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Full Length Research Paper

The antibacterial activity of *Azadarichta indica* (neem) seeds extracts against bacterial pathogens associated with eye and ear infections

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The effects of crude extracts of seeds of *Azadarichta indica* against pathogenic *Staphylococcus aureus, Staphylococcus pyogenes, Escherichia coli* and *Pseudomonas aeruginosa* obtained as clinical isolates from patients diagnosed with eye and ear infections were investigated using the agar well diffusion method . The growth of all the isolates were inhibited, though to varying degrees, with gram-positive more susceptible than gram-negative bacteria. The control laboratory strains were more sensitive to the toxic effects of the crude extracts than the corresponding test bacteria. Hexane extracts were more effective, producing larger zones of growth inhibition sizes and smaller MIC and MBC values, than the aqueous extracts. The MIC values ranged from 1.59 - 25 mg/ml while the MBC values ranged from 3.17 - 50 mg/ml. The extracts were more effective under elevated temperature and acidic conditions. The ability of the crude extracts to inhibit the growth of such pathogenic bacteria that frequently cause eye and ear infections as those used in this study is an indication that the neem seed has the potential and can be used as a source for new broad spectrum oral antibiotics. The result obtained in this study validates the use of the neem seeds in traditional medicine to treat infectious conditions especially those involving the eye and ear.

Key words: *Azadarichta indica*, neem seeds, pathogenic, disc diffusion, validates, infectious condition.

INTRODUCTION

Eye and ear infections are amongst the most common diseases encountered in medical practice today, affecting people of all ages from neonate to geriatric groups. Occurring both in the community and hospital settings, Eye and ear infections are among the common reasons that compel an individual to seek medical attention. The normal flora of the conjunctiva of the eye includes the *Staphylococci, Streptococci and Haemophilus* species, while that of the middle ear are mainly coagulase-negative *Streptococci, Escherichia coli, Staphylococci, Haemophilus* species and *Moraxella* species (Natarajan et al., 2003). Colonization with these organisms usually begins immediately after birth, and is usually life-long. Complications due to eye and ear infections begin with the colonization of the affected sites by some pathogenic strains of bacteria. As with other infections, these infections of the eye and ear are usually treated with antibiotics, but in the last couple of years, there has been a lot of reports on treatment failures due to emergence and spread of bacterial resistance (Mordi and Erah, 2006). Bacterial resistance to antibiotics represents a serious problem for clinicians and the pharmaceutical industry and great efforts are being made to reverse this trend, and one of them is the widespread screening of medicinal plants from the traditional system of medicine

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hoping to get some newer, safer, and more effective agents that can be used to fight infectious diseases (Natarajan et al., 2003). The traditional medical practitioners use a variety of herbal preparations to treat different kinds of diseases including microbial infections (Mann et al., 2008). The scientific literature is full of reports of studies on roots, stem bark, seeds, flowers and fruits of higher plants having bioactive substances such as peptides, alkaloids, tannins, phenols, sterols, flavonoids, glycosides amongst others which confer healing properties for their use in medicine (Levin et al., 1979: Benli et al., 2008; El-Mahmood et al., 2008).

Azadirachta indica is one of such medicinal plants belonging to the *Meliaceae* family and is indigenous to southern Asia (Akula et al., 2003). It is an extensively popular tree in Nigeria and are commonly referred to as Neem (English), "*Dogon Yaro*" (Hausa), "*Gaadina*" (Fulfulde) and "*Akun shorop*" (Igbo). *A. indica* is a multi-purpose timber tree from which high value products are extracted for use as an insecticide, fertilizers and multi-purpose medicines. In India, it is popularly known as the village dispensary (Akula et al., 2003). The therapeutic efficacy of the *A. indica* have been described by practitioners of traditional medicine. Some of the ethnomedicinal uses include treatment of skin disorders, rashes and boils, stomach ulcers, rheumatism, respiratory tract infections, sore gums and throat, eye and ear infections, leprosy and diabetes (Isman et al., 1990; Kaura et al., 1998; Akula et al., 2003). Also, the medicinal uses has been reported by several workers and these includes having antipyretic (Okpanyi and Ezenkwa, 1981), antimalarial (Tella, 1977), antitumour (Fujiwara et al., 1982), antiulcer (Pillai and Santhakumari, 1984), antidiabetic (Shukla et al., 1984) and cardio-vascular properties (Thompson and Anderson, 1978). In a previous survey of plants used for the treatment of ear and eye infections, amongst the practitioners of traditional medicine and other knowledgeable rural dwellers in the northern parts of Nigeria, the neem seed was listed as one of the most popular source of medi-caments.

The seeds are crashed and the juices coming out are pressed several times on the infected ear and or the eye to obtain an effective dosage. Since a large pro-portion of rural people cannot afford the cost of modern health care, they simply stay at home and nurse themselves back to health using local herbs and plants as remedies.

There is still little evidence on the anti-microbial properties of the neem seeds against majority of the microbes particularly clinical isolates from patients suffering from eye and ear infections. It would be interesting to assess the efficacy of the crude extracts of the neem seeds against some pathogenic bacteria associated with ear and eye infections in order to establish scientific rationale for its uses as antimicrobial agents. The effects of pH and temperature on the efficacy of the extracts were also investigated to simulate the conditions in the stomach and the intestines since the decoctions are usually taken orally.

MATERIALS AND METHODS

Collection of plant materials

Fresh fruits of *A. indica* were collected from the Yola campus, Federal University of Technology, Yola, Nigeria, in the month of May, 2008. The fruits were identified at the Department of Biological Sciences by Prof. E. B. Alo.

Preparation of the plant material

The fruits were manually separated into their seeds and seed hulls (kernels). The seeds were dried in a shade under room temperature for sixty days and then crushed into coarse powdery substance by using mortar and pestle. The coarse powdery substance was dried again and was then micronized to fine powder using an electric blender (Kenwood Limited, Harvant, United Kingdom) to form some fine powder which was then stored in an air tight bottle in the laboratory until required (Dahot , 1998 ; De and James , 2002).

Extraction procedures

One hundred grams of the powdered sample of *A. indica* seed kernel was soxhlet extracted for 24 h using 500 ml each of distilled water and hexane as solvents following the methods described by Jaber and Al-Mossawi, (2007). The extracts were evaporated to dryness using a rotary evaporator (Model 349/2, Corning Limited) maintained at 40^{o}C. The dried extracts were weighed and kept in a well labeled sterile specimen bottles. Different concentrations of the extracts at 100, 50, 25, 12.5, 6.25, 3.17, 1.58 and 0.79 mg/ml were freshly prepared by redissolving the dried powder in the same solvent which was used for the extraction.

Preparation of antibiotic dilution

The antibiotic chloramphenicol was purchased at Mahmuda Pharmaceutical store in Yola, Adamawa 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 30 µg/ml. The prepared dilution of the antibiotic was used for subsequent antimicrobial test and served as a positive control.

Collection of specimens

Patients consent was first obtained before collection of specimens which were done with assistance of the hospital staff. Using a sterile cotton wool swab moistened in peptone water, the inner surfaces of the infected eye and ear were gently swabbed and then the swabs promptly taken to the hospital laboratory in a container of Amies medium (Oxoid) for further analysis. All the media used were of Oxoid grade, unless otherwise stated.

Isolation and Identification of bacteria

In the laboratory, the swabs were gently rolled on to surfaces of previously prepared Mueller-Hinton, MacConkey and Chocolate agar plates. After incubation for 24 h, some distinct colonies resembling the selected bacteria were picked and further subcultured three times on agar plates so as to obtain pure

colonies. After isolation, the bacteria were identified using standard microbiological procedures as described by Cowan and Steel (1974) and Cheesborough (2006). All the identified bacteria were immediately transferred to the Federal University of Technology, Yola, Microbiology laboratory where they were stored in agar slants in a refrigerator maintained at 4^{o}C until required. Purity of the isolates was checked at regular intervals by plating and staining (El-Mahmood and Amey, 2007). The test organisms were labeled as A1 *E. coli*), B1 (*Pseudomonas aeruginosa*), C1 (*Streptococcus pyogenes*),*and D1* (*Staphylococcus aureus*) respectively . A parallel experiment involving some sensitive laboratory strains labeled A2 (*E. coli*), B2 (*P. aeruginosa*), C2 (*S. pyogenes*) and D2 (*S. aureus*) were used as control organisms.

Preparation of inoculum

The standardization of culture was done according to the method of Baker and Thomsberg (1983) and Clinical and Laboratory Standard Institute (2006). Briefly 2 mm diameter colonies of the 18 h culture of an organism were picked with a sterile wire loop and immersed into a sterile bottle containing Mueller Hinton broth (Hi Media) and was incubated for 5 h .Normal saline was added gradually to it so as to compare the turbidity to that of 0.5 McFarland standard corresponding to approximately 1.0 × 10^{8} cfu / ml. This was done for each of the test and control 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 an 18 h culture of bacteria adjusted to 0.5 MacFarland standard was inoculated into 90 mm sterile Petri plate, then 19 ml sterile Mueller Hinton agar at 45^{o}C added, and the plate rocked gently for 1 min. for even mixing of the contents. The plate was kept on a flat bench for 30 min. to dry. Five wells (4 mm) deep were punched on the agar plate by using a sterile 6 mm diameter cork borer. Then 0.5 ml of the reconstituted extract at a concentration of 50 mg/ml was pipetted into the first hole, second and third holes, 0.5 ml of 30 µg/ml of chloramphenicol solution was pipetted into the fouth hole to serve as a positive control and 0.5 ml of pure solvent into the fifth hole to serve as a negative control. The plate was allowed to stand on flat bench for 30 min to allow diffusion into the agar before incubation at 37^{o}C for 24 h. The experiment was done in triplicate 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 and control bacteria.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the crude extract was determined using the doubling dilution method of Sahm and Washington (1990). Briefly, 1 ml of the reconstituted crude extract at a concentration of 100 mg/ml was added to 1 ml of sterile Mueller Hinton broth. 1 ml of this extract concentration was transferred to another test tube and this dilution continued until an 8th test tube was reached, giving extract concentrations of 100, 50, 25, 12.5, 6.25, 3.17, 1.58 and 0.79 mg/ml in different test tubes. Then 1 ml of an 18 h culture of bacteria previously adjusted to 0.5 McFarland standard (1.0 X 10^{8} cfu/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 contained 1 ml of pure solvent but no extract and served as a negative control. The 10th test tube contained a solution of 30 µg/ml of chloramphenicol solution and served as positive control. The above procedure was done for each of the test and control bacteria. The test tube with 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 24 h at the temperature of 37^{o}C. After incubation, the MBC was determined at the dilution at which there was no visible growth on the plate (De and Ifeoma, 2002).

Effect of pH on activity

This was carried out as described by EL-Mahmood et al. (2008). Briefly 1 ml of the extract at a concentration of 50 mg/ml was pipetted into separate test tubes each containing 1 ml of Mueller Hinton broth. To the first test tube, few drops of 1N HCL acid was added drop wise until pH 2 was attained and to the second test tube, few drops of 1 M NaOH was added drop wise until pH 10 was achieved. 1 ml of an 18 h culture of bacteria was added to each of the test tubes and the contents were thoroughly mixed and then allowed to stand for 1 h after which they were neutralized to pH 7 again using either acid or base as the case may be. Another test tube containing only extract (untreated) was left for the same period and it served as a control. Other experimental procedures were as described previously. These experiments were done for each of the test and control bacteria and mean of three results taken.

Effect of temperature on the activity of the crude extract

The method as described by EL-Mahmood et al. (2008) was used. Briefly, 1 ml of the extract at a concentration of 50 mg/ml was pipetted into two different test tubes containing 1ml Mueller Hinton broth. One of the test tubes was treated at the temperature of 10^{o}C, and the other test tube was treated at the temperature of 100^{o}C respectively. Another test tube containing the extract was left at room temperature 32 - 35^{o}C (untreated) for 1 h and it served as a control. After the heat treatment, antibacterial susceptibility test of both the treated and untreated cultures were carried out as previously described.

RESULTS

The result of the extraction processes with hexane yielded some 28.4% w/w and water yielded 16.1% w/w. Percentage extract yield (w/w) was estimated as dry extract weight/dry material weight multiplied by 100. The results of the antibacterial screening of the seed extracts are presented in Figure 1. Antibacterial activity was recorded if the zone diameters were equal to or larger than 6 mm. Hexane extracts produced larger zone diameters, between 13 - 22 mm while the water extracts had a zone diameter range of 8-17 mm. The effects of pH on the efficacy of the crude aqueous extracts are shown in Figure 2. The activity of the crude extracts of *A. indica* was optimal under acidic pH (pH 2). The effectiveness of

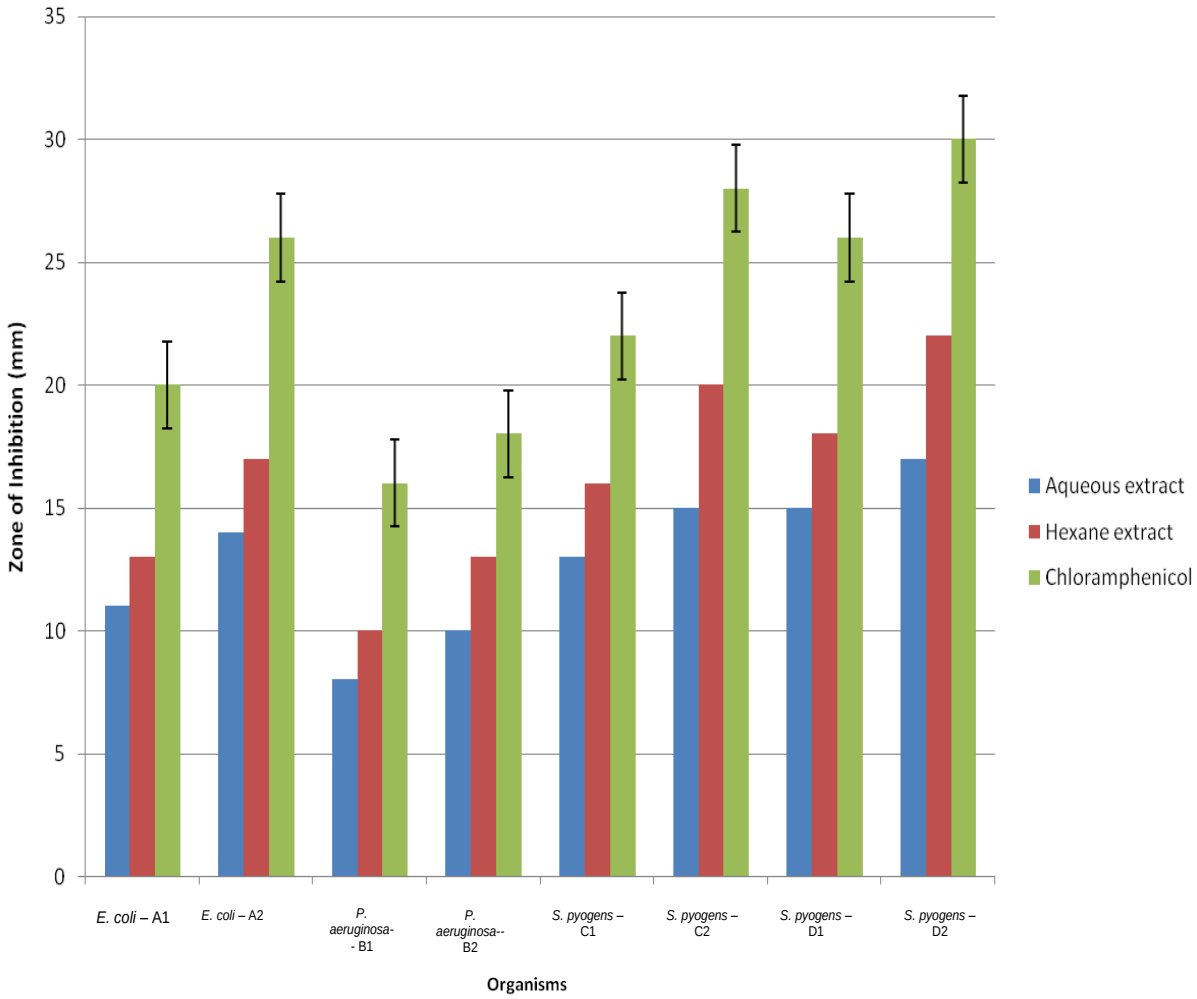

Figure 1. Antibacterial activity of the crude seed extracts of *Azardirachta indica.*

the crude extracts was less at alkaline (pH 10). For example when water was used as a solvent for *S. aureus* (D1) at pH 2, the zone diameter was 18 mm and at pH 10, the zone diameter was 15 mm. Increase in temperature enhanced the antibacterial activity of the extracts as shown in Figure 3. The effect of temperature on the efficacy of the aqueous extract on *S. aureus* (D1) as measured by the diameters of zone of inhibition of growth was 16 mm at 10°C and 18 mm at 100°C. In general, this trend of higher efficacies at higher temperatures and lower efficacies at lower temperatures is similar for all the other bacteria, regardless whether water or hexane was used as a solvent. The MIC and MBC values of the crude seed extracts are presented in Table 1. The MIC of the test organisms ranged between 6.25-25 mg/ml for aqueous extract and 3.17-12.5 mg/ml for hexane extract, while MBC values ranged from 12.5-50 mg/ml for aqueous extract and 6.25-25 mg/ml for the hexane extract. The MIC and MBC values for the control organisms followed similar trend to that of the test bacteria, though with lower values.

DISCUSSION

Plants contain many biologically active compounds which have potential for development as medicinal agents. Herbal medicines already form the basis of therapeutic use in the developing countries, but of recent, there has been an increase in the use of herbal medicines in the developed world too (De and Ifeoma, 2002. 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; Bhomick and Choudhary, 1982; 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

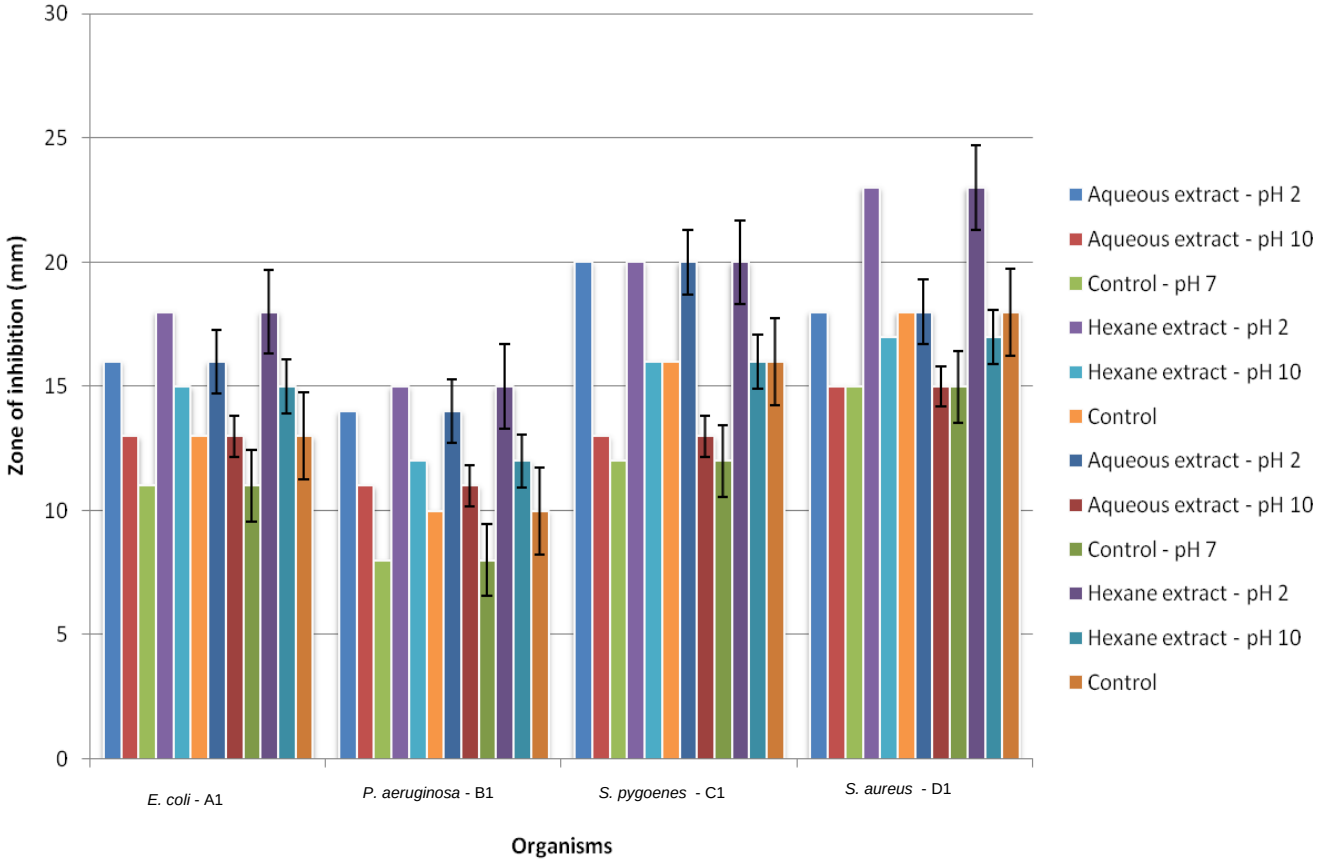

Figure 2. Effect of pH on the antimicrobial activity of *Azadirachta indica* seed extracts.

Table 1. The MIC and MBC values of the crude seed extracts of *Azadirachta indica.*

Organisms	Extract concentration (mg/ml)			
	Aqueous extracts		Hexane extracts	
	MIC	MBC	MIC	MBC
E. coli A1	12.5	25	6.25	12.5
E. coli A2	12.5	12.5	6.25	6.25
P. aeruginosa B1	25	50	12.5	25
P. aeruginosa B2	25	25	12.5	25
S. pyogenes C1	6.25	12.5	6.25	6.25
S. pyogenes C2	3.15	6.25	3.17	3.17
S. aureus D1	6.25	12.5	3.17	6.25
S. aureus D2	3.17	6.25	1.59	3.17

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 (Ogbonnia et al., 2008).

In this study, a variety of pathogenic bacteria implicated as causative agents of eye and ear infections were selected for the screening for antibacterial activity of the crude neem seed extracts to perceive the efficacy, antibacterial spectrum as well as authenticate some of the ethnomedicinal claims. The susceptibility of the test bacteria [(A1 (*Escherichia coli*), B1 (*P. aeruginosa*), C1 (*S. pyogenes*), and *D1* (*S. aureus*)] were compared with laboratory strains [A2 (*E. coli*), B2 (*P. aeruginosa*), C2 (*S.*

pyogenes) and D2 (*S. aureus*)] of known sensitivity. Extracts from both hexane and aqueous solvents inhibited the growth of both the test and control bacteria, though to varying degrees. In a similar study involving some dermatophites, Natarajan et al. (2003) did not record any activity for their aqueous extracts, contrary to the data presented in this study. De and Ifeoma (2002) also did not record any antibacterial activity with the aqueous extracts of both the bark and the leaves of the neem bark extracts against their test bacteria. 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. Some antibacterial effects of the neem seeds have also been reported against *S. mutans, S. faecalis, M. tuberculosis, V. cholera, S. pyogenes and K. pneumoniae* (Biswas et al., 2002). In another study, neem seed extracts have also been observed to inhibit the growth and development of asexual and sexual stages of drug-sensitive and resistant human malarial parasite (*P. falciparum*), some fungi and some viruses (Natarajan et al., (2003).This broad spectrum activity of crude neem extracts have been linked to the presence of bioactive compounds notably azadiractin, gedunin, nimbidin, mahmoodin and nimbolide (Biswas et al., 2002) which make the neem plant useful for the treatment of various infectious conditions including those of the eye and ear. Amongst the test bacteria, *S. aureus* (D1) was the most susceptible, closely followed by *S. pyogenes* (C1), while *E. coli* (A1) and *P. aeruginosa* (A1) were less susceptible as shown by their relatively smaller sizes in Fig. 1. Several authors have also reported that plant extracts are more effective against gram-positive than gram-negative bacteria and attributed this to the differences in their cell wall structures (Rabe and Van Staden, 1997, Parekh and Chanda, 2006). The control bacteria were more susceptible to the toxic effects of the crude extracts than the test bacteria though the sensitivity also varied according to strains.

The effects of pH on the efficacy of the crude extracts are shown in Figure 2. The activity of the crude seed extracts of *A. indica* was optimal under acidic pH (pH 2). The effectiveness of the crude extracts was observed to decrease as the pH was raised to alkalinity (pH 10). This pattern is similar for all the other bacteria and also when hexane was used as a solvent. Activity at acidic pH is indication of acid stability while diminished activity at alkaline pH indicates less efficacy under alkaline conditions. Practitioners of traditional medicine usually add some additives like *"Kanwa"* (Potash) which is a basic salt, when treating their crude extracts. Acid and alkaline treatment was carried out in order to simulate the situations in the stomach and the gastrointestinal tract, because the crude seed extracts are also taken orally, in addition to their being crashed and the juicy contents squeezed at the infectious sites.

The effect of temperature on the efficacy of the crude seed extracts on the test bacteria as shown in Figure 3 indicate that activity were more under elevated temperatures and this trend is similar for all the bacteria, regardless whether water or hexane was used as a solvent. This supports practices of traditional healers who most of th times boils the plant parts before issuing out to patients, which is a normal practice in herbal medicine. Treatment of plant extracts to high temperature could inactivate volatile compounds, but could also increase the release of active compounds and free radicals (Majumdar et al., 1998).The traditional medicine practitioners were reported to sometimes crash the seeds before squeezing the fresh juices in to the affected eye and or ear and after several applications, may achieve the antibacterial dosage at the infectious sites. This repeated application at an infectious site is due to the lower activity of the extracts at lower temperatures as depicted in Figure 1.

The standard antibiotic chloramphenicol, demonstrated highest activity than the crude extracts as shown in Figure 1. This is because the antibiotic is in pure state and has undergone some refining processes that have established it as standard antibiotic (Prescott et al., 2005).The observed difference in efficacy may also be due to the fact the extracts were in a crude form and would contain some inert substances which do not have any antibacterial activity. The organisms used for the purposes of controls were consistently more susceptible than the test organisms.

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 as shown in Table 1. The growths of the organisms were inhibited at concentrations that ranged between 3.17-25 mg/ml for aqueous extract and 1.59-12.5 mg/ml for hexane extract. The study showed that *E. coli* (A1) *and P.* aeruginosa (B1) had higher MIC values, meaning that higher concentration of the extracts are required to inhibit the growth of these bacteria, while *S. pyogenes* (C1) and *S. aureus* (D1) had lower MIC values and would require low extract concentration to inhibit their growth and these coraborated with data presented in Table 1. Plant based antimicrobial substances generally have higher MIC and MBC values when compared to antimicrobial substances obtained from microorganisms or those that are synthetically produced because of the presence of impurities. The MBC of the plant seed extracts ranged between 6.25-50 mg/ml for the aqueous extract and 3.17-25 mg/ml for hexane extracts. In this study, *P. aeruginosa* (B1) and *E. coli* (A1) had higher MBC values, thus suggesting lower susceptibility to the crude extracts and lower MBC values for *S. aureus* (D1) and *S. pyogenes* (C1) thus suggesting higher activity of the extracts

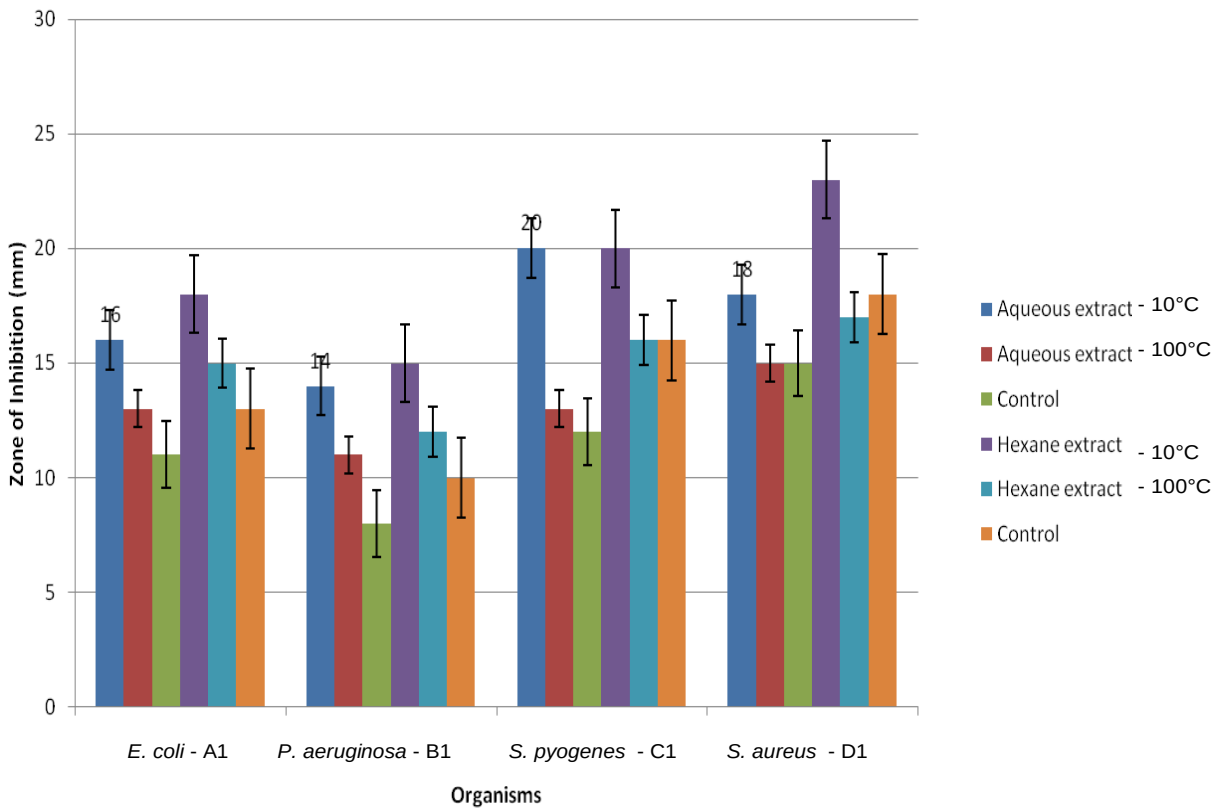

Figure 3. Effect of temperature on antimicrobial activity of the extracts of *Azardirachta indica* seed extracts.

against the organisms. 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 250 µg/ml by Natarajan et al. (2003) which are far lower than the MIC and MBC values obtained in this study. However, 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.* This MIC values are particularly important where people depend on the usage of this medicinal plant. In all the experiments conducted, sterile distilled water and a solution of hexane that were used as negative controls did not show any significant activity.

The observation that the extracts were effective against both gram-positive and gram-negative bacteria suggests that the active principles have a broad spectrum of activity and these confirm the use of crude extracts seeds of *A. indica* in folkloric medicines to treat eye and ear infections.

Conclusion

The results of this study, supports the use of the crude seed extracts of the neem plant in traditional practice to treat infections especially those that affects the ear and eye, but only if adjusted to suitable conditions of pH and temperature, and provided the infections are caused by susceptible bacteria.

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