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Antibacterial Activity of Azadirachta indica (Neem) Seed Oil against Bacteria Associated with Ear Infection

Halima Isa,* Alice A. Mbidomti
Department of Microbiology, Modibbo Adama University of Technology, Yola, Adamawa State, Nigeria
*Corresponding author contact. halimaisa@mautech.edu.ng

***Abstract*—** *Plants have been a source of herbal remedies throughout the history of mankind. Various medicinal plants have been used for years in daily life to treat diseases all over the world. In this research, antimicrobial activity of Azadirachta indica seed oil was evaluated against bacteria associated with ear infection using agar well diffusion method. Azadirachta indica (Neem) seed oil was extracted using soxhlet extraction setup with hexane and isopropanol as solvents. Ear specimens were obtained from patients with ear infections attending Specialist Hospital, Yola, and specimens were cultured, sub cultured until pure cultures were obtained and isolates identified, morphologically, following gram staining and biochemical procedures, identified isolates were Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli. Hexane extracts were more effective against the isolates with diameter of zone of inhibition ranging from 18-24mm than the isopropanol extract with 12-20mm. Staphylococcus aureus was most sensitive to the oil followed by E.coli then P. aeruginosa The MIC and MBC of the oil extracts was also determined using double dilution method, the MIC of hexane extracts ranges from 6.25-12.5% and 12.5-25% for isopropanol extracts, while the MBC range from 12.5-25% and 25-50% for hexane and isopropanol extracts respectively. The results obtained in this study indicate the potential of Azadirachta indica seeds oil (crude extracts) to inhibit the growth of such pathogenic organisms that frequently cause ear infections.*

***Keywords*—** *Agar well diffusion; Azadirachta indica; Ear infection; Infectious condition; Inhibition; Neem oil Pathogenic; Seed*s.

I. INTRODUCTION

In recent times, there is an increase in the resistance of pathogenic bacteria to antibiotics. Increase in microbial pathogens which are resistant to drug have become a major threat to human society. Therefore natural remedies have been put forward [1]. *Azadirachta indica* (Neem) is an omnipotent tree and a sacred gift of nature. Neem is being considered as the most important and useful medicinal plant [1]. It is an evergreen, tall, fast-growing tree, which can reach a height of 25m and 2.5m in girth which has an attractive crown of deep green foliage and honey scented flowers. Neem has more than hundred unique bioactive compounds, which have potential applications in agriculture, animal care, public health, and for even regulating human fertility [3].

Neem oil is a vegetable oil pressed from the fruits and seeds of the neem. It is the most important of the commercial available products of neem for organic farming and medicines [4]. Neem oil varies in color; it can be golden yellow, yellowish brown, reddish brown, dark brown, greenish or bright red. It has a rather strong odor that is said to combine the odors of peanut and garlic. It is composed mainly of triglycerides and contains many triterpenoid compounds, which are responsible for the bitter taste. It is hydrophobic in nature; in order to emulsify it in water for application purposes, it must be formulated with appropriate surfactants. Neem oil is not used for cooking purposes [4]. In India, it is used for preparing cosmetics (soap, hair products, body hygiene creams, hand creams) and in Ayurvedic, Unani and folklore traditional medicine, in the treatment of a wide range of afflictions [1]. The most frequently reported indications in ancient Ayurvedic writings are skin diseases, inflammations and fevers, and more recently rheumatic disorders, insect repellent and insecticide effects [5].

There are several methods for obtaining Neem oil from the seeds. These include: mechanical pressing, supercritical fluid extraction, and solvent extraction. Mechanical extraction is the most widely used method to extract oil from Neem seed. However, the oil produced with this method usually has a low price, because it is turbid. Supercritical extraction of oil produces oil with very high purity; however the operating and investment cost is high. Extraction using solvent has several advantages, it gives higher yield and it is less turbid than oil obtained from mechanical extraction, it also has low operating cost compared with oil from supercritical fluid extraction [6]. Neem oil extract, which is the fatty acid-extract of Neem tree seeds, is the most widely used product of the Neem tree. Neem seeds contain about 25 - 45% oil and provide the major source of Neem chemicals [7].

The normal micro biota of the external ear resembles those of the skin with Coagulase negative *Staphylococcus* and *Escherichia coli* are found occasionally, *Streptococcus pyogenase*, *Pneumococci*, *Haemophilus influenza*, and *Moraxella caterrhalis* are most frequently isolated bacteria from the middle and internal ear [8]. Infections of the external ear is caused by *Pseudomonas aeruginosa* which are opportunistic hospital-acquired, affecting those already in poor health and immune suppressed, infections are difficult to eradicate due to *P. aeruginosa* being resistance to many antimicrobials [9]. An infection of the middle ear or internal ear is known as 'otitis media' (inflammation of the middle ear) [9]. In acute otitis media, 30-50% of aspirated fluids are bacteriological sterile but most frequently isolated bacteria *are Pneumococci*, *H. influenza* and *Streptococci* (Brooks *et al*., 1998*). Streptococcus pneumonia* causes acute otitis media

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affecting children and adults worldwide. These bacteria pathogens are the cause of approximately 40% of acute otitis media. Acute otitis media do not commonly progress to inactive disease but they do contribute significantly to the burden and cost of pneumococcal disease (Akula *et al*., 2003). As neem seed oil consists of so many beneficial properties to fight against several diseases, this work aimed at determining its antibacterial effect (invitro) on some of the pathogens associated with ear infection.

II. METHODOLOGY

A. Collection of Neem seeds Page Layout

Dry fruits of *Azadirachta indica* were obtained within Modibbo Adama University of Technology Yola campus.

B. Preparation of Seeds

The seeds were thoroughly washed and then placed in a room at the temperature of 30^0C for 30 days; the seeds were then crushed into coarse powdery substance by using mortar and pestle. The coarse powdery substance was dried again and then subjected to electric blender to form powder. The powdered sample was then stored in an air tight, in the laboratory until the commencement of other procedures [12], [13].

C. Extraction procedure

The oil was extracted at the Chemistry Department of MAUTECH, Yola and soxhlet extraction setup was used for the extraction of *A. indica* oil [14]

150g of the crushed sample of *Azadirachta indica* seeds powder was weighed and then placed into a dry soxhlet thimble that is made of filter paper for each of the solvent used. 100 ml of hexane and isopropanol were used as solvents. The percentage of the extract yield was calculated at the end of the extraction process using the formula below; *% yield of the extract = Weight of sample after extraction divide by Weight of sample before extraction multiply by 100 [14]*.

D. Some Phytochemical analysis of Neem (Azadirachta indica) oil

Some of the phytochemical analysis that was carried out are; saponins, tannins, phenols, flavonoids and alkaloids [15].

E. Collection of specimen to obtain Test organisms

Sterile swap sticks were used to obtained swaps from the inner and middle ear of patients with ear infection attending Specialist Hospital Yola, Adamawa State Nigeria, and were then transported to microbiology laboratory of MAUTECH immediately for culturing, isolation and identification of organisms. The organisms were cultured on blood agar, nutrient agar and McConkey agar plates. The plates were then incubated at 37^0C for 48 hours.

F. Identification of the bacteria

After 48 hours, plates were observed for growth and other physical characteristics, a colony was picked from each plate and then sub cultured three times on MacConkey agar, nutrient agar and blood agar at 37^0C for 24 hours to get a pure culture for further identification. The organisms were identified as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* according to their morphological appearance (colony) on the plates, following gram staining procedure and biochemical tests.

G. Determination of Antimicrobial Activity of Neem oil

The Antimicrobial activity of Neem oil was evaluated against the three ear pathogens mentioned above by agar well diffusion technique. Broth cultures (standardized inoculum) of the test organisms compared to McFarland's standard 0.5 were prepared [14]. Lawn culture of the test organisms were made on nutrient agar plates using sterile cotton swab and the plates were dried for 15 minutes. Four Wells measuring 4mm in depth were made on the agar using sterile cork borer. Using a pipette, 1 ml of the hexane extract (Neem oil) was introduced into the first hole, 1 ml of the isopropanol extract on the second hole, 1ml of 10. 0 mg/ml of chloronphenicol (Elisca) solution into the third hole to serve as a positive control and 1 ml of distilled water into the fourth hole to serve as a negative control. The plates were allowed to stand on flat bench for 30 minutes to allow diffusion into the agar before incubation. The plates were incubated at 37^0C overnight and the diameter of zone of inhibition of growth was measured in mm. The entire tests were done in triplicate on all the three isolates to minimize test error.

H. Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the crude extract was determined using the doubling dilution method [16]. Exactly 2.5 ml of hexane extract (Neem oil) solution was added to 2.5 ml of sterile nutrient broth contained in a 10 ml test tube to obtain different extract concentrations of 50%, and subsequently, 25, 12.5, 6.25, 3.125, and 1.56% (double dilutions) were made in different test tubes. 1 ml of 18 hours culture of *Staphylococcus aureus* that was previously adjusted to 0.5 McFarland standards (1. 0 x 10^8 cfu /ml) was inoculated into each of the test tubes and the contents was thoroughly mixed. The tubes were then incubated at 37^0C for 24 hours [17]. The above procedure was repeated for each of the other isolates; *Pseudomonas aeruginosa*, *Escherichia coli*. The lowest concentration of the oil that did inhibit the visible growth of a microorganism after overnight incubation was observed and recoded as minimum inhibitory concentration [18].

I. Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) was determined by the dilution at which there was no visible growth on the media [19]. For each of the test tube in the MIC determination that did not show any visible growth, a loop full streaked on a sterile nutrient agar surface (in a Petri dish). The inoculated plates were incubated at the temperature of 37^0C for 24 hours. After incubation, the lowest concentration of the oil extract that kills a particular bacterium was recorded as minimum bactericidal concentration of the bacteria.

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III. RESULTS

A. Characteristics and Percentage Yield

Results of characteristics and percentage yield of the oil extracts are shown in Table 1. The results showed that *Azadirachta indica* oil extracted using hexane has higher percentage yield than that of isopropanol; extracts are in liquid form and golden yellow in colour.

TABLE 1. Characteristics and percentage yield of *Azadirachta indica* oil extracts.

Physical characteristics	Isopropanol extract	Hexane extract
Color	Greenish yellow	Golden yellow
Consistency	Liquid form	Liquid form
Weight of sample used (g)	150	150
Percentage yield (%)	29	43

B. Phytochemical Analysis

The extracts also contain specific bioactive components based on the phytochemical analysis carried out. The bioactive components found in both hexane and isopropanol were saponins, alkaloids, tannins, and phenols while flavonoid was found only in hexane extract as shown in Table 2.

TABLE 2. Phytochemical constituents of *Azadirachta indica* oil extracts

Chemical constituent	Isopropanol extract	Hexane extract
Saponins	+	+
Tannins	+	+
Phenols	+	+
Flavonoids	-	+
Alkaloids	+	+

Key: (+)-Present
(-) Absent

C. Antimicrobial Activity of the Seed Oil Extract of Azadirachta Indica

The results as shown in table 3 revealed that all the isolates were susceptible to the Neem oil extracts. Both the hexane and isopropanol extracts showed activity against the isolates as shown in Table 4.

TABLE 3. Antimicrobial activity of the seed oil extract of Azadirachta indica

Organisms	Diameter of Zone of inhibition (mm)			
	Isopropanol Extract	Hexane Extract	Pos. control	Neg. control
S. aureus	18	20	26	0
P. aeruginosa	12	16	20	0
E. coli	16	18	24	0

Key: (mm) = Millimeter

D. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Azadirachta Indica Seed Oil

The MIC and MBC of the crude oil extract are given in Table 4. Isopropanol extract showed MIC at 25% on *E. coli* and 12.5 % on *S. aureus* and *P. aeruginosa*. Hexane extract showed MIC at 6.25% on both *P. aeruginosa* and *E. coli* and12.5% on *S. Aureus*. Hexane extract showed MBC at 25 ml against *P. aeruginosa* and at 12.5% against *E. coli* and *S. aureus*. Isopropanol oil extract showed MBC activity at concentration of 50ml for *E. coli*, 25% for *P. aeruginosa* and *S. aureus*. This means that at concentration of 50%, the seed oil extract exerts bactericidal effects on *E. coli* and at 25% on *S. aureus* and *P. aeruginosa*.

TABLE 4: MIC and MBC of *Azadirachta indica* oil extracts on the selected bacteria

Organisms	Concentration of oil extract (%)			
	Isopropanol extract		Hexane extract	
	MIC	MBC	MIC	MBC
S. aureus	12.5	25.0	6.25	12.5
P. aeruginosa	12.5	25.0	6.25	12.5
E. coli	25.0	50.0	12.5	25.0

IV. DISCUSSION

Azadirachta indica is a common plant with rich medicinal attribute and is known to possess bioactive components which confirm their uses in pharmaceutical and local medicines [20]. The extraction process used was soxhlet extraction method [14], which uses chemical solvents to extract oil from tissues having a relatively low proportion of oil. It retrieves the last traces of oil through repeated washing or proportion under reflux in special glassware from the result obtained, the hexane solvent has higher percentage yield (42%) than isopropanol. This may be due to differences in the boiling points and polarity of the solvents as it has been reported that different degree of phyto-constitutes have different degree of solubility in different types of solvents. Phytochemical screening of the seed extracts revealed the presence of alkaloids, tannins, phenols, flavonoids and saponins. These secondary metabolites are linked to antimicrobial activity of the plant material [21]. The inhibitory activities exhibited by the extract tends to agree with the reports of [22] and [23], all of whom linked antimicrobial properties of plants to the presence of bioactive secondary metabolites.

All the crude extract inhibited the growth of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* although to varying degrees may be because of different solvent. The isopropanol extract demonstrated the least activity against the test organisms compared to hexane extract. It was reported that the activity of extracts depends on the solvent employed in the extraction process [24]. When plant materials are grounded in water or the plant cells are damaged, some phenolases and hydrolases are often on the activity of the active compounds in the extract or there may be incomplete extraction of the active principles thus explaining the law activity. Traditionally, however crude plant extracts are prepared with water as infusions, decoctions and poultices, therefore, it is very unlikely that the herbalist is able to extract these compounds, which are responsible for the activity observed in hexane extracts and isopropanol extracts [23].

Results also showed that activity of all the extracts are concentration dependent. Several researchers have reported similar results. The standard antibiotic chloronphenicol (Elisca) demonstrated highest activity (positive control). This may be because the standard antibiotic is in pure state and has been refined processes that have established it as standard antibiotic [9].

The organisms used for the purposes of these investigations are associated with ear infections [9]. Results of this investigation therefore have shown that *Azadirachta indica* is a potential source of antibiotic substance for drug

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development for use against this group of organisms. The MIC of the plant seed extracts ranged between 12.5-25% (for isopropanol) and 6.25-12.5% (for hexane extract). The MBC of the plant seed extracts ranged between 12.5-50% (for both isopropanol and hexane extract). High MIC and MBC values are indications of low activity while low MIC and MBC are indications high activity.

V. CONCLUSION

The study has confirmed that crude extract of *A. indica* seed oil possessed reasonable activity against some bacteria associated with ear infection and if the crude extract undergoes further purification, it can be used to treat ear infection. The finding therefore supports the use of *A. indica* by traditional medicine practitioners in the treatment of ear infection.

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