PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 9(4): 970–976
https://doi.org/10.14719/pst.1874

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RESEARCH ARTICLE

Anti-bacterial potential of (*Acacia nilotica, Trigonella foenum-graecum, Punica granatum* and *Commiphora myrrha*) crude extracts against diverse drug sensitive and resistant bacterial species

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OPEN ACCESS

ARTICLE HISTORY
Received: 30 April 2022
Accepted: 05 July 2022

Available online
Version 1.0 : 28 August 2022
Version 2.0 : 01 October 2022

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS etc.
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CITE THIS ARTICLE

Osman MFA, Abdalla SS, Abdelghani S, Mangi AA, Eltayeb LB. Anti-bacterial potential of (*Acacia nilotica, Trigonella foeum-graecum, Punica granatum* and *Commiphora myrrha*) crude extracts against diverse drug sensitive and resistant bacterial species. Plant Science Today. 2022; 9(4): 970–976. https://doi.org/10.14719/pst.1874

Abstract

The alarming increase in bacterial resistance to antibiotics caused some authors to state that we are approaching a post-antibiotic era and medical catastrophe, the study aimed to assess the antimicrobial effects of selected plant extracts against several sensitive and resistant bacterial isolates. Experimental cross-sectional study was conducted, 70% ethanol crude *Acacia nilotica, Trigonella foenum-graecum, Punica granatum* and *Commiphora myrrha* extract was prepared and several commercial antimicrobials agent tested, the antibacterial activity was investigated using the disc diffusion method. The inhibition zones' diameters (mm) were calculated and interpreted by Zone Diameter Interpretative Standards. Data were analyzed by using (SPSS) software version 22. About 200% of *A. nilotica* and *T. foenum-graecum* showed bactericidal effects against *Enterococcus faecalis,* means ±SD (12.3 ± 2.8 and 12.5 ± 2.1). The activity of 200% *C. myrrha* extract was highest against all diverse bacterial. Despite a relatively high inhibition zone among all plant ethanol extracts, the findings demonstrate that there is no statistical significance in the inhibitory activity impact of varying concentrations of 70% ethanol extracts of all plants extract against bacterial isolates (P. value ≥ 0.05). The outcomes of the ethanol extracts of the used plant under study demonstrated that the herbal extract can be a superior antimicrobial potential than the result of the commercial broad spectrum antimicrobial agent utilized. *C. myrrha* extract was potent antimicrobial activity against all diverse bacterial species.

Keywords

Plant extract, antimicrobial effects, bacterial species, multi-drug resistant

Introduction

Acacia nilotica is a common herb that is found in tropical and subtropical areas. Traditional ayurvedic global best practices using *A. nilotica* leaves, bark and pods to treat cancer, cough, gastroenteritis, pyrexia, small pox, piles and menstrual cramps. It is also confirmed to have antimicrobial properties wide range of gram positive and gram-negative bacteria and fungi (1). *T. foenum-graecum*, also known as fenugreek, is among the ancient therapeutic plants, with various potential health benefits including hepatoprotective, anti-inflammatory, antiulcer, antilithigenic, antitumor, antimicrobial and neuroprotective effects (2). *P. granatum*, also widely recognized as

pomegranate, is a plant whose various parts have been used in traditional medicine to treat a variety of disorders such as inflammatory processes, gastroenteritis, helminthiasis, cough and fertility problems (3). *C. myrrha* (Myrrh) has been traditionally used in perfumes, balms for mummification, skin disease treatments and for healing wounds due to it anti‐inflammatory and antimicrobial effects for the treatment of oral ulcers, gingivitis, sinusitis, glomerulonephritis, brucellosis and parasitic infections (4). Interestingly, infectious diseases are a leading cause of morbidity and mortality all over the world (5), through use of antimicrobial drugs to treat such diseases has resulted in an increase in antibiotic-resistant pathogens, necessitating the use of substitute medications that are readily available, reasonably priced and efficacious with few adverse effects (6, 7). The reemergence of antibiotic-resistant pathogens such as *Staphylococcus aureus, Mycobacterium tuberculosis, Enterobacter sp.*, *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* complicates the problem of antibiotic resistance (8, 9). On the other hand, development of methicillin and other antibiotics against infectious diseases, led to the development of a pharmaceutical industry in the last half of this century that has done much to combat disease in man (10, 11), so herbals produce and contain a variety of chemical substances that act upon the body (12). Plant-derived antimicrobials have immense clinical efficacy. They are efficacious in the management of communicable diseases since reducing many of the adverse events associated with synthetic antimicrobials. The medicinally important effects of herbal materials are simply the result of secondary product combinations plant derived. These derivatives mostly are secondary metabolites in plants, such as alkaloids, steroids, tannins and phenol compounds, flavonoids, steroids, resins, fatty acids and gums, all of which have significant patterns effects on humans. Compounds derived from various plant parts is being used to relieve symptoms, intestinal pathogens, sore throat, common cold, cholera, pyrexia and pneumonia (13, 14). The main purpose of the screening and chemical studies of medicinal plants is to assess the antimicrobial effects of selected plant extracts against several sensitive and resistant bacterial isolates, which may contribute to an invention of new drugs that would be more valuable than drugs currently being used.

Materials and Methods

Study design

Experimental cross-sectional study was conducted at Microbiology laboratory at faculty of Medical Laboratory Sciences, National University, Sudan Khartoum state, Sudan, during the period from September to November 2021 on 2 Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) and 2 Gram negative bacteria (*Klebsiella spp.* & *Pseudomonas aeruginosa*) isolated from clinical samples. This study does not include human subjects or interaction with patients neither any private information to be disclose. The decision of the ethical clearance required was sought from the Ethical Committee of the National University, Sudan.

Sample size and Specimen collection

Fifty (50) isolated bacteria from clinical samples were collected from different hospitals. Gram-negative and gram-positive bacteria isolates was collected from different hospital locality include (Elribat University Hospital, Royal Care Hospital, Yastabshiroon Hospital). Information about the bacterial isolates was collected from records using informed questionnaire.

Plant collection and Extraction

The *A. nilotica* (seeds), *T. foenum-graecum* (seeds), *P. granatum* and *C. myrrha* was collected from local Market in Khartoum in October 2021. The plants were cleaned with distilled water, dried with air in the shade with proper ventilation and then they were lightly ground using pestle until their use for extraction.

Preparation of crude extracts

Plants seeds extraction was achieved for the seeds by using maceration techniques. 100 g of each grounded material was weighed by sensitive balance, then each plant was soaked in 400 ml of 70% ethanol.

Preparation of 70% ethanol extraction

To extract 10 g of the plant, it was immersed in 70% ethanol for five days with daily filtration. To evaporate the solvent, it was subjected to low pressure until completely dried with air until we could evaporate, then it was weighed and stored at 4 °C until use. The following are produced percentages:

$$\frac{\text{Weight of extract obtained}}{\text{Weight of the plant sample}} \text{ X } 100$$

Specimen processing

The bacterial isolates were collected from different hospital, delivered to NUSU Microbiology laboratory, gram positive bacteria were inoculated on Blood agar, while gram negative ones were inoculated on MacConkey agar for purification and then for identification using different biochemical tests.

Sample inoculation

The isolates gram positive was inoculated in blood agar and gram negative was inoculated in MacConkey agar. A single colony from the growth was taken using bacteriological loop. The loop was flamed, sterilized and cooled down before used. The inoculums were spread to make another three to four areas, sterilization was done between every area and the next one to give clear single colonies, the plate was incubating at 37 °C overnight 24 hrs in aerobic condition.

Identification of gram positive and gram-negative bacterial isolates

The isolated organism was identified by cultural characteristics “colonial morphology”, Gram stain, and catalase test, coagulase test, Manitol Salt Agar (MSA), litmus Milk

reduction test, bile aesculine hydrolysis test if it was gram positive. Biochemical test including (Triple Sugar Ion agar, urease test, citrate utilization test, indole test and motility test) if it was gram negative.

Gram stain

In dry clean slide smear prepared from a colony of cultured plate and fixed by passing over the flame after being air dried. The slid was flooded with crystal violet stain for 1 min, then washed off in running tap water then flooded with iodine for 1 min, washed off in running tap water and decolorized with 70 % alcohol 3 time for few seconds, washed of immediately and finally the smear was flooded with saffranine for 2 min washed, blot with filter paper, dried and examined under the microscope using oil immersion lens (15).

Identification the isolates gram positive

Catalase test

A total of 3ml of 3% hydrogen peroxide acid was transferred into test tube, and many colonies of tested bacteria were added in the hydrogen peroxide solution using a sterile wooden stick. Positive outcomes are revealed by prompt bubble formation.

Coagulase test

In a glass slide, a thick suspension of bacteria is made by mixing it with a physiological saline solution, then a drop of plasma was added and mixed gently with the suspension by continuous rotation. Positive result indicated as clot which appeared after about 15 seconds (16).

Mannitol Salt Agar (MSA)

A bacterial colony was inoculated on mannitol salt agar containing 75g/1sodium chloride and incubated aerobically at 37 ^{0}C for overnight. *S. aureus* ferments mannitol forming yellow colonies as result.

Litmus milk reduction test

This test was used to differentiate between *Enterococci* and other *Streptococci*. Two to 3 colonies of test organisms were suspended in the litmus milk and incubated aerobically at 37 ^{0}C for 18-24 hrs, change in color indicating positive test.

Bile Aesculin hydrolysis test

This test was used to differentiate between Enterococci and other Streptococci. Several colonies of test organisms were streaked on the surface of the bile aesculin agar slant and incubated aerobically at 37 ^{0}C for 18-24 hrs, a diffuse blacking of more than half of the slant indicating positive test.

Identification of Gram-negative isolates

oxidase test

Oxidase reagent (tetramethyl para-phenylene diamine) was added in to filter paper and colonies of tested organism were transferred into the filter paper, rapidly develops a purple color developed at the colonies of oxidase positive organism.

Citrate utilization test

The organism was inoculated in Koser's citrate medium by making zigzag on the surface of the slope and incubate at 37 ^{0}C for 24 hrs, the color of the incorporated bromothymol blue indicator changes from green to blue, due to citrate utilization and production of alkali.

Urease test

The test was performed by inoculating the bacteria by zigzag on the surface of a slope medium containing urea along with suitable pH indicator (phenol red), color changed from yellow to pink with ammonia production, indicating positive urease test.

Triple Sugar Ion (TSI)

The organism was stabbed in the butt and zigzag form on the slant. A yellowish butt and reddish slope imply just glucose fermentation. while yellow color of all media means fermentation of glucose and lactose. No change of media color implies fermentation of neither glucose nor lactose. Cracks and bubbles indicate gas production where blackening indicates hydrogen sulphide production.

Indole test

It was done by inoculation of peptone water with organism and incubation for 24 hrs at 37 ^{0}C and addition of Kovac's reagent, a red color indicates the production of indole.

Motility test

A tube of semi solid media is inoculated by stabbing to a depth of about 5 cm. it is incubated at 37 ^{0}C for 18-24 hrs, if the organism spread out from the line of inoculation the test is positive (motile organism), if the organism grows only along the line of stab the test is negative (non- motile organism). The isolates bacteria were preserved by inoculated on nutrient agar, incubated at 37 ^{0}C overnight then the purified culture was kept in refrigerator at 4 ^{0}C until use (15).

Antimicrobial susceptibility testing method

Disk diffusion method for Crude Plant Extract

The disc diffusion method, which was conducted on Mueller Hinton agar, was used to screen the antimicrobial activity of plant extracts (MHA). Bacterial suspension was diluted to 108 CFU/ml with sterile normal saline solution (turbidity = McFarland standard 0.5). Then bacterial suspension was swabbed evenly over the entire of MHA and left to dry for 5 min. Sterile filter paper discs" blank discs" (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extracts with different concentration diluted serial dilution as 200%, 100%, 50% and 25% (2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml) respectively, after resuspended by methanol. Addition to this used blank filter paper soaked in methanol as control. The inoculated plates were incubated at 37 °C for 24 hrs. The diameters (mm) of the inhibition zones were measured including the diameters of the disc (15) (Fig. 1).

For commercial antimicrobials agent

After swabbing of entire petri dish with bacterial suspension, an antimicrobial disc was dispensed onto the surface

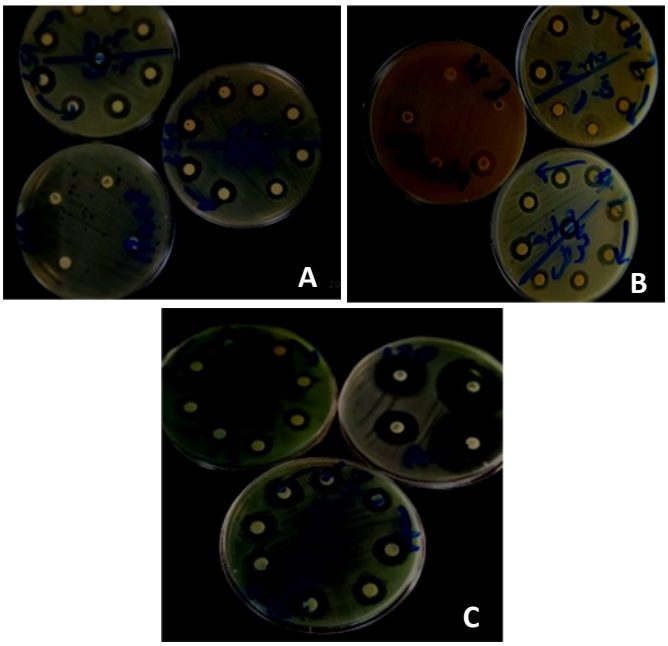

Fig. 1. Zone of Inhibitions of antibacterial activity of herbals extract used in the study using disk diffusion method with different concentration. (**a**) *Klebsiella pneumonia*, (**b**) *Enterococcus faecalis*, (**c**) *Pseudomonas aeruginosa.*

of the inoculated agar plate. Following antibiotics used were: (Vancomycin – VA 30 mcg, Gentamicin – GEN 10 mcg, Ciprofloxacin – CIP 5 mcg, Trimethoprim/Sulpha methoxazole – SXT 25 mcg, [Meropenem – MEM 10 mcg (just for gram negative)], Methicillin – ME 5mcg (just for gram positive). The inoculated plates were incubated at 37 °C for 24 hrs. The diameters (mm) of the inhibition zones were measured including the diameters of the disc. The size of the zones of inhibition were interpreted using Hi media interpretative chart.

Data analysis

Data were analyzed by using Statistical Packaged for Social Science (SPSS) software version 22.

Results and Discussion

The occurrence and increase incidence of antimicrobial resistance to antimicrobial drugs is a global crisis. Phytochemical analysis revealed the presence of several classes of secondary metabolites that are effective against microbial pathogens, including alkaloids, polyphenols, flavonoids, anthraguinones, coumarins, saponins, tannins, triterpenes and steroids (16). The existence of these chemical compounds in the tested plant extracts could provide a preliminary justification for their antibacterial and antimicrobial activities. In the present study, we studied antimicrobial potential of selected plant extracts (Table 1) against sensitive and multidrug resistant clinical bacterial isolates.

Current study revealed that ethanol extract of *A. nilotica* has bactericidal activity that able to inhibit the growth of all clinical isolates investigated which normally resistant to variety of antibiotic. Interestingly, it showing high degree of inhibition zone against *E. faecalis*. This finding is correlated with an earlier report (17) who studied activity of water extract of *A. nilotica* seeds based on phenolic agent varies on the microorganism against ATCC of *E. coli, S. typhimurium, Y. enterocolitica, K. pneumonia, B. cereus, S. aureus*. Table 2 demonstrate the frequency of clinical bacterial isolates, where *K. pneumonia* and *P. aeruginosa* account for majority of bacterial species 16 (32%). Table 2 illustrated the frequency of isolates according to type of clinical samples, 60% of S. aureus isolated from wound, 37.5% isolated from blood and wound samples, 56.6% *P. aeruginosa* isolated from blood.

Table 1. Yield percentages of extraction

Name of plant	Family	Part of plant used	Weight of plant in g	Weight of extract in g	Yield %
Acacia nilotica	Mimo-saceae	Seeds	100	25.3	25.3
Trigonella foeum-graecum	Fabace-ae	Seeds	100	10.7	10.7
Punica granatum	Lyth-raceae	Fruit	100	27.5	27.5
Commiphora myrrha	Burser-aceae	Tree Sap	100	17.4	17.4

Table 2. Frequency of Clinical bacterial isolates

Organisms	Frequency	Percent
Staphylococcus aureus	10	20.0
Enterococcus faecalis	8	16.0
Klebsiella pneumoniae	16	32.0
Pseudomonas aeruginosa	16	32.0
Total	50	100.0

The results revealed that 70% ethanol extract of the four herbals showed remarkable antibacterial activity against all, gram positive (*S. aureus, E. faecalis*) and gram negative (*K. pneumonia, P. aeruginosa*).

Findings revealed that *A. nilotica* against bacterial species, the means ±SD is (*S. aureus*, *E. faecalis*, *K. pneumonia* and *P. aeruginosa* bacteria11.4 ± 1.6, 12.3 ± 2.8, 11.6 ± 2.3 and 11.8 ± 2.5) respectively. *T. foenum-graecum* showed more bactericidal effects against *E. faecalis,* means ±SD (12.5 ± 2.1) than other isolates. 200% of *P. granatum extract* has antimicrobial efficacy against *E. faecalis*, and *P. aeruginosa* bacteria MIC means is (12.9 ± 2.9 and 12.6 ± 2.9) consequently. The activity of *C. myrrha* extract was highest against all diverse bacterial. Despite a relatively high inhibition zone among all plant ethanol extracts, the findings demonstrate that there is no statistical significance in the inhibitory activity impact of varying concentrations of 70% ethanol extracts of *A. nilotica*, *T. foenum-graecum*, *P. granatum* and *C. myrrha* against bacterial isolates (P. value≥ 0.05) all data described in (Table 3-6).

The possible justification attributed to *A. nilotica* seed which provides tannins, flavonoids, polyphenolic compounds, glycosides, volatile oils, organic acids and coumarins and polyphenolic compounds may be mainly accountable for the plant's antimicrobial properties because of phyto-constituents are responsible for plants' antibacterial activity and polyphenolic compounds and/or volatile oils inhibit broad range of organisms. As a

Table 3. Frequency of isolates according to type of samples

Organism	Type of sample	Frequency	Percent
Staphylococcus aureus	Blood	4	40.0
	Wound	6	60.0
	Total	10	100.0
Enterococcus faecalis	Blood	3	37.5
	Wound	3	37.5
	Urine	2	25.0
	Total	8	100.0
Klebsiella pneumonia	Blood	4	25.0
	Wound	9	56.3
	Urine	2	12.5
	CSF	1	6.3
	Total	16	100.0
Pseudomonas aeruginosa	Blood	9	56.3
	Wound	3	18.8
	Urine	4	25.0
	Total	16	100.0

Table 4. Inhibition zone of different concentrations of *Acacia nioltica* 70% ethanol extracts (mm)

Organism	*Acacia nilotica*				P. value
	25%	50%	100%	200%	
Staphylococcus aureus	11.4 ± 1.4	11.5 ± 1.6	11.0 ± 1.7	11.4 ± 1.6	0.894
Enterococcus faecalis	11.3 ± 0.9	11.6 ± 1.6	12.3 ± 1.9	12.3 ± 2.8	0.670
Klebsiella pneumoniae	9.8 ± 4.0	10.6 ± 3.1	11.2 ± 3.5	11.6 ± 2.3	0.414
Pseudomonas aeruginosa	11.7 ± 3.5	11.7 ± 3.6	11.7 ± 4.0	11.8 ± 2.5	0.999

Table 5. Inhibition zone of different concentrations of *Trigonella foenum-graecum* 70% ethanol extracts (mm)

Organism	*Trigonella foenum*				P. value
	25%	50%	100%	200%	
Staphylococcus aureus	10.0 ± 3.9	9.8 ± 3.9	10.7 ± 3.8	10.7 ± 3.9	0.933
Enterococcus faecalis	12.8 ± 1.6	12.3 ± 1.7	12.0 ± 1.3	12.5 ± 2.1	0.834
Klebsiella pneumoniae	10.1 ± 4.3	10.7 ± 3.3	11.8 ± 3.9	11.9 ± 4.0	0.466
Pseudomonas aeruginosa	12.3 ± 1.9	12.6 ± 1.5	12.5 ± 2.3	12.1 ± 2.1	0.920

Table 6. Inhibition zone of different concentrations of *Punica granatum* 70% ethanol extracts (mm)

Organism	*Punica granatum*				P. value
	25%	50%	100%	200%	
Staphylococcus aureus	9.9 ± 3.9	11.0 ± 4.3	10.1 ± 3.8	9.9 ± 3.9	0.912
Enterococcus faecalis	10.8 ± 1.2	11.0 ± 1.9	11.3 ± 2.4	12.9 ± 2.9	0.220
Klebsiella pneumoniae	9.5 ± 4.9	8.4 ± 5.1	10.0 ± 5.3	10.6 ± 5.6	0.686
Pseudomonas aeruginosa	10.9 ± 3.6	11.8 ± 3.8	11.9 ± 2.4	12.6 ± 2.9	0.561

chemical antiseptic, phenol is well documented in literature, moreover tannins have astyptic consequence, their existence may have hastened healing of wounds (18). With regard to *T. foenum-graecum* seeds ethanol extract, our finding demonstrated that fenugreek seed extracts had antimicrobial activity against all bacterial isolates, and highest bactericidal activity against *Enterococcus* and *P. aeruginosa*. These finding were matched with an earlier study (19), who showed the antibacterial activity of *T. foenum-graecum* seeds methanol, ethanol, chloroform and aqueous extracts against tested pathogenic bacterial strain, this activity attributed to exist of alkaloids, falvonoids, saponins. It was also noted that fenugreek seed aqueous extract demonstrated antimicrobial property against six clinical isolates of bacteria (20). The antibacterial activity of fenugreek seed extracts on many pathogenic bacterial strains suggests that they are probable sources of new antimicrobial properties. several studies conclude that the antibacterial activity of fenugreek seed extracts on many pathogenic bacterial strains suggests that they are probable sources of new antimicrobial properties and its antibacterial activity was strongest against *P. aeruginosa* and lowest against *K. pneumonia* and *S. sonnei* (21, 22).

An evidenced antibacterial activity against *E. faecalis* and *P. aeruginosa* MIC is about (12.9 ± 2.9) for two bacteria, both of which are seen to be antibiotic resistant to at least common antibiotics. This result evidenced the antibacterial potential of *Punica granatum* extract against bacterial strain causing foodborne diseases (23). Reports are on the antimicrobial effects of *P. granatum* peel extract *in vivo* and invite and eventually proven that *P. granatum* has antibacterial activity against Salmonella (24). Based on this encouraging conclusion. They documented that *P. granatum* peel extract has the potential to be a potent antimicrobial therapy for salmonellosis.

All commercial antimicrobial agent assessed reveal a verity of sensitivity and resistance against the used microorganism. Gentamicin is the only one that has broad spectrum antibiotic and has bactericidal effects against all used organisms as described in (Table 7, 8). Zone of inhibitions of antibacterial activity of herbals extract used in the

Table 7. Inhibition of different concentrations of *Commiphora myrrha* 70% ethanol extracts (mm)

Organism	*Commiphora myrrha*				P. value
	25%	50%	100%	200%	
Staphylococcus aureus	11.2 ± 4.3	11.2 ± 4.2	12.4 ± 1.9	12.6 ± 4.8	0.797
Enterococcus faecalis	12.8 ± 1.6	12.8 ± 0.9	13.4 ±1.2	13.8 ± 2.3	0.518
Klebsiella pneumoniae	10.6 ± 4.5	10.6 ± 5.5	10.7 ± 5.3	10.7 ± 6.6	0.966
Pseudomonas aeruginosa	12.7 ± 1.9	12.7 ± 1.7	12.9 ± 1.4	14.8 ± 2.6	0.669

Table 8. Antimicrobial activity of commercial antimicrobial against gram positive bacteria

Gram positive Organism	Antibiotics				
	VA	GEN	CIP	SXT	ME
Staphylococcus aureus	16.2 ± 6.6 (I)	22.7 ± 10.5 (S)	23.6 ± 13.1 (S)	19.3 ± 8.6 (S)	0.8 ± 0.8 (R)
Enterococcus faecalis	15.1 ± 11.3 (I)	13.6 ± 12.5 (S)	6.6 ± 3.4 (R)	12.3 ± 5.3 (I)	1.4 ± 1.3 (R)

study using disk diffusion method with different concentration has been given.

P. aeruginosa is well documented for its antibiotic resistance and is thus an extremely hazardous pathogen. Because of the permeability hindrance provided by its outer lipopolysaccharide membrane, the bacterium is naturally resistant to many antibiotics (LPS). In present study, the maximum antimicrobial property of *C. myrrha* was demonstrated against *P. aeruginosa*. Our findings agreed with an earlier report where the maximum antibacterial effect was exhibited by *C. myrrha* against bacteria responsible for periodontitis (25).

Variability in the antibacterial activities of the extracts were evidenced, which could be attributed to discrepancies in their chemical composition as well as the mechanism of action of their bioactive substances. All of the extracts have considerable bioactive compounds; nevertheless, activity is entirely reliant not only on the existence of bioactive molecules in phyto-constituents, but also on their concentration and possible interactions with other elements.

The findings of our study show that organic solvent extracts had excellent antimicrobial efficacy since the fundamentals of antimicrobials were either polar or non-polar and were obtained only using organic solvents. As a result, the current fact implies that organic solvent extraction was appropriate for substantiating the antibacterial effects of plant extracts, which is endorsed by many other researchers (25-27). The above outcomes can serve as the foundation for ongoing studies into toxicology testing, separating active compounds, evaluating and clarifying the constituents of these plants extract against a broader spectrum of resistance bacterial and fungal strains in order to discover new therapeutic principles.

Conclusion

The current study revealed authentic solution for drug resistance through the use of naturally derived extracts. The 4 plant extracts used in this study have antimicrobial activity that inhibit or at least has bacteriostatic effects against 4 microbial isolates. *C. myrrha* extract was potent antimicrobial activity against all diverse bacterial; so those extracts may be used as food additives and food preservatives to control such microbial population and conserve human and animal health.

Acknowledgements

This publication was supported by the Deanship of scientific research at Prince Sattam Bin Abdul Aziz University. Authors appreciated to Microbiology laboratory at faculty of medical laboratory sciences National University Sudan Khartoum state, Sudan.

Authors contributions

All authors evaluated the article, significantly contributed to the debate of the content, wrote the article, checked and revised the manuscript before submission.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical issues: None.

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