPH scavenging activity of each antler extract was measured according to a slightly modified version of the method of Blois [19]. DPPH solutions (1.5 × 10^{-4} M, 100 \( \mu \)L) were mixed with and without each extract (100 \( \mu \)L), after which the mixtures were incubated at room temperature for 30 min. After standing for 30 min, absorbance was recorded at 540 nm using a microplate reader. The scavenging activity was calculated as a percentage using the following equation:

\[
\text{Inhibition} \% = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100, \quad (1)
\]

where \( A_{\text{control}} \) was the absorbance of the reaction mixture without an RVA sample and \( A_{\text{sample}} \) was the absorbance of the reaction mixture with an RVA sample.

2.4.2. Hydrogen Peroxide Radical Scavenging Activity. Hydrogen peroxide scavenging activity was determined according to the method of Muller [20]. A 100 \( \mu \)L of 0.1 M phosphate buffer (pH 5.0) was mixed with each extract in a 96-microwell plate. A 20 \( \mu \)L of hydrogen peroxide was added to the mixture and then incubated at 37°C for 5 min. After the incubation, 30 \( \mu \)L of 1.25 mM ABTS and 30 \( \mu \)L of peroxidase (1 unit/mL) were added to the mixture and then incubated...
at 37°C for 10 min. The absorbance was recorded at 405 nm by microplate reader and the percentage of scavenging activity was calculated using (1).

2.4.3. Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of each antler extract was determined according to the method of Chung et al. [21]. Hydroxyl radicals were generated by the Fenton reaction in the presence of FeSO₄. A reaction mixture containing 0.1 mL of 10 mM FeSO₄, 10 mM EDTA, and 10 mM 2-deoxyribose was mixed with 0.1 mL of the extract solution, after which 0.1 M phosphate buffer (pH 7.4) was added to the reaction mixture to reach a total volume of 0.9 mL. Subsequently, 0.1 mL of 10 mM H₂O₂ was added to the reaction mixture, which was incubated at 37°C for 4 h. After incubation, 0.5 mL of 2.8% TCA and 1.0% TBA were added to each mixture, after which each mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm. Hydroxyl radical scavenging activity was calculated as a percentage using (1).

2.4.4. ABTS Radical Scavenging Activity. The ABTS scavenging activity of each antler extract was assessed following the method of Arnao et al. [22]. Stock solutions included ABTS⁺⁺ solution and potassium persulfate solutions. A working solution was prepared by mixing the 2 stock solutions in equal quantities and allowing them to react for 12 h. The working solution was diluted with fresh ABTS⁺⁺ solution and mixed with or without each extract. After incubation for 2 h, the absorbance of each solution was recorded at 735 nm. The scavenging activity was calculated as a percentage using (1).

2.4.5. FRAP Assay. The FRAP assay was performed according to the method of Benzie and Strain [23]. Fresh working solution was prepared by mixing acetate buffer, TPTZ solution, and FeCl₃·6H₂O solution and warmed at 37°C before use. Each extract was allowed to react with the FRAP solution in a dark room at room temperature for 30 min. The absorbance of the colored product was measured at 595 nm. Scavenging activity was calculated as a percentage using (1).

2.4.6. ORAC Assay. For ORAC assay, the method of Ou et al. was used with some slightly modification [24]. The working solution of FL and AAPH radical was prepared daily. Sample, blank, or standard was placed in 96-microwell plate, and the plate was heated to 37°C for 15 min prior to the addition of AAPH. The fluorescence was measured immediately after the AAPH addition and measurements with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm were taken every 5 min until the relative fluorescence intensity was less than 5% of the value of the initial reading.

The ORAC values, expressed as μM Trolox equivalents (μM TE/mg) were calculated by applying the following formula:

\[
\text{ORAC (μM TE)} = \frac{(C_{\text{Trolox}} \times (\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}) \times k)}{\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}},
\]

where \(C_{\text{Trolox}}\) is the concentration of Trolox (20 μM), \(k\) is the sample dilution factor, and \(\text{AUC}\) is the area below the fluorescence decay curve of the sample, blank, and Trolox, respectively, calculated by applying the following formula in a Microsoft Excel spreadsheet (Microsoft, Washington, USA):

\[
\text{AUC} = \left(0.5 + \frac{f_5}{f_{0}} + \frac{f_{10}}{f_{0}} + \cdots + \frac{f_n + 5}{f_{0}}\right) \times 5,
\]

where \(f_{0}\) is the initial fluorescence and \(f_n\) is the fluorescence at time \(n\).

2.5. Statistical Analysis. The results shown are summaries of the data from at least 3 experiments. All data are presented as mean ± SEM (standard error of the mean). Statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC, USA). Treatment effects were analyzed using one-way ANOVA followed by Dunnett’s multiple range test. Results of \(P < 0.05\) indicated statistical significance.

3. Results and Discussion

3.1. Bioactive Composition. The biologically active substances contained in the 3 RVA segments, including uronic acid, sulfated GAGs, sialic acid, uridine, uracil, and hypoxanthine, are listed in Tables 1 and 2.

The uronic acid content, sulfated GAGs content, and sialic acid content of the T-RVA and M-RVA sections were significantly greater than those of the B-RVA section \((P < 0.05)\). The DW extract of the T-RVA section contained 36.25 mg/g uronic acid, 555.76 mg/g sulfated GAGs, and 111.28 mg/g sialic acid (Table 1). The DW extract of the RVA contained 0.957 mg/g uridine, 1.084 mg/g uracil, and 1.263 mg/g hypoxanthine (Table 2).

Uronic acid has been reported to improve circulation and decrease stroke risk [25]; therefore, our chemical analyses indicate that the DW extract of T-RVA might possess similar activities. Sulfated GAGs, particularly CS (chondroitin sulfate), are of particular interest to physicians and pharmacists. Sulfated GAGs are composed of units of amino sugar, including D-glucosamine and D-galactosamine, and bond with core proteins to form proteoglycans. Cartilage proteoglycans regulate water retention and are integral to the differentiation and proliferation of chondrocytes. The most prominent sulfated GAG in velvet antler tissue is chondroitin sulfate [26]. Sialic acid is a water soluble component that was efficiently extracted by DW and showed significant accumulation in the T-RVA section. Our findings are similar to those of a previous report [27], which showed that the T-RVA, or “wax piece,” contains sialic acid levels higher than those of other antler regions. Uracil is a primary mediator of MAO (monoamine oxidase) inhibition by velvet antler extract [28]. Our data indicate that the T-RVA section may contribute the majority of the inhibitory effect on MAO activity produced by velvet antler. In a report by Wang et al. [29] aimed at identifying the active compound in velvet antler responsible for inhibiting MAO-B activity, the author suggested that the main antiaging compound in velvet antler is hypoxanthine. Zhou et al. [28] showed that uridine was...
Evaluated by assessing DPPH, H2O2 methods were used to evaluate different aspects of the extracts. Therefore, the DW extract of T-RVA is expected to show strong antioxidant activity due to its abundance of uridine. Zhou and Li [18] investigated the amounts of uridine, uracil, and hypoxanthine from ethanol extracted velvet antler, and the values were 3.7, 3.6 and 3.9 mg/g, respectively. Their values were higher than ours. This may be attributed to extraction method.

### Table 1: Sulfated GAGs, sialic acid, and uronic acid contents of regrowth velvet antler extracts.

<table>
<thead>
<tr>
<th></th>
<th>T-RVA</th>
<th>M-RVA</th>
<th>B-RVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfated GAGs</td>
<td>555.76 ± 12.48</td>
<td>369.30 ± 19.81</td>
<td>228.98 ± 24.42</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>111.28 ± 4.27</td>
<td>79.50 ± 5.20</td>
<td>72.96 ± 1.50</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>36.25 ± 2.96</td>
<td>25.90 ± 2.29</td>
<td>23.11 ± 2.40</td>
</tr>
</tbody>
</table>

### Table 2: Hypoxanthine, uridine, and uracil contents of regrowth velvet antler extracts.

<table>
<thead>
<tr>
<th></th>
<th>T-RVA</th>
<th>M-RVA</th>
<th>B-RVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine</td>
<td>1.08 ± 0.03</td>
<td>1.00 ± 0.01</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>Uridine</td>
<td>1.26 ± 0.03</td>
<td>1.14 ± 0.04</td>
<td>1.06 ± 0.03</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.96 ± 0.08</td>
<td>0.092 ± 0.03</td>
<td>0.845 ± 0.04</td>
</tr>
</tbody>
</table>

#### 3.2. Antioxidant Activity

The antioxidant activities of RVA may not be attributed to a single mechanism. Therefore, 6 methods were used to evaluate different aspects of the antioxidant activities of RVA.

The antioxidant activities of the DW extracts of RVA were evaluated by assessing DPPH, H2O2, ABTS, and hydroxyl radical scavenging activity. In addition, FRAP and ORAC were estimated.

The antioxidant activity of the DW extract of T-RVA was significantly better than those of the M-RVA and B-RVA sections (P < 0.05) and appeared to be dose-dependent. The DPPH radical scavenging activity was highest for the T-RVA section (53.44 μM TE/mg; IC50 0.853 mg/mL) and lowest for the B-RVA section (Figure 1). H2O2 (32.20 μM TE/mg; Figure 2) and ABTS (60.31 μM TE/mg; Figure 3) radical scavenging activities were also highest for the T-RVA section. The hydroxyl radical scavenging activity was highest for the T-RVA section (23.09 μM TE/mg), whereas the activities of M-RVA and B-RVA were similar (Figure 4). The T-RVA section was the most effective section in the FRAP assay (35.81 μM TE/mg), whereas the activities of B-RVA and M-RVA were similar (Figure 5). In the ORAC assay, 1,000 mg/mL T-RVA showed excellent activity (121.58 μM TE/mg) (Figure 6).

DPPH radical scavenging activity is often used as a method of evaluating antioxidant activity. DPPH is a stable radical that accepts an electron and/or hydrogen radical from donor molecules to form a stable diamagnetic molecule. Therefore, the extracts of velvet antler may have provided an electron and/or hydrogen radical to neutralize DPPH [30]. In a report by Lee and Chung [31], the DPPH radical scavenging

![Figure 1: The effect of RVA on DPPH radical scavenging activity.](image1)

![Figure 2: The effect of RVA on H2O2 radical scavenging activity.](image2)

![Figure 3: The effect of RVA on hydroxyl radical scavenging activity.](image3)
activity of velvet antler extract obtained from the upper section was reported to be 67.1% at an extract concentration of 100 mg/mL, which was lower than the activity measured in our analysis. \( \text{H}_2\text{O}_2 \) is a reactive nonradical and a clinically important compound due to its ability to penetrate biological membranes. \( \text{H}_2\text{O}_2 \) can be converted into more reactive species, such as singlet oxygen and hydroxyl radicals, thereby causing lipid peroxidation or toxicity to cells. Therefore, scavenging of hydrogen peroxide can decrease prooxidants’ levels. Our analysis of \( \text{H}_2\text{O}_2 \) scavenging by velvet antler produced results similar to those reported by Je et al. [30]. Hydroxyl radicals are extremely reactive and easily react with amino acids, DNA, and membrane components. In this study, the hydroxyl radical scavenging activity of RVA was higher than that of velvet antler as reported by Je et al. [32]. In addition, our analysis of ABTS radical scavenging activity by RVA identified activity higher than that reported by Zhao et al. [33]. The FRAP assay treats the antioxidants contained in the samples as reductants in a redox-linked colorimetric reaction, allowing assessment of the reducing power of antioxidants [34]. Zhao et al. [33] reported activity of 85.8 ± 0.02% by 5 mg/mL velvet antler extract in the FRAP assay, which was lower than the activity measured in our analysis. The ORAC assay has been applied extensively to evaluate the antioxidant activities of fruits, vegetables, leaves, stems, herbs, and spices. As a result, the ORAC assay is commonly mentioned in scientific publications and health food publications [35]. However, the antioxidant activity of RVA has not been evaluated using the ORAC assay. Therefore, this is the first report of an assessment of the antioxidant activity of RVA using the ORAC assay. ORAC value of gallic acid was shown 161 ± 4.8 by Zulueta et al. [36], which was higher than the activity of RVA found in our study.

4. Conclusions

In the present study, we provided the first comprehensive evaluation of the biologically active substances of RVA and the antioxidant potential of different RVA segments. Future studies are required to further elucidate the other biological activities of the T-RVA, M-RVA, and B-RVA sections and the biological mechanisms underlying their effects.

Conflict of Interests

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

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