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RESEARCH ARTICLE

In vitro Antibacterial Evaluation of Four Selected Medicinal Plants against *Staphylococcus aureus* Isolated from Bovine Mastitis in Mieso District West Hararghe Zone, Oromia Regional State, Ethiopia

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Abstract:

Background:

Bovine mastitis is a major disease mostly caused by bacterial infection and associated with losses in the global dairy industry. Since mastitis-causing bacterial developing multidrug resistance to conventional antibiotics, there is an admirable supplementary study on medicinal plants to use them as an alternative therapy. This study aimed to evaluate the antibacterial activity and phytochemical screening of four selected medicinal plants against *Staphylococcus aureus*.

Methods:

An experimental study was done to evaluate the antibacterial activity of crude methanolic extracts of four traditionally used medicinal plants against *S. aureus*. Standard phytochemical screening tests were conducted to detect the bioactive principle of plants. Agar well diffusion assay was used to evaluate the antimicrobial activity of crude methanolic plant extract. The broth dilution method was also used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of plant extracts.

Results:

The percentage yields obtained from the root of *Clerodandrum myricoides, Kalanchoe densiflora, Kalanchoe marmorata* and leaf of *Kalanchoe marmorata and Datura stramonium* were 22.6%, 37.2%, 51.6%, 32.3% and 50.7%, respectively. Phytochemical screening tests revealed the presence of secondary metabolites such as tannins, phenols, and steroids in all plant extract, except in *D. stramonium*, while others like alkaloids, flavonoids, quinones, and saponins were fairly detected in all samples. The agar well diffusion results showed significant ($p < 0.05$) differences in the mean zone of inhibition (ZOI) between each plant at different concentrations with significant potency comparable to gentamicin. *C. myricoides* and *D. stramonium* revealed the broadest spectrum of action yielding the highest ZOI (27.0 ± 0.58 mm), whereas *K. marmorata* leaf showed less activity with the lowest ZOI (22.3 ± 0.33 mm). The broth dilution method indicated that the MIC value of plant extracts against *S. aureus* ranged between 3.90 and 7.80 mg/ml while its corresponding MBC value ranged between 7.80 and 15.6 mg/ml. According to the MIC/MBC ratio, all tested plants (except *K. densiflora*) against standard *S. aureus* while C. *myricoides* and *D. stramonium* against clinical *S. aureus* isolate were determined to be bactericidal.

Conclusion:

This finding confirmed that all tested plants had a potential anti-staphylococcal effect. Thus, further study on *in vivo* experiments and cytotoxicity analyses must be conducted to suggest these plants as alternative mastitis treatments.

Keywords: Antibacterial activity, Crude extracts, *In vitro*, Mastitis, Medicinal plants, Phytochemicals, *Staphylococcus aureus*.

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1. INTRODUCTION

Ethiopia is the country that possesses the largest livestock population in Africa with an estimated cattle population of 59.5 million [1], and annual milk production of 1.5 million tons [2]. Contrariwise, low annual per capita consumption of milk in Ethiopia revealed that current milk production in the country is not convincing compared to the country's requirements [3]. It was also suggested that livestock diseases, mainly mastitis, are a major factor contributing to reduced milk production [4].

Bovine mastitis is one of the most critical diseases of dairy cows correlated with reduced milk production globally [5].

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Studies have estimated its prevalence is approximately 30% of African countries [6]. According to the report by Ndahetuye *et al.* [7] in East Africa, the prevalence of subclinical mastitis (SCM) was 86.2% in Uganda, 75.9% in Tanzania, and 62% in Ethiopia [7]. The disease also accounted for 78% of total milk production losses and 984.64 Eth Birr financial loss for each cow per lactation in Ethiopia [8].

The most common cause of bovine mastitis is *Staphylococcus aureus* [3]. It is very difficult to manage *S. aureus* associated disease with conventional therapies due to its pathogenesis and emerging resistance [9, 10]. The isolates of *S. aureus* in Africa are known to have high rates of resistance to erythromycin, tetracycline, cotrimoxazole, clindamycin, oxacillin, and vancomycin due to frequent prescription of these drugs [11, 12]. The meta-analysis finding of Deyno *et al.* [13] in Ethiopia revealed that *S. aureus* has gotten notoriously resistant to almost all of the antimicrobial agents in use. Shiluli *et al.* [14] reported that 67% of *S. aureus* strains were resistant to ampicillin, oxacillin, ceftazidime, vancomycin and amoxicillin, while Tadesse *et al.* [15] recorded 100% resistance to ampicillin. The capability of *S. aureus* for the β-lactamase enzyme production enables it to resist β-lactam drugs [16], while staphyloxanthin production improves its cell resistance to UV radiation and reactive oxygen species [17].

The development of resistance to presently available antibiotics is a worldwide concern, especially in veterinary medicine. It is, therefore, required to go for new therapeutic choices by determining the antimicrobial properties of medicinal plants such as *Clerodandrum myricoides, Kalanchoe densiflora, Kalanchoe marmorata and Datura stramonium,* which contain various secondary metabolites that are used for antibiotics synthesis [18, 19]. It is also known that over 50% of all modern drugs are of natural product origin which implies the role of medicinal plants in drug development programs [20]. Plant-based antibiotics are considered to be safer due to their natural origin when compared with synthetic drugs [21, 22]. According to the Medicinal Plant Names Services, around 7.5% of all plant species on Earth are recorded as being used medicinally, however, only 1.2% are cited in medicinal regulatory publications [23].

In general, proper use of medicinal plants can have spectacular health effects. The scientific evidence available regarding the antimicrobial activities of traditionally used medicinal plants against bacterial pathogens in the country is limited. For this reason, it is found necessary to provide scientific justification for the traditional uses of medicinal plants to the research community. For this study, four medicinal plants were selected by using an ethno-pharmacological approach. Information on herbal uses and preparation of these plants was collected from resident farmers (traditional healers who have been utilizing these plants as traditional medicines for the treatment of different disease conditions), using various sources aiming to evaluate *in vitro* antibacterial activity and determine phytochemicals constituents of crude methanolic extract of these plants against *S. aureus* isolated from bovine mastitis.

2. MATERIALS AND METHODS

2.1. Description of Study Area

The study was conducted in Mieso districts of West Hararghe Zone, Oromia Regional State, Ethiopia. Mieso district is one of the pastoral distincts found in the Oromia region located 300 km east of Addis Ababa and 25 km west of Chiro town, the capital of the zone. Mieso is a district where the pastoral/agro-pastoral farming system prevails. The district is located in the Eastern low lands of Ethiopia between 4009’30.1” and 40056’44”E longitude and 9019’52” and 8048’12”N latitude, with an altitude ranging from 1107 to 3106 meters above sea level. Most parts of the district are situated at about 1700 meters above sea level, which receive an average annual rainfall of 635-945 mm, which is bimodal as the short rain season is between March and April while the main rain is between July and September. Its mean annual temperature is 21 °C [24].

According to basic data of the West Hararghe Zone of Agriculture and Natural Resource Office (2017), Mieso district covers an area of 186,716 ha, and the economic base of the population is mixed agriculture, which is crop and livestock production. It is home to approximately 157,861 heads of cattle; 62,374 heads of camels; 114,721 heads of goats; 42,528 heads of sheep; 37,166 heads of donkeys, and in total, 414,650 heads of the animal population. The major crops grown in the district are sorghum, maize, and haricot bean [25].

2.2. Study Design

In vitro antibacterial activity evaluation of crude methanol extract of traditionally used medicinal plants (the root of *Clerodandrum myricoides* and *Kalanchoe densiflora,* and from the leaf of *Kalanchoe marmorata* and *Datura stramonium)* (Experimental study) was conducted against the standard and clinically isolated *S. aureus* from bovine mastitis (Table **1**). The crude extracts from each plant were tested at five graded concentrations (1000, 750, 500, 250 and 125 mg/ml). Gentamicin (0.025 mg/ml) was used as a positive control, while 5% DMSO was used as a negative control. In the morning, before milking the cows, the udder and teats were cleaned with immaculate clothes immersed in hot water and the tips of the teat were cleaned with sterile dry clothes. Then,5ml of milk samples were aseptically collected from each teat quarter using sterile universal bottles labeled with cattle and teat quarter from a local breed of cattle brought to Mieso woreda veterinary clinic for clinical mastitis management. The collected samples were transported in an ice box to Microbiology Laboratory at Haramaya University College of Veterinary Medicine for microbiological processing.

2.3. Collection and Identification of Plant Materials

Fresh roots of *K. densiflora* and *C. myricoides* and fresh leaves of *D. stramonium* and *K. marmorata* were collected from the open fields of Dida nini area around Asebot town found at a location of 910'32.1”N and 4040'05.5”E, having a distance of 40 km from Chiro, the capital town of western Hararghe zone, 33 km from Aledeghi Wildlife Reserve, 229 km from Dire dawa city, 220 km from Haramaya university,

237 km from Harari city, 285 km from Addis Ababa in Mieso district, Western Haraghie Zone of Oromia regional state, at April 2021.

Table 1. The significant ethno-botanical information of the study plant.

No.	Scientific Name	Family	Indigenous Name of the Plant	Part used
1.	*Kalanchoe densiflora*	Crassulaceae	Biixxu	Root
2.	*Kalanchoe marmorata*	Crassulaceae	Pippii	Leaf & root
3.	*Datura stramonium*	Solanaceae	Banjii	Leaf
4.	*Clerodandrum myricoidos*	Lamiaceae	Harmal	Root

Taxonomic identification of plant specimens to the genus and species level was done using herbarium materials and taxonomic keys described in various volumes on the flora of Ethiopia, and a voucher specimen was deposited at the Plant Science Department of Botany (Herbarium) of Haramaya University as stated in Table **1**. The fresh roots and leaves of the collected plants were washed with tap water and air dried under shade in the open air at room temperature (23 C or 73 F) for four weeks to reduce deterioration of the plant drug material. The dried samples were ground into powder separately using an electrical grinding mill (GM1001Retsh, Germany) and stored at room temperature in air-tight containers until extraction.

2.4. Preparation of Crude Extract

The extraction of each plant material was done following methods formerly used by Nigussie *et al.* [26]. Every 100 grams of air-dried powder of *K. marmorata, K. densiflora, C. myricoides*, and *D. stramonium* was extracted with 500 ml of 99.9% methanol. 100 grams of corresponding material was weighed and put into sterile flasks, and 500 ml was filled with 99.9% methanol then, it was kept on an Orbital shaker (GFL 3006, Germany) at 100 rpm for 96hrs. Thereafter, it was filtered using a laboratory funnel with Whatman No.1 filter paper (Whatman Ltd., England) in sterile flasks; then, it was kept in the universal bottles. Removal of the organic solvent was performed using a rotary evaporator (RE-5299 Henan, China). Thus, the solvent was evaporated to make the final volume one-fifth of the original volume, then kept in a water bath (40 °C). The consequential volume yield of each extract was weighed separately, then bottled and stored in a refrigerator at -4 °C until utilization for further study. Sterility of the plant extracts was tested by inoculating 0.5 ml from each extract on sterile Mueller Hinton Agar and incubated at 37 ˚C for 18–24hrs, then observed for the growth of microorganisms. The absence of growth in the extracts after incubation indicates sterility, and evaluation of the plant extracts was done for their antimicrobial activity following the guideline mentioned in CLSI guidelines [27, 28]. The crude extracts of *K. marmorata*, *K. densiflora*, *C. myricoides,* and *D. stramonium* were then diluted in sterilized 5% DMSO separately to get the second and subsequent concentrations (750 mg/ml, 500 mg/ml, 250 mg/ml, and 125 mg/ml).

2.5. Phytochemical Screening

Standard Phytochemical screening tests were conducted using the procedure and methodology discussed by Kebede *et al.* [19] to detect the presence of bioactive principles believed to have antibacterial activities: alkaloids, terpenoids, steroids, glycosides, saponins, phenols, tannins, flavonoids, and quinones. The concentration of phytochemicals in each plant was categorized into high concentration (+++), moderate concentration (++), low or slight concentration (+) and absence of phytochemicals (-).

2.5.1. Test for Alkaloids (Dragendorff Test)

Around 200mg of plant material was boiled in 10mL methanol and filtered. Then, 1% HCl was added, followed by 6 drops of Dragendorff reagent. The brownish-red precipitate was taken as a piece of evidence for the presence of alkaloids.

2.5.2. Test for Saponins (Froth Test)

1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 min. The mixture was then filtered, and 2.5ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

2.5.3. Test for Terpenoids (Salkowski Test)

Five (5 ml) of each extract was mixed with 2 ml of chloroform, and 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish-brown coloration of the interface was formed to show positive results for the presence of terpenoids.

2.5.4. Test for Tannins (Braymer's Test)

About 2.5mg of each plant extract was boiled in 5ml of water in a test tube and then filtered through Whatman No.1 filter paper. 2-3 drops of 0.1% ferric chloride were added and read for brownish-green or a blue-black precipitate, indicating a positive result.

2.5.5. Test for Phenols (Ferric Chloride Test)

The crude extract of the plant material was treated with 3 to 4 drops of ferric chloride solution, or dissolved 5 mg of dry extract in 0.5ml of 1% ferric chloride solution. The formation of bluish-black color indicates the presence of phenolic compounds.

2.5.6. Test for Flavonoids (Shinoda Test)

2 ml filtrate was added to concentrated HCl, and magnesium ribbon was put into the solution. Pink-tomato red to yellowish color indicated the presence of flavonoids.

2.5.7. Test for Quinones (Borntrager's Test)

About 1mg of each extract was reacted with 2ml benzene, shaken properly, and filtered through Whatman No.1 filter paper. Then, the filtrates were allowed to react with 2.5ml of 10% ammonia solution and shaken properly. The presence of

pink, red, or violet color in ammonia solution in the lower phase indicates a positive result.

2.5.8. Test for Steroids (Libermannburchardt)

Crude extract was mixed with 2ml of chloroform and concentrated H_2SO_4 was added sidewise. If red color is produced in the lower chloroform layer, it indicates the presence of steroids.

2.5.9. Test for Glycosides (Keller-Kiliani Test)

About 1.25mg of each extract was allowed to react with 0.5ml chloroform and mixed carefully. About 0.5 ml of concentrated H_2SO_4 was then carefully added to form a lower layer. The reddish-brown color at the interface indicates the presence of a steroidal ring, the glycone portion of glycosides.

2.6. Isolation and Identification of *Staphylococcus aureus* from Bovine Mastitis

Isolation of *S. aureus* was undertaken following the method described by Salauddin *et al.* [29]. A loop full of milk portion from each sample was aseptically inoculated onto a sterilized blood agar plate (BAP) endowed with 5% heparinized sheep blood, then the plates were incubated aerobically at 37 °C for 24hrs. Then the plates were examined for the presence of *S. aureus* based on the growth, and morphologic features such as colony size, shape, color and hemolytic characteristics. Thus the assumed colonies of *S. aureus* were relatively large, round, convex, golden yellow with entire edge colonies and hemolysis pattern.

The presumptive colonies were selected and subcultured on nutrient agar (Oxoid, Hampshire, England) and incubated aerobically at 37 °C for 24-48hrs to get pure culture. After incubation, the suspected colonies of *S. aureus,* a circular entire edge, the convex golden yellow color colony, were examined for their gram reaction. The catalase tests were then conducted for the Gram-positive colonies, and the *S. aureus* species are assumed to be catalase positive (gas bubbles formation). The colonies that have identified as Gram-positive, Catalase-positive, and Oxidase-negative cocci were sub-cultured on Mannitol Salt Agar (MSA), then incubated at 37 °C and examined after 24-48hrs for growth and change in the color of the medium. The presence of growth and change of pH in the media (red to yellow in color) will be regarded as confirmative identification of the salt-tolerant staphylococci.

The colonies of staphylococci that generated a yellow pigment on the MSA plate were subjected to coagulase tests. Accordingly, the colony was sub-cultured on nutrient medium broth and incubated at 37 °C for 24hrs. Then, a drop of the 24hrs old colony was mixed with 0.5 ml of rabbit plasma and incubated for 4-24hrs at 37 °C. The visible clotting in the suspension that was evaluated at 30 minutes intervals for the first 4hrs of the test, then after 24hrs of incubation, was considered coagulase-positive. The coagulase-positive staphylococci were subcultured on purple base agar (with 1% maltose). Finally, *S. aureus* was identified as the colony that rapidly ferment maltose and change the color of the medium (dark purple to yellow), and the colonies appear to be yellow.

2.7. Sources of Standard Strains

The standard laboratory strains of *S. aureus* (ATCC 25923) obtained from the Ethiopia Public Health Institute (EPHI) were used as quality control organisms in this study. The purity and viability of the organisms were justified by plating, gram staining, and conducting primary and secondary biochemical tests.

2.8. Antibacterial Activity Test

2.8.1. Inoculum Preparation and Standardization

For the antimicrobial assay, inoculums were prepared from overnight cultures by the direct colony method, as mentioned by Kwiatkowski *et al.* [17], with slight modification. The standard strain and clinical *S. aureus* isolate were cultivated for 18-24hrs at 37 °C on blood agar. After 18-24hrs of growth, two–three colonies were harvested using the bacteriological loop, suspended into 4 ml of sterile saline solution 0.85% in a test tube and vortexed for 2 min. The turbidity of the suspension to be inoculated was adjusted in line with 0.5 McFarland standards which corresponds to approximately 1×10^8 CFU/ml using spectrophotometry.

2.8.2. Agar Well Diffusion Assay

Agar well diffusion method elucidated by Owusu *et al.* [30] was employed with slight modification to evaluate the antibacterial activity of crude methanolic plant extract. Fresh cultures of respective pathogens (approximately 1×10^8 CFU/ml) were streaked evenly over the surface of the sterile Mueller Hinton agar plates with a sterile cotton swab; then, the plates were left to dry for ten minutes. After drying, equidistant wells were made on each plate using a sterilized cork borer measuring 6 mm in diameter. Then 100 µl of 1000 mg/ml, 750 mg/ml, 500 mg/ml, 250 mg/ml, and 125 mg/ml test extracts were dispensed into the labelled wells. For the comparison, gentamicin (0.025 mg/mL) and 5% DMSO (100 µl/well) were used as positive and negative controls, respectively.

The plates were then kept in the refrigerator for 2hrs to allow the extract to diffuse properly into the medium. The plates were then incubated at 37 °C for 24hrs, and the ZOI was measured by using a sliding digital microcaliper in millimeters. The experiment was performed in three independent tests for both standard and isolated bacteria, and the mean of ZOI was calculated for each test extract and the standard antibiotic. Antibacterial activities of crude extracts were interpreted by calculating the area of the inhibition zone around the well. Antimicrobial activity evaluation of each test plant was assessed in triplicates (n=3) by measuring the size of the inhibition zone to the nearest mm, and the values were expressed as the mean of three repetitions ± standard error of the mean (SEM). Fractions exhibiting strong antimicrobial inhibitory potential were considered for further MIC and MBC determination.

2.8.3. Determination of Minimum Inhibitory Concentration (MIC) and Total Activity (TA)

The broth dilution method was used to determine the minimum inhibitory concentration (MIC) of plant extracts in accordance with the method that was described by Kowalska - Krochmal & Dudek - Wicher [31] with slight modification.

Based on the results of antibacterial testing, the most efficient crude extract was chosen. Hence, MIC was determined for extracts that showed a growth inhibition diameter of ≥14 mm at 500 mg/ml concentration. Two-fold serial dilutions of the extracts were made with nutrient broth. Extract solution of 500 mg/ml was serially diluted in eight (8) test tubes to the concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 mg/ml. A microbial suspension of 1 ml was added to each of the tubes and incubated at 37 °C for 24 hrs. The control tubes did not have test extract but contained the test bacteria, and DMSO was used to dissolve the extracts. After incubation, the visual turbidity was visualized compared with the negative control. However, the turbidity of suspension in the inoculum preparation was analyzed by using spectrophotometry adjusted in line with 0.5 McFarland standards (1×108 CFU/ml) and then recorded. The lowest concentration in which the turbidity was not observed was considered a MIC of the plant extracts. In addition to MIC, the total activity in (ml/g) which indicates the volume to which the extract derived from 1g of plant material can be diluted and still inhibits the growth of the microorganism was determined. The total activity was calculated as the total mass in mg extracted from 1 g of plant material divided by the MIC value [32].

2.8.4. Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of plant extracts was determined by the broth dilution method in accordance with the method described by Parvekar *et al.* [33] with slight modification. Minimum bactericidal concentration (MBC) was recorded as the lowest extract concentration killing 99.9% of the bacterial inocula after incubation at 37 °C for 24 hrs. After the MBC determination of the plant extract, aliquots of 100 µl from all the tubes, which showed no visible bacterial growth and equal volume from the control tubes without extracts, were inoculated on Mueller-Hinton agar and incubated at 37 °C for 24 hrs. When 99.9% of the bacterial population is killed at the lowest concentration of an antimicrobial agent, it is termed as the MBC endpoint. This was done by observing pre- and post-incubated agar plates for the presence or absence of bacteria. When the ratio of MBC/MIC was ≤2, the extract was considered bactericidal; otherwise, it was considered bacteriostatic; but if the ratio ≥16, the extract was considered ineffective [34].

2.9. Data Analysis

Data gained from the tests were entered into Microsoft Excel, 2013 Spread Sheet and analyzed using SPSS Version 20 software. Results were expressed as mean ± standard error of the mean (M±SEM). The statistical differences of the mean ZOI of plant extract were carried out by employing one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. MIC and MBC values were computed using descriptive statistics. The analysis was performed at a 95% confidence level and a *P* value <0.05 denoted statistical significance.

3. RESULTS

3.1. Determination of Extraction Yield and Physical Characteristics of Plants Extract

The extract yields, percent yields, the mass in mg extracted from 1g of plant material and the color appearance of the studied plants' methanol extracts are shown in Table **2**. Maximum yield was obtained from *K. marmorata* root (51.6%), followed by *D. stramonium* (50.7%). The minimum yield was obtained from *C. myricoides* (22.6%). The color appearance was assessed by visualization compared with the standard color reference in the laboratory.

3.2. The Plant's Preliminary Phytochemical Constituents

The preliminary phytochemical analysis for the methanolic extract of studied plants showed the presence of all tested phytochemicals in the roots of *K. marmorata* and *C. myricoides* as indicated by the data presented in Table **3**. An appreciable amount of steroids and phenols were detected in all plant extracts (except *D. stramonium*). Alkaloids are highly detected in *D. stramonium,* whereas the glycosides in the root of *K. marmorata*, flavonoids in *K. densiflora*, and terpenoids in *C. myricoides*. The strong detection of anthraquinones and saponins was observed in *K. marmorata* leaf and *C. myricoides*. Both parts of *K. marmorata* and *K. densiflora* were rich in tannins, whereas *C. myricoides* and both parts of *K. marmorata* were rich in phenols. We may speculate that these bioactive constituents are responsible for the antimicrobial activities that were demonstrated by crude extracts of medicinal plants.

Table 2. Extract yield and crude extract color of the studied plant using 99.9% methanol.

Plant Species	Sample (g)	Yield (g)	Yield (%)	ME (mg)	Extract Color
C. myricoides	100	22.6	22.6	226	Red orange
K. densiflora	100	37.2	37.2	372	Bright green
K. marmorata Root	100	51.6	51.6	516	Pinkish red
K. marmorata Leaf	100	32.3	32.3	323	Deep green
D. stramonium	100	50.7	50.7	507	Dark green

Note: ME= Mass in mg extracted from 1g of plant material.

Table 3. Phytochemical constituent of methanolic plant extract.

Phytochemicals	2ndry Metabolite Test	Plant Species & Results				
		C. Myricoides	*K. Densiflora*	*K. Marmorata Root*	*K. Marmorata Leaf*	*D. Stramonium*
Glycosides	Keller-Kiliani test	++	+	+++	-	-
Quinones	Borntrager's test	+++	+	++	+++	+
Alkaloids	Dragendorff test	++	+	+	++	+++
Saponins	Frothing test	+++	-	++	+++	++
Tannins	Braymer's test	++	+++	+++	+++	++
Steroids	Libermann burchardt	+++	+++	+++	+++	-
Phenols	Ferric chloride test	+++	++	+++	+++	-
Flavonoids	Shinoda test	+	+++	++	++	++
Terpenoids	Salkowski test	+++	+	+	+	+

Note: '+++' = high concentration; '++' = moderate concentration; '+' = slight concentration; ' –' = absence of phytochemical.

3.3. Antibacterial Activity

3.3.1. Agar Well Diffusion Assay

The results of agar well diffusion for antibacterial activity of methanolic extracts of the selected four medicinal plants at the concentration ranging from 125-1000 mg/ml revealed that all extracts were found to exhibit a considerable antibacterial potency against *S. aureus*. Among the four plants screened, the broadest spectrum of action was recorded by *C. myricoides* and *D. stramonium*, yielding the highest ZOI (27±0.58 mm) against clinical isolate and standard strains of *S. aureus*, respectively. The result was significant (p <0.05) compared to that of gentamicin which showed ZOI ranged between 25±0.6 and 28±0.7 mm. The lowest activity was observed in *K. marmorata* leaf, yielding a ZOI ranging between 22.3±0.33 and 9.7±0.33 mm against *S. aureus*. The mean ZOI of triplicate experiments for all tested plant extracts at the five different concentrations is summarized in Table **4**. Generally, it was observed that the highest concentration of the extracts exhibited a significantly (P <0.05) higher ZOI as compared to the respective lowest concentration. Gentamicin showed a significant (p <0.05) ZOI compared to the test extracts, while the negative control showed no antibacterial activity. Fortunately, all tested plants showed significant antibacterial activity against *S. aureus*. Thus, all were further chosen for the MIC and MBC determination.

3.3.2. MIC, MBC, and TA Value of Plant Crude Extract

The MIC and MBC values of each plant extract were determined to evaluate their antibacterial to demonstrate their antibacterial potency. The ANOVA test results showed a significant (P < 0.05) difference in the inhibitory effects of each plant extract. The MIC value of plant extracts ranged from 3.9 to 7.8 mg/ml, and its corresponding MBC values ranged between 7.8 and 15.6 mg/ml. *C. myricoides*, *D. stramonium*, and *K. marmorata* root demonstrated the strongest activity with MIC values of 3.9 mg/ml, followed by *K. marmorata* leaf 6.50±1.30 mg/ml. The lowest activity was recorded by *K. densiflora* with significant MIC values of 7.8 mg/ml. The significantly higher MBC values (7.8 mg/ml) were recorded by *C. myricoides*, and *D. stramonium* against both strains and *K. marmorata* root against the standard strains of *S. aureus*. The lowest MBC values (15.6 mg/ml) were observed in *K. densiflora* against both strains and the leaf of *K. marmorata* against clinical *S. aureus* isolate (Table **5**). The MBC result was directly proportional to its respective MIC value. The bacteria species inhibited at the lower concentration of the plant extracts also showed no growth in subculture media with relatively lower concentrations.

Table 4. The mean zone inhibition result of selected plant's methanolic extracts against *S. aureus.*

Plant Species	S. Aureus	Mean Zones of Inhibition (mm) ± SEM (n=3) at the concentration of				
		1000 mg/ml	750 mg/ml	500 mg/ml	250 mg/ml	125 mg/ml
C. myricoides	*Cl. iso*	27.0 ± 0.58^{abcde}	22.7 ± 0.67^{bcf}	20.0 ± 0.58^{abc}	15.0 ± 0.58^{abef}	12.3 ± 0.33^{adef}
	ATCC	24.0 ± 0.58^{abcde}	20.7 ± 0.33^{abcdf}	18.0 ± 0.58^{abef}	13.0 ± 0.58^{adef}	10.7 ± 0.33^{adef}
K. densiflora	*Cl. iso*	23.0 ± 0.58^{abcde}	19.0 ± 0.58^{abc}	16.0 ± 0.58^{abef}	12.3 ± 0.33^{adef}	10.0 ± 0.58^{adef}
	ATCC	21.0 ± 0.58^{abcde}	18.3 ± 0.33^{abcf}	16.7 ± 0.33^{abef}	14.0 ± 0.58^{abdef}	11.7 ± 0.33^{adef}
K. marmorata R	*Cl. iso*	25.0 ± 0.58^{abcde}	20.3 ± 0.33^{abcdf}	16.7 ± 0.67^{abcef}	13.7 ± 0.33^{adef}	11.3 ± 0.33^{adef}
	ATCC	26.7 ± 0.58^{abcde}	23.0 ± 0.58^{abcdf}	19.0 ± 0.58^{abef}	16.7 ± 0.67^{abef}	13.0 ± 0.58^{acdef}
K. marmorata L	*Cl. iso*	22.3 ± 0.33^{abcd}	19.7 ± 0.33^{abc}	17.0 ± 0.58^{abcf}	13.0 ± 0.58^{adef}	10.3 ± 0.33^{adef}
	ATCC	21.3 ± 0.33^{abc}	18.0 ± 0.58^{abcdf}	15.0 ± 0.58^{abcef}	11.7 ± 0.33^{adef}	9.7 ± 0.33^{acdef}
D. stramonium	*Cl. iso*	26.7 ± 0.58^{abcde}	22.0 ± 0.58^{abcdf}	19.0 ± 0.58^{abcef}	15.7 ± 0.33^{abef}	12.0 ± 0.58^{acdef}
	ATCC	27.0 ± 0.58^{abcde}	24.0 ± 0.58^{abcdf}	20.3 ± 0.33^{abcef}	16.3 ± 0.33^{abdef}	12.3 ± 0.33^{acdef}

(Table 4) contd.....

Plant Species	S. Aureus	Mean Zones of Inhibition (mm) ± SEM (n=3) at the concentration of				
		1000 mg/ml	750 mg/ml	500 mg/ml	250 mg/ml	125 mg/ml
Gentamycin (0.025 mg/ml)	*Cl. iso*	25.0 ± 0.6				
	ATCC	28.0 ± 0.7				

Note: The values are mean ± SEM (n=3); significant at P <0.05; [a] compared to gentamycin, [b] compared to 125 mg/ml, [c] compared to 250 mg/ml, [d] compared to 500 mg/ml, [e] compared to 750 mg/ml, [f] compared to 1000 mg/ml; the negative control has shown no antibacterial activity; ATCC= standard strains, Cl. iso= clinically isolated strains.

Table 5. The minimal inhibitory concentration, minimal bactericidal concentration (MIC, MBC in mg/ml) and total activity (TA in ml/g) value of crude extract.

Plant Species	MIC		MBC		MBC/MC		TA
	Cl. iso	ATCC	*Cl. iso*	ATCC	*Cl. iso*	ATCC	
C. myricoides	3.90 ± 0.0	3.90 ± 0.0	7.80 ± 0.0	7.80 ± 0.0	2.0	2.0	58
K. densiflora	7.80 ± 0.0	7.80 ± 0.0	15.6 ± 0.0	15.6 ± 0.0	2.5	3.4	48
K. marmorata root	3.90 ± 0.0	3.87 ± 0.3	10.4 ± 2.6	7.80 ± 0.0	2.7	2.0	132
K. marmorata leaf	6.50 ± 1.3	6.50 ± 1.3	15.6 ± 0.0	13.0 ± 2.6	2.4	2.0	50
D. stramonium	3.90 ± 0.0	3.90 ± 0.0	7.80 ± 0.0	7.80 ± 0.0	2.0	2.0	130

Note: The values are mean ± SEM (n=3); significant at P<0.05; Cl. iso = clinical isolate; ATCC = standard strains; TA= total mass in mg extracted from 1 g of plant material (ME) divided by MIC and it represents average value of both strains.

4. DISCUSSION

The percentage yield obtained in *C. myricoides* was 22.6%. The result was comparable with the previous finding of Kediso *et al.* [35], who reported 19.9% from ethanol leaf extract. The MICs result in this study was 3.9 mg/ml. This finding was congruent with the previous report of Tadele [36], who reported 5 mg/ml against *S. aureus*. Qualitative tests for secondary metabolites in *C. myricoides* revealed the strong detection of terpenoids, saponins, phenols, quinones and steroids, which may be responsible for the antibacterial activity demonstrated. Similar results were recorded in the previous study by Yimer *et al.* [37]. The extract showed significant antibacterial efficacy against *S. aureus* with 27.0±0.58 mm ZOI. The current finding was somehow more pronounced than the previous study by Njeru *et al.* [38], who recorded 20.3 mm. In spite of the potent MIC value of 0.015mg/ml against *S. aureus,* which is far from the current finding. The possible explanation for the difference between the reports might be variation in the quality of the isolate, stage of maturation and climate of the plant site [39].

The percentage yield obtained from methanol leaf extracts of *D. stramonium* was 50.7%. The result was incongruent with the previous finding of Bernard *et al.* [40], who got 28% using a soxhlet extractor. The great variation observed between the investigation results may be due to considerable variation in the method of extraction [41]. An appreciable amount of alkaloids, flavonoids and saponins in the plant was demonstrated by phytochemical screening tests that might be responsible for the observed pharmacological activities. The previous report of Tiwari *et al.* [42] also recorded the same results. The plant showed efficient antibacterial activities in this study, yielding 26.7±0.58 and 27±0.58 mm ZOI against isolate and standard strains of *S. aureus,* respectively. The current results of ZOI were comparable with the previous finding of Ali *et al.* [19] and Kothai [43], who recorded 22mm and 27mm ZOI against *S. aureus,* respectively. The broadest spectrum of action with MIC (3.9 mg/ml) and MBC (7.8 mg/ml) value of *D. stramonium* against *S. aureus* was demonstrated in this study. The previous findings of Baynesagne *et al.* [44], who reported the MIC (6.25 mg/ml) and MBC (12.5 mg/ml) values, were in line with the results of the current study. However, another study by Dike-Ndudim *et al.* [45] on the ethanol leaf extract of *D. stramonium* recorded the MIC and MBC values of 3.12 and 25 mg/ml, respectively, which was incongruent with the current finding. The differences in MBC results may be from the difference in the types of solvent used.

The percentage yield obtained from methanol root extracts of *K. marmorata* was 51.6% and ranked the highest results obtained from all tested plants. Phytochemical screening results of both root and leaf extracts revealed the strong detection of tannins, phenols, steroids, saponins and glycosides (not in the leaf), which might be responsible for its fantastic pharmacological activities. Similar results were recorded in the previous report [46]. The root extract showed a potent antibacterial activity yielding a ZOI ranging between 27±0.58 and 11.3±0.33 mm against both strains of *S. aureus*. Significant associations were detected within the extracts at different concentrations as well as within the positive control. The MIC values (3.87 mg/ml) in the current study were more pronounced than the previous reports of Fisaha [47], who recorded a 12.5 mg/ml MIC value against *S. aureus.* The great variation observed in MIC values in both investigations may be due to considerable variation in the method of extraction, the solvent used, and parts of the plant used [48].

The percentage yield obtained from methanol leaf extracts of *K. marmorata* was 32.3% and in line with the previous finding (28.2%) [49]. The previous antibacterial activities finding (20.3 mm) against MRSA recorded by Abdelkhalek *et al.* [50] on ethanol leaf extracts at 100 mg/ml were more pronounced than the current finding that showed the inhibition zone of 10.3 mm against *S. aureus* at 125 mg/ml. The reason for the variation in the result may be due to considerable variation in plant origins, method of extraction, the solvent used, and bacterial strains involved.

The percentage yield obtained from methanol root extracts

of *K. densiflora* was 37.2%. Qualitative phytochemical screening of the extracts demonstrated that the plant was rich in secondary metabolites such as flavonoids, steroids, tannins and phenols. The prior study of Anywar *et al.* [51] also recorded the same finding. The present study revealed effective anti-*Staphylococcus* activity of *K. densiflora* showing a significant ZOI ranging between 23±0.58 and 10±0.58 mm against *S. aureus* with MIC and MBC values of 7.80 and 15.6 mg/ml respectively. The current findings were more pronounced than the previous finding on methanol leaf extract by Kirui *et al.* [52], who reported 17 mm ZOI and MIC value of 82-127.5 mg/ml against *S. aureus*. The noticeable variation in ZOI, MIC and MBC results demonstrated in some investigations may be due to considerable variation in the method of extraction, the solvent used, and parts of the plant used.

A significant relationship was observed between phytochemical constituents and the antimicrobial activity of plant extracts. The mechanisms of action for the antibacterial activity of flavonoids, as elucidated by Khotimah *et al.* [53], are cytoplasmic membrane function inhibition, nucleic acids synthesis inhibition, and energy metabolism. Saponins are connected to bacterial cell membranes' penetrability, weakening cellular mechanisms, controlling biofilm formation, inhibiting bacterial capsule production, and reducing microbial toxin production [21]. It was suggested that terpenoids, alkaloids and phenolic compounds interact with enzymes and proteins of the microbial cell membrane causing its disruption to disperse a flux of protons towards the cell exterior, which induces cell death or may inhibit enzymes necessary for amino acids biosynthesis [54]. Tannins can inactivate several enzymes, microbial adhesion, and cell envelope transport proteins [55]. Quinones are able to form irreversible complexes with amino acids in proteins, which lead to their inactivation, implying that they target cell wall constituents, including surface exposed adhesions, cell wall polypeptides, and membrane-bound enzymes [28].

Generally, the highest anti-*Staphylococcus* activity against ATCC was recorded by *C. myricoides,* although a minimum yield was obtained from it. The maximum yield and the second most potent activity against both strains were recorded by *K. marmorata* root. The second appreciable yield and the strongest efficacy (> gentamicin) against the clinical isolate of *S. aureus* were demonstrated by *D. stramonium*. The trace number of secondary metabolites and the lowest anti-*Staphylococcus* activity was observed in *K. densiflora*. According to MIC/MBC ratio, all tested plants (except *K. densiflora*) against standard *S. aureus* while *C. myricoides* and *D. stramonium* against clinical *S. aureus* isolate were determined to be bactericidal. Therefore, using the extracts of these plants could favor alternative controls in *S. aureus* causing mastitis, thereby reducing multi-resistance pathogen while enabling the animal products free from antimicrobial residues.

CONCLUSION

The current study revealed a potent antibiotic activity of *C. myricoides, K. densiflora, K. marmorata,* and *D. stramonium* compared with gentamicin. This finding implies that these tested plant extracts may contain bioactive phytochemical compounds with therapeutic potential compared to the antibiotic for the treatment of numerous infections. The finding of this study provides a piece of evidence to encourage the standardized use of these plants as potential candidates for the development of novel antibacterial formulations with stronger efficacy that can be used to promote animal health and productivity. Thus, further study on the antibacterial activities of all parts of the plant, combination of active plants, and extraction using other solvents should be performed. Determination of biological activities, particularly *in vivo* experiments, and antioxidant, cytotoxicity, and affordability analyses of those plant extracts should also be investigated.

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of Variance
ATCC	=	American Type Culture Collection
CFU	=	Colony Forming Units
CLSI	=	Clinical Laboratory Standard Institute
CSA	=	Central Statical Agency
DMSO	=	Dimethyl sulfoxide
MDR	=	Multi-drug Resistant
MBC	=	Minimum Bactericidal Concentration
MIC	=	Minimum Inhibitory Concentration
MSA	=	Mannitol Salt Agar
SCM	=	Subclinical Mastitis
SEM	=	Standard Error of Mean
TA	=	Total Activity
UV	=	Ultraviolet
ZOI	=	Zones of Inhibition.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author, [B.Y] on special request.

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None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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