IN VITRO ANTIBACTERIAL EFFECTS OF DIFFERENT SOLVENT EXTRACTS OF THE LEAVES OF *NICOTIANA TABACUM* LINN (SOLANACEAE) ON CLINICAL ISOLATES FROM OTITIS MEDIA PATIENTS

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ABSTRACT

Background: Treatment failures in Otitis media may be due to high cost of treatment and/or resistance to common antibiotics, which may result to complications in children. The possible discovery of effective and cheap phytomedicine with antibacterial activity - commonly might be a life-saving measure.
Objective: This study investigated the phytochemistry and antibacterial activity of *Nicotiana tabacum* Linn (Solanaceae) leaves on clinical isolates from Otitis media patients.
Materials and Methods: The powdered leaves of *N. tabacum* were extracted with 70% methanol and further fractionated into dichloromethane and n-butanol fractions. The isolated and purified organisms from the ear swabs of otitis media patients were characterized by biochemical tests and then standardized with 0.5 McFarland to obtain *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcal pneumonia, Bacillus cereus, Escherichia coli, and Moraxella catarrhalis* bacterial strains. The antibacterial activity of the different extracts was done using Kirby – Bauer disc diffusion assay.
Results: The n-Butanol fraction at 20- 100µg/ml exhibits a broad spectrum of activity against all the clinical isolates when compared to standard antimicrobial agents. The phytochemical tests carried out on the crude extracts of *N. tabacum* showed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids and steroids.
Conclusion: *N. tabacum* leaves has *in vitro* antibacterial activity which can be attributed to the presence of secondary metabolite.

Keywords: Otitis media, antibacterial, *Nicotiana tabacum*, phytomedicine, clinical isolates.

INTRODUCTION

Otitis media is defined as an infection of the middle ear but most prevalent in children (0-17yrs). Otitis media is the most common reason for medical office visit in childhood. The financial impact of otitis media is substantial [1] and if poorly managed or untreated, could lead to serious complications [2]. The prevalence of this disease (1.3 - 31.3%) is higher in children from uneducated, low socio - economic background and poor living standards [3,4]. Bacteria has been implicated as the main causative agent in almost all cases of otitis media [5], although a few cases have been reported to be of viral origin (6). The common organisms implicated in otitis media include *staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenza, Proteus mirabilis* among others [5,6]. Worldwide, bacterial resistance to antibiotics is rapidly expanding thus becoming a growing concern. Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistant strain [7–9]. A vast number of medicinal plants have been

recognized as valuable resources of natural source of antimicrobial compounds [10]. Medicinal plant extracts offer considerable potential for the development of new agents effective against infections currently difficult to treat [11]. A wide range of phytochemicals present in plants are known to inhibit bacterial pathogens [12].

Nicotiana tabacum Linn (tobacco) belongs to the family Solanaceae, native to the Americans but currently cultivated and used worldwide [13] . Traditionally it is used as an anthelmintic, insecticide, diuretic, emetic, antimicrobial, and antispasmodic. In southern Nigeria, it is used in the treatment of toothache, while in the western part of Nigeria it is used to treat chronic purulent ear infections of any origin. Previous studies of the genius *Nicotiana* showed that it possessed antibacterial activity [14–16]. The current research work aims at validating the local use of the *Nicotiana tabacum* for purulent otitis media by comparing its antibacterial activity against antibacterial agents used in clinical practice.

Materials and methods

Materials

The chemicals used in this study were of analytical grade (BDH). Ciprofloxacin® and Augmentin® were obtained from Fidson Pharmaceuticals and GSK (Nigeria). The bacterial strains used in this study: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Bacillus cereus* and *Moraxella catarrhalis* were all clinical isolates from patients in Braithwaite Memorial Specialist Hospital, Port Harcourt. Rivers State, Nigeria.

Collection of Plant Material

Fresh leaves of *Nicotiana tabacum* Linn (Solanaceae) were collected from Akure, Ondo state in Southwestern Nigeria and shade dried for 7 days. The fresh leaves were authenticated at Department of Plant Science and Biotechnology, University of Port Harcourt, where voucher sample was deposited and voucher number allocated.

Extraction

The powdered leaves of *Nicotiana tabacum* (200 g) was macerated with 70 % methanol (2 L) for 72 hours with intermittent shaking at ambient temperature. The sample was thereafter filtered and concentrated *in vacuo* using a rotary evaporator to achieve a 10% w/v concentration. The sample was dried over a water bath set at 39 °C to obtain a brownish semisolid crude extract. The crude extract obtained was suspended in a specific amount of distilled water. The aqueous solution of the crude extract was partitioned sequentially with dichloromethane (500 ml x 5) and n-butanol (500 ml x 5).

Isolation and chemical characterization of microorganisms

Preparation of Culture Media

The following agar: Nutrient, MacConkey, Blood and Chocolate were prepared according to standard methods [18,19]. These agar plates were dried in a 37 °C incubator/oven (Memmert®, West Germany).

Preparation of inoculum

Ear swabs were collected from ten (10) patients (1-20 yrs.) diagnosed and confirmed to have otitis media by cleaning the patients' ear with a cotton wool soaked in normal saline and swabbing the ears with sterile cotton swab stick. The inoculations of the organisms with cotton swabs were carried out aseptically by streaking the surface of dried agar plates in three different directions and the plates incubated (37 °C) for 24 h.

Gram staining test
The physical properties (color, smell and texture) of the microbial growth were noted. Gram staining test was conducted to identify and classify (Gram positive or Gram negative) each of the organism. The organisms were then sub-cultured on different media (nutrient agar, MacConkey Agar, blood agar and chocolate agar and incubated (37 °C) for 24h. The sub-cultured organism were isolated on a slant to obtain a pure culture and incubated ((37 °C) for 24 h.

Biochemical tests
Conventional biochemical tests (Catalase, Oxidase, Indole, Coagulase, Citrate and Nitrate tests) were employed to identify the organisms.

Standardization of Isolates
The isolated organisms on agar slants were activated by sub-culturing into agar plates and incubated. This was standardized by transferring each colony of organism using sterilized wire loops (flamed and cooled) into 4 mL of peptone water. The inoculum suspension was incubated at 35°C until it achieves the turbidity of 0.5 McFarland standard which is approximately 1.0 x 10^8 CFU/ml.

Antibacterial activity test
The antibacterial activities of the crude extract and the fractions were assayed using the standard Kirby–Bauer disc diffusion method [19]. The test cultures were swabbed on the surface of the solidified media and allowed to dry for 10 minutes. The tests were conducted in triplicates at 100.0, 50.0 and 20.0 µg/disc concentration of crude extract and the fractions. The treated discs were separately placed on the surface of the medium and left to stand for 30 minutes at room temperature. A negative control was prepared using methanol, n-butanol and dichloromethane. Ciprofloxacin® (35 µg/ disc) and Augmentin® (35 µg/ disc) were the positive controls. The plates were incubated upside down for 24 h at 37°C and zones of inhibition were recorded. All of the experiments were done in triplicate. Activity index of each extract was calculated by using the formula
Activity Index (AI) = Inhibition zone of the sample/ inhibition zone of the standard

Phytochemical Screening
According to standard methods [17] the phytochemical screening of crude methanol extract, n-butanol and dichloromethane fractions were carried out.

Ethical Approval
The ethical approval for this study was obtained from University of Port Harcourt Teaching Hospital Ethical committee with number – UPTH/ADM/90/S.II/VOL XI/10.

Statistical analysis
In vitro antibacterial results were obtained from three independent experiments (n = 3) and Individual bar graph shows the mean value presented as mean ± SD of the inhibition zone diameter. One way analysis of variance (ANOVA) was used to analyze the differences among the groups followed by Tukey's post-test using Graph Pad Prism 5.0 software (www.graphpad.com/scientific-software/prism). Values $P < 0.05$ were considered significant. Values marked with an * differ from Ciprofloxacin® standard drug ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) while those marked with # differ significantly from Augmentin® standard drug ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$).

Results

The Biochemical Tests

The bacterial samples were identified morphologically and classified according to their species using the biochemical tests (Table 2). The biochemical analysis revealed the presence of six (6) causative organisms of otitis media: *B. cereus, S. aureus, P. aeruginosa, S. pneumoniae, M. catarrhalis* and *E. coli*.

Table 2. Identification of the Bacterial Species using Biochemical Tests

	Species of Clinical Pathogens					
Name of Test	*S. aureus*	*B. cereus*	*P. aeruginosa*	*S. Pneumoniae*	*M. catarrhalis*	*E.coli*
Catalase	+	+	+	–	–	+
Indole	–	–	–	–	–	+
Oxidase	–	–	+	–	+	–
Nitrate	+	–	–	+	+	–
Citrate	–	+	–	–	–	–
Coagulase	–	–	–	–	–	–
Gram - stain	+	+	–	+	–	–
Shape	Cocci	Rods	Rods	Cocci	Diplococci	Rod

Antibacterial activity of Plant Extracts

The antibacterial activity of the different concentrations of the extract/fractions of *N. tabacum* were compared with those of standard antibiotics (Ciprofloxacin® and Augmentin®) at 35 µg/mL (Figures 1- 6). The antibacterial activity of different concentrations of n-butanol fraction, 70% methanol extract and dichloromethane fraction of *N. tabacum* on the clinical isolate of *B. cereus* (Fig 1: A, B, C), shows that the antibacterial activities of 50 and 100 µg/L of n- butanol (31.50 ± 1.50 mm and 34.50 ± 4.72 mm respectively) was significantly ($p < 0.01$) higher than that obtained with Ciprofloxacin® (20.00 ± 1.63 mm) and Augmentin® (24.00 ± 4.32 mm). Methanol extracts (50 and 100 µg/ml) had an antibacterial activity (17.00 ± 4.35 and 21.60 ± 2.08 mm respectively) which is comparable to the control antibiotics while dichloromethane fraction had no activity against *B. cereus*.

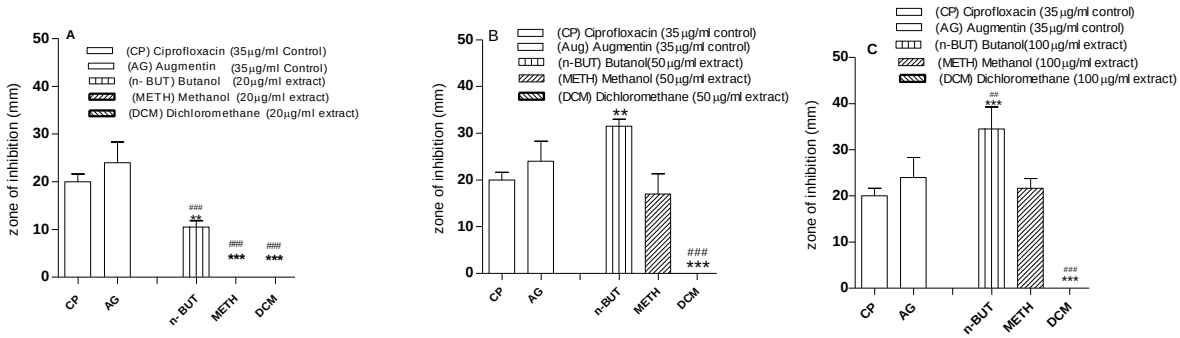

Fig. 1: The effect of different plant extract/ fractions against *B. cereus.*

Figure 2 (A, B, C) shows the antibacterial activity of different concentrations of n-butanol fraction, methanol 70% extract and dichloromethane fraction of *N. tabacum* on the clinical isolate *of S. aureus*. The methanol 70% extract at all the concentrations determined had zone of inhibition (18.50 ± 2.51, 20.50 ± 4.04 and 21.00 ± 2.64 mm respectively) that did not differ significantly from that of the Ciprofloxacin® (27.30 ± 3.35 mm) and Augmentin® (19.40 ± 4.57 mm) at 35µg/ml concentration respectively while the n-butanol fraction at 50

and 100 µg/L (37.50 ± 4.37 and 39.00 ± 0.50 mm respectively) were significantly higher ($p < 0.01$ and 0.001) than the standards.

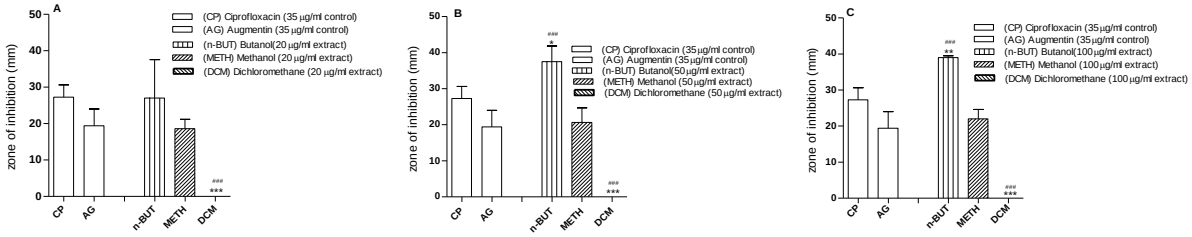

Fig. 2: The effect of different plant extract/fractions against S. *aureus.*

The antibacterial activity of 100 µg, 50 µg and 20 µg of n-butanol fractions, methanol 70% extracts and dichloromethane fraction of *N. tabacum* on the clinical isolates *of P. aeruginosa* (Fig 3: A, B, C) shows that 50 and 100 µg/L of n- butanol fraction and methanol extracts had zone of inhibition of (13.50 ± 4.44 and 14.50 ± 0.50 mm) and (12.00 ± 4.00 and 14.00 ± 3.46 mm) respectively which is not significantly different from that of Augmentin® (19.00 ± 1.15 mm) but different from Ciprofloxacin (25.50 ± 3.69 mm) both at concentrations of 35 µg/ml ($p < 0.001$).

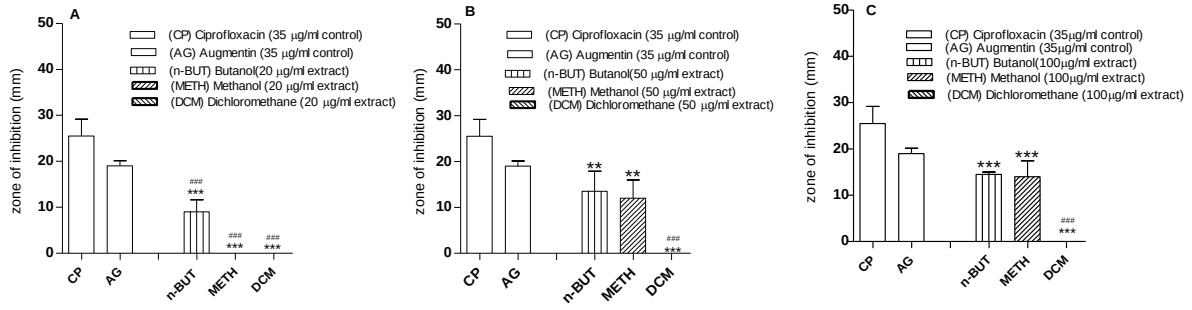

Fig. 3: The effect of different plant extract/fractions against *P. aeruginosa.*

The results of Fig 4 (A, B, C) shows the antibacterial activity of 100 µg, 50 µg and 20 µg of n-butanol fraction, methanol 70% extract and dichloromethane fraction of *N. tabacum* on the culture growth *of S. pneumoniae*. The antibacterial activities of different concentrations of the extract and fractions under consideration were of comparable antibacterial activity to that of standard antibiotics, except that of dichloromethane fraction at concentration of 100 µg/ml showed an insignificant activity against *S. pneumoniae* when compared to the standard drugs.

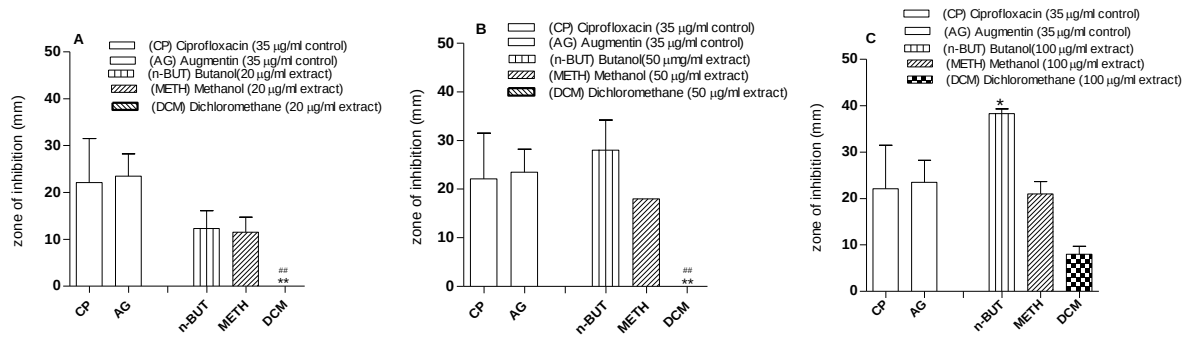

Fig. 4: The effect of different plant extract/fractions against *S. pneumonia.*

The results of Fig 5 (A,B,C) shows the antibacterial activity of 100 µg, 50 µg and 20 µg of n-butanol, methanol 70% and dichloromethane extracts of *N. tabacum* on the culture growth of *M. catarrhalis.* The dichloromethane and methanol 70% extracts at 100 µg/ml concentrations had activities significantly similar to that of standard Ciprofloxacin® and Augmentin® (35 µg/ml respectively).

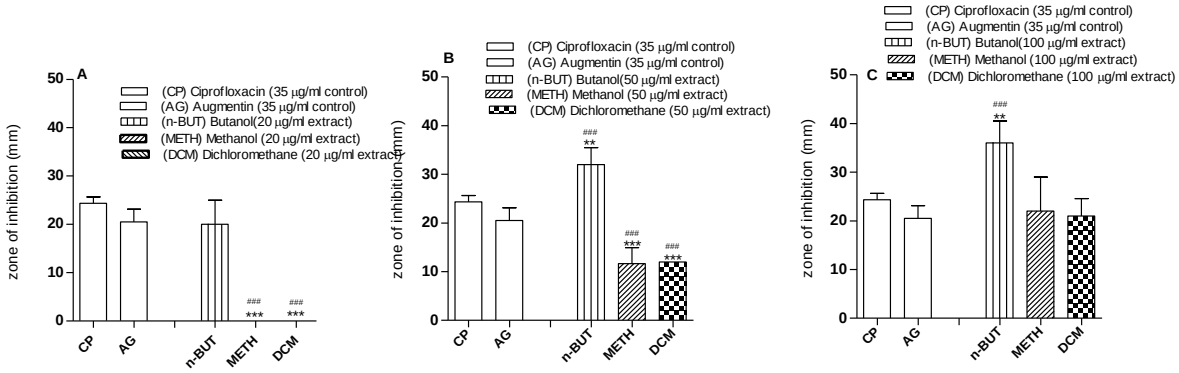

Fig. 5: The effect of different plant extract/fractions against *M. catarrhalis.*

The results of Fig 6 (A,B,C) shows the antibacterial activity of 100 µg, 50 µg and 20 µg of n-butanol, methanol 70% and dichloromethane extracts of *N. tabacum* on the culture growth of *E.coli.* The n-butanol extract at 20, 50 and 100 µg/ml had antibacterial activity (20.00 ± 4.16, 27.00 ± 4.00 and 32.00 ± 2.00 mm respectively) which is significantly higher than the standard drug Augmentin® (8.30 ± 2.93 mm, $p< 0.01$) and Ciprofloxacin (21.30 ± 2.50 mm)

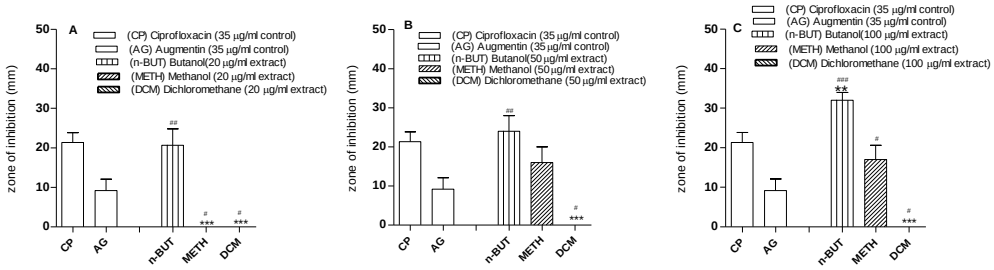

Fig. 6: The effect of different plant extract/fractions against *E.coli.*

Activity Indices

The activity indices of *Nicotiana tabacum* using ciprofloxacin® and Augmentin® is shown in Tables 3 and 4 respectively.

Table 3: Activity indices of the *Nicotiana tabacum* extracts using Ciprofloxacin® as standard and control

Name of organism	Different extracts of *N. tabacum*								
	n- Butanol (µg/ml)			Methanol 70% (µg/ml)			Dichloromethane (µg/ml)		
	20	50	100	20	50	100	20	50	100
B. cereus	0.53	1.58	1.73	-	0.86	1.10	-	-	-
S. aureus	0.99	1.37	1.43	0.68	0.75	0.77	-	-	-
S. pneumoniae	0.56	1.27	1.73	0.52	0.81	0.95	-	-	0.36
P. aeruginosa	0.35	0.53	0.57	-	0.47	0.55	-	-	-
M. catarrhalis	0.82	1.32	1.48	-	0.49	0.91	-	0.49	0.86
E.coli	0.94	1.27	1.50	-	0.80	0.75	-	-	-

KEY: - = No inhibition

Table 4: Activity indices of the *Nicotiana tabacum* extracts using Augmentin® as standard and control

Name of organism	Different extracts of *N. tabacum*								
	n- Butanol (µg/ml)			Methanol 70% (µg/ml)			Dichloromethane (µg/ml)		
	20	50	100	20	50	100	20	50	100
B. cereus	0.44	1.31	1.44	-	0.71	0.92	-	-	-
S. aureus	1.39	1.93	2.01	0.95	1.06	1.08	-	-	-
S. pneumoniae	0.53	1.20	1.64	0.49	0.77	0.90	-	-	0.34
P. aeruginosa	0.47	0.71	0.76	-	0.63	0.74	-	-	-
M. catarrhalis	0.98	1.56	1.75	-	0.58	1.07	-	0.58	1.02
E.coli	2.41	3.25	3.85	-	1.92	2.05	-	-	-

KEY: - = No inhibition

Phytochemical analysis

The phytochemical screening result of the methanol 70 % leaf extracts, dichloromethane and n-butanol fractions of *N tabacum* (Table 5)

Table 1. The result of the Phytochemical screening of *N tabacum* leaf extract.

Phytochemical constituent	Extract		
	Methanol 70%	Dichloromethane	n- butanol
Alkaloids	+	+	+
Tannins	+	+	+
Steroids	-	+	-
Phenolics	+	-	+
Flavonoids	+	-	+
Cardiac glycosides	+	+	+

1. Key: +,= positive and -, = negative

DISCUSSION

In this study, the causative bacteria: *S. pneumoniae, M. catarrhalis, P. aeruginosa and S. aureus, B. cereus and E. coli* were identified from the ear swabs of study patients in Port Harcourt, Nigeria indicating a full representation of both Gram positive and Gram negative organisms in the pathogenesis and etiology of otitis media, this is in agreement with literature on bacteria causing otitis media (21,22), while *P. aeruginosa* has also been implicated in most chronic cases (23). The medicinal plant *N. tabacum* has been used as an alternative therapy in otitis media traditionally it is therefore necessary by this study to validate the *in vitro* antibacterial activity of *N. tabacum* by comparing its antibacterial activity to some current standard drugs (23,24).

The n- butanol extract of *N. tabacum* at all the concentrations (20-100 µg) used for this study showed significant *in vitro* antibacterial activity (in a dose dependent manner) against the entire tested microorganism which is comparable to the activity of the standard drugs (Ciprofloxacin and Augmentin® (35µg)). This is followed by the 70 % methanol extract and finally the dichloromethane fraction which showed the least activity but has activity against only *M. catarrhalis* and *S. pneumoniae*. This implies that the main antibacterial activity resides in the n-butanol fractions and the polar fractions of *N. tabacum* and this could be due to the phytochemicals present in this fraction. The Gram negative organism *P. aeruginosa* showed the least sensitivity to all the extracts. This may be due to easy development of drug resistance by *P. aeruginosa* to most currently available antibiotics resulting to the refractory nature of otitis media and therefore frequent visits to the doctor (23,25,26). The organism *P. aeruginosa* also has developed the intrinsic ability to withstand harsh physical conditions such as extreme temperature, salts and antiseptics due to low outer membrane permeability and extensive efflux pump system (27) . The high sensitivity of different *N. tabacum* extracts against both Gram positive and Gram negative organism (Fig. 1-6) shows a broad spectrum of activity. The activity index is an expression of potency of the plant extract compared to the standard drug and it is obtained as a ratio of plant zone of inhibition to the standard drug zone of inhibition. From the activity index (AI) results (Tables 3 and 4), the potency of Ciprofloxacin is higher than that of Augmentin, because the drug Ciprofloxacin gave lower values for AI indicating a bigger zone of inhibition for the denominator which is the standard drug. This confirms that there is more resistance by the bacteria to Clavulanic acid augmented Amoxicillin which is a β-lactam Penicillin than to the drug Ciprofloxacin which is a fluoroquinolone derivative (28). The activity index of ≥1 shows that the plant *N. tabacum* has activity same or even better than the orthodox medicine at that concentration and for that particular bacteria. These reveal that these microorganism are very susceptible to the antibacterial activity of *N. tabacum*. Investigating therefore other alternative sources of medicine is of importance because there is hardly any antibiotic today that have not been challenged by micro-organisms to some level of resistance (29,30). The phytochemical screening revealed the presence of alkaloids, tannin and cardiac glycosides in the different extracts**.** Due to the polar nature of the phenolics and the flavonoids they are elaborated in both methanol extract and n-butanol fraction, while steroid is only elaborated by dichloromethane fraction. This is in agreement with earlier study conducted by some researchers (31). The observed antibacterial activity against the tested bacteria could be explained by the presence of these potentially bioactive classes of secondary metabolites with a strong ability to penetrate or disrupt the multilayered structure of both Gram positive and Gram negative organisms (Table 5). Flavonoids, polyphenols, tannins and alkaloids have been reported to possess antimicrobial properties and thus could be responsible for the antibacterial activity (12,32). The observed activity strongly suggests that *Nicotiana tabacum*

can be an effective topical herbal remedy in the treatment of infections caused by the tested pathogens (otitis media) as some plant extracts have shown from other research (33).

CONCLUSION

This study revealed that *Nicotiana tabacum* leaf has selective antibacterial activity against the tested bacteria. The antibacterial activity of the n-Butanol fraction against the tested bacteria showed its potential to be used for the development of the therapeutic agent for the treatment of otitis media as it is been practiced locally. A further study to characterize the active principle in the n-Butanol fraction and to elucidate its mechanism of its action is needed to confirm its ethno-medicinal usage.

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Conflict of interest: The authors declare that they have no conflict of interests.

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