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Antibacterial activity of leaf extract of *Breonadia salicina* (Rubiaceae), an endangered medicinal plant of Saudi Arabia

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ABSTRACT. Wild plants can contain bioactive compounds with potential activity against disease-causing microorganisms. In the Kingdom of Saudi Arabia, there are many plant species that may have antibacterial, antifungal, or antiviral activities, among other properties. We extracted bioactive compounds with methanol as well as with water from leaves of *Breonadia salicina*, which is an endangered plant found in the wild in Saudi Arabia. These extracts were tested against the bacteria *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Shigella sonnei*, *Escherichia coli*, and *Staphylococcus aureus*. Both extracts showed antibacterial activity against all of the microorganisms, and thus, *B. salicina* leaf extract has potential as an antimicrobial agent for the preservation of foods, instead of synthetic chemical compounds. We found that the methanolic leaf extract was more effective than the aqueous crude extract against *B. subtilis*, *P. aeruginosa*, and *S. aureus*.

Key words: Minimal inhibitory concentration; Natural preservative; Food poisoning; Endangered plant

INTRODUCTION

Breonadia salicina is a tree species that reaches up to 20 m in height and 80 cm in diameter, growing along high escarpments of 500 to 2000 m in Saudi Arabia. Recent explorations indicate its presence in southwestern Saudi Arabia in Jizan, Rabua, and Al-Baha Provinces. It has medicinal as well as economic value, where it is widely used to treat cancer, gastrointestinal illness, fevers, headaches, arthritis, diabetes, inflamed wounds, ulcers, and bacterial and fungal infections. It also has antidiarrheal properties (Sibandze et al., 2010). The bark of *B. salicina* is reported to be an astringent (Doke and Vilakazi, 1972). All these medicinal properties of *B. salicina* are due to the presence of various groups of bioactive compounds, which are produced in different parts of the plant in response to various environmental conditions.

Wild plants have different medicinal value due to the presence of various phytoconstituents that are produced in response to different environmental factors. Medicinal and aromatic plants are sources of essential oils, pharmaceuticals, colorants, dyes, cosmetics, and biocides (Lubbe and Verpoorte, 2011). These medicinal plants have wide applications in industry as antioxidants (Puangpronpitag and Sittiwet, 2009) and antimicrobials (Weerakkody et al., 2010). The extracts of different parts of various plant species have antioxidant properties (Sulaiman and Ooi, 2012; Nair et al., 2012; Jaberian et al., 2013). Green tea and grape seed extract are widely used for their antimicrobial, anticarcinogenic, and anti-inflammatory properties (Perumalla and Hettiarachchy, 2011). Nowadays, the food industry uses various chemical preservatives to protect foods from contamination by various microorganisms (Natta et al., 2008). However, synthetic preservatives could be toxic, carcinogenic, or teratogenic and may cause various health problems (Agatemor, 2009; Pundir et al., 2010). Such additives should be avoided in the food industry and other alternatives be adopted (Agatemor, 2009).

The selection of solvent for extraction is very important, so that more compounds and in larger amounts can be obtained. Various solvents are used for the extraction of bioactive compounds; however, most of the solvents used for extraction are chloroform, ethanol, hexane, methanol, and diethyl ether (Gamboa et al., 2003; Guerrero-Rodriguez et al., 2007; Jasso de Rodriguez et al., 2007). All bioactive compounds have different solubility in different solvents. Therefore, the selection of the organic solvent is very important to obtain more extract with different types of compounds. These new solvents must be recognized as safe to be used under organic production systems (Castillo et al., 2010).

Staphylococcus aureus and *Escherichia coli* are common food-poisoning bacteria (Furukawa et al., 2009; Argudin et al., 2010; Di Giannatale et al., 2011; Hennekinne et al., 2012; Xue and Zhang, 2013). *S. aureus* produces a wide variety of toxins. They are active in high nanogram to low microgram quantities and resistant to conditions such as heat treatment and low pH, hence retaining their activity in the digestive tract after ingestion (Evenson et al., 1988). *Bacillus subtilis* is associated with outbreaks of food poisoning (Kramer et al., 1982); however, how it causes food poisoning is not clear. *Pseudomonas aeruginosa* also causes food poisoning (Meitert et al., 1984). Shigellosis is a major public health concern worldwide, especially in developing countries (Wold Health Organization, 2006). It is an acute intestinal infection caused by bacteria of the genus *Shigella* (Koh et al., 2012). Infection is most frequent in children, the elderly and the immunocompromised (Wold Health Organization, 2006; Pichel et al., 2007). The antimicrobial activity of *B. salicina* leaf extract has not been previously evaluated against foodborne pathogens. However, this plant is one of the most endangered species

in Saudi Arabia, with a dwindling population size that may lead to its extinction, and therefore, there is urgent need for conservation efforts using biotechnological approaches (Khan et al., 2012). The aim of the present study was to test different leaf extracts of *B. salicina* against some pathogens known to cause food poisoning.

MATERIAL AND METHODS

Plant material

In the present study, *B. salicina* was collected from three places in Saudi Arabia, including Jizan, Asir, and Al-Baha, for the evaluation of antibacterial activities (Figure 1). All populations sampled were identified morphologically by a botanist at King Saud University, Riyadh, Saudi Arabia. The collected leaves were dried at room temperature for pulverization and solvent extraction.

Figure 1. Photograph of *Breonadia salicina* taken from wild condition.

Preparation of plant extract

The leaves were finely ground with a mortar and pestle. The powered leaves (50 g) were suspended in 100 mL methanol and extracted for 24 h on a rotary shaker at 25°C. The mixture was filtered and the above step repeated to extract more bioactive compounds. The two filtrates were pooled and placed in a sterile conical flask and kept at room temperature for evaporation of methanol. Finally, the extract was dissolved in 1 mL methanol for the evaluation of its antimicrobial activity. Another 50-g portion of leaf powder was extracted with 100 mL water for 1 h in a boiling water bath. The mixture was filtered with a 0.2-mm Whatman filter paper, and the residue was suspended in the same volume of water and kept for 1 h at the same temperature in a water bath. The two filtrates were pooled and dried at 40°C on a water bath. The extract obtained was

filtered through a 0.2-mm filter paper, and the solvent was removed by rotary evaporation under reduced pressure. The dried powder was dissolved in 1 mL autoclaved distilled water to test for antimicrobial activity. The resulting crude extract was then stored at 4°C until further analysis.

Bacterial strain culture

The foodborne pathogens selected for evaluating the antimicrobial activity of *B. salicina* leaf extract were the Gram-negative bacteria *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853), *Shigella sonnei* (ATCC 11060), and *E. coli* (ATCC 442) and the Gram-positive *B. subtilis* (ATCC 10400). LB Miller broth was used for bacterial cultures. All bacteria were grown at 37° ± 1°C to obtain 3 x 10^6 cells per mL in exponential phase. The experiments were carried out in triplicate.

Determination of minimum inhibitory concentrations (MIC)

Both methanolic and aqueous leaf extracts were used for the determination of MIC. MIC was determined using the disk diffusion method (Indian Pharmacopoeia Committee, 1996). Both extracts were tested at concentrations of 0.5, 1, 1.5, 2, 2.5, and 3 mg per disk. A paper disk without leaf extract was taken as the negative control. The plates were incubated at 37° ± 1°C for 24 h, and the presence of growth was determined with the naked eye. The minimum concentration that inhibited bacterial growth was determined as the MIC for that extract.

Antibacterial activity

The crude extract was tested against six bacteria. The extracted bioactive compounds were dissolved and diluted with extract solvent. Serial dilution was performed for each concentration. The inhibition zone was measured after a 16-h incubation at 37° ± 1°C using the paper disk method (Whatman No. 3, 5 mm) (Indian Pharmacopoeia Committee, 1996). The filter paper disks were sterilized by autoclaving and dried. The methanolic and aqueous extracts were applied to sterile filter paper disks at a fixed concentration of 3 and 6 mg, respectively. The microorganisms tested were spread with sterile glass rod on sterile plates containing nutrient agar. The filter paper disks containing the crude extracts were placed on the seeded plates. Paper disks with known crude extract concentrations were placed on the surface of the agar. The test was performed in triplicate to obtain reliable results.

Statistical analysis

Analysis of variance of the data was carried out using the statistical package SPSS, and significant differences between the treatment means were determined using the Duncan multiple range test (Crawley, 2005).

RESULTS AND DISCUSSION

Crude plant extracts are of great importance in the preservation of foodstuff by avoiding use of synthetic chemical compounds. All bacteria including *P. aeruginosa*, *S. aureus*,

B. subtilis, *E. coli*, and *S. sonnei* cause food poisoning, and their activities have been inhibited by the application of synthetic chemical compounds (preservatives). Sometimes, however, these preservatives are harmful to human health (Anselmi et al., 2002; Stefanidou et al., 2003), while natural preservatives are free from toxicity. Therefore, the *B. salicina* extract could be used as an alternative for the preservation of foods in place of synthetic compounds, since both leaf extracts showed antibacterial activity against these bacteria. Such natural antibacterial agents have more advantages over synthetic compounds and are more beneficial for human health. Plants have numerous secondary metabolites that are produced in response to various abiotic and biotic stresses (Zangerl and Berenbaum, 1987; López-Gresa et al., 2011) and plant development (Walker et al., 2012). For the evaluation of the antibacterial activity of *B. salicina* leaf extract, all observations of bacterial growth were performed with the naked eye, and the growth-inhibition zone was measured in millimeters. Using several extract concentrations, the MIC was determined against these bacteria, and all bacteria showed variation in antibacterial activity at the same concentrations of leaf extract. The inhibition zone appeared at 1.5 mg methanolic extract (per disk; 15 µL) against *B. subtilis* and *S. sonnei*, which was a very low concentration compared to that for other bacteria (Table 1). The MIC was 3 mg (crude methanolic extract) for *E. coli*, *P. aureus*, and *S. aureus*. The same concentration of aqueous extract was applied for all bacteria and the MIC was found to be 2.5 mg (Table 2). Thus, we can conclude that all bacteria were more susceptible to the methanolic extract compared to the aqueous extract, which may be due to the greater solubility of the compounds in methanol.

Table 1. Antibacterial activity of methanolic leaf extract of *Breonadia salicina* expressed as minimal inhibitory concentration.

Bacteria	Concentrations of crude extract per disc					
	0.5 mg (5 µL)	1.0 mg (10 µL)	1.5 mg (15 µL)	2.0 mg (20 µL)	2.5 mg (25 µL)	3.0 mg (30 µL)
Bacillus subtilis (ATCC 10400)	-	-	+	+	+	+
Escherichia coli (ATCC 442)	-	-	-	-	+	+
Shigella sonnei (ATCC 11060)	-	-	+	+	+	+
Pseudomonas aeruginosa (ATCC 27853)	-	-	-	-	-	+
Staphylococcus aureus (ATCC 29213)	-	-	-	+	+	+

(-) = no inhibition zone was observed; (+) = inhibition zone was observed.

Table 2. Antibacterial activity of aqueous leaf extract of *Breonadia salicina* expressed as minimal inhibitory concentration.

Bacteria	Concentrations of crude extract per disc					
	0.5 mg (5 µL)	1.0 mg (10 µL)	1.5 mg (15 µL)	2.0 mg (20 µL)	2.5 mg (25 µL)	3.0 mg (30 µL)
Bacillus subtilis (ATCC 10400)	-	-	-	-	+	+
Escherichia coli (ATCC 442)	-	-	-	-	+	+
Shigella sonnei (ATCC 11060)	-	-	-	-	+	+
Pseudomonas aeruginosa (ATCC 27853)	-	-	-	-	+	+
Staphylococcus aureus (ATCC 29213)	-	-	-	-	+	+

(-) = no inhibition zone was observed; (+) = inhibition zone was observed.

The inhibition zone of the methanolic extract was greater against *P. aeruginosa*, *S. aureus*, and *B. subtilis* compared to other bacteria at the same concentration (3 mg/disk) (Table 3). Similarly, when the concentration of the same crude extract (methanol) was increased (6 mg/

disk), the diameter of the inhibition zone also increased (Table 3). A similar result was also obtained with these bacteria when the aqueous leaf extract was applied (Table 4). Thus, *P. aeruginosa*, *S. aureus*, and *B. subtilis* were more susceptible compared to *E. coli* and *S. sonnei*, showing greater antibacterial activity for the leaf extracts.

Table 3. Antibacterial activity of methanolic leaf extract of *Breonadia salicina* measured by inhibition zone.

Bacteria	Inhibition zone diameter (mm)	
	Concentration of 30 mg (30 µL)	Concentration of 60 mg (60 µL)
Bacillus subtilis (ATCC 10400)	$6.867 \pm 0.3215^{b,c}$	9.767 ± 0.2082^{a}
Escherichia coli (ATCC 442)	6.467 ± 0.5508^{c}	8.567 ± 0.4041^{b}
Shigella sonnei (ATCC 11060)	6.500 ± 0.5000^{c}	8.900 ± 0.3606^{b}
Pseudomonas aeruginosa (ATCC 27853)	7.767 ± 0.2517^{a}	9.833 ± 0.2887^{a}
Staphylococcus aureus (ATCC 29213)	$7.500 \pm 0.5000^{a,b}$	9.667 ± 0.5774^{a}

Data are reported as means ± SD of three replicates. Different superscript letters mean significance at $P < 0.05$ (Duncan multiple range test).

Table 4. Antibacterial activity of aqueous leaf extract of *Breonadia salicina* measured by inhibition zone.

Bacteria	Inhibition zone diameter (mm)	
	Concentration of 30 mg (30 µL)	Concentration of 60 mg (60 µL)
Bacillus subtilis (ATCC 10400)	$6.667 \pm 0.5774^{a,b}$	$7.667 \pm 0.4163^{a,b}$
Escherichia coli (ATCC 442)	5.733 ± 0.0577^{c}	6.933 ± 0.1155^{c}
Shigella sonnei (ATCC 11060)	6.333 ± 0.2887^{b}	7.067 ± 0.1155^{c}
Pseudomonas aeruginosa (ATCC 27853)	$6.667 \pm 0.1528^{a,b}$	$7.500 \pm 0.5000^{b,c}$
Staphylococcus aureus (ATCC 29213)	7.100 ± 0.1732^{a}	8.100 ± 0.1732^{a}

Data are reported as means ± SD of three replicates. Different superscript letters mean significance at $P < 0.05$ (Duncan multiple range test).

In our study the antibacterial activity shown by both extracts of *B. salicina* was unknown; however, it can be further studied by gas chromatography-mass spectrometry (GC-MS) and isolation of the single active compounds. The methanolic as well as aqueous extract of many plant species have shown antibacterial activity against *S. aureus*, *P. aeruginosa*, and *E. coli* (Taye et al., 2011; Raja et al., 2011; Mendez et al., 2012; Martins et al., 2013). Among the bacteria studied here, *P. aeruginosa*, *S. aureus*, and *B. subtilis* were found to be very susceptible to 6 mg methanolic extract. Similarly, *S. aureus* was very susceptible to the aqueous extract at the same concentration, but this antibacterial activity was less than with the methanolic extract. However, all microorganisms studied showed different levels of susceptibility to the leaf extract of *B. salicina*, which may be used against other foodborne pathogens.

CONCLUSION

The leaf extract of *B. salicina* showed antibacterial activity against Gram-negative and -positive bacteria, which varied between bacteria at the same concentration of extracts. This variation in activity might have been due to the presence of various groups of secondary metabolites. In future study, compounds could be identified from these leaf extracts with GC-MS and can be used for the preservation of foods in place of chemical compounds.

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