ANTI BACTERIAL ACTIVITY OF NEEM (*Azadirachta indica)* LEAF EXTRACT ON *Klebsiella pneumoniae* AND *Staphlococcus aureus* IN ZARIA, KADUNA STATE

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Abstract: *Azadirachta indica* commonly known as neem is a medicinal plant belonging to the family Meliaceae. The research was carried out to determine the antimicrobial effects of aqueous extracts of the leaves of *Azadirachta indica* using minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts as indices. Clinical isolates of *Klebsiella pneumoniae* and *Staphylococcus aureus* were used as test organisms. For the aqueous extract of the leaves, a minimum concentration of 125 mg/ml was found to inhibit the growth of *K. pneumoniae* and *S. aureus* in nutrient broth. MBC for *K. pneumoniae* and *S. aureus* are 250 and 250 mg/ml, respectively. The aqueous extracts of *A. indica* showed relatively strong antimicrobial activity, inhibiting the growth of bacterial isolates used which therefore indicates that the plant has antibacterial properties. It may be attributed to the high active compounds from the sample unlike the low volatility of some extracts such as ethanol. It is recommended that management studies should be carried out on the plant to explore its usage as a possibility of treatment for pathogenic bacterial infections.

Keywords: Antibacterial, aqueous extracts, infections, maximum inhibitory

Introduction

Plants contain certain biological active components which are potential for development as medicinal agents (Aslam *et al*., 2009; Heleno *et al*, 2011). Product of the neem tree (*Azadirachta indica*) from all parts of the plant has demonstrated efficacy against many pest species including arthropod pests and diseases of crops. Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. Plants have generally served traditionally as the most important weapon against pathogens to man (Sofowora, 1992; 1993; Garg *et al*., 1993; Cimolai, 2008; Faiza *et al*., 2009). The ancient man is known to have utilized plant materials as drugs against many diseases. He was totally dependent on green plants for his daily need of medicament (Trease and Evans, 2002; Nwanjo, 2005). The early man was able to distinguish food, medicinal and poisonous plants based on trial and error led by instinct, taste, experience and observation of animal's behaviours. For example, chimpanzees have been observed to ingest the leaves of *Vernonia amygdalina* (bitter leaf) when suffering from parasitic infections (Hawaze *et al*., 2012) hence man was eventually able to categorize plants into edible and non-edible. Botany and medicine have been closely linked throughout history. Prior to this century, medical practitioners whether allopath (medical doctors), homeopaths, naturopaths, or herbalist had to know the plants in the area and how to use them since many of their drugs were derived from plants (Nweze *et al*., 2004). Around 1900s, 80% of the drugs were derived from plants. However, in the decades that followed, the development of synthetic drugs from petroleum products caused a sharp decline in the pre-eminence of drugs from live plant sources (Nweze *et al*., 2004). With the recent trend of high percentage resistance of microorganisms to the present day antibiotics, efforts have been intensified by researchers towards the search for more sources of antimicrobial agents (Hawaze *et al*., 2012; Rogers, 20 Enterobacteriaceae, the enteric bacteria, are facultative anaerobic Gram-negative rods that live in the intestinal tract of human and animals in health and diseases. The Enterobacteriaceae among the most important bacteria medically, a number of generations within the family are human and animal intestinal pathogens (for examples *Salmonella, Shigella* and *Yersinia*). Several others are normal colonist of the human gastrointestinal tract (for example, *Escherichia coli*, *Enterobacter*, *Klebsiella*) but these bacteria as well as, may occasionally be associated with diseases in humans and animals (Tona, 2004; Ogston, 1984; Schlegal, 1995; Okemo *et al*., 2001; Shafiel *et al*., 2011).

The genus *Staphylococcus* is made up of gram positive cocci with diameter of 0.5 – 1.5 urn. *Staphylocoocus aureus* is the most pathogenic spp of the genus *Staphylococcus.* It is implicated in both communities acquired and nocosomial infections. It often symptomatically colonizes the skin and mucus membrane of healthy individuals in particular the anteronaires. Due to importance of *Staphylococcus aureus* on the increasing prevalence of antibiotic resistance stains, these bacteria have become the most studied *Staphylococcus species.* The increased prevalence of microorganisms which are resistant to the available antibiotics is one of the major challenges for the healthcare systems worldwide. Antibiotic-resistant infections are associated with one to two-fold increases in mortality compared to antibiotic-susceptible infections (Ebi, 2001).

In general, it can be observed that the treatment of virus, bacteria, fungi and protozoa with the existent drugs is increasingly difficult due to rapid mutation of these organisms into new genetic variants which result in their being resistant to the antibiotics (Jarraud *et al.*, 2001; Gillet *et al*., 2002; Sing *et al*., 2008; Iwase, 2010; Ezeokeke *et al*., 2015). Moreover, antibiotic resistance imposes enormous health expenditure due to the higher treatment costs and longer hospital stays. Phenolic acids including benzoic and cinnamic acid derivatives have been found in plants. Among benzoic acid derivatives, p-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids were identified in different plant species and tannins like allergic acid (Sharma *et al*., 2009; Shravan and Kannapan, 2011; El-mahmood *et al.*, 2010).

In vitro and epidemiologic studies suggest that consumption of foods rich in phenolic compounds might significantly decrease the risk of some health problems due to their antioxidant, anti-mutagenic, anti-inflammatory and antibacterial properties (Almas *et al*., 1995; Badam *et al*., 1999; Dinges *et al*., 2000; Debola, 2002; Deepak *et al*., 2013). Antimicrobial activities of the aqueous and ethanolic extracts of plant potentials were evaluated both in vitro and in vivo against *Aspergillus niger* and *Escherichia coli.* The percentage yields of aqueous extracts were greater than that of

ethanolic extract. Both extracts showed a potentially good antimicrobial activity, however aqueous extract had more activity than ethanolic activity. The activities increased with increasing concentration. Maximum antifungal activity was shown by aqueous extract of *A. conyzoides* against *A. niger* and *A. ustus* with the average inhibition of 20 mm each while the least activity were recorded against *A. fumigatus* at the concentration of 800 mg/mL with 7 mm zones of inhibition. The MIC values of extracts ranged from 50 to 794 mg/mL (El-mahmood *et al.,* 2010). However, not many reports are available on the exploitation of antifungal or antibacterial property of plants for developing commercial formulations for application in crop protection.

Historically, plants extracts have been used as a safe, effective and natural remedy for ailment and diseases in traditional medicine (Kluytmas *et al*., 1997; Sonia and Srinivasan, 1999; Mandell, 2009). They have also played significant role in providing active ingredients in controlling and reducing diseases in humans when edible species are eaten. Traditionally, the screening of bioactive compounds involves, a brute force approach that demands huge investment of significant time and resources to identify a single promising lead compound from chemical libraries consisting of up to several million entities, finding an efficacious drug to bring to market have little or no guarantee. Therefore this study was aimed to evaluate the antimicrobial efficacy of aqueous extracts of *Azadirachta indica* against *Klebsiella pneumoniae* and *Staphylococcus aureus,* (Cole, 2001; Cook *et al*., 2007; Wuyep *et al*., 2017)

Materials and Methods

Collection and processing of plant material

The neem leaves were obtained from Samaru town, and were exposed to room temperature at 37^0C to dry (for about four days) before further analysis. The test organisms were *Klebsiella pneumonia* and *Staphylococcus aureus.* They were maintained on nutrient agar and MacConkey slant under refrigerated at 4^0C temperature for further analysis of neem leaf extract.

Culture media

MacConkey Agar and nutrient slant was prepared following the manufactures instruction and was used to subculture *Klebsiella pneumonia and staphylococcus aureus* and incubated at 37^0C for 24 h after which colonial morphology was observed and biochemical test was conducted to confirm the organisms.

Biochemical identification of the test organism

The following test was carried out to biochemically identify the clinical isolate (*Klebsiella pneumonia*)

Simmon"s citrate utilization test

The isolate was inoculated into a Simmon"s Citrate agar slant in a bijou bottle and incubated at 37ºC for 48 h. Development of a deep blue colour indicated a positive test while a green colour (neutral colour of the medium) indicated a negative test. *Klebsiella pneumonia* is citrate positive (Biswas *et al*., 2002; Wuyep *et al*., 2017)

Urease test

The isolate was inoculated into a urea agar slant in a bijou bottle and incubated at 37ºC for 48 h. Development of a red colour indicated a positive reaction. No red colour indicated a negative urease test. *Klebsiella pneumonia* is urease negative (Kuete *et al*., 2009; El-mahmood *et al.,* 2010).

Methyl red test

The isolate was inoculated into 5 ml of freshly prepared MR-VP broth and incubated for 48 h at 35ºC. 1 ml of the broth was transferred into a small test tube and 3 drops of methyl reagent was added. Development of red colour indicated an organism while the positive MR test while yellow colour indicated negatives a negative test. *Klebsiella* is MR negative (Bandyopadhayay *et al*., 2004; El-mahmood *et al.,* 2010).

Voges Proskauer test

To the rest of the broth in the original tube above, 15 drops of 5% α-napthol in ethanol followed by 5 drops of 40% KOH were added, mixed properly by shaking, capped loosely and placed in a slopping position. Development of a red colour change indicated a negative VP test. *Klebsiella* is VP negative (Becker *et al*., 2003; Heleno *et al*., 2011, 2012).

Motility test

The isolate was incubated into a motility media by making a fine stab with a sterile needle to a depth of 1 – 2 cm short of the bottom of the tube. It was incubated at 37ºC for 24 h. Line of inoculation was defined and the medium was clear for non-motile organism while the line of inoculation was not defined and the rest of the medium was somewhat cloudy for the motile organisms (Kuete *et al*., 2011; Wuyep *et al*., 2017).

Indole test

Klebsiella isolate was inoculated into 5 ml of peptone water in a bijou bottle using a sterile wire loop. It was then incubated at 37^0C for 24 h. Thereafter, 3 drops of kovac's reagent was then added. Development of a red colour in the reagent layer above the broth indicated a positive reaction (Kuete *et al*., 2011).

Triple sugar iron (TSI) test

Each isolate was cultured using a sterile wire loop in a TSI medium. The butt was stabbed twice and the surface of the slant was streaked. The tubes were capped loosely and incubated a 37ºC for 24 h. The reactions were read immediately after 24 h. Gas formation was determined by the appearance of several bubbles in the butt, and cracks in the butt and pushing of the butt from the bottom of the tube. Hydrogen sulphite formation was determined by the blackening of the whole butt or streak or ring of blackening at the slant-butt junction. Reactions could be read as *Klebsiella* gives an A/AG reaction on TSI.

Determination of minimum inhibitory concentration (MIC)

A plot of the square of radius diameter of the zones of inhibition against log concentration of the dilutions was done and a suitable curve drawn from the plots of each extracts. Extrapolation of the curves was done to determine the log of MIC. From this log, the MIC was calculated as the antilog (Tona *et al*., 1998; Feng *et al*., 2002). The MIC is defined as the lowest concentration that will prevent the growth of the test organisms.

Ethanolic, acetonic and aqueous extraction (by Maceration)

Ethanolic extraction: Extraction of leaves was carried out using modified procedures described by Okigbo and Omodamiro (2007). About 371 g of *Azadirachta indica* were each soaked in ethanol and aceton. The plant powders to ethanol were maintained at the ratio of 1:5 (w/v). The suspensions were kept for 3 d in tightly sealed vessels at room temperature, stirred several times daily with a sterile glass rod. The suspension were first filtered through sterile muslin cloth, decanted and then filtered using sterile Whatman No. 1 filter paper inserted in a funnel. The filtrates were concentrated under vacuum to dryness under reduced pressure using rotary evaporator at 40^0C to obtain the crude extracts.

Aqueous extraction: The same procedure described above was used for the aqueous extraction. About 228 g of *A. indica* plant powder were soaked in distilled water (cold macerated). The ratio of plant to water was maintained at 1:10 (w/v). The filtrates were concentrated by evaporation on water bath at 45^0C to dryness, not exceeding the boiling point of the solvent (water) (Ezeokeke *et al*., 2015). The extracts obtained were stored in a refrigerator at 4°C until required for use.

The dry weight of the plant extracts was obtained by the solvent evaporation and weighted. Some portions were used

for phytochemical screening, other parameters and for the susceptibility test.

Data collection and statistical analysis

Data obtained were subjected to Analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) using statistical package for social science SPSS to know the significance in the zone of inhibition, effectiveness of each extract and the susceptibility of the test organism. Least significant difference (LSD) of p=0.05 was used to compare means. This was applicable to acetonic and ethanolic extracts.

Results and Discussion

The results obtained from the phytochemical screening of the leaf extract, Biochemical of the clinical isolates, antibacterial screening of the neem leaf extract and also the determination of the minimum inhibitory concentration and minimum bactericidal concentration of the neem leaf extract are in Tables 1, 2, 3, and 4, respectively. The cultural characterization of the obtained isolates on Mannitol Salt Agar and on MacConkey Agar is typical of *Staphylococcus aureus* and *Klebsiella pneumonia.*

The biochemical characterization profile is also typical of *Klebsiella. Klebsiella* is indole negative, methyl red negative, and Vogesproskauer (VP) negative. *Klebsiella pneumonia* was able to utilize citrate making it citrate positive, urease negative, is a motile organism and gave an acid/alkaline with gas production (A/AG) reaction on Triple Sugar Iron (TSI) indicating that it produces acid and gas in TSI medium.

Table 1: Phytochemical analysis of the Neem leaves extract

Components	Inference
Alkaloids	+
Anthraquinones	-
Carbohydrates	+
Cardiac glycosides	+
Deoxy sugars	+
Flavonoids	+
Saponins	+
Steroids	+
Triterpenes	+

+ = present; - = absent

Table 2: Antimicrobial activity of neem leaves extracts on bacterial isolates with zone of inhibition

Concentration (mg/ml)	*Klebsiella pneumoniae* (mm)	*Staphylococcus aureus* (mm)
500	12	16
250	8	14
125	4	9
62.5	NI	6

NI=No Inhibition

The results of the phytochemical screening reveals that the extract (aqueous) had carbohydrates (Molisch test), Triterpenoids, Glycosides (Keller-killani test), cardiac glycosides, Tannin, Saponin (frothing test), and Alkaloids. There was absence of phenol and anthraquinones. These findings agree with the reports of Parida, *et al*. (2002). Many of the existing synthetic drugs cause various site effects. Hence, drug development plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects (Srivastava *et al*., 2000; Neely *et al*., 2000; Nordmann *et al*., 2009). *Azadirachta indica* leaves possessed good anti-bacterial activity, confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care (Saradhajyothi and Subbarao, 2011). The extracts of Neem when used as medicinal plant, the phytoconstituents alkaloids, glycosides, flavonoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens (Hafiza, 2000).

The consumption of Neem leaf as a vegetable should be encouraged in Nigeria as it is done in India. Neem tree is native to South East Asia and also found in most tropical countries including Nigeria. If the practise of processing and consuming neem leaves as a vegetable is carried out in Nigeria, it will reduce a lot of health challenges as neem have been researched to have a broad spectrum activity against microorganisms and also improve the health standard of individuals. (Chattopadhyay *et al.*, 2005).

Table 3: Minimum inhbitory concentration of the Neem leaves extract

Concentrations (mg/ml)	*K. pneumonia*	Concentration (mg/ml)	*S. aureus*
500	-	500	-
250	-	250	-
125	-	125	-
62.5	+	62.5	+
31.3	+	12.5	+

+ = Growth, - = No Growth

Table 4: Minimum bactericidal concentration of the Neem leaves extract

Concentrations (mg/ml)	*K. pneumonia*	*S. aureus*
500	-	-
250	-	-
125	+	+

+ = Growth, - = No Growth

From the antibacterial screening tests of the crude extract of *Azadirachta indica* (neem) leaves carried out on the selected bacterial pathogens (*klebsiella pneumonia* and *Staphylococcus aureus),* the aqueous extract of the leaves was not able to inhibit the bacterial isolate (*Klebsiella*) on Mueller-Hinton Agar. The growth was inhibited by the least concentration of 125 mg/ml of the aqueous extract of the neem leaves. The growth of *Staphylococcus aureus* in nutrient broth was inhibited by the least concentration of 125 mg/ml of the aqueous extract of the neem leaves. This was followed by *Klebsiella pneumonia* at 125 mg/ml. *Staphylococcus aureus* and *Klebsiella pneumoniae* had their MBC at concentration of 250 mg/ml of the aqueous extract of the neem leaves.

Conclusion and Recommendation

From the result obtained in this research, it can be concluded that neem leaves possess antibacterial activities which is capable of inhibiting the growth of some bacterial isolate (*K. pneumonia* and *S. aureus).*Thus, at the end of this study, it was found that the neem leaves extract was able to inhibit the growth of the isolates at MBC of 250 mg/ml which is the best result of the study. Based on this research, it is therefore recommended that further study should be carried out on neem leaves especially the phytochemical constituents, so that the main active component(s) that inhibited the growth of the bacterial isolates used will be extracted, purified and used for drug production by the pharmaceutical industries. Also other solvent, such as petroleum ether, ethanol, ethyl acetate and chloroform should be used in the extraction of neem leaves to determine their level of effectiveness.

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