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Proximate, Phytochemical and Antibacterial Analysis of *Persea americana* Obtained from Nigeria

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Abstract: The proximate analysis and antimicrobial activities of *Persea Americana* seed (Avocado pear) against pathogenic *Escherichia coli* and *Staphylococcus aureus* was carried out to ascertain the unique properties that makes it confer antibacterial effect. This research work was carried out in the Microbiology Project Laboratory of Chukwuemeka Odumegwu Ojukwu University, Uli. The organisms used were obtained from urine samples of students and characterized using microbiological and biochemical tests. The phytochemical and proximate analysis of the seed extract of the plant was determined quantitatively using chemical and spectrophotometric methods. The inhibitory activity test was carried out using agar well diffusion method also tube dilution technique using double-fold serial dilution method was employed for assaying the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at various concentrations (31.25, 62.5, 125, 250 and 500 mg/ml). The phytochemical analysis revealed the presence of alkaloids, tannins, flavonoids, saponins, phenols, cyanogenic glycosides and steroids. The proximate content revealed that the seed contained more of moisture than fat and fibre. The ethanolic extract of *Persea americana* seed showed a pronounced activity (11.00 mm and 9.40 mm) against *Escherichia coli* and *Staphylococcus aureus* were significantly higher ($P<0.05$) than that of the aqueous extract (5.90 mm) which showed for only *Escherichia coli*. This activity differed significantly ($P>0.05$) from that of Ciprofloxacin (22.10 mm and 16.40 mm). The results of MIC for the ethanolic extract against *E. coli* and *S. aureus* were 500 mg/ml and 250 mg/ml respectively, while the MBC result was pronounced for only *E. coli* at 500 mg/ml. The spectra of antimicrobial activities displayed by the extracts could be attributed to the presence of these phytochemicals and signifies the potential of *Persea americana* as a source of therapeutic agent.

Keywords: *Escherichia coli*, *Staphylococcus aureus*, *Persea Americana* Seed, MIC, MBC

1. Introduction

Medicinal plants have continued to attract attention in the global search for effective antimicrobial agents that can combat resistant pathogens that have been rendering many conventional drugs recalcitrant in the treatment of infections. The potential of higher plants being used for new drug is still largely unexplored. Plants are the most exclusive sources of drugs for the majority of the world as people in the developing countries use medicinal plants for their primary health care [1]. Before scientists made inroads into research of drugs that cure human infections, traditional means of treating diseases involved using concoctions from plants, either in single form or in mixtures. Plants have the major advantage of still being used as the most effective and cheaper alternative sources of drugs. The use of medicinal plants as sources of relief from illnesses can be traced back to several millennia.It is an art as old as mankind [12].

According to Bibitha *et al*. [4] the local use of natural plants as primary health remedies due to their pharmacological properties is quite common in Asia, Latin America and Africa. The production of medicines and the pharmacological treatment of diseases began with the use of herbs [28]. Many plants are consumed as food, without the in-depth knowledge of their exact chemical composition and contribution to health, although their utilization had passed through several ancestral generations who probably realized from experience that those plant food materialswere beneficial [6]. According to Nwachukwu *et al*., [17] plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and acute diseases and both traditional healers and pharmaceutical drug companies exploit these attributes[19]. The seeds of *Persea americana* has a diverse application in ethnomedicine, ranging from treatment for diarrhea, dysentery, toothache, intestinal parasites to the area of the skin treatment and beautification [24]. *Persea americana* leaves have been reported to possess anti-inflammatory and analgesic activities. The seeds are rich in tannins and carotenoids, and tocopherols from the fruit were shown to inhibit the *in-vitro* growth of prostate cancer cell lines and "persin" from avocado leaves was shown to have antifungal properties and to be toxic to silkworms. Antioxidant activity and phenolic content of the seed was found to be greater than 70% [26]. In this study, the phytochemical and antimicrobial activity of *Persea americana* was carried out with a view to providing scientific basis on the claim by traditional healers of its uses in traditional medicine in the context of the continued search for active therapeutic agents from plants. The goal of this study is to phytochemically analyze the constituents and determine the proximate and antibacterial properties of *Persea americana* on *Escherichia coli* and *Staphylococcus aureus*.

2.Materials and Methods

2.1. Sample Collection

The fresh fruits of *Persea americana* were collected from Oba, Idemili-South Local Government Area, Anambra State, Nigeria.

Figure 1.*Leaves and fruit of Persea Americana.*

2.2. Preparation of Sample for Extraction

The seeds of Persea americana was removed from the fruit. They were thoroughly washed with distilled water and kept at room temperature for one month to dry. The dried sample were ground with a sterile hand grinder, weighed and kept ready for extraction of active ingredients [18].

2.3. Extraction Procedure

A 20 g portion of the sample was extracted by solvent extraction in 200 ml of ethanol and water shaken intermittently respectively for 3 days. The resulting extracts were filtered using Whatman No 1 filter paper. The extracts were evaporated to dryness at room temperature in a steady current [18].

2.4. Concentration of the Extracts

One hundred milliliters (100 ml) of ethanolic extract was exposed at room temperature for 7 days. While that of aqueous extract was concentrated using electric shaker bath at a temperature of about 40°C for 5 days. The concentrates were used to prepare the actual concentration of the extracts (500 mg/ml), used for the analysis.

2.5. Determination of the Extractive Value

The determination of the plant seed extractive value was carried out to get the exact weight of the extract in the prepared concentration which was used for the analysis. One gram (1.0 g) each of the extracts was evaporated in an evaporating dish of known weight in an oven to dryness at 30°C and weighed. The dish containing the residue was allowed to cool and then reweighed. The weight of the residue was obtained by subtracting the initial weight of the empty dish from the weight of the dish and residue. This process was carried out in duplicates [30].

2.6. Preparation of the Test Samples

In this study the concentration of 500mg/ml of the extract was used to screen for the antimicrobial activity. This was done by using the methods of [30]. Here, 2.5 g of the extract was dissolved in 5.0 ml of peptone water.

2.7. Phytochemical Analysis

The ground sample was subjected to qualitative and quantitative phytochemical screening, using methods of [34].

Quantitative determination of the presence of phytochemicals

Alkaloids: Five millilitres (5 ml) of the sample was mixed with 96% ethanol and 20% tetraoxosulphate (vi) acid (1:1). One milliliter (1 ml) of the filtrate from the mixture added to 5 ml of 60% H_2SO_4 allowed to stand for 5 minutes. Then, 5 ml of 0.5% formaldehyde was added and allowed to stand for 3 hours. The reading was taken at an absorbance of 565 nm.

Glycoside: This was carried out using Buljet's reagent one gram (1 g) of the fine powder of the sample was soaked in 70% ethanol for 2 hours and then filtered. The extract was

then purified using lead acetate and disodium hydrogen tetraoxosulphate (vi) (Na_2HPO_4) solution before addition of freshly prepared Buljet's reagent. Theabsorbance was taken at 550 nm.

Saponins: Five millilitres (5 ml) of the sample was dissolved in aqueous methanol. Then 0.25 ml of aliguot was taken for spectrophotometric determination for total saponins at 544 nm.

Tannins: Ten millilitres (10 ml) of the sample was pipette into 50 ml plastic bottle containing 50 ml of distilled water. This is shaked 1 hour on a mechanical shaker. The solution was filtered and 5 ml of the filtrate was mixed with 2 ml $FeCl_3$in 0.1 NH_4Cl. The absorbance was measured at 120 nm.

Steroids: The extract was diluted with normal NH_4OH solution. Two millilitres (2 ml) of the dilute was mixed with 2 ml of chloroform in a test tube. Three millilitres (3 ml) of ice-cold acetic only dried was added to the mixture and two drops of concentrated H_2SO_4 was continually added to the mixture and allowed to cool. The absorbance was taken at 420 nm.

Phenolics: Ten millilitres (10 ml) of the sample was boiled with 50 ml acetone for 15 minutes. Five millilitres (5 ml) of the solution was pipette into a 50 ml flask. Then 10 ml of distilled water was added. This was followed by the addition of $2MnNH_4OH$ and 5 ml concentrated amyl alcohol.The mixture was left for 30 minutes and the absorbance was taken at 505 nm.

Flavonoids: Five millilitres (5 ml) of the extract was mixed 5 ml of dilute hydrochloric acid (HCl) and boiled for 30 minutes. The boiled extract was allowed to cool and then filtered. One millilitre (1 ml) of the filtrate was then added to 5 ml of ethyl acetate and 5 ml 1% ammonia solution. The absorbance was taken at 420 nm

2.8. Proximate Analysis

Moisture content: A dried crucible was weighed (W_1) and 5 g of the sample was transferred into it, and the weight (W_2) was also taken. The crucible and its content was heat in an oven at 105°C for 4 hours, and the final weight (W_3) was taken.

$$\text{Percentage Moisture Content} = \frac{W2-W3}{W2-W1} \times \frac{100}{1} \quad (1)$$

Ash Content: A dried crucible was weighed (W_1) and 5 g of the sample was transferred into it and the weight (W_2) of the crucible and its content was taken. This was heated in a furnace at 550°C for 3 hours, and the final weight (W_3) was taken.

$$\% \text{ Ash Content} = \frac{W3-W1}{W2-W1} \times \frac{100}{1} \quad (2)$$

Fat Content: The weight (W_1) of the dried flask was taken and 5 g of the sample was transferred into the thimble of the extractor. The sample was extracted using organic solvent. The moisture content of the extract was evaporated in an oven at 105°C and the final weight of the flask and its content was taken.

$$\% \text{ Fat} = \frac{W3-W1}{\text{Weight of sample}} \times \frac{100}{1} \quad (3)$$

Fibre Content: The weight (W_1) of the chaff from extraction was taken and transferred into a beaker. Water and sodium hydroxide was added and boiled for 30 minutes. This was filtered and the residue was re-transferred into a beaker and acidified. This was also boiled for 30 minutes. This was finally filtered and the residue generated was transferred into a crucible and weighed (w_2), dried in an oven at 105°C. The residue was transferred into a furnace and ash at 550°C. this was cooled and the weight (W_3) of the crucible and its content was taken.

$$\% \text{ Fibre} = \frac{W3-W2}{W1} \times \frac{100}{1} \quad (4)$$

Protein Content: The digestible flask was dried, and 0.5 g sample was transferred into it, and digested with Sulphuric acid and Selenium powder. The sample generated was diluted, and 5 ml of the diluted sample was transferred into a distillation flask, and 5 ml NaOH was added and distillated into a conical flask containing 10 ml boric acid, bromocresol green and methyl red. The resultant mixture generated after distillation was titrated against hydrochloric acid (burette). The titre value was taken and recorded.

$$\% \text{ Nitrogen} = \text{Titre value} \times \text{Molarity of acid used} \times \text{Atomic mass of } N_2 \quad (5)$$

$$\% \text{ Protein} = \% \text{ Nitrogen} \times \text{Factor} \quad (6)$$

Carbohydrate Content:

$$\% \text{ Carbohydrate} = 100 - (\% \text{ Moisture} + \% \text{ Fat} + \% \text{ Ash} + \% \text{ Fibre} + \% \text{ Protein}) \quad (7)$$

2.9. Sterilization of Glassware

The glassware were sterilized using electric oven. The glassware were washed with detergent and rinsed with clean water. These were air dried and placed inverted inside the stair case of the oven and set the thermostat at 160°C for 3 hours.

2.10. Isolation and Identification of Test Organisms

The test organism used for this work was collected from urine samples of students from Chukwuemeka Odumegwu Ojukwu University, Uli Campus. A sterile universal container was used for the sample collection, and the samples were aseptically inoculated into petri dishes containing Eosine Methylene Blue (EMB) agar, Mannitol salt agar and MacConkey agar respectively. The plates were incubated inverted at 37°C for 24 hours, the growth on the different media plates were subcultured aseptically into Nutrient media using streaking method as described by Cheesbrough, [5]. The pure cultures generated were characterized and identified using their colonial descriptions; gram staining, microscopy and biochemical reaction [5].

2.10.1. Gram Staining

This was carried out using the modified method of Willey *et al*. [33]. In this process, a thin smear of the culture was prepared on a clean grease free slide, air dried and heat fixed. The smear was flooded with crystal violet solution for 60 seconds and rinsed with water. It was then covered with Gram's iodine for 60 seconds and rinsed with water. Alcohol (95% w/w ethanol) was used to decolourize the slide content for 10 seconds and rinsed with water. The smear was then counter stained with safranin solution for 60 seconds, rinsed and air dried. The stained smear with a drop of immersion was then observed under the light microscope using oil immersion objective lens.

2.10.2. Motility Test

This was carried out using the method described in Willey *et al.* [33]. The medium used was semi-solid agar. It was prepared by mixing 17.0g of bacteriological agar with 20.0g of nutrient broth in 1 litre of distilled water. Heat was applied to dissolve the agar and 10.0ml amounts were dispensed into test tubes and sterilized by autoclaving. The test tubes were allowed to set in a vertical position. Inoculation was done by making a single stab down the center of the test tube to half the depth of the medium using a standard stabbing needle. The test tubes were incubated at 37°C for 24 hours, motile bacteria swarm and gave diffused spreading growth that was visible to the naked eye.

2.10.3. Indole Test

This was carried out using the method described by Willey *et al.* [33]. Indole is a nitrogen containing compound formed when the amino acid tryptophan is hydrolyzed by bacteria that have the enzyme tryptophanase. This is detected by using Kovac's reagent. For this test, isolates were grown in peptone water in 500.0 ml of deionized water. Ten millilitres of peptone water was dispensed into the test tubes and sterilized. The medium was then inoculated with the isolates and incubated at 37°C for 48 h. Five drops of Kovac's reagent were carefully layered onto the top of 24 h old pure culture. The presence of indole was revealed by the formation of red layer colouration on the top of the broth culture.

2.10.4. Methyl Red Test

This was carried out as described in the Manual of Microbiology by Cheesbrough [5]. The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37°C for 48 h. After incubation, five drops of 0.4 % solution of alcoholic methyl red solution was added and mixed thoroughly, and the result was read immediately. Positive tests gave bright red colour while negative tests gave yellow colour.

2.10.5. Voges-Proskauer Test

This was carried out as described in the Manual of Microbiology by Cheesbrough [5]. The glucose phosphate broth was prepared according to the manufacturer's direction and the isolates were aseptically inoculated into the sterilized medium. This was incubated at 37°C for 48 h. After incubation, 1.0 ml of 40% potassium hydroxide (KOH) containing 0.3% creatine and 3 ml of 5% solution of α-naphthol was added in the absolute alcohol. Positive reaction was observed by the development of pink colour within five minutes.

2.10.6. Catalase Test

This was carried out as described in the Prescott, [25] hydrogen peroxide (May and Baker) was the reagent used. A loopful of the *Staphylococcus aureus* from a 42 hours old pure culture was transferred to a clean grease-free microscopic slide and a drop of hydrogen peroxide (H_2O_2) was added to the bacteria on the slide. Prompt effervescence indicated catalase production as a result of the liberation of free oxygen as gas bubbles. Catalase is an enzyme capable of decomposing hydrogen peroxide to water and oxygen.

2.10.7. Oxidase Test

Oxidase reagent containing 1.0% (w/v) tetramethyl-p-phenylenedimine dihydrochloride was prepared by dissolving 0.1g of this compound in 10ml of distilled water. Strip off filter paper was soaked with this reagent, smear of the isolates from the pure culture were made on the oxidase paper strips and observed for colour change from grey to purple or violet colour lateen 2-5 seconds for oxidase-positive organism. For oxidase-negative bacterial there is usually no colour change. The change of colour is due to the possession of cytochrome [33].

3. Maintainance of Test Organisms

The isolated organism was used for the antibacterial sensitivity testing prior to the test, the organism was sub cultured on nutrient agar at 37°C for 24hours.Then the 24hours culture was transferred in nutrient broth and incubated at 37°C for 24hours.

3.1. Reparation of Standard Antibiotics

The standard antibiotics used for this work was ciprofloxacin. This was because it is effective for the treatment of bacterial infections. 500 mg/ml of standard antibiotic was prepared by dissolving 5 g in 10 ml of distilled water.

3.2. Sensitivity Testing Using Agar-Well Diffusion Method

Each labeled plate was uniformly inoculated with the test organism (a 24 hours broth culture of *Escherichia coli* and *Staphylococcus aureus*) using pour plating method. A sterile cork borer of 5 mm diameter was used to bore wells on the medium. One tenth millilitre (0.1 ml) of various concentrations of the extracts were dropped into each labeled well and then incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (mm) was used as control.

3.3. Determination of Minimum Inhibitory Concentration

Here, various concentrations of the test extract were obtained using double fold serial dilution. Each dilution was assayed against the test organism using tube dilution method. One millilitre of the test organism was added into each dilution and incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth. This was determined and recorded.

3.4. Determination of Minimal Lethal Concentration

Here, equal volume of various concentrations of those tubes that did not show any visible growth for MIC were subcultured on sterile poured plate and incubated at 37°C for 24 hours. The lowest concentration of the extract that showed no visible growth is the MLC.

3.5. Statistical Analysis

The results of the data generated were expressed as mean ± standard deviation (SD). The statistical analysis of data generated from protective study was carried out using student "t" test at 95% confidence limit [31].

4. Results

The characteristic properties of the test organisms used for this study is shown in Table 1. The test organisms; *Staphylococcus aureus* and *Escherichia coli* were isolated from urine sample. They were characterized and identified based on their colonial description, gram reactions and biochemical reactions.

Table 1. *Characteristics and identities of the test bacteria.*

Parameter	*Staphylococcus aureus*	*Escherichia coli*
Appearance on agar plate	Golden yellow	Green metallic sheen
Margin	Entire	Entire
Gram reaction	+	-
Shape	Coccus	Rod
Catalase test	+	-
Motility test	-	+
Indole test	-	+
Coagulase	+	-
MR test	-	+
VP test	-	+

+=Positive
-=Negative

The quantitative and qualitative phytochemical determination of the seed extract is shown in Tables 2 and 3 respectively. The analysis revealed the presence of alkaloid, tannins, flavonoid, saponins, phenolics, cyanogenic glycosides and steroid in the plant seed; *Persea americana.* The study showed that *Persea americana* contained these phytochemicals in appreciable quantities. Proximate content revealed that the seed contained more of moisture then fat and fibre. The table 4 contains the nutritive content of *Persea americana* seed.

Table 2. *Qualitative phytochemical screening of the seed extract.*

Phytochemical	*Persea americana* seed
Alkaloid	+
Tannins	+
Flavonoids	+
Saponins	++
Phenolics	+
Glycosides	+
Steroids	+

++=High content+=Moderate content
-=Nil

Table 3. *Phytochemical constituents of Persea americana seed extract.*

Parameter	Value (mg/100 g)
Alkaloid	0.84±0.03
Tannins	0.36±0.04
Flavonoids	1.87±0.41
Saponins	17.18±0.81
Phenols	7.02±0.37
Cyanogenic glycosides	0.08±0.01
Steroids	0.04±0.00

Table 4. *The nutritive constituents of Persea americana seed.*

Parameter	Value (%)
Moisture	57.14±0.82
Fat	14.45±0.58
Protein	2.17±0.17
Fibre	16.12±0.78
Ash	1.21±0.03
Carbohydrate	8.91±0.41

The diameter zones of inhibitions of ethanolic and aqueous extracts of *Persea americana* against *Escherichia coli and Staphylococcus aureus* are shown in Table 5.

Table 5. *Diameter of zones of inhibition of the inhibitory substances against the test organisms using 5 mm cork borer.*

Extract	*Staphylococcus aureus*	*Escherichia coli*
EEP	9.40 ±0.16	11.00± 0.82
AEP	-	5.90± 0.16
CPX	16.40±0.16	22.10± 0.82

EEP=Ethanolic extract of *Persea americana*
AEP=Aqueous extract of *Persea americana*
CPX=Ciprofloxacin

The study revealed that the extracts inhibited *Escherichia coli* more *Staphylococcus aureus.* The result also showed that ethanolic extract exhibited the highest activity when compared to aqueous extract.

The Minimum Inhibitory Concentration (MIC) of the studied plant seed against the tested organisms are shown in table 6 below.

Table 6. *Minimum Inhibitory Concentration (MIC) of the inhibitory substances (mg/ml).*

Substance	*Staphylococcus aureus*	*Escherichia coli*
EEP	500	250
AEP	-	-
CPX	125	62.5

EEP=Ethanolic extract of *Persea americana*
AEP=Aqueous extract of *Persea americana*
CPX=Ciprofloxacin

The Minimum bactericidal concentration (MBC) of the studied plant seed against the tested organisms is shown in table 7 below.

Table 7. *Minimum Bactericidal Concentration (MBC) of the inhibitory substances (mg/ml).*

Substance	*Staphylococcus aureus*	*Escherichia coli*
EEP	-	500
AEP	-	-
CPX	250	125

EEP=Ethanolic extract of *Persea americana*
AEP=Aqueous extract of *Persea americana*
CPX=Ciprofloxacin

The result of this study revealed that ethanolic and aqueous seed extract of the plant studied showed pronounced activity against the tested organisms and the inhibitory effect differed significantly ($P< 0.05$) from the control (Ciprofloxacin). The study further revealed that the ethanolic seed extracts of the plant studied exhibited most pronounced activity than the aqueous extract when comparing the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal concentration (MBC) as shown in Tables 6 and 7.The result showed that *Persea Americana* exhibited the highest activity on *Escherichia coli.*

5. Discussion

About 80% of the world population in Africa depends on traditional medicine for primary health care [20]. *Persea Americana* (Avocado) is among the useful plant used for traditional medicine in Africa. Different parts of these plant bodies, extracted with different types of solvent have been used by researchers for investigating its properties. In this present study; water and ethanol were used as the extractive solvents. Water was used because of its isotonic and physiological features that are favourable for extracting water soluble bioactive components [8]. The non-polar bioactive components present in the seed extract were extracted using ethanol. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way.In this present study, antimicrobial activities of *Persea Americana* seed extract were evaluated. The phytochemical compounds present in the ethanolic and aqueous extract were qualitatively determined. The following were found to be present in the seed; steroids, glycosides, phenolics, flavonoids, saponins, tannins and alkaloids as shown in Table 3. These phytochemicals are responsible for the antimicrobial activities of the seed as stated by many researchers [29]. The proximate parameter includes moisture content, fat, protein, fibre, ash and carbohydrate of which the result showed that the seed contain more moisture, fat and fibre.The present study revealed that the seed extracts of *Persea americana* showed more pronounced activity against *E. coli* than *Staphylococcus aureus.* The reason could be that the seed extract contained more of the potent bioactive molecules and that *E. coli* was more susceptible than *S. aureus.* It was observed that the ethanolic extract of this seed extract was highest while aqueous extract were the least.This indicates that the active constituents of the plant have more ability to be dissolved in ethanol and least in aqueous extract as used in the study. The result of this study highlights that the organic solvent extracts exhibited greater antimicrobial principles were either polar or non-polar and they were extracted only through the organic medium. This present research work suggests that organic solvent extraction is suitable to verify antimicrobial activities of medicinal plants and the findings were supported by many researchers [29]. The results of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the seed extract showed that the ethanolic and aqueous extracts exhibited similar activity MIC compared to Ciprofloxacin (CPX) (control) which had more pronounced activity. Also the ethanolic extract exhibited similar bactericidal activity with CPX. This means that infections caused by this organism could be managed effectively using a single dose of this seed extract. Also, further research involving *in vivo* assays will be needed to establish the relationship between the MICs and MBCs obtained in this study and the effective dosage hat should be administered in ethnomedicinal practice.

6. Conclusion

The study revealed that *Persea Americana* seed extract exhibited pronounced antimicrobial activities against the tested pathogenic organism; *Escherichia coli* and *Staphylococcus aureus.* From the above evidence, it is clear that the seed extract of the studied plants possesses antimicrobial therapeutic properties especially when extracted with ethanol and could serve as alternative therapy for some microbial infections. This therefore supports the folklore usage of the studied plant and the traditional knowledge of local users for treating bacteria and suggests that medicinal plants could be more economical and safe in treating these bacteria.

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