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Antibacterial, Antioxidant Activities, Purification and GC-Ms Analysis of *A. nilotica* (L.) Ethanolic Pods Extract

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***Abstract**: Acacia nilotica is a plant in Sudan known as Gard, Gum Arabic, and Sunut. It has many uses in folk medicine, utilized as therapeutic remedies to manage various illnesses including microbial infections. This study is designed to evaluate the antibacterial, and antioxidant activities of* A. nilotica *ethanolic pod extract, to purify and to determine the components of the extract. The coarse powder of unseeded* A. nilotica *was extracted by cold maceration using 50% Ethanol and successively fractionated to produce petroleum ether, ethyl acetate, and the remaining aqueous extracts.* A. nilotica *extracts were used against* Staphylococcus aureus *ATCC25923,* Escherichia coli *ATCC25922, and* Pseudomonas aeruginosa *ATCC27853. The antimicrobial and antioxidant activities and active ingredients were determined using standard methods. Antibacterial activity of the extract showed that all the tested strains were sensitive to ethyl acetate fraction with MIC and MBC (12.5 and 25) mg/ml respectively, which is similar to the crude extract. In the DPPH radical scavenging assay of the extract and the standard quercetin at concentrations (250, 125, 50, 10, and 5) µg/ml. The radical scavenging activity was 95.05%, 94.91%, 93.19%, 19.88%, and 17.12% for the extract, which is higher compared to quercetin which gave 89.7%, 85.8%, 62.1%, 55.5%, and 45% respectively. The GC-MS analysis of the total constituents exhibited that 1,2,3-Benzentriol (59.86%), followed by phenol, 2,2'-methylenebis[6-(1,1-dimethy (14.92%) are the major components of 74.78% abundance. Finally, these findings provided useful information on the therapeutic potential of* A. nilotica*. The major constituents can play a promising role in antibacterial against* P. aeruginosa, E. coli*, and* S. aureus *to serve as a platform for formulating effective herbal drugs.*

Keywords: *Acacia nilotica,* Antibacterial, Antioxidant, GC-MS, *In-silico*, Antimicrobial susceptibility

Introduction:

Bacterial infections are a common clinical dilemma in both acute and chronic cases. The treatment of bacterial infections should only be started after the proper diagnosis to minimize antibiotic resistance (1). In this era of the Antimicrobials revolution, although there is an immoderate production of new Antimicrobial agents there is an extravagant increase in the Emergence of antimicrobial resistance, that become a significant health problem (2). Antibacterial resistance is developing rapidly and threatens to exceed the rate at which new antimicrobials an introduced. The use of Antimicrobial agents creates a selective evolutionary pressure, which increases the resistance. Antimicrobial stewardship, best use, seeking alternative Antimicrobials, and infection prevention are the most effective ways to slow the development of antimicrobials resistance (3). According to the World Health Organization (WHO), estimation there was about 80% of the population depends upon herbal remedies for the management of different types of diseases due to their affordability, availability especially in low incomes countries like Sudan, better patient acceptance, and fewer adverse effects compared to the chemicals (4). Some previous studies demonstrated the use of medicinal plants as an alternative for the management of health problems ranging from minor to moderate discomfort including infections, diabetes, cancer, and many other diseases (5) (6) (7) (8) (9) (10) (11) (12). *Acacia nilotica* is one of the medicinal plants that belong to the family of *Fabaceae* and the subfamily *Mimosoideae* (13). The common name of the Acacia tree is the Talh tree (14), Gard and Gum Arabic, and Sunut which is grown in many different countries including the central and northern parts of Sudan (15), Saudi Arabia, India, Egypt, and Sri Lanka (14). It has many uses in folk medicine, where various parts of the plant including the fruit, stem bark, and mature pods are utilized as therapeutic remedies to manage many different illnesses as tonic and astringent also are used for the treatment of common colds, bronchitis, pneumonia, and dysentery (15). Some studies verified the antimicrobial activity of *A. nilotica* (14) (15) (16). In addition, acacia is rich in antioxidant phenolic compounds, mainly condensed

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tannin and phlobatannins, and some previously published data revealed that Acacia extract possesses many therapeutic effects including antimicrobial, anticancer, antimutagenic, antipyretic, anti-inflammatory, antiulcer, antihypertensive, antispasmodic, and antioxidant activities (14) (17) (18) (19) (20) (21) (22). Some previous studies showed that the methanolic extracts of *A. nilotica* pod exposed higher total phenolic contents and good antioxidant activities compared to other different extracts. They possessed good bioactive ingredients for counteracting various ailments (14) (18) (23). Hence, this study was undertaken to assess the Antibacterial activity of the *A. nilotica* pod extract, to determine the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) against the selected Bacterial strains, to evaluate the antioxidant activity using DPPH radical scavenging activity, and to conduct the phytochemical analysis by GC-MS for determination of the major components.

Materials and Methods

Plant Materials

Collection of the plant material and preparation of the extract:
The fruit of *A. nilotica* was collected on March 2022 from the botanical garden of the Faculty of Pharmacy, University of Gezira, Wad Medani City, Gezira State, Sudan. Then authenticated by Prof. Alhadi Mohamed Mohamed Ahmed Ali, with the voucher number: (A 1) deposited in the Herbarium unit of the Medicinal and Aromatic Plants Research Center (MAPRC) at the University of Gezira, Sudan. After thoroughly washing, the fruits were air-dried at room temperature. Crushed into a coarse powder, then 500g of the unseeded powder of *A. nilotica* pods was extracted by maceration using ethanol 96% (1500 ml) at room temperature for 7 consecutive days with intermittent shaking. The plant extract was filtered through a Whatman No. 1 filter paper using a Buchner funnel vacuum. The filtrate was collected and evaporated using a rotary evaporator at 60ºC under reduced pressure to produce a dry extract following the procedure mentioned by Amber, (24).

Determination of the extract Yield
The evaporated dried extract yield on a dry weight basis (crude extract) was calculated by the following equation:

$$(X = \frac{N}{N0} X100\%)$$

Where N0: the weight of the dry plant material loaded for extraction
N: the weight of the extract after the solvent evaporation as mentioned by Amber, (24).

Chemicals and reagents

The Antibiotics used for this study include Vancomycin 30 MCG, Item No SD045-5CT, Cat HIMEDIA, Ceftriaxone 30 MCG, Item No SD065-5CT, Cat HIMEDIA and Meropenem 10 MCG, Item NO SD727-5CT, Cat HIMEDIA. All solvents used for this experiment including Ethanol (99.9%) and Methanol (99.9%) were of high purity (Research lab, India). The purified distilled water was prepared in the Quality control laboratory, Faculty of Pharmacy, University of Gezira, Sudan. All other chemicals were of analytical grade.

Equipment and instruments

The sensitive balance (BOECO, Germany), the autoclave (Modle: YX-280A, Volume: 18 L, Pressure: 0.14MPa-0.16MPa), the water bath (Scott Science, U.K), the incubator (BACTERIOLOGICAL INCUBATOR i-therm Al-7741) and the hot air oven (ELECTROTHERMAL Thermostat Dryer Model: G2X-DH-300 BS, Power: 1200 W, Voltage: 220, V: 50 Hz, Date: JUL 2011).

Bacterial strains

Standard strains of Gram-negative Bacteria ***E. coli*** ATCC 25922 and ***P. aeruginosa*** ATCC 27853, and Gram-positive ***S. aureus*** ATCC 25923, were obtained from the Medical Laboratory and blood transfusion safety services administration, General Directorate of curative medicine, Ministry of Health, Khartoum, Sudan (March 2022). The choice of bacterial pathogens is based on the WHO recommendations for the priority pathogens according to their antimicrobial resistance to encourage research and development of new antibiotics, Jubeh, (25).
Preparation of bacterial inoculums

The 24 hours old culture of each Bacterial standard strain was emulsified in sterile nutrient broth media.

In vitro antimicrobial test

The antimicrobial screening was performed by using the agar well diffusion method following the previously reported procedure with some modification (26), where sterile Mueller Hinton's agar media (HIMEDIA) was aseptically dispensed into sterile Petri dishes and uniformly, seeded with 100 µl of a suspension containing 1.5×10^8 CFU/ml of appropriate standard strains of Gram-negative Bacteria *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, Gram-Positive *S. aureus* ATCC 25923, using a swab stick, as stated by Salihu (23). The inoculums were previously refreshed from overnight cultures by the direct colony method. Where a single colony was picked up directly from the plate with a sterile wire loop and suspended into the sterile nutrient broth. The turbidity of the inoculated suspension was previously adjusted to be equivalent to 0.5 McFarland's standard solution. After that, the tested organisms were uniformly seeded over the surface of Mueller-Hinton agar. Then punched with the back for sterile blue tips of a graduated pipette to form 7 mm diameter wells, which were filled with the 100 µl of the appropriate extract of a known concentration of 100 mg/ml which was prepared by dissolving (1g of the dried crude extract into 10 ml of 50% methanol in distilled water) and solvent blank (Methanol 50% in distilled water) used as a negative control where the positive control used standard Antibiotics disk placed on the surface of the medium (7) (26). Ceftriaxone for ***E. coli*** ATCC 25922, Meropenem for ***P. aeruginosa*** ATCC 27853, and Vancomycin was used for ***S. aureus*** ATCC 25923. The plates were left on the bench top at room temperature for 30 minutes and then incubated at 37ºC overnight. After incubation, the growth inhibition zone was measured in millimeters (mm). Each experiment was performed in triplicate to validate the findings statistically (26).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antimicrobial activities of *A. nilotica* were further investigated by tube-dilution method to determine the minimum inhibitory concentration (MIC) where the method stated by Ehaimir, (7) Parvekar, (27) Cheng, (28) Hussain, (29) Carrol, (30) was adopted with few modifications. For this purpose, stock solutions of *A. nilotica* crude extract 100 mg/ml, Mueller-Hinton broth, and Bacterial strain suspension equivalent to 0.5 MacFarland's standard solution were prepared following standard methods (31). Serial dilution of (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml) was made in the tubes containing the broth media except for the last one which inoculated with 100 µl of the solvent to be considered as a negative control and the all different tubes were inoculated with 100 µl of the bacterial suspension. Subsequently, all concentrations that showed no change in color were transferred onto nutrient agar and incubated at 37°C overnight, also the concentration exposed turbidity was subcultured in the agar plate as a confirmatory test, the lowest concentration with no growth of bacteria was considered as MBC (32). The MBC was determined as the lowest concentration that eliminate 99.9% of the initial bacterial population (27). Further, the crude extract (4.5g) was dissolved in 400 ml of Ethanol (50%) and then was sequentially fractionated with petroleum ether (3×100ml) using a separatory funnel to obtain 0.38g of petroleum ether fraction (the upper layer) after evaporation of the solvent. The aqueous layer was further fractionated by ethyl acetate (3×100ml) to obtain ethyl acetate fraction (upper layer) which was 2.02 g after solvent evaporation and the remaining aqueous fraction (lower layer) was 2.10 g after evaporation of the solvent. Solvents have been evaporated by rotary evaporator at 60℃ under reduced pressure (10).

Evaluation of antioxidant characteristics

DPPH assay

DPPH radical scavenging activity of *A. nilotica* pods extract was measured following the method reported by Hussain, (29), with some modifications. Sample stock solutions (0.1 g/100ml) were diluted to final concentrations of 250, 125, 50, 10, and 5 µg/ml in methanol. 1.0 ml of a 0.3 mM 2, 2 diphenyl-2-picryl hydrazyl (DPPH) in methanol solution was added to a 2.5 ml solution of the different concentrations of the extracts and allowed to react at room temperature for 30 minutes. The absorbance of the resulting mixture was measured at 518 nm and converted to percentage antioxidant activity (AA %), using the formula below:

$$(Antioxidant\ Activity\ (inhibition\ \%) = \frac{AC - AS}{AS} \times 100\%$$

where AC: The absorbance of a control solution,

AS: The absorbance of standard or sample solution.

Each sample and standard were measured in triplicate.

Methanol (1.0 ml) plus plant extract solutions (2.5 ml) was used as a blank. DPPH solution (1.0 ml; 0.3 mM) plus methanol (2.5 ml) was used as a control. Stock solution (1 mg/ml) of Quercetin was diluted to final concentrations of 250, 125, 50, 10, and 5 µg/ml in methanol and used as a positive control.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The ethanolic extract of *A. nilotica* pods was analyzed for its chemical composition using GC-MS systems. The GC-MS analysis was performed on Shimadzu (GC\MS 2010) Helium used as carrier gas and the separation was achieved using a column (TG-SQG-15 ms×0.25 mm×0.25 µm). The starting oven temperature was programmed at 60°C with an increasing temperature10°C until reached 280°C. The crude extract was injected with split mode. Mass spectra were taken at 70eV; fragments from 40 to 1000 Dalton. The final confirmation of constituents was made by computer matching the mass spectra peaks with the Wily and National Institute Standard and Technology (NIST) Libraries mass spectra database (Biomedical Research 2017).

Data organization and statistical analysis

The Data was organized and tabulated by using Microsoft Word 2016 and Microsoft Excel 2016. The experiments were carried out in triplicates and the average of the clear zone of inhibition and standard deviations (SD) were obtained as mean and standard deviation (M±SD).

Results and Discussion

The extractive value/ yield from *A. nilotica* pods obtained by maceration in alcohol was found to score (38.80%). Ethanol was used as a solvent for its ability to extract a wide range of compounds with different polarities Saha (32). The high yield obtained indicated that alcohol (96%) is capable to extract phenolic compounds in which *A. nilotica* is rich. The Antibacterial activity of *A. nilotica* ethanolic extract (100 mg/ml) was evaluated against three standard strains: *E. coli* ATCC 25922*, P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923 using well diffusion method. The results appeared that all three tested strains *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were sensitive to the extract (100 mg/ml) with a diameter of (29.00±1.41 mm 27.66±1.52 mm and 20.50±2.29) respectively in similarity to the sensitivity reported by Carrol, (30) the positive control used for *E. coli* and *P. aeruginosa* and *S. aureus* (Ceftriaxone 30mcg, Meropenem 10mcg, and Vancomycin 30mcg) respectively, which exposed (29.33±2.08 mm, 29±1.70 mm, and 19±0.50 mm) respectively (Table 1), also on the sensitive rage reported in CLSI (33). All the strains were further tested by reducing the concentration of the crude, petroleum ether, ethyl acetate and the remaining aqueous extract to 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml for the determination of the MIC which was conducted by tube dilution technique and the MBC by agar diffusion method, present that the MIC of *A. nilotica* ethyl acetate fraction against all the tested strains was 12.5 mg/ml similar to that of the crude extract and the MBC was 25 mg/ml (Fig. 1, Fig. 2, Fig. 3). The above-mentioned findings vitrified the uses of *A. nilotica* for the treatment of wound infections, urinary tract infections, dermatological infections, abscesses, and respiratory diseases. Also, the results harmonized with previous studies presented that the pods of *A. nilotica* are used traditionally for bacterial infections according to Sadiq (16) and Karim (34). The DPPH radical scavenging assay of *A. nilotica* pods extract showed dose-dependent activity with EC50: µg/ml (Table 2), compared to that of the standard Quercetin. Results coincide with those reported earlier by Foyzun (17), Al-Rajhi (20) and confirmed the good antibacterial potential of *A. nioltica* extract as stated by Rao (19), Al-Rajhi (20), Ali (21) and Elamary (22). The chemical composition of *A. nilotica* pod extract was analyzed by using GC-MS systems. The separation of the total constituents showed that 1,2,3-Benzentriol (59.86%), followed by phenol, 2,2'-methylenebis[6-(1,1-dimethy (14.92%) are the major constituents of 74.78% abundance (Table 3). These identified components in *A. nilotica* pods extract are known to possess high antioxidant activity (14) (17) (20) (35) as well as antibacterial effects (35). The presence of the Benzentriol derivative which is known as bioactive with antibacterial activity according to (36) (37) phenol derivatives has antibacterial activity stated by (38) (39) as major components which is in the same line with the previous study carried out by (40). Benzenetriol and its derivatives possessed antibacterial activity as the study conducted by Cavalca (37). phenol was proven to have bioactivities including antimicrobial and antioxidant activities (41) (42).

Table 1: Antimicrobial Susceptibility of *A.nilotica* Ethanol Extract dissolved in Methanol 50% against different Bacterial Strains vs suitable Antibiotics

Bacterial strain	**Mean Zone of inhibition (mm) ±SD**				
	***A. nilotica* Extract Conc.**	**Mean Zone of inhibition (mm) ±SD**	**Susceptibility S/R**	**Antibiotic (+ve control)**	**Mean Zone of inhibition (mm) ±SD**
E. coli ATCC 25922	100 mg/ml	29.00±1.41	S	Ceftriaxone 30 mcg	29.33±2.08
P. aeruginosa ATCC 27853	100 mg/ml	27.66±1.52	S	Meropenem 10 mcg	29±1.70
S. aureus	100 mg/ml	20.50±2.29	S	Vancomycin 30 mcg	19±0.50

Bacterial strain	Mean Zone of inhibition (mm) ±SD				
	A. nilotica Extract Conc.	Mean Zone of inhibition (mm) ±SD	Susceptibility S/R	Antibiotic (+ve control)	Mean Zone of inhibition (mm) ±SD
ATCC 25923					

*S: Sensitive R: Resistance

*N. B: Mean Zone of Inhibition in CLSI (mm): *E. coli* ATCC 25922= 29-35, *P. aeruginosa* ATCC 27853 = 27-33, *S. aureus* ATCC 25923=17-21

Table 2: Radical scavenging activity of *A. nilotica* and Standard (Quercetin)

Concentration	Scavenging activity (%)	
	A. nilotica pods Extract	Standard (Quercetin)
250 µg/ml	95.05%	89.7%
125 µg/ml	94.91%	85.8%
50 µg/ml	93.19%	62.1%
10 µg/ml	19.88%	55.5%
5 µg/ml	17.12%	45%

Table 3: Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of *A. nilotica* pods extract major constituents

Peak	***A. nilotica* pods extract**			
	R. Time	Area	Area %	Name
1	11.322	53.17	54.59	1,2,3-Benzenetriol
2	11.673	3.34	5.27	1,2,3-Benzenetriol
17	22.812	6.05	14.92	Phenol, 2,2’-methylenebis[6-(1,1-dimethy

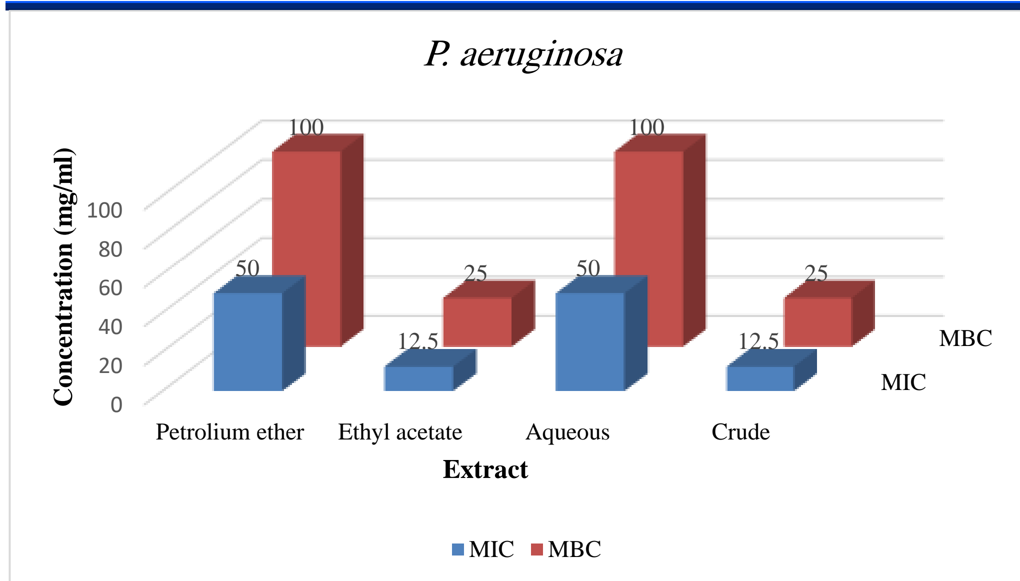

Figure 1: Determination of the MIC and MBC of the three different fractions vs crude extract against *P. aeruginosa*

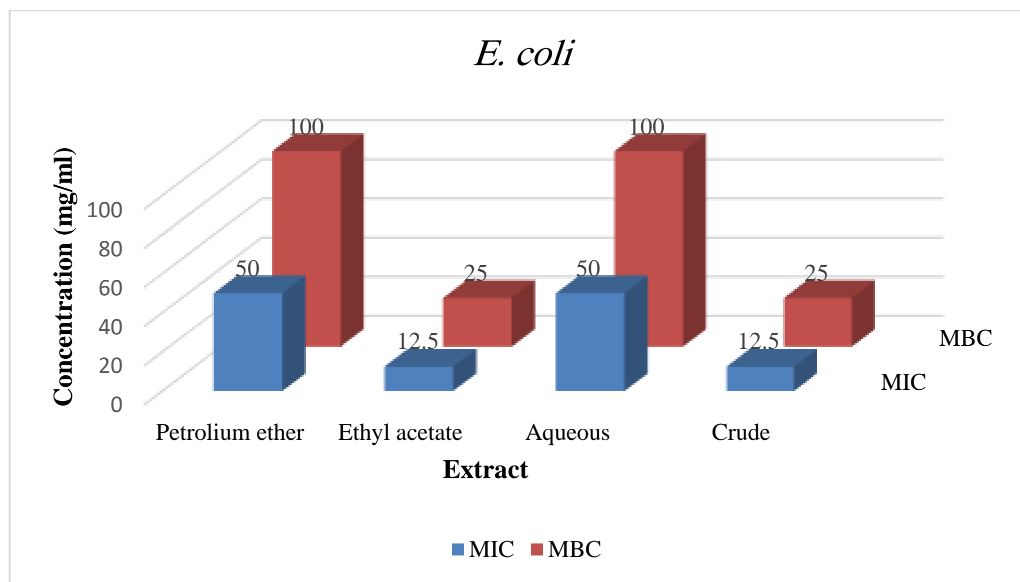

Figure 2: Determination of the MIC and MBC of the three different fractions vs crude extract against *E. coli*

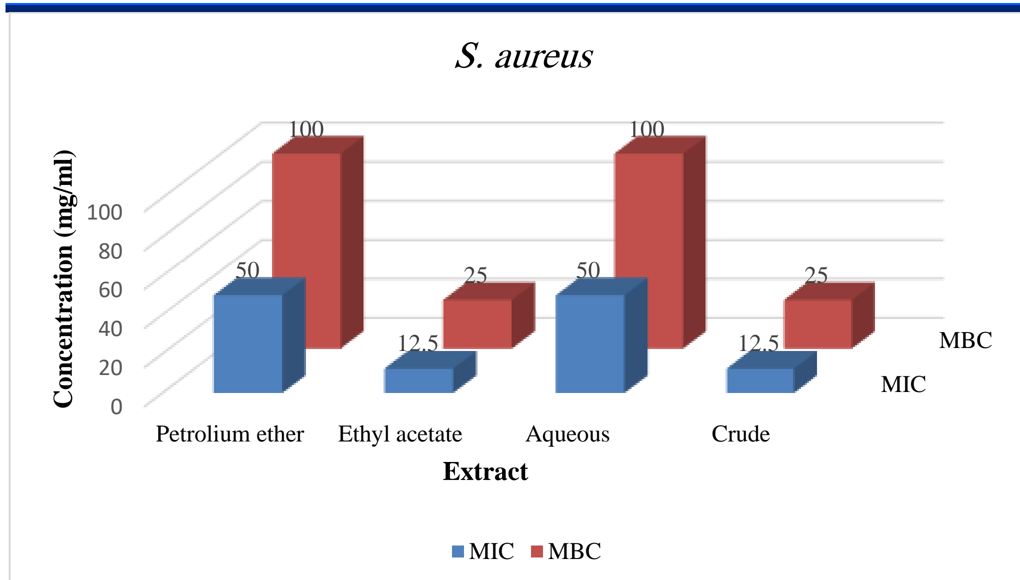

Figure 3: Determination of the MIC and MBC of the three different fractions vs crude extract against *S. aureus*

Conclusion

The study on *A. nilotica* revealed that the ethanolic extract exhibits prominent antioxidant and antibacterial activities. The presence of 1,2,3-Benzentriol and phenol, 2,2'-methylenebis[6-(1,1-dimethy are the major components that could be considered potential antibacterial agents. *In vitro* tests on the antibacterial activities, of *A. nilotica* ethanolic extract confirmed that *E. coli, P. aeruginosa*, and *S. aureus* all are susceptible to the extract. Thus *A. nilotica* can play a promising role in antimicrobial against these stains and serve as a platform for formulating effective herbal drugs.

Conflict of interest

The authors declare no conflict of interest

Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Figure 1: Antimicrobial susceptibility of *A. nilotica* 100 mg/ml vs Vancomycin against *Staph aureus* ATCC* 25923

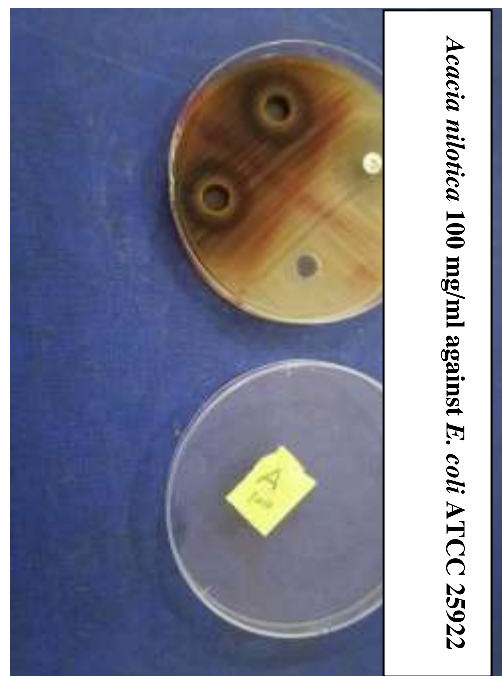

Figure 2: Antimicrobial susceptibility of *A. nilotica* 100 mg/ml vs Ceftriaxone against *E. coli* ATCC* 25922

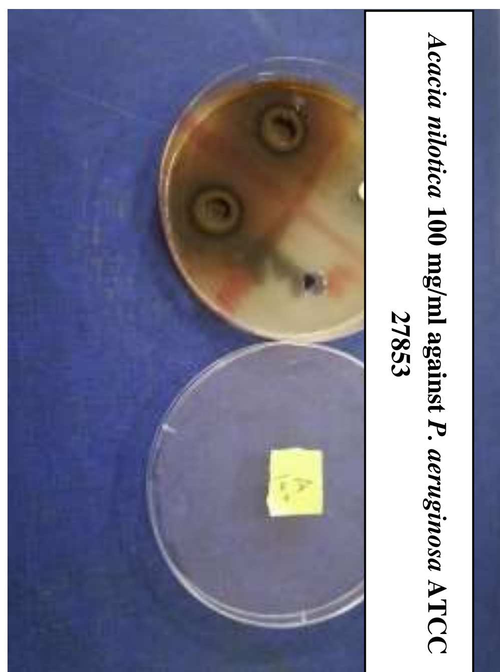

Figure 3: Antimicrobial susceptibility of *A. nilotica* 100 mg/ml vs Meropenem against *Pseudomonas aeruginosa* ATCC* 27853