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Natural Products for Infectious Diseases

Guest Editors: Kang-Ju Kim, Xiangqian Liu, Takashi Komabayashi, Seung-Il Jeong, and Serkan Selli

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Editorial Board

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Editorial

Natural Products for Infectious Diseases

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Infectious diseases have represented a threat to human lives since the beginning of human existence. Many infectious diseases have been conquered through the discovery of antibiotics and antiviral agents. However, the antibiotic-resistant strains and mutant microorganisms that are now emerging are more powerful than the existing ones. In addition, some existing microorganisms have developed resistance to antibiotics, leading to infections that are more difficult to treat. Moreover, microbial biofilms cannot be treated by antibiotics and can cause chronic infections. Infectious diseases continue to pose a threat to humans, and continued efforts are needed to develop effective treatments.

In recent times, natural products have been as widely used as chemical drugs against clinical diseases. Most chemical drugs that are widely used today were isolated from natural products, and thus natural products will continue to be important raw materials for the development of new drugs. However, since natural products are the byproducts of empirical medicine, they lack scientific validation. Currently, various scientific experiments are being conducted to fill this gap by evaluating the efficacy of natural product.

This special issue includes 7 research articles and 1 review article addressing the efficacies of natural products for treating infectious diseases, such as infection by multidrug-resistant bacteria, viral influenza, coccidiosis, leishmaniasis, infectious septic shock, and biofilm formation. These articles represent pharmacological activity tests, investigation of action mechanisms of natural products, clinical trials with scientific statistical analyses, and phytochemical analyses of bioactive components in medicinal plants, which are important for scientific validation of the use of natural products in alternative and complimentary medicine.

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We express our great gratitude to all authors for their contributions and reviewers for their great help. We convey our sincere thanks to the Editorial Board for their approval on this topic and continuous support of successful publication of this special issue. The Lead Guest Editor would like to thank the Guest Editors for their enthusiastic assistance. We hope this special issue will bring readers a useful academic reference in their research.

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Research Article

Antibacterial and Antibiofilm Activity of Methanolic Plant Extracts against Nosocomial Microorganisms

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Biofilm is a complex microbial community highly resistant to antimicrobials. The formation of biofilms in biotic and abiotic surfaces is associated with high rates of morbidity and mortality in hospitalized patients. New alternatives for controlling infections have been proposed focusing on the therapeutic properties of medicinal plants and their antimicrobial effects. In the present study the antimicrobial and antibiofilm activities of 8 methanolic plant extracts were evaluated against clinical isolated microorganisms. Preliminary screening by diffusion well assay showed the antimicrobial activity of *Prosopis laevigata*, *Opuntia ficus-indica*, and *Gutierrezia microcephala*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined ranging from 0.7 to >15 mg/mL. The specific biofilm formation index (SBF) was evaluated before and after the addition of plant extracts (MBC × 0.75). *Opuntia ficus-indica* caused the major reduction on SBF in dose-dependent manner. Cytotoxic activity of plant extracts was determined using brine shrimp lethality test (*Artemia salina* L.). Lethal Dose concentration (LD_{50} values) of the plant extracts was calculated. LD_{50} values for *P. laevigata* and *G. microcephala* were 141.6 and 323.3 μg/mL, respectively, while *O. ficus-indica* showed a slight lethality with 939.2 μg/mL. Phytochemical analyses reveal the presence of flavonoids, tannins, and coumarines.

1. Introduction

Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces [1]. Biofilm-associated cells have the ability to adhere irreversibly on a wide variety of surfaces, including living tissues and indwelling medical devices as catheters, valves, prosthesis, and so forth [2].

They are considered an important virulence factor that causes persistent chronic and recurrent infections; they are highly resistant to antibiotics and host immune defenses [3]. Bacteria protected within biofilm exopolysaccharides are up to 1,000 times more resistant to antibiotics than planktonic cells (free-floating) [4], which generates serious consequences for therapy and severely complicates treatment options [5]. An estimated 75% of bacterial infections involve biofilms that are protected by an extracellular matrix [6].

Biofilm resistance is due to several reasons, like restricted diffusion of antibiotics into biofilm matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability, and the action of antibiotic-modifying enzymes [7]. The increased biofilm resistance to conventional treatments enhances the need to develop new control strategies [8].

Biofilm inhibition is considered as major drug target for the treatment of various bacterial and fungal infections, and pharmacological development of this drugs is now extensively studied [9]. In recent years, several green nonlethal strategies for biofilm control have been developed, because the mode of action of these novel antibiofilm agents is

Table 1: Overview of the collected plants used in this investigation.

Scientific name	Common name	Family	Part used	Voucher number
Sophora secundiflora (Ortega) Lag. Ex DC.	Mountain laurel	Fabaceae	Aerial parts	027770
Sphaeralcea ambigua A. Gray	Desert globemallow	Malvaceae	Bark	027771
Prosopis laevigata (Humb. et Bonpl. ex Willd) M.C. Johnston	Smooth mesquite	Fabaceae	Bark and leaves	027772
Opuntia ficus-indica Mill.	Nopal cactus	Cactaceae	Cladode	027773
Marrubium vulgare L.	White horehound	Lamiaceae	Aerial parts	027774
Scutellaria drummondii Benth	Drummond's skullcap	Lamiaceae	Aerial parts	027775
Nothoscordum bivalve Britton.	Crowpoison	Alliaceae	Bulb	027776
Gutierrezia microcephala (DC.) Gray	Sticky snakeweed	Asteraceae	Aerial parts	027777

much less susceptible to the emergence of resistance [10]. However although they are promising strategies, they have disadvantages because none have been totally effective [5].

One promising alternative is the search for naturally occurring compounds of plant origin capable of blocking biofilm formation [11]. Historically, plant extracts and their biologically active compounds have been a valuable source of natural products, which have played a central role in the prevention and treatment of diseases, helping to maintain human health [12]. Furthermore, they are widely accepted due to the perception that they are safe and have a long history of use in folk medicine to cure diseases and illnesses since ancient times [13].

Considering the above and based on previous results obtained in our laboratory, in the present study we propose to evaluate the antibiofilm effect of 8 extracts plants against 5 clinical isolated pathogens.

2. Material and Methods

2.1. Plant Material. Fresh and healthy plants growing wild around the Casablanca community, located in Santa Catarina, Nuevo León, Mexico (25°39′11.33″N 100°42′41.09″W), were collected between March and April 2014. Voucher samples were deposited at the herbarium of the Botanical Department of Biological Sciences School, Universidad Autónoma de Nuevo León, for identification purposes. Collected plants (Table 1) were washed thoroughly in tap water, followed by successive washing in distilled water. Washed plants were cut into small pieces and air-dried at room temperature (25±2°C) under shade. Finally, dried material was grounded to coarse powder in a manual grain mill and stored in plastic containers for further analysis.

2.2. Bacterial Strains and Culture Conditions. The microorganisms used in this study were 5 nosocomial pathogens, 4 Gram-negative (*Klebsiella pneumoniae*, *Enterococcus faecalis*, *Escherichia coli*, and *Stenotrophomonas maltophilia*) and 1 Gram-positive (*Staphylococcus aureus*). All strain were kindly provided by Dra. Elvira Garza Gomzález, School of Medicine, UANL. Strains were maintained in Mueller-Hinton (MH) agar (Difco) at 4°C. Active cultures were obtained by inoculation of a loopful of each strain into separated 5 mL MH broth (Difco) and incubated for 18 h at 37°C.

2.3. Preparation of Plant Extracts. Extracts were prepared following the methodology proposed by Sánchez et al. [14], with minor modifications. Briefly, one hundred grams (100 g) of dried plant material was soaked with 500 mL of methanol for 24 h at room temperature (25 ± 2°C), under occasional shaking. Extraction was repeated three times, and the extracts obtained were combined and filtered through Whatman filter paper number 1. After that, they were concentrated to dryness under reduced pressure using a rotary evaporator at 45°C. Stock solutions (200 mg/mL) were prepared in methanol and stored at 4°C in the dark for further experiments.

2.4. Qualitative Phytochemical Screening. The extracts were subjected to standard phytochemical tests in order to evaluate their chemical composition for different active constituents; for this extracts (3–5 mg/mL) they were separately suspended in 1 mL of absolute ethanol or distilled water (carbohydrate determination) using clean test tubes.

2.5. Bayer's Test for Unsaturation. In this case aqueous 1% $KMnO_4$ was added dropwise to the extract solution. A positive test was evidenced by the disappearance of the purple color of $KMnO_4$ and the appearance of a brown solid precipitate (MnO_2) [15].

2.6. Detection of Triterpenes/Steroids (Liebermann-Burchard Reagent). One mL of acetic anhydride and 5 drops of concentrated sulfuric acid (H_2SO_4) were added to the extract. A color change from violet to blue confirms the presence of steroids [16] and formation of blue-green ring indicated the presence of terpenoids [17].

2.7. Coumarins. Three mL of 2 N NaOH was added to 2 mL of aqueous extract. Formation of yellow color indicated the presence of coumarins. Confirmation test was performed by adding 1 mL of 5 N HCl; in this case a colorless solution formed at the upper layer is considered positive [18].

2.8. Alkaloids. Ethanolic extracts (20 μL) were applied on TLC plates (Silica Gel 60G, 5 × 10 cm) and eluted using toluene-ethyl acetate-diethylamine (70 : 20 : 10) as solvent system. Alkaloids were detected after spraying Dragendorff's reagent as orange-brown spots on TLC plates [19].

2.9. Screening for Sesquiterpene Lactones. The Baljet reaction (1% Picric acid in 10% sodium hydroxide) was used to detect sesquiterpene lactones in the extracts. Reagents were mixed at a 1 : 1 ratio and added to 1 mL of extracts (2-3 mg). The transformation of the sodium picrate solution's yellow color to orange-red color confirmed the positive reaction [20].

2.10. Test for Quinones. Extracts suspended in ethanol (1 mL) were treated with 1 mL of concentrated sulfuric acid. Formation of red color shows the presence of quinones [21].

2.11. Carboxyl Group. The presence of carboxyl groups was evidenced by adding 10 drops of 10% sodium bicarbonate solution; visible bubbles of carbon dioxide were considered a positive reaction [21].

2.12. Test for Tannins. Extracts were treated with 1 mL of 5% ferric chloride which was added. The presence of tannins was indicated by the formation of bluish black or greenish black precipitate [22].

2.13. Shinoda Test. Few fragments of magnesium metal ribbon (3-4 pieces) were added to 1 mL of ethanolic extract, followed by dropwise addition of concentrated hydrochloric acid. Formation of pink or red color indicated the presence of flavonoids [23].

2.14. Saponin. Two mL of distilled water was added to extracts suspended in ethanol and was shaken vigorously. The formation of copious foam layer indicates the presence of saponins [23].

2.15. Carbohydrates. For carbohydrates test, extracts (10 mg) were suspended in 1 mL of distilled water; afterward 2 mL of 0.2% anthrone reagent and 5 drops of concentrated sulfuric acid were added. Dark green color showed the presence of carbohydrates [21].

2.16. Evaluation of Antimicrobial Activity. Antimicrobial activity of plant extracts was performed using the agar-well diffusion bioassay. Briefly, 100 μL of fresh culture (approximately 10^6 CFU/mL) was uniformly spread onto Mueller-Hinton agar (MHA) plates by sterile Driglasky loop. Then, inoculated plates were allowed to dry at room temperature for 20 min. After that, wells of 6 mm in diameter were made in the agar using a sterilized cup-borer and 100 μL of each extract was poured in the wells. Methanol was used as control. Plates were incubated at 37°C for 18 h. Antibacterial activity was evidenced by the presence of clear inhibition zone around each well. The diameter of this zone was measured and recorded [14].

2.17. Assessment of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The MIC and MBC were determined on plant extracts that showed antimicrobial activity, by a broth microdilution method proposed by Novy et al. [24], with minor modifications. Briefly, 100 μL of Mueller-Hinton Broth (Difco) plus different concentrations of plant extracts was prepared and transferred to each microplate well to obtain dilutions of the active extract, ranging from 1.0 to 25 mg/mL. Then, 10 μL of a fresh culture (final concentration of 1×10^6 CFU/mL) of test organisms was added. Microplates were incubated at 37°C for 24 h [25]. MIC was defined as the lowest concentration of the extract that restricted the visible growth of microorganism tested.

To determine MBC, 100 μL from each well that showed no visible growth was reinoculated on MH agar plates; then the plates were incubated at 37°C for 24 h. MBC was defined as the lowest extract concentration showing no bacterial growth. Methanol was used as blank and tetracycline (Sigma Aldrich, Mexico City, Mexico) as positive control. Once the MBC was recorded, the sublethal activity on bacterial growth was determined; for this, concentrations of 75, 50, and 25% of MBC were tested in a 96-well microplate and the counts of microbial cells were done by plate count technique, as previously mentioned.

2.18. Biofilm Formation Inhibition. The effect of extracts on biofilm formation was evaluated in 96-well polystyrene flat-bottom plates [26]. Briefly, 300 μL of inoculated fresh tryptic case soy yeast broth (TSY) (final concentration 10^6 CFU/mL) was aliquoted into each well of microplate and cultured in presence of sublethal concentrations (75, 50, and 25% of MBC) previously determined. Wells containing medium and those without extracts and only with methanol were used as controls. Plates were incubated at 37°C for 48 h. After incubation, supernatant was removed and each well was washed thoroughly with sterile distilled water to remove free-floating cells; thereafter plates were air-dried for 30 min and the biofilm formed was stained during 15 min at room temperature with 0.1% aqueous solution of crystal violet. Following incubation, the excess of stain was removed washing the plate three times with sterile distilled water. Finally, the dye bound to the cells was solubilized by adding 250 μL of 95% ethanol to each well and after 15 min of incubation, absorbance was measured using microplate reader at a wavelength of 570 nm. Biofilm determination was made using the formula SBF = (AB − CW)/G, where SBF is the specific biofilm formation, AB is the OD570 nm of the attached and stained bacteria, CW is the OD570 nm of the stained control wells containing only bacteria-free medium, and G is the OD630 nm of cell growth in broth [27].

2.19. Toxicity Bioassay. Brine shrimp (*Artemia salina*) lethality bioassay was carried out in accordance with methodology proposed by Meyer et al. [28] to determine the toxicity of extract plants. For this, brine shrimps cysts were hatched in a shallow rectangular container, which was divided into two unequal compartments, filled with sterile artificial seawater

Table 2: Diameter of inhibition zone of methanolic extracts against clinical isolated bacteria.

Plant	Inhibition zone (cm)				
	K. pneumoniae	*E. faecalis*	*E. coli*	*S. maltophilia*	*S. aureus*
S. secundiflora	NI	NI	NI	NI	2.1 ± 0.3
S. ambigua	NI	NI	NI	NI	1.2 ± 0.1
P. laevigata	1.4 ± 0.3	1.7 ± 0.3	1.5 ± 0.3	NI	2.6 ± 0.3
O. ficus-indica	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.3	NI	1.6 ± 0.3
M. vulgare	NI	0.7 ± 0.01	NI	NI	1.8 ± 0.2
S. drummondii	NI	0.6 ± 0.01	NI	NI	1.7 ± 0.2
N. bivalve	NI	NI	NI	NI	NI
G. microcephala	NI	NI	1.6 ± 0.1	NI	2.3 ± 0.2

Values are means ± standard deviations. NI: no inhibition.

(prepared by dissolving sea salt 38 g/L and adjusted to pH 8.5 using 1 N NaOH) under constant aeration and proper light. Cyst (ca. 50 mg) was sprinkled into the larger compartment, which was darkened, while the smaller was illuminated. Yeast solution 0.06% was added to the hatching chamber to feed the larvae. After 48 h the phototropic free nauplii were collected from the lighted side.

Lethality bioassay was performed using 10 collected nauplii, which were transferred into vials contained tested crude plant extract, at 10, 100, and 1000 μg/mL, and artificial seawater. Appropriate quantities of methanol were used as negative control.

After 24 h of incubation, live nauplii were counted and the LC_{50} values were estimated using a Probit regression analysis. Extracts giving LC_{50} values above 1000 μg/mL were considered nontoxic.

2.20. Statistical Analysis. All experimental results were expressed as mean ± standard deviation (SD) for analysis performed in duplicate at least three times. Statistical analysis of the data was performed by Analysis of Variance (ANOVA) and mean comparison using Student's t-test, using SPSS software version 17.0. The LC_{50} for bioassay with *A. salina* was determined according to the Probit statistical method. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

A total of 8 methanolic plant extracts were tested against 5 clinical bacterial isolates. Methanol was selected as extraction solvent, because it is one of the best solvents used for the extraction of antimicrobial substances [29, 30]. Moreover, methanol polarity ensured the extraction of polar and moderately polar active compounds from plants against microorganisms like terpenoids, tannins, flavones, and polyphenols [31].

Results of preliminary antimicrobial tests, performed by the well diffusion method, were quite variable between each plant extract ranging from 0 to 2.8 cm (Table 2). *P. laevigata* extract was active against all the clinical isolates, while *N. bivalve* bulb did not show activity against any microorganism. The highest diameter of inhibition was obtained with *P. laevigata* extract (2.8 ± 0.5 cm), against *S. aureus* strain, followed by *G. microcephala* (2.3 ± 0.2 cm) and *O. ficus-indica* (1.6 ± 0.3 cm) also against *S. aureus*. Meanwhile *E. coli* was less susceptible to these extracts showing diameters of 1.7 ± 0.3, 1.4 ± 0.1, and 1.6 ± 0.1 cm, respectively. *K. pneumoniae* and *E. faecalis* were more resistant to the extracts, only inhibited by *P. laevigata* and *O. ficus-indica* with inhibition zones ranging from 0.7 ± 0.08 to 1.3 ± 0.2; on the other hand, *S. maltophilia* was the only microorganism that was not inhibited by the extracts.

However, the well diffusion assay is considered a qualitative technique and is mainly used for selecting extracts with antimicrobial activity, mostly when diameters zones of inhibition are ≥10 mm [32]. It is important to recognize that the size of inhibition zones of different extracts could be due to the compounds polarity obtained, since a more diffusible but less active extract could give a bigger diameter of inhibition than a nondiffusible but more active extract [33].

Minimum inhibitory concentration (MIC) results are comparable to those obtained in the agar-well diffusion technique, because the lowest MIC were obtained using the extracts showing the best antimicrobial activity (data not shown). Meanwhile results of minimum bactericidal concentrations (MBC) are listed in Table 3, where *P. laevigata* extract had the lowest MBC with a value of 2 mg/mL for *E. coli*, 2.8 mg/mL for *E. faecalis*, 3.8 mg/mL for *K. pneumoniae*, and 0.7 mg/mL for *S. aureus*. Extracts and *O. ficus-indica* got the highest CMB ranging from 1.0 to ≥15 mg/mL. CMBs of *G. microcephala* were 2.8 and 8.3 mg/mL against *S. aureus* and *E. coli*, respectively. MBC results show that *S. aureus* was the more sensitive microorganism, being inhibited for 8 methanolic extracts, while *S. maltophilia* was not inhibited by any extract. Broadly, our results agree with previous reports, which mention greater activity of extracts towards Gram-positive microorganisms compared to Gram-negative microorganisms [34]. These differences can probably be attributed to the structural and compositional differences in the cell wall and membranes [25]. The Gram-negative bacteria have an outer membrane that serves as barrier for many molecules; also, the presence of efflux pump system has been demonstrated, which can mediate the resistance to natural compounds [35]. *Escherichia coli* was the most susceptible of the Gram-negative bacteria; this finding also agrees with previous reports [36].

Table 3: Minimum bactericidal concentration (MBC) of methanolic extracts against clinical isolated bacteria.

Plant	MBC (mg/mL)				
	K. pneumoniae	*E. faecalis*	*E. coli*	*S. maltophilia*	*S. aureus*
S. secundiflora	NE	NE	NE	NE	9.1 ± 0.4
S. ambigua	NE	NE	NE	NE	>15
P. laevigata	3.8 ± 0.1	2.7 ± 0.1	1.5 ± 0.2	NE	0.7 ± 0.01
O. ficus-indica	>15	>15	4.0 ± 0.3	NE	1.0 ± 0.2
M. vulgare	NE	0.7 ± 0.01	NE	NE	3.9 ± 0.3
S. drummondii	NE	0.6 ± 0.01	NE	NE	7.3 ± 0.2
N. bivalve	NE	NE	NE	NE	NE
G. microcephala	NE	NE	8.3 ± 0.2	NE	2.8 ± 0.3

Values are means ± standard deviations. NE: not evaluated.

Table 4: Phytochemical screening results of selected methanolic extracts.

Compounds	*P. laevigata*	*O. ficus-indica*	*G. microcephala*
Unsaturation	—	++	++
Triterpenes/steroids	++/Steroids	++/Triterpenes	+++/Triterpenes
Coumarins	+++	+	+++
Alkaloids	+++	—	—
Sesquiterpene lactones	—	—	++
Quinones	—	+	+
Carboxyl group	—	—	—
Tannins	+++	++	+++
Saponins	—	—	—
Carbohydrates	++	+++	—
Flavonoids	++	+	++

+: low intensity reaction, ++: medium intensity reaction, and +++: strong intensity reaction.

According to the previously mentioned results, it was decided to select 3 plant extracts (*P. laevigata*, *O. ficus-indica*, and *G. microcephala)* which were active against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive); moreover these extracts showed the lowest MBC.

Phytochemical screening results of selected plant extracts are summarized in Table 4 and show the presence of different functional groups. Coumarins, alkaloids, tannins, and flavonoids were found in *P. laevigata* extract. Similar compounds have been reported in different species of this plant like *P. juliflora*, where the presence of tannins, phenolics, flavonoids, steroids, terpenes, and alkaloids has been reported [37]. Likewise, reports of *Prosopis* spp. mentioned that this plant contains harmine, prosopine which is an alkaloid reported in several papers, tyramine, prosopinine, and juliflorine, which are alkaloids that intercalate into DNA and could explain the antimicrobial activity of this extract [31, 38].

In case of *O. ficus-indica*, results indicate the presence of triterpenes, coumarins, quinones, tannins, carbohydrates, and flavonoids; flavonoids cause bacterial death by inhibiting DNA or RNA synthesis and tannins including possible inhibition of extracellular microbial enzymes [39, 40].

Meanwhile, triterpenes, coumarins, quinones, tannins, flavonoids, and sesquiterpene lactones were found in *G. microcephala*. According to Gören et al. [41] sesquiterpene lactones are the main secondary metabolite responsible for the antimicrobial activity in Asteraceae family. While McDaniel and Ross [42] report the presence of alkaloids and saponins conferring some toxicity at this plant.

Biofilm formation inhibition results by addition of subinhibitory concentrations (75, 50, and 25% of MBC) of plant extracts against *E. coli* and *S. aureus* indicated that the obtained effect was dose-dependent. The best biofilm reduction is observed in higher concentrations of the extracts (75% of WBC). Similar results were reported by Issac Abraham et al. [43], who reported that methanolic caper extract significantly inhibited biofilm formation and EPS production in *E. coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. As well Ravichandiran et al. [44] reported that ethanolic extract of the bark of *Melia dubia* caused a strong suppression of hemolysis, swarming motility, and biofilm formation in *E. coli*. Results of the effect of concentrations corresponding to 75 and 50% of MBC caused significant ($P < 0.05$) reduction of the specific biofilm formation (SBF) of *E. coli* (Figure 1) from approximately 3 (strong biofilm) to levels of 0.2 (weak biofilm, 75% MBC) and 1.2 (moderated biofilm, 50% MBC). The SBF classification categories were mentioned by Mittal et al. [45] who mention that strong biofilm producers (SBF index > 2.00), intermediate biofilm producers (SBF index between 1 and 2), and weak biofilm producers (SBF index < 1.00). Similar results were obtained with

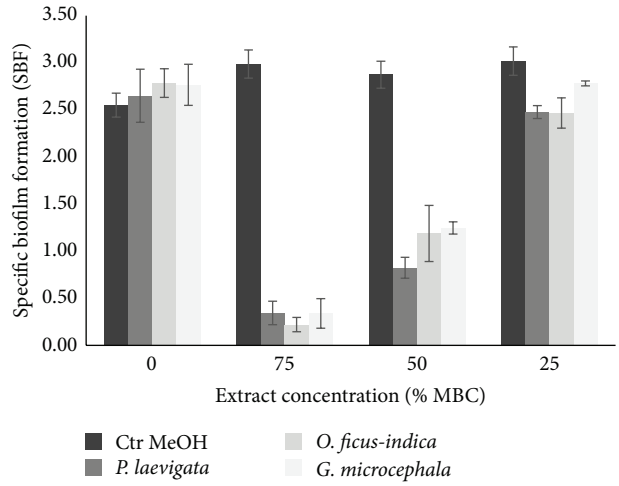

Figure 1: Inhibition of biofilm formation by different concentrations of plant extracts against *E. coli*.

S. aureus (data not shown). Inhibition of biofilm formation can be explained by the presence of flavonoids, previously reported such as quercetin, kaempferol, naringenin, and apigenin, which are capable of reducing biofilm synthesis because they can suppress the activity of the autoinducer-2 responsible for cell-to-cell communication [46].

A. salina bioassay is used to evaluate the toxicity of plant extracts and has the advantage of being inexpensive, reliable, and reproducible [47]. In a previous study, Ahmed et al. [48] determined the toxicity of methanol extract of *Prosopis spicigera* reporting 60% survived nauplii at 100 μg/mL which is consistent with the results obtained in this work, because LD_{50} obtained of *P. laevigata* was 141.6 μg/mL indicating that the extract is moderately toxic; this may be due to the presence of certain bioactive compounds which may be related to the antibacterial activity. For *G. microcephala* was moderately toxic with LD_{50} of 323.3 μg/mL, some studies mentioned that this toxicity may be due to the presence of saponins, essential oils, mono- and sesquiterpenes, tannins, and alkaloids [42, 49]. Results of *O. ficus-indica* indicate slight toxicity (939.2 μg/mL); this is consistent as reported by Déciga-Campos et al. [50]. Low toxicity could be explained with the common use of this plant in traditional medicine. Furthermore, in vivo and in vitro experiments of cladodes and fruits show a beneficial effect on health due to the presence of flavonoids, which have health-related properties, which are based in their antioxidant activity [51, 52].

4. Conclusions

Some of the plant extracts evaluated in present research had potential antimicrobial and antibiofilm activities against isolated nosocomial bacteria, which can be an alternative to control the formation of microbial biofilms or can be used as model to the search for new drugs.

Competing Interests

The authors declare that they have no competing interests.

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Review Article

Herbal Remedies for Coccidiosis Control: A Review of Plants, Compounds, and Anticoccidial Actions

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Coccidiosis is the bane of the poultry industry causing considerable economic loss. *Eimeria* species are known as protozoan parasites to cause morbidity and death in poultry. In addition to anticoccidial chemicals and vaccines, natural products are emerging as an alternative and complementary way to control avian coccidiosis. In this review, we update recent advances in the use of anticoccidial phytoextracts and phytocompounds, which cover 32 plants and 40 phytocompounds, following a database search in PubMed, Web of Science, and Google Scholar. Four plant products commercially available for coccidiosis are included and discussed. We also highlight the chemical and biological properties of the plants and compounds as related to coccidiosis control. Emphasis is placed on the modes of action of the anticoccidial plants and compounds such as interference with the life cycle of *Eimeria*, regulation of host immunity to *Eimeria*, growth regulation of gut bacteria, and/or multiple mechanisms. Biological actions, mechanisms, and prophylactic/therapeutic potential of the compounds and extracts of plant origin in coccidiosis are summarized and discussed.

1. Introduction

Each year, over 50 billion chickens are raised as a source of meat, accounting for over one-third of protein food for humans [1]. However, poultry production is often confronted by avian coccidiosis, flu, and other infectious diseases [1]. Avian coccidiosis is characterized as an infectious protozoan disease caused by gut parasites of the genus *Eimeria* (Coccidia subclass) [2]. So far, nine *Eimeria* species, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. mitis*, *E. tenella*, *E. mivati*, and *E. hagani*, have been identified from chickens [3]. These parasites can infect and multiply within the mucosal epithelia in different parts of bird guts via oral route. As a result, they cause gut damage (i.e., inflammation, hemorrhage, diarrhea, etc.), morbidity, and mortality in poultry [4]. This disease annually causes a global loss of over 2.4 billion US dollars in the poultry industry, including poor growth performance, replacement of chicks, and medication [1]. Current approaches to constrain avian coccidiosis include anticoccidial chemicals, vaccines, and natural products. Anticoccidial chemicals, coccidiocides, coccidiostats, and ionophores, have long been used as a mainstream strategy to control avian coccidiosis in modern poultry production [5]. Although this strategy is cost-effective and successful, the presence of drug resistance and public demands for residue-free meat has encouraged development of alternative control strategies [5]. Moreover, in European countries, the prophylactic use of anticoccidial chemicals as feed additives has been strictly limited since 2006 and a full ban has been proposed to be effective in 2021 (Council Directive of 2011/50/EU of the European Council). To cope with this global situation, vaccination, composed of one or more strains of wild-type or attenuated *Eimeria* species, is successfully developed as another approach to prevent coccidiosis though their cross-species protection and efficacy may need to be improved. Natural products are emerging as an attractive way to combat

coccidiosis. Currently, there are at least four plant products commercially available on the market and they can be used as anticoccidial feed additives in chickens and/or other animals, including Cocci-Guard (DPI Global, USA) [6], a mixture of *Quercus infectoria*, *Rhus chinensis*, and *Terminalia chebula* (Kemin Industries, USA) [7], Apacox (GreenVet, Italy) [8], and BP formulation made up of *Bidens pilosa* and other plants (Ta-Fong Inc., Taiwan) [9]. Besides, investigation of the compounds and/or their derivatives present in anticoccidial plants may inspire the research and development of anticoccidial chemicals. One successful example is halofuginone, a synthetic halogenated derivative of febrifugine, which was initially identified from the antimalarial plant, Chang Shan (*Dichroa febrifuga*) [10]. Despite the aforesaid merits of natural products, several challenges in the anticoccidial use of natural products such as anticoccidial efficacy, identification of active compounds, mechanism, safety, and cost-effectiveness of plant extracts and compounds need to be overcome prior to further applications.

Several reviews were published on the plant extracts and their phytochemicals for avian coccidiosis [1, 11, 12]. To complement and update recent progress on the anticoccidial properties of plant extracts and/or compounds, here we have updated the plants and compounds related to anticoccidial activity, chemistry, and prophylactic/therapeutic potential. New views on the anticoccidial action of plants and compounds are also discussed. Common terms and their definitions used in this paper are presented as follows.

Anticoccidial Chemicals. These are chemicals which kill coccidia (coccidiocides) or slow their growth (coccidiostats). Ionophores are a class of polyether chemicals which interfere with ion transport, leading to the death of coccidia.

Phytoextract/Plant Extract. They are a collection of plant ingredients obtained by means of solvent extraction.

Phytocompound/Phytochemical. This refers to any chemical synthesized by plants.

Natural Products. These are compounds/chemicals found in nature including plants, animals, and minerals.

Coccidia. It is a subclass of microscopic, spore-forming, single-celled protozoan parasites belonging to the apicomplexan class.

Eimeria. This is a genus of apicomplexan parasites that include various species capable of causing gut disease in poultry and other animals.

1.1. Life Cycle of Eimeria Species. As illustrated in Figure 1, *Eimeria* species have a complex life cycle, consisting of three developmental stages (sporogony, schizogony/merogony, and gametogony). Oocysts excreted in poultry feces sporulate in a favourable environment with high humidity at 25–30°C. Once sporulating oocysts are ingested by birds, physical and chemical agents in their digestive tracts are released and mature infectious sporozoites form sporocysts. The sporozoites enter epithelial cells in the gut, depending on the specific *Eimeria* species, and form trophozoites and, later, schizonts during schizogony/merogony [13]. Merozoites, released from the schizont, can penetrate into the epithelia and continue this merogony stage 2 to 3 times in order to increase the cell number of merozoites at the asexual stage of reproduction. Alternatively, merozoites may enter the sexual stage of reproduction by forming male microgametes, the equivalent of sperm, and female macrogametes, the equivalent of oocytes, in host cells. Following fertilization, the zygotes develop into oocysts and are excreted into poultry stool. *Eimeria* species may require 4–7 days to complete their entire life cycles [14]. Thus, the asexual and sexual stages of reproduction in the *Eimeria* life cycle have been targeted by anticoccidial compounds [4].

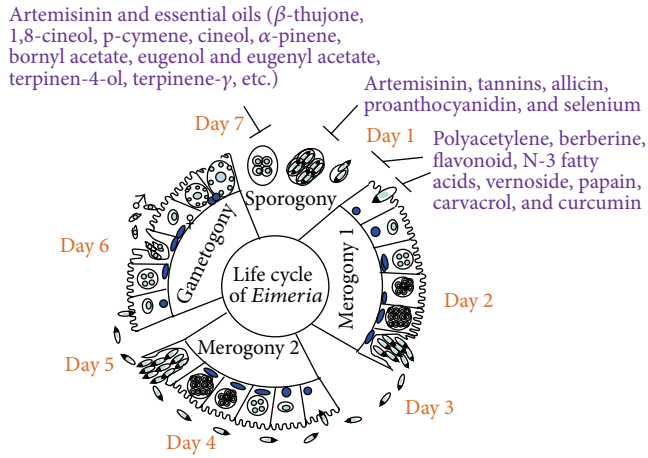


Figure 1: Plant compounds target different stages of the life cycle of *Eimeria* species. *Eimeria* species take 4–7 days to complete their life cycles. They have 3 different developmental stages in poultry: sporogony, merogony, and gametogony. This scheme is modified from the previous publication [40]. Different phytocompounds inhibit the growth of *Eimeria* species at sporogony and merogony stages.

1.2. Prophylaxis and Therapy for Avian Coccidiosis. Coccidiosis is one of the major problems in intensive poultry farming [1]. In contrast to anticoccidial chemicals and vaccines, the use of medicinal plants and phytocompounds as natural remedies for avian coccidiosis has become an alternative strategy that is easily embraced by eco- and health-conscious consumers. Of course, this strategy is also in compliance with the "anticoccidial chemical-free" regulation enacted by the European Union (Council Directive of 2011/50/EU). Plants produce a broad-spectrum variety of phytochemicals such as phenolics, polyacetylenes, alkaloids, polysaccharides, terpenoids, and essential oils with a large number of bioactivities [12, 15, 16]. Different reports on the use of medicinal plants for diseases suggest that they have the potential to "kill several birds with one stone" because they contain multiple phytochemicals and can intervene in multiple disease-related signalling pathways [17].

2. Plants and Compounds for Avian Coccidiosis

Over 300,000 species of flowering plants have been recorded worldwide. So far, less than 1% of them have been explored for use against protozoan diseases [18]. In this section, a total of 68 plants and phytocompounds, which were scientifically tested for suppression of *Eimeria* species, are described and discussed [1, 9, 11, 12]. Table 1 lists 32 anticoccidial plants whose active compounds and modes of action need to be elucidated: *Sophora flavescens* (Fabaceae), *Sinomenium acutum* (Menispermaceae), *Quisqualis indica* (Combretaceae), *Pulsatilla koreana* (Ranunculaceae), *Ulmus macrocarpa* (Ulmaceae), *Artemisia asiatica* (Asteraceae), *Gleditsia japonica* (Fabaceae), *Melia azedarach* (Meliaceae), *Piper nigrum* (Piperaceae), *Urtica dioica* (Urticaceae), *Artemisia sieberi* (Asteraceae), *Lepidium sativum* (Brassicaceae), *Foeniculum vulgare* (Apiaceae), *Morinda lucida* (Rubiaceae), *Commiphora swynnertonii* (Burseraceae), *Moringa oleifera* (Moringaceae), *Origanum* spp. (Lamiaceae), *Laurus nobilis* (Lauraceae), *Lavandula stoechas* (Lamiaceae), *Musa paradisiaca* (Musaceae), *Moringa stenopetala* (Moringaceae), *Solanum nigrum* (Solanaceae), *Mentha arvensis* (Lamiaceae), *Moringa indica* (Moringaceae), *Melia azadirachta* (Meliaceae), *Tulbaghia violacea* (Amaryllidaceae), *Vitis vinifera* (Vitaceae), *Artemisia afra* (Asteraceae), *Quercus infectoria* (Fagaceae), *Rhus chinensis* (Anacardiaceae), and *Terminalia chebula* (Combretaceae). Details about active phytocompounds present in the other anticoccidial plants and their mechanisms are summarized in Tables 2–4.

2.1. Plants and Compounds That Inhibit the Life Cycle of Eimeria. In this section, the phytochemicals and plants, which suppress coccidiosis via intervention with the developmental stages of life cycle in *Eimeria* species in poultry, are discussed. They are summarized in Table 2. Their chemistry and mechanism of action of phytochemicals and plants are also described below and summarized in Table 2 and Figure 1.

2.1.1. Artemisia annua and Artemisinin. *A. annua* and its constituent active compound artemisinin have been reported to have anticoccidial action (Table 2). Mechanistic studies show that this compound generated reactive oxygen species (ROS) via degradation of iron-implicated peroxide complex and, therefore, induced oxidative stress. Of note, ROS was documented to directly inhibit sporulation and cell wall formation in *Eimeria* species, leading to interference with the life cycle of *Eimeria* [19–23]. In addition, *A. annua* has lots of phytochemicals, flavonoids, and phenolic compounds which can help birds maintain commensal microflora and take up large amounts of nitrogen. Commensal bacteria play a significant role in enhancing digestion of food and absorption of nutrients and improve innate and acquired immune response in poultry [24].

2.1.2. Condensed Tannins and Pine Bark. The extract from the bark of the pine tree (*Pinus radiata*), which is rich in condensed tannins, was reported to inhibit the life cycle of Coccidia as evidenced by decreased sporulation of the oocysts of *E. tenella*, *E. maxima*, and *E. acervulina* [25]. The mode of action of condensed tannins was suspected to be penetration of the wall of the oocyst and damage to the cytoplasm since the tannins could inactivate endogenous enzymes responsible for the sporulation process. This was further supported by the appearance of abnormal sporocysts in oocysts [25].

2.1.3. Garlic (Allium sativum) and Allicin. Garlic and its sulfur compounds, allicin, alliin, ajoene, diallyl sulfide, dithiin, and allylcysteine, are reported to have broad antimicrobial activities which can eliminate negative factors of microbial infections. An *in vitro* study has shown that allicin inhibits sporulation of *E. tenella* effectively [26–28]. The anticoccidial mechanism of garlic and its sulfur compounds remains elusive.

2.1.4. Selenium, Phenolics, and Green Tea (Camellia sinensis). Green tea extracts have been shown to significantly inhibit the sporulation process of coccidian oocysts [29, 30]. Accordingly, the selenium and polyphenolic compounds in green tea are thought to be active compounds to inactivate the enzymes responsible for coccidian sporulation [29, 30].

2.1.5. N-3 Fatty Acids, Flavonoids, Vernoside, and Their Plant Sources. Upon the invasion of *Eimeria* sporozoites into the intestinal epithelium, reactive nitrogen species (RNS) and reactive oxygen species (ROS) are often produced by host cells, leading to the death of sporulating oocysts [21]. Similarly, another study demonstrated that the extracts from *Berberis lyceum*, in which berberine was enriched, inhibited the sporozoites of *E. tenella* in chickens via induction of oxidative stress. Other studies indicated that extracts from *Linum usitatissimum* [21], *Ageratum conyzoides* [31], and *Vernonia amygdalina* [32] controlled coccidian infection via induction of oxidative stress. Moreover, N-3 fatty acids, flavonoids, and vernoside were identified as active compounds present in *L. usitatissimum*, *A. conyzoides*, and *V. amygdalina*, respectively. These compounds were shown to elicit oxidative stress (Table 2). Oxidative stress is known to cause imbalance of oxidant or antioxidant species in the host and is often observed in a wide range of microbial and parasitic infections including coccidiosis [19]. Moreover, these natural extracts not only enhanced chicken growth but also had no noticeable toxicity.

2.1.6. Carica papaya and Papain. Two studies have reported that extracts from *C. papaya* leaves significantly inhibit coccidiosis [32, 33]. Little is known about the anticoccidial mechanism. Proteolytic destruction of *Eimeria* by papain and/or inflammatory suppression by vitamin A were proposed as possible mechanisms by which *C. papaya* and its active compounds acted to suppress coccidiosis [32, 33].

2.1.7. Saponin, Betaine, and Their Plant Sources. Hassan et al. demonstrated that dietary supplementation of guar bean (*Cyamopsis tetragonoloba*) suppressed coccidiosis in chickens [34]. This suppression was proposed to be achieved by saponins, presumably the active compounds, which bind with

Table 1: Anticoccidial properties of plants.

S. number	Plant species (usage)	Usage*	*Eimeria* species	Parameter measured	Reference
1	*S. flavescens*	Decoction	Et	WG ↑, OC ↓, BD ↓, LS ↓, and M ↓	[41]
2	*S. acutum*	Decoction	Et	WG ↑, OC ↓, BD ↓, LS ↓, and M ↓	[41]
3	*Q. indica*	Decoction	Et	WG ↑ and M ↓	[41]
4	*P. koreana*	Decoction	Et	WG ↑ and LS ↓	[41]
5	*U. macrocarpa*	Decoction	Et	LS ↓	[41]
6	*A. asiatica*	Decoction	Et	WG ↑ and LS ↓	[41]
7	*G. japonica*	Decoction	Et	LS ↓	[41]
8	*M. azedarach*	Decoction	Et	WG ↑ and LS ↓	[41]
9, 10	*P. nigrum* and *U. dioica*	Ethanolic extract	Mixed species	OC ↓	[42]
11	*A. sieberi*	Petroleum ether extract	Et	OC ↓, BD ↓, LS ↓, and M ↓	[43–45]
12	*L. sativum*	Ethanolic extract	Et	OC ↓, LS ↓, M ↓, and WG ↑	[46]
13	*F. vulgare*	Ground leaves powder	Et	OC ↓, LS ↓, M ↓, WG ↑, and BD ↓	[47]
14	*M. lucida*	Ground leaves powder	Mixed species	WG ↑ and OC ↓	[48]
15	*C. swynnertonii*	Ethanolic resinous extract	Oocysts	OC ↓, M ↓, and WG ↑	[49]
16	*M. oleifera*	Acetone leaves extract	Mixed species	WG ↑ and OC ↓	[50]
17	*Origanum* spp.	Essential oil	Mixed species	OC ↓, LS ↓, M ↓, and WG ↑	[51]
18	*L. nobilis*	Essential oil	Mixed species	OC ↓, LS ↓, M ↓, and WG ↑	[51]
19	*L. stoechas*	Essential oil	Mixed species	OC ↓, LS ↓, M ↓, and WG ↑	[51]
20	*M. paradisiaca*	Methanolic extract	Et	OC ↓ and PCV ↑	[52]
21	*M. stenopetala*	Ground leaves powder	Et	OC ↓, LS ↓, and WG ↑	[53]
22	*S. nigrum*	Decoction	Et	WG ↑ and FC ↑	[54]
23	*M. arvensis*	Decoction	Et	WG ↑ and FC ↑	[54]
24	*M. indica*	Decoction	Et	WG ↑ and FC ↑	[54]
25	*M. azedarach*	Fresh juice	Mixed species	OC ↓	[55]
26	*T. violacea*	Acetone extract	Et	OC ↓, LS ↓, and FC ↑	[56]
27	*V. vinifera*	Acetone extract	Et	OC ↓, LS ↓, and FC ↑	[56]
28	*A. afra*	Acetone extract	Et	OC ↓, LS ↓, and FC ↑	[56]
29	*G. rhois*	Ground powder	Et	OC ↓, LS ↓, M ↓, and WG ↑	[57]
30, 31, 32	*Q. infectoria*, *R. chinensis*, and *T. chebula*	?	Et, Em, and Ea	OC ↓, LS ↓, and M ↓	[7]

Et: *E. tenella*; Ea: *E. acervulina*; Ema: *E. maxima*; Eb: *E. brunetti*; En: *E. necatrix*; Emi: *E. mivati*; WG: body weight gain; OC: oocyst count; FC: feed consumption; M: mortality; EO: essential oil; BD: bloody diarrhea; FCR: feed conversion ratio; LS: lesion scores; ↑: improvement/increase/higher; ↓: decrease/lower; PCV: packed cell volume; ∗: whole plants and/or aerial parts of plants were used for tests unless indicated otherwise; ?: unknown.

sterol molecules present on the cell membrane of the parasites [34]. Another study also reported that the extracts of *M. cordifolia*, *M. citrifolia*, and *M. arboreus* showed anticoccidial effects in chickens [35]. Saponins were presumed to be the active compounds which could lyse oocysts. In contrast, another report described betaine, an active compound isolated from beet or other plants, as contributing to the stabilization and protection of the epithelial cells in which *Eimeria* multiply [36].

2.1.8. Essential Oils and Their Plant Sources. Essential oils derived from plants showed inhibition of *Eimeria* species at different developmental stages (Figure 1). Essential oils are an important natural product resource, which are rich in many phytocompounds. Both *in vitro* and *in vivo* studies reported that essential oils can be used as feed additives in chickens to control coccidiosis [1, 11, 12]. Bioactive compounds present in the essential oils extracted from *Oreganum compactum*, *A. absinthium*, *Rosmarinus officinalis*, *Anredera cordifolia*, *Morinda citrifolia*, *Malvaviscus arboreus*, *Syzygium aromaticum*, *Melaleuca alternifolia*, *Citrus sinensis*, and *Thymus vulgaris* were able to destroy the parasites, including oocysts and sporozoites (Table 1).

2.1.9. Maslinic Acid. Maslinic acid, an active compound in the leaves and fruit of the olive tree (*Olea europaea*), was

Table 2: Phytochemicals interfering with the life cycle of *Eimeria* species.

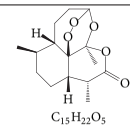
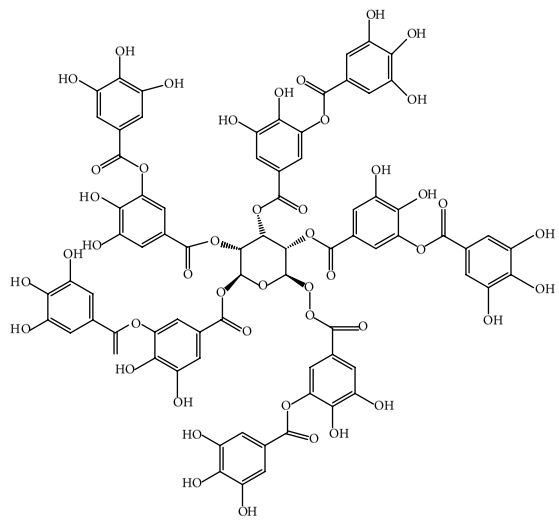
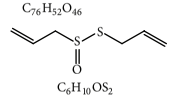
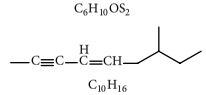
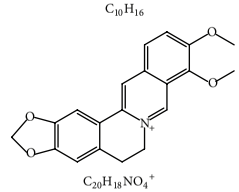
S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
1	Artemisinin	 $C_{15}H_{22}O_5$	*A. annua*	Inhibition of oocyst wall formation and sporulation via oxidative stress	Et, Ea, and Ema	[20–23, 58]
2	Tannin	 $C_{76}H_{52}O_{46}$	*P. radiata*	Inhibition of sporulation	Et, Ea, and Ema	[25]
3	Allicin	 $C_6H_{10}OS_2$	*A. sativum*	Inhibition of sporozoites	Et	[26, 28]
4	Polyacetylene	 $C_{10}H_{16}$	*B. pilosa*	Inhibition of sporozoites; immune modulation	Et	[8, 9]
5	Berberine	 $C_{20}H_{18}NO_4^+$	*A. lyceum*	Inhibition of sporozoites by oxidative stress	Et	[59]

Table 2: Continued.

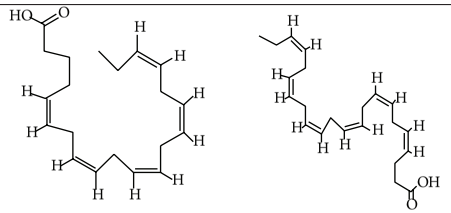
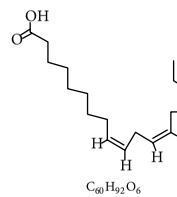
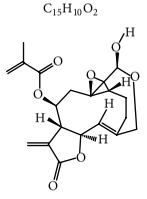
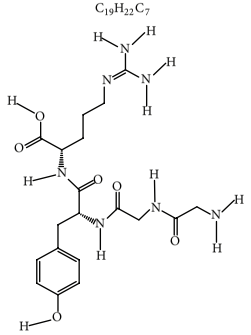
S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
6	N-3 fatty acids		*L. usitatissimum*	Oxidative stress	Et	[21]
7	Flavonoid	 $C_{15}H_{10}O_2$	*A. conyzoides*	Oxidative stress	Et	[31]
8	Vernoside	 $C_{19}H_{22}C_7$	*V. amygdalina*	Oxidative stress	Et	[32]
9	Papain	 $C_{21}H_{33}N_7O_4$	*C. papaya*	Digestion of sporozoites in caeca	Et	[32]

Table 2: Continued.

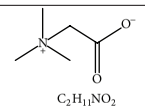
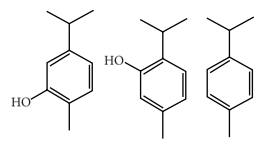
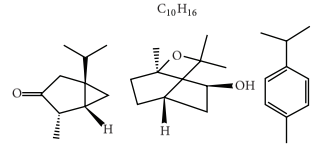
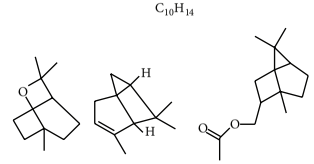
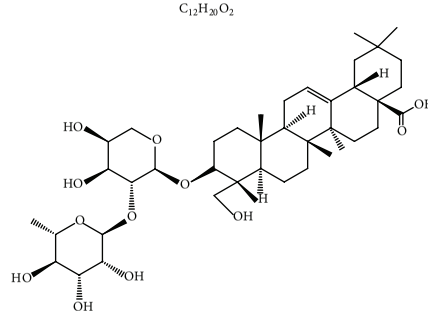
S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
10	Betaine	 $C_2H_{11}NO_2$	*O. vulgare*	Stabilizing intestinal structure and function	Et, Ea, and Ema	[25, 36]
11	Essential oil (carvacrol, thymol, and terpinene-γ)	 $C_{10}H_{14}O$, $C_{10}H_{14}O$, $C_{10}H_{16}$	*O. compactum*	Destruction of sporozoites	*Eimeria* species	[12, 60]
12	Essential oil (β-thujone, 1,8-cineol, and p-cymene)	 $C_{10}H_{16}O$, $C_{10}H_{18}O_2$, $C_{10}H_{14}$	*A. absinthium*	Prevention of oocyst development	*Eimeria* species	[61, 62]
13	Essential oil (cineol, α-pinene, and bornyl acetate)	 $C_{10}H_{18}O$, $C_{10}H_{16}$, $C_{12}H_{20}O_2$	*R. officinalis*	Antioxidant and destruction of oocysts	*Eimeria* oocysts	[61, 63, 64]
14	Saponin	 $C_{41}H_{66}O_{12}$	*M. cordifolia*, *M. citrifolia*, *M. arboreus*, and *C. tetragonoloba*	Destruction of oocysts and parasites	Et	[35]

Table 2: Continued.

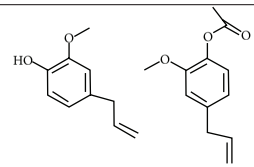
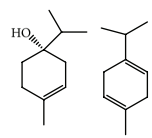
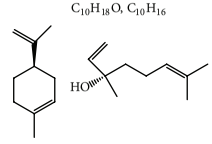
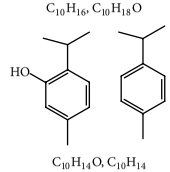
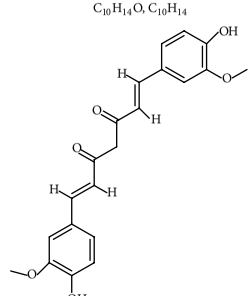
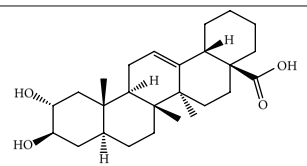
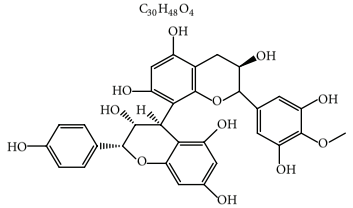
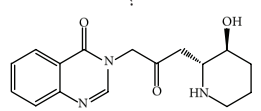
S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
15	Essential oil (eugenol and eugenyl acetate)	 $C_{10}H_{12}O_2$, $C_{12}H_{14}O_3$	*S. aromaticum*	Destruction of oocysts	Et	[51, 61]
16	Essential oil (terpinen-4-ol and terpinene-γ)	 $C_{10}H_{18}O$, $C_{10}H_{16}$	*M. alternifolia*	Destruction of oocysts	*Eimeria* oocysts	[61, 65]
17	Essential oil (limonene and linalool)	 $C_{10}H_{16}$, $C_{10}H_{18}O$	*C. sinensis*	Destruction of oocysts	*Eimeria* oocysts	[61, 66]
18	Essential oil (thymol and p-cymene)	 $C_{10}H_{14}O$, $C_{10}H_{14}$	*T. vulgaris*	Destruction of oocysts	*Eimeria* oocysts	[12, 61]
19	Curcumin (diferuloylmethane)	 $C_{21}H_{20}O_6$	*C. longa*	Inhibition of sporozoites; immune modulation	Et and Ema	[21, 67]

Table 2: Continued.

S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
20	Maslinic acid	 $C_{30}H_{48}O_4$	*O. europaea*	?	Et	[37]
21	Proanthocyanidin	 $C_{31}H_{28}O_{12}$	Grape seed	Antioxidant	Et	[38]
22	Selenium	?	*C. sinensis*	Inhibition of sporulation	Et, Ea, and Ema	[30]
23	Febrifugine	 $C_{16}H_{19}N_3O_3$	*D. febrifuga*	Inhibition of multiplication	Et	[10, 39, 68]

Et: *E. tenella*; Ea: *E. acervulina*; Ema: *E. maxima*; Eb: *E. brunetti*; En: *E. necatrix*; Emi: *E. mivati*; ?: unknown.

Table 3: Phytochemicals regulating host immunity against *Eimeria* species.

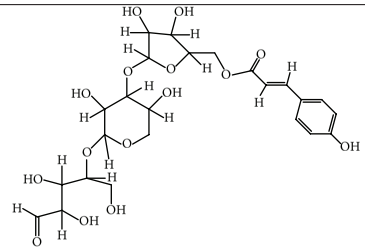
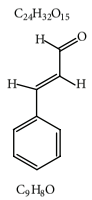
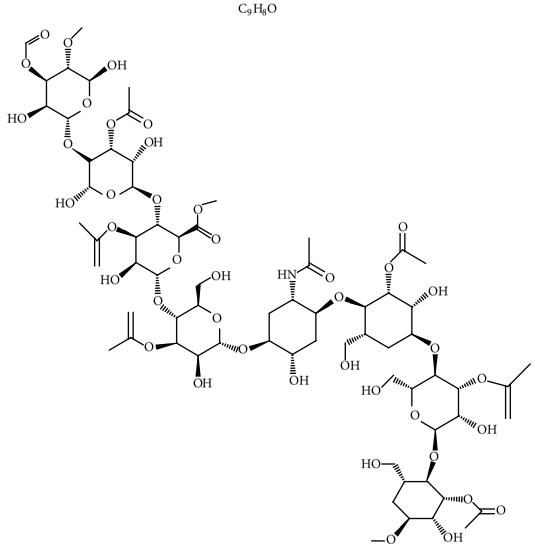
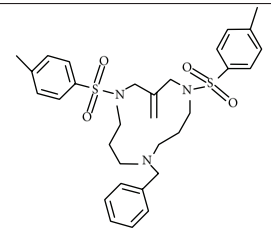
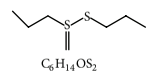
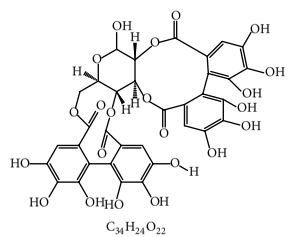
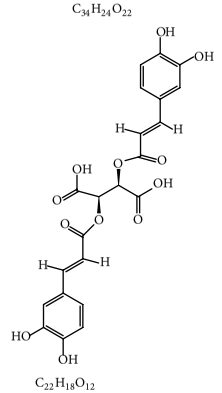
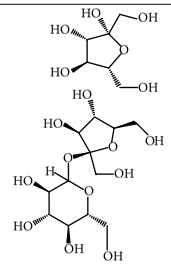
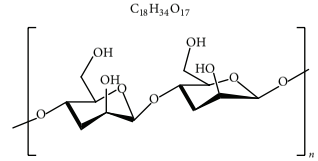
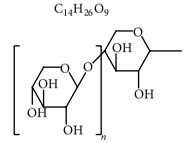
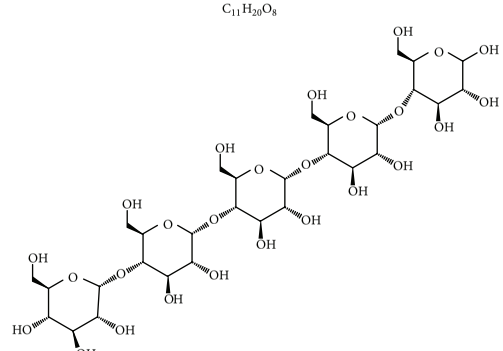
S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
24	Arabinoxylans		*T. aestivum*	Immune stimulation	Et, Ea, Ema, and En	[69]
25	Cinnamaldehyde	 $C_{24}H_{32}O_{15}$ C_9H_8O	*C. cassia*	Immune modulation	Et, Ea, and Ema	[70, 71]
26	Acemannan	 $C_{66}H_{100}NO_{49}^-$	*A. vera*	Immune stimulation	*Eimeria* spp.	[72, 73]

Table 3: Continued.

S. number	Compound	Structure and formula	Plant	Mechanism	*Eimeria* species	Reference
27	Lectin	 $C_{31}H_{39}N_3O_4S_2$	*F. fraxinea*	Immune stimulation	Ea	[74]
28	Propyl thiosulfinate	 $C_6H_{14}OS_2$	*A. sativum*	Protective immunity	Ea	[75, 76]
29	Tannin (pedunculagin)	 $C_{34}H_{24}O_{22}$	*A. officinalis*	Immune stimulation	*Eimeria* spp.	[77]
30	Chicoric acid	 $C_{22}H_{18}O_{12}$	*E. purpurea*	Immune stimulation	*Eimeria* spp.	[78, 79]
31	Mushroom polysaccharide	?	*L. edodes* and *T. fuciformis*	Immune stimulation	Et	[80]
32	Phenolics compounds	?	*P. salicina*	Immune stimulation	Ea	[81, 82]

Et: *E. tenella*; Ea: *E. acervulina*; Ema: *E. maxima*; Eb: *E. brunetti*; En: *E. necatrix*; ?: unknown.

Table 4: Phytochemicals with prebiotic function for gut microbiota.

S. number	Prebiotics	Chemical structure	Plant	Effects on poultry microbiota	Reference
33	Inulin	 $C_{18}H_{34}O_{17}$	Chicory	Enhancing gut microflora, morphology, and immunity	[83, 84]
34	Mannan-oligosaccharides	 $C_{14}H_{26}O_9$	Fungi and yeast	Increasing digestion and gut microbiota	[85, 86]
35	Xylooligosaccharides	 $C_{11}H_{20}O_8$	Bamboo shoots, fruits, vegetables, and wheat bran	Increasing *Lactobacillus* in colon	[87]
36	Isomaltooligosaccharides	 $C_{30}H_{52}O_{26}$	Starch	Increasing cecal probiotics and fatty acids	[88, 89]
37	Soy oligosaccharides	?	Soybean	Changing microbiota	[90]
38	Pyrodextrins	?	Sucrose	Increasing gut microbiota and growth performance	[91]
39	Oligofructose	?	Asparagus, sugar beet, garlic, onion, chicory, and artichoke	Increasing digestion and gut microbiota	[85, 86, 92]
40	Arabinoxylooligosaccharides	?	Wheat bran	Increasing digestion and gut microbiota	[93–95]

?: unknown.

originally identified as a novel anticoccidial compound as indicated by the lesion index, the oocyst index, and the anticoccidial index [37]. However, its anticoccidial activity remains unknown.

2.1.10. Proanthocyanidin and Grape Seed. Proanthocyanidin is a naturally occurring polyphenolic antioxidant widely distributed in grape seed and other sources. Grape seed proanthocyanidin extract was shown to reduce *E. tenella* infection as shown by gut pathology, body weight, and mortality [38]. Accordingly, this extract decreased nitric oxide but increased superoxide dismutases in the plasma of chickens [38]. These data suggest that proanthocyanidin from grape seed diminishes coccidiosis via downregulation of oxidative stress.

2.1.11. Dichroa febrifuga. *D. febrifuga*, also known as Chang Shan, is a Chinese medicinal herb for protozoan diseases. Zhang and coworkers showed that crude extract of *D. febrifuga* was effective against *E. tenella* infection in chickens [39]. Febrifugine, an alkaloid, was isolated from this plant and its halogenated derivative, halofuginone, was developed as anticoccidial chemical [10].

2.2. Plants and Compounds That Modulate Host Immunity against Eimeria. From an evolutionary point of view, birds have a complete immune system consisting of innate and adaptive immune responses [96]. Both immune responses are responsible for coccidial clearance and vaccine immunization [12, 97]. Medicinal plants often have immunomodulatory compounds which boost antimicrobial immune responses to uphold homeostasis of poultry health [91, 98]. Therefore, immunoregulatory plant extracts and compounds could be utilized as an alternative method to reinforce immune response against avian coccidiosis. Immunoregulatory phytochemicals for avian coccidiosis are described in Table 3 and Figure 2.

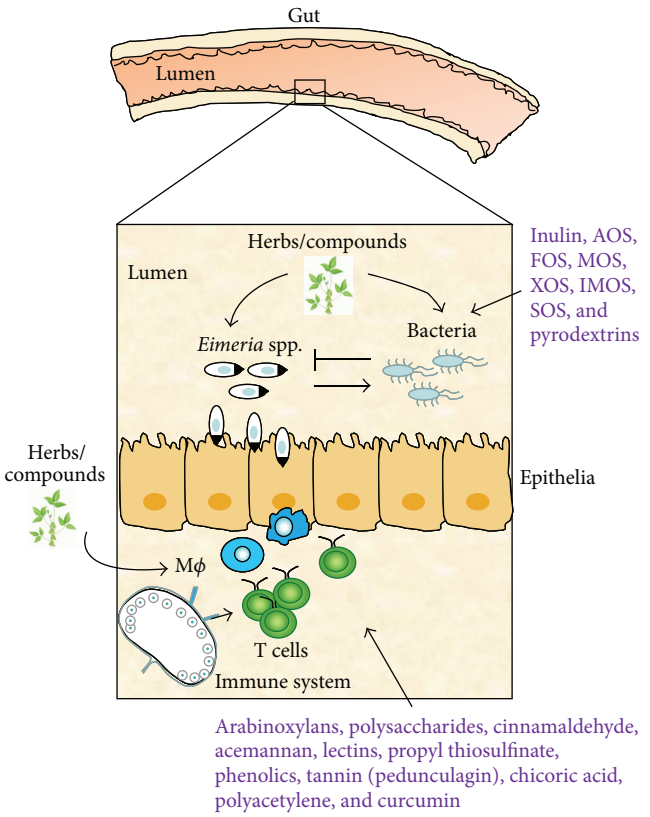


Figure 2: Immune and prebiotic modulation underlying anticoccidial compounds. In the lumens of bird guts, bacteria and *Eimeria* species interact with each other. Particularly, some beneficial bacteria can reduce gut lesions caused by *Eimeria* species. Gut-associated T cells, macrophages, and other immune cells can mount immune responses to harmful *Eimeria* and bacteria. Phytocompounds from plants can inhibit the multiplication of *Eimeria*, expand the growth of beneficial bacteria, and/or boost immunity, leading to controlling *Eimeria* infection in the gut of poultry.

2.2.1. Arabinoxylans, Wheat (Triticum aestivum), and Sugar Cane (Saccharum officinarum). Akhtar and colleagues showed that arabinoxylan, a bioactive compound from wheat bran, improved coccidiosis in chickens as indicated by body weight, oocyst count, and gut lesions [69]. In contrast, Awais and Akhtar reported that different extracts of sugar cane juice and bagasse protected against coccidiosis in chickens as shown by body weight gain, oocyst shedding, lesion score, and anticoccidial indices [99]. The data from both studies revealed that wheat bran arabinoxylan and sugar cane conferred host protection against *Eimeria* infection via natural and adaptive immune response. Cell-mediated immunity seemed to be a key factor in response to coccidiosis in chickens when compared to humoral immunity [69, 99].

2.2.2. Polysaccharides from Astragalus membranaceus Radix, Carthamus tinctorius, Lentinus edodes, and Tremella fuciformis. Guo and colleagues reported that the polysaccharides derived from the herb *A. membranaceus Radix* and the mushrooms *L. edodes* and *T. fuciformis* effectively controlled *E. tenella* infection in chickens [80]. Concurrent with the anticoccidial protection, the polysaccharides could enhance anticoccidial antibodies and antigen-specific cell proliferation in splenocytes via cellular and humoral immunity to *E. tenella* in chickens [80]. Their mechanism appeared to stimulate cell proliferation of the lymphocytes via regulation of DNA polymerase activity.

2.2.3. Cinnamaldehyde, Carvacrol, Capsicum Oleoresin, and Turmeric Oleoresin. Two phytonutrient mixtures, VAC (carvacrol, cinnamaldehyde, and *Capsicum* oleoresin) and MC (*Capsicum* oleoresin and turmeric oleoresin), were tested for coccidiosis in chickens [70]. The data proved that both combination treatments effectively protected against *E. tenella* infection. Moreover, both treatments exhibited an increase in NK cells, macrophages, $CD4^+$ T cells, $CD8^+$ T cells, and their cytokines (IFN-γ and IL-6) and a decrease in TNFSF15 and IL-17F, leading to induction and elevation of host immunity to kill *E. tenella* in chickens [70].

2.2.4. Aloe and Acemannan. Gadzirayi et al. showed that *A. excelsa* possesses anticoccidial activity in chickens [36, 43,

100, 101] despite lack of information about its mode of action and its active compound(s). Another study stated that aqueous and ethanolic extracts from *A. vera* mounted a cell-mediated immune response as well as a humoral response against coccidiosis in chickens. The immunomodulatory compounds could include aloe polysaccharide acemannan (Figure 2), which binds the mannose receptor on macrophages, stimulating them to produce inflammatory cytokines such as IL-1 through IL-6 and TNF-α and eventually suppress coccidiosis as shown by greater weight gain and lower fecal oocyst counts [72, 100].

2.2.5. Oriental Plum (Prunus salicina) and Phenolics. One report showed that dietary supplementation of plum fruit powder, rich in phenolic compounds, added to chicken feed significantly diminished *E. acervulina* infection in chickens as demonstrated by increased body weight gain and reduced fecal oocyst shedding [81]. Accordingly, plum fruit powder greatly augmented the transcription of IFN-γ and IL-15 and splenocyte proliferation, indicating that plum fruit can boost immune response to coccidiosis.

2.2.6. Mushroom (Fomitella fraxinea) and Lectin. One study showed that the lectin derived from a mushroom, *F. fraxinea*, protected chickens from *Eimeria* challenge via enhancement of both cellular and humoral immune responses [74]. This work also suggested that this mushroom could enhance both immune responses to *Eimeria* species in chickens. This study implies that immunoregulatory botanicals such as mushroom can improve poultry growth and development via immune protection from infectious pathogens and toxins. Moreover, botanicals, containing micro- and macronutrients, can increase growth performance in poultry.

2.2.7. Propyl Thiosulfinate and Propyl Thiosulfinate Oxide. One study showed that garlic compounds, propyl thiosulfinate (PTS) and propyl thiosulfinate oxide (PTSO), could alter the expression levels of 1,227 transcripts related to intestinal intraepithelial lymphocytes (IEL) in chickens. PTSO/PTS was shown to activate transcription factor, NF-κB, which plays a key role in regulating the immune response upon infection. Therefore, it seems that a combination of PTSO and PTS rendered chickens more resistant to experimental *E. acervulina* infection and augmented adaptive immunity, including a higher antibody response and greater splenocyte proliferation, compared with control chickens [75]. Another *in vitro* study showed that PTS could stimulate splenocyte proliferation and directly kill the sporozoites, pointing to the same conclusion [75].

2.2.8. Tannins and Chicoric Acid from Emblica officinalis and Echinacea purpurea. Tannins and chicoric acid, isolated from *E. officinalis* [77] and *E. purpurea* [102], respectively, were reported to effectively elicit humoral immune response against coccidial infection in chickens. However, the mechanism by which both compounds boost anticoccidial immunity is not clear.

2.3. Plants and Compounds That Possess Prebiotic Properties. Like in humans, gut microbiota are important for health and disease in poultry [103]. Gut microbiota perform multiple functions involved in nutrient digestion, gut development and growth, establishment/maintenance of the immune system, suppression of pathogenic microbes, microbial infections, and so forth [8, 104–109]. Thus, promotion of beneficial microbes and reduction of harmful microbes contribute to growth performance and health in poultry [103]. Several studies have indicated that probiotics, containing one or multiple species of *Lactobacillus*, *Enterococcus*, and/or *Bifidobacterium*, can reduce coccidiosis and enhance growth performance in chickens [57, 97, 104, 110, 111]. Currently, little is known about the anticoccidial mechanisms of probiotics. These modes of action have been proposed: maintaining a healthy balance of bacteria by competitive exclusion and antagonism, promoting gut maturation and integrity, modulating immunity and preventing inflammation, altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production, improving feed intake and digestion, and neutralizing enterotoxins and stimulating the immune system [91, 108]. On the other hand, coccidiosis is frequently accompanied by secondary bacterial infection [71, 105, 106, 112].

Prebiotics refer to nondigestible feed ingredients that promote the growth of probiotics and their activities in guts [113]. As illustrated in Table 4, the most common prebiotics, used in poultry, include inulin, arabinoxylooligosaccharides (AOS), fructooligosaccharides (FOS), mannan-oligosaccharides (MOS), xylooligosaccharides (XOS), isomaltooligosaccharides (IMOS), soy oligosaccharides (SOS), and pyrodextrins [92, 114]. These oligosaccharides are derived from the plants such as chicory, onion, garlic, asparagus, artichoke, leek, bananas, tomatoes, and wheat [92]. Dietary supplementation of these prebiotics to chicken feed has enhanced immune defence against pathogen infection and reduced the mortality rate [91, 115]. The mechanism of prebiotics is yet to be revealed, but they selectively stimulate beneficial bacteria in the intestinal system of the bird. The increasing number of beneficial microbiota excludes the harmful pathogens from colonization in the intestinal track of the bird. Subsequently, healthy hosts can produce a wide variety of bacterions and other immunomodulators that can stimulate macrophages to neutralize the pathogens [114]. Thus, prebiotic-mediated immunological changes may be partially due to direct interaction between prebiotics and gut immune cells as well as due to an indirect action of prebiotics via preferential colonization of probiotics and their products that interact with immune cells [85]. Therefore, prebiotics exert their functions mainly via increasing gut probiotics to suppress pathogens and boosting immune response in chickens to constrain gut pathogens [91]. Moreover, Bozkurt et al. reported that prebiotics diminished coccidial infection in chickens but kept marginal oocyst production that might serve as a source of live vaccine for uninfected chickens [116]. Two other publications emphasized that probiotics composed of *Bifidobacterium animalis* and *Lactobacillus salivarius* alleviated the detrimental impact of the *Eimeria* infection on chickens and improved growth performance [110, 111]. These

findings suggest that prebiotics suppress coccidiosis plausibly via indirect regulation of increased probiotics and host immunity. Apparently, prebiotics share many similar anticoccidial mechanisms with probiotics. Overall, dietary supplementation of prebiotics is emerging as a novel approach to control coccidiosis.

2.4. Plants and Compounds with Multiple Mechanisms to Inhibit Coccidiosis

2.4.1. Curcumin and Curcuma longa. As described in Table 2, turmeric (*C. longa*) has long been used as a spice and medicinal herb. One publication stated that *C. longa* showed anticoccidial activity [67, 117]. Another reported that curcumin (diferuloylmethane), an active compound in *C. longa*, consistently destroyed sporozoites of *E. tenella* [118]. Similarly, a combination of *A. annua* and *C. longa* showed anticoccidial efficacy in broilers challenged with a mixture of *E. acervulina* and *E. maxima* [119]. In addition, curcumin was shown to enhance coccidiosis resistance as evidenced by increased BW gains and reduced oocyst shedding and gut lesions [21, 67]. Consistently, curcumin elevated host humoral immunity to *Eimeria* species and diminished gut damage in poultry [21, 67].

2.4.2. Polyacetylenes and Bidens pilosa. As described in Table 2, Yang et al. demonstrated that *B. pilosa* has exhibited anticoccidial activity in chickens infected with *E. tenella* as evidenced by survival rate, fecal oocyst count, gut pathology, body weight, and bloody stool [9]. Although the active compounds in *B. pilosa* responsible for anticoccidial action are unknown, this plant is a rich source of phytochemicals, such as 70 aliphatics, 60 flavonoids, 25 terpenoids, 19 phenylpropanoids, 13 aromatics, 8 porphyrins, and 6 other compounds [120]. Interestingly, one polyacetylene (1-phenyl-1,3-diyn-5-en-7-ol-acetate) and one flavonoid (quercetin-3,3-dimethoxy-7-0-rhamnoglucopyranose) in this plant have been proposed to be active compounds against the protozoan parasite, *Plasmodium* [121].

However, the identity of the active compounds needs to be further ascertained. The mechanism of *B. pilosa* and its active compounds is not clear. It is possible that this plant and its active compounds intervene in the initial phases of the *Eimeria* life cycle because the phases may be liable to chemical attack when compared with the oocyst whose wall is very resistant to physical and chemical insults. In addition, *B. pilosa* was shown to modulate host immunity [122], which might have impact on coccidiosis.

Compared to anticoccidial drugs, *B. pilosa* was shown to produce little or no drug resistance in *Eimeria* [9]. Botanicals developed low resistance in *Eimeria* probably because different compounds target multiple pathways related to drug resistance [9, 12].

It should be noted that the plant- and phytochemical-based remedies can be used *per se* or in combination with other anticoccidial agents. This idea was further confirmed by one publication indicating that *Echinacea*, an immunotherapeutic herbal extract, was used to boost the immunization efficacy in chickens in combination with anticoccidial vaccines [78].

3. Conclusions and Perspectives

Coccidiosis is a deadly and debilitating infectious disease in poultry, which is caused by enteric protozoan parasites, *Eimeria species*, in the guts of birds. These parasites damage the guts of the birds, leading to moderate clinical symptoms such as sick bird appearance, bloody stool, hemorrhage, and gut lesions and death. Pathogens (*Eimeria* species, swallowed oocyst counts, etc.), host genetics, and environmental factors could influence the clinical outcome of avian coccidiosis. Current prophylaxis and therapy for coccidiosis comprise anticoccidial chemicals, vaccines, and natural products. Plants are a rich source of phytochemicals against coccidiosis. Here, we summarized the chemistry and biology of over 68 plants and compounds which prevented and treated avian coccidiosis via the regulation of the life cycle of *Eimeria* species and host immunity and gut microflora in experimental and field trail studies. Emphasis was placed on recent advances in the understanding of the potential of these plants and compounds to prevent and/or treat avian coccidiosis. Moreover, the actions, mechanisms, and therapeutic potential of these plant compounds and/or extracts in avian coccidiosis and new insights into the advantage of plant extracts and compounds that simultaneously regulate *Eimeria*, bacteria, and immune cells were discussed. Comprehensive information about the structure, activity, and modes of action of these compounds can aid research and development of anticoccidial remedies.

Competing Interests

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

Authors' Contributions

Thangarasu Muthamilselvan and Tien-Fen Kuo equally contributed to this study.

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Research Article

Ultrastructural Changes and Death of *Leishmania infantum* Promastigotes Induced by *Morinda citrifolia* Linn. Fruit (Noni) Juice Treatment

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The search for new treatments against leishmaniasis has increased due to high frequency of drug resistance registered in endemics areas, side effects, and complications caused by coinfection with HIV. *Morinda citrifolia* Linn., commonly known as Noni, has a rich chemical composition and various therapeutic effects have been described in the literature. Studies have shown the leishmanicidal activity of *M. citrifolia*; however, its action on the parasite has not yet been elucidated. In this work, we analyzed leishmanicidal activity and ultrastructural changes in *Leishmania infantum* promastigotes caused by *M. citrifolia* fruit juice treatment. *M. citrifolia* fruit extract showed a yield of 6.31% and high performance liquid chromatography identified phenolic and aromatic compounds as the major constituents. IC_{50} values were 260.5 μg/mL for promastigotes and 201.3 μg/mL for intracellular amastigotes of *L. infantum* treated with *M. citrifolia*. Cytotoxicity assay with J774.G8 macrophages showed that *M. citrifolia* fruit juice was not toxic up to 2 mg/mL. Transmission electron microscopy showed cytoplasmic vacuolization, lipid inclusion, increased exocytosis activity, and autophagosome-like vesicles in *L. infantum* promastigotes treated with *M. citrifolia* fruit juice. *M. citrifolia* fruit juice was active against *L. infantum* in the *in vitro* model used here causing ultrastructural changes and has a future potential for treatment against leishmaniasis.

1. Introduction

Due to the continental dimensions of Brazil there are various parts of its territory with difficult access. Consequently, there is a limit to public health resources and a tendency for the inhabitants of these remote regions not to get the necessary government health benefits. This geographical isolation contributes to strengthening the local traditional medical practices and other natural resources to treat diseases, including parasitic diseases such as leishmaniasis [1].

Leishmaniasis is caused by protozoan parasites transmitted through the bites of infected female sandflies (usually *Phlebotomus* or *Lutzomyia*). The disease appears in three clinical forms: the visceral form, also known as kala-azar, is usually fatal within 2 years if left untreated; the cutaneous form, causing skin ulcers; and the mucocutaneous form, which invades the mucous membranes of the upper respiratory tract, causing gross mutilation by destroying soft tissues in the nose, mouth, and throat [2]. The disease, which is prevalent in 98 countries and 3 territories on 5 continents, has approximately 1.3 million new cases annually, of which 300,000 are visceral and 1 million are cutaneous or mucocutaneous. These numbers show the importance of this disease in public health, including Brazil [3].

Treatment for leishmaniasis was first introduced by Vianna in 1912. Organic compounds of antimony are the drugs of choice in treating this disease, and amphotericin B was introduced recently. Both treatments present several side

effects, are highly toxicity, and have an elevated cost which has led to the search for new alternatives. The search for a leishmanicidal agent of low toxicity and high efficiency is a challenge and has involved several research groups around the world [4]. Herbal remedies have gained a lot of attention in this area as a potential source to obtain new compounds with therapeutic activities.

Morinda citrifolia Linn. is a small plant native to Southeast Asia, commonly known as Noni, and one of the most significant sources of traditional medicine in those countries. Due to the various ethnopharmacological activities associated with this plant, it is now cultivated all over the world, including Brazil. Studies have shown the efficacy of Noni in the treatment of pain and inflammatory reactions [5] and antitumoral activity [6]. Activity against bacteria [7] and fungi [8] has also been observed. Recently, the *in vitro* activity of morindicone and morinthone isolated from the stem of *M. citrifolia* was described against *L. major*. Moreover, a clinical study was carried out to determine the efficiency of a topical ointment with *M. citrifolia* stem extract against cutaneous leishmaniasis, and there was an excellent response in 50% and a good improvement in 30% of the 40 patients evaluated [9]. Therefore, to demonstrate the action of *M. citrifolia* against promastigotes of *Leishmania* and evaluate the ultrastructural changes caused by such treatment, this study evaluated promastigotes forms of *Leishmania infantum* treated with *M. citrifolia* fruit juice by electron microscopy.

2. Materials and Methods

2.1. Plant Material. M. citrifolia fruits were collected in November 2011 from São Luís (S2°31 W44°16), Maranhão, in the Brazilian Legal Amazon at 24 m above sea level. Fruits were collected when the exocarp was translucent. The plant material was identified by Ana Maria Maciel Leite, and the voucher specimen number 2000346 was deposited at the Herbário Professora Rosa Mochel, Universidade Estadual do Maranhão. In the laboratory, the fruits were washed with distilled and sterilized water, dried at 25°C, and placed in sterile glass bottles for 3 days to drain off the extract released. This liquid was centrifuged twice at 4000 rpm for 15 minutes; the supernatant was lyophilized and stored at −20°C [8]. The lyophilized *M. citrifolia* fruit juice was dissolved in DMSO and dilutions with different concentrations in culture medium were made immediately before use. The concentration of DMSO in medium did not exceed 1%.

2.2. High Performance Liquid Chromatography Coupled with Diode Array and Evaporative Light Scattering Detectors (HPLC-DAD-ELSD). The HPLC chromatographic profile of the *M. citrifolia* fruit juice was performed on a Shimadzu LC-10Avp equipped with two LC-8Avp pumps, controlled by a CBM-10A interface module, an automatic injector with two detectors, a diode array detector SPD-M10A (DAD), and an evaporative light scattering detector (ELSD) with a drift tube temperature setting of 40°C, using nitrogen as the nebulizer gas and gain at 4.0. HPLC grade solvents and Milli-Q water were used and the analysis was performed on a reversed phase LiChrospher C18 column (4.6 mm × 250 mm; 5 μm, Waters). The mobile phase was water (A) and methanol (B), with the following gradient composition: (0–20 min) 5–20% (B), (20–30 min) 20%–35% (B), and (30–35 min) 35% (B). The UV chromatogram was obtained at 365 nm. The sample injection volume was 10 μL. A constant flow of 1 mL/min was used during the analysis. Before analysis 5.0 mg of the extract was dissolved in 1.0 mL of Milli-Q water and the mixture was centrifuged.

2.3. Parasites. Promastigote forms of *L. infantum* (MCAN/BR/2008/1112) were cultured at 26°C in Schneider's Insect medium (Sigma-USA) supplemented with 10% fetal bovine sera (Gibco-USA), 100 U/mL of penicillin (Gibco-USA), and 100 μg/mL of streptomycin. The cultures used had a maximum of ten *in vitro* passages.

2.4. Animals. Female BALB/c mice of 4–6 years old were purchased from Centro de Criação de Animais de Laboratório do Instituto Oswaldo Cruz, Rio de Janeiro, and maintained under pathogen-free conditions. The animals were handled in accordance with Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal. The local Ethics Committee on Animal Care and Utilization approved all procedures involving the animals (CEUA FIOCRUZ-LW72/12).

2.5. Cells Culture. The macrophage J774.G8 line was cultured in RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal bovine sera, penicillin (100 U/mL), and (100 μg/mL) streptomycin, at 37°C and 5% CO_2. Female BALB/c mice were inoculated with 3 mL of sodium thioglycolate 3% and after 72 hours peritoneal macrophages were harvested with PBS solution. The harvest was centrifuged at 4000 rpm and the cells suspended in RPMI medium supplemented as described before and cultured at 37°C and 5% CO_2.

2.6. Activity against Promastigote Forms. Promastigote forms of *L. infantum* (10^6 parasites/mL) from a 2–4-day-old culture were placed in 96-well plates in the presence of different concentrations of *M. citrifolia* fruit juice (960–30 μg/mL), in a final volume of 200 μL per well, for 72 hours. Wells without parasites were used as blank and wells with only parasites were used as control. After the treatment, the viability of parasites was evaluated by the tetrazolium-dye (MTT) colorimetric modified method [10]. MTT (5 mg/mL), a volume equal to 10% of the total, was added to each well. After 2 hours, the plate was centrifuged at 4000 rpm; then the supernatant was removed from each well and 100 μL of DMSO was added to dissolve the formazan. The absorbance was analyzed on a spectrophotometer at a wavelength of 540 nm. The data was normalized according to the formula

$$\%\ \text{survival} = \text{DO sample} - \frac{\text{DO blank}}{\text{DO control}} - \text{DO blank} \times 100. \quad (1)$$

The results were used to calculate IC_{50} (50% inhibition of parasite growth). Amphotericin B was used as the reference drug.

Table 1: Activity against promastigotes and intracellular amastigotes of *Leishmania infantum*, cytotoxicity in peritoneal macrophages from BALB/c and selectivity index of *Morinda citrifolia* fruit extract treatment and amphotericin B.

Compounds	IC_{50} (μg/mL)		CC_{50}	SI
	Promastigote	Intracellular amastigote	J774.G8	
Morinda citrifolia fruit juice	260.5 ± 0.044	201.3 ± 0.175	>2000	>9.9
Amphotericin B	3.1 ± 0.230	0.9 ± 0.121	2.7 ± 0.156	3.0

IC_{50}: inhibitory concentration of 50% parasites. CC_{50}: cytotoxicity concentration of 50% cells. SI: selectivity index. Data are presented as the mean ± SD of three independent experiments realized at least in triplicate.

2.7. Activity against Intracellular Amastigotes. Peritoneal macrophages were cultured in 24-well plates (10^5 cells/well), with coverslips, at 37°C and 5% CO_2. The cells were infected with promastigote forms of *L. infantum* using a ratio of 10 : 1 parasite/cell, and after 2 hours the cells were washed three times with PBS to remove free parasites. The infected cells were treated with different concentrations of *M. citrifolia* fruit juice (480–30 μg/mL) in triplicate for 24 hours. The coverslips with the infected and treated cells were fixed with Bouin, stained with Giemsa, and examined by light microscopy. The inhibition percentage was calculated and IC_{50} was calculated with the GraphPad Prism software. Amphotericin was used as the reference drug.

2.8. Cytotoxicity Assay. J774.G8 macrophages were cultured in 96-well plates (5×10^5 cells/mL) with different concentrations of *M. citrifolia* fruit juice (2000–1.8 μg/mL) to a final volume of 200 μL per well, at 37°C and 5% CO_2. Wells without cells were used as blank and wells with only cells were used as control. After 24 hours, the cells were fixed with 10% trichloroacetic acid for 1 hour at 4°C, stained with Sulforhodamine B (Sigma, USA) solution 0.4% in 1% acetic acid for 30 minutes, and washed with 1% acetic acid solution. Sulforhodamine B was solubilized in 200 μL of 10 mM tris-base solution and the plate was read in a spectrophotometer at 540 nm wavelength [11]. The data was normalized following the formula described earlier. The results were used to calculate the cell cytotoxicity by 50% (CC_{50}) with the GraphPad Prism 5.

2.9. Transmission Electron Microscopy. Promastigote forms of *L. infantum* were treated with *M. citrifolia* fruit juice at concentrations of 480, 240, 120, 60, and 30 μg/mL for 24 hours. The parasites were fixed with 2.5% glutaraldehyde (Sigma, USA) in 0.1 M sodium-cacodylate buffer, pH 7.2 overnight. Parasites were washed three times with 0.1 M sodium-cacodylate buffer and postfixed in a solution containing 1% osmium tetroxide, 0.8% ferrocyanide, and 5 mM calcium chloride, washed in 0.1 M sodium-cacodylate buffer, dehydrated in graded acetone, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope JEM-1011 (JEOL, Japan).

2.10. Statistical Analysis. The values were expressed as mean ± SD. The results were analyzed statistically by Analysis of Variance (ANOVA) followed by the Tukey test. The analyses were performed with the software GraphPad Prism 5.0.4. Differences were considered significant when $p < 0.05$.

3. Results and Discussion

The *M. citrifolia* fruit juice was brown, translucent, and of medium viscosity, with its characteristic odor and pH 3.94. After lyophilization, the juice yielded 6.31% of a highly hygroscopic powder. The constituents of *M. citrifolia* fruit juice were analyzed by HPLC-DAD-ELSD and the analysis of the chromatograms obtained from both detectors showed peaks related to compounds with sensitivity in the UV region. The LC-DAD and LC-ELSD chromatograms are presented in Figure 1. The major peaks of the UV-365 nm chromatogram were associated with the characteristic UV spectra of flavonoid (peak 8) and anthraquinones (peak 11). The ELSD fingerprint showed an intense peak at 3.2 min which was not observed in the DAD chromatogram. This signal could be related to the polysaccharides previously reported in *M. citrifolia* that have significant antitumoral activity [12]. Polysaccharides from *Echinacea purpurea* showed activity against *Leishmania enriettii* [13] and the presence of these substances in the phytochemical fingerprint showed the probable chemical potential of the fruit extract against protozoa such as the *Leishmania* genus. This potential was demonstrated through *in vitro* leishmanicidal activity of the *M. citrifolia* fruit juice against *L. infantum*.

The effect of *M. citrifolia* fruit juice on the promastigote forms of *L. infantum* was monitored for 72 hours. *M. citrifolia* fruit juice produced a dose-dependent reduction in the proliferation of the parasite (Figure 2(a)), with growth inhibition of 50% of the promastigotes at a concentration of 260.5 μg/mL (Table 1). The values are considered promising when compared with other fruit extracts. The crude extract from the fruit *Momordica charantia* showed an IC_{50} under 600 μg/mL for *L. donovani* promastigotes [14].

There are few data about *in vitro* leishmanicidal activity of *M. citrifolia* constituents in the literature. A clinical trial on the antileishmanial activity of *M. citrifolia* showed good activity for two anthraquinones isolated from stem extract, morindicone and morinthone [9]. Anthraquinones also have been isolated from *M. lucida*, a plant of same gender of *M. citrifolia,* and presented activity against the growth of *Plasmodium falciparum* and promastigotes of *L. major in vitro* [15].

Trying to find compounds responsible for *in vitro* leishmanicidal activity, the *M. citrifolia* fruit juice was submitted to a column partition and, interestingly, the partitions showed IC_{50} values above the value obtained for the full juice (data not shown). This result indicates that various substances present in the *M. citrifolia* fruit juice contribute to the leishmanicidal activity, probably, synergistically, corroborating

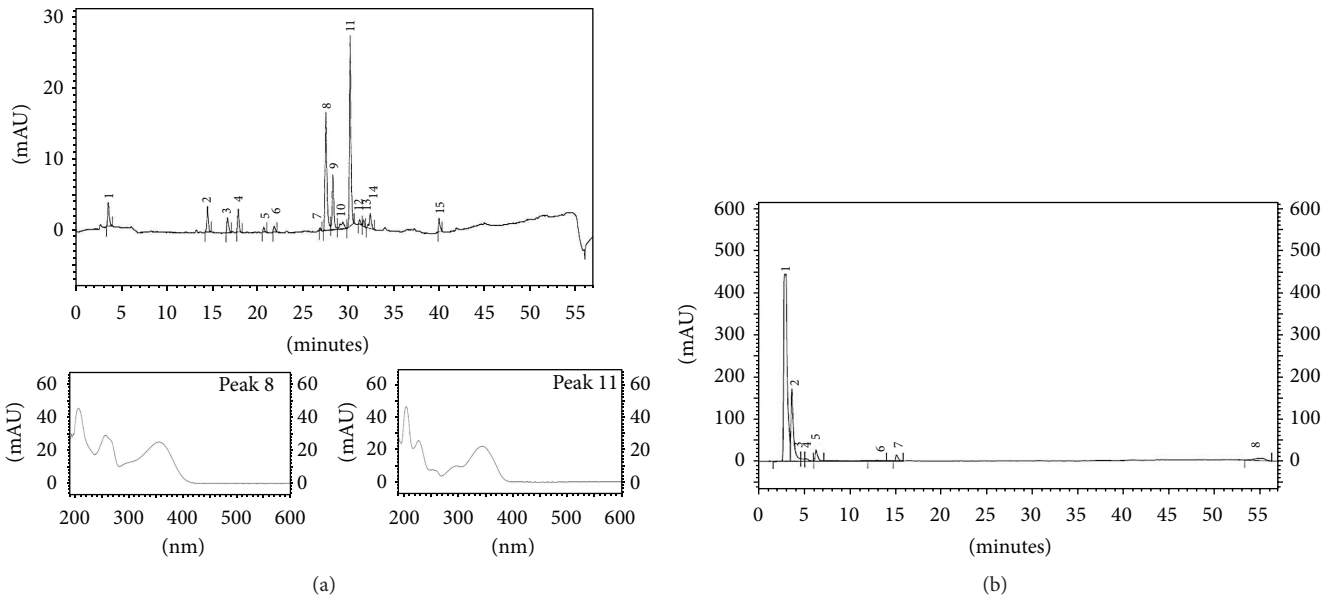


Figure 1: High Performance Liquid Chromatography coupled with diode array detector (a) and evaporative light scattering detector (b) of *Morinda citrifolia* fruit juice at 365 nm. (a) Peaks 8 and 11: highlight of the UV spectra.

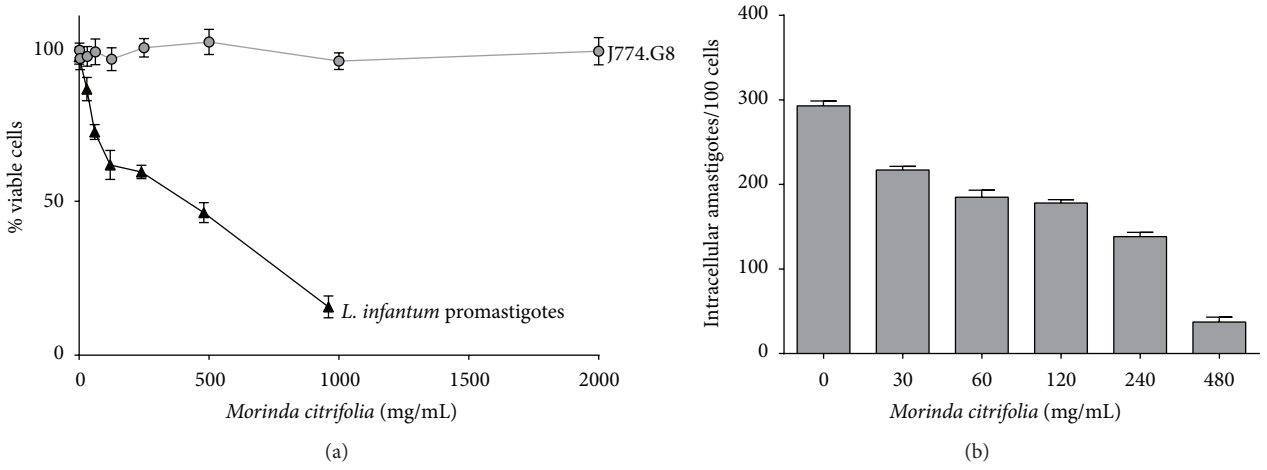


Figure 2: Leishmanicidal activity and *in vitro* cytotoxicity of *Morinda citrifolia* fruit juice. (a) Viability of *Leishmania infantum* promastigotes and J774.G8 macrophages treated with *M. citrifolia* for 72 and 24 hours, respectively. (b) Intracellular amastigotes in BALB/c peritoneal macrophages treated for 24 hours. Data represent mean ± SD of at least three independent experiments realized in quintuplicate.

previous studies where more than one molecule presented biological activity [16, 17].

Although the leishmanicidal activity *in vitro* against promastigotes is used by many researchers as a screening test to search for new drugs for the treatment of leishmaniasis, the positive result of this test alone cannot be considered as an indicator of potential drug action. Activity against intracellular amastigotes is necessary and is perhaps the most effective way to relate the *in vitro* activity of a substance with its possible effectiveness *in vivo*. Thus we also evaluated *M. citrifolia* activity against intracellular amastigotes (Figure 2(b)).

As shown in Table 1, there is an increase in activity of the *M. citrifolia* fruit juice against intracellular amastigotes compared with the activity against promastigotes. The IC_{50} value decreased for intracellular amastigotes with a value of 201.3 μg/mL. When observed by light microscopy, macrophages showed vacuoles with probable remains of intracellular amastigotes (Figure 3). This result indicates the possible action of the *M. citrifolia* fruit juice on macrophage activation and modulation, as already shown in previous works, such as the decreased production of IL-4 and increased production of TNF-α, IL-1β [12], INF-γ, and NO [18].

To ensure that *M. citrifolia* fruit juice was only acting on intracellular amastigotes, without causing damage to the host cell, the cytotoxicity in J774.G8 lineage macrophages was investigated by the Sulforhodamine B method (Figure 2(a)).

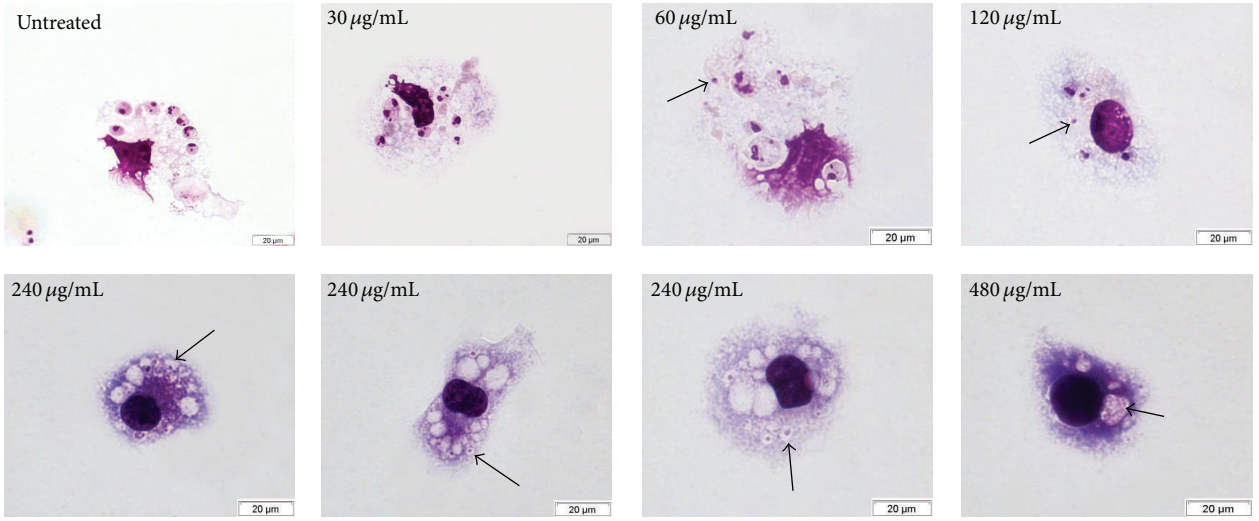


Figure 3: Light microscopy of BALB/c peritoneal macrophages infected with *Leishmania amazonensis* and treated with *Morinda citrifolia* fruit juice. Arrows indicate vacuoles with probable remains of intracellular amastigotes.

This colorimetric method is based on the quantification of total protein by binding anionic Sulforhodamine B electrostatic crystals with cellular proteins. No cytotoxicity was observed at the concentrations analyzed and the selectivity index for *M. citrifolia* fruit juice was at least 3.3-fold higher than amphotericin B (Table 1).

Macrophages were used to assess the toxicity *in vitro* for being target cells of infection by *Leishmania*. The analysis of cytotoxicity against macrophages J774.G8 shows the low cytotoxicity of *M. citrifolia* fruit juice. This data becomes more relevant when analyzed together with IC_{50} to intracellular amastigote forms, generating SI higher than 9.9 that falls within the generic hit selection criteria of SI to new compounds for infectious diseases from Japanese Global Health Innovative Technology [19]. Indeed, as the generic hit criteria must be applied to phytotherapy with some reservations, the selective index is the most reliable criterion to assess the safety of extracts, essential oils, or others natural products. Besides, the leishmanicidal activity of *M. citrifolia* must be analyzed in addition to immunomodulatory effects and toxicity in posterior studies.

The transmission electron microscopy analysis of *L. infantum* promastigotes treated with the *M. citrifolia* fruit juice was performed to determine the ultrastructural changes. Photomicrographs of promastigotes (Figures 4, 5, 6, and 7) showed the degree of damage after 24 hours of treatment. The parasites without treatment showed normal morphology (Figure 4(a)).

The observation of *L. infantum* promastigotes treated with 30 μg/mL of juice showed vacuolization of the cytoplasm, some with electron-dense regions inside, and this became more evident at higher concentrations (Figures 4(b) and 4(c)). Similar structural changes have also been described in *L. amazonensis* treated with essential oils [20]. In these, the vacuoles are associated with entry of substances by simple diffusion, caused by increased permeability of the membrane due to the compounds in the essential oils.

Vesicles in the flagellar pocket were observed in promastigotes treated with 30 and 60 μg/mL of *M. citrifolia* fruit juice (Figures 4(d), 5(b), and 5(c)). The presence of vesicles in flagellar pockets indicates an intense exocytic activity in the region of the flagellar pocket. These changes have also been reported in promastigotes of *L. amazonensis* treated with inhibitors of ergosterol synthesis, such as 22,26-azasterol [21]. The increased activity in the region of the exocytic flagellar pocket may be the result of an abnormal secretion of lipids, which accumulate as a consequence of drug action or indicate an exacerbated production of proteins by cells in an attempt to survive [22].

Membrane structures in the cytoplasm were observed in treatments with 30, 60, 120, and 240 μg/mL of *M. citrifolia* fruit juice (Figures 4(b)–4(d), 5(a), 6(a) and 6(b)). These structures are membranes of the endoplasmic reticulum dispersed in the cytoplasm and are probably involved in the recycling of abnormal organelles. The presence of autophagosome-like vesicle material in promastigotes treated with 240 μg/mL of *M. citrifolia* fruit juice shows an autophagic process (Figures 6(c) and 6(d)), suggesting a remodeling of organelles irreversibly damaged by the treatment. Autophagy can serve as a protective mechanism by recycling macromolecules and removing damaged organelles, but excessive autophagy can result in cell death [23]. To try to survive the effects caused by *M. citrifolia* fruit juice treatment, parasites may react triggering autophagic events, and this exacerbated autophagic response could lead to death, as observed in parasites treated with the 480 μg/mL of juice.

The treatment with 480 μg/mL of *M. citrifolia* fruit juice for 24 hours induced severe cellular damage (Figures 7(a) and 7(b)), with the extravasation of cytoplasmic contents and loss of cellular integrity. The progression of ultrastructural changes is related to increasing drug concentrations, reaching its apex with parasite destruction at higher drug concentrations, and showing the direct action of *M. citrifolia*

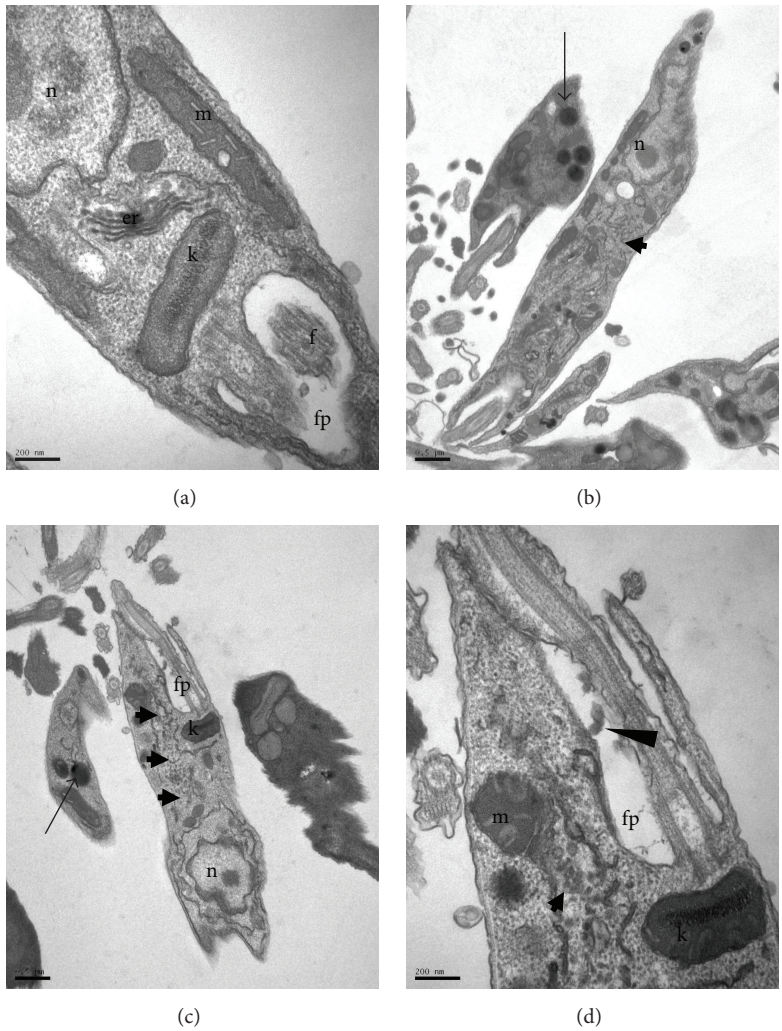


Figure 4: Ultrastructure of *Leishmania infantum* promastigotes incubated for 24 hours, at 26°C, with *Morinda citrifolia* fruit juice. (a) Control. (b–d) Promastigote treated with 30 μg/mL. (b-c) Electron-dense vesicles (arrows) and granular material throughout the cytoplasm (block arrows). (d) Membranes in flagellar pocket (arrowhead). k: kinetoplast, m: mitochondria, n: nucleus, pf: flagellar pocket, f: flagellum, and er: endoplasmic reticulum.

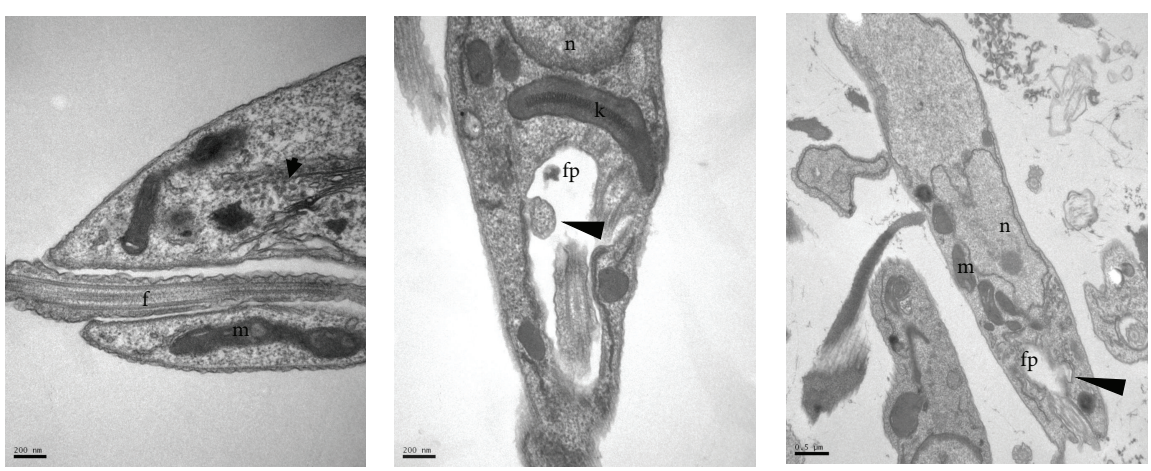
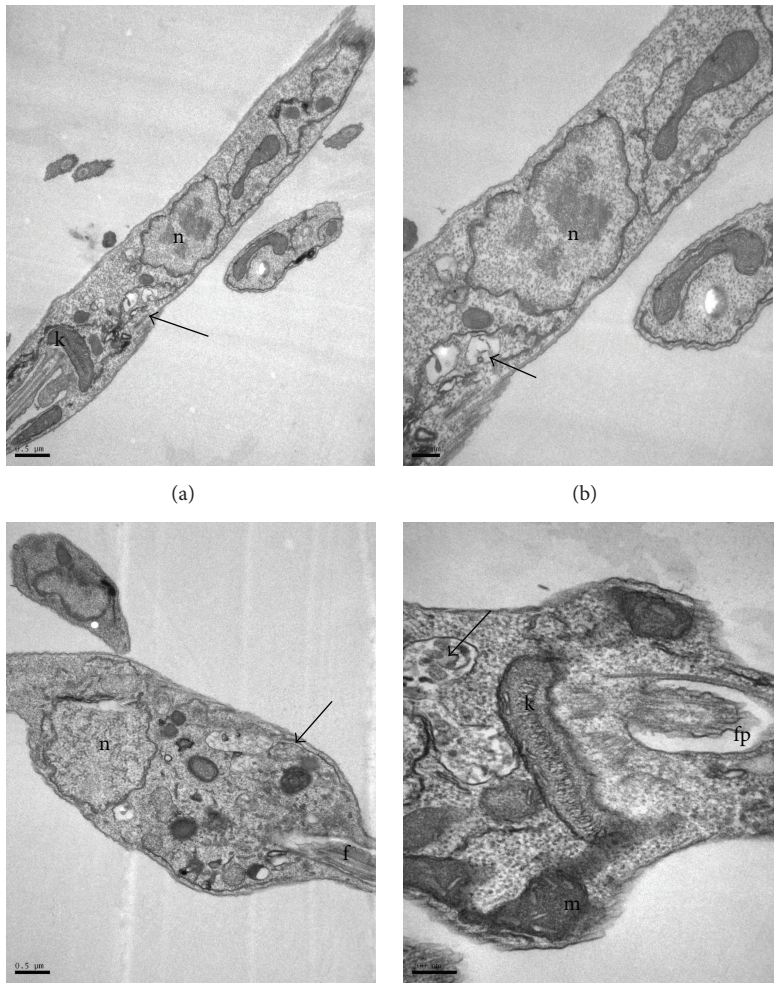


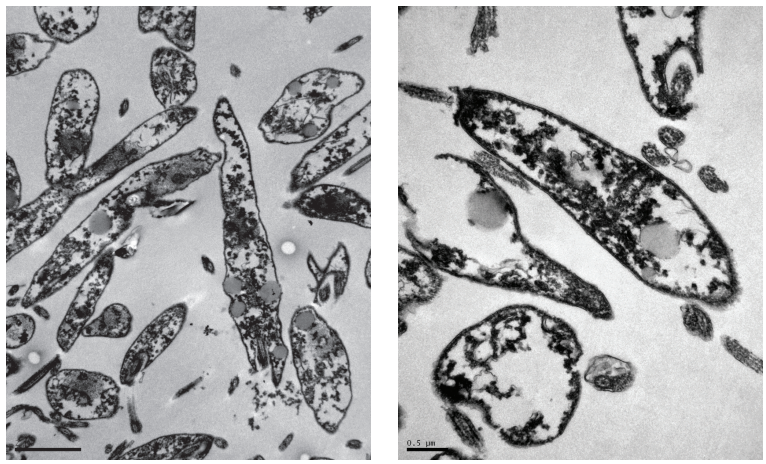
Figure 5: Ultrastructure of *Leishmania infantum* promastigotes incubated for 24 hours, at 26°C, with *Morinda citrifolia* fruit juice. (a–c) Promastigote treated with 60 μg/mL. Electron-dense vesicles (arrows) and granular material throughout the cytoplasm (block arrows). Vesicles breaking up in flagellar pocket (arrowhead). k: kinetoplast, m: mitochondria, n: nucleus, pf: flagellar pocket, and f: flagellum.



(a) (b)

(c) (d)

Figure 6: Ultrastructure of *Leishmania infantum* promastigotes incubated for 24 hours, at 26°C, with *Morinda citrifolia* fruit juice. (a-b) Promastigotes treated with 120 μg/mL. (c-d) Promastigotes treated with 240 μg/mL. Vesicles with granular material throughout the cytoplasm (arrows). Vesicles with autophagosome-like material (asterisks). k: kinetoplast, m: mitochondria, n: nucleus, pf: flagellar pocket, and f: flagellum.



(a) (b)

Figure 7: Ultrastructure of *Leishmania infantum* promastigotes incubated for 24 hours, at 26°C, with 480 μg/mL of *Morinda citrifolia* fruit juice. (a-b) Promastigotes with loss of membrane integrity.

fruit juice on the parasite and its dose-dependent action. No changes were observed in the nucleus, the mitochondria, the flagellum, the kinetoplast, or the subpellicular microtubules.

4. Conclusion

M. citrifolia fruit juice showed leishmanicidal activity against *L. infantum* promastigote, causing ultrastructural changes such as cytoplasmic vacuolization, lipid inclusion, increased exocytosis activity, autophagosome-like vesicles, loss of cellular integrity, and death of the parasite. Considering the activity and the alterations observed against promastigote forms of *L. infantum*, further studies must be conducted to evaluate the potential of *M. citrifolia* fruit juice in leishmaniasis treatment.

Disclosure

Ana Lúcia Abreu-Silva (CNPq no. 306218/2010-0) is senior researcher.

Competing Interests

The authors report no conflict of interests.

Authors' Contributions

Kátia da Silva Calabrese and Ana Lúcia Abreu-Silva contributed equally to this work.

Acknowledgments

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Research Article

Chamaecyparis obtusa Suppresses Virulence Genes in *Streptococcus mutans*

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Chamaecyparis obtusa (C. obtusa) is known to have antimicrobial effects and has been used as a medicinal plant and in forest bathing. This study aimed to evaluate the anticariogenic activity of essential oil of *C. obtusa* on *Streptococcus mutans*, which is one of the most important bacterial causes of dental caries and dental biofilm formation. Essential oil from *C. obtusa* was extracted, and its effect on bacterial growth, acid production, and biofilm formation was evaluated. *C. obtusa* essential oil exhibited concentration-dependent inhibition of bacterial growth over 0.025 mg/mL, with 99% inhibition at a concentration of 0.2 mg/mL. The bacterial biofilm formation and acid production were also significantly inhibited at the concentration greater than 0.025 mg/mL. The result of LIVE/DEAD® BacLight™ Bacterial Viability Kit showed a concentration-dependent bactericidal effect on *S. mutans* and almost all bacteria were dead over 0.8 mg/mL. Real-time PCR analysis showed that gene expression of some virulence factors such as *brpA, gbpB, gtfC,* and *gtfD* was also inhibited. In GC and GC-MS analysis, the major components were found to be α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%). These results show that *C. obtusa* essential oil has anticariogenic effect on *S. mutans*.

1. Introduction

Dental caries is the most common infectious oral disease that has afflicted humans including children and adolescents [1]. It is a multifactorial disease, which is caused by detrimental changes in bacterial ecology due to formation of a biofilm that adheres to the tooth surface [2]. During the past few decades, many reports worldwide showed an overall decreasing trend of dental caries. However, recent studies have reported an alarming increase in caries prevalence, especially among the underprivileged groups [3].

S. mutans can colonize the oral cavity and form bacterial biofilm. It has the ability to survive in an acidic environment and interact with other microorganisms colonizing this ecosystem [2].

Caries results from an imbalance between demineralization and remineralization of tooth structure. Acidogenic bacteria ferment dietary carbohydrates, thereby producing organic acids, which initiate dissolution of tooth enamel and breakdown of dental tissue [4]. The extent of the pH fall is influenced by numerous factors, including the composition of the microflora, as well as the type and frequency of sugar intake [5].

S. mutans produce glucosyltransferase (GTF) enzyme which is recognized as virulence factors in the etiology of dental caries. GTF enzymes synthesize extracellular glucans

and contribute significantly to the dental plaque matrix's polysaccharide formation [6]. The sucrose-dependent mechanism of plaque formation is based on GTF produced by *S. mutans* in combination with glucan-binding proteins (GBPs). The synthesized glucans provide the possibility of both bacterial adhesion to the tooth enamel and adhesion of the microorganisms to each other [2].

Demineralization can be reversed by calcium and phosphate, together with fluoride, diffusing into the tooth and depositing a new veneer on the crystal remnants in the noncavitated lesion, and is known as remineralization [4]. Fluoride has been used as the "first choice" for the prevention of dental caries [7], and other anticariogenic natural products or compounds like xylitol have also been introduced [8].

C. obtusa is a tropical tree species found in Japan and the southern region of South Korea, and essential oil is extracted from leaves and twigs of the *C. obtusa* tree. The essential oil has several types of terpenes and has been commercially used in soaps, toothpaste, and cosmetics as a functional additive [9]. The essential oil of *C. obtusa* is a concentrated hydrophobic liquid containing volatile compound with natural antibiotic properties that protect against harmful insects, animals, and microorganisms. Inhalation of this essential oil is known as *C. obtusa* aromatherapy or *C. obtusa* forest bathing [10] and has been shown to exert antibacterial and antifungal effects [11].

This study was performed to analyze anticariogenic effect of *C. obtusa* on *S. mutans* and to determine its chemical composition using a gas chromatography (GC)/gas chromatography-mass spectrometry (GC-MS) analysis.

2. Materials and Methods

2.1. Plant Material and Essential Oil. *C. obtusa* was collected in October 2013 from the Jeollanam-do province, South Korea. Fresh leaves and twigs of *C. obtusa* (1 kg) were ground mechanically and hydrodistilled for 3 hours using a Clevenger-type apparatus. The yield of the *C. obtusa* essential oil was 1.08% of yellow pale oil, based on the fresh weight of the plant. The *C. obtusa* essential oil was stored in a deep freezer (−70°C) to minimize the loss of volatile compounds.

2.2. Inhibition of Bacterial Growth. *S. mutans* (ATCC 25175) was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in brain heart infusion (BHI; Difco, Detroit, MI, USA) broth under aerobic condition at 37°C. The growth of *S. mutans* was examined at 37°C in 0.95 mL of brain heart infusion broth containing various concentrations of the *C. obtusa*. These tubes were inoculated with 0.05 mL of an overnight culture grown in the BHI broth (final: 5×10^5 colony-forming units [CFU]/mL) and incubated at 37°C for 24 h. The optical density (OD) of cells was measured at 550 nm using a spectrophotometer. Each concentration of the extract was tested in triplicate.

2.3. Acid Production. Acid production by *S. mutans* was examined to evaluate the effect of *C. obtusa* essential oil using a modification of previously described method [12]. The *C. obtusa* essential oil was filter-sterilized using membrane filter with 0.2 μm pore size and added to 0.95 mL of the phenol red broth containing 1% glucose, which was then inoculated with 0.05 mL of the seed culture of *S. mutans*. After 24 h of cultivation, the pH of the cultures was determined using a pH meter (Corning, Inc., Corning, NY, USA). Three replicates were measured for each concentration of the test extract.

2.4. Biofilm Formation Assay. The biofilm assay was based on a previously described method [13, 14]. Biofilm formation was measured by staining with safranin and observed by scanning electron microscopy (SEM). *C. obtusa* essential oil was added to BHI broth containing 0.1% sucrose in 35 mm polystyrene dishes. The cultures were then inoculated with a seed culture of *S. mutans* (final: 5×10^5 CFU/mL) and incubated for 24 h at 37°C. After incubation, the supernatants were removed, and the dishes were rinsed with distilled water. Biofilm formation were stained with 0.1% safranin and photographed. The bound safranin was released from the stained cells with 30% acetic acid and the absorbance of the solution was measured at 530 nm. In addition, biofilm on 35 mm polystyrene dishes was observed by SEM [15]. The biofilm formed on the dishes was rinsed with distilled water, fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 24 h, and dehydrated with ethanol gradient series (60%, 70%, 80%, 90%, 95%, and 100%). Then, the samples were freeze-dried, sputter-coated with gold (108A sputter coater; Cressington Scientific Instruments, Inc., Watford, England, United Kingdom), and observed by SEM (JSM-6360, JEOL, Tokyo, Japan).

2.5. Confocal Laser Scanning Microscopy. To determine the bactericidal effect of *C. obtusa* essential oil on *S. mutans*, staining with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit was performed and examined by confocal laser scanning microscopy. The cultured *S. mutans* in BHI was diluted using BHI media to 1×10^7 CFU/mL and treated with the essential oil at 37°C under aerobic conditions. After 30 min of incubation, the bacteria were washed with PBS and stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA), prepared according to the manufacturer's protocol. After 15 min of staining, bacteria were observed using confocal laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany).

2.6. Real-Time Polymerase Chain Reaction Analysis. A real-time PCR was performed to examine the effect of *C. obtusa* essential oil on virulence factor gene expression of *S. mutans*. The subminimal inhibitory concentration (0.025–0.1 mg/mL) of the essential oil was used. After 24 h of culture, total RNA was isolated from *S. mutans* using a TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized. The amplification was performed using a StepOnePlus Real-Time PCR system with SYBR® Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). 16S rRNA was used as an internal control.

2.7. GC and GC-MS Analysis. GC analysis was performed on a Hewlett-Packard model 6890 series gas chromatograph with a flame ionization detector (FID) and a split ratio of 30 : 1 using DB-5HT fused silica capillary columns (30 m × 0.32 mm, i.d., 0.10 μm film thickness). The temperature of column was programmed from 40°C to 230°C, at 2°C/min and then kept constant at 230°C for 20 min. The injector and detector temperatures were 230°C and 250°C, respectively. The gas carrier used was nitrogen, at a flow rate of 0.80 mL/min. Peak areas were measured by electronic integration and the relative amounts of the individual components were determined based on the peak areas. The GC-MS analysis was carried out on an Agilent Technologies 7890A GC and 5975C mass selective detector (MSD) operating in EI mode at 70 eV, fitted with a DB-5MS fused silica capillary column (30 m × 0.25 mm, i.d., 0.25 μm film thickness). The column temperature was programmed from 40°C to 230°C at 2°C/min and then kept constant at 230°C for 20 min. The injector and ion source temperatures were 250°C, respectively. The gas carrier was helium at a flow rate of 1.0 mL/min. The identification of individual components was based on comparisons with Wiley 7n/NIST 05 mass spectra libraries and retention indices with reference to literature data. Linear retention indices were calculated against those of n-paraffin (C6–C26) series [16].

2.8. Statistical Analysis. All experiments were performed in triplicate. Data were analyzed using SPSS software 12.0 (Chicago, IL, USA). The data are expressed as the mean ± standard deviation values. The statistical analysis was done using Student's t-test. Values of $p < 0.01$ were considered statistically significant.

3. Results

3.1. Inhibition of Bacterial Growth. After extraction of *C. obtusa* essential oil by hydrodistillation, the antibacterial activity of the oil was tested against *S. mutans*. *C. obtusa* essential oil significantly inhibited the growth of *S. mutans* in a concentration-dependent manner. The bacteria were treated with 0.025, 0.05, 0.1, and 0.2 mg/mL of *C. obtusa* essential oil. When treated with 0.025 mg/mL of the essential oil, the bacterial growth was significantly inhibited in comparison to the control group ($p < 0.05$). The positive control (0.1% NaF) also showed antibacterial activity (Figure 1).

3.2. Inhibition of Acid Production. To investigate whether *C. obtusa* essential oil can inhibit *S. mutans* organic acid production, the bacteria were cultured in the presence of various concentrations (0.025–0.2 mg/mL) of the essential oil and the change in pH was measured. The pH of the control declined to 5.30 after bacterial culture, while the initial pH of the media before bacterial culture was 7.40. However, the addition of 0.025, 0.05, 0.1, and 0.2 mg/mL of *C. obtusa* essential oil resulted in pH levels of 5.38, 5.73, 7.12, and 7.40, respectively. These results indicate that *C. obtusa* essential oil may inhibit the organic acid production by *S. mutans*.

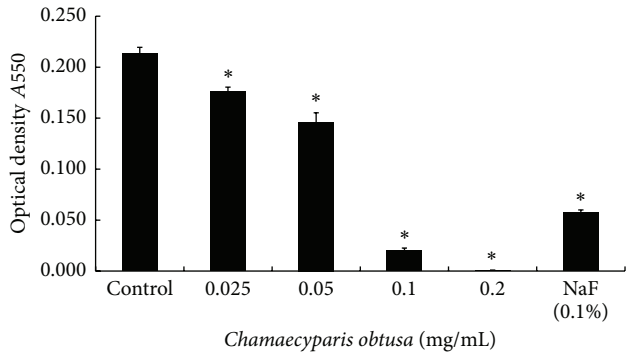

Figure 1: Bacterial growth inhibition of *Streptococcus mutans* (*S. mutans*) by *Chamaecyparis obtusa* (*C. obtusa*) essential oil. The optical density (*A*550) was read using a spectrophotometer. $^*p < 0.01$ compared to the control group.

Table 1: Inhibitory effect of *C. obtusa* essential oil on acid production by *S. mutans*.

Con. (mg/mL)	pH (before incubation)	pH (after incubation)
Control	7.40 ± 0.00	5.30 ± 0.00
0.025	7.40 ± 0.00	5.38 ± 0.01*
0.05	7.39 ± 0.00	5.73 ± 0.00*
0.1	7.40 ± 0.00	7.12 ± 0.00*
0.2	7.40 ± 0.00	7.40 ± 0.00*
0.1% NaF	7.40 ± 0.00	7.10 ± 0.00*

Data (pH) are represented as mean ± standard deviation.
$^*p < 0.01$ when compared with the control group after incubation.

NaF (0.1%) used for the positive control also inhibited acid production, resulting in a pH of 7.10 (Table 1).

3.3. Inhibition of Biofilm Formation. To determine whether *C. obtusa* essential oil inhibits biofilm formation by *S. mutans*, the bacteria were cultured in the presence of various concentration of *C. obtusa* essential oil in polystyrene dishes. Biofilm formation was studied using safranin staining, and absorbance was measured at 530 nm. The biofilm formation by *S. mutans* was significantly inhibited by treatment with *C. obtusa* essential oil in a dose-dependent manner over 0.025 mg/mL of *C. obtusa* essential oil. When treated with 0.1% NaF (positive control), complete inhibition was shown (Figure 2). SEM results were consistent with those of safranin staining (Figure 3).

3.4. Bactericidal Effect. To evaluate bactericidal effect of *C. obtusa* essential oil, *S. mutans* were cultured in presence of high concentrations (0.2–1.6 mg/mL) of the essential oil, stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit and observed using confocal laser scanning microscopy. Treatment with *C. obtusa* essential oil decreases living bacteria (green fluorescence labeled cell stained by SYTO® 9) and increases dead bacteria (red fluorescence labeled cell stained by PI). The bactericidal effect of *C. obtusa* essential oil was also observed in a dose-dependent manner (Figure 4).

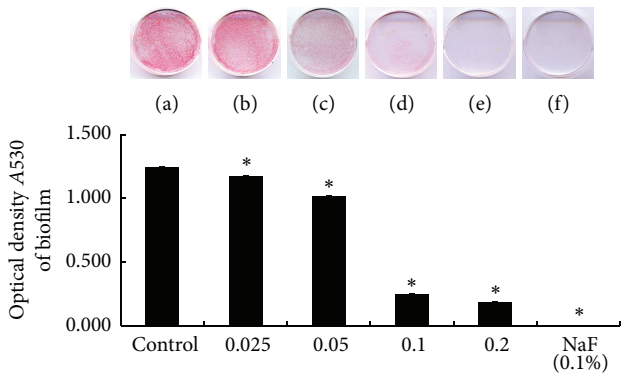

FIGURE 2: Inhibitory effect of *C. obtusa* essential oil on biofilm formation by *S. mutans*, as observed by safranin staining. (a) Control; (b) 0.025 mg/mL; (c) 0.05 mg/mL; (d) 0.1 mg/mL; (e) 0.2 mg/mL essential oil of *C. obtusa*; and (f) positive control (0.1% NaF). *Significance was determined at $^{*}P < 0.01$ compared to the control group.

However, low concentration (lesser than 0.2 mg/mL) of the essential oil did not show bactericidal effect (data not shown).

3.5. Inhibition of Virulence Factor Gene Expression. To evaluate the influence of *C. obtusa* on the gene expression of virulence factors in *S. mutans*, the bacteria were treated with subminimal inhibitory concentration (0.025–0.1 mg/mL) of *C. obtusa*, and the gene expressions of various virulence factors were assessed by real-time PCR. We evaluated the genetic expressions of *brpA*, *gbpB*, *gtfC*, and *gtfD*. The expressions of *brpA*, which contribute to the control of the phosphotransferase system (PTS) and are related with acid tolerance, and *gbpB* which contribute to bacterial adherence, were significantly decreased at 0.025 mg/mL of *C. obtusa*. The expression of *gtfC* and *gtfD* which encode GTFase C and D proteins was significantly decreased when *S. mutans* was treated with 0.025 mg/mL of *C. obtusa* (Figure 5).

3.6. The Composition of C. obtusa Essential Oil by GC and GC-MS Analysis. Fifty-nine constituents were identified in the *C. obtusa* essential oil, accounting for up to 97.38% of the total material. Thus, all unidentified compounds were only minor components. The major components included α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%) (Table 2).

4. Discussion

Fluoride plays an important role in the prevention and control of dental caries. However, an unfortunate side effect of fluoride is fluorosis. Ingestion of fluoride before 2 to 3 years of age is considered critical for possible fluorosis in the permanent dentition [17]. Therefore, natural products are currently receiving special attention as a good alternative to synthetic chemical substances for the prevention of dental caries [18].

Essential oils are volatile components mainly obtained by distillation of plant and consist of a mixture of various terpenoids. Terpenes are the therapeutic chemical substances present in medicinal plants [19]. Essential oils extracted from various plants are known to have antimicrobial activity [20]. Some natural derivatives like extracts from *Myristica fragrans*, *Lippia sidoides*, *Hyptis pectinata*, *Curcuma longa*, and *Baccharis dracunculifolia* have been proved to be effective against *S. mutans* [21–25]. This study was performed to evaluate anticariogenic activity of *C. obtusa* essential oil on *S. mutans*.

To evaluate anticariogenic properties of *C. obtusa* essential oil, *S. mutans* was used because these bacteria is considered as a major cause for the formation of dental caries [2]. Our results showed that growth of *S. mutans* was suppressed by treatment with *C. obtusa* essential oil. Furthermore, the results of LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit also showed that *C. obtusa* essential oil has a bactericidal effect against *S. mutans*. These results suggested that *C. obtusa* essential oil has a potential for anticariogenic effect, which is interesting since the inhibition of the growth of *S. mutans* is one of the strategies for prevention of dental caries.

Growth of *S. mutans* was suppressed by treatment with *C. obtusa* essential oil in a concentration-dependent manner above the concentration of 0.025 mg/mL. In dental plaque formation, pH is one of the major factors, since low pH leads to demineralized hydroxyapatite and favors the cariogenicity [26]. *S. mutans* can metabolize dietary sugars and produce organic acid. Low-pH environment in the biofilm matrix results in dissolution of enamel, thus initiating the pathogenesis of dental caries. Therefore, the alteration of pH is used as an indicator to determine the effect of anticariogenic agents [27]. In this study, *C. obtusa* essential oil inhibited the decrease of pH induced by *S. mutans* and the result suggests that *C. obtusa* essential oil may inhibit dental caries through inhibition of acid production by *S. mutans*.

Biofilms are communities of microorganisms that adhere to biological or abiotic substrata and produce an extracellular matrix typically comprising of polysaccharides and proteins. Dental plaque is a kind of biofilm found on a tooth surface, embedded in a matrix of host and bacterial polymers [28]. In biofilm, known as plaque in the oral cavity, the interaction of specific bacteria with constituents of the diet results in caries [27]. Biofilm formation by *S. mutans* was also inhibited by treatment with *C. obtusa* essential oil cultured on polystyrene dishes. These results suggested that *C. obtusa* essential oil directly inhibits the biofilm formation by *S. mutans*.

Furthermore, several virulence gene factors of *S. mutans* are associated with various aspects of cariogenicity such as acid tolerance, bacterial adhesion, and biofilm formation [2]. The *brpA* has been shown to contribute to biofilm formation and plays a major role in cell envelope biogenesis/homeostasis and regulation of stress response as well as in acid tolerance [29, 30]. The g*bpB*, which encodes surface-associated glucan-binding protein (GBP), mediates binding of bacteria to glucans and enables development of biofilm. The g*tfC* and g*tfD* encode glucosyltransferases (GTFase) which are essential virulence factor in plaque development and are responsible for glucans formation from sucrose [2].

Table 2: Gas chromatography and gas chromatography-mass spectrometry (GC/GC-MS) analysis of the essential oil isolated from *C. obtusa*.

Retention time (min)	Retention index[a]	Compound	Peak area (%)
8.849	801	n-Hexanal	0.18
11.982	852	*trans*-2-Hexenal	0.28
13.110	870	n-Hexanol	0.06
15.125	902	Bornylene	0.12
16.419	919	Tricyclene	0.77
16.763	925	α-Thujene	0.12
17.742	936	α-Pinene	11.38
18.650	952	Camphene	2.87
20.228	975	Sabinene	0.46
21.345	980	δ-3-Carene	0.37
22.047	983	β-Pinene	7.22
24.483	1023	α-Terpinene	40.60
24.927	1029	Limonene	0.57
25.474	1036	β-Phellandrene	3.45
27.317	1061	γ-Terpinene	0.21
28.393	1072	*cis*-Sabinene hydrate	0.35
29.742	1089	α-Terpinolene	3.40
29.983	1092	Dehydro-*p*-cymene	0.53
30.874	1102	*trans*-Sabinene hydrate	0.09
31.769	1115	1-Octen-3-yl acetate	0.06
33.199	1133	*trans*-*p*-2-Menthen-1-ol	0.05
36.312	1173	Cryptone	0.11
36.461	1175	p-Cymen-8-ol	0.10
36.965	1181	Terpinen-4-ol	0.23
37.889	1193	α-Terpineol	0.11
38.960	1207	Verbenone	0.09
39.699	1217	Fenchyl acetate	0.07
42.535	1256	Linalyl acetate	0.08
45.552	1296	Bornyl acetate	12.45
49.502	1352	α-Terpinyl acetate	0.62
51.422	1380	α-Copaene	0.28
53.780	1414	α-Cedrene	0.52
54.015	1417	β-Caryophyllene	0.16
54.331	1422	β-Cedrene	0.25
55.009	1431	*cis*-Thujopsene	0.07
55.419	1434	β-Gurjunene	0.06
56.378	1453	α-Humulene	0.37
56.976	1462	*cis*-Muurola-4(14),5-diene	0.24
57.656	1472	β-Cadinene	0.06
57.927	1476	Germacrene D	0.13
58.150	1490	β-Himachalene	0.10
59.003	1492	α-Muurolene	0.16
59.519	1497	α-Chamigrene	0.05
60.024	1508	γ-Cadinene	0.38
61.079	1525	δ-Cadinene	0.15
61.522	1530	Cadina-1,4-diene	0.09
61.937	1539	Selina-3,7(11)-diene	0.07
63.011	1551	Elemol	0.07
63.406	1562	*trans*-Nerolidol	0.25
64.651	1578	Spathulenol	0.05
64.916	1586	Caryophyllene oxide	0.10
66.435	1603	Cedrol	0.97
67.079	1622	1-*epi*-Cubenol	0.08
67.835	1635	γ-Eudesmol	0.58
68.123	1239	τ-Cadinol	0.57
68.964	1648	α-Cadinol	0.09
69.973	1670	Bulnesol	0.07
70.829	1685	α-Bisabolol	0.13
82.676	1898	Rimuene	0.27
84.836	1926	Hibaene	2.33
85.085	1945	Pimaradiene	0.10
86.747	1977	13-Isopimaradiene	1.42
88.068	1998	Dolabradiene	0.09
90.446	2051	Dehydroabietane	0.07
Total			97.38

[a]Retention index on DB-5HT column.

In this study, to evaluate correlation between inhibitory effect by *C. obtusa* essential oil and virulence factors expression, we determined the mRNA expression level of several virulence factors using a real-time PCR analysis. We evaluated the gene expression level of *brpA*, *gbpB*, *gtfC*, and *gtfD*. *C. obtusa* essential oil significantly inhibited the transcription level of *brpA*, *gbpB*, *gtfC*, and *gtfD*.

Based on our results of GC/GC-MS analysis, the major components included α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%) (Table 2). Although the biological activities of *C. obtusa* essential oil are not yet fully understood, some previous study reported that several types of terpenes of *C. obtusa* have been shown to exert antibacterial and antifungal effect [11]. Recent studies also reported other beneficial properties of *C. obtusa*, such as pharmacological activities for the treatment of atopic dermatitis [31], improvement on cognitive function of the central nervous system on rat experimental model [10], and promotion of hair growth [32]. In addition, *C. obtusa* has antinociceptive and anti-inflammatory properties, which increases its applicability in oral care [9, 33].

Based on our results, we conclude that *C. obtusa* may have a possible practical use against the cariogenic bacteria within the mouth and recommend further investigation of *C. obtusa* on periodontopathic and cariogenic bacteria.

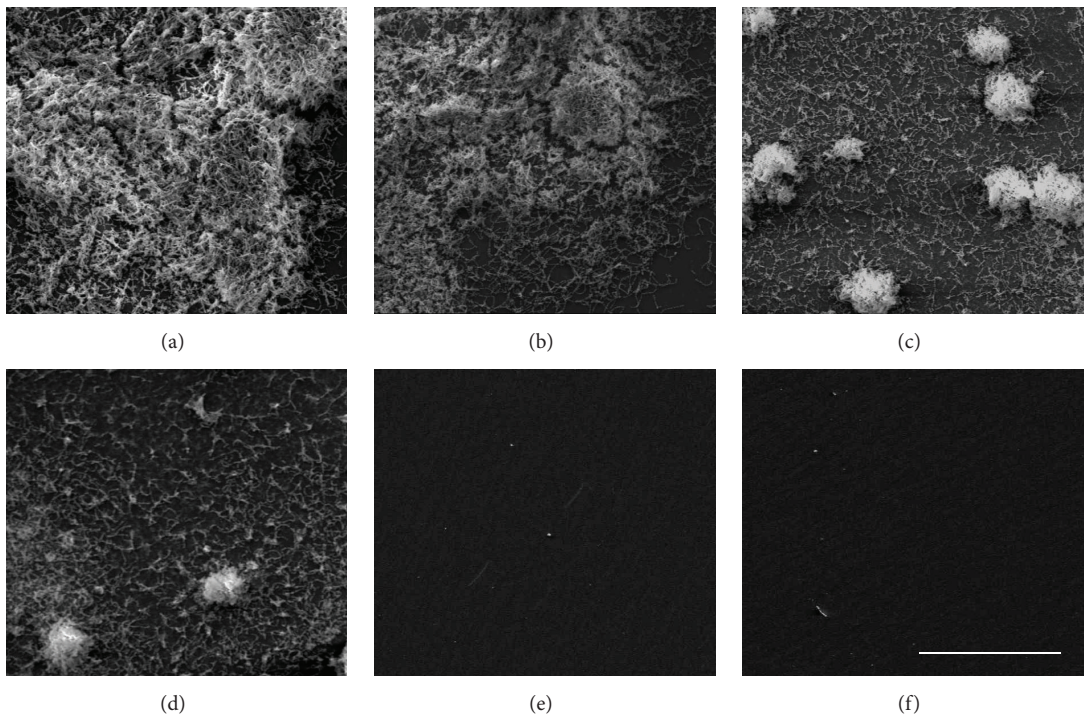

Figure 3: Inhibitory effect of *C. obtusa* essential oil on biofilm formation by *S. mutans*, as observed by scanning electron microscopy. (a) Control; (b) 0.025 mg/mL; (c) 0.05 mg/L; (D) 0.1 mg/mL; (e) 0.2 mg/mL; and (f) positive control (0.1% NaF). Scale bar = 10 μm.

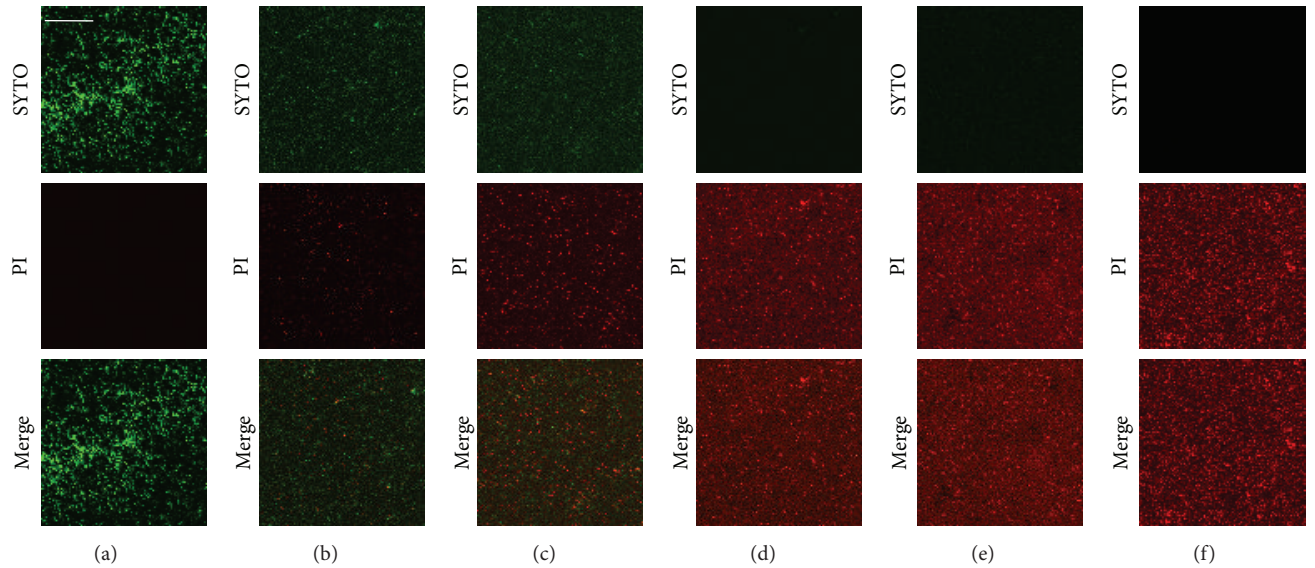

Figure 4: Bactericidal effect of *C. obtusa* essential oil. Cultured *S. mutans* was treated with *C. obtusa* extract (0.2–1.6 mg/mL) and stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. The stained bacteria were observed by confocal laser scanning microscopy. Treatment with *C. obtusa* decreased green-labeled living bacteria (SYTO® 9 stain) and increased red-labeled dead bacteria (PI stain) in a dose-dependent manner. (a) Control; (b) 0.2 mg/mL; (c) 0.4 mg/mL; (d) 0.8 mg/mL; (e) 1.6 mg/mL; and (f) positive control (0.1% NaF). Bar = 100 μm.

5. Conclusions

This study has proved that *C. obtusa* essential oil exhibited significant inhibition of bacterial growth, acid production, and biofilm formation by *S. mutans*. Also *C. obtusa* essential oil showed bactericidal effect. Furthermore, *C. obtusa* essential oil also inhibited the transcription level of several virulence factors such as *brpA*, *gbpB*, *gtfC*, and *gtfD* of *S. mutans*. In GC and GC-MS analysis, the major components were α-terpinene (40.60%), bornyl acetate (12.45%), α-pinene (11.38%), β-pinene (7.22%), β-phellandrene (3.45%), and α-terpinolene (3.40%). Therefore, the results of this study

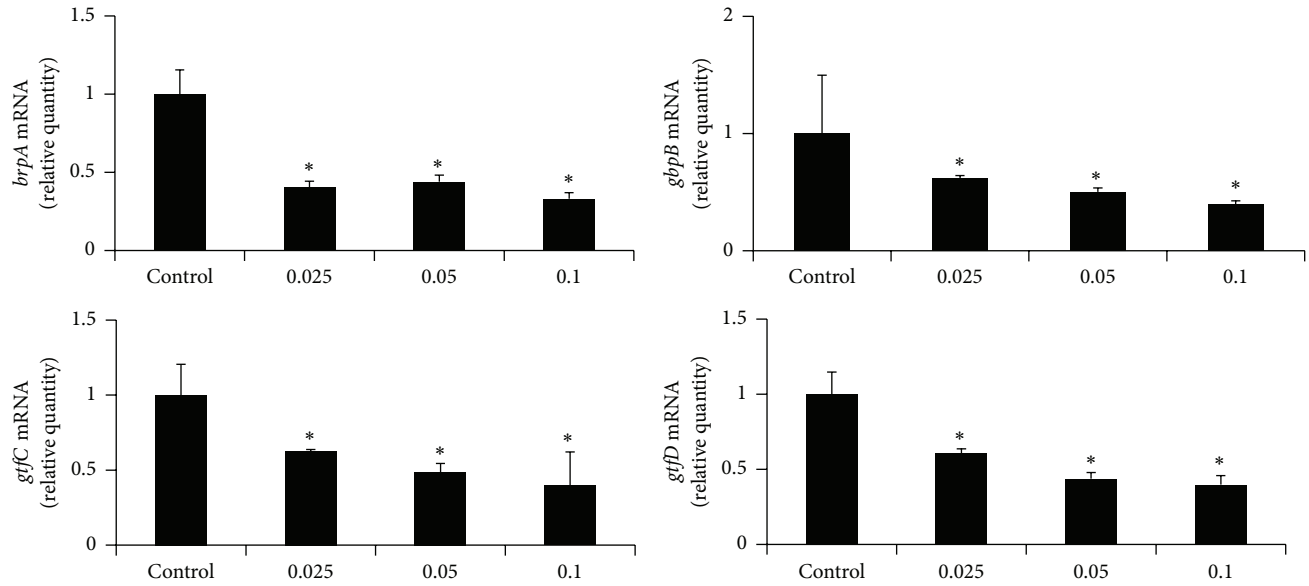

Figure 5: Inhibitory effect of *C. obtusa* essential oil on virulence factor gene expression. *S. mutans* was cultured and treated with subminimal inhibitory concentration (0.025–0.1 mg/mL) of *C. obtusa* extract, and real-time polymerase chain reaction (PCR) analysis was performed. The *brpA, gbpB, gtfC,* and *gtfD* expressions were significantly inhibited at 0.025 mg/mL of *C. obtusa*. Each value is expressed as mean ± standard deviation. *Significance was determined at $^{*}P < 0.01$ when compared with the control group.

indicate that *C. obtusa* essential oil showed good anticariogenic effect on *S. mutans* and appear to be a promising new agent that may prevent dental caries. Further studies are needed to develop the new medicine for clinical use.

Competing Interests

The authors declare that there are no competing interests.

Acknowledgments

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Research Article

Composition Analysis and Inhibitory Effect of *Sterculia lychnophora* against Biofilm Formation by *Streptococcus mutans*

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Pangdahai is a traditional Chinese drug, specifically described in the Chinese Pharmacopoeia as the seeds of *Sterculia lychnophora* Hance. Here, we separated *S. lychnophora* husk and kernel, analyzed the nutrient contents, and investigated the inhibitory effects of *S. lychnophora* ethanol extracts on cariogenic properties of *Streptococcus mutans*, important bacteria in dental caries and plaque formation. Ethanol extracts of *S. lychnophora* showed dose-dependent antibacterial activity against *S. mutans* with significant inhibition at concentrations higher than 0.01 mg/mL compared with the control group ($p < 0.05$). Furthermore, biofilm formation was decreased by *S. lychnophora* at concentrations > 0.03 mg/mL, while bacterial viability was decreased dose-dependently at high concentrations (0.04, 0.08, 0.16, and 0.32 mg/mL). Preliminary phytochemical analysis of the ethanol extract revealed a strong presence of alkaloid, phenolics, glycosides, and peptides while the presence of steroids, terpenoids, flavonoids, and organic acids was low. The *S. lychnophora* husk had higher moisture and ash content than the kernel, while the protein and fat content of the husk were lower ($p < 0.05$) than those of the kernel. These results indicate that *S. lychnophora* may have antibacterial effects against *S. mutans*, which are likely related to the alkaloid, phenolics, glycosides, and peptides, the major components of *S. lychnophora*.

1. Introduction

The Chinese traditional medicine Pangdahai consists of the dried mature seeds of *Sterculia lychnophora* Hance, a deciduous tree that belongs to the Sterculiaceae family. The trees grow mainly in tropical zones including Vietnam, India, Malaysia, Thailand, Indonesia, Gwangdong, and the Hainan Island in China [1]. The immature seed has an oval shape and looks similar to an olive while the mature seed has a length and approximate width of 2–2.5 and 1.2–1.7 cm, respectively, and weighs about 2 g (Figure 1). The outer layer of the seed is very thin and brittle, and when soaked in water its exposed interior is yellowish brown with a spongy mucus rich consistency [2].

In the dictionary of traditional Chinese medicine, *S. lychnophora* is odorless, has a viscous consistency when chewed at length, is cool or cold in nature, and has a slightly sweet or bittersweet taste. It has been used to treat the pharyngitis, constipation, and tussis in most cases [3, 4]. In China, *S. lychnophora* is commonly boiled or soaked in hot water and consumed as beverage for the treatment of sore throat or bloating. Recent report has shown neuroprotective effect [4]. However, anticariogenic effect or antibacterial activity of *S. lychnophora* is not well known. The main constituents are bassorin in the outer seed layer as well as galactose and pentose (mainly arabinose), 15.06% and 24.7%, respectively, in the peel [5].

Dental caries constitutes the most common chronic ecological disease in dentistry and is known as tooth decay or cavities. It is an infectious disease in which the hard tissues of the teeth such as the enamel, dentin, and cementum are gradually and irreversibly destroyed [6, 7]. Dental caries is caused by some types of oral *Streptococci* including the Gram-positive *Streptococcus mutans*, which is the most important

Figure 1: Photographs of *Sterculia lychnophora* Hance plant parts.

cariogenic bacteria and primary causative agent of this disease [8, 9]. *S. mutans* metabolizes the carbohydrates contained in consumed foods and produces organic acids, which initiate tooth enamel decay. Although fluoride compounds have been used to inhibit the formation of dental caries, high levels are cytotoxic [10]. Therefore, the development of new and safe agents that protect against the formation of dental caries is important. Natural products are good candidates for drug discovery including anticariogenic cariogenic agents. In the present study, we show *S. lychnophora* inhibits the growth as well as acid production and biofilm formation of *S. mutans*. This is the first report of anticariogenic effect of *S. lychnophora*. Furthermore, we analyzed the composition of phytochemicals and nutrient components of *S. lychnophora*.

2. Materials and Methods

2.1. Materials. Brain heart infusion (BHI) broth was purchased from Difco Laboratories (Detroit, MI, USA). Glucose and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). *Streptococcus mutans* ATCC 25175 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA).

2.2. Plant Material and Extraction. Pangdahai was obtained from the Oriental Drug Store Dae Hak Yak Kuk (Iksan, South Korea) and was authenticated by Young-Hoi Kim at the College of Environmental and Bioresource Sciences, Chonbuk National University (Jeonju, South Korea). A voucher specimen (number 18-03-12) has been deposited at the Herbarium of the Department of Oral Biochemistry in Wonkwang University. The husk and kernels of the *S. lychnophora* plant material were separated prior to use. The ethanol extract was prepared using 300 g of plant material, which was placed in a 3000 mL flask and macerated with 3000 mL of 70% ethanol for 72 h at room temperature. The ethanol extract samples were then dried, weighed, and stored at −20°C and the yield was 10.68 g (3.56%). The ethanol extract was dissolved in DMSO to obtain the desired stock solution for the experiments. The final concentration of DMSO was adjusted to 0.1% (v/v) in the culture systems, which did not interfere with the test while control groups were treated with medium containing 0.1% DMSO.

2.3. Inhibition of Growth and Acid Production. Bacterial growth inhibition was determined using a modification of methods described previously [11]. The cell growth evaluation was performed at 37°C in tubes with 0.95 mL of BHI broth containing varying concentrations of *S. lychnophora* extracts. The tubes were inoculated with 0.05 mL of an overnight culture of *S. mutans* grown in BHI broth at a final density of 5×10^5 colony-forming units (CFU)/mL and incubated at

37°C. After a 24 h incubation, the minimum inhibitory concentration (MIC), defined as the lowest concentration that inhibited the visible growth of *S. mutans* following overnight incubation, was determined by measuring the optical density (OD) of the growth media at 550 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices Co., Sunnyvale, CA, USA). In addition, the pH of the culture media was determined using a pH meter (HANNA Instrument, Philippines). The measurements were performed in triplicate for each concentration of the test extracts and sodium fluoride (NaF, 1%) was used as a positive control.

2.4. Biofilm Assay. The biofilm assay used in this study was based on a method described previously [12]. *S. lychnophora* extract was added to BHI broth containing 1% glucose in 35 mm polystyrene dishes or 24-well plates (Nunc, Copenhagen, Denmark). The culture media were then inoculated with a seed culture of methicillin-resistant *S. mutans* at a final density of 5×10^5 CFU/mL. After culturing for 48 h at 37°C, the supernatant was removed completely, and the dishes, wells, or wells containing the composite resin teeth were rinsed with distilled water. The amount of biofilm formed in the wells was measured by staining with 0.1% safranin, followed by treatment with 30% acetic acid to release the bound safranin from the stained cells, and the absorbance of the solution was measured at 530 nm. The biofilm formed on the surface of the resin teeth was also stained with 0.1% safranin and photographed.

2.5. Scanning Electron Microscopy (SEM). The biofilm formed on the 35 mm polystyrene dishes was also examined using scanning electron microscopy (SEM) using a modification of a previously described method [13]. The biofilm formed on the dishes was rinsed with distilled water and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C for 24 h. After sequential dehydration with graded concentrations of ethanol (60, 70, 80, 90, 95, and 100%), the samples were freeze-dried, sputter-coated with gold (108A sputter coater, Cressington Scientific Instruments Inc., Watford, England, UK), and observed using a scanning electron microscope (JSM-6360 SEM, JEOL, Tokyo, Japan).

2.6. Confocal Laser Scanning Microscopy. The bactericidal effect of the *S. lychnophora* extracts was determined using confocal laser scanning microscopy. The *S. mutans* culture (in BHI) was diluted with additional BHI medium to a density of approximately 1×10^7 CFU/mL and then treated with high concentrations (8–64 mg/mL) of *S. lychnophora* extracts at 37°C under aerobic conditions. After a 30 min incubation, the bacteria were washed with PBS and stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions, for 15 min. Stained bacteria were observed using a confocal laser scanning microscope (LSM 510, Zeiss, Germany). This method is based on two nucleic acid stains: green fluorescent SYTO 9 and red fluorescent propidium iodide stains, which differ in their ability to penetrate healthy bacterial cell and label live bacteria and those with damaged membranes, respectively.

2.7. Phytochemical Screening. The ethanol extracts of *S. lychnophora* were analyzed using phytochemical test [14]. The alkaloids, phenolics, glycosides, peptides, flavonoids, steroids, and organic acids were determined using Mayer's reagent, ferric chloride reagent, Molisch test, Biuret reagent, Mg-HCl reagent, Liebermann-Burchard reagent, and silver nitrate reagent, respectively.

2.8. Analytical Assays. The proximate components (moisture, protein, fat, carbohydrate, and ash) and mineral contents of the *S. lychnophora* extracts were analyzed using the Association of Official Analytical Chemists (AOAC) methods [15]. The following analyses were performed for the proximate nutrient determination. Moisture loss was determined after exposing the extract samples to a temperature of 110°C for 5 h in a forced draft oven. Total nitrogen was determined using the Kjeldahl method with a semiautomatic nitrogen analyzer. The ash content was determined by extracting ether soluble material from the extract samples with petroleum ether in a Soxhlet extractor for 8 h. Then, 2 g of the sample was charred and ashed to a constant weight at 550°C for 5 h in a muffled furnace. The carbohydrate content was calculated using the fresh weight-derived data, according to the following equation:

$$\begin{aligned} \text{g/100 g carbohydrate} &= 100\,\text{g/100 g} \\ &\quad - (\text{g/100 g moisture} + \text{g/100 g protein} \\ &\quad + \text{g/100 g fat} + \text{g/100 g ash}). \end{aligned} \tag{1}$$

2.9. Mineral Content Analysis. The *S. lychnophora* extract contents of Ca, Fe, Na, K, Mg, and Zn were analyzed using an atomic absorption spectrophotometer while the P content was determined using a spectrophotometer.

2.10. Dietary Fiber Analysis. The *S. lychnophora* husk and kernel extracts were separately analyzed for their insoluble dietary fiber (IDF), soluble dietary fiber (SDF), and total dietary fiber (TDF) using the relevant AOAC methods [15].

2.11. Statistical Analysis. All the experiments were performed in triplicate and the data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 18.0 program. The data are expressed as the mean ± standard error (SE). The differences between the means of the experimental and control groups were evaluated using Student's t-test and p values < 0.05 were considered statistically significant.

3. Results

3.1. Inhibition of Bacterial Growth and Acid Production. The ethanol extract of *S. lychnophora* showed significant dose-dependent antibacterial activity against *S. mutans* at concentrations of 0.01, 0.02, 0.03, and 0.04 mg/mL (Figure 2). Furthermore, compared to the control group, the inhibitory effects observed in the extract-treated groups were significant at concentrations higher than 0.01 mg/mL ($p < 0.05$) while the 0.1% NaF positive control exhibited antibacterial activity as well. A comparison of the extract-treated and control

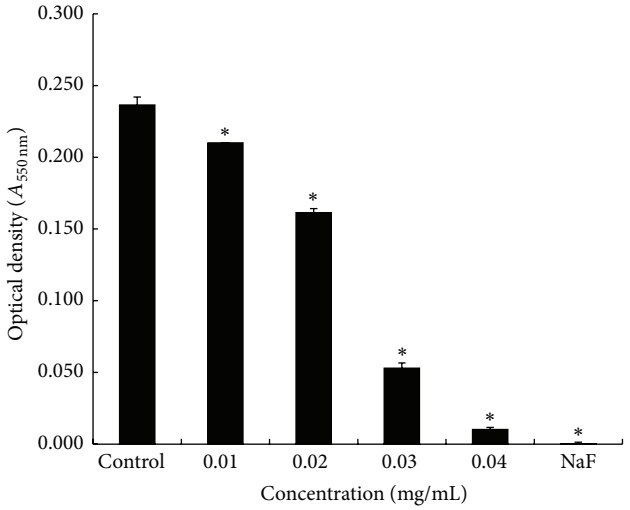

Figure 2: Optical density of *S. mutans* culture supernatants following treatment with varying concentrations of ethanol extracts of *S. lychnophora*. * $p < 0.05$ when compared with the control group after incubation.

Table 1: The pH of *S. mutans* incubated with ethanol extract of *S. lychnophora*.

Conc. (mg/mL)	pH (before incubation)	pH (after incubation)
Control	7.24 ± 0.06	5.37 ± 0.01
0.01	7.26 ± 0.06	5.42 ± 0.02*
0.02	7.25 ± 0.00	5.62 ± 0.02*
0.03	7.25 ± 0.00	6.55 ± 0.03*
0.04	7.25 ± 0.00	7.23 ± 0.02*
0.1% NaF	7.30 ± 0.06	7.29 ± 0.00*

* $p < 0.05$ when compared with the control group after incubation.

groups at the concentrations tested (0.01, 0.02, 0.03, and 0.04 mg/mL) revealed antibacterial effects of 11.02, 31.78, 77.54, and 95.76%, respectively.

The inhibitory effects of *S. lychnophora* ethanol extract against acid production by *S. mutans*, determined by the effects of the extract on pH, are shown in Table 1. The pH was significantly decreased in the untreated control group (pH 5.37 ± 0.01) but this effect was significantly inhibited in the positive control group (0.1% NaF, pH 7.29 ± 0.00). *S. lychnophora* extract (0.01–0.04 mg/mL) showed significant inhibition. These results indicate that the ethanol extract of *S. lychnophora* may inhibit organic acid production by *S. mutans*.

3.2. Inhibitory Effect of S. lychnophora on Biofilm Formation. The inhibitory effects of the extract of *S. lychnophora* on the biofilm formation by *S. mutans* evaluated using safranin staining are shown in Figure 3. The extract of *S. lychnophora* (0.01–0.04 mg/mL) inhibited the formation of *S. mutans* biofilm, which was also inhibited by the positive control (0.1% NaF). Furthermore, the *S. lychnophora* extract induced significant dose-dependent changes in the color intensity (OD) of the stained biofilm.

Table 2: Phytochemical screening of the ethanol extract from *S. lychnophora*.

Plant constituent	Contents
Alkaloid	+++
Phenolics	+++
Glycosides	+++
Peptides	+++
Steroids, terpenoids	+
Flavonoids	+
Organic acids	+

+++: strong; ++: moderate; +: weak; −: absent.

The SEM photographs (Figure 4) illustrate the results obtained using safranin staining. *S. mutans* attached and aggregated to the surface of the polystyrene 35 mm dishes and formed the visible biofilm in the control group. However, the biofilm formation was decreased in the presence of *S. lychnophora* at concentrations higher than 0.03 mg/mL and in the presence of the positive control (0.1% NaF).

In addition, we observed biofilm formation on the surface of the resin teeth following safranin staining (Figure 5). The extract of *S. lychnophora* (0.01–0.04 mg/mL) inhibited the formation of biofilm on the surface of resin teeth, and the inhibition was particularly potent at concentrations higher than 0.03 mg/mL.

3.3. Bactericidal Effect of S. lychnophora against S. mutans. The bactericidal effect of *S. lychnophora* is showed in Figure 6. The bactericidal effect of *S. lychnophora* was determined by staining the cultured bacteria with LIVE/DEAD BacLight Bacterial Viability Kit followed by confocal laser scanning microscopy. Bacterial viability decreased at high concentrations (0.04, 0.08, 0.16, and 0.32 mg/mL) of *S. lychnophora* extract, dose-dependently. This result suggests that high concentration of *S. lychnophora* extract may be bactericidal against *S. mutans*.

3.4. Phytochemical Screening. The results of the phytochemical tests for the ethanol extract are shown in Table 2. The preliminary phytochemical analysis is performed on the ethanol extracts. The ethanol extract revealed a strong presence of alkaloid, phenolics, glycosides, and peptides while the presence of steroids, terpenoids, flavonoids, and organic acids was low.

3.5. Proximate Composition. The results of the proximate composition analysis of *S. lychnophora* extracts are shown in Table 3. Moisture (11.97% ± 0.19) and ash (5.38% ± 0.10) of husk were higher than moisture (7.83% ± 0.05) and ash (2.65% ± 0.03) of kernel. Protein (3.12% ± 0.07) and fat (0.02% ± 0.01) of husk were lower than protein (17.24% ± 0.23) and fat (6.47% ± 0.25) of kernel. No significant difference was found in the levels of carbohydrate between the husk (79.54% ± 0.21) and kernel (65.79% ± 0.17).

3.6. Minerals Analysis. The results of the mineral content analysis of *S. lychnophora* extracts are shown in Table 4. The

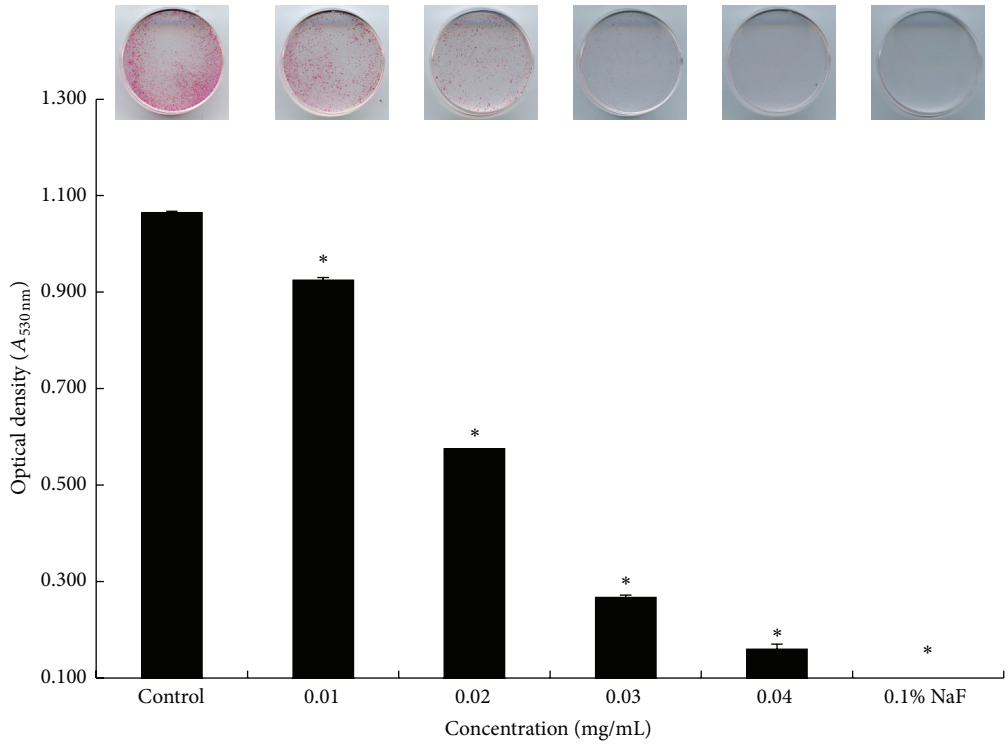

Figure 3: Safranin staining of *S. mutans* biofilm formation. Control, 0.01, 0.02, 0.03, and 0.04 mg/mL ethanol extract of *S. lychnophora* and positive control 0.1% sodium fluoride (NaF). * $p < 0.05$ when compared with the control group after incubation.

Table 3: The proximate composition of *S. lychnophora*.

	Husk (%)	Kernel (%)
Moisture	11.97 ± 0.19*	7.83 ± 0.05*
Protein	3.12 ± 0.07*	17.24 ± 0.23*
Fat	0.02 ± 0.01*	6.47 ± 0.25*
Ash	5.38 ± 0.10*	2.65 ± 0.03*
Carbohydrate	79.54 ± 0.21	65.79 ± 0.17

* $p < 0.05$ when compared with the husk and kernel.

Table 4: Mineral contents of *S. lychnophora*.

	Husk (mg/100 g)	Kernel (mg/100 g)
Calcium	367.64 ± 6.39*	85.84 ± 3.07*
Copper	0.89 ± 0.01*	1.81 ± 0.08*
Iron	6.57 ± 0.47*	4.15 ± 0.11*
Magnesium	405.41 ± 15.68*	218.94 ± 5.46*
Phosphorus	11.5 ± 0.01*	76.81 ± 6.49*
Potassium	1734.76 ± 33.75*	1111.32 ± 4.77*
Sodium	2.9 ± 0.05	2.46 ± 0.25

* $p < 0.05$ when compared with the husk and kernel.

husk showed Ca, Fe, Mg, and K contents that were significantly higher ($p < 0.05$) than those of the kernel. The husk had copper levels of 0.89 ± 0.01 mg/100 g and phosphorus contents of 11.50 ± 0.01 mg/100 g, which were both significantly lower ($p < 0.05$) than those of kernel. No significant difference was found in the levels of Na between the husk and kernel.

Table 5: Content of dietary fiber in husk and kernel of *S. lychnophora*.

	Husk (mg/100 g)	Kernel (mg/100 g)
Insoluble dietary fiber (IDF)	58.08 ± 0.06*	1.31 ± 0.01*
Soluble dietary fiber (SDF)	14.05 ± 0.23*	0.42 ± 0.02*
Total dietary fiber (TDF)	72.13 ± 0.29*	1.73 ± 0.03*

* $p < 0.05$ when compared with the husk and kernel.

3.7. Dietary Fiber Analysis. Table 5 shows the IDF, SDF, and TDF of the *S. lychnophora* husk and kernel. The IDF, SDF, and TDF of husk are higher than those of kernel significantly.

4. Discussion

There have been numerous research studies on the prevention and treatment of dental caries, which is one of the most frequently contracted and chronic dental diseases in humans. It is known that this disease affects 85.7% of people on average. Dental caries, once contracted, is not self-limiting and, therefore, cannot be cured without treatment [16]. Furthermore, if left untreated dental caries may develop into pulpitis, which can cause severe pain and eventually result in the necessary extraction of teeth [16].

S. mutans is commonly found in the dental plaque of humans and is the most cariogenic bacteria against the tooth

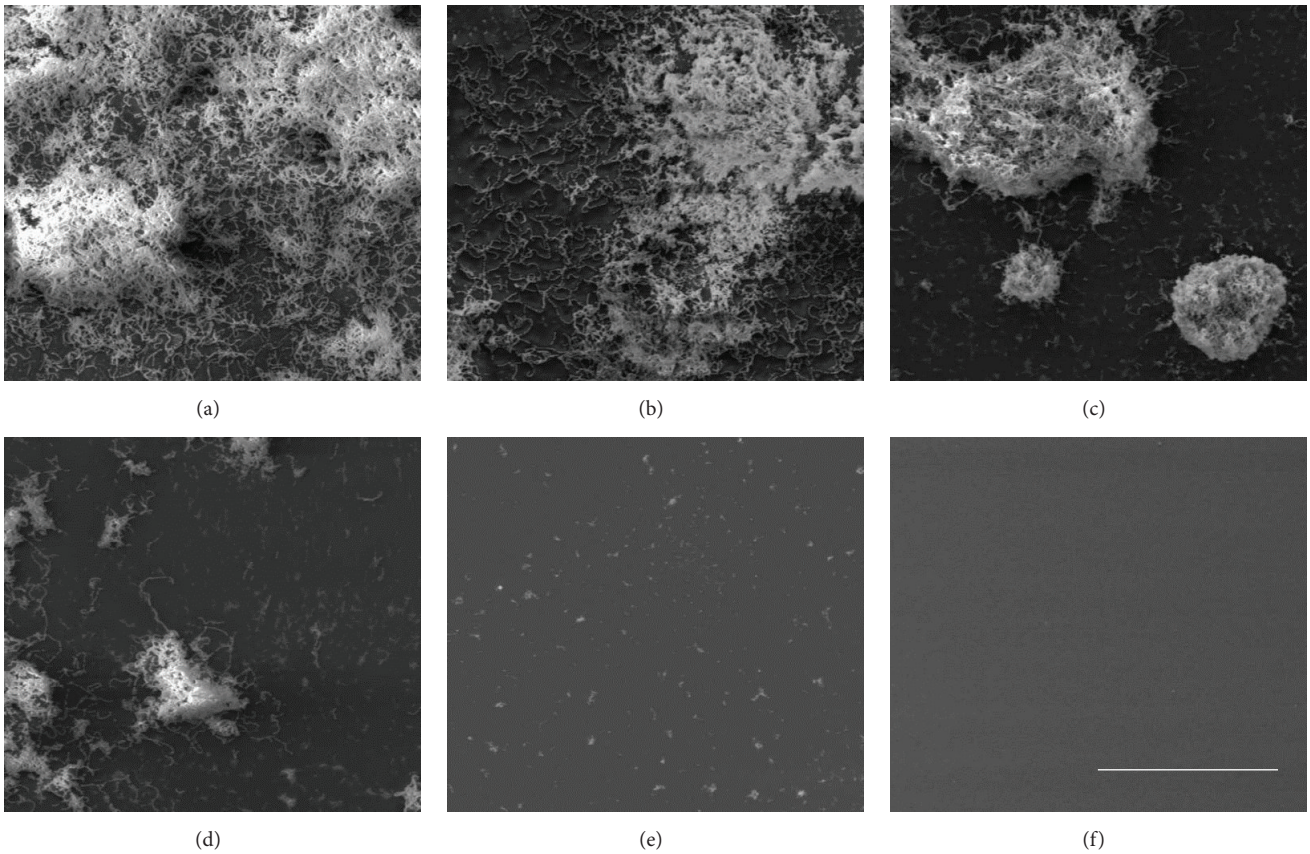

Figure 4: Scanning electron microscopy of *S. mutans* biofilms grown in the presence of ethanol extract of *S. lychnophora:* (a) control, (b) 0.01, (c) 0.02, (d) 0.03, and (e) 0.04 mg/mL extract and (f) positive control (0.1% NaF), scale bar = 10 μm.

enamel. It metabolizes dietary sugars and produces organic acids such as propionic, butyric, lactic, and formic acids as metabolic products, which can lower the pH of dental plaque and demineralize the tooth enamel and thereby initiate dental caries [17, 18].

Adhesion and colonization of *S. mutans* on the acquired enamel pellicle coated tooth surface are the initial step of the formation of dental plaque, which is a type of biofilm. The biofilm formation enhances bacterial resistance to both the host defense system and antimicrobials. Several natural substances have been developed for the treatment and prevention of dental diseases. The methanol extracts from leaves of green perilla and mugwort [19] as well as white ginseng [20] were reported to have excellent antibiotic effect against *S. mutans*. However, it was reported that ethanol extracts of *S. lychnophora* have more outstanding antibiotic effects than these substances. The methanol extracts of *Aralia continentalis* [21] as well as *Dianthus superbus* [22] were reported to have effects at 2 mg/mL and 4 mg/mL, respectively, against *S. mutans*. In addition, the ethanol extracts of *S. lychnophora* have a superior antibiotic effect to these substances as well. We aimed to provide scientific evidence of how *S. lychnophora* extracts can reduce the growth of germs that cause dental caries.

In this study, we prepared the ethanol extract of *S. lychnophora* and investigated its potential effects against the cariogenic properties of *S. mutans*. The extract of *S. lychnophora* inhibited the growth of *S. mutans*. It is commonly known as the main bacteria responsible for the formation of dental plaque and dental caries [7]. The ethanol extract of *S. lychnophora* inhibited the decrease in pH induced by *S. mutans*. These results suggest that the ethanol extract of *S. lychnophora* may inhibit organic acid production by *S. mutans*. Furthermore, the ethanol extract of *S. lychnophora* inhibited biofilm formation by *S. mutans* at concentrations ranging from 0.01 to 0.04 mg/mL and the SEM data on the biofilm formation corroborated the safranin staining data. The extract of *S. lychnophora* (0.01–0.04 mg/mL) also inhibited the biofilm formation on the resin tooth surface. In the present study, we have used 0.1% NaF as a positive control. It exhibited antibacterial activity and inhibited the decrease of pH and biofilm formation of *S. mutans* as *S. lychnophora*. However, previous reports have shown that fluoride compounds have cytotoxicity when fluoride compound was used at concentrations higher than 80 ppm [23]. Fluoride compounds have been investigated to inhibit the dental caries, but dental caries still remains the major cause of tooth loss. Therefore, it is necessary to develop new agents

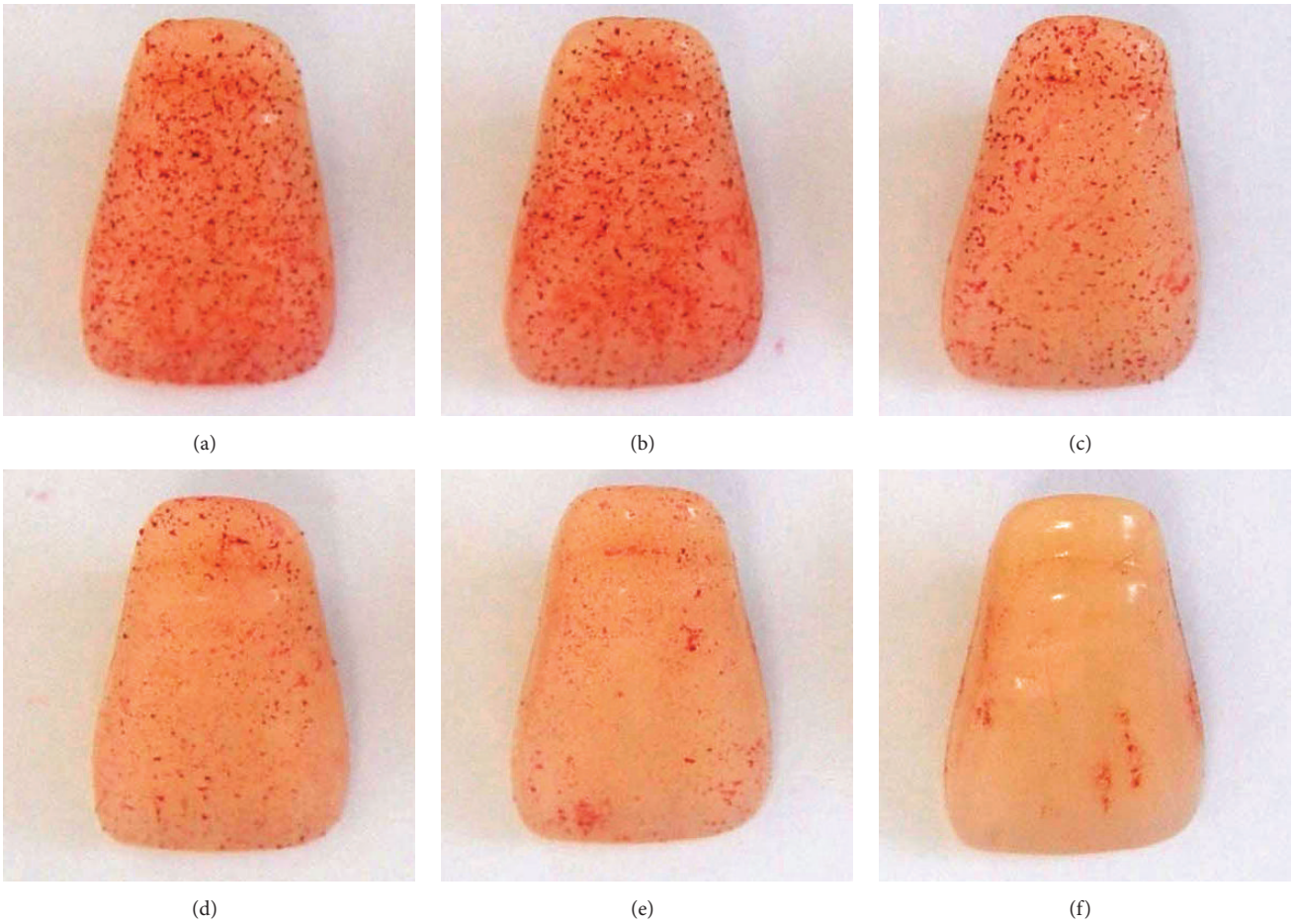

Figure 5: *S. mutans* biofilms on resin tooth surface following incubation with ethanol extract of *S. lychnophora:* (a) control, (b) 0.01, (c) 0.02, (d) 0.03, and (e) 0.04 mg/mL extract and (f) positive control, 0.1% sodium fluoride (NaF).

having better effect against dental caries. In this experiment, we found a strong presence of alkaloid, phenolics, glycosides, and peptides and a low presence of steroids, flavonoids, and organic acids. These compounds may have been responsible for the anticariogenic activity observed in the present study [24, 25].

The *S. lychnophora* ethanol extract showed levels of alkaloid, phenolics, glycosides, and peptides that were greater than those reported for the ethanol extract of *Aralia continentalis* [21]. In the results of the ethanol extract of *Aralia continentalis*, it had the strong presence of flavonoids and organic acids, moderate presence of phenolics and steroids, and the weak presence of alkaloids.

Then, we investigated the proximate composition of the *S. lychnophora* husk and kernel. The husk had moisture and ash contents that were significantly higher ($p < 0.05$) than those of the kernel as well as protein levels and fat contents that were significantly lower ($p < 0.05$) than those of kernel. According to the study by Li and Chen [26], the levels of Fe and P were 183.0 and 2786.0 mg/kg in *S. lychnophora* from China, and P was 2017.0 mg/kg in samples from Cambodia, which was more than that observed in our study. The levels of Ca, Mg, and Na at 1210.0, 3323.0, and 8.1 mg/kg, respectively, were higher in our study. The IDF level of the husk was higher than that in the peel of persimmon (15.71%), jujube (16.88%), citron (7.32%), cereal, and potato samples (7.36%) [27, 28]. The dried soymilk residue contained about 16% of SDF, which is higher than that of the husk of *S. lychnophora* [29]. The ISF level of the kernel was lower than that of the pulp of persimmon (1.95%), jujube (1.95%), and citron (2.61%) [27].

In conclusion, we demonstrated that the ethanol extract of *S. lychnophora* may inhibit acid production and biofilm formation, which may be due to the organic acids and glycosides, which are the major components of the extract of *S. lychnophora*. Therefore, we provided scientific evidence of the potential efficacy of the ethanol extract of *S. lychnophora* in the treatment of dental caries and a basis for its continued ethnomedicinal application and future development as a standard treatment.

Competing Interests

The authors declare that they have no competing interests.

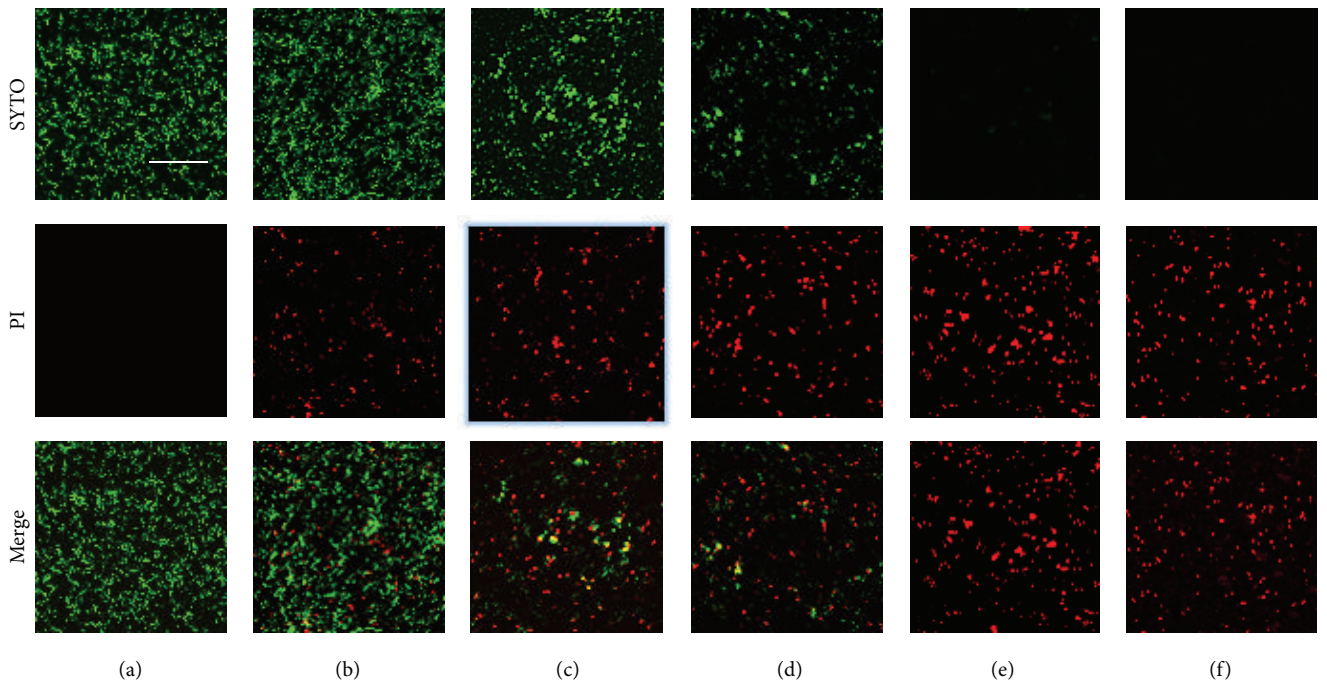

Figure 6: Bactericidal effect of *S. lychnophora* cultured *S. mutans* treated with *S. lychnophora* and stained with LIVE/DEAD BacLight Bacterial Viability Kit. Stained bacteria were observed using confocal laser scanning microscopy. Treatment with *S. lychnophora* decreased green-labeled living bacteria (SYTO 9 stain) and increased red-labeled dead bacteria (PI stain) dose-dependently. (a) Control, (b) 0.04, (c) 0.08, (d) 0.16, and (e) 0.32 mg/mL extract and (f) positive control sodium fluoride 0.1% (NaF), scale bar = 50 μm, objective lens (×100).

Acknowledgments

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Research Article

Effects of Safflower Yellow on the Treatment of Severe Sepsis and Septic Shock: A Randomized Controlled Clinical Trial

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Objective. To evaluate the clinical effect of safflower yellow on the treatment of severe sepsis and septic shock. *Methods.* 85 patients with severe sepsis and septic shock were randomly selected to receive either therapy according to the international guidelines for management of severe sepsis and septic shock (Surviving Sepsis Campaign 2012) (control group, $n = 45$) or conventional therapy plus safflower yellow (study group, $n = 40$). The 28-day mortality and 28-day Kaplan-Meier survival curves were compared as primary outcomes. *Results.* The 28-day mortality from all causes and in-hospital mortality were significantly lower in the study group (50%, 17.5%) as compared to the control group (78.58%, 54.76%) ($P = 0.007$, all causes, $P < 0.001$, in-hospital), and the 28-day Kaplan-Meier survival curve was higher in the study group than in the control group ($P = 0.008$, all causes, $P < 0.001$, in-hospital, Log Rank). 72 hours after treatment, secondary outcomes including heart rate, leukocyte counts, lactate levels, and platelet counts of patients in the study group were ameliorated significantly as compared with the control group. *Conclusion.* This study offers a potential new strategy employing safflower yellow to more effectively treat patients with severe sepsis and septic shock. This trial is registered with identifier ChiCTR-TRC-14005196.

1. Introduction

Severe sepsis and septic shock are common in critical care medicine and are usually associated with high mortality. There are an estimated 751,000 cases (3.0 cases per population of 1000 persons) of sepsis or septic shock in the United States each year [1] and even very high morbidity of 7.68% in Netherlands [2], which is accompanied by high mortality in many clinical trials [3–5]. The rapid progression, poor overall prognosis, and high mortality of severe sepsis and septic shock have stimulated many researchers and intensivists in critical care medicine to search for better means of treatment. However, improvement of outcome is still a complex issue, and no "magic bullet" has to date been found. The Surviving Sepsis Campaign (SSC) which offers international guidelines for management of severe sepsis and septic shock has been updated in the 2012 edition [6], and many clinical trials [7, 8] have been carried out to assess the effects of such suggested intervention on patients with severe sepsis and septic shock. However, many intensivists feel there is still lack of effective treatment for severe sepsis and septic shock. It is clear that development of new medication and treatment strategies is urgently needed.

The clinical importance of herbal medicine has drawn substantial attention in recent years. Safflower, which is the dried flower of *Carthamus tinctorius* L., has been used extensively in Chinese medicine for treating gynecological disease and coronary heart disease [9]. Safflower yellow is the main effective component derived from *Carthamus tinctorius* L., and it has been reported to exhibit anticoagulative, vasodilatory, antioxidative, and anti-inflammatory effects [10–16]. In patients with severe sepsis and septic shock, phenomena such

as activation of the coagulation system, hypercoagulability of the blood, and release of inflammatory mediators and cytokines, as well as adhesion and aggregation of neutrophils, are commonly found [17, 18]. However, safflower yellow has not previously been employed for treatment of severe sepsis or septic shock. According to the pharmacologic effects of safflower yellow noted above, we hypothesized that intervention with safflower yellow may decrease the mortality in patients with severe sepsis and septic shock. Here, for the first time, a prospective randomized controlled trial was conducted on patients with severe sepsis and septic shock in a poverty-stricken area in western China.

2. Methods

2.1. Approval of Study Design. This prospective randomized controlled study was approved by the Medical Ethics Committee (number 2012-A-1) of the People's Hospital of Pujiang County, Sichuan Province, China. This study is registered with http://www.chictr.org.cn/ (ref. ChiCTR-TRC-14005196).

2.2. Eligibility. Eligible adult patients signed informed consent forms before they were enrolled in this randomized controlled trial. Written informed consent was obtained from all participants. Inclusion criteria included patient age of 18 to 85 years, with a diagnosis of severe sepsis or septic shock according to the diagnostic standards of the 2012 severe sepsis and septic shock treatment international guidelines. Exclusion criteria were as follows: (1) hypovolemic shock, cardiogenic shock, distributive shock, or obstructive shock; (2) pregnancy or lactation in female patients; (3) patient allergy to safflower yellow; (4) current patient enrollment in other medical research; (5) severe disease of the liver and/or kidney.

All participants were informed about the two methods to be used in this trial including conventional therapy according to the international guidelines for management of severe sepsis and septic shock 2012 (control group) and conventional therapy plus safflower yellow treatment (study group).

2.3. Treatment. All patients were assigned to either the study group or the control group according to a number extracted at the beginning of the study from a table of random numbers generated by the Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA). This was done only once, and there was no subsequent modification of number assignment during the trial. The study group assignments were placed in sealed, opaque, randomly assorted envelopes. The envelope was not opened until the patient was enrolled in the trial. Patients and statisticians were both blinded to the use of safflower yellow.

Patients in the two groups received 3-hour and 6-hour bundles of conventional therapy according to the international guidelines for management of severe sepsis and septic shock 2012 [9]. Patients in the study group received intravenous injection of safflower yellow at a dose of 100 mg [19] every 12 hours for 72 hours in addition to therapy.

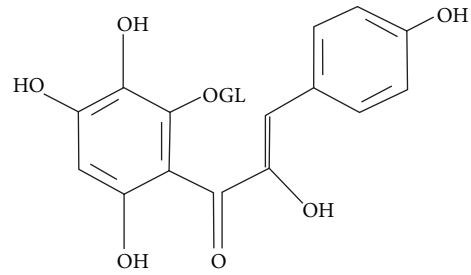

Figure 1: The structure of safflower yellow.

According to Surviving Sepsis Campaign (SSC) international guidelines for management of severe sepsis and septic shock 2012, we collected samples for the culture isolation of the pathogen in each case within 1 hour after patient's arrival in the ICU, or within 3 hours after arrival in the emergency department.

2.4. Pharmacology and History of Safflower Yellow. Safflower yellow [20] is the main effective constituent of Flos Carthami. The molecular structure is shown in Figure 1 [20]; it has a molecular weight of 612.53, and the chemical formula is $C_{27}H_{32}O_{16}$ [20]. The major chemical ingredient of the safflower yellow injectable extract used in this study was hydroxyl safflower yellow A (HSYA) or safflomin A. Flos Carthami, a traditional Chinese herbal medicine, was extensively employed to deal with menstrual problems, cardiovascular disease, pain, and swelling associated with trauma [21]. Flos Carthami is the flower of *Carthamus tinctorius* Linn., a diploid oilseed crop which has been domesticated in the Fertile Crescent region over 4,000 years ago. Full botanical plant name is *Carthamus tinctorius* Linn., Asteraceae [22, 23].

2.5. Outcomes. The 28-day mortality from all causes and in-hospital mortality, as well as Kaplan-Meier survival curves, were evaluated in the two groups as primary outcomes.

Secondary outcomes included patient respiratory frequency (F), heart rate (HR), urine output, blood pressure, arterial partial pressure of oxygen (PaO_2), lactate level, bilirubin level, and serum creatinine level, which were measured and assessed every 12 hours for 72 hours. Arterial blood gas values, lactate concentrations, coagulation-related variables, and clinical variables required for determination of the Acute Physiology and Chronic Health Evaluation (APACHE II) score (on a scale from 0 to 71, with higher scores indicating more severe organ dysfunction) were obtained at baseline (0 hour) and at 72 hours. Patients were followed up clinically for 28 days. The number of days of ICU hospitalization, length of time on mechanical ventilation, and renouncement rate of voluntary patient withdrawal from treatment in the two groups were also examined.

2.6. Statistical Analysis. In order to initially calculate the sample size required for this study, we first posited that the mortality of the study group with safflower yellow intervention would be 30% lower than the control group.

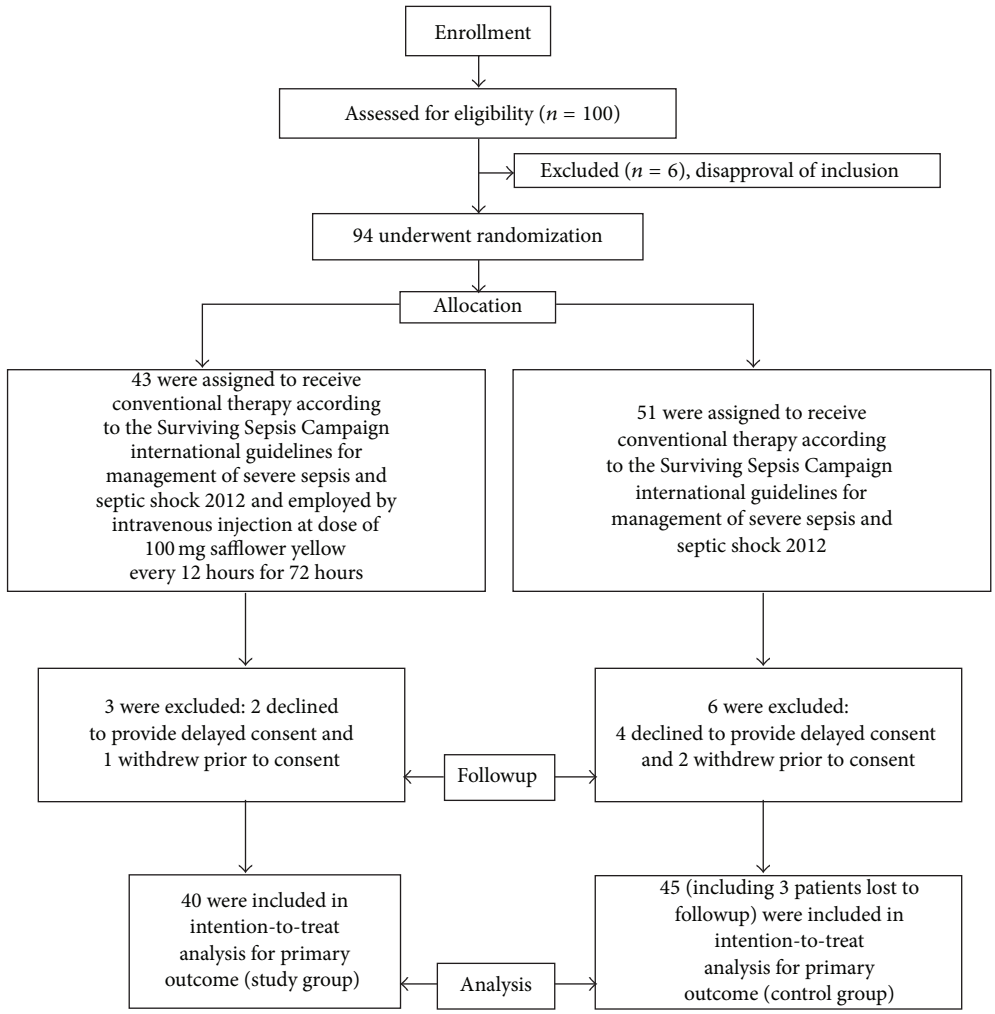

Figure 2: Patients flow diagram.

We used data from a previous septic sepsis trial with 52.5% mortality in the control group [24]. Assuming a rate of patient withdrawal from the trial of 20%, to achieve a two-sided type I error rate of 5% and a power of 80%, we calculated that a sample size of 100 patients was required to detect differences in mortality between these two groups. Numbers (%) for categorical variables were compared using Pearson chi-square test or Fisher's exact test. Normally distributed continuous variables were presented as mean ± standard deviation. Statistical significance was determined by Pearson chi-square test, Student's t-test, Wilcoxon Rank-Sum Test, and Log Rank Kaplan-Meier analyses. P values less than 0.05 were considered to be significant. The Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA) was used for analysis.

3. Results

The trial began on 18 March 2012 and ended on 8 September 2015. It thus lasted almost 41 months. A total of 100 patients were enrolled in this study (Figure 2). Of these patients, 40 were assigned to the study group and 45 were assigned to the control group. All of these 85 patients had medical followup by telephone for 28 days. Three patients were lost to followup in the control group. Thirteen patients in the study group and 10 patients in the control group voluntarily terminated treatment for personal reasons such as financial burdens or poverty in family members. The renouncement rate of voluntary patient self-termination of treatment was not significantly different in the two groups (Table 3).

Basic causes of severe sepsis and septic shock in these patients, critical illness severity scores, and demographic data are summarized for both groups, and both groups showed similar features (Table 1).

After treatment, primary outcomes such as 28-day mortality from all causes and in-hospital mortality (Table 2) were significantly lower in the study group than in the control group ($P = 0.007$ and $P < 0.001$), and the 28-day Kaplan-Meier survival curve was higher in the study group than in the control group (Figures 3 and 4).

Some secondary outcomes, including heart rate, respiratory frequency, leucocyte counts, platelet counts, lactate level, and serum creatinine, decreased, and PaO_2, mean arterial pressure, and urinary production per hour increased in study

Table 1: Characteristics of the trial patients at baseline.

Demographics	Study group (n = 40)	Control group (n = 45)	P value
Age (years)	64.53 ± 14.89	69.03 ± 12.62	0.189
Weight (kg)	53.90 ± 6.60	53.08 ± 8.75	0.675
Sex (male : female)	28 : 12	28 : 17	0.450
APCAHE II	29.67 ± 7.68	30.39 ± 7.10	0.693
SOFA (sepsis-related organization failure assessment)	12.93 ± 2.46	11.71 ± 2.85	0.190
Comorbidities			
Severe pneumonia	15	13	0.399
Peritonitis	6	11	0.277
Acute exacerbation of chronic obstructive pulmonary disease	10	13	0.549
Severe acute pancreatitis	4	1	0.165
Biliary tract infection	1	3	0.327
Urinary system infection	2	2	1.000
Fracture with infection	1	0	0.488
Multiple organ dysfunction syndrome	1	1	0.741
Burn	0	1	0.512

Table 2: Clinical primary outcomes.

Primary outcome measures	Study group (n = 40)	Control group (n = 42)	Relative risk (95% CI)	P value
28-day mortality (all causes)	20/40 (50%)	33/42 (78.58%)	0.636 (0.449–0.901)	0.007
28-day mortality (hospitalization)	7/40 (17.5%)	23/42 (54.76%)	0.320 (0.154–0.661)	<0.001

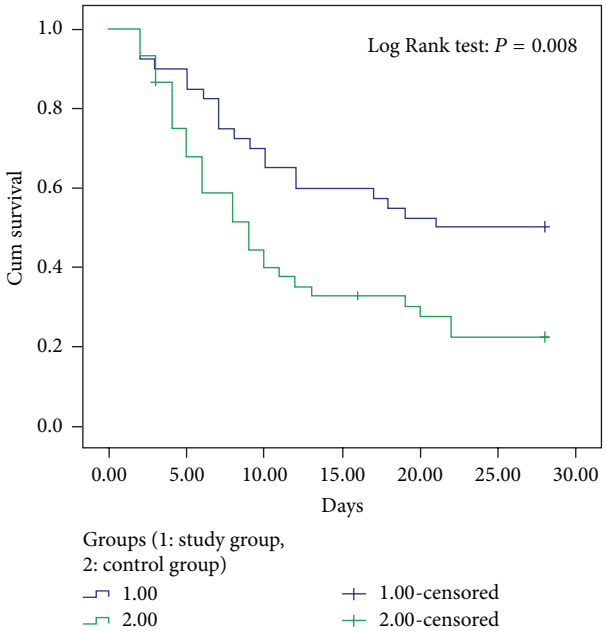

Figure 3: Kaplan-Meier survival curve elevated in study group compared with control group (all causes).

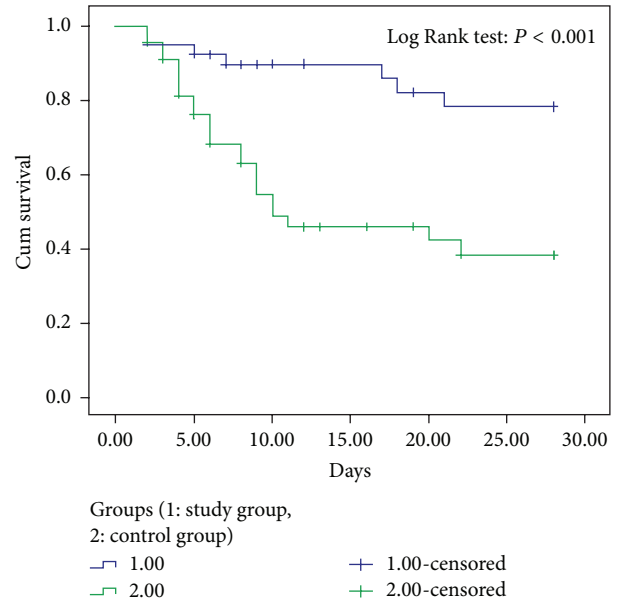

Figure 4: Kaplan-Meier survival curve elevated in study group compared with control group (in-hospital).

group patients as compared to the control group (Table 3). Days of ICU hospitalization and mechanical ventilation showed no significant difference between the two groups (P = 0.951, P = 0.928) (Table 3).

An allergic reaction was found in 1 patient in the study group, who had erythema, rash, and swelling all over the body. These signs of allergic reaction improved after immediate termination of the safflower yellow infusion and intravenous injection of dexamethasone and calcium glucose.

Table 3: Clinical secondary outcomes.

Secondary outcome factors	Study group ($n = 40$)	Control group ($n = 42$)	P value
Heart rate (beats/min)			
Before intervention	122.80 ± 20.89	113.22 ± 27.25	0.106
72 h after intervention	91.63 ± 17.96	112.38 ± 29.13	0.042
Mean artery pressure (mmHg)			
Before intervention	70.43 ± 19.84	74.94 ± 19.25	0.297
72 h after intervention	81.35 ± 15.48	75.02 ± 18.02	0.093
Respiratory frequency (breaths/min)			
Before intervention	25.55 ± 8.19	25.78 ± 6.65	0.282
72 h after intervention	19.87 ± 5.39	23.36 ± 6.05	0.019
Arterial partial pressure of oxygen (PaO_2, mmHg)			
Before intervention	82.15 ± 45.99	74.51 ± 35.37	0.651
72 h after intervention	107.83 ± 41.51	81.41 ± 39.97	0.015
Leucocyte counts (10^9/L)			
Before intervention	16.22 ± 8.72	14.82 ± 8.57	0.926
72 h after intervention	12.05 ± 7.75	17.21 ± 8.16	0.013
Urinary production per hour (mL/hour)			
Before intervention	68.28 ± 40.40	74.12 ± 52.99	0.555
72 h after intervention	130.14 ± 93.53	65.7 ± 41.52	0.002
Platelet counts (10^9/L)			
Before intervention	163.38 ± 120.02	155.83 ± 84.91	0.454
72 h after intervention	156.10 ± 104.79	108.26 ± 64.13	0.018
Lactate level (mmol/L)			
Before intervention	4.68 ± 3.32	4.61 ± 3.55	0.937
72 h after intervention	2.48 ± 2.26	4.40 ± 3.41	0.006
Bilirubin (μmol/L)			
Difference before and 72 h after intervention	13.65 (4.50, 53.10)	17.00 (3.50, 249.63)	0.844
Creatinine (mmol/L)			
Before intervention	137.22 ± 58.62	136.80 ± 65.47	0.976
72 h after intervention	126.52 ± 68.61	160.73 ± 92.27	0.06
Rate of voluntary termination of treatment (%)	32.5% (13/40)	23.80% (10/42)	0.381
Days of ICU hospitalization (days)	8.23 ± 5.25	8.12 ± 10.19	0.951
Days of mechanical ventilation (days)	3.60 ± 4.14	3.70 ± 4.29	0.928

Table 4: Culture isolation of the pathogen.

Isolation of the pathogen	Study group ($n = 40$)	Control group ($n = 45$)	P value
Positive culture from blood or sterile specimen, number (%)	12 (30%)	14 (31.11%)	0.912
Klebsiella pneumoniae	4	3	0.665
Haemophilus influenzae	2	3	1
Escherichia coli	4	5	1
Staphylococcus aureus	1	2	1
Others	1	1	1

There were no other serious adverse reactions such as allergic shock. Bilirubin levels did not increase after safflower yellow treatment ($P = 0.844$) (Table 3).

Cultures of blood, sputum, abdominal drainage fluid, urine, and pus were performed, with percentages of isolation of 30% and 31.11% in the study and control groups, respectively. These pathogens included *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Escherichia coli*, and *Staphylococcus aureus*. This information about cultured isolation of the pathogens is showed in Table 4.

4. Discussion

As we originally hypothesized, this study demonstrated that safflower yellow significantly reduced 28-day mortality and increased survival in patients with severe sepsis and septic shock. Failure of multiple organ systems brings about the high mortality in sepsis and shock, and deterioration of cardiorespiratory function is particularly critical. In this study, we show safflower yellow acts mainly by improving respiratory and cardiovascular function and tissue perfusion, as well as by decreasing inflammatory reaction.

Safflower yellow improved the hemodynamic index of patients with severe sepsis and septic shock as reflected by increases in BP and decreases in the HR and in turn improves the tissue and organ perfusion index. The increase of BP improving tissue perfusion of vital organs was reflected in significant decrease of blood lactate levels compared with those in the control group. Blood lactate levels may be used as an indicator for tissue perfusion during management of severe sepsis and septic shock [25]. In the 3-hour and 6-hour protocols in the guidelines [9], it is necessary to reverse anaerobic metabolism and low tissue perfusion in these patients as quickly as possible. After treatment with safflower yellow, blood lactate levels in the study group decreased significantly, suggesting safflower yellow improves the ischemia hypoxia and anaerobic glycolysis in septic shock.

Previous research reported that administration of esmolol decreased the cardiac workload and safely preserved myocardial function by reducing the heart rate in patients with severe sepsis and septic shock [26]. Similarly, administration of safflower yellow in patients with severe sepsis and septic shock was associated with decreases in the heart rate, which confer benefits such as lengthening of coronary diastolic perfusion time, improvement of coronary perfusion, and alleviation of myocardial ischemia and hypoxia. Consistent with improvement of organ perfusion in, for example, the kidney, urine volume per hour significantly increased with the use of safflower yellow.

Safflower yellow also improved respiratory function. After treatment, respiratory frequency decreased, and respiratory frequency in the safflower yellow group was significantly lower than in the control group. This decrease in respiratory frequency reduced the work of breathing, alleviated respiratory distress, and decreased oxygen consumption. Furthermore, the patients in the study group showed evident increases in PaO_2 as compared with the control group, representing an increased tissue oxygen supply.

Disordered inflammatory and coagulation function also contribute to the adverse clinical effects in severe sepsis and septic shock. However, safflower yellow improved inflammation indices and coagulation function in these patients. It has been found that safflower yellow effectively inhibits expression of mRNA for proinflammatory factors such as TNF-α, IL-1β, and IL-6 and promotes expression of anti-inflammatory factors such as IL-10 [27]. Through anti-inflammatory mechanisms, damage to the lung tissue of patients with sepsis and septic shock caused by inflammatory reaction may be reduced, and increases in permeability of blood capillaries may be inhibited. Safflower yellow may also act by blocking the cascade reaction of cytokines that are activated by this disease and thus inhibit activation and adhesion of neutrophils [12, 13, 21]. Leukocyte counts in the study group were significantly decreased as compared with those in the control group after treatment. The platelet counts in the study group were not significantly decreased, but those in the control group decreased significantly, suggesting safflower yellow inhibits platelet activation and aggregation [28].

Serum creatinine was improved in the study group together with urine volume increases as compared with the control group, consistent with safflower yellow acting to improve renal blood flow and perfusion while conferring no injury to renal function. Safflower yellow had no effects on liver function, and after treatment there were no statistically significant differences in bilirubin levels between groups, demonstrating safflower yellow has no hepatotoxicity (Table 3). Employment of safflower yellow thus appears to be safe and effective in the treatment of severe sepsis and septic shock.

During clinical management of patients with severe sepsis and septic shock, safflower yellow improved clinical indices, such as circulation, breath, oxygenation, inflammation response, microcirculation perfusion, and coagulation function, and reduced 28-day mortality and increased 28-day survival. Mortality from all causes was selected as the primary outcome in this study and was as high as 78.58% in the control group and 50% in the study group. In the past, mortality in this disease was very high. The overall mortality from a meta-analysis including 131 studies from 1958 to 1997 was 49.7% [29]. Under current standards of treatment in the developed countries, the overall mortality in the three famous large clinical trials published was 18.70%, 32.14%, and 29.36% at 90 days, respectively [30–32].

There are several possible reasons for this mortality rate from all causes of 78.58% in the control group, which is markedly high as compared with other current studies, as well as the mortality rate from all causes in the study group which is significantly lower than our control group but is still higher than other current studies [30–32]. First, most of the patients in this study were from the countryside and were living in poverty. After an initial period of treatment, if therapeutic intervention did not achieve the level of success expected by the family, or the family could not afford continuing treatment costs, patient family members would give up on all therapy and leave the hospital. Some patient family members from both groups gave up on therapy, including 13 cases in the study group and 10 cases in the control group. All of these 23 patients died within 28 days of returning home, which increased the mortality from all causes. If these 23 patients were excluded, the in-hospital mortality would be 54.76% and 17.5% in the control and study groups, respectively, which is close to other current studies [30–32]. Second, most enrolled patients had severe clinical disease from the outset of treatment, with average APACHE II scores of 29.67 and 30 for the study group and the control group, respectively. Moreover, the score for the sepsis-related organization failure assessment was as high as 12, which is much higher than scores found in similar research [33, 34]. This all argues that these patients had more severe illness and higher mortality risk factors, ultimately giving rise to higher mortality as compared with other studies. Third, this research was conducted in a poverty-stricken area, an agricultural county named Pujiang County, in the west of China. Although we performed this research according to international guidelines for treatment of sepsis and septic shock, the medical conditions, medical facilities, and medical level of the hospital and staff all lagged behind the current international level. However, despite the comparatively high mortality rate in the control group and suboptimal hospital

conditions for patient treatment, the critical highlight of this study is that after treatment with safflower yellow in-hospital mortality was reduced to 17.5%, which is closely similar to the mortality reported in studies abroad [35, 36].

This study had some limitations. First, this was a single-center study, and the patients and physicians were from a poverty-stricken area in the west of China. Therefore, the processing of samples was limited. Second, a significant number of family members gave up on therapy after 3 days, influencing the primary outcome (28-day mortality). Third, due to limited conditions, samples were not collected for cellular or molecular experiments to study the molecular mechanisms associated with safflower yellow. Nevertheless, study of the molecular mechanism for safflower yellow in treatment of severe sepsis and septic shock with a larger patient study pool is warranted.

In conclusion, with the use of safflower yellow for therapeutic intervention in severe sepsis and septic shock, the 28-day mortality from all causes and in-hospital mortality were reduced by 28.57% and 37.26%, respectively. Although the rates of mortality for safflower yellow are higher than those reported for conventional treatment in some current studies, they are significantly lower than those for the control group in our study. To our knowledge, this is the first study to evaluate a natural extract of a traditional Chinese herbal medicine for treatment of critical infectious illness, and it is the first time safflower yellow has been used to effectively treat severe sepsis and septic shock. We plan to perform a multicenter RCT clinical trial with a larger number of patients to further study and verify the role and mechanism of safflower yellow in treating severe sepsis and septic shock. However, findings in this study offer a potential new strategy for effectively treating patients with severe sepsis and septic shock.

Conflict of Interests

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

Acknowledgments

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Research Article

Modes of Antiviral Action of Chemical Portions and Constituents from Woad Root Extract against Influenza Virus A FM1

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Woad root has been used for the prevention of influenza for hundreds of years in many Asian countries. In this study, the antiviral modes of clemastanin B (CB), epigoitrin, phenylpropanoid portion (PEP), and the mixture of phenylpropanoids, alkaloids, and organic acid portions (PEP + ALK + OA) from wood root extract against influenza virus A FM1 were investigated. The results revealed that CB, epigoitrin, PEP, and PEP + ALK + OA exert their anti-influenza activity via inhibiting the virus multiplication, prophylaxis, and blocking the virus attachment. The primary mode of action of PEP and PEP + ALK + OA is the inhibition of virus replication. The inhibitory effect on virus attachment and multiplication is the main modes for epigoitrin. All the compounds or chemical portions from woad root extract tested in this study do not have direct virucidal activity. Our results provided the comprehensive analysis of the antiviral mechanism of wood root extract.

1. Introduction

Influenza or flu is one of the most significant acute respiratory diseases caused by the infection of influenza virus. Seasonal influenza affects millions of people in the world every year, leading to a serious threat to public health especially to children and the elderly. In addition, influenza virus has the potency to cause a severe pandemic and economic loss [1]. The outbreak of avian influenza A in China in 2013 caused nearly $6.5 billion in losses to the economy.

Currently, the synthetic antiviral drugs or vaccines have limited use in developing countries due to the emergence of resistant strains, the high cost, and the harmful side effects [2, 3]. However, anti-influenza agents derived from herbs have many advantages such as low cost and toxicity, extensive source, and ease of access [4, 5]. Moreover, herbal drugs usually have multitarget effects, which not only act as antiviral agents but also stimulate immunity [6]. Therefore, medicinal plant extracts and phytochemicals are attracting more and more attention as the potential sources for the development of new antiviral drugs during the recent decade.

Woad root (common name: Ban Lan Gen) is the dry root of plant *Isatis indigotica* Fort. Woad root was first documented as the herbal drug in *The Divine Husbandman's Herbal Foundation Canon*, a famous ancient medical book in the Han Dynasty of China (200 AD). It has been used in the treatment of cold, sore throat, and headache for hundreds of years in China [7, 8]. Woad root was used for the prevention of severe acute respiratory syndrome (SARS) in 2003 and swine flu pandemic in 2009 in China, Hong Kong, Taiwan, and Japan [9, 10].

Flu vaccines are the main prophylactic treatment in winter to protect against the influenza viruses. It was estimated that more than $3.2 billion was spent on flu vaccines production every year by the federal government of USA [11]. Therefore, it is necessary to develop the inexpensive drugs

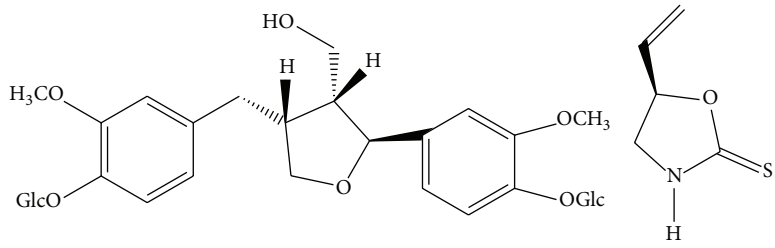

Figure 1: Chemical structure of clemastanin B (CB) and epigoitrin.

with effective prophylactic activity. The use of water extract of woad root to prevent cold has been documented for hundreds of years.

Recently, the antiviral effect of the methanol, water, and ethyl ester extract of woad root was confirmed through *in vitro* test [10, 12]. However, the modes of antiviral actions of these extract are still not clear. Additionally, there is little information on the differences of antiviral action between the single pure compound and the extract where the compound is isolated from.

Phenylpropanoids (PEP), alkaloids, and organic acids are three major chemical portions in woad root. Clemastanin B (CB) is the most abundant compound which belongs to phenylpropanoid. Epigoitrin is the main alkaloid compound isolated from the woad root (Figure 1) [13, 14]. A previous screening showed that CB and epigoitrin have the strong inhibitory effect on influenza A1 virus FM1 [15]. The objective of the present study was to elucidate the possible anti-influenza mechanisms of CB and epigoitrin and compare with the phenylpropanoids portion and the mixture of phenylpropanoids, alkaloids, and organic acid portions (PEP + ALK + OA).

2. Materials and Methods

2.1. Viral Strains, Cell Lines, and Reagents. Mouse lung-adapted variant of influenza virus A FM1 strain was obtained from the Department of Microbiology and Immunology at Shandong University. The virus was propagated twice in the allantoic cavity of 9- to 10-day-old embryonated hen's eggs at 35°C for 48 h to enhance the virulence. The allantoic fluid was harvested for the measurement of its hemagglutinating activity. Once the hemagglutination titer reached 1 : 640, the virus was aliquoted and stored at −80°C until use. The Madin-Darby canine kidney (MDCK) cells and human cervical cancer (HeLa) cells were obtained from Institute of Cell Biology, Chinese Academy of Sciences. The positive control ribavirin (Batch number: 101018) was purchased from Baili Pharmaceutical Co. Ltd. in Sichuan province, China.

2.2. Preparation of Plant Extracts. The woad root was collected from Anhui province, China. Herb identification was confirmed through morphological and microscopic analysis according to the Chinese Pharmacopeia [16]. The extraction of pure compounds and chemical portions of woad root was conducted by Tianjin SunnyPeak Biotech. Co. Ltd. The mixture of phenylpropanoids, alkaloids, and organic acid portions (PEP + ALK + OA) was prepared by mixing each of the above portions at a ratio of 1 : 2 : 2 (w/w/w) [17]. The lyophilized materials were directly resuspended in the cell culture medium and filter sterilized through the 0.22 μm membrane. For those compounds which cannot be dissolved in the medium, they were first dissolved in DMSO and then diluted with the fresh medium. The final DMSO concentration in the medium was less than 1%. The concentration of CB and epigoitrin was 50 μg/mL and the initial concentration of PEP and PEP + ALK + OA was 100 μg/mL.

2.3. Modes of Anti-Influenza Action. The anti-influenza action of CB, epigoitrin, and chemical portions from woad root extract was investigated in four different modes: therapeutic action, prophylaxis, direct virus inactivation, and inhibition of virus attachment.

2.4. Preincubation with Virus (Therapeutic Action of the Drugs). The cells were preinfected with the virus before the pure compounds or chemical portions of plant extract were added. The therapeutic action of the drugs was evaluated by both cytopathic effects (CPE) reduction assay and cell MTT assay.

The CPE reduction assay was conducted according to the previous report with slight modifications [18]. Briefly, quadruplicate MDCK monolayer cells in 96-well plates were infected with 0.1 mL suspension containing 100 $TCID_{50}$ (50% Tissue Culture Infective Dose) of virus for 2 h. The unabsorbed virus was then washed off using PBS. Quadruplicate cell monolayers were subsequently overlaid with 0.1 mL medium containing different nontoxic twofold serial dilutions of pure compounds, chemical portions of woad root extract. Cells with virus infection without drug treatment and the cells without virus and drugs were used as controls. The plates were incubated at 37°C under 5% CO_2 for 72 h. The virus-induced CPE was observed under a light microscope in comparison with the parallel virus control and cell control.

The MTT reduction assay was performed according to the standard protocol [19]. In short, the experimental setup was the same with the procedures in CPE assay. After 3 days of incubation, 20 μL of MTT was added to each well

and incubated at 37°C for 4 h. Subsequently, DMSO was added and the absorbance was measured at 570 nm. The cells protection rate (%) was calculated by the following formula:

$$\left[\frac{\mathrm{OD}_{\mathrm{exp}} - \mathrm{OD}_{\mathrm{virus\ control}}}{\mathrm{OD}_{\mathrm{cell\ control}} - \mathrm{OD}_{\mathrm{virus\ control}}}\right] \times 100\%. \quad (1)$$

See [16].

2.5. Pretreatment with Drugs (Prophylaxis). To evaluate the effects of pure compounds and chemical portions from woad extract on prophylaxis of cell infection, the MDCK monolayer cells in 96-well plates were overlaid with different nontoxic twofold serial dilutions of pure compounds, chemical portions of woad root extract. Four replicates were set up for each treatment and control. After 4 h, the test substances were removed from the wells and the monolayer cells were then infected with 100 TCID_{50} of influenza virus A FM1 at 37°C for 2 h to allow virus absorption. Subsequently, the unabsorbed virus was washed off using PBS and the equal amount of maintenance medium was added into each well. The plates were incubated at 37°C under 5% CO_2 for 72 h. The virus-induced CPE was observed under light microscope.

2.6. Direct Virucidal Assay. The direct virucidal activity of the pure compounds and chemical portions from woad extract was tested according to the methods described by Carlucci et al. [20]. One hundred microliters of 100 TCID_{50} of the virus was treated with equal volumes of twofold diluted pure compounds or extract portions for 2 h at 37°C. The samples were then tenfold serially diluted. When the confluent monolayer of MDCK cells was formed, the surviving virus in the mixtures was determined in CPE assay and titers (TCID_{50} values) were calculated according to the Reed-Muench method.

2.7. Inhibition of Virus Attachment Assay. The monolayer of MDCK cells was cultured in 96-well plates. One hundred microliters of different nontoxic twofold serial dilutions of pure compounds, chemical portions of woad root extract, and the equal volume of 100 TCID_{50} of the virus were simultaneously added to MDCK cells [21]. After incubation of 2 h at 37°C, the virus/extract mixture was removed from the wells after which maintenance medium was added. The plates were incubated at 37°C under 5% CO_2 for 72 h. The virus-induced CPE was observed under light microscope.

2.8. Statistical Analysis. All the experiments were repeated three times, each with quintuplicate determinations. The data were expressed as mean ± SD. Student's t-test was performed to compare between the control and treatments. A value of $p < 0.05$ was considered as significant difference (∗) and $p < 0.01$ was considered very significant (∗∗).

3. Results and Discussion

3.1. Therapeutic Action of the Pure Compounds and Chemical Portions from Woad Root Extract. In order to investigate the therapeutic effect on influenza virus A, the cells were preinfected with the virus for 2 h followed by the addition of the antiviral compounds and chemical portions from woad root extract. The solvent blank, CB, epigoitrin, and chemical portions of woad root extract had no obvious cytotoxicity (data was not shown). The therapeutic action was evaluated by both CPE assay and MTT assay. Clear cytopathic effects were observed in MDCK cells infected with FM1 after 72 hours such as increased gaps between cells, rupture of the cell nucleus, and the partial or complete collapse of cells (Figure 2(a), virus control). In virus control group, 50%–75% of CPE was observed (see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2537294). However, MDCK cells grow well in the drug treatment groups (Figure 2(a), ribavirin, and PEP + ALK + OA portion groups) and CPE formation was completely inhibited in all the dilutions (Table S1).

MTT reduction assay showed that the addition of CB, epigoitrin, PEP, and PEP + ALK + OA portions from woad root extract significantly increased the viability of MDCK cells preinfected with the virus compared with the virus control group in all the dilutions ($p < 0.01$) (Figure 2(b)). The molecular mechanisms of clemastanin B and epigoitrin for their antiviral activities have not been fully understood. A recent report showed that clemastanin B might target on viral endocytosis and retain the ribonucleoprotein (RNP) of the influenza virus in the nucleus [22]. Interestingly, the protection rate in four treatment groups was significantly higher than that in positive control ribavirin group under the same dilution ($p < 0.05$). This indicated that compounds and extract portions from woad root have better therapeutic action against influenza A virus FM1 than the current commercial synthetic antiviral drug ribavirin. Additionally, the protective effect of CB, epigoitrin, PEP, and PEP + ALK + OA portions was not dose-dependent. The highest protection rate was observed in 1 : 4 dilution of CB, epigoitrin, or the mixture of PEP + ALK + OA portions, while PEP diluted 1 : 8 resulted in the highest cell viability (Figure 2(c)). In comparison to different treatment groups, the mixture of PEP + ALK + OA portions (1 : 4) has the highest cell protection rate.

3.2. Prophylactic Action of the Pure Compounds and Chemical Portions from Woad Root Extract. In order to evaluate the effects of pure compounds and chemical portions from woad root extract on prophylaxis of influenza A virus, the MDCK cells were treated with serial dilution of CB, epigoitrin, PEP, and PEP + ALK + OA portions, respectively. After 4 h, the drugs were removed from the wells and the cells were infected with 100 TCID_{50}. The CPE formation was observed and the cell viability was measured by MTT assay. CPE assay showed that there was no obvious CPE formation in MDCK cells pretreated with CB, epigoitrin, PEP, and PEP + ALK + OA portions in all the dilutions (Table S2).

As shown in Table 1, pretreatment with either pure compounds or chemical portions of woad root extract in all the dilutions significantly improved the viability of MDCK cells ($p < 0.01$). Moreover, compared with ribavirin, natural compounds or extracts from the wood root have higher

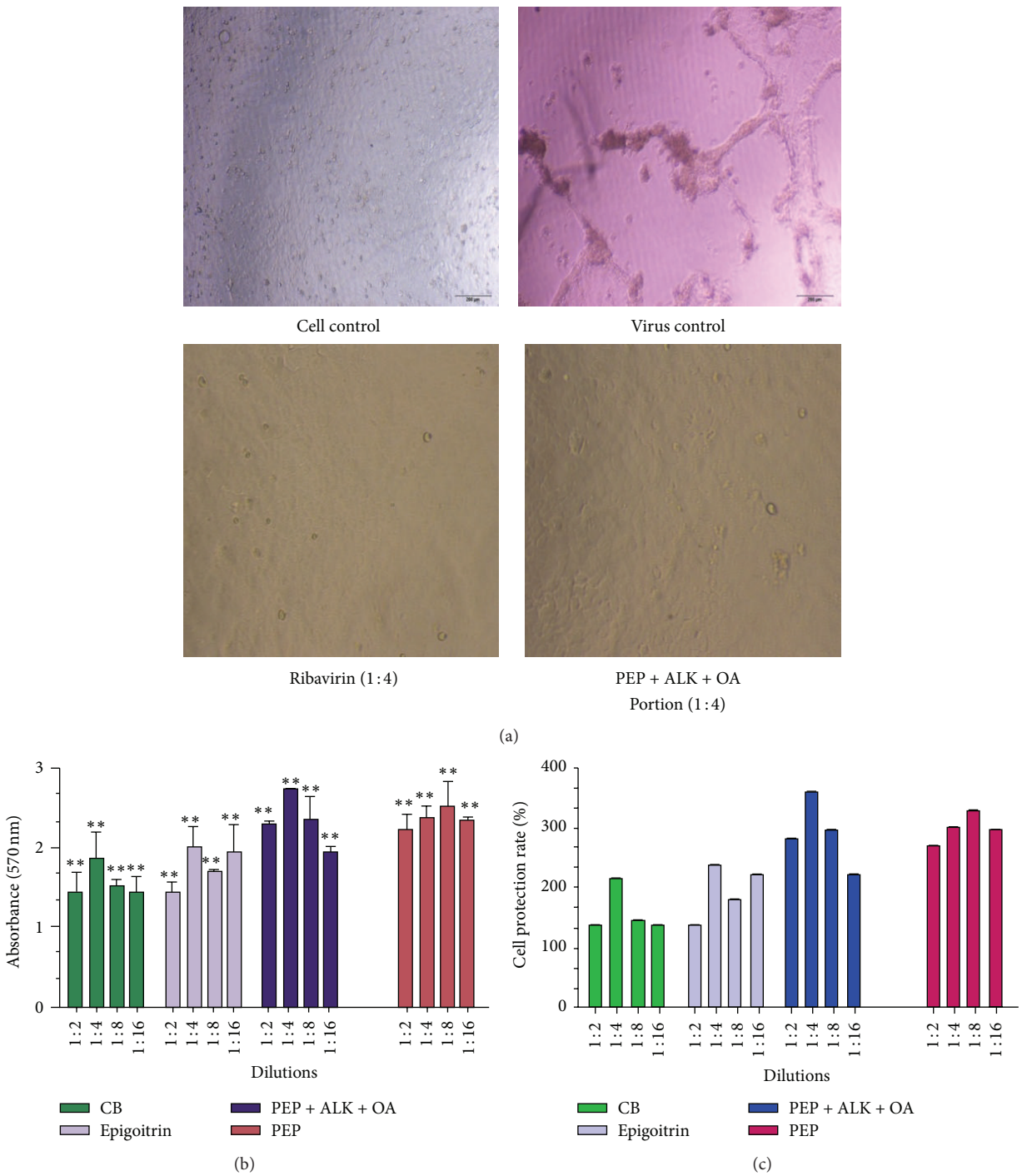

Figure 2: Effect of different dilutions of pure compounds and chemical portions from woad root on the viability of MDCK cells preinfected with 100 $TCID_{50}$ of influenza A H1N1 virus: (a) microscopic analysis; (b) MTT assay; (c) cell protection rate (%). CB: clemastanin B; PEP + ALK + OA: the mixtures of phenylpropanoids, alkaloids, and organic acid portions. Cell control: normal MDCK cells without virus infection and drugs treatment; virus control: cells infected with the virus without drug treatments. Ribavirin (1 : 4): error bars represent standard deviation. The asterisks indicate a significant difference between the test samples and the virus control according to Student's t-test.

prophylactic activity against influenza virus A FM1 ($p < 0.01$). The cell viability was dose-dependently increased by PEP and PEP + ALK + OA portions (Table 1). In contrast, the protection rate and cell viability were not significantly changed by the dilution of CB and epigoitrin (from 1 : 2 to 1 : 16). Among the four different natural products used in this study, PEP portion showed the most significant protective effect with the cell protection rate of 263.467%.

Table 1: Effect of prophylactic treatment on the viability and protection rate of MDCK cells.

Groups	OD_{560}				Protection rate (%)			
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 2	1 : 4	1 : 8	1 : 16
CB (50 μg/mL)	$2.126 \pm 0.034^{**}$	$2.228 \pm 0.167^{**}$	$2.284 \pm 0.017^{**}$	$2.218 \pm 0.011^{**}$	188.348	199.08	205.023	198.08
Epigoitrin (50 μg/mL)	$2.197 \pm 0.152^{**}$	$2.239 \pm 0.050^{**}$	$2.202 \pm 0.102^{**}$	$2.041 \pm 0.036^{**}$	195.871	200.29	196.344	214.435
PEP portion (1 mg/mL)	$2.840 \pm 0.150^{**}$	$2.464 \pm 0.024^{**}$	$2.407 \pm 0.048^{**}$	$2.149 \pm 0.069^{**}$	263.467	223.91	217.912	190.821
PEP + ALK + OA (1 mg/mL)	$2.698 \pm 0.082^{**}$	$2.090 \pm 0.042^{**}$	$2.045 \pm 0.089^{**}$	$2.171 \pm 0.028^{**}$	248.58	184.61	179.879	193.135
Ribavirin (100 μg/mL)	$1.866 \pm 0.251^{**}$	$1.924 \pm 0.306^{**}$	$1.614 \pm 0.086^{**}$	$1.869 \pm 0.016^{**}$	161.047	167.1	134.245	161.31
Virus control		0.335 ± 0.073						
Cell control		1.286 ± 0.277						

PEP: phenylpropanoids portion; PEP + ALK + OA: the mixtures of phenylpropanoids, alkaloids, and organic acid portions. Cell control: normal cells without virus infection and drug treatments. Virus control: cells infected with influenza A FM1 virus. Data of OD_{560} was mean ± SD. The asterisks indicate a significant difference between the test samples and the virus control according to Student's t-test. $^{**}p < 0.01$.

Table 2: Inhibitory effect of pure compounds and chemical portions from woad root extract on influenza A FM1 virus attachment.

Groups	OD_{560}				Protection rate (%)			
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 2	1 : 4	1 : 8	1 : 16
CB (50 μg/mL)	$1.940 \pm 0.262^{**}$	$1.665 \pm 0.058^{**}$	$1.599 \pm 0.161^{**}$	$1.368 \pm 0.210^{**}$	201.267	161.455	151.973	118.458
Epigoitrin (50 μg/mL)	$2.192 \pm 0.108^{**}$	$1.980 \pm 0.246^{**}$	$1.833 \pm 0.049^{**}$	$1.639 \pm 0.347^{**}$	237.749	207.058	185.849	157.763
PEP portion (1 mg/mL)	$2.437 \pm 0.190^{**}$	$2.340 \pm 0.123^{**}$	$2.020 \pm 0.319^{**}$	$1.695 \pm 0.041^{**}$	273.218	259.175	212.921	165.798
PEP + ALK + OA (1 mg/mL)	$2.043 \pm 0.072^{**}$	$2.055 \pm 0.009^{**}$	$1.743 \pm 0.282^{**}$	$1.599 \pm 0.069^{**}$	216.178	217.988	172.819	151.900
Ribavirin (100 μg/mL)	$1.614 \pm 0.093^{**}$	$1.736 \pm 0.307^{**}$	$1.441 \pm 0.001^{**}$	$1.279 \pm 0.018^{**}$	154.144	171.734	129.099	105.646
Virus control		0.549 ± 0.690						
Cell control		1.24 ± 0.675						

PEP: phenylpropanoids portion; PEP + ALK + OA: the mixtures of phenylpropanoids, alkaloids, and organic acid portions. Cell control: normal cells without virus infection and drug treatments. Virus control: cells infected with influenza A FM1 virus. Data of OD_{560} was mean ± SD. The asterisks indicate a significant difference between the test samples and the virus control according to Student's t-test. $^{**}p < 0.01$.

3.3. Direct Virucidal Action of the Pure Compounds and Chemical Portions from Woad Root Extract. Next, we investigated the effect of CB, epigoitrin, PEP, and PEP + ALK + OA portions on the inactivation of influenza virus A FM1 at 37°C. The cells without treatment were shown in Figure 3(a) and the virus control was in Figure 3(b). The MDCK cells infected with influenza virus and cocultured with CB, epigoitrin, PEP, or PEP + ALK + OA showed pyknosis condensation and even lysis (Figures 3(c)–3(f)). The surviving virus titer was 0.01 $TCID_{50}$ in all the experimental groups. However, positive control ribavirin inhibits the CPE formation completely in all the dilution levels (Figure 3(g)). These observations revealed that CB, epigoitrin, PEP, and PEP + ALK + OA could not directly inactivate influenza virus A FM1 even at the concentration of 1 : 2 dilution. In a previous study, Hsuan et al. also found that the inhibition of pseudorabies virus by the methanol extract of woad leaves extract was not due to the direct virus inactivation [12].

3.4. Inhibitory Activity of the Pure Compounds and Chemical Portions from Woad Root Extract on Influenza Virus A FM1 Attachment. Influenza A virus attachment is primarily mediated by two types of glycoproteins called hemagglutinin and neuraminidase [23]. In this study, we investigated whether natural occurring compounds and chemical portions from woad extract inhibit virus attachment to the host cells. The results of CPE assay were listed in Table S3. There was no CPE formation in all the drug treatment groups even at the lowest concentration (1 : 16 dilution). The results suggested that CB, epigoitrin, PEP, and PEP + ALK + OA portions have a strong inhibitory effect on binding of influenza A virus to MDCK cells.

The viability of MDCK cells at 72 h after the infection of virus and simultaneous treatment with natural compounds was determined by MTT reduction assay. As shown in Table 2, the viability of MDCK cells was significantly increased as the result of drug treatments compared with virus control ($p < 0.01$). The inhibition of virus adsorption to the host cells by CB, epigoitrin, and PEP was in a dose-dependent manner. The maximum inhibitory effect of CB, epigoitrin, and PEP was observed with a 1 : 2 dilution, which resulted in 3.53, 3.99, and 4.43 times' increase in cell viability, respectively. In contrast, the highest cell protection rate in PEP + ALK + OA and positive control ribavirin groups was found in a 1 : 4 dilution. Additionally, it was shown that CB, epigoitrin, PEP, and PEP + ALK + OA are more effective than ribavirin on the inhibition of influenza A FM1 virus attachment (Table 2). When comparing four natural compounds and chemical portions, it was observed that PEP portion diluted 1 : 2 possessed the maximum inhibitory effect of virus absorption, which led to the highest protection of 273.218%.

The classically defined antiviral mechanisms for medicinal plants include inhibiting virus replication, blocking virus

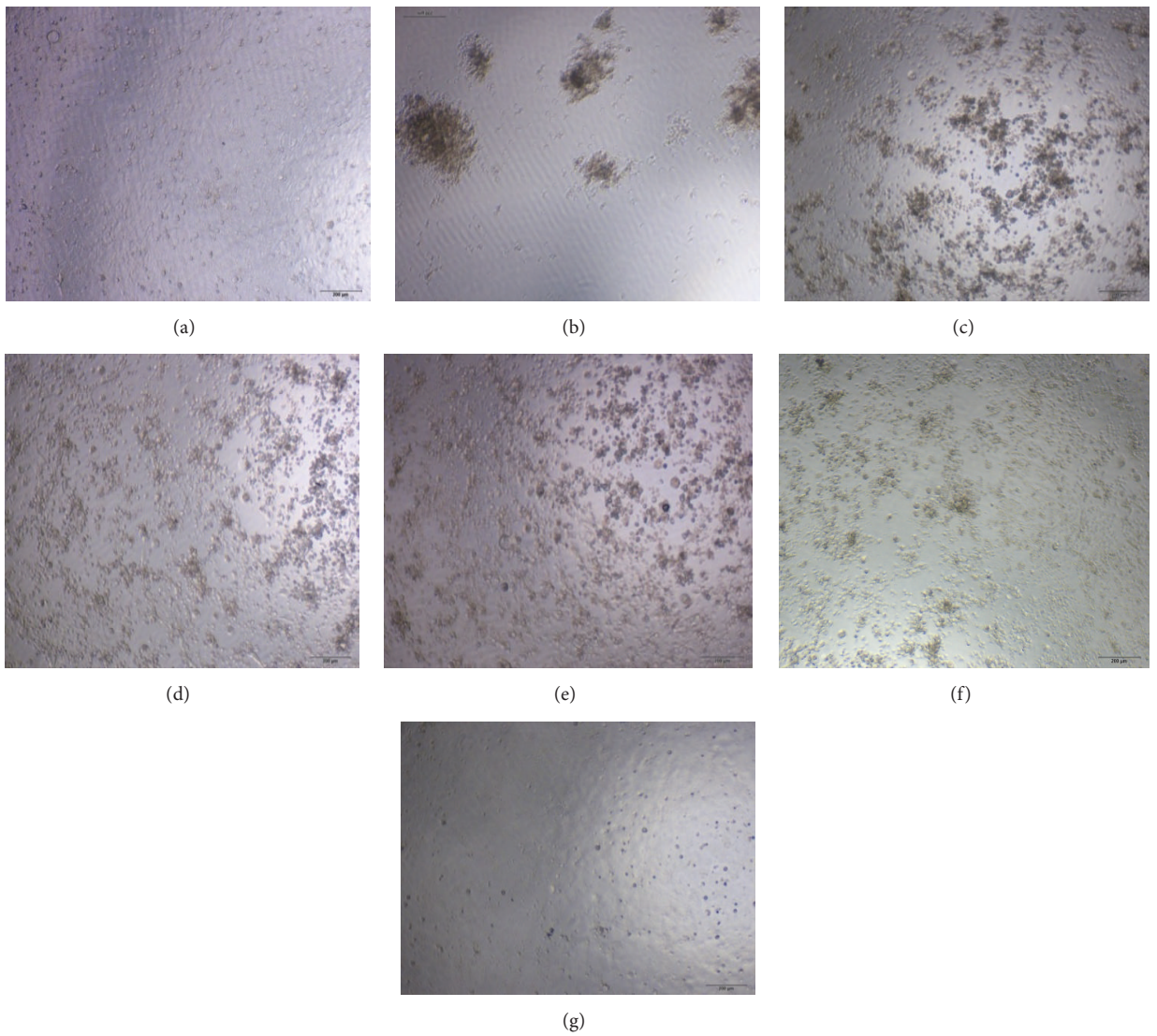

Figure 3: Direct virucidal effect of pure compounds and chemical portions from woad root extract on influenza A1 virus FM1: (a) cell control; (b) virus control; (c) CB (1 : 2); (d) epigoitrin; (e) PEP (1 : 2); (f) PEP + ALK + OA (1 : 2); (g) ribavirin (1 : 4).

attachment, direct inactivating the virus, and preventing from virus infection [24]. In this study, it was clearly demonstrated that CB, epigoitrin, PEP, or PEP + ALK + OA showed the anti-influenza activities by therapeutic action (inhibition of virus multiplication), prophylaxis, and inhibition of virus attachment. However, differences were observed on the major modes of antiviral action in different compounds and chemical portions (Figure 4). For instance, the highest cell protection rate in PEP or PEP + ALK + OA was from its therapeutic action (Figure 4, PEP, A1; PEP + ALK + OA, A1). The main anti-influenza modes for epigoitrin are the inhibition of virus multiplication and virus attachment (Figure 4, epigoitrin A1, epigoitrin A3). In contrast, three modes of antiviral action of CB contribute equally on the cell protection rate.

CB is the major phenylpropanoid compound in woad root and epigoitrin is the abundant alkaloid and indicator for the quality control of woad root [16]. Previous studies reported that the overall virus inhibitory effect of green tea is stronger in the plant total extract than the single pure compound from the extract due to the possible synergistic interactions between the ingredients in the extract [18, 25]. However, our results suggested that the change of antiviral activity might be because of the differences of antiviral mechanisms between the single compound and the mixture of the extract.

4. Conclusion

In the present study, the modes of anti-influenza action of the chemical portions and constituents from woad root extract were investigated. Our results revealed that CB, epigoitrin, PEP, or PEP + ALK + OA demonstrated their anti-influenza activities by therapeutic action, prophylaxis of cells,

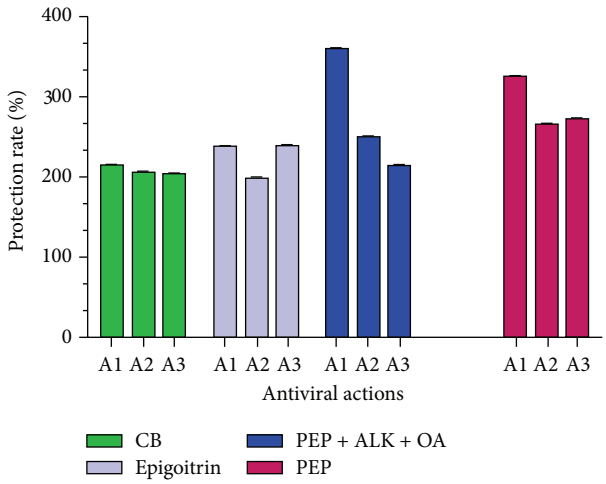

Figure 4: Differences on the major modes of antiviral action in the pure compounds and chemical portions from woad root extract. A1: therapeutic action; A2: prophylaxis; A3: inhibition of virus attachment. CB: clemastanin B; PEP + ALK + OA: the mixtures of phenylpropanoids, alkaloids, and organic acid portions. The concentration for CB: A1 (1 : 4 dilution), A2 (1 : 8 dilution), and A3 (1 : 2 dilution); epigoitrin concentration: A1 (1 : 4), A2 (1 : 16), and A3 (1 : 2); PEP + ALK + OA concentration: A1 (1 : 4), A2 (1 : 2), and A3 (1 : 4); PEP concentration: A1 (1 : 8), A2 (1 : 2), and A3 (1 : 2).

and inhibition of virus attachment. All the compounds or chemical portions tested do not have direct virucidal activity. The main antiviral mode for PEP and PEP + ALK + OA is the therapeutic action, while epigoitrin mainly inhibits the virus multiplication and attachment. To our knowledge, this is the first report on the antiviral mechanism of the compounds and chemical portions from woad root extract.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Jia-Hang Su and Rui-Gang Diao contributed equally to this work.

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Research Article

Antibacterial Activity of *Salvadora persica* L. (Miswak) Extracts against Multidrug Resistant Bacterial Clinical Isolates

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Much effort has focused on examining the inhibitory effect of *Salvadora persica* (miswak) on oral microorganisms, but information concerning its antibacterial activity against other human pathogens, particularly multidrug resistant (MDR) isolates, is scarce. Therefore, this study aimed to assess the in vitro antibacterial activities of *Salvadora persica* L. extracts against 10 MDR bacterial clinical isolates other than oral pathogens. The antibacterial activity of aqueous and methanol miswak extracts was assessed using the agar dilution and minimum inhibitory concentration (MIC) methods. Overall, the 400 mg/mL of miswak extract was the most effective on all strains. The methanol extract exhibited a stronger antibacterial activity against Gram-negative (3.3–13.6 mm) than Gram-positive (1.8–8.3 mm) bacteria. The lowest MIC value was seen for *E. coli* (0.39, 1.56 μg/mL), followed by *Streptococcus pyogenes* (1.56 μg/mL). The highest MIC value (6.25, 12.5 μg/mL) was recorded for methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*. This study demonstrates, for the first time, the moderate to strong antibacterial activity of miswak extracts against all tested MDR-pathogens. Methanol extract appears to be a potent antimicrobial agent that could be considered as complementary and alternative medicine against resistant pathogens. Further studies on a large number of MDR organisms are necessary to investigate and standardize the inhibitory effect of miswak extracts against these emerging pathogens.

1. Introduction

The emergence of multidrug resistant (MDR) human bacterial pathogens during 1990s and more recently the extensively resistant clinical isolates is hampering efforts to control and manage human infections by these organisms [1]. The development of antimicrobial resistance due to misuse of antibiotics is worrisome [2]. Moreover, continuous increase in the global isolation rates of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and carbapenem-resistant Gram-negative bacilli clinical isolates poses a serious therapeutic problem because no new antimicrobial agents are currently available for treatment of infected patients [2–4].

Plants are important in human's life and fulfill his every day's needs. They are used as cosmetics, food, flavors, ornamental, and medicine. Medicinal plants have become part of complementary medicine worldwide, because of their potential health benefits. Various plant extract has great potential against infectious agents and can be used for therapeutic purposes [5, 6].

The toothbrush tree, *Salvadora persica* L, also called miswak, belonging to the Salvadoraceae family, is one of the most important ones among 182 species of plants being used as chewing sticks. It has been widely used in many Asian, African, and Middle Eastern countries. The roots, twigs, and stems of this plant have been used for oral hygiene and small

miswak sticks have been used as toothpicks for maintaining oral hygiene [7, 8]. It has been reported that the aqueous and methanol extracts of miswak possess various biological properties against organisms considered important for the development of dental plaque and periodontitis [9].

Previous in vitro studies have reported the antibacterial and antifungal effects of miswak on cariogenic bacteria and periodontal pathogens including *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Lactobacillus acidophilus*, *Pseudomonas aeruginosa*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Haemophilus influenzae*, and *Candida albicans* [10–16]. Moreover, data from controlled clinical studies showed that *Salvadora persica* extract is also an effective antimicrobial agent when utilized clinically as an irrigant in the endodontic treatment of teeth with necrotic pulps [17–20].

Much effort has focused on examining the inhibitory effect of *Salvadora persica* on oral organisms, but information concerning the antibacterial activity of *Salvadora persica* against other human pathogens, particularly MDR isolates, is scarce [14, 15]. Therefore, this study aimed to assess the in vitro antibacterial activities of *Salvadora persica* L. extracts against 10 MDR bacterial clinical isolates (other than oral pathogens) including methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), penicillin-resistant *Streptococcus pyogenes*, *Enterococcus faecalis*, and 6 carbapenem-resistant Gram-negative bacilli: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*.

2. Materials and Methods

This in vitro descriptive study was conducted during the period from March to August 2015. A total of 10 pathogenic MDR bacterial strains other than oral pathogens were included in this study.

2.1. Bacterial Clinical Isolates. All bacterial strains were isolated from clinical specimens of hospitalized patients with nosocomial infections identified according to the Centers for Disease Control and Prevention/National Healthcare Safety Network (CDC/NHSN) criteria [22]. The selected organisms were MRSA, MRSE, penicillin-resistant *Streptococcus pyogenes*, *Enterococcus faecalis*, and 6 carbapenem-resistant Gram-negative bacilli: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*. All isolates were identified to the species level using standard methods and verified using the VITEK-2 system (Biomérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Seven nosocomial strains were isolated from urinary tract infection, two strains were isolated from blood stream infection, and one strain was isolated from ventilator-associated pneumonia. Antimicrobial resistance patterns of all isolates were determined using the reference broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [21]. Methicillin resistance among *S. aureus* and *S. epidermidis* strains was identified by PCR amplification of the *mec*A gene as previously described [23]. Carbapenem resistance among Gram-negative bacilli was confirmed using the modified Hodge test following the CLSI guidelines [21]. Multidrug resistance (MDR) of the isolates was identified by resistance to ≥3 of the following antimicrobial classes: penicillins, cephalosporins, aminoglycosides, carbapenems, and quinolones [21].

2.2. Miswak Collection. *Salvadora persica* chewing sticks were purchased from the local market of Jazan Province in Saudi Arabia during spring 2015 when plants were flowering. The sticks were washed with distilled water, cut into small species, and allowed to dry at room temperature for 2 weeks. Then, they were grounded to powder using electrical blinder.

2.3. Extracts Preparation. Preparation of aqueous and methanol extracts was carried out by mixing 100 g of *Salvadora persica* powder with 1 L of distilled water (for aqueous extract) and 95% methanol (Sigma-Aldrich, St. Louis, USA), with methanol extract for 24 h. The mixture was then filtered using Whatman number 1 filter paper, and the filtrate was then evaporated in vacuum evaporator at 60°C (for aqueous) and 40°C (for methanol). The extracts were stored in sterile bottles and kept frozen at −20°C until further use [24]. Before testing, the miswak extracts were freshly reconstituted in methanol (for methanolic extract) and water (for aqueous extract) at a final concentration of 400 mg/mL which was used to further prepare serial dilutions (400–50 mg/mL).

2.4. Inoculum Preparation. All bacterial isolates were grown to the exponential phase in tryptic soy broth (TSB) (Difco Laboratories, Detroit, USA) at 37°C for 18 h. The bacterial growth was estimated as turbidity using spectrophotometer to measure the light absorption of the microbial mass as determined by the optical density readings at 620 nm (OD_{620}). Growth was checked every 30 minutes, and the exponential phase of bacterial growth was identified by the increased OD_{620} reading. Then, the inoculum density of each bacterial suspension was adjusted to a final density equivalent to 0.5 McFarland Standard (1.5×10^8 CFU/mL) in sterile saline (0.84% NaCl).

Antimicrobial Testing. The antimicrobial activity of miswak extracts was carried out using the agar diffusion and minimal inhibitory concentration (MIC) methods.

2.4.1. Agar Diffusion Method. The antimicrobial testing was performed on Mueller Hinton agar plates (Difco Laboratories) using the agar diffusion method. Briefly, 100 μL of bacterial suspension was spread smoothly on the agar plates. The required numbers of wells, each 3 mm in diameter, were cut out of the agar using a sterile glass capillary ensuring proper distribution of holes in the periphery and one in the center for each agar plate. Then, wells were filled with 50 μL of sterile extract (aqueous or methanol) made from *Salvadora persica* stock solution (400, 200, 100, and 50 mg/mL). This

Table 1: The antibacterial activities of aqueous and methanol extracts of miswak against 10 MDR pathogenic organisms.

MDR bacterial isolates*	The diameter of inhibition zones (mm)									
	Aqueous extract (mg/mL)				Methanol extract (mg/mL)				Positive control**	
	400	200	100	50	400	200	100	50	VAN (30 μg)	TOB (10 μg)
MRSA	6.2	5.1	3.8	3.0	8.3	5.1	3.4	2.0	18	NT
MRSE	6.0	4.8	3.2	2.7	7.6	5.8	3.1	1.8	19	NT
Streptococcus pyogenes	6.4	4.5	3.5	2.6	7.4	5.4	3.0	2.2	21	NT
E. faecalis	6.1	5.2	4.0	3.2	7.8	6.1	5.0	2.9	19	NT
E. coli	12.3	9.7	7.2	5.3	13.6	9.2	6.5	4.8	NT	21
K. pneumonia	11.8	8.5	7.0	4.3	12.7	8.7	5.8	4.6	NT	20
P. aeruginosa	10.5	8.0	5.9	4.0	10.2	8.8	6.2	4.2	NT	17
S. marcescens	12.0	9.2	5.6	4.8	12.5	8.1	5.0	4.5	NT	18
A. baumannii	8.5	7.3	4.6	3.2	9.8	8.0	5.2	3.5	NT	16
S. maltophilia	8.2	7.0	4.8	3.0	9.5	7.6	4.9	3.3	NT	16

*MRSA: methicillin-resistant *staphylococcus aureus*, MRSE: methicillin-resistant *staphylococcus epidermidis*.
**VAN: vancomycin, TOB: tobramycin, and NT: Not tested.
The CLSI zone diameter interpretive criteria for vancomycin (VAN): ≥15 mm: susceptible, 13-14 mm: intermediate, and ≤12 mm: resistant [21].
The CLSI zone diameter interpretive criteria for tobramycin (TOB): ≥17 mm: susceptible, 15-16 mm: intermediate, and ≤14 mm: resistant [21].

was followed by 2 h preincubation at room temperature for proper diffusion of the plant extract into the media. Then, the plates were incubated at 37°C for 24 h [25]. The mean diameter of complete growth inhibition zone (in mm) was measured without the well's diameter and considered as the inhibition zone. Antibiotic discs (Difco Laboratories, Detroit, USA), 30 μg vancomycin for Gram-positive isolates and 10 μg tobramycin for Gram-negative strains, were used as positive controls, and water (for aqueous extract) or methanol (for methanol extract) was used as negative control. The test for each microorganism was repeated three times to ensure reproducibility. The average zones diameter values from three repeats were taken in determination of the final inhibition zones. This was done to ensure that all inhibition zones within each experiment were obtained under the same experimental conditions.

2.4.2. The Minimal Inhibitory Concentration (MIC). The MIC of the *Salvadora persica* extracts was determined using the standard microdilution method in 96 multi-well microtiter plates, as previously described [26], with slight modifications. Briefly, the dissolved extracts were first diluted to a concentration of 50 mg/mL, then 50 μL from each of the aqueous and methanol extracts was pipetted into the first well of each microtiter plate row, and 50 μL of TSB was distributed from the 1st to the 12th well of each row. Twofold serial dilution was achieved by transferring 50 μL of scalar dilution from the first to the subsequent wells of each row. The final concentration of the extracts adopted to evaluate antibacterial activity was included from 25 mg/mL to 0.003 mg/mL. Finally, 10 μL of each bacterial suspension was added to each well. Two row lines in each plate were used as controls: one row line with vancomycin as a positive control for Gram-positive isolates and another row line with tobramycin for Gram-negatives strains (in a serial dilution of 32–0.015 μg/mL). Plates were incubated at 37°C for 18–24 h. The lowest concentration at which no turbidity occurred was taken as the MIC value. Plates were analyzed individually to determine MIC and the average MIC values from three repeats were taken in determination of the final MIC values for each extract to ensure accuracy and reproducibility.

3. Results

The antibacterial activities of aqueous and methanol extracts of *Salvadora persica* against 10 MDR pathogenic organisms are listed in Table 1. Overall, the 400 mg/mL of miswak extracts was the most effective ones on all strains. The methanol extract of miswak had growth inhibitory effects on the tested pathogens more than aqueous extract. The methanol extract exhibited a stronger antibacterial activity against Gram-negative (3.3–13.6 mm) than Gram-positive (1.8–8.3 mm) bacteria. The highest growth inhibition was recorded against *E. coli* (4.8–13.6 mm), followed by *K. pneumoniae* (4.6–12.7 mm) and *Serratia marcescens* (4.5–12.5 mm). *Streptococcus pyogenes* was the most susceptible one to methanol extract among all Gram-positive pathogens with inhibition zone diameter of 2.2–7.4 mm.

The MIC values of aqueous and methanol extracts are presented in Table 2. The lowest MIC value was seen for *E. coli* (0.39 mg/mL for methanol extract and 1.56 mg/mL for aqueous extract), followed by *Streptococcus pyogenes* (1.56 mg/mL for both extracts). The highest MIC value (12.5 mg/mL for aqueous extract and 6.25 mg/mL for methanol extract) was recorded for MRSA, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*.

4. Discussion

Antimicrobial resistance has always been a global health concern challenging treatment of human infections caused by MDR-bacterial pathogens [1]. This problem has opened a wide range of research studies investigating the possible use of natural plant extracts in traditional medicine. In the present

Table 2: The MIC values of aqueous and methanol extracts of miswak against 10 MDR pathogenic organisms.

MDR bacterial isolates*	MIC values (mg/mL) Miswak extracts		MIC values (μg/mL)** Positive control	
	Aqueous	Methanol	VAN	TOB
MRSA	12.5	6.25	1.0	NT
MRSE	6.25	3.125	0.5	NT
Streptococcus pyogenes	1.56	1.56	0.125	NT
E. faecalis	3.125	3.125	0.5	NT
E. coli	1.56	0.39	NT	0.25
K. pneumonia	3.125	0.781	NT	0.5
P. aeruginosa	6.25	1.56	NT	1.0
S. marcescens	1.56	1.56	NT	2.0
A. baumannii	12.5	6.25	NT	8.0
S. maltophilia	12.5	6.25	NT	4.0

*MRSA: methicillin-resistant *staphylococcus aureus*, MRSE: methicillin-resistant *staphylococcus epidermidis*.
**VAN: vancomycin, TOB: tobramycin, and NT: not tested.
The CLSI MIC Interpretive Criteria for vancomycin (VAN): ≤2 μg/mL: Susceptible, 4–8 μg/mL: Intermediate, ≥16 μg/mL: Resistant [21].
The CLSI MIC interpretive criteria for tobramycin (TOB): ≤4 ug/mL: susceptible, 8 μg/mL: intermediate, and ≥16 μg/mL: resistant [21].

study, a variety of MDR Gram-positive and Gram-negative bacteria were used in screening antimicrobial activity of aqueous and methanol extracts of *Salvadora persica*.

In this study, the 400 mg/mL of miswak extracts was the most effective one on all pathogens and the methanol extract exhibited a stronger antibacterial activity against Gram-negative than Gram-positive bacteria. These results are in agreement with previous findings from other studies [10, 11, 13, 16, 17]. Contrary to these findings, Al-Bayati and Sulaiman investigated the aqueous and methanol extracts of *Salvadora persica* for antimicrobial activities against seven oral pathogens [12]. In their study, the aqueous extract inhibited all isolated microorganisms and was more efficient than the methanol extract. It is well known that the antimicrobial property of *Salvadora persica* extracts is attributed to the different phytochemical constituents. Mohammed investigated the phytochemical constituents of *Salvadora persica* extracts and revealed the presence of flavonoids, sterols, saponins, tannins, basic alkaloids, and reducing components in methanol extract and saponins, tannins, and reducing components in aqueous extract which could be responsible for the observed antimicrobial property of methanol extract compared with aqueous extract [16]. Sofrata et al. identified a volatile compound: benzyl isothiocyanate (BITC) in *Salvadora persica* extracts [27]. In their study, the BITC exhibited rapid and strong bactericidal effect against Gram-negative bacteria but low effect on Gram-positive bacteria. The authors speculated that BITC might penetrate through the outer bacterial membrane and possibly interfere with the bacterial redox systems and thus hamper the ability of the bacterium to maintain its membrane potential [27].

In this study, the methanol extract had promising MIC values against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. marcescens* among Gram-negative pathogens and *Streptococcus pyogenes* among Gram-positive bacteria. Previous studies have reported that *Salvadora persica* extracts were effective against *S. aureus*, *Streptococcus mutans*, *Streptococcus pyogenes*, *E. faecalis*, and *P. aeruginosa* [9–13, 16, 17]. However, high MIC values were reported in this study against MRSA, *A. baumannii*, and *S. maltophilia*. To the best of our knowledge, this is the first study investigating the antibacterial activity of miswak extracts against these MDR-pathogens. Our finding is of considerable concern. These emerging pathogens become a significant health problem because of their remarkable ability to innate and acquire resistance to multiple antimicrobial classes and to survive in nosocomial environments [1, 3, 4].

5. Conclusion

This study demonstrates, for the first time, the strong to moderate antibacterial activity of methanol and aqueous miswak extracts against all tested MDR-pathogens. However, methanol extract has stronger antibacterial effect, and it appears to be a potent antimicrobial agent that could be considered as complementary and alternative medicines against resistant pathogens. Further in vitro and in vivo studies on a large number of clinical isolates of MRSA, *A. baumannii*, and *S. maltophilia* are necessary to further investigate and standardize the inhibitory effect of miswak extracts against these emerging pathogens.

Disclosure

Mohamed Saeed Zayed Al-Ayed is Dean of College of Applied Medical Sciences and Ahmed Morad Asaad is Coordinator of Microbiology Department, College of Medicine, Najran University.

Conflict of Interests

None of the authors had a conflict of interests.

Authors' Contribution

Ahmed Morad Asaad and Abduljabbar Hadi AlMarrani designed this study, for which Abduljabbar Hadi AlMarrani secured miswak plant. Ahmed Morad Asaad, Hany Goda Attia, and Mohamed Ansar Qureshi performed laboratory work. Mohamed Saeed Zayed Al-Ayed and Abduljabbar Hadi AlMarrani collected data and references. Ahmed Morad Asaad and Mohamed Ansar Qureshi wrote the paper, which was revised by Mohamed Saeed Zayed Al-Ayed. All authors read and approved the final paper.

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