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Original Research Article

Preliminary phytochemical screening of methanolic extract of *Azadirachta Indica* and antibacterial activities against foot borne and contaminated bacteria

Ama S. O., Ushie O. A.*, Aasegh T. J., Nyikyaa J. T., Aikhoje E. F. and Terseer D. N.

Department of Chemical Science, Federal University, Wukari, Nigeria.

Corresponding Author E-mail: afiushie@yahoo.com

Abstract

Wound and food borne causes an increasing burden to healthcare systems as the availability of drugs capable of stimulating the process of wound repair is still limited in order to treat the patient. In addition, the increasing prevalence of antibiotic resistance bacteria is widely recognized and poses a continuing burden to healthcare. Therefore, the need of novel antibiotics for effective treatment for wound infection is necessary in order to eliminate the threat of the infections. Objective of this study was to investigate the in vitro antibacterial activity of medicinal plant, *Azadirachta indica* or its local name 'neem', on some selected infectious bacterias. Methanolic *Azadirachta Indica* extract was used to test on the bacteria at five different concentrations. Antimicrobial susceptibility testing (AST) were used in this study. Zone of inhibition have been shown to *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E.coli* and were considered significant. This study showed that methanolic *Azadirachta Indica* extract possess antimicrobial activity and further studies need to be done in order to exploit the bioactive compounds of *Azadirachta Indica* in order to reveal its potential as a new source of antibacterial agent.

Keywords: Azadirachta Indica, antimicrobial, foodborne, phytochemical, Medicinal plants.

INTRODUCTION

Herbal remedies are known to treat many infectious diseases throughout the history of mankind. Plant material continues to play a major role in the primary health care as therapeutic remedies in many developing countries. Thus, the discovery of medicinal plants as antimicrobial agents is useful in expanding the wide varieties of antibiotics available (Zaidan *et al.,* 2005). Medicinal plants are part and parcel of human science from the dawn of civilization. In India they form the backbone of several indigenous traditional medicines (Nayak *et al.,* 2011). Medicinal plants are various plants used in herbalism and thought by some to have medicinal properties. Plants have a great potential for producing new drugs for human benefit. Neem is used in traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. Its twigs provide a chewing stick and are widely used in the Indian sub-continent (Almas and Ansallafi, 1995). *Azadirachta indica* extract is used as a herbal remedy and as a source of many therapeutic agents in different countries. *A. Indica* is used for the treatment of diabetes because of its potential anti-diabetes properties (Sharvan *et al.,* 2011).

Azadirachta Indica seeds have also been used as traditional medicine to treat infections, particularly in the eyes. *A. Indica* aqueous extract has also been implicated as a possible chemotherapeutic and viral agent (Amer *et al.,* 2010). Due to the fatal effect of food borne and wound causing bacteria on humans, this research work seeks to use less toxic plant extract of medicinal plant to

check the inhibition effect on bacteria growth. This study is aimed at determining the antimicrobial activity of *A. Indica* against some selected bacteria strains (*Staphylococcus aureus, Pseudomonas aeruginosa,* and *E.coli*).

MATERIALS AND METHODS

Sampling collection and Preparation

Mature leaves of *Azadirachta indica* were used for this study which were collected from their natural habitat within Federal university Wukari, Nigeria. The completely dried leaves were coarsely powdered and 100 g was used for successive extraction in 250 ml methanol for three days with periodic shaking. Then, the extract was filtrated and the filtrate was collected. The filtered liquid extracts were subjected to rotary evaporation and subsequently concentrated under reduced pressure (in vacuum at 40°C). Then, the extracts were evaporated to dryness and stored at 4°C in an airtight bottle and kept in a refrigerator until it is required for analysis.

Preliminary Phytochemical Screening

Phytochemical tests were carried out on all the extracts using standard procedures to identify the constituents. Qualitative analysis of the crude extracts were carried out as described by literature reports (Tiwari *et al.,* 2011, Sagayaraj *et al.,* 2015, Ushie *et al*., 2013 , Ushie *et al*., 2013a, Ushie *et al*., 2013b) to identify the presence of the classes of secondary metabolites (alkaloids, anthraquinones, flavonoids, tannins, saponins, glycosides, cardiac glycosides, terpenes, steroids, phenol, etc).

Detection of Alkaloids

The individual extracts were dissolved in a dilute hydrochloric acid and filtered. The filtrate was further tested with the following reagents for the presence of alkaloids:

Dragendroff's test: filtrates were treated with potassium bismuth iodide solution (Dragendroff's reagent). The formation of an orange red precipitate indicated the presence of alkaloids.

Hager's test: filtrates were treated with saturated aqueous solution of picric acid (Hager's reagent). Presences of alkaloids were confirmed by the formation of yellow coloured precipitate.

Mayer's test: Filtrates were treated with potassium mercuric iodide (Mayer's reagent). Formation of a whitish yellow or cream coloured precipitate indicated the presence of alkaloids.

Detection of Saponins

Froth test: Extract was diluted with distilled water to 20ml and shaken in a graduated test tube for 15minutes. Formation of 1cm layer of foam indicated the presence of saponins.

Foam test: small quantity of extract was shaken with 2ml of water; persistence of foam produced for ten minutes indicated the presence of saponins.

Detection of phytosterols: Small quantity of extract was dissolved in 5ml of chloroform salkowski's test on adding a few drops of conc. Sulphuric acids. Allow the solution to stand formation of brown ring indicated the presence of phytosterols.

Liermannburchards test: The chloroform extracted solution was treated with few drops of acetic anhydride. Boil and cooled then concentrated sulphuric acid was added. Formation of a bluish green colour confirms the presence of phytosterols.

Detection of Phenolic compounds

Ferric chloride test: The extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

Lead acetate test: The extracts were treated with 3ml of 10% lead acetate solution. A bulky white precipitate indicated the presence of phenolic compounds.

Detection of tannins

0.5g of the dried powdered neem leaves was boiled in 20ml of water in a test tube, Filtered then few drops of 0.1% ferric chloride was added to the mixture. Development of a brownish green or blue black colouration indicated the presence of tannins.

Detection of flavonoids

The extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on further addition of dilute

acid, indicated the presence of flavonoids.

Alkaline reagents test: Extract was treated with few drops of lead acetate solution. Formation of yellow precipitate indicated the presence of flavonoids.

Lead acetate test: Few drops of ferric chloride solution were added to the extract solution. Development of intense green colour indicated the presence of flavonoids.

Procedure for preparation of nutrient agar

28g of nutrient agar was dissolved in 1dm^3 distilled water and heated on a hot plate for the agar to dissolve, and then transferred to autoclave for sterilization at 121^{0}cfor 15minutes. It was allowed to cool and 20ml was transferred to plates until solidification took place. The the entire surface of nutrient agar was streaked on the plate with the bacteria. Perforated paper were dissolved in the extract at different concentrations to determine the inhibition of each bacteria and the results obtained were recorded by taking the mean, and it standard deviation to discuss the result of inhibition by these bacteria's.

Microorganism and Inoculums Preparation

Gram-positive strains of *Staphylococcus aureus* ATCC 29737, and Gram-negative strains of pseudomonas ATCC 14028 and *Escherichia coli* ATCC 10536, were obtained from the microbiology Laboratory, Benue State University Teaching Hospital Makurdi. Each strain was inoculated in nutrient agar medium and incubated at 37° C for 24 hours and the resulting bacteria were used as inoculums. A sterile wire loop was used to pick a singly formed colony and uniformly streaked on the agar plates.

Antibacterial activity of *Azadirachta indica* Extract

To determine the antimicrobial activity of *Azadirachta indica* neem methanolic extract, the agar well-diffusion method was used. Nutrient agar plates were swabbed using a cotton swab from an 8 hour-old broth culture of Gram-positive or Gram negative bacteria. By using a cork borer, wells (8-mm diameter and approximately 2-cm apart) were made in each of these plates. A stock solution of each *Azadirachta indica* methanolic extract was prepared at a concentration of 0.1g/ml in methanol. A control well comprising methanol without plant extract was also made. The plates were incubated at 37°C for 18-24 h for bacterial pathogens. The zone of inhibition (marked as either positive (+) or negative (-) was used as indicator for the effect of the extract against bacterial species. According to Yehia *et al.*, overnight cultures of the Gram-positive strains *Staphylococcus aureus,* and the Gram-negative strain *Pseudomonas* and *E. coli* were suspended in Ringer's solution to a turbidity equivalent of 0.5 McFarland (1.5 × 10 CFU/ml 8) and 100 µl was spread onto Mueller-Hinton agar plates.

RESULTS AND DISCUSSION

Result

Bar chart indicate that the hot extract works more effective in inhibition of the bacteria E.coli. and as the concentration increases the inhibition of the bacteria E.coli also increases from 0.1g/ml to 0.5g/ml. (Table 1 and 2)(Figure 1)

Here the average inhibition of the bacteria has shown characteristics which are different from other selected bacteria's in the fact that at first concentration of 0.1g/ml works more effective in the hot extract and as the concentration increases the inhibition increases for both, at 0.3g/ml it was observed that both of cold and hot extract become same in the inhibition of the bacteria growth. (Figure 2)

Bar chart shows that the hot extract inhibit bacteria growth more effective than the cold extract and also as the concentration is increased the inhibition also increased. (Figure 3 and 4)

Discussion

The result of the qualitative analysis of *Azadirachta indica* is presented in Table 1. Saponins were present in extract. Tannins, alkaloids, flavonoids, phenols and sponnins were found to be present in the leaf extract *Azadirachta indica*. The presence of tannin in the medicinal plant suggests the ability of these plants to play key roles as antifungal antidiarrheal, antioxidant and antihemorrhoidal agent (Asquith and Butter, 1986). Alkaloids have been found to have microbiocidal effect and the major anti-diarrheal effect is probably due to their effects on small intestine and antihypertensive antifungal, anti-inflammatory, anti-fibrogenic effect (Ghosal *et al.,* 1996). Alkaloids in plants are used in medicine as anaesthetic agents (Herourat *et al*., 1998). The result revealed the presence of flavonoid in the extracts and hence, mistletoe plant can be use to modifies the body's reaction toallergens, virus and caranogens. It has been reported to show anti-inflammatory, antifungi, antibacterial and antimicrobial activities based on the literature (Cushnie and Lamb, 2005).

The presence of saponins in the seeds can be useful in treating inflammation. Some of the characteristics of saponins include formation of foams in aqueous

Table 1. Result for phytochemical screening of *Azadirachta Indica* leaf extract.

Phytochemical test	Hot extract Test	Cold extract Test
Alkaloids	+	+
Saponins	+	+
Flavonoids	+	+
Tannins	+	+
Phenolic compounds	+	+

Table 2. Antimicrobial Susceptibility Testing Zone Inhibition of Bacteria.

Concentration (g/ml)	E.coli		Pseudomonas		Staph	
	Hot	Cold	Hot	Cold	Hot	Cold
0.1	2.3	1.8	2.0	1.5	1.5	1.0
0.2	2.5	2.1	2.0	1.8	2.2	1.5
0.3	2.6	2.3	2.0	2.0	2.4	1.6
0.4	2.8	2.6	2.0	2.0	2.6	1.8
0.5	3	2.8	2.0	2.0	2.8	2.8
Average	2.64	2.3	2.32	1.58	1.6	2
SD	0.52	0.9	0.76	0.66	0.42	1.0

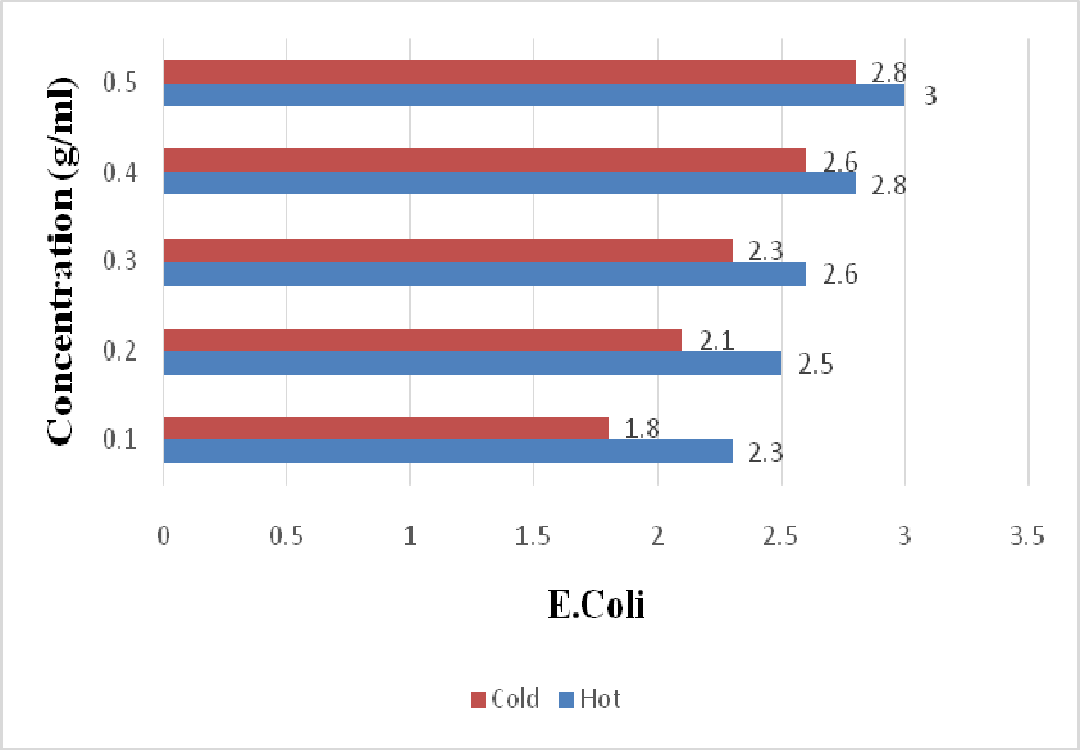

Figure 1. Bar chart of varied concentration (g/ml) of neem extract on *E.coli* inhibition for both cold and hot extracts.

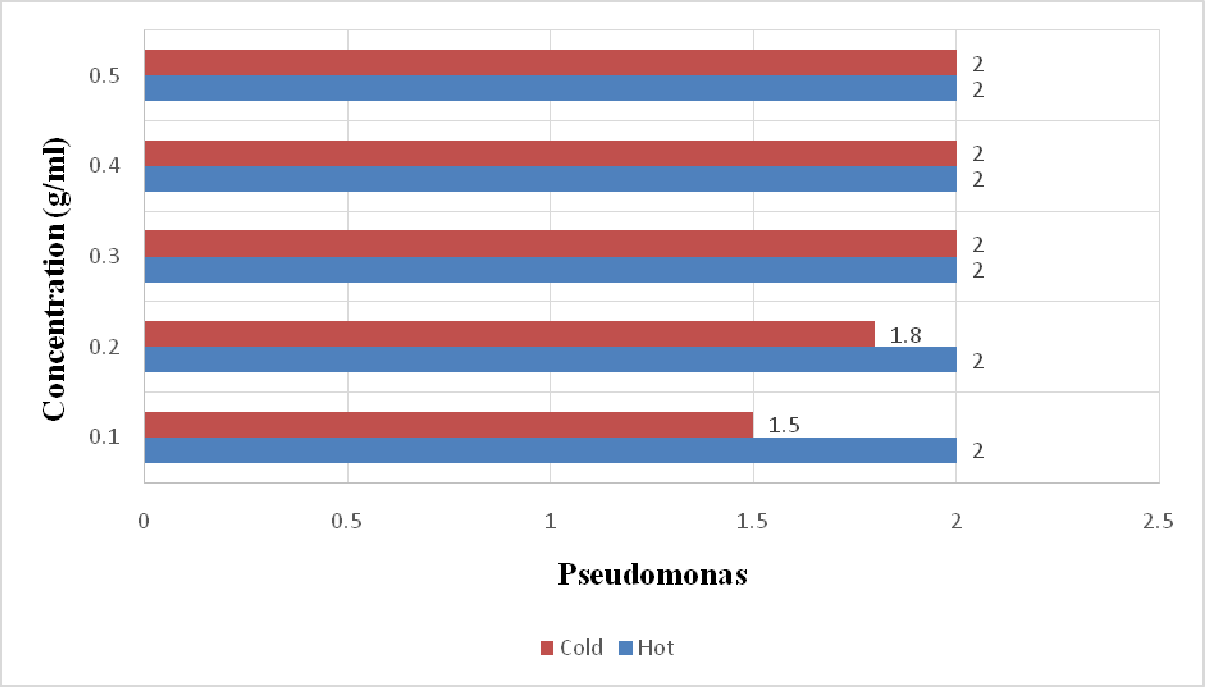

Figure 2. Bar chart for varied concentration (g/ml) of neem extracts on *pseudomonas* inhibition.

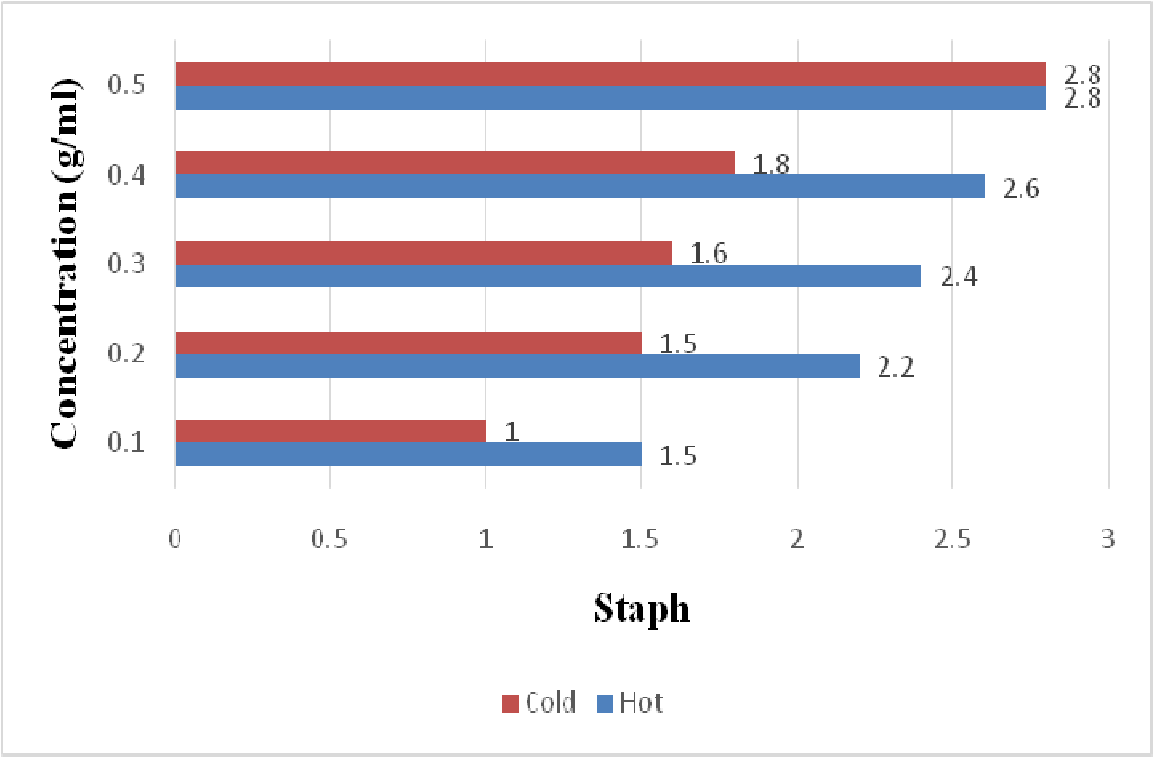

Figure 3. Bar chart for varied concentration (g/ml) of neem extracts on *Staph* inhibition.

Figure 4. Zone of inhibition of neem extract on *Staphylococcus aureus, E.coli, and Pseudomonas aeruginosa.*

solutions, haemolytic activity, cholesterol binding properties and bitterness (Rita *et al*., 2015). Also in nature, saponins appear to act as antibiotics that protect plants from microbes (Opara *et al*., 2019). Phenols are present in the extracts of mistletoe plant thus can normally be involved in defense against ultraviolet radiation or aggression by pathogens, parasites and predators, as well as causative to plants colours. They are ubiquitous in all plant organs and are therefore an integral part of the human diet (Dai and Mumper, 2010). Also, phenolic compounds, can inhibit the absorption of amylase in the treatment of carbohydrate absorption, such as diabetes (Sales *et al*., 2012)

Methanolic neem extract in this study had shown to possess antibacterial activities against *Staphylococcus aureus, E.coli* and *Pseudomonas aeruginosa*. *Staphylococcus aureus, E.coli* and *Pseudomonas aeruginosa* were inhibited by the concentration ranging from 0.1g/ml to 0.5g/ml. However, very poor growth of *these bacteria were found on nutrient* agar using the cold extract, which showed that the growth criteria did not met, thus the antimicrobial effect of neem extract against the above named bacterial as compared to the hot extract could not be concluded. *Staphylococcus aureus* was susceptible to the methanolic neem extract starting at concentration 0.1g/ml through 05g/ml. The result in Table 2 showed that the higher concentration of neem extract, the larger the diameter of inhibition zones, thus, this exhibit the concentration dependant activity of the bacterium. At concentration 0.4g/ml and 0.5g/ml, diameter of inhibition zone as seen in Figure 2 were considered significant if compared with the values at the initial concentration of extract, this is clear that the higher concentration the more significance antimicrobial effect over positive control against *Staphylococcus aureus*, E.coli and pseudomonas.

In this study, neem extracts had shown to have antibacterial effect on Gram positive bacteria but not on Gram negative bacteria. This was supported by the previous study performed that had found that plant extracts inhibited the Gram-positive microorganisms more than the Gram-negative ones. *Pseudomonas aeruginosa* showed antibiotic resistant because it may be chromosomally or plasmid mediated and may also be affected by changes in the cellular membrane or intracellular environment. This could possibly be explained by the fact that the antibacterial activity of neem had been found to be mainly due to the inhibition of cell membrane synthesis within the bacteria where the “L” forms of *Pseudomonas aeruginosa* was develop thus

becoming resistant strains. However, the other study found that methanolic extract of neem leaves at 100mg/ml exhibit 10 to 23 mm diameter of inhibition. This variation may be due to the different method where agar well diffusion method was used instead of the susceptibility testing. Hence the Table 2 shows mean and standard deviation of values.

CONCLUSION

In this study, the antibacterial activity of neem extracts towards significant microbes that causes wound infections and/or food borne has been investigated. The methanolic extract of neem leaf is remarkable because it inhibited the growth of the tested bacteria, they were *Staphylococcus aureus, E.coli and pseudomonas* In this study, *E.coli* exhibit an antimicrobial effect with significant zone of inhibition looking at its mean values, but the hot extract was observed to work more effectively as compared to cold or crude extract at all concentrations of 0.1g/ml through 0.5g/ml.

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