www.ajbrui.org

Afr. J. Biomed. Res. Vol. 26 (September 2023); 439- 445

Research article

Phytochemical Study and Antibacterial Activity of Ethanolic Extracts of *Azadirachta indica* Leaves on Previously Profiled Multidrug Resistant Aerobic Bacteria

***Adeluola, A.O.[1], Nwaeze, K.U.[2], Akindele, A.E.[1] and Okusami, O.O.[1]**

[1.] *Dept. of Pharmaceutical Microbiology & Biotechnology, Faculty of Pharmacy. Univ. of Lagos.*

[2.] *Dept. of Pharmaceutical Chemistry, Faculty of Pharmacy. Univ. of Lagos*

ABSTRACT

The challenge of antimicrobial resistance of pathogenic microbes, continues to be a global issue and sourcing of new antimicrobial compounds is still more challenging. The study of active plant components can be a lead to aid in the discovery of new efficacious antimicrobial agents. *Azadirachta indica*, known as Neem, is used in Phytotherapy and Herbal medicine. It possesses an array of biologically active compounds having anti-inflammatory, antimalarial, antifungal, antibacterial, antiviral properties amongst other therapeutic activities. This study is aimed at the qualitative and quantitative determination of the phytochemical constituents and the extent of inhibition by the ethanolic extract of Neem leaves on selected Gram positives organisms; all known to be Methicillin resistant Staphylococcus aureus (MRSA), from a previous study and Gram-negative organisms, which are known to be multidrug resistant (MDR) also from a previous study. The presence of alkaloids, saponins, phenolics, flavonoids, tannins, cardiac glycosides, steroids were detected. Cardiac glycosides quantity was highest (1.569mg/g) followed by alkaloids (0.42mg/g) and saponins (0.32mg/g).Others were below 0.1mg/g. A.indica ethanolic leaf extract exhibited antibacterial activity on only one strain of Klebsiella pneumonia, Med 1(2) with the maximum zone of inhibition (25 mm) obtained at 400mg/ml and MIC value of 160 mg/ml. All the Staphylococcus species except Med. 4k, gave varying zones of inhibition (14mm – 34mm) over the range of concentrations (400mg/ml to 100mg/ml) used for the test and MIC values (5.12 mg/ml to 40 mg/ml). This study indicates that the ethanolic leaf extracts of *Azadirachta indica* have good potentials as Antimicrobials against infections of MRSAs.

Keywords: *Azadirachta indica, Phytochemical, Multi-drug resistant, Herbal medicine, Anti-microbials*

**Author for correspondence: Email: aadeluola@unilag.edu.ng; debodelu@gmail.com; Tel: +234 7056004859*

Received: January 2023; Accepted: July 2023

DOI: https://doi.org/10.4314/ajbr.v26i3.20

INTRODUCTION

The prevalence of antimicrobial drug resistance by many microbial organisms, and the challenge of discovery of new antibiotics, is a great threat to antimicrobial chemotherapy all over the world. This has led to a relentless search for safe therapeutic agents, of herbal origin with antimicrobial properties, to be used against these microorganisms (K. Dhama *et al*, 2014). Among bacterial organisms widely implicated in this problem of anti-bacterial drug resistance, are the Methicillin-resistant Staphylococcus aureus (MRSA). *Staphylococcus xylosus* is a coagulase negative species of bacteria, belonging to the genus *Staphylococcus*. This species is virtually defined as a non-pathogenic S*taphylococcus*, but a few strains of *S. xylosus* are related to animal and human opportunistic infections (Boamah, *et al*., 2017). However, they are capable of acquiring and harbouring the resistance genes for the MRSA thus making them potentially hazardous.

Gram-negative bacteria especially the enterobacteriaceae, are among the most notable public health problems in the world, due to its great capacity of causing diseases in man and its level of resistance to antimicrobials (Hormozi *et al*., 2018; Oliveira and Reygaert, 2019). They can infect almost all the systems including the digestive system, nervous system, urinary system and bloodstream (Wenzler *et al*., 2016; Oliveira and Reygaert, 2019). They have been implicated in diseases in man, such as pneumonia, diarrhoea, sepsis, urinary tract infections, meningitis amongst others. Species under Enterobacteriaceae that often cause diseases in humans include Escherichia, Klebsiella, Enterobacter, Proteus, Shigella, Citrobacter, Salmonella (Oliveira and Reygaert, 2019). Some of these such as *Escherichia coli, Klebsiella pneumonae*, Enterobacter spps., Citrobacter spps among others can produce extended-spectrum β-lactamase (ESBL) enzymes (Jacoby and Meideros, 1991; Tzelepi *et al*., 2000; Coudron *et al*., 1997, Magwenzi *et al*., 2017). The ESBL enzymes confer the characteristics of multidrug resistance

(MDR) to such organisms against antibiotics such as penicillins, third generation cephalosporins and even carbapenems. Carbapenem-resistant, ESBL-producing bacteria are listed in the critical section of the global priority pathogens by the WHO in 2017, with the intention of aiding the research into new and effective antibacterial agents (Asokan *et al.,* 2018; Banin *et al*., 2017). These MDR species have been isolated from both community acquired (CA) and hospital acquired (HA) cases of infections (Magwenzi *et al*., 2017).

Plants produce compounds to protect themselves from microbial attacks (Maleki *et al*., 2017).

A medicinal plant is any plant which, in one or more of its parts, contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs. Medicinal plants provide health promoting characteristics, temporary relief of symptomatic problems or have curative properties. Medicinal plants are good sources of antimicrobial agents (Mahesh and Satish, 2008). They are therapeutically significant for several illnesses or diseases.

They are widely used by all sections of people either directly as folk remedies indigenously or indirectly in the pharmaceutical preparation of modern medicines (Farnsworth *et al*., 1985).

Herbal medicines consist of plant or its part, used to treat injuries, disease or illnesses and used to prevent and treat diseases and ailments or to promote health and healing. It is a drug preparation or product made from a plant or plants and used for any such purpose. Herbal medicines are still the mainstay of about 75-80% of the world's population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with human body and with less side effects. Herbal medicines are the oldest form of health care remedies known to mankind. (Mohsin and Rajmahammad, 2017).

A medicinal plant named *Azadirachta indica* (Meliaceae), commonly known as Neem was used for this study. Neem is a member of the mahogany family, Meliaceae. It is mainly cultivated in the Indian subcontinent and the sub-saharan Africa like Nigeria. More than 140 compounds have been isolated from different parts of the neem tree. All parts of the neem tree:- leaves, flowers, seeds, fruits, roots and bark have been used traditionally for the treatment of inflammation, infections, fever, skin diseases and dental disorders. Recent studies have shown that *Azadirachta indica* (Neem) plant possesses anti-inflammatory, antibacterial, antipyretic, antifungal, antigastric, antitumor, hypoglycemic, and anti-arthritic properties. The neem plant has a wide spectrum of antibacterial activity against Gram positive and Gram negative microorganisms (Muhammad *et al* 2019).

Neem leaves popularly known as "Dogonyaro" leaves in Nigeria also known as 'Wonder leaves', is a herb known to have numerous health benefits and has been used for beauty regimes for thousands of years now. Neem leaves are well known not only for its medicinal properties but also for taking care of the environment. (Bhowmik, *et al.* 2010; Srivastava, *et al*., 2020.)

This study at aimed to evaluating and comparing the micro-biological activities of Neem leaf on known MDR bacterial isolates. Mankind has made very good use of these antimicrobials in its fight against infectious diseases. Although many drugs are now completely synthetic, the natural drug may be manipulated to change its structure to give better chemical and biological properties. They are then referred to as semi-synthetics.

MATERIALS AND METHODS

Collection of Neem leaf samples and extraction by maceration: Fresh samples of *Azadirachta indica* were collected from 2 locations. One from a farmland at Ijaiye, Lagos Nigeria, this was identified and authenticated at The University of Lagos herbarium, Department of Botany, Lagos Nigeria and given a herbarium specimen number, LUH 8727. The second one, was obtained in large quantities from a local herb seller at Mushin market in Lagos state. It was also identified at the Herbarium of the Department of Botany, University of Lagos Nigeria, with tag number; LUH 8991. The plant material collected was rinsed to remove debris from the surface of the leaves, and the leaves were then sorted and detached from the stem of the whole plant. The leaves were dried using a Hot air oven for 5 days at 40ºC. The plant materials were kept away from sunlight. After drying, the plant material was pulverized into powder, using a milling machine specifically for grinding plant materials. The milled leaves were transferred into 5L glass container for cold maceration by soaking 500g of pulverized leaves sample in 1.5 L of 70% ethanol for 72 h before filtering using clean muslin cloth and later Whatman filter paper No 2 (110 mm). The mixture was agitated to allow even spread of the solvent through the surface area of the plant material and the procedure was carried out for five days. More solvent was added to completely cover the plant material and the cold maceration procedure was carried out thrice (Adebayo *et al.,* 2006). At the end of the maceration process, the filtrate was pooled together and concentrated using rotatory evaporator. The sample was oven dried at 40^0 C until complete evaporation of solvent to obtain the ethanol extract. The yield of extract was calculated and kept in a sterile sample bottle at 4℃, in a refrigerator for preservation until when needed.

Qualitative and quantitative phytochemical screening of leaf extracts: Qualitative and quantitative screening of the extract, of the Neem leaves for alkaloids, flavonoids, saponins, steroids, tannins, terpenoids, glycosides and phenolic compounds was carried out using standard analytical methods stipulated by the Association of Official Analytical Chemists (AOAC).

Preparation of working concentrations of the *Azadirachta indica* leaf extracts: 4g of *Azadirachta indica* leaf extracts was dissolved in 10ml of distilled water in a conical flask to give a concentration of 400mg/ml and labelled Sample A in a sample bottle. 2ml of sample A was added to 2ml of the sterile diluent (sterile distilled water) in another sample bottle to give a concentration of 200mg/ml and labelled as Sample B. 2ml of sample B was then added to 2ml of sterile diluent (sterile distilled water) in yet another sample bottle to give a concentration of 100mg/ml and labelled as Sample C. The

samples A, B and C were pasteurized and then used as the working samples of the extract.

Levofloxacin was used as the Reference standards.A stock solution of Levofloxacin with a concentration of 5mg/ml, which is also equivalent to 5000µg/ml was prepared and labelled as sample I. 1ml of sample I was transferred into a sterile sample bottle containing 9ml of Sterile water, to give a concentration of 500µg/ml and labelled, Sample II. From sample II, 1ml was measured into a sterile sample tube containing 9ml of the sterile diluent to give a concentration of 50µg/ml, labelled Sample III. A double dilution of Sample III was done by measuring 4ml of its content and transferring into a sterile sample tube containing 4ml of the sterile diluent to give a concentration of 25µg/ml, labelled Sample IV. Further double dilutions were serially carried out to give lower concentrations of 12.5µg/ml (Sample V) and 6.25µg/ml (Sample VI) respectively.

Bacterial Isolates: Isolates from our laboratory stock, which had been previously profiled and revalidated, for identity and antimicrobial susceptibility in previous studies, were used in this study (Adeluola *et.al.* 2017; Adeluola *et.al.* 2018).

Gram-positive isolates used: Five isolates of *Staphylococcus aureus* (ES 1, MED 4K, ES 21, ES 7 and ATCC 25923) and one strain of *Staphylococcus xylosus* (ES 16) were used for the study.

Gram-negative isolates used: Three strains of *Klebsiella pneumoniae*: N3, Med 1 (2) and OG 1(4), one *Enterobacter aerogenes:* N 14 and one *Escherichia coli*: ATCC 25922.

Antimicrobial susceptibility test: Similar to Bauer *et al.* (1966), overnight Tryptone-soya broth cultures of the isolates were seeded on Mueller Hinton agar (MHA) plates using sterile swabsticks to spread the isolates like a carpet over the entire surface of the MHA. Each plate appropriately labelled with the identity of the seeded isolate was marked out in four segments, with each segment assigned to a particular test concentration of the extract and/or the standard antimicrobial agent being used. After a few minutes for drying on the bench elapsed, a cork borer of 9 mm in diameter was used to make wells in the marked out segments of the MHA petri dishes. About 150µL of each concentration of the extracts (400mg/ml, 200mg/ml and 100mg/ml), levofloxacin (50µg/ml, 25gµ/ml and 6.25µg/ml) were aseptically introduced into their assigned bored holes in the labelled quadrants. While 150µL of sterile distilled water was dispensed into the fourth quadrant labelled "Control". After dispensing, the plates were allowed to stand for 2 hours on the bench before incubating at 37^{o}C for 24hrs. Thereafter, they were observed for zones of inhibition.

Tests for Minimum Inhibitory Concentration: A combination of the Agar dilution technique recommended by Hugo and Russell and the Clinical and Laboratory Standard Institute, CLSI guidelines, 2008, were used in order to establish the minimum concentration that inhibited a particular responsive assay organism. The sixteen working concentrations used were 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 40, 80 and 160mg/ml. 7 stock concentrations of 5, 10, 20, 40, 80, 160 and 400mg/ml were prepared from the extracts labelled A, B and C above for use. 7 stock concentrations of 5, 10, 20, 40, 80, 160 and 400mg/ml were prepared from the extracts labelled A, B and C above for use and as highlighted in Table 1.

These concentrations were prepared by measuring specified volumes of agar into universal glass bottles labelled A-P and the agar was sterilized in the autoclave at 121°C for 15 minutes. The required volumes of extracts were added into the labelled petri-dishes before adding the required volume of molten MHA with thorough mixing. (Table 1)

.

Table 1
Constituents of MHA Plates for MIC Test

Bottle label	Stock concentration of extract (mg/ml)	Volume of extract (ml)	Volume of MHA (ml)	Conc. Of extract in MHA (mg/ml)
A	5	0.01	19.99	0.0025
B	5	0.02	19.98	0.005
C	5	0.04	19.96	0.01
D	5	0.08	19.92	0.02
E	5	0.16	19.84	0.04
F	5	0.32	19.68	0.08
G	5	0.64	19.36	0.16
H	5	1.28	18.72	0.32
I	5	2.56	17.44	0.64
J	20	1.28	18.72	1.28
K	20	2.56	17.44	2.56
L	80	1.28	18.72	5.12
M	80	2.56	17.44	10.24
N	400	2	18	40
O	400	4	16 (1.5x strength)	80
P	400	8	12 (Double Strength)	160

The overnight culture of each test organism, was standardised by diluting with sterile normal saline to give the equivalent turbidity of 0.5 McFarland reference standard, equivalent to 1.5×10^{8} bacterial cells/ml.
Each organism was spotted three times with the aid of an inoculating loop on their respectively assigned sites on the MHA plates, in an aseptic zone. The plates were then incubated at 37°C for 24 hours. The MICs were recorded as the least concentration of the extract with no apparent growth of the test isolates on the MHA plates.

Tests for Minimum Bactericidal Concentration (MBC): About 19mls of single strength MHA was poured into petri dishes and the agar allowed to set. The first 3 minimum inhibitory concentration plates that showed no growth, were selected to be sub-cultured onto blank single strength MHA plates. With the use of an inoculating loop, containing a loopful of 4% tween 20, the spot where there was no growth of microorganisms, on the selected MIC plates were rubbed and streaked onto the blank agar plates. The fresh plates were incubated at 37°C for 18-24hours. For each test isolate, the least concentration that still did not show any growth at this stage, is taken as the MBC.

RESULTS

Collected Neem leaves and yield of extracts: Upon weighing, a total yield of 500g + 820g of the milled extract was obtained. An 8.37% yield of the ethanol extract of the *A. indica* obtained, appeared blackish green, sticky in texture with a pungent odour and a bitter taste.

Ethanolic Extract	Colour	Odour	Taste	Texture	Percentage Yield % W/V
A.*indica* leaf	Blackish green	Pungent	Bitter	Sticky	8.37

TABLE 3:
Phytochemical test results

Phytochemicals	Qualitative Test Result	Quantitative Test Result (Mg/G)
Saponin	+	0.32
Reducing sugar	-	ND
Phenolic compound	+	0.03
Tannin	+	0.01
Flavonoid	+	0.06
Cardiac glycosides	+	1.569
Anthraquinones	-	ND
Steroids	+	ND
Terpenoid	-	ND
Alkaloid	+	0.42

'+' - Presence of phytochemical '-' - Absence of phytochemical
ND - Not Done

Phytochemical screening: Qualitative and quantitative tests carried out on Neem leaf extract, gave result showing a preponderance of cardiac glycosides (1.569mg/g), alkaloids (0.42mg/g) and saponin (0.32mg/g). Other phytochemicals present like Phenolic compounds, Tannins, Flavonoids and Alkaloids were less than 0.1mg/g in concentration.

Table 4:
Zones of inhibition of Neem leaf extract on the Gram-positive bacterial isolates.

Organisms	Zones Of Inhibition At Various Concentrations Of Extract (In Mm)			
	400 mg/ml	200 mg/ml	100 mg/ml	Control
ES 1 (*Staphylococcus aureus*)	32	28	16	--
MED 4K (*Staphylococcus aureus*)	0	0	0	0
ES 21 (*Staphylococcus aureus*)	34	24	20	0
ES 7 (*Staphylococcus aureus*)	20	16	14	0
ES 16 (*Staphylococcus xylosus*)	30	26	20	0
ATCC 25923	30	25	20	0

All the Gram positive test isolates including ATCC 25923, gave zones of inhibition at all concentrations against the levofloxacin used as reference standard; seen in Table 6.

Whereas out of the Gram negative test organisms, Figure 1 shows that only the ATCC 25922 gave zones of inhibiton against levofloxacin at all concentrations tested. Only *Enterobacter aerogenes* (N 14) gave appreciable zone of inhibition at 12.5 µg/ml while others only showed signs off susceptibility from 25 µg/ml (Figure 1).

Table 5:
Zones of inhibition of Neem leaf extract on the Gram-negative bacterial isolates.

Organism	Diameter (mm) of the zone of inhibition of organisms at the concentrations of *A.indica* extract			
	400 mg/ml	200 mg/ml	100 mg/ml	Control
Klebsiella pneumoniae (Med 1(2))	**25**	**15**	-	-
Enterobacter aerogenes (N14)	-	-	-	-
Klebsiella pneumoniae [OG1 (4)]	-	-	-	-
Klebsiella pneumoniae (N3)	-	-	-	-
Escherichia coli (ATCC 25922)	-	-	-	-

'-' represents no zone of inhibition

Table 6:
Zones of inhibition of Levofloxacin standard on Gram - positive bacteria isolates

Organisms	Zones Of Inhibition Of Different Concentrations Of Levofloxacin(in mm)			
	50µg/ml	25µg/ml	12.5µg/ml	6.25gµ/ml
ES 1 (*Staphylococcus Laureus*)	15	12	10	5
MED 4K (*Staphylococcus aureus*)	34	29	26	20
ES 21 (*Staphylococcus aureus*)	28	24	20	12
ES 7 (*Staphylococcus aureus*)	34	30	25	20
ES 16 (*Staphylococcus aureus*)	30	28	20	17
ATCC 25923 (*Staphylococcus aureus*)	32	30	20	10

Antimicrobial susceptibility test: With reference to Table 4, each of the Gram positive test isolates, including the standard strain used (ATCC 25923), gave zones of inhibition ranging from 14 mm to 34 mm over the range of extract concentrations, 100 mg/ml, 200 mg/ml and 400 mg/ml and all in ascending order. This is evidence of some level of susceptibility. Only one Staphylococcus aureus (Med 4k) showed complete resistance to the extract at all concentrations tested.

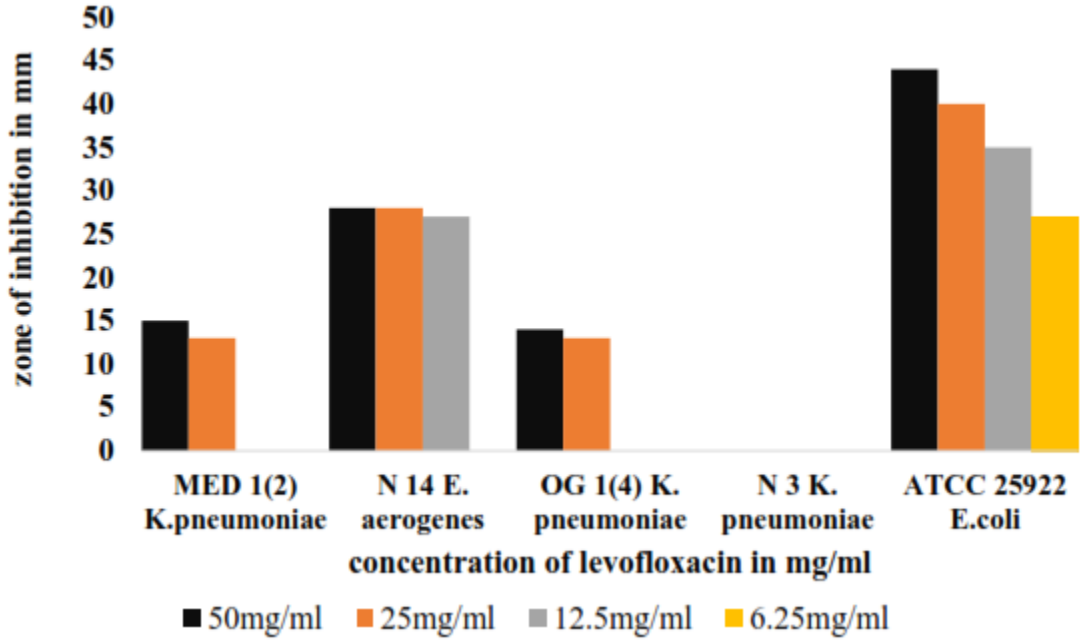

Figure 1:
The zones of inhibition of Gram - negative isolates against the concentrations of Levofloxacin

Table 7:
The Minimum Inhibitory Concentrations (MICs) of the *A. indica* leaf extracts against the Gram - positive isolates

Organisms	ES 1 (*Staphylococcus aureus*)	MED 4K (*Staphylococcus aureus*)	ES 21 (*Staphylococcus aureus*)	ES 7 (*Staphylococcus xylosus*)	ES 16 (*Staphylococcus aureus*)	ATCC 25923 (*Standard Staphylococcus aureus*)
MIC values for Neem leaf extract	5.12mg/ml	--	40mg/ml	40mg/ml	40mg/ml	40mg/ml

Of all the Gram negative isolates tested, only one *Klebsiella pneumonae* (Med 1 (2) gave zones of inhibition of 15 mm and 25 mm against 200 mg/ml and 400 mg/ml of the extract respectively as shown on Table 5. All the others including the Standard strain (ATCC25922), did not show any zone of inhibition that is non-susceptible.

MIC test result: From the results shown on Table 7, only one *Staphylococcus aureus* (Med 4k), which gave no zone of inhibition during susceptibility test (Table 4), also did not give any MIC value for the *A. indica* extract.

Following the pattern of results from the susceptibility test result from Table 5, only *K.pneumoniae* (Med 1(2)) and the standard strain, ATCC 25922 gave MIC result of 160 mg/ml as shown on Table 8.

Table 8:
The Minimum Inhibitory Concentrations (MICs) of the *A. indica* leaf extracts against the Gram - negative isolates.

ORGANISM	*A .indica* leaf extract (mg/ml)
K.pneumoniae (Med 1(2))	160
K.aerogenes (N14)	-
K.pneumoniae (OG 1(4))	-
K.pneumoniae (N3)	-
E.coli (ATCC 25922)	160

Minimum Bactericidal Concentration (MBC) test results: All the MBC test results obtained from the Gram positive strains, on Table 9, were consistent with the results from the MIC test results. However, the MDR K.pneumoniae (Med 1(2)) and the standard E.coli (ATCC 25922) did not give MBC beyond what was obtained for MIC; Table 10.

Table 10:
The minimum bactericidal concentration (MBC) Results for *A. indica* leaf extract against the Gram - negative isolates.

Organism	*A. indica* leaf extract
K.pneumoniae (Med 1(2))	-
E.aerogenes (N14)	-
K.pneumoniae (OG 1(4))	-
K.pneumoniae (N3)	-
E.coli (ATCC 25922)	-

'-' represents no MBC recorded.

DISCUSSION

The percentage yield obtained from the ethanolic extract of the leaves of A. indica after extraction was 8.37 % w/w which is higher than the 6.8 ± 0.12 obtained from the ethanolic extract of the bark of A. indica by Anokwuru *et al*., 2011. Low yield obtained from medicinal plants is one of the challenges

encountered in the discovery of novelle drugs of herbal origin. Large quantities of the plant parts are therefore required to obtain the needed phytochemical compounds. Factors which could affect yield of plant products are specie of medicinal plant, source of plant material, method of extraction, time of extraction, extraction medium, extent of processing, temperature of extraction, level of moisture as well as particle size. (Sandeep *et al*., 2016, Biondi *et al*., 2021).

The phytochemicals obtained from this study e.g. Saponins, Phenolic compounds, Tannins, Flavonoids, Cardiac glycosides, steroids and alkaloids, are known to show medicinal activity as well as exhibiting physiological activity (Sofowora, 1993). The presence of the secondary metabolites revealed in the plant extracts, are responsible for the therapeutic activities of most plants (Geyid *et al*., 2005). These metabolites / phytochemicals usually exhibit stronger antimicrobial properties than others (Hutchinson *et al*., 1963).

The ethanolic leaf extract of A.indica from this study, contained phytochemicals similar to findings from Sharma *et al*., 2018 with minor differences. Prashanth (2014) reported the absence of both triterpenoids and steroids which agrees with and contradicts the findings of this study respectively where terpenoids were absent but steroids were detected. According to another study (Vinoth *et al*., 2012), it was reported that Alkaloids, terpenoids and glycosides were absent whereas in this study, alkaloids and Cardiac glycosides were detected. The absence of Terpenoids agree with findings reported by Prashanth (2014) but contradicts that of Vinoth *et al* (2012). Prashanth (2014) and Vinoth *et al* (2012) also reported the presence of reducing sugars in the ethanolic leaf extract of A.indica which was absent in this study. The diversities in types of phytochemical compounds obtained from the different studies can be attributed to species of medicinal plants, method, time, medium, extent and temperature of extraction among other factors as stated above (Biondi *et al*., 2021).

Each of the Gram positive test isolates, including the standard strain used (ATCC 25923), gave zones of inhibition. This is evidence of some level of susceptibility to the ethanolic leaf extracts of *A.indica* by these isolates including those which are known to be Methicillin resistant *Staphylococcus aureus* (MRSA) from previous studies by Adeluola *et al*., 2017. Only one *Staphylococcus aureus* (Med 4k) showed complete resistance to the extract at all concentrations tested. Of all the MDR Gram negative isolates tested, only one *Klebsiella pneumonae* (Med 1 (2)) gave measurable zones of inhibition at higher levels of concentration, of extracts of *A.indica* leaves used. Only the *E.coli* standard strain, ATCC 25922 gave appreciable zones of inhibition in the test against ciprofloxacin as evidence of high level of resistance by the other Gram negative test organisms used, which are also known to possess high efflux pump activity (EPA) (Adeluola *et al.,* 2018). These antibacterial test results show that *A.indica* have a higher potential for antibacterial activity against MRSA than MDR Gram negative isolates.

The MIC results for Neem leaves as shown in Table 8 revealed that the MIC for MDR *Staphylococcus xylosus* (ES 16), MRSA (ES 21, ES 7) and *Staphylococcus aureus* (ATCC 25923) was 40mg/ml, while that of MDR *Staphylococcus aureus* (ES 1) was found to be 5.12mg/ml. Below these concentrations, there was no inhibition of growth as seen in Table 8. At higher concentrations, the MRSA (MED 4K), still exhibited organism growth which further confirmed the results obtained from the AST as MED 4K being Resistant to the extract. The MBC results for Neem leaf extract for the Gram-positive test organisms was found to be consistent with the MIC results obtained. This indicates that the Leaf extract of *Azadirachta indica* is bactericidal against the studied Gram positive organisms at the tested concentrations.

It was observed that the Leaves of A. *indica* showed remarkable inhibitory activity against all the MRSA organisms (ES 1, ES 21, ES 7, ES 16) except MED 4K which was considered resistant and ATCC 25923. Comparing the activities of the extracts with that of levofloxacin standard, the MRSAs (ES1, ES 21, ES 7, and ES 16) and ATCC 25923 can be presumed to be Susceptible to the leaves of Neem leaf ethanolic extract, while MED 4K was observed to be Resistant.

Findings reported by Mohammad (2017) stated antibiotic activity of *A. indica* ethanolic leaf extract against *E.coli* and *K.pneumoniae* strains but there is no appreciable antibacterial activity of *A. indica* ethanolic leaf extract on the MDR organisms used in this study except one MDR strain of *K.pneumoniae* (Med 1(2)). Although in this study, the ethanolic extract of *A. indica* leaves was not effective on the *E. coli* standard (ATCC 25922), the MIC test results from the Gram negative test isolates, was consistent with the AST results earlier obtained from the ethanolic leaf extact of *A. indica*. Only the MDR *K. pneumoniae* (Med 1(2)) and the *E. coli* standard strain (ATCC 25922) gave MIC result of 160 mg/ml. However, upon sub-culturing for the minimum bactericidal concentration (MBC), Med 1 (2) and ATCC 25922 grew again. This suggests a weak bacteriostatic effect of the antibacterial component of A.indica leaves on both Med 1 (2) and ATCC 25922 and not a bactericidal effect. Other similar studies showed both ethanol extracts of A. indica leaves and twigs were found to have potent antibacterial activity against MSSA and *E. Coli* (Ravi *et al*, 2015.

In conclusion, it can be deduced from the tests carried out and the results obtained, that ethanolic leaf extracts of A.indica possess metabolic compounds which may be useful in tackling the problem of drug resistance in MRSA infections. Further study with a more appreciable yield of the phytochemical compounds available for test, may reveal and confirm the presence of more of the compounds with antibacterial activity against MRSAs. However, with the MDR Gram negative infections, the best that may be obtained, may be a retardation of infections caused by these types of organisms. Better results with the use of higher concentrations which are non-toxic cannot be ruled out.

Acknowledgements:

- *The authors gratefully acknowledge the Head of the Department of Pharmaceutical Microbiology & Biotechnology, Faculty of Pharmacy, University of Lagos, for use of the laboratory space.*
- *The authors also acknowledge the kind assistance of the laboratory technologist, Mr. Abdulrahaman Usman and his laboratory staff, of the Department of Pharmaceutical Microbiology & Biotechnology, Faculty of Pharmacy, University of Lagos, for their kind assistance in making the laboratory facilities available in good condition for use.*
- *The authors appreciate Dr. Nodza George of the Herbarium of the Department of Botany, University of Lagos, for assisting in the plant identification.*

REFERENCES

Adebayo EA, Ishola OR (2009a). Phytochemical and antimicrobial screening of crude extracts from the root, stem bark, and leaves of Terminalia glaucescens *Afr. J. Pharm. Pharmacol*. 3(5): 217-221.

Adeluola, A.O., Oyedeji, K.S., Mendie, U.E., Johnson, J.R. and Porter, J.R., 2017. Detection of efflux pump activity among clinical isolates of *Staphylococcus*and Micrococcus species. *Tropical Journal of Pharmaceutical Research*, 16(11), pp.2697-2704.

Adeluola, A.O., Oyedeji, K.S., Mendie, U.E., Johnson, J.R. and Porter, J.R., 2018. Detection, inhibition and molecular analysis of multidrug resistant aerobic gram-negative clinical isolates from a tertiary hospital in Nigeria. *African Journal of Biomedical Research*, 21(1), pp.15-21.

Anokwuru, C.P., Anyasor, G.N., Ajibaye, O., Fakoya, O. and Okebugwu, P., 2011. Effect of extraction solvents on phenolic, flavonoid and antioxidant activities of three nigerian medicinal plants. *Nature and Science*, 9(7), pp.53-61.

Asokan, G.V. and Vanitha, A., 2018. WHO global priority pathogens list on antibiotic resistance: an urgent need for action to integrate One Health data. *Perspectives in public health*, 138(2), pp.87-88.

Banin, E., Hughes, D. and Kuipers, O.P., 2017. Bacterial pathogens, antibiotics and antibiotic resistance. *FEMS microbiology reviews*, 41(3), pp.450-452.

Bhowmik, D., Chiranjib, Y.J., Tripathi, K.K. and Kumar, K.S., 2010. Herbal remedies of *Azadirachta indica* and its medicinal application. *J Chem Pharm Res*, 2(1), pp.62-72.

Biondi, F., Balducci, F., Capocasa, F., Visciglio, M., Mei, E., Vagnoni, M., Mezzetti, B. and Mazzoni, L., 2021. Environmental conditions and agronomical factors influencing the levels of phytochemicals in Brassica vegetables responsible for nutritional and sensorial properties. *Applied Sciences*, 11(4), p.1927.

Boamah, V.E., Agyare, C., Odoi, H., Adu, F., Gbedema, S.Y. and Dalsgaard, A., 2017. Prevalence and antibiotic resistance of coagulase-negative Staphylococci isolated from poultry farms in three regions of Ghana. *Infection and drug resistance*, 10, p.175.

Coudron, P.E., Moland, E.S. and Sanders, C.C., 1997. Occurrence and detection of extended-spectrum beta-lactamases in members of the family Enterobacteriaceae at a veterans medical center: seek and you may find. *Journal of clinical Microbiology*, 35(10), pp.2593-2597.

Dele, O.S., Sunday, A. and Ajoke, O.B., Phytochemical profile and proximate composition of *Azadirachta indica* (A. Juss) harvested at different times of the day. *International Journal of Herbal Medicine* 2020; 8(2): 07-11

Dhama, K., Tiwari, R., Chakraborty, S., Saminathan, M., Kumar, A., Karthik, K., Wani, M.Y., Amarpal, S.S. and Rahal, A., 2014. Evidence based antibacterial potentials of medicinal plants and herbs countering bacterial pathogens especially in the era of emerging drug resistance: An integrated update. *Int J pharmacol*, 10(1), pp.1-43.

Geyid, A., Abebe, D., Debella, A., Makonnen, Z., Aberra, F., Teka, F., Kebede, T., Urga, K., Yersaw, K., Biza, T. and Mariam, B.H., 2005. Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. *Journal of ethnopharmacology*, 97(3), pp.421-427.

Hormozi, S.F., Vasei, N., Aminianfar, M., Darvishi, M. and Saeedi, A.A., 2018. Antibiotic resistance in patients suffering from nosocomial infections in Besat Hospital. *European journal of translational myolog*y, 28(3).

Hutchinson, J., Dalziel, J.M. and Hepper, F.N., 1963. Flora of West Tropical Africa. Volume II. Flora of West Tropical Africa. Volume II., (2nd ed.(rev.)).

Jacoby, G.A. and Medeiros, A.A., 1991. More extended-spectrum beta-lactamases. Antimicrobial agents and chemotherapy, 35(9), pp.1697-1704.

Magwenzi, M.T., Gudza-Mugabe, M., Mujuru, H.A., Dangarembizi-Bwakura, M., Robertson, V. and Aiken, A.M., 2017. Carriage of antibiotic-resistant Enterobacteriaceae in hospitalised children in tertiary hospitals in Harare, Zimbabwe. *Antimicrobial Resistance & Infection Control*, 6(1), pp.1-7.

Muhammad, A., Lawan, I.M., Abubakar, A. and Dangora, I.I., 2019. Antimicrobial Activity of *Azadirachta indica* (Neem) Leave Extract Against Some Clinical Isolates. *Dutse J Pure Appl Sci*, 5(1b), pp.97-104.

Oliveira, J. and Reygaert, W.C., 2019. Gram negative bacteria.[Updated 2019 Mar 4]. StatPearls [Internet]. Treasure Island (FL). StatPearls Publishing (Jan-).

Prashanth GK, Krishnaiah GM. Chemical composition of the leaves of *Azadirachta indica* Linn (Neem), International Journal of Advancement in Engineering Technology, *Management and Applied Science*. 2014; 1(5):21-31.

Ravi, K., Bharavi, K. and Kumar, R., 2015. Phytochemical screening and antibacterial activity of ethanol extract of leaves and twigs of *Azadirachta indica* A. Juss. *Pharmaceutical and Biosciences Journal*, pp.56-59.

Sandeep, I.S., Kuanar, A., Akbar, A., Kar, B., Das, S., Mishra, A., Sial, P., Naik, P.K., Nayak, S. and Mohanty, S., 2016. Agroclimatic zone based metabolic profiling of turmeric (Curcuma Longa L.) for phytochemical yield optimization. *Industrial Crops and Products*, 85, pp.229-240.

Sharma, J., Rahul, S.N., Tiwari, S., Joshi, M.D., Pundhir, A. and Kumar, R., 2018. Phytochemical analysis and Antibacterial properties of *Azadirachta indica* (Neem) leaves extract against E. coli. *Journal of Pharmacognosy and Phytochemistry*, 7(4), pp.1368-1371.)

Sofowora, A., 1993. Recent trends in research into African medicinal plants. *Journal of ethnopharmacology*, 38(2-3), pp.197-208.

S**rivastava, S.K., Agrawal, B., Kumar, A. and Pandey, A., 2020.** Phytochemicals of *Azadirachta indica* source of active medicinal constituent used for cure of various diseases: A Review. *Journal of Scientific Research*, 64(1), pp.385-90.

Tzelepi, E., Giakkoupi, P., Sofianou, D., Loukova, V., Kemeroglou, A. and Tsakris, A., 2000. Detection of extended-spectrum β-lactamases in clinical isolates of Enterobacter cloacae and Enterobacter aerogenes. *Journal of clinical microbiology*, 38(2), pp.542-546.

Vinoth B, Manivasagaperumal R, Rajaravindran M. Phytochemical analysis and antibacterial activity of *Azadirachta indica* A. Juss. *International Journal of Research in Plant Science*. 2012 Sep;2(3):50-5.

Wenzler, E., Fraidenburg, D.R., Scardina, T. and Danziger, L.H., 2016. Inhaled antibiotics for Gram-negative respiratory infections. *Clinical microbiology reviews*, 29(3), pp.581-632.