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Water Maceration of Black Mahlab Effectively Inhibits the Growth of *Staphylococcus aureus* Associated with Skin Infections

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ABSTRACT

Background: With the rising risk of antibiotic resistance, our ability to combat *Staphylococcus aureus* (S. *aureus*) in skin and soft tissue infections (SSTIs) is being compromised. **Purpose:** This study aimed to determine the activity of several aqueous extracts of black mahlab seeds (maceration, infusion, and decoction) against S. *aureus*-associated SSTIs. **Methods:** We employed the disc diffusion Kirby-Bauer method to evaluate the anti-staphylococcal activity of the extracts. Furthermore, we used various extract concentrations to calculate each extract's minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) against *S. aureus*. **Result:** The results revealed enhanced anti-staphylococcal activity, with inhibition zones measuring 11.5, 22.5, and 26.5 mm at concentrations of 25, 50, and 100 mg/ml, respectively. These effects were similar to ampicillin against S. *aureus* and significantly higher ($p \leq 0.05$) than gentamicin. The extracts showed antibacterial activity with MICs and MBCs ranging from 12.5 to 25 mg/ml. **Conclusion:** Overall, compared to the clinically prescribed antibiotics, ampicillin, and gentamicin, black mahlab seed extract obtained by water maceration exhibits increased anti-staphylococcal activity associated with SSTIs.

Keywords*:* Antibiotic Resistance, Black Mahlab, Gentamicin, Maceration, *Staphylococcus aureus.*

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BACKGROUND

Skin and soft tissue infections (SSTIs) frequently encounter infectious diseases that require antibiotic treatment. These conditions can vary from mild skin abscesses and superficial tissue infections to severe skin and soft tissue diseases that pose a risk to life.[1] There is a growing global trend in SSTIs, with the United States experiencing an annual impact on approximately 14 million individuals. Notably, this condition is most prevalent among individuals aged 50 years or older.[2] *Staphylococcus aureus* (S. *aureus*) is a gram-positive bacterium that is facultatively anaerobic and has a spherical form. It is an opportunistic pathogen in SSTIs, and its risk is well-documented. Chronic wounds, cardiovascular disease, peripheral vascular disease, diabetes mellitus, and renal disease are among the risk factors for S. *aureus* in SSTIs.[3] Antibiotics are common for treating Staphylococcal infections in SSTIs. However, antibiotic-resistant S. *aureus* has quickly emerged, reducing treatment choices.[4,5] Biofilm secretion weakens antibiotics like β-lactams, fluoroquinolones, and cephalosporins against S. *aureus* in skin infections. This causes strong resistance and severe infections.[6] Therefore, we need better antibiotic therapies for S. *aureus* infection in SSTIs. These treatments should be more effective and easier to mass produce.

Certain Sudanese medical herbs have been used to treat bacterial skin infections and wounds for a long time. Black mahlab (*Mooneyham ciliatum*, family *Acanthaceae*) is a medicinal herb.[7] The Nubian ethnic group in Sudan uses it as folk medicine at Nubian Mountain. The Nubian people prepare traditional cosmetics as lotions from the powdered seeds of the black mahlab herb. These lotions assist in cleansing, smoothing, and softening the skin. Additionally, they are utilized for alleviating inflammation, treating wounds, and managing eczema.[8] A prior investigation illustrated black mahlab seed extracts' potential antioxidant and anti-inflammatory properties in vitro.

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The flavonoids, alkaloids, and tannins found in black mahlab are the most abundant and biologically active compounds contributing to their anti-inflammatory and antibacterial effects. Consequently, the total flavonoid and alkaloid content was assessed relative to the overall extractable compounds in black mahlab seeds. [9] In this study, we estimated the total tannin in black mahlab seeds. This is an initial instance of its kind.

Our study explored how seed extracts from black mahlab effectively combat S. *aureus* associated with SSTIs. We specifically utilized water as the solvent for our extracts. Unlike organic solvents, water was selected for its ability to support the growth of microorganisms. Moreover, water's high polarity enhances the extraction of valuable plant compounds.[10] We also aim to mimic the Nubian people's method of preparing lotions from black mahlab seeds using water.

METHODS

Each of the experiments in this research was carried out at the Department of Pharmaceutics laboratories and by the Faculty of Pharmacy's guidelines at Omdurman Islamic University, Sudan. HI Media Laboratories in India provided the Mueller-Hinton agar and broth. We bought ampicillin from Bristol-Myers Squibb, USA, gentamicin from General Medicine Co., Sudan, and ethanol from Scharlau Chemie, Spain. All other reagents and solvents were of the highest analytical grade commercially available. *S. aureus* samples used in this study were obtained from the Khartoum Hospital, Sudan, and all samples were then processed and kept at MAPRI, Khartoum Sudan.

The seeds were from the Nubian Mountain region. They were first identified by Mr. Bakry Abdelhalim Hasanain, a respected herbalist and healer in Sudan who sadly passed away due to the coronavirus in 2021. The Department of Pharmacology at the College of Pharmacy, Omdurman Islamic University, Sudan, later confirmed their taxonomy.

After letting the seeds dry at room temperature, we ground them into powder using a mortar and pestle. Twenty grams of the coarsely ground seed powder were added to a flask that contained 400 mL of sterile distilled water. The flask was left to stand at 37 °C for 24 hours while being shaken occasionally. The resulting aqueous mixture was filtered and placed in an oven set at 40 °C for three days. The filtered extracts were then concentrated using a rotary evaporator at 70 °C for 4 hours. Lastly, we re-suspended the extracted extract was re-suspended in 1X phosphate buffered saline to obtain a concentration of 500 mg/mL. It was then stored at 4°C in sterile glass dark bottles until needed for the antibacterial activity test

The obtained seeds were dehydrated at room temperature and ground with a mortar and pestle to a powder. In a separate 400 ml of sterile boiled distilled water, we put 20 g of the ground seed and left it for five minutes. We employed both cold and hot infusion extraction procedures. Then, the aqueous mixtures were filtered and placed in an oven at 40°C for two days. Afterward, the extracted material was reconstituted in 1X phosphate buffer saline to a 500 mg/mL concentration and stored at 4°C in sterile glass dark bottles until needed for the antibacterial activity test.

The dried black mahlab seeds were pounded into a coarse powder using a mortar and pestle. Twenty grams of coarse powder were weighed, put into a flask with 400 mL of boiled water, and left to stand for 10 minutes. We then processed the extract as described above. A loopful of *S. aureus* was transferred to 10 mL of optical densities (OD) and was measured using a spectrophotometer after 24 hours of bacterial culture. We adjusted the OD to 0.5 by adding Mueller-Hinton broth to the bacterial suspensions and increased the concentration of bacterial suspensions to 10^8 cells/mL.

We used ten milliliters of 4% sodium hydroxide and 20 mL of 0.01N iodine to dissolve the black mahlab seed water extract. The resultant mixture was put into a gelatin solution with different tannin concentrations. The liquid was then evaluated for tannin content after being diluted with water and acidified with 10 mL of 4% sulfuric acid.[11] We obtained an inoculum of around 10^5 CFU/mL by diluting the 18-hour culture with sterile physiological saline solution (0.85% (w/v) sodium chloride supplemented with (0.2%) Tween 80. One microliter of bacterial inoculum was applied to the Mueller-Hinton Agar surface once it had already dried, and it was allowed in contact with the surface until it had dried entirely.

Through the use of the disc difussion Kirby Bauer method, the antibacterial activity of water and ethanol maceration of black mahlab seeds at various concentrations was evaluated. Each plate was prepared with five m wells, into which was put 0.1 mL of each extract, and the mixture was left to stand at 37 °C for 24 hours. A broad-spectrum antibiotic (gentamicin and ampicillin) was the positive control for measuring the diameter of each extract's zone of inhibition in millimeters.[12,13]

The exclusion criteria were: 1) patients who used or were now using any kind of topical antibiotic (for the isolation of S. aureus samples); 2) plants treated with solvents other than water. The investigation used the 96-well microdilution technique to calculate the minimum inhibitory concentrations (MICs) for each extract. To each well, 100 µL of an overnight culture containing 6.0 log10 CFU/mL of each bacteria was introduced. Every extract was made into a two-fold serial dilution using DMSO with concentrations ranging from 25 mg/mL to 150 mg/mL. After adding 100 µL of each serial dilution to each well to make a total volume of 200 µL, the plates were sealed and incubated for a full day at 37 °C. The technique was also used to create control plates that were devoid of plant extracts. After 24 hours, the minimum

concentration at which no discernible growth was seen was identified as each plant's MIC.[14.15]

Two loops full of culture were collected from each broth tube that did not exhibit any growth in the MIC tubes to determine the MBC. We then inoculated these loops onto new nutrient agar plates period onto new nutrient agar plates. Following a 24-hour incubation period, the plates were checked for bacterial growth. The MBCs were the concentrations of the extracts that did not exhibit any growth. Every experiment was conducted three times.[14.15]

Ethical Declaration This research has been reviewed by the Ethics Committee at the Department of Pharmaceutics, College of Pharmacy, Omdurman Islamic University (No: 012/2018OIU_DPh).

Statistical Analysis Every data point is shown as the mean ±SD, and the number of samples for each antibacterial test is 3 (n = 3). The Prism software (version 6.1) was used for statistical analysis, and one-way ANOVA was used. A value of $p < 0.05$ indicated significant differences between treatments.

RESULT

Table 1 shows the yield percentage and various organoleptic parameters for the three aqueous seed extracts utilized in this investigation. Our findings indicated that the greatest percent yield was achieved when black mahlab seeds were extracted by decoction (10.13%), followed by infusion (9.5%), and lastly by maceration (9.02%). The extraction yield represents the efficacy of the extraction process in obtaining particular elements from the source material. Moreover, it also provides information on how extractable the plant is in various settings. [17]

Table 2 displays the total tannin content of the black mahlab seed water maceration (usually expressed as a percentage of wet weight). The overall tannin content was 9.7%. Indeed, this high tannin content could provide the water maceration with a potential anti-staphylococci action. Tannin effectively inhibits bacteria by inactivating microbial adhesions, enzyme production (e.g. coagulase, staphylokinase, and exoenzymes), cell envelope transport proteins, and mineral absorption.[18]

Table 3 and Fig. 1. provide an evaluation of the anti-staphylococcal activity of all the water extractions and standard antibiotics. The water maceration displays considerable antibacterial action (p <0.01) at 100 and 150 mg/mL, with an inhibition zone of 18 and 20 mm, according to the *S. aureus* susceptibility rates to different dilutions. However, at the 50 mg/mL dilution, we observed a reduced susceptibility with an inhibitory zone of 10 mm. Unfortunately, no susceptibility to *S. aureus* was found at the extract dilution of 25 mg/mL. On the other hand, neither the water infusion nor the decoction contained any *S. aureus* activity at any of the dilutions tested (Table 1).

In this investigation, we used MICs and MBCs to further assess the anti-staphylococcal activity of the three black mahlab seed extracts. With an MIC of 6.25 mg/mL and an MBC of 3.125 mg/mL, respectively, the water maceration suppressed *S. aureus*. These values were equivalent to the MICs and the MBCs of ampicillin and gentamicin, the most clinically used antibiotic medicine in SSTIs. Table 4 and 5 is a list of the outcomes. Unfortunately, extracts (water infusion and water decoction) did not provide statistically significant MICs or MBC effects compared to ampicillin and gentamicin (data not shown).

Table 1. Organoleptic properties of the three aqueous seed extracts

Extract type	Yield %	Color	Odor	Taste	Consistency
Infusion	9.50	Brown	Aromatic	Bitter	Solid
Maceration	9.02	Yellow	Aromatic	Bitter	Solid
Decoction	10.13	Brown	Aromatic	Bitter	Solid

Table 2. The total tannin content of water maceration of black mahlab seeds

Test No.	Yield %	Tannin %
1.	9.05	9.3
2.	9.10	9.7
3.	9.00	10.1
Mean	9.02	9.7

Table 3. The anti-staphylococcal activity of the water extracts against *S. aureus*

Extraction type	Part used	Concentration mg /mL	*Staphylococcus aureus*
Water infusion	Seeds	25	-
		50	-
		100	-
		150	-
Water maceration	Seeds	25	-
		50	10
		100	18
		150	20
Water decoction	Seeds	25	-
		50	-
		100	-
		150	
Distilled water (negative control)	-	-	-

Key: (> 6 mm) sensitive, (< 6mm) resistant, (-) no inhibition zone

Table 4. Antibacterial activity of standard antibiotics against *S. aureus*

Antibiotic Used	Concentration mg /mL	*S. aureus*
Ampicillin	0.005	14
	0.01	22
	0.02	26
	0.04	27
Gentamicin	0.005	13
	0.01	14
	0.02	17
	0.04	18

Key: (> 6 mm) sensitive, (< 6mm) resistant, (-) no inhibition zone

Table 5: MICs of water maceration against *S. Aureus*

Conc. (µg/mL)	Medium added (mL)	Inoculum added (mL)	MICs	MBCs
3.125	20	0.01	+	*
6.25	20	0.01	*	-
12.5	20	0.01	-	-
25	20	0.01	-	-
50	20	0.01	-	-

Key: (-) no growth, (+) light growth, (*) MIC.

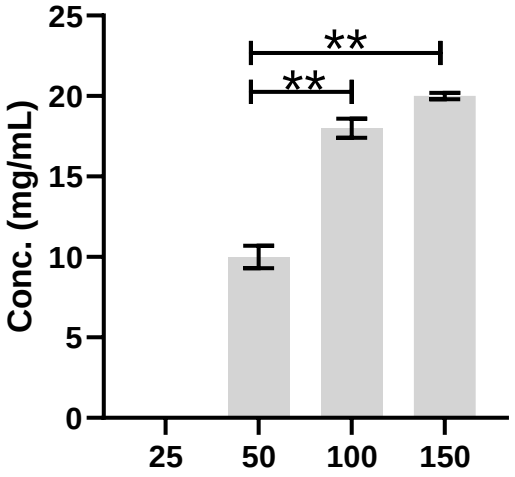

Fig. 1: Antibacterial activity of water maceration against *S. aureus*. Data are presented as mean ± SD (n = 3), ** $p < 0.01$

DISCUSSION

This study used 3 different techniques to extract the black mahlab seeds: decoction, maceration, and infusion. Water was the solvent system. We used these extraction methods because they significantly affect the type and number of biomolecules present in the extract.[19,20] Furthermore, we are trying to obtain an extract that is similar to the local Nubian customs used to treat skin irritation and wounds. The disc diffusion Kirby-Bauer and disc diffusion Kirby-Bauer techniques were used to ascertain each extraction technique's anti-staphylococcal activity. The results of the water maceration demonstrated the strongest anti-staphylococcal activity (Table 3 and Fig. 1). Moreover, Tables 3 and 4 demonstrate that the anti-staphylococcal activity of the water maceration was significantly ($p < 0.05$) greater than that of gentamicin, one of the most widely used antibiotics in clinical practice for treating SSTIs caused by *S. aureus.* This activity was comparable to the ampicillin activity effect, one of the antibiotics used frequently therapeutically to treat SSTIs caused by *S. aureus*. The use of heat throughout the decoction and infusion stages of this investigation may cause the active phytochemical components that eradicate *S. aureus* to break down. On the other hand, the maceration method is used when the substances to be extracted are heat-sensitive.[7]

According to several prior qualitative and quantitative phytochemical investigation findings, flavonoids and tannins were found in the black mahlab seeds. According to several prior qualitative and quantitative phytochemical investigation findings. We measured quantitatively the total flavonoid content of

the black mahlab seeds was measured quantitatively.[8] We then determined the total tannin concentration in the black mahlab seeds for this investigation, which was not disclosed in any other studies (Table 2). It has been shown that tannin, a phenolic molecule rich in natural and process-derived compounds, is a potent phytochemical against bacteria and viruses. Specifically, tannin may disrupt the cell wall integrity of *S. aureus* bacteria by attaching itself to its peptidoglycan.[21] It is noteworthy to add that many earlier studies have shown flavonoids' capacity to prevent the growth of *S. aureus*.[22]

Further research is necessary Utilizing the MICs and MBCs procedures, we quantitatively assessed the anti-staphylococcal activity of the black mahlab seed extracts obtained from the maceration, decoction, and infusion processes. The MICs of the water maceration on S. *aureus* were 6.25 mg/mL (Table 5), which is the same as the MICs of two therapeutically used antibiotics, ampicillin, and gentamicin. There were no significant MICs and MBCs against *S. aureus* in any other extracts. Consequently, we suggested that the most effective extraction technique for using black mahlab seed extract in SSTIs associated with *S. aureus* infection is water maceration.

We used the crude extract of black mahlab seeds for the test against *S. aureus* infection in SSTIs rather than an isolated chemical. This is due to its potential for synergistic activity. Plant crude extracts have a pharmacological activity that is greater than that of their separated active principles because of the synergistic effects of several components in the entire extract.[23,24,] The water maceration of black mahlab seed may kill *S. aureus*-linked *STTIs*; we strongly advise using it for skin ointment formula, wound wash, antiseptics, or surgical wound dressing pads. When using black mahlab seed water maceration for medicinal purposes, there was no expectation of any cytotoxic effects. Nonetheless, a combination of tannins, phenolics, or flavonoids often makes up the water maceration's constituents. Each of these elements might affect how long healthy eukaryotic cells survive. Therefore, before using the black mahlab seed maceration in therapeutic settings, we highly recommend establishing its cell safety.

The reported significant incidence of SSTIs points to rising morbidity and the ongoing spread of the antibiotic-resistant *S. aureus* bacteria. In contrast to water infusion and decoction, this research showed that black mahlab seed maceration in water could control *S. aureus, which is* linked to SSTIs. Even at lower concentrations, the extract type is particularly more appropriate against *S. aureus*. Further research is necessary to determine the precise bioactive chemicals, their mechanism of action, their harmless nature in vivo, and their final formulation to the appropriate dose form.

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