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Antimicrobial activity of crude extract from *Millettia ferruginea* leaves and barks against selected bacterial pathogens and *Candida albicans*

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The progressive increase in antimicrobial resistance was the major attribute among human pathogens that has given rise to the need to investigate other sources of therapy from various sources such as medicinal plants. The aim of this study was to assess the antibacterial activity of crude extract of leaves and bark of *Millettia ferruginea* against human pathogenic bacteria (*Staphylococcus aureus and Shigella boydii*) and *Candida albicans*. Maceration technique was employed for extraction of the plant by using solvents of different polarity levels. Antibacterial and antifungal activities of each crude extracts were evaluated at a concentration of 100, 200 and 300 mg/ml via agar well diffusion assay. The standard drugs of 30 µg/ml and 1 ml of DMSO were used as positive and negative control respectively. Minimum inhibitory concentration was also determined. The result of the study indicated that all the three solvent extracts of leaf of *M. ferruginea* did not show antibacterial activity for test pathogens. Acetone and chloroform bark extracts showed antibacterial activity at all three concentrations. Minimum inhibitory concentration (MIC) for acetone bark extracts were 12.5 and 25 mg/ml for *S. aureus* and *S. boydii* respectively and also for chloroform extracts 25 and 50 mg/ml for *S. aureus* and *S. boydii* respectively. Ethanol bark extracts showed antibacterial activity only for *S. aureus* at higher concentrations (200 and 300 mg/ml) with minimum inhibitory concentration (MIC) of 200 mg/ml. Acetone extract at 300 mg/ml was insignificantly different from the antibiotic Gentamicine (P = 0.12). The present results concluded that out of three leaf extracts and three bark extracts, only chloroform and acetone extracts of bark have antibacterial activity against either *S. aureus* or *S. boydii*, while none of the prepared extracts had antifungal action.

Key words: *Millettia ferruginea*, MIC, in vitro natural antimicrobial assay, *Staphylococcus aureus*, *Shigella boydii.*

INTRODUCTION

Ethiopia is a center of diversity for a number of flora and fauna, the sixth center of biodiversity in the world. The country is endowed with rich flora, having more than 6,500 species of vascular plants out of which an estimated 12% are endemic and about 887 species are used as medicinal plants. The majority (80%) of Ethiopian

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people depends on traditional medicine for their health care, and more than 95% of traditional medicinal preparations made from plant origin (Ashenif, 2017). Medicinal plants contain many special substances and chemicals (micro components) having therapeutic activities used for medicinal purpose achieving curing different diseases (Bajpai and Agarwal, 2015). About 80% of the world's population, especially Asian and African countries use plant extracts or their active constituents as folk medicine for some aspect of primary health care in traditional therapies (Wangkheirakpam, 2018), since they tend to have less toxicity, less environmental effects and are more affordable than purchasing expensive conventional drugs. So, with its economic importance, alternative treatment has gained a respectable position (Karunamoorthi et al., 2012).

Millettia ferruginea is endemic to Ethiopia (Thulin, 1983; Azene, 2007; Belachew, 1993; Biruhalem et al., 2011, Choudhury et al., 2016), belonging to the family *Leguminaceae*, sub-family *Papilionnodeae* (Azene, 2007). In Ethiopia, two sub species of *Millettia* are found namely, *M. ferruginea* and *M. darassana* (Thulin, 1983). The distribution of *M. ferruginea* is between 1,000 - 2,500 m above sea level (MacLachlan, 2002), commonly found in the northern part of the country (Thulin, 1983) with multipurpose tree as all parts of *M. ferruginea* (fruit, leaves, stems and root) possess traditional medicinal action to treat amoeba, earache and nails infection, tooth aches and gonorrhea (Belachew, 1993; Fisseha et al., 2009) or skin infection (mujele) having antimicrobial and insecticidal activity (Eyob et al., 2010; Choudhury et al., 2016).

Despite its seed oil is used as nutritive source for human food rich in amino acids (Berhanu and Amare, 2012, 2013), this seed extract has a toxic effect on adult ticks *in vitro* (Choudhury and Yoseph, 2015), while leaves, flowers and twigs of *M. ferruginea* are important feed resource for ruminants; cattle, sheep and goats (Takele et al., 2014). Therefore, the main aim of this study was to assess the antibacterial activity of the crude extract from *M. ferruginea* leaves and bark against selected bacterial pathogens and *Candida albicans.*

MATERIALS AND METHODS

Description of study area

The study was conducted at Dilla, the administrative center of Gedio Zone in the SNNPR, Ethiopia. The town is located at 89 km far from Hawassa, the capital city of SNNPR and 359 km far from Addis Ababa, which is situated at 1123.47 km^2 at 6° 21' - 6° 24' north latitude and 38° 17' - 38° 20' east longitude. It is found in kola agro ecological zone with an altitude of 1400 km above sea level and annual temperature ranging from 22 to 29°C (Eshetu and Basha, 2015).

Plant sample collection

Fresh leaves and bark of *M. ferruginea* were collected around Dilla University main campus in December 2018 from natural habitat and the plant was identified by a botanist. Thereafter, fresh leaves and bark of the plant were separately washed with tap water followed by distilled water to remove dirt and soil particles. The plants were cut into small pieces and shade dry at room temperature under complete aseptic conditions with a careful and continuous follow up to avoid any contamination. The dried samples were then grinded by using sterilized electric grinder and the powder was sieved with (0.5 mm) mesh to obtain fine powder. Finally, the powder was weighted and kept for further use.

Crude extraction

Crude extraction was carried out by maceration protocol described by Felhi et al. (2017) with slight modification. Extraction was started from high polar solvent chloroform, followed by acetone and ethanol by keeping the polarity level. 200 g of finely grinded leaf and steam bark of *M. ferruginea* were soaked in different flasks containing solvents such as acetone (Sigma Aldrich, Germany), chloroform (Sigma Aldrich, Germany) and ethanol (Merck, United Kingdom) in 1 to 5 (w/v) ratios separately. The mixtures were left at room temperature for a period of at least 48 h with frequent agitation on orbital shaker with 180 rpm until the soluble matters were dissolved. Each macer (the damp solid material) was filtered using sterile cotton followed by filter paper (Whatmann No.1). After filtration, solvents were evaporated using Rota vapor at 40°C and crude extracts were obtained. The resulting crude extracts were collected in capped labeled bottles and dried at room temperature. Finally, the extracts were measured and kept in refrigerator at 4°C until use for further experiments.

Antimicrobial activity test

Bacterial strains

Three different media: Salmonella Shigella Agar (SSA), Mannitol Salt Agar (MSA) and Muller Hinton Agar (MHA) were used for this study according to Quinn et al. (2002), to re isolate clinical isolates of *S. aureus* (ATCC13311) and *S. boydii* (ATCC9207) were collected from Ethiopian Public Health Institute, Addis Ababa, Ethiopia.

Fungal strains

Potato Dextrose Agar (PDA) was used for this study according to Packiyalakshmi et al. (2017) to re-isolate clinical isolates of *C. albicans* collected from Ethiopian Public Health Institute, Addis Ababa, Ethiopia.

Preparation of test solution

The standard crude extracts of leaf and bark of *M. ferruginea* were further diluted to make three different concentrations in a separate flask according to Packiyalakshmi et al. (2017) with modification as the working stock solutions of different concentrations were 100, 200 and 300 mg/ml. The first working solution was prepared by transferring 100 mg of each extracts to sterile test tube containing 1 ml of DMSO to give a concentration of 100 mg/ml; the second and the third working concentrations were prepared by similar manner. Gentamicine, Ceftriaxone and Clotrimazole of each were used as positive control for *Staphylococcus aureus*, *Shigella boydii* and *C. albicans,* respectively while DMSO of 1 ml was used as negative control and the stocks were stored at 4°C for further use.

Table 1. Results of percent yield of *M. ferruginea*.

Plant part	Solvent used	Weight of dry powder (g)	Yield (%)	Extract colour
Leaf	Acetone	200	5.32	Dark green
	Chloroform	200	3.96	Dark green
	Ethanol	200	9.27	Dark green
Bark	Acetone	200	4.12	Dark green
	Chloroform	200	2.56	Dark green
	Ethanol	200	5.02	Dark green

Antimicrobial test

The antibacterial potential of the crude extract against the bacterial pathogens were also done by using the method described in Packiyalakshmi et al. (2017) via agar well diffusion technique. Firstly, bacterial pathogens were grown on their selective media, that is, MSA and SSA for *S. aureus* and *S. boydii* respectively and incubated at 37°C for 24 h. A few colonies of each strain were transferred with a sterile inoculating loop to a broth culture until turbidity was adjusted to that of 0.5 McFarland turbidity standards. Then, culture of test isolates were streaked on independent surface of already prepared MHA plates by using a sterile swab stick that was used to apply the suspension uniformly. Similar protocol was employed as the antifungal test. Each procedure was repeated three times and average inhibition zone diameter was taken to present results. The measurement of the linear growth of bacterial pathogens and fungus were taken by measuring the linear length of bacteria and fungus growth (mm) from point of origin toward the end of growth. Growth inhibitions were calculated with reference to positive control. Each test was conducted in triplicate to confirm the reproducibility of the observed data.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration (MIC) of acetone, chloroform and ethanol crude extract of leaves and bark of *M. ferruginea* on the tested fungal and bacterial isolates were determined using bi-fold dilution (Akinsete et al., 2017) with slight modification, where sterile 6-mm cork borer was used to bore five wells onto prepared MHA plates seeded with the test isolates. Different concentrations of the extracts (200, 100, 50, 25, 12.5, and 6.25 mg/ml) were dispensed into each well and labeled. The preparations were left to diffuse before incubating at 37°C for 48 h. The lowest concentration of the agent that has prevented the growth of the bacteria was taken as the MIC. The zones of inhibition was observed and recorded.

RESULTS

The percent yield of extracts obtained from sample using acetone, chloroform and ethanol gave different yields (Table 1). The highest yield was obtained from ethanol extract of leaf (9.27%) followed by acetone extract of leaf (5.32%), ethanol extract of bark (5.02%) and acetone extract of bark (4.12%).

Antibacterial activity of crude extracts of *M. ferruginea* against *S. aureus* and *S. boydii* were tabulated in Table 2, while all prepared extracts had no any antifungal action against *C. albicans*.

Antibacterial activity of crude acetone, chloroform and ethanol extracts of leaf and bark of *M. ferruginea* against *S. aureus* and *S. boydii*

The results showed that, all the three different concentrations of acetone leaf extracts were inactive and do not inhibit the growth of both selected bacterial pathogens. On the other hand, the acetone crude extracts of bark of *M. ferruginea* showed antibacterial activities. The mean zones of growth inhibitions against *S. aureus* at a concentration of 100, 200 and 300 mg/ml are 11.67±0.58, 14.50±0.18 and 16.17±0.62 mm, respectively (Figure 1). Similarly, the mean zones of growth inhibitions against *S. boydii* at a concentration of 100, 200 and 300 mg/ml were 9.33±0.58, 12.40±0.58 and 15.33±0.58 mm respectively (Figure 1). Standard antibiotics, Gentamicine and Ceftriaxone, which served as positive controls, also inhibit the growth of test bacterial pathogens at a concentration of 30 µg/ml with maximum mean inhibition values of 20.33±0.58 and 22.67±0.289 mm against *S. aureus* and *S. boydii* respectively.

Also, the chloroform extract of bark of *M. ferruginea* showed growth inhibition of *S. aureus* and *S. boydii* at different rates for different concentrations but less than its acetone extract bark showing inhibition zone of 15.67±0.58, 12.33±0.58 and 9.83±0.29 mm at 300, 200 and 100 mg/ml (Figure 2).

While no growth inhibitions were recorded for all test pathogens at the three concentrations of ethanol extract either of leaf or bark of *M. ferruginea* (Table 2), the size of growth inhibition zones ranged from 9.67 ± 0.29 mm at 300 mg/ml and 8.00 ± 0.00 mm at 200 mg/ml (Figure 3).

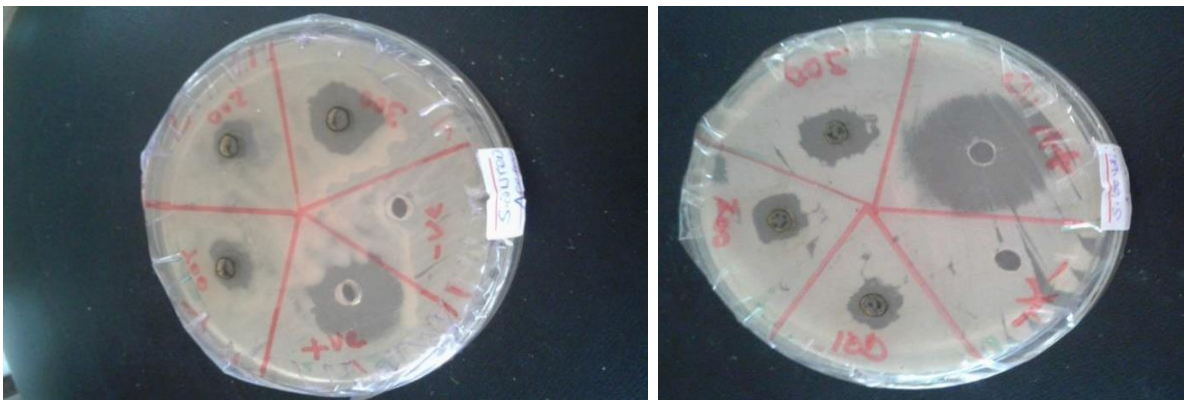
DISCUSSION

M. ferruginea has traditional use against different pathogens, and its extracts require solvents based on the polarity level (Altemimi et al., 2017), since chloroform and ethanol extracted both polar and non-polar compounds respectively from the plant, thus with no limitation of the amount of extractable compounds. Acetone was also

Table 2. Growth inhibitory level of bark crude extracts on the tested bacterial pathogens as compared to the antibiotics.

Test materials	Concentration	Test organisms		Remarks
		Staphylococcus aureus	***Shigella boydii***	
DMSO	1 ml	-	-	
Antibiotic	30 µg/ml	++++	++++	
BMA	100mg/ml	++	+	
	200 mg/ml	+++	++	
	300 mg/ml	++++	+++	
BMC	100 mg/ml	+	+	
	200 mg/ml	++	+	
	300 mg/ml	+++	++	
BME	100 mg/ml	-	-	Antibiotics used: Gentamicine against (*S. aureus*) and Ceftriaxone against (*S. boydii*)
	200 mg/ml	+	-	
	300 mg/ml	+	-	
LMA	100 mg/ml	-	-	
	200 mg/ml	-	-	
	300 mg/ml	-	-	
LMC	100 mg/ml	-	-	
	200 mg/ml	-	-	
	300 mg/ml	-	-	
LME	100 mg/ml	-	-	
	200 mg/ml	-	-	
	300 mg/ml	-	-	

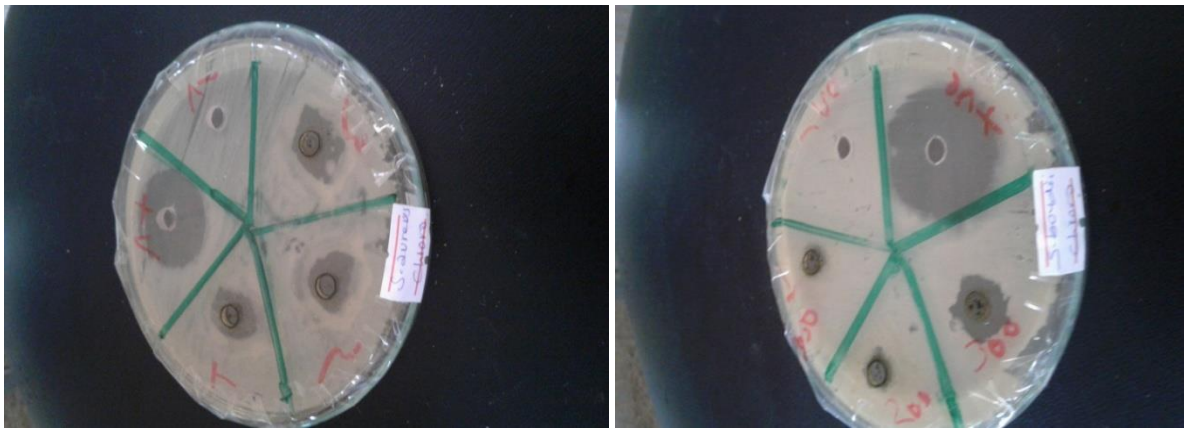
BMA and LMA = Bark and Leaf of *M. ferruginea* Acetone extract respectively. BMC and LMC = Bark and Leaf of *M. ferruginea* Chloroform extract respectively. BME and LME = Bark and Leaf of *M. ferruginea* Ethanol extract respectively. ++++ = Strong effect; +++ = Moderate effect; ++ = Low effect; +=Weak effect; - = no effect.



A B

Figure 1. Effect of crude acetone bark extract of *M. ferruginea* against (A) *S. aureus* and (B) *S. boydii.*

specifically included as it is an intermediately polar solvent. The differences in extract yield of the crude extracts using the same extraction solvent varied significantly from one part of the plant to the other. The amount of the antioxidant components that can be extracted from a plant material is mainly affected by the strength of the extraction procedure and efficiency of the extracting solvent to dissolve compounds (Sultana et al., 2009). Plant secondary metabolites are used as source of microbicides, pesticides, for production of pharmaceutical drugs etc. *M. ferruginea* has been investigated extensively in documents recommending different plant parts extracts application against skin infection, tooth ache, STD (gonorrhea) or storage insect



A B

Figure 2. Effect of crude chloroform bark extract of *M. ferruginea* against (A) *S. aureus* and (B) *S. boydii.*

Figure 3. Effect of crude ethanol extract of *S. aureus.*

pests (Askale 2015; Marick et al., 2017; Tamirat et al., 2015). In the present study, the antibacterial and antifungal activities of the leaf and bark of *M. ferruginea* extracts were done against different test bacterial pathogens *S. aureus* and *S. boydii* and fungus *C. albicans*. Agar well diffusion method was employed for antimicrobial assays since it was more sensitive than other methods. Results of the current study revealed that, the application of acetone, chloroform and ethanol leaf extracts of *M. ferruginea* shows neither antibacterial nor antifungal activity. Hence, leaf of *M. ferruginea* is not effective against the tested pathogens. This result was in agreement with the report of Choudhury and Yoseph (2015) who studied the toxicity of *M. ferruginea* (Hochst) Baker against the larvae and adult ticks of *Boophilus decoloratus,* a one-host tick in cattle, observed the result and reported that the toxic constituents are totally absent in petroleum ether extract of *M. ferruginea* leaf; however, the solvent and test pathogen differ. In contrast, the methanol and aqueous extract of *M. ferruginea* leaf at a concentration of 500 mg/ml inhibits the growth of *S. aureus* and *S. boydii* at growth inhibition zone of 13 and 8 mm, respectively (Biruhalem et al., 2011). These differences might have appeared by the change of solvents, the use of standard strain pathogens rather than clinical isolates of test pathogens and the increase of peak of extract concentration. Acetone and chloroform extracts of *M. ferruginea* bark were active against the tested bacterial pathogens at all the specified concentrations while ethanol extract was less active in *S. aureus* and totally inactive in *S. boydii*. All the extracts had no activity against *C. albicans*, showing that the plant totally have no antifungal activity. The effects of extracts were concentration dependent on the growth of test bacterial pathogens. Higher and lower mean growth inhibition zones were recorded at the higher and lower concentrations of extracts respectively. The higher degree of inhibition observed from increased concentration of extracts might be due to the increased availability of the antibacterial compounds in the media. Similar observations were reported for increased inhibitory activity of *Millettia aboensis* extracts to the

Table 3. Means of MICs values of crude acetone, chloroform and ethanol extracts of *M. ferruginea* bark against the growth of test pathogens.

Test pathogens	Test materials	Concentration	Mean ±SD	P-value
	DMSO	1 ml	0.00 ± 0.00	0.00
	Gentamicine	30 µg/ml	20.33 ± 0.58	
		100 mg/ml	11.67 ± 0.58	0.00
	Acetone extract	200 mg/ml	14.50±0.18	0.00
		300 mg/ml	16.17±0.62	0.12
S. aureus		100 mg/ml	9.83±0.29	0.00
	Chloroform extract	200 mg/ml	12.33±0.58	0.00
		300 mg/ml	15.67±0.58	0.00
		100 mg/ml	0.00±0.00	0.00
	Ethanol extract	200 mg/ml	8.00±0.00	0.00
		300 mg/ml	9.33±0.58	0.00
	DMSO	1 ml	0.00±0.00	0.00
	Ceftriaxone	30 µg/ml	22.67±0.58	
		100 mg/ml	9.32±0.58	0.00
	Acetone extract	200 mg/ml	12.40±0.58	0.00
		300 mg/ml	15.33±0.58	0.00
S. boydii		100 mg/ml	8.17±0.29	0.00
	Chloroform extract	200 mg/ml	9.67±0.29	0.00
		300 mg/ml	12.67±0.29	0.00
		100 mg/ml	0.00±0.00	0.00
	Ethanol extract	200 mg/ml	0.00±0.00	0.00
		300 mg/ml	0.00±0.00	0.00

Values represent means of triplicate, ± denotes standard deviation, p-value indicates statistical differences between the effect of each extracts with the respective concentration used, p-value <0.05was taken as significance difference.

growth of test pathogens with increasing extract concentration (Onyegeme-Okerenta and Okafor, 2014).

The antibacterial activities of bark extracts of the three solvents assayed here possessed different levels of antibacterial activities. Among the three solvent extracts tested against *S. aureus* and *S. boydii*, acetone extract was found to be the best and more effective than chloroform and ethanol extracts. On the other hand, the crude extracts showed antibacterial activities with higher inhibition zone for the two solvent (acetone and chloroform) extracts at each test concentrations for *S. aureus* (Gram negative bacteria) than *S. boydii* (Gram negative bacteria). This can be due to the existence of the murein layer in Gram negative bacteria covered by a lipopolysaccharide layer which makes the bacterial cells more resistant to antibiotics. Ethanol extract did not compare since it has no inhibition effect on *S. boydii*. Antibacterial activity of each extracts of *M. ferruginea* bark against *S. aureus* and *S. boydii* were tested at a concentration of 100, 200 and 300 mg/ml. At each concentration, acetone extracts showed higher inhibitory activity than chloroform extracts on the growth of test pathogens while ethanol showed the least inhibitory activity. As reported above, the activities of all solvent crude extracts against the test bacterial pathogens were varied and concentration dependent. The increase in concentration of the medicinal plant crude extracts, as well as the greater inhibitions of growth of test bacterial pathogens was observed. Acetone crude extracts of *M. ferruginea* bark observed highest inhibition zone of 16.17±0.62 mm and 15.33±0.58 mm against *S. aureus* and *S. boydii* respectively and also chloroform crude extracts inhibits 15.67±0.58 mm and 12.67±0.29 mm recorded at maximum concentrations against *S. aureus* and *S. boydii* respectively. The result was related with Tamirat et al. (2015), who reported that the antimicrobial activity of methanol and diethyl ether extract showed significant inhibition activity against *Pseudomonas aeruginosa* with inhibition zone of 15 and 10 mm respectively though the solvents and test pathogen were varied. This shows that the bark of this plant extract contains broad spectrum compounds of natural origin. Hence, further study is suggested to identify and characterize these biological active compounds since the community uses this plant for the treatment of different types of diseases. Antibacterial effect of *M. ferruginea* bark involved a comparison of its acetone extract at 300 mg/ml with commercially available standard antibacterial

drug (Gentamicine) by comparing the inhibition zone. As reported above, the standard drug showed larger inhibitory effect than the acetone crude extract. This does not reinforce the position of standard antibacterial drugs that should be used in treatments whenever available. The manifestation of activity of the extract against the bacteria was an indication of the broad spectrum of activity and thus it can be used as source of antibiotic substances for drug development. MIC assays were employed to evaluate the effectiveness of the crude extracts that showed significant antibacterial activities. The MIC value of acetone, chloroform and ethanol of crude extracts showed different minimum inhibition effect against the tested bacterial pathogens which ranged from 200 to 6.25 mg/ml. Among these, acetone extract had lowest MIC value of 12.5 mg/ml for *S. aureus*. Similar MIC value (25 mg/ml) was recorded for chloroform and acetone extracts on *S. aureus* and *S. boydii* respectively (Table 3). Highest MIC values were exhibited by ethanol and chloroform extracts with 50 mg/ml for *S. aureus* and *S. boydii* respectively (Table 3). Extracts with lower MIC scores are very effective antimicrobial agents. The entire reports were used to validate the inhibitory effect of *M. ferruginea* bark extracts in this research.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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