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Antibacterial Activities and Phytochemical Screening of Extracts of Flower of *Azadiracthta indica* (Neem) against Some Selected Clinical Isolates

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Abstract

A study on the phytochemistry and antibacterial effects of petroleum ether, chloroform and methanol extract of flower of *Azadirachta indica* on five clinical isolates viz: *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae* was carried out using deep well agar diffusion and broth dilution methods. The results obtained showed that methanol flower extract possessed the highest antibacterial activity against *P. miravilis* with inhibition zone of 23mm at the concentration of 4000µg/ml. The minimum inhibitory concentration (MIC) of the petroleum ether flower extract was 62.5µg/ml for all the test organisms while *P. miravilis, S. aureus* and *K. pneumoniae* had MIC value 62.5µg/ml with the exception of *E. coli* and *P. aeruginosa.*Methanol flower extract recorded 62.5µg/ml against *P. miravilis, S. aureus.* While *E. coli*, *P. aeruginosa and K. pneumoniae* had no MIC value across all the concentrations. the minimum bactericidal concentration (MBC) for the petroleum ether extracts was 125µg/ml, for chloroform extracts was 250µg/ml and for methanol extracts was 62.5µg/ml. Phytochemical analysis of the flower extracts showed the presence of reducing sugars, alkaloids, tannins, flavonoids, resins, and saponins, in all the extracts. Petroleum ether flower extract (PFE) showed antibacterial activities against all the test organisms while Methanol flower extract (MFE) had antibacterial activities against only two test organisms.

Key words: *Phytochemistry, Azadirachta indica, extracts, Clinical isolates ,MIC, MBC*

INTRODUCTION

Medicinal plants have a long history of use and that is widespread in both developing and developed countries. According to reports of the World Health Organization, 80% of the world's population relies mainly on traditional therapies which involve the use of plant extracts or their active substances (WHO, 1993). Microorganisms have developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs (Ahmad *et al.,* 1998). Furthermore, antibiotics are sometimes associated with side effects (Cunha, 2001), whereas there are some advantages of using antimicrobial compounds of medicinal plants, such as fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature (Vermani and Garg, 2002). It is known that more than 400, 000 spp. of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine (Odugbemi, 2006). Some plant decoctions are of great value in the treatment of diarrhoea or gastrointestinal disorder, urinary tract infections, skin infections, infertility, wound and cutaneous abscesses (Ergene et al., 2006). The tree, *Azadirachta indica* of the family Maliaceae*;* popularly known as neem tree or darbejiya (Hausa) is an evergreen tree, native to the Southeast Asia and found in most tropical countries.

It has been in use since ancient times, to treat a number of human ailments and also as household pesticide (Chattopadhyay*et al.,* 1993; Chattopadhyay 1996; Chattopadhyay and Bandyopadhyay, 2005). Extracts from the bark, leaves, fruits and roots have been used to control leprosy, intestinal helminthosis and respiratory disorders (Ketkar and Ketkar, 1995). Every part of the neem tree has been used as traditional medicine for house-hold remedy against various human ailments from antiquity.

Chemical investigation on the products of the neem tree extensively undertaken in the middle of the isolation of nimbin, the first bitter compound isolated from neem oil, more than 135 compounds have been isolated from different parts of neem (Ganguli, 2002). Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex (Subapriya and Nagini, 2005).

Infectious diseases are the world's leading cause of premature deaths, killing almost 50 000 people every day. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (Piddock and Wise, 1989; Singh et al., 1992; Mulligen et al., 1993; Davis, 1994; Robin et al., 1998). However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in immune compromised, AIDS and cancer patients (Rinaldi, 1991; Diamond, 1993). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants.Thus, the choice of *azadirachta indica*as a plant of interest for this research work was done based of its diverse and immense therapeutic ethno medicinal values. In view of the forgoing, this research is aimed at studying the antibacterial activity and phytochemical properties of extracts of flower of *Azadirachta indica* (neem) on some selected clinical isolates.

MATERIALS AND METHODS

Collection of plant materials

The flowers of *Azadirachta indica* was obtained from Audu Bako Zoological and Botanical Garden, Kano. The collected flowers were identified and authenticated in Herbarium, department of Plant Biology, Bayero University Kano. Voucher No 118 was given to the plant.

Preparation of the plant materials

The collected sample was washed thoroughly with running tap water and finally with sterile distilled water. The material was chopped into small pieces and then air dried on a sterile blotter under shade for 20-30 days.

Extraction procedures

Plant extract was prepared by the maceration method as demonstrated by Alade and Irobi (1993). 200 g of the air dried powdered form of the flower of *Azadirachtaindica* was soaked in 250 ml of petroleum ether for three days. The mixture was stirred every 24 h using a sterile glass rod. The plant sediment was allowed to dry and soaked in 250ml of chloroform and finally in methanol solvent for three days respectively. At the end of extraction each extract was passed through Whatman filter paper No. 1 (Whatman, UK). The procedure was repeated three timeswith fresh volumes of the solvents used.The alcoholic filtrates obtained were concentrated in water bath at 30°C and stored at 4°C until further use. The dried plant extracts/fractions obtained were weighed and labeled as PFE(Petroleum ether flower extract), CFE(Chloroform flower extract), and MFE(Methanol flower extract).

Phytochemical screening

- **Test for saponins**

Exactly 2ml of petroleum ether extract was vigorously shaken with distilled water and allowed to stand for a while. A persistent frothing indicates the present of saponins. Same procedure was repeated using chloroform and methanolic extracts (Sofowora, 1984).

- **Test for alkaloids**

Half gram of petroleum ether extract was stirred with 5ml 1% HCL on steam bath.

The solution was cooled and filtered.1ml of the filtrate was treated separately with drops of Mayer's dragendoff's and wagner's reagents; and formation of dirty/dark brown, yellow-brown or reddish brown precipitated respectively indicates the presence of alkaloid (El-olemy et al., 1994).

- **Test for phlobactannins**

A 2ml of each extract was added to 5ml HCL.Formation of turbidity/precipitation indicates the presence of phlobactannins. (Sofowora, 1984).

- **Test for tannins**

Exactly 2ml of each extract was treated with 3 drops of 5% ferric chloride. A dark black colored precipitate in a very dark solution, which gives a green-black to blue black colouration on dilution indicates the presence of tannins (Sofowora,1984).

- **Test for reducing sugar**

One gram of each extract was weighed and diluted with 2ml distilled water. Fehling's solution(A and B) were added and the mixture warmed. A brick- red precipitate at the bottom of the test tube indicates reducing sugars (Brain and Turner, 1975).

- **Test for flavonoids**

Two grams of the petroleum ether extract was weighed and placed in a test tube, followed by the addition of 10ml of DMSO. The mixture was heated, followed by the addition of magnesium metal and six drops of concentrated hydrochloric acid. The appearance of red colour was indicatives of the presence of flavonoids. Same procedure was repeated with chloroform and methanol extracts respectively (Sofowora, 1993).

- **Test for resins:**

A 2.0g of each extract was dissolved in 10ml of ethanioc acid anhydride. One drop of concentrated sulphuric acid was added. The appearance of purple colour which rapidly changes to violet indicates the presence of resins (Evans, 1995).

- **Test for antraquinone**

Two mills of petroleum ether extractwere treated with 5ml of benzene. This gave two layers. The clear colorless upper layer was pipette and the organic layer treated with 3ml of 10% aqueous ammonia. Change of colour from rose-pink to red indicates anthraquinone. Same procedure was repeated using chloroform extracts and methanolic extracts (Ciulie, 1984)

- **Test for steroids(Salkowaski's test)**

Two mills of concentrated sulphuric acid was added to 2ml of each extract .Appearance of effervescence after which a clear reddish brown color appeared at the interface confirms the presence of steroidal ring (Harbone,1973).

Test organisms

Clinical isolates of bacteria were used for the bioassay studies. The isolates include *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,*and *Klebsiella pneumoniae*. The isolates were obtained from microbiology laboratory of Murtala Mohammad Specialist Hospital (MMSH), Kano, Nigeria. They were further confirmed using standard biochemical test as described by Cheesbrough (2002).The isolates were maintained on freshly prepared nutrient agar (oxoid) slants and kept in a refrigerator at 4^{o}c until required for use.

Preparation of extract concentrations

This was carried out using standard method described by Cheesbrough, (2002). Stock solution of the petroleum ether crude extract, chloroform crude extract and methanol crude extract were prepared by weighing 0.008g of each and dissolved in 2ml of dimethlysulfoxide (DMSO) in glass vial bottles.

This gave an extract concentration of 8000µg/ml (stock solution).Four varied extracts concentrations (4000µg, 2000µg, 1000µg and 500µg) were prepared from the stock solution (8000µg) using double serial dilution for each extract.

Preparation of antibiotic dilution (Standard)

The antibiotic chloramphenicol was purchased at a registered Pharmaceutical store in Kano State, Nigeria and was reconstituted by dissolving 0.3 mg of powder in a 100 ml of distilled water so as to get a concentration of 30µg/ml. The prepared dilution of the antibiotic was used

for subsequent antimicrobial test and serve as a positive control

Preparation of inoculums

The standardization of culture was done according to the method of Baker and Thomsberg (1983) and CLSI in2006.Two mm diameter colonies of the 18 h culture of an organism was picked with a sterile wire loop and immersed into a sterile bottle containing Mueller Hinton broth (Hi Media) and incubated for 5 hours .Normal saline was added gradually to it so as to compare the turbidity to that of 0.5 McFarland standard corresponding to approximately 1.5 × 10^8cfu / ml. This was done for each of the test bacteria.

Assay for antibacterial activity

The Boakye-Yiadom (1987) agar well diffusion method was used to evaluate the antimicrobial activity of the crude extracts. Briefly, 1.0 ml of the standard inoculum was inoculated into 90 mm sterile Petri plate, then 19 ml sterile Mueller Hinton agar was added and the plate rocked gently for 1 minute for even mixing of the contents. The plates were kept on a flat bench for 30 minutes to gel. The six wells were made on respective agar plate by using cork borer of 4mm in diameter size. Two drops of petroleum ether, chloroform, and methanol extracts at different concentration of 4000µg/ml, 2000µg/ml, 1000µg/ml, and 500µg/ml equivalent to a potency of 400µg, 200µg, 100µg, and 50µg respectively were introduced to their respective wells. Two drops of 30µg/ml of chloramphenicol solution was served as a positive control and 0.1 ml of DMSO as a negative control. The plates were allowed to stand on flat bench for 30 minutes to allow diffusion into the agar before incubation at 37^{o}C for 24 h. The experiment was done for each of the extract against each of the test bacteria and mean zone diameter was recorded. Antibacterial activity was evaluated by measuring the diameters of zones of growth inhibition (Hugo and Russell, 1983; WHO, 2003).These experiments were repeated for each of the test bacteria.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the crude extracts was determined using the doubling dilution method of Saham and Washington (1990).Two milliliters of the reconstituted crude extract at a concentration of 500µg/ml was added to two milliliters of sterile Mueller Hinton broth. Two milliliters of this extract concentration was transferred to another test tube and this dilution continued until an 8th test tube is reach, giving extract concentrations of 250, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95 and 0.98µg/ml in different test tubes. 0. 1 ml of an 18 h culture of bacteria previously adjusted to 0.5 McFarland standard (1.5 X 10^8cfu/ml) was inoculated into each of the test tubes and the contents thoroughly mixed. The tubes were incubated at 37^{o}C for 24 h. The 9th test tube containing 2 drops DMSO was served as a negative control. The 10th test tube containing a solution of 30µg/ml of chloramphenicol solution was served as positive control. The above procedure was followed for each of the test bacteria. The lowest concentration of the extract that did not show any detectable growth was taken as the MIC.

Determination of the minimum bactericidal concentration (MBC)

From each of the test tubes in the MIC determination that did not show any visible growth, 100 µl of the broth was aseptically inoculated on to a sterile Mueller Hinton agar surface and gently spread all over the surfaces with a sterile bent glass rod. The inoculated plates were incubated for 24hrs at 37^{o}C. After incubation, the MBC is the lowest concentration of the extract that showed no was determined at the dilution, at where there was no growth (colony) on the plate (De and Ifeoma, 2002).

STATISTICAL ANALYSIS

Data obtained was subjected to statistical analysis using one way ANOVA

RESULTS

Table 1 shows the results of Preliminary physicochemical characteristics of neem flower crude extracts which yielded 5.6g,

7.8g and 14g of the petroleum ether, chloroform and methanolic extracts respectively from the initial crude of 200g. A physical characteristic of the extracts fractions indicates that there were variations in the colour, odour and texture of the extracts.

Table 1: Physicochemical characteristics of the flower extracts of *A. indica*

Plant part	solvent	Initial weight (g)	Final weight(g)	Colour	Odour	Texture
Flower	Petroleum ether	200	5.6	Dark-brown	Fruity	Oil/gummy
	Chloroform	186	7.8	Light-green	Fruity Pleasant	Gummy
	Methanol	176	14	Light-brown	Pleasant fruity	Gummy

Table 2, illustrates the phytochemical analysis of the neem flower extracts which indicates presence of reducing sugars and alkaloids in all the plant extracts. Tannins were present in MFE only, while Steroids and Anthraquinone were absent in both. Flavonoids were found in PFE, MBE, and MFE, while Resins and Saponins were present in PFE fractions only.

Table 2: Result of the phytochemical analysis

Secondary metabolite group	Extracts PFE	CFE	MFE
Reducing sugar	+	+	+
Tannins	-	-	+
Steroids	-	-	-
Flavonoids	+	-	+
Resins	+	-	-
Saponins	+	-	-
Anthraquinone	-	-	-
Phlabactannins	-	-	-
Alkaloids	+	+	+

Key: Present (+), Absent (-)

Table 3, demonstrates the antibacterial activity patterns of different fractions of flower extracts of *A. indica.* The result showed that petroleum ether flower extracts (PFE) have antibacterial activity against all the test organisms. Chloroform flower extract (CFE) have antibacterial activity against all isolates with the exception of *E. coli* and *P. aureginosa* while methanol flower extract (MFE) have antibacterial activity against only*P. miravilis*and *S. aureus* with the exception of *E. coli, P .aureginosa* and *K.pneumoniae* across all concentrations. Moreover, from the result methanol flower extract possessed the highest antibacterial activity against *P. miravilis* with inhibition zone of 23mm at the concentration of 4000µg/ml.

Table 3: Antibacterial activity of flower extracts of *Azadirachta indica* against the bacterial isolates

	Diameter of zone of Inhibition (mm)/Extract concentration(μg/ml)											
	PFE				CFE				MFE			
Isolates	500	1000	2000	4000	500	1000	2000	4000	500	1000	2000	4000
P. miravilis	15	17	19	21	13	16	17	19	17	19	20	23
E. coli	08	10	14	15	00	00	00	00	00	00	00	00
S. aureus	14	16	17	19	12	15	16	20	13	14	15	17
P. earuginosa	07	08	09	11	00	00	00	00	00	00	00	00
K. pneumonia	08	08	09	15	08	15	20	21	00	00	00	00

Table 4, showed the activities of Chloramphenicol (antibiotic) as positive control and DMSO as negative control on the test organisms. It indicated that all the test organisms were susceptible to chloramphenicol at 30μg/ml and resistant to DMSO.

Table 4: Activities of Chloramphenicol (antibiotic) as positive control and DMSO as negative control on the test organisms.

	Diameter of zone of Inhibition (mm)/ Anbiotic concentration(μg/ml)	
Isolates	**Chloramphenicol 30μg/ml**	**DMSO**
P. miravilis	14	0
E. coli	16	0
S. aureus	19	0
P. earuginosa	14	0
K. pneumonia	9	0

Table 5, shows the Minimum Inhibitory Concentration (MIC) of different fractions of *A. indica* extracts against the selected clinical isolates. The results showed that all the test organisms have the same MIC of 62.5μg/ml in the petroleum ether extract while *P. miravilis, S. aureus and K. pneumonia* tested with Chloroform flower extracts have also the same MIC of 62.5μg/ml while *E. coli* and *P. aeruginosa* were resistant. Moreover, all the isolates tested with methanolic extracts of flower of *A. indica* also have MIC value of 62.5μg/ml with the exception of *E.coli,P. earuginosa* and *K. pneumoniae* which all of them showed resistance to methanol flower extract.

Table 5: Minimum Inhibitory Concentration of Petroleum ether, Chloroform and Methanol flower extracts of *A. indica*

	Extracts concentration(μg/ml)														
	PFE					CFE					MFE				
Isolates	250	125	62.5	31.3	15.6	250	125	62.5	31.3	15.6	250	125	62.5	31.3	15.6
P. mirabilis	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
E. coli	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
P. aureginosa	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
K. pneumoniae	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+

Key: + = Growth not inhibited; - Growth inhibited

Table 6, showed the minimum bactericidal concentration of Petroleum ether, Chloroform and Methanol flower extracts against the test organisms. Minimum Bactericidal Concentration of each isolate was *determined* at the lowest concentration which inhibits bacterial growth. From the result it showed that *E. coli*, *P. aeruginosa* and *K. pneumoniae* had the same MBC of 125µg/ml while *S. aureus* and *P. mirabilis,*have no MBC value across all concentrations of petroleum ether flower extracts(PFE).In CFE only *P. mirabilis* had the MBC value of 250µg/ml. However in MFE *S. aureus* had the MBC value of 250µg/ml while *P. mirabilis* had the MBC value of 62.5µg/ml and the remaining tests organisms were resistant.

Table 6: Minimum Bactericidal Concentration of Petroleum ether, Chloroform and Methanol flower crude extract of *A. indica*

	Extracts concentration(µg/ml)								
	PFE			CFE			MFE		
Isolates	250	125	62.5	250	125	62.5	250	125	62.5
P. miravilis	+	+	+	-	+	+	-	-	-
E. coli	-	-	+	+	+	+	+	+	+
S. aureus	+	+	+	+	+	+	-	+	+
P. aeruginosa	-	-	+	+	+	+	+	+	+
K .pneumoniae	-	-	+	+	+	+	+	+	+

Key: + = Growth not inhibited; - Growth inhibited

Discussion

In this study the phytochemical screening of the flower extracts of Neem plant revealed the presence of reducing sugars, alkaloids, Tannins, Steroids, Anthraquinone, Flavonoids, Resins, phalabactannins and Saponinsin in the extracts . The phytochemical components of the *A. indica* have been established in previous studies and these include tannins, saponins, alkaloids, carbohydrates, phenols, flavonoids, anthraquinones, cardiac glycosides, sterols and resins (Sundarasivara and Nazma, 1977; Rao *et al*., 1986, Natarajan*et al.,* 2003; De and Ifeoma, 2002; Biswas *et al*., 2002). Several studies have linked presence of these bioactive compounds in plant materials to antimicrobial activity. The presence of these secondary metabolites in plants, produce some biological activity in man and animals and it is responsible for their use as herbs. These compounds also serve to protect the plant against infection by microorganisms, predation by insects and herbivores, while some give plants their odors and or flavors and some still are responsible for their pigments (Ketkar *et al.,* 1995, El-Mahmood *et al,*. 2008). In some cases, the activity has been associated with specific compounds or classes of compounds. These active constituents can be used to search for bioactive lead compounds that could be used in the partial synthesis of more useful drugs (Ogbonniaet al., 2008).

A .indica flower extracts of both petroleum ether, chloroform and methanol solvents inhibited the growth of the test bacteria, though to varying degrees. Petroleum ether flower extracts showed low to moderate activity where PFE had inhibition zone (IZ) between 07-21mm. Chloroform flower extract showed low to moderates activity with (IZ) between 8-21mm with the exception of *E. coli*, and *P. aeruginosa* respectively. Methanolic extract showed fairly higher degree of activity in which MFE had (IZ) of between 13-23mm.From the result it shows that methanolic extract was the most effective with the wider zone of inhibition of 23mm (Table.3).The effectiveness of these solvents may be due to their polarity where methanol was the most polar among the three solvents used.

The zones of growth inhibition recorded for the methanol and acetone extracts by De and Ifeoma (2002) were also smaller in size than those obtained in this study. Several factors are known to influence yield and biological activities of plant based products, including the age of the plant, time of harvest, drying and processing of the materials, methods of extraction and the solvents used

The quantitative measure of the *in*-vitro activity of antibiotics and non-antibiotic antibacterial agents including those agents of plant origin with antibacterial potentials are the MIC and MBC. The minimum inhibitory concentration was defined as the lowest concentration of the compound to inhibit the growth of microorganisms .Table 5 shows the minimum inhibitory concentration (MIC) of *A. indica* flower extracts against the selected clinical isolates and the results showed that all the test organisms have the same MIC of 62.5µg/ml in PFE, while in CFE *P. mirabilis, S. aureus* and *K.pneumoniae* had the same MIC of 62.5µg/ml. In MFE *P. mirabilis,* and *S. aureus* had the same MIC values of 62.5µg/ml . The study showed that *E. coli* and *P. aeruginosa* had no MIC value in CFE while *E. coli*, *P. earuginosa* and *K. pneumonia* also have no MIC values in MFE at all concentrations, meaning that higher concentration of the extracts are required to inhibit the growth of these bacteria. The MBC of the PFE extracts was 125µg/ml against E. *coli, P. aeruginosa* and K. pneumonia respectively while *P. mirabilis* and *S. aureus* have no MBC across all the concentration. In CFE only P. mirabilis had MBC value of 250µg/ml while the remaining test organisms have no MBC across all concentrations. Meanwhile in MFE *P. mirabilis* had MBC value of 62.5µg/ml while*, S. aureus* had MBC value of 250µg/ml while *E. coli, P. aeruginosa* and *K. pneumoniae* had no MBC values at all the concentrations used. The higher the MBC values is the lower the susceptibility of microorganism to the crude extracts and the lower the MBC values is the higher the activity of the extracts against the organisms. In this study, *P. mirabilis*, had the lowest MBC value (125µg/ml) in methanolic flower extracts (MFE) (Table 6). The quantity of the active ingredients required to effect complete kill may not matter since medicinal plants have been reported to have little or no side effects (Hassain-Eshrat, 2002; Ogbonnia *et al.,* 2008). The MIC and MBC values for neem leaves against some fungal isolates were reported to be 250µg/ml by Natarajan *et al.,* (2003) which are in accordance with the MBC values of *P. mirabilis,* in Petroleum ether neem flower extract and S.aureus in MFE obtained in this study. De and Ifeoma (2002) reported that at a concentration of 10 mg/ml their crude extracts were unable to inhibit the growth of some bacteria, particularly *P. aeruginosa* and *E. coli.* However, the result of this study showed that PFE of *A. indica* inhibited the growth of *P. aeruginosa* and *E. coli* even at lower concentration of 125µg/ml.

The varied zone of inhibition of MBE on *E. coli* at concentrations of 500, 1000, 2000 and 4000µg in table3 disagreed with reports by Yagoub et al.,(2007) who in their preliminary screening for anti-microbial activity of different plants against different organisms, methanolic extracts of *A.indica* produced zero zone of inhibition against *E. coli.*

Conclusion

Based on the pharmacological results of the study, it could be confirmed that the extracts contain chemical constituents of pharmacological significance. The observation that the extracts were effective against the test bacteria suggests the use of crude extract of flower of *A. indica* against infection caused by clinical isolates like *P. mirabilis, Escherichia coli, S. aureus,P. aeruginosa*, and *K. pneumonia*.It is therefore recommended for the isolation and purification of bioactive compounds in Neem tree responsible for the antimicrobial activity.

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