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Original Article

Liquid-Liquid Successive Fractionation of Stem Bark Alcoholic Extract from *Acacia polyacantha*: GC-MS Analysis and Antibacterial Activities

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Abstract

Antimicrobial resistance is considered as one of the top problematic issues facing world health system. Fortunately, medicinal plants, considered as ongoing source of antimicrobial agents can resolve this health problem *Acacia polyacantha* tree (AL-Kakamout) is widely available in Sudan. It is one of the main sources of Gum Arabic and used traditionally for treating many bacterial diseases. This study aimed to analyze the fractions of hydro-ethanol extract of *A. polyacantha* stem bark obtained by liquid-liquid successive fractionation by GC-MS method and to determine the antibacterial activity of these fractions against two standard bacterial strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and Gram-negative bacteria *Pseudomonas aeroginosa* (ATCC 27853) by well diffusion technique. The powdered *A. polyacantha* stem bark was extracted by cold maceration using 70% Ethanol and successively fractionated to produce petroleum ether, butanol and aqueous fraction. The butanol fraction in which Cyclolanostanol acetate (28.29%), Dopamine, N,N-dimethyl-, dimethyl ether (21.94%), were dominating, was the most active against both bacterial strains. Petroleum ether fraction which is rich in Lupeol, trifluoroacetate (37.64%) and stigmasterol (13.05%) triterpenoids was found to be more active against *P. aeroginosa* and with less activity against *S.aureus.* While the remaining aqueous fraction where hordenine alkaloid (47.21%) and 3-O-Methyl-d-glucose (%30.46) were dominant components showed no activity against *S.aureus* and with low activity against *P. aeroginosa*.

In conclusion, *A.polyacantha* bark extract fractions are rich in phytochemical compounds having antibacterial activity and highly recommended to be further investigated as potential antibacterial agents.

Keywords

Medicinal plants, Antibacterial activity testing, GC-MS analysis, *S. aureus, P.aeroginosa.*

1 Introduction

Medicinal plants have been used since ancient times as a rich source of therapeutic agents for the prevention and treatment of diseases. Also, they provide the main source of useful structures for the development of novel therapeutic agents [1]. WHO has reported that most of the world's population use herbal medicine for primary health care. Because they belief that, herbal drugs are safe, cheap and affordable [2]. Many modern drugs have been reported to show resistance in bacterial infections. These drugs are also more expensive. At the same time most of the African population lives below poverty line and cannot afford expensive modern drugs. These challenges call for renewed strategies on treatment, especially in the development of antimicrobials. According to World Health Organization (WHO), medicinal plants can provide the best alternative source to obtain variety of drugs [3]. So, they can give new options for solving antimicrobial resistance [4]. Sudan is a virgin area for phytomedicine research because, it contains a mixture of Islamic, Arabic and African traditions, besides the diversity of climates in Sudan results in a rich variety of plant species [5].

Acacia polyacantha Willd. (Mimosaceae) synonym of Senegalia polyacantha (Willd.) Seigler & Ebinger [6], is widely spread in tropical Africa [7]. It is called Kakamout in Sudan [3] and represents one of the main sources of gum Arabic [8]. The leaves are twice compound with 14- 35 pairs

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of pinnae and 20-60 leaflets per pinna. Leaves are fairly large and arranged singly along the shoots. The upper surface of the leaves is darker than the lower, and mostly with hairs on the margins and on the leaf stalk [9]. The plant has been used in traditional medicine for the treatment of snakebite, livestock diseases such as Salmonellosis and gastrointestinal diseases [10], for venereal diseases and stomach disorders [3]. Also used as an infusion to bath children who are restless at night [11]. Furthermore, it is used as antimalarial [12]. Pharmacological studies of extracts and compounds from the plant included antibacterial activity [10,11,13,14], larvicidal activity [3], radical-scavenging activity [7,10], hypoglycemic effect [15] and anti-leishmaniasis [16]. Previous phytochemical investigations of Kakamout leaves methanolic extract led to the isolation of polyacanthoside A, oleanolic acid, stigmasterol, epicatechin, quercetin, Chiroinositol, and oleanolic acid [11]. This study aimed to identify the chemical constituents of alcohol extract of A. polyacantha stem bark and test its antibacterial activity against the selected bacterial strains.

2 Methods

Chemicals and reagents

The antibiotics Ceftriaxone 30MCG, Item No SD065-5CT, Cat HIMEDIA* and Vancomycin 30MCG, Item No SD045-5CT, Cat HIMEDIA*. The experimental solvents Methanol (Purity 99.9%), Ethanol (Purity 99.9%), Petroleum ether (Purity 99%) and Butanol (Purity 99%) were obtained from (SDFCL India). The purified distilled water obtained by distillation The filter papers were Whatmann No 1. (Sigma-Aldrish, China).

Glassware and Instruments

Glassware beakers, conical flasks, round bottom flasks, open glass column, Perti-dishes, cylinders, test tubes etc., were from (SanaiLab BORO 3.3 KSA). Electrical blender (Moulinex Blender the genuine 400 W, France), GC-MS (GC/MS-QP2010SE, Shimadzu, Japan Serial number (O20535400496SA)) and freeze dryer (LYO GT2, SRK-Systemtechnik GmbH, Germany).

Microorganisms

The standard strains of Gram-positive Bacteria *Staphylococcus aureus* (ATCC 25923), Gram negative *Pseudomonas aeruginosa* (ATCC 27853) were obtained from Faculty of Microbiology, Gezira University, Sudan.

Plant materials

The stem Bark of the plant Kakamout (*Acacia polyacantha* L.) was collected from Bazoora area, South of Algadaref State in Sudan on 2 Feb. 2022. The plant sample had been identified and authenticated by the taxonomist Fatima Abdelrahman Yassin at Medicinal & Aromatic Plants and Traditional Medicine Research Institute (MAPRI), Khartoum, Sudan on 23 Feb. 2022 and the voucher specimen (code number: A-1975-17-MAPTRI-H) was deposited in the institute herbarium.

Extraction and Processing

The stem Bark materials were cut, shade dried and milled to give coarse powder using electrical blender. 100g of powdered materials were macerated for 72 hours with 1L of 70% ethanol. The extract was filtered using Buchner apparatus and the solvent was evaporated under reduced pressure to dryness using rotary evaporator. Finally, the crude extract was further freeze dried and kept in a refrigerator until use. The crude stem bark extract (4.5g) was dissolved in 400 ml of Ethanol (70%) and then was sequentially fractionated with petroleum ether (3×100ml) using separatory funnel to obtain 0.42g of petroleum ether fraction (the upper layer) after evaporation of solvent. The aqueous layer was further fractionated by butanol (3×100ml) to obtain butanol fraction (upper layer) which was 1.78g after solvent evaporation and the remaining aqueous fraction (lower layer) was 2.44g after evaporation of solvent. Solvents have been evaporated by rotary evaporator at 60℃ under reduced pressure. The three fractions were sorted in a refrigerator till use.

GC-MS Analysis

The GC-MS analysis of fractions obtained from the stem bark was performed in Medicinal & Aromatic Plants and Traditional Medicine Research Institute, Department of Pharmaceutics, Central Lab. The GC capillary column (Rtx-5MS-30m×0. 25mmI.D×0.25µm). Injector temperature was 300°C.The injector was operated in the split mode. The oven temperature was programmed from 60°C to 300 °C at 10°C/min. The Carrier gas was helium at flow rate (1.6 mL/min), Volume of injection was 1µL. The MS conditions (ion source temperature 200 °C and the Interface temperature was 250°C). The mass scan range (m/z) was 40–500 m/z, the total of run time is 34 minutes. The resulted chromatograms of the crude extract fractions (petroleum ether, butanol and aqueous), were compared by matching their retention times and mass fragmentation patterns with known components stored in the library of the National Institute of Standards and Technology (NIST) [14].

Antibacterial activity test

The Well diffusion method was used to screen the antibacterial activity of crude extract and its fractions obtained from the stem bark of *A. polyacantha*. Mueller Hinton agar (MHA) was used as nutrient media [17]. Colonies from sub-cultured bacteria were diluted with sterile normal saline (0.9%) to give 10^8 cfu/ ml (turbidity equivalent to McFarland's standard solution 0.5) [18]. About 20ml of melted MHA medium was poured into sterile plates and 200µl of bacterial suspension was added to the agar

plates. Then was mixed gently to achieve equal distribution. After complete solidification of the media at room temperature, circular wells, 8 millimeter in diameter, were punched with the back of the sterile blue tips of graduated pipette [19]. Two wells were filled with 100 µl of the extracts using different concentrations of crude extract (500, 250, 100, and 50 mg/ml) and 50mg/ml for each fraction. The third well was filled with 100 µl of methanol 50% as negative control. The positive control was Ceftriaxone disk 30 µg for *P. aeroginosa* ATCC 27853 and Vancomicin 30 µg disk for *S. aureus* ATCC 25923 were placed on the surface of the media. The plates were left at room temperature for one hour and then incubated at 37°C for an overnight. The test was done in duplicate for each extract concentration. Finally, the mean diameters of the inhibition zones were obtained in (mm) using the following standard: < 9 mm zone was considered as inactive; 9-12mm as partially active while 13-18mm as active and > 18mm as very active [20].

3 Results

Yield percent of A. polyacantha stem bark extract and its fractions

The yield percent of alcoholic crude extract was 14.96% and it was dark brown in color. Then 4.5 g from the crude extract was subjected to liquid-liquid fractionation. The intense yellow petroleum ether fraction was (9.3%), and butanol fraction was (39.5%), while the dark brown aqueous fraction was (51.2%).

GC-MS analysis of petroleum ether fraction

As reported in Figure 1 and Table 1, the major identified compounds in the petroleum ether fraction were Lupeol, trifluoroacetate (37.64%), Stigmasterol (13.05%) and Diisooctyl phthalate (7.61%).

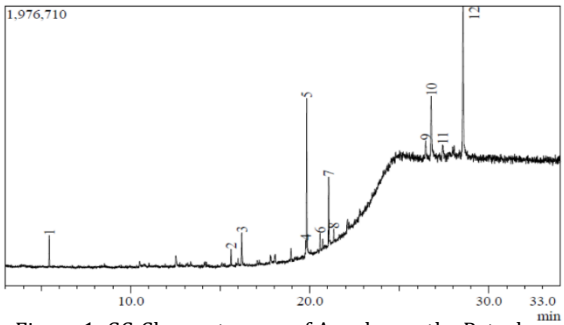


Figure 1: GC-Chromatogram of A. polyacantha Petroleum ether fraction

GC-MS analysis of butanol fraction

As shown in figure 2 and table 2, the major compounds identified in butanol fraction were 9,19-Cyclolanostan-3-ol, acetate, (3.beta.)- (28.29%), Dopamine, N,N-dimethyl-, dimethyl ether (21.94%), 2-Propenoic acid, 3-(4-methoxyphenyl)-, (E)- (5.43%) and 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol (3.26%).

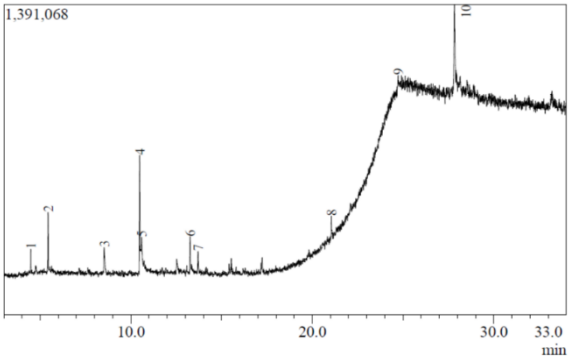


Figure 2: GC-Chromatogram of *A. polyacantha* butanol fraction.

GC-MS analysis for stem bark aqueous fraction

as described in figure 3 and table 3, Hordenine (47.21%), 3-O-Methyl-d-glucose (30.46%), 1,4,7-Triazacyclononane, 1-benzoyl- (7.49%) and Adenosine, N6-phenylacetic acid were the major identified compounds in the stem barks aqueous fraction.

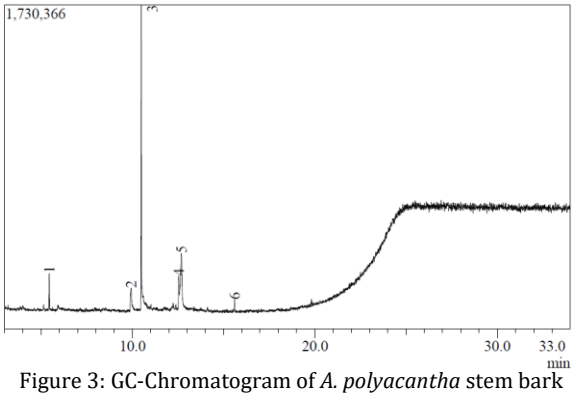


Figure 3: GC-Chromatogram of *A. polyacantha* stem bark aqueous fraction

Antibacterial activity

As illustrated in Figures 4-8, the antibacterial activity of A. polyacantha extract, and its fractions, were tested against Gram positive bacteria Staphylococcus aureus (ATCC 25923) and Gram-negative bacteria Pseudomonas aeroginosa (ATCC 27853) and the results showed that the crude extract and its fractions exhibited good antibacterial activity except aqueous fraction which demonstrate inactivity towards the S. aureus.

4 Discussion

Hydro-ethanol as 70% Ethanol was used as a solvent for extraction being eco-friendly and less expensive compared to other solvents [7]. Besides, hydro-alcoholic mixtures with a high amount of ethanol (70 - 90%) are the best commonly

Table 1: Major Phytochemical components identified in petroleum ether fraction of *A. polyacantha* by GC-MS

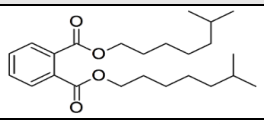
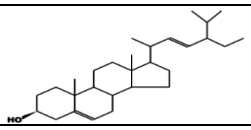
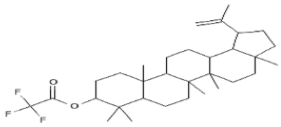
Peak No.	**Compound Name**	**Structure**	**Retention time**	**Area %**
7	Diisooctyl phthalate		21.051	7.61
10	Stigmasterol		26.7749	13.05
12	Lupeol, trifluoroacetate		28.546	37.64

Table 2: Major Phytochemical components identified in butanol fraction of *A. polyacantha* by GC-MS stem bark

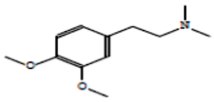
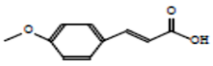
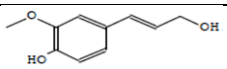
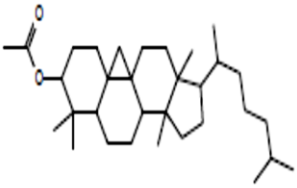
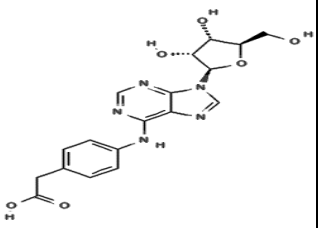
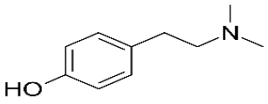
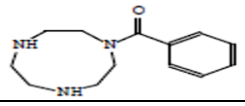
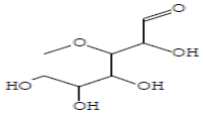
Peak No.	**Compound Name**	**Structure**	**Retention time**	**Area %**
4	Dopamine, N,N-dimethyl-, dimethyl ether		10.483	21.94
6	2-Propenoic acid, 3-(4-methoxyphenyl)-, (E)-		13.265	5.43
7	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol		13.701	3.26
10	9,19-Cyclolanostan-3-ol, acetate, (3.beta.)-		27.831	28.29

Table 3: Major Phytochemical components identified with GC-MS of *A. polyacantha* stem bark aqueous fraction

Peak No.	Compound Name	Structure	Retention time	Area %
2	Adenosine, N6-phenylacetic acid		9.928	7.97
3	Hordenine		10.484	47.21
4	1,4,7-Triazacyclononane, 1-benzoyl-		12.541	7.49
5	3-O-Methyl-d-glucose		12.686	30.46

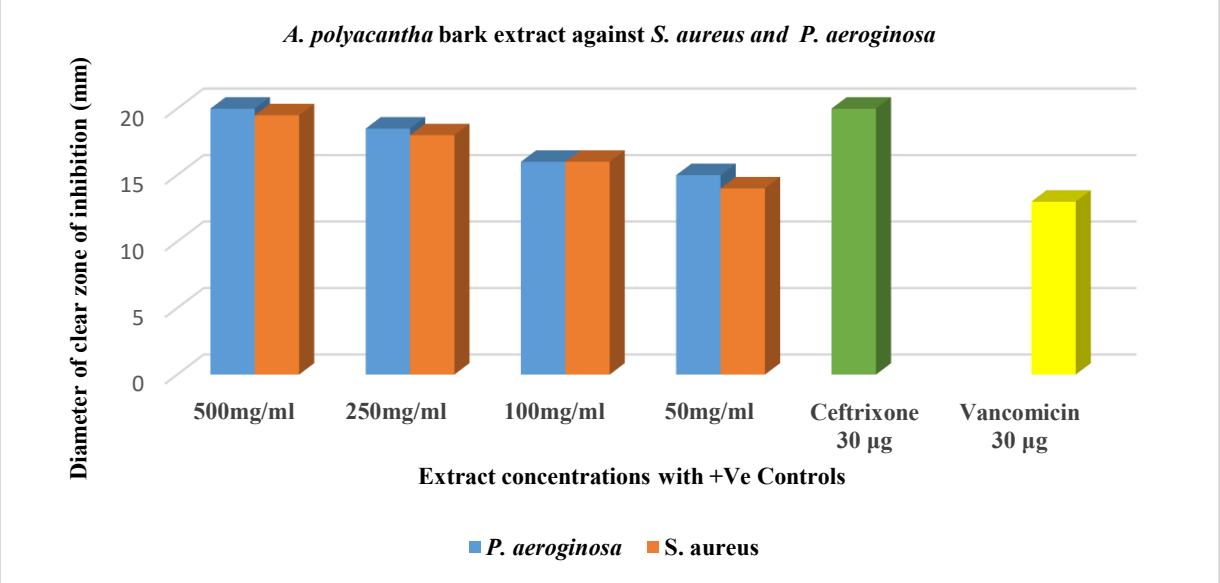


Figure 4: The Susceptability of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeroginosa* (ATCC 27853) to *A. polyacantha* stem bark extract at different concentrations

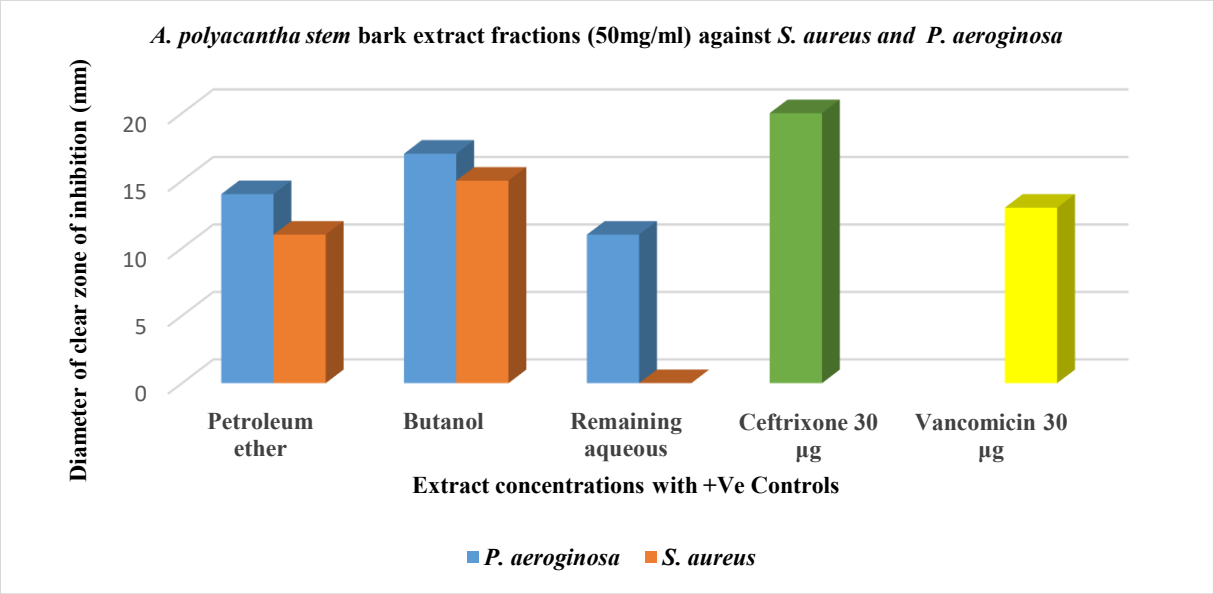


Figure 5: The Susceptability of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeroginosa* (ATCC 27853) to *A. polyacantha* stem bark extract fractions (petroleum ether, butanol and remaining aqueous) at concentration of 50mg/ml

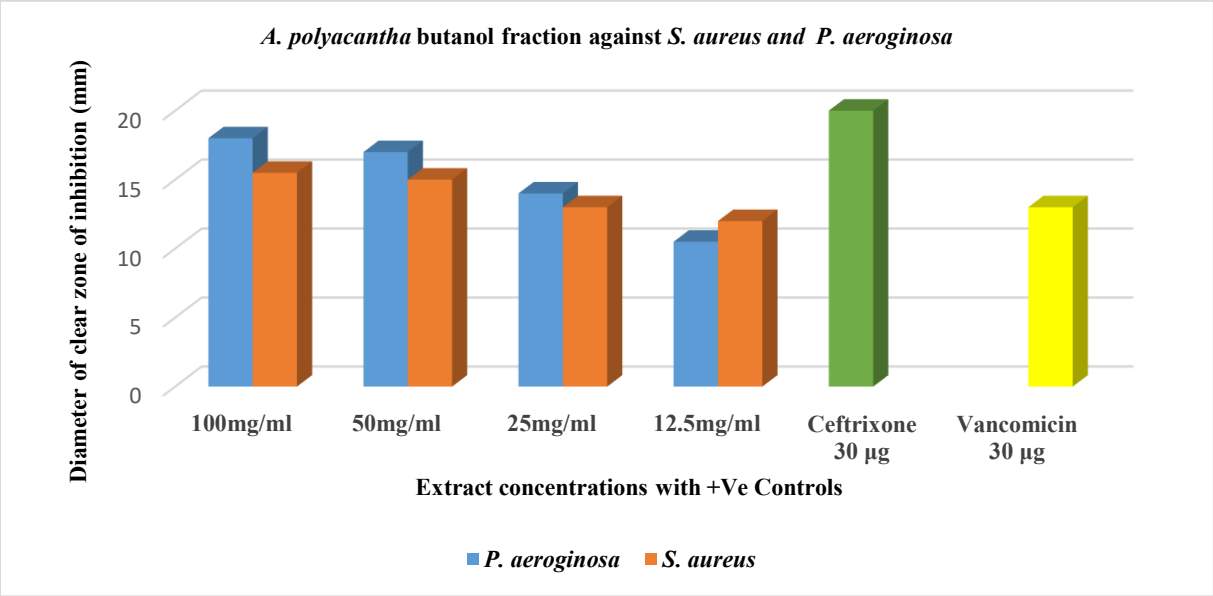


Figure 6: The Susceptability of *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeroginosa* (ATCC 27853) to *A. polyacantha* stem bark butanol fraction at different concentrations

N.B: Diameter of clear zone of inhibition < 9 mm zone is considered as inactive, 9-12mm as partially active, 13-18mm as active and > 18mm as very active [20].

used solvents for the extraction for their ability to extract a vast range of compounds of different polarities [21]. Herein, The yield percentage of hydroethanolic maceration (Ethanol 70%) for the stem bark of A. polyacantha gave 14.96 % which is very close to the yield% (15.2%) produced by Koudoro et al, [10], who used hydro-ethanol as a solvent for extracting the stem bark of A. polyacantha by maceration to give the highest yield (yield%=15.2%) compared to ethanol alone (yield%=6.8%) or water alone (yield%=14%).

The chemical analysis of A. polyacantha stem bark fractions was carried out using GC-MS for the first time as a best approach for identifying bioactive compounds [22].

The stem bark extract exhibited significant activity against both bacterial strains at a concentration as low as 50mg/ml. This result agrees with other studies of A. polyacantha stem barks extract against S. aureus [10,11,23] On liquid-liquid successive fractionation using petroleum ether and butanol, the butanol fraction was the most active against both strains in a dose dependent manner in which Cyclolanostanol acetate (28.29%), Dopamine, N,N-dimethyl-, dimethyl ether (21.94%), Propenoic acid-methoxyphenyl (5.43%) and Hydroxy-propenyl-methoxyphenol (3.26%) were dominating. This activity agreed with Shaza et al, [13]. Petroleum ether fraction which is rich in Lupeol, trifluoroacetate (37.64%), stigmasterol (13.05%) triterpenoids and Diisoctyl phthalate (7.6%) was more active against P. aeroginosa and less active against S.aureus. While the remaining aqueous fraction where hordenine alkaloid (47.21%) and 3-O-Methyl-d-glucose (%30.46) were dominant, showed no activity against S.aureus and with low activity against P.aeroginosa, this result is similar to that study of A. polyacantha aqueous bark extract [10].

The existence of phytoconstituents in A. polyacantha aerial parts extracts are known to have antibacterial activity alone or in synergism, these components include: steroidal compounds [24–26], triterpenoids [11,23,27–29] phthalates [30], fatty acids of different types [14], alkaloid of dopamine type [31,32], phenyl propanoids [33], other phenolic componds [34,35] and methyl pyranoside derivatives [36,37].

5 Conclusion

In conclusion, these findings support and validate the traditional use of the plant for treating bacterial diseases. Further phytochemical and antibacterial investigations are warranted.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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