ISSN: 2754-5008

Journal of Pharmaceutical Research and Reports

SCIENTIFIC Research and Community

Research Article

Open Access

Antimicrobial Activity of Acacia Nilotica Pods Against Carbapenem Resistant Gram-Negative Bacteria Isolated from Different Hospitals in Khartoum State

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ABSTRACT

This study was aimed to investigate the antimicrobial activity of ethanolic extract of Acacia nilotica against carbapenem resistant bacteria during the period from February 2018 to December 2018. Agar disc diffusion method was used to determine the antimicrobial activity of Acacia nilotica and the ethanolic extract was examined against carbapenem resistant Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Enterobacter cloacae and Citrobacter freundii isolated from different hospital in Khartoum state and the sample include (urine, wound swab, sputum, blood and body fluid) for 91 clinical isolates. The extract show activity against all tested microorganism and the inhibition zone were17±3 mm for E. coli, 18.3±4mm for Klebsiella pneumoniae, 16.9±4mm for Proteus mirabilis, 17±3 mm for Pseudomonas aeruginosa, 18.3±0.5mm for Enterobacter cloacae and 16±5mm for Citrobacter freundii and the activity of the extract at 100 mg/ml concentration show sensitive (82.4%), (72.6%) for 50mg/ml and (51.6%) for 25 mg/ml for all tested bacteria. Ethanol extract of Acacia nilotica was found to be effective as antibacterial against different bacterial pathogens, providing the scientific basis for its traditional application in Sudanese folk medicine against many bacterial diseases, extract had an in vitro antibacterial activity against carbapenem resistant bacteria, further studies are required to confirm this result to identify active ingredient and toxicity.

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Received: September 14, 2021; **Accepted:** September 21, 2021; **Published:** September 30, 2021

Keywords: Acacia Nilotica Pods, Antimicrobial Agents, Carbapenem Antibiotic, Ethanol Extract, Agar Disc Diffusion Test

Introduction

Since 1970, resistance to antimicrobial has become an escalating problem [1]. Effective antimicrobial strategies developing drive from the fact that the bacteria were uniquely suited for survival in toxic environment, the ability of bacteria to subsist on antibiotics and the potential to acquire resistance gene as a growing concern [2].

Plants can be an effective source of antimicrobials and had been used for centuries traditionally to inhibit microbial growth [3]. Acacia nilotica contains secondary metabolites including amines and alkaloids such as cyanogenic glycosides, cyclitols, fatty acids and seed oils, fluoroacetate, gums, nonprotein amino acids, terpenes, flavonoids and condensed tannins [3]. The emerging phenomenon of multidrug resistant (MDR) Gram-negative bacilli (GNB) is a pressing contemporary concern. This challenge has been compounded by the paucity of new antibiotics in late-stage development for MDR- GNB [4].

The carbapenems are considered the last resort antibiotics used for infections caused by MDR-GNB due to their stability against beta-lactamases, penicillinases and cephalosporinases, and their broad spectrum of action [5]. The global spread of carbapenemase-producing Enterobacteriaceae, Pseudomonas aeruginosa, and Acinetobacter species (i.e., multidrug-resistant organisms [MDROs] is a critical medical and public health issue [6]. These bacteria are often resistant to all beta-lactam agents and are frequently co-resistant to multiple classes of other antimicrobial agents, leaving very few treatment options [6].

The problem of microbial resistance is growing and the outlook for use of antimicrobial drugs in the future is still uncertain, therefore, action must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetic or natural [6]. Furthermore, antibiotic resistance is a global challenge that impacts all pharmaceutically used antibiotic, in recent years pharmaceutical companies have almost stopped producing new antibiotics which have led researchers to look for alternative antimicrobial, Herbs were used for the treatment of infectious diseases in many developing countries [7]. Plant possesses active ingredient for defense against plant pathogen many of these antimicrobial substances were found to produce the same effect against human pathogen, there for

the researchers started to screening plenty of plant extract and essential oil against various human pathogen, screening medical plant for antimicrobial sensitivity has led to encouraging result however it is essential to investigate the toxicity of plant as well [7]. Acacia nilotica possesses antibacterial activity because it used in rural medical care for treatment of many infectious and chronic diseases, thus, to verify the antibacterial activity of those plant against resistant bacteria isolated from deferent clinical sample.

In Sudan, with high percentage of multidrug resistant bacteria, we in urgent need to develop new drug from our traditional medicine. Therefore, our study was attempt to solve problem of resistant, found good treatment for bacterial disease.

Materials and Methods

This study was a descriptive cross-sectional study, During the period from February to December 2018 a total of ninety-one clinical isolates were obtained from clinical specimens of Hospitalized patients in different hospitals in Khartoum state including Military hospital, Omdurman Teaching Hospital, Fedial hospital, Ultra laboratory, Alacadimy Hospital and Police Education Hospital. All Gram negative carbapenem resistant bacteria were Included in this study, all isolates that are ither non-Gram negative or carbapenem susceptible are excluded from this study.

Data Collection

Data were collected from the isolates using data collection form containing all study variables

Ethical Consideration

Permission was issued by the Medical Laboratory Science Collage's Ethical Committee; Sudan University of Science and Technology the consent was taken from Laboratory Manager to collect specimens with ensuring all ethical consideration for conducting the research in a way that protect the patient's privacy

Collection Of plant Samples

The plant extracts were collected and authenticated at the Medicinal and Aromatic plant Research Institute (MAPRI).

Preparation of the Extract

Extraction was carried out according to method descried by Sukhdev et.al, Hundred gram of the plant samples were grounded using mortar and pestle and extracted by soaking in 80 % ethanol for about five days with daily filtration and evaporation. Solvent was evaporated under reduced pressure to dryness using rotary evaporator apparatus and the extract allowed to air till complete dryness and the yield percentages were calculated [8].

Collection of samples

Various clinical samples including (wound swab, urine, blood and sputum) were collected, inoculated in basic media and selective media then identified and preserved in glycerol peptone water, the initial collection and processing of the specimen was done in hospital then further investigation and sensitivity was done at Research Laboratory in Sudan University for Science and Technology.

Identification

Biochemical test for Gram's negative rods were carried out according by Chesbrough [9].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing of Gram-Negative Bacilli isolates will be performed on Muller-Hinton Agar (MHA) plate (Oxoid, UK) by the KirbyBauer disk diffusion method following the Clinical and Laboratory Standards Institute guidelines [10]. The antimicrobial agents which were tested from different categories including: extended spectrum cephalosporins [ceftazidime (30 µg), antipseudomonal penicillin's with βlactamase inhibitors carbapenems [imipenem (10µg), meropenem (10 µg)], aminoglycosides [gentamicin (10 µg), amikacin (30 µg)], fluoroquinolones [ciprofloxacin (5 µg), folate pathway inhibitor [co trimoxazole (25 µg)], and polymyxin [colistin (10 µg)] (Oxoid, UK). E. coli ATCC 25922 were used as control strains and tested each time when susceptibility testing was performed, zone diameters of each of the antibiotics will be interpreted as per CLSI recommendations (10).

Sensitivity Testing

Muller Hinton media susceptibility of common and rapid growing bacteria using antimicrobial by Kirby – Bauer method, antimicrobial to be included in sensitivity test well depend on pathogen and rang of locally available antimicrobial [9].

Preparation of Bacterial Suspension

The inoculum density was compared with McFarland standard solution of $BaSO_4$ (0.1ml of 1% $BaCl_2$ + 9.9ml of 1% H_2SO_4). The suspension was stored in the refrigerator at 4ºC until used.

Modified Kirby Bauer Method

Three to five colonies of similar appearance were touch and emulsified in 3to 4 ml of normal saline or nutrient broth , in good light the turbidity of the suspension were matched with turbidity of McFarland stander against piece of paper Muller Hinton agar was seed by using sterile cotton swab and the surface of the media allow to dry , then by sterile forceps apply the disc about 15mm from the edge and 25mm, then incubate the plate in incubator at 37C0 for 18-24 hour interpretation of zone size by interpretative chart either to be sensitive ,intermediate and resistant [9].

Determination of Ethanolic Extract of Acacia nilotica Activity

By Disc diffusion method with some modifications; In aseptic conditions, 20 ml of warm Mueller Hinton agar (Watin-Biolife, KSA), was poured on sterile disposable plates (Jalil Medicals) and left at room temperature to solidify, then turned upside down and kept in the refrigerator for about halve hour. 100 µl of the bacterial suspensions (previously adjusted) were swapped onto the Mueller Hinton plates, using sterile cotton swaps. Sterile blank discs of 6 mm were previously prepared from Whatman No.1 filter paper (Sigma-Aldrich) and saturated with 100,50 and 25 mg/ml to trap about 8 and 4 mg/disc, respectively (Pre-experimental measurements showed that the 6 mm disc absorb about 20 µl). Saturated discs were placed onto inoculated plates, the plates were allowed to stand for a while at room temperature, and then incubated at 37°C for 24 hrs. The susceptibility of the tested bacteria to the extract was indicated after incubation by zones of growth inhibition in millimeter (mm) using a transparent ruler. Gentamicin discs (10 µg/disc) (Oxoid), were used as standard antibacterial (positive control), other discs saturated with the solvent (ethanol) were loaded in a separate inoculated plate and served as negative control [11].

Phenotypic Detection of Carbapenemase

All isolates resistant to meropenem and imipenem in Kirby-Bauer disk diffusion method would be confirmed for carbapenemase production by Modified Hodge Test (MHT). In the test, inoculum of E. coli ATCC 25922 (comparable to 0.5 McFarland standard),

would be inoculated on MHA. Two discs of meropenem and imipenem (10 µg) would be placed on the surface of MHA 30 mm opposite to each other in a straight line, the test organisms will be streaked from the edge of one disk meropenem to edge of the other imipenem disk. The plates would be incubated at 37 °C for 24 h. They would be examined for a clover leaf type indentation or flattening at the intersection of the test organism and E. coli ATCC 25922 within the zone of inhibition of the carbapenem susceptibility disc as described by [12].

Phenotypic Detection of Carbapenemase Producer for MBL

Isolates that will be found resistant to imipenem, meropenem or third generation cephalosporins (ceftazidime) in Kirby Bauer disk diffusion method will presumptively considered MBL producers and will be confirmed by the Imipenem-EDTA Combined Disc Test (CDT). Briefly, the test inoculums (comparable to 0.5 McFarland standards) will be inoculated MHA plates. Two imipenem (10ug) disks will be placed on the surface of agar plate and 10 µl EDTA solutions will be added to one of them to obtain a desired concentration of 750 µg. The plates will be incubated for 24 hours at 35°C. An increase in zone size of ≥7 mm for imipenem-EDTA disk compared to imipenem disk alone will indicate MBL producer strain as described by [13].

Data Analysis

The Data analyzes was carried out through statistical package for the social science (SPSS) version 20 (one sample T-test and other statistical method e.g., mean and stander deviassion percentage and frequency), and excel programs.

Results

The bacteria are isolated and identified were 91 (31.9%) and were carbapenem resistant bacteria, E. coli were most abundant 40 (44%), followed by Klebsiella pneumoniae 25(27.8%) followed by Proteus mirabilis were 14 (15.6), Pseudomonas aeruginosa were 5 (5.5), Enterobacter cloacae were 3 (3.3 %) and Citrobacter freundii were 3 (3.3%) table (1). Most predominant sample type contain carbapenem resistant bacteria was wound swab 36 (40%) then followed by urine sample 35 (38.9%), sputum sample 15 (16.7), fluid sample (2.2 %) and blood sample represent 2 (2.2 %). Table (2). The result of antimicrobial agents showed that most effective antibiotic against carbapenem resistant isolated bacteria was colistin 75 /91(83.3 %), followed by Amikacin 40/91 (54.4%), cotrimoxazol 18/91 (20%) and more resistant antibiotic was ceftazidime 86 /91 (95. %) followed by ciprofloxacin 84/91 (92.3%) table (3). Modified hodge test (Figure 2) show 57/91(62.7%) was positive and 34/91 (37,3 %) was negative and Imipenem-EDTA Combined Disc Test (figure3) show 63/91 (69.2%) was positive and 28/91 (30.8%) was negative . the extract activity (Figure1-4) against all tested microorganisms and the inhibition zone were E.coli 17±3 mm, Klebsiella pneumoniae 18.3±4,mm Proteus mirabilis 16.9±4mm, Pseudomonas aeruginosa 17±3 mm , Enterobacter cloacae 18.3±0.5mm, Citrobacter freundii 16±5mm and the activity of the extract at 100 mg/ml concentration show Sensitive 75/91 (82.4%) and resist 15(17.6%), 66/91 (72.6%) for 50mg/ml resistant represent 25/91 (27.4%) and 47/91 (51.6%) for 25 mg/ml and the resistant was 44/91 (48.3%) for all tested bacteria (table 4). Antimicrobial activity of Acacia nilotica against all bacterial type are presented in Table (5) Effectivity of Acacia nilotica verses drugs(antibiotic) are presented in Table (6)

Table 1: Percentage and frequency of isolated bacteria

Bacterial isolate	Frequency	Percentage %
E. coli	40	44%
Klebsiella pneumoniae	25	27.8%
Proteus mirabilis	14	15.6%
Pseudomonas aeruginosa	5	5.5%
Enterobacter cloacae	3	3.3%
Citrobacter freundii	3	3.3%
Total	91	100%

Table 2: Frequency and percentage of clinical sample

Sample	Frequency	Percentage
Blood	2	2.2%
Fluid	2	2.2%
Sputum	15	16.7%
Urine	35	38.9%
Wound swab	36	40 %

Table 3: Percentage and frequency of drug sensitivity

Antibiotic	Sensitive	Resistant
Ceftazidime	4 / 91 (4.4%)	87/91 (95.6%)
Cotrimoxazole	18/91 (19.8%)	73/91 (80.2%)
Ciprofloxacin	7/91 (7.7%)	84/91 (92.3%)
Imipenem	0/91 (0%)	91/91 (100%)
Amikacin	40/91 (54.0 %)	51/91 (56%)
Colistin	75/91 (82.4%)	16/91 (17.6%)

Table 4: Antimicrobial activity of Acacia nilotica against isolated bacteria

AN concentration	Sensitive	Resistant	p.value
100%	75/91 (82.4%)	16/91 (17.6%)	0.0
50%	66/91 (72.6%)	25/91 (27.4%)	0.491
25%	47/91 (51.6%)	44/91 (48.3%)	0.006

Key: (AN: *Acacia nilotica*, SD: stander deviation)

Table 5: Bacterial isolate and plant concentration (sensitive and Resistant)

Isolates	Sensitive/ Concentration						Resistant/ Concentration		
	100%	p. value	50%	p. value	25%	p. value	100%	50%	25%
E. coli	33/40 (82.5%)	0.001	26/40 (65%)	0.363	21/40 (52.5%)	0.047	7/40 (11.5%)	14/40 (35%)	19/40 (47.5%)
Klebsiella pneumoniae	25/25 (100%)	0.001	21/25 (84%)	0.027	17/25 (68%)	0.410	0/25 (0%)	4/25 (16%)	8/25 (32%)
Proteus mirabilis	13/14 (92.9%)	0.104	10/14 (71.4%)	0.197	4/14 (28.6%)	0.002	1/14 (7.1%)	4/14 (28.6%)	10/14 (71.4%)
Citrobacter freundii	2/3 (66.7%)	0.775	1/3 (33.3%)	0.504	1/3 (33.3%)	0.390	1/3 (33.3%)	2/3 (66.7%)	2/3 (66.7%)
Enterobacter cloacae	3/3 (100%)	0.010	3/3 (100%)	0.423	1/3 (33.3%)	0.122	0/3 (0%)	0/3 (0%)	2/3 (66.7%)
Pseudomonas aeruginosa	5/5 (100%)	.075	4/5 (80%)	1.000	2/5 (40%)	0.463	0/5 (0%)	1/5 (20%)	3/5 (60%)

Table 6: Effectivity of Acacia Nilotica Against Drugs (Antibiotics)

Isolates	Acacia Nilotica			Ceftazidime	Ciprofloxacin	Cotrimoxazole	imipenem	Amikacin	Colistin
	100%	50%	25%						
E. coli	33/40 (82.5%)	26/40 (65%)	21/40 (52.5%)	2/40 (5%)	2/40 (5%)	8/40 (20%)	0/40 (0%)	18/40 (45%)	40/40 (100%)
Klebsiella pneumoniae	25/25 (100%)	21/25 (84%)	17/25 (68%)	0/25 (0%)	2/25 (8%)	4/25 (16%)	0/40 (0%)	14/25 (56%)	22/25 (88%)
Proteus mirabilis	13/14 (92.9%)	10/14 (71.4%)	4/14 (28.6%)	2/14 (14.3%)	1/14 (7.1%)	4/14 (28.5%)	0/40 (0%)	5/14 (35.7%)	7/14 (50%)
Pseudomonad as aeruginosa	5/5 (100%)	4/5 (80%)	2/5 (40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/40 (0%)	2/5 (40%)	2/5 (40%)
Enterobacterr cloacae	3/3 (100%)	3/3 (100%)	1/3 (33.3%)	0/3 (0%)	2/3 (66.7%)	2/3 (66.7%)	0/40 (0%)	1/3 (33.3%)	2/3 (66.7%)
Citrobacter freundii	2/3 (66.7%)	1/3 (33.3%)	1/3 (33.3%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/40 (0%)	0/3 (0%)	2/3 (66.7%)

Figure 1: *Acacia nilotica* pods

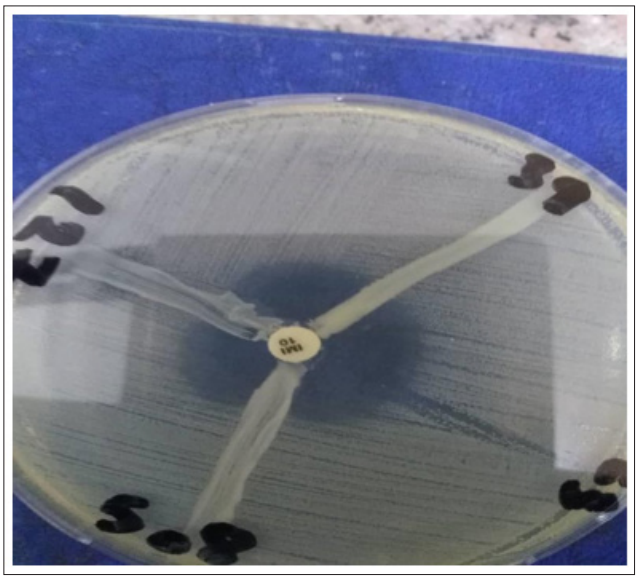

Figure 2: The Modified hodge test (MHT) performed on a Muller Hinton Agar plate. (508,127) MHT positive result (39) MHT negative result.

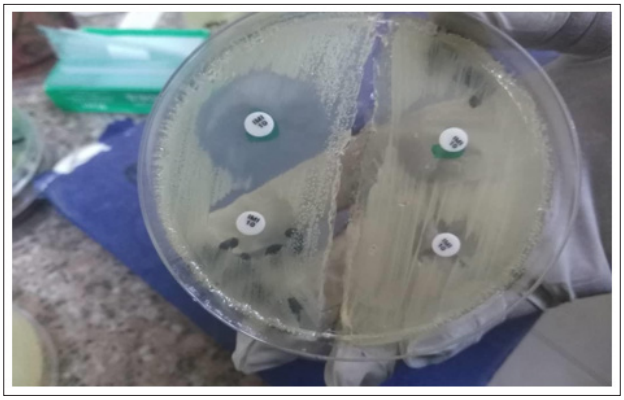

Figure 3: Positive imipenem-EDTA combined disc test for one of the isolated Gram-negative bacteria. The imipenem + EDTA discs shows larger zone of inhibition than imipenem disc alone.

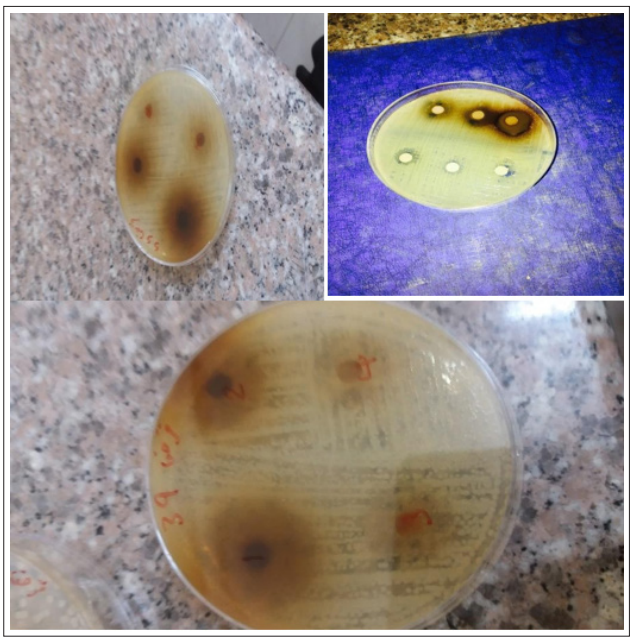

Figure 4: *Acacia nilotica* antimicrobial activity

Discussion

Antimicrobial drugs provide the main basis for treatment of various microbial infections; however, the high genetic variability of some microorganisms enable them to rapidly develop antimicrobial resistance. Thus, there has been a continuing search for new potent antimicrobials [14].The results of this study indicate that the ethanol extract of *Acacia nilotica* pods contains active compounds capable of killing a range of bacteria types, including *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Citrobacter ferundii* which are carbapenem resistant bacteria. And the activity effective at the highest concentration of plant extract and lower at the lower concentration for all tested bacteria that agreement with study although it use differ bacterial species reported that *Escherichia coli*, *followed by Staphylococcus aureus , Acinetobacter baumanni*, and *Bacillus cereus* ATCC 11778 bactericidal effect at high concentrations and bacteriostatic at lower concentrations, and other study also agreement with my study found and the most effective against *E.coli*. But my study done on carbapenem resistant bacteria show that all the test organisms were found to be sensitive to all the extracts prepared in the study. Maximum activity with respect to zone of inhibition was recorded for *E. coli* followed by *S. pyogenes*, *B. cereus*, *V. cholerae*, *S. aureus*, *B. subtilis*, *Aeruginosa*, *S. dysenteriae*, *C. perfringens*, *S. typhi*, *L. monocytogenes*, while C [11].

Jejuni was found to be least sensitive with no activity recorded with acetone extract (P<0.001. and also, study done on multi drug resistant [15]. Also, agreement with study (16) but differ in the solvent used and the bacterial type show the antibacterial activity of aqueous extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Standard drug used as control recorded zone of inhibition on the test bacteria, Chloroform extract was found to be the least effective as compared to aqueous extract with diameter zone of inhibition and compared the finding against *S. aureus* and *P. aeruginosa* this study use different type of the extract but it show the activity of *Acacia nilotica* against the bacterial isolate even use other type of extract also The activity of the standard control was more effective, also agreement with Acacia nilotica leaves and park extraction against *S. aureus*, *E.coli*, *P.aeruginosa*, *P. mirablis*, *Acinetobacter spp* and *Candida albicans* in the acetonic leave extract the zone of inhibition of *S. aureus* followed by *P. mirablis E.coli* and *Acinetobacter spp*, *Candida albicans* and *Pseudomonas* spp, this study done only on park not in leaves therefore get differ in the result but also give activity against bacterial isolate .

My study disagreement with. In the activity concentration (lower more active than highest one) and in the type of control antibiotic used the lowest concentration (10%) of the extract, showed a high antibacterial activity against *Staphylococcus aureus* and a low antibacterial activity against *Klebsiella pneumoniae* highest activity was due to the action of ciprofloxacin and gentamycin against *Pseudomonas aeruginosa* and *K. pneumoniae*. While the lowest activity was due to the action of amoxicillin against *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* There was an insignificant difference ($p > 0.05$) [16-18].

The present study was done on carbapenem resistant other study done on ESβL the lysates showed remarkable bactericidal properties, killing almost 100 % of the bacteria they were tested against, including neuropathogenic *Escherichia coli*, MRSA, and *Klebsiella spp*. The bactericidal activity was heat-resistant and showed minimal cytotoxic effects on human brain microvascular endothelial cells. FPLC revealed eight peaks, with three of them representing compounds that had maximum bactericidal activity against all the tested isolates, but showed < 30 % host cell cytotoxicity determination of cytotoxicity ranged from 8.1 – 29.0 %, depending on the fraction [19]. And it not detected in my research.

The differences may be attributed to the fact that effectiveness of the extracts largely depends on the kind of solvent used. Further, the concentration of the extract and kind of bacteria may also account for the difference in the susceptibility pattern of the test organism. There were few researches about effect of *Acacia Nilotic* a on carbapenem resistant bacteria. Therefore, this study determined the value of *Acacia nilotica* plant as alternative treatment for bacterial infections and carbapenem resistant bacteria that can be used to completely overcome or minimize the resistance of bacteria observed in synthetic antimicrobial agents.

Conclusion

Ethanolic extract of *Acacia nilotica* was found to be effective as antibacterial against different bacterial pathogens, providing the scientific basis for its traditional application in Sudanese folk medicine against many bacterial diseases. Good activity of Ethanol

extract of *Acacia nilotica* at highest concentration (100 mg/ml), Poor activity at lower concentration at 25mg /ml. Acacia nilotica extract had an *in vitro* antibacterial activity against carbapenem resistant bacteria which are *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, *Proteus mirabilis*, *Citrobacter ferundii* and Enterobacter cloacae. It may be used as an alternative treatment of infections associated with these organisms.

Recommendation

Further investigation into pharmacological properties of secondary metabolites of *Acacia nilotica*, more research is required to understand the mode of actions of these plants. Further studies should be carried out for the isolation and characterization of the bioactive compounds. Determination of minimum inhibition concentration (MICS) and toxicity for the active ingredients of each bacteria including in this study. More studies about carbapenem resistance bacteria, and can be supported by molecular detection of resistant genes.

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