Nigerian Journal of Pharmaceutical and Applied Science Research, 9(1):1-9, January 2020 (ISSN 1485-8059 (Available at www.nijophasr.com)

Nymphaea lotus and *Phyllanthus amarus*: Thin Layer Chromatography, Alkaloidal Fractions and Antimicrobial Activities on Multidrug Resistant Organisms Associated with Middle Ear Infection

[*1]Akinjogunla, O. J., [2]Umo, A.N., [1]Okon, M. U. and [1]Akaka, B.C.

[1]Department of Microbiology, Faculty of Science, University of Uyo, P.M.B. 1017, Uyo, Akwa Ibom State.
[2]Department of Medical Microbiology and Parasitology, Faculty of Basic Clinical Sciences, University of Uyo, Akwa Ibom State.

ABSTRACT

Phytochemical analysis and antimicrobial potential of ethanolic extracts of *Phyllanthus amarus* (ELEPA) and *Nymphaea lotus* (ELENL) on isolates from otitis media were evaluated using conventional methods, thin layer chromatography and disc diffusion technique. Alkaloids, saponins, tannins, flavonoids, anthraquinones, terpenes, deoxy-sugar, phenolic and cardiac glycosides were phyto-constituents detected in ELEPA and ELENL. Two alkaloidal components (R_1 0.5, 0.3) were detected in ELENL, while only one alkaloidal component of 0.65 retention factors were detected in ELEPA. The results also showed that between 121 (53.7%) to 134 (59.6%), 136 (60.4%) to 153 (68.0%) and 154 (68.4%) to 168 (74.7%) of the bacterial isolates were sensitive to the ELEPA (decoction) and ELENL (decoction) at 20 $mgml^{-1}$, 40 $mgml^{-1}$ and 80 $mgml^{-1}$ concentrations, respectively, Equal ratio by volume of ELEPA and ELENL (concoction) exhibited stronger antimicrobial activity with relatively higher zones of inhibition against all the organisms tested compared to decoction of ELEPA and ELENL. Among the Gram negative bacteria, lowest inhibitory zone (7.7±1.0 mm) was obtained in *S. marcescens*, while the highest inhibitory zone (18.9±1.7 mm) was obtained in *Enterobacter* spp**.** The results showed that between 47.3% and 62.4% fungal isolates were sensitive to different concentrations of ELENL (decoction) and ELEPA (decoction), between 62.4% and 74.2% fungal isolates were sensitive to concoction of ELEPA and ELENL at different concentrations, while alkaloidal fractions of ELEPA and ELENL were most effective on *C. albicans* and *A. flavus*. This study has shown the necessity to consider these potent ethanolic extracts of *P. amarus* and *N. lotus*, judging by the antimicrobial activity, for formulation of synthetic drugs against middle ear infection caused by both bacteria and fungi.

Key Words: Otitis media, *Phyllanthus amarus, Nymphaea lotus,* Alkaloidal Fraction, Sensitivity.

INTRODUCTION

Otitis media is the infection associated with the inflammation of the middle ear due to pathogenic micro-organisms in the middle ear (Damoiseaux, 2005; Akinjogunla *et al*., 2011). Sources of infection in otitis media are solely dependent on the route by which infection reaches the middle ear (Daly, 1997). The organisms found in the middle ear include *Candida albicans*, *Aspergillus* spp., *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (Ekpo *et al*., 2009). The growing resistance to antimicrobial agents of all respiratory tract pathogens has made the management of acute otitis media more difficult (Itzhak and Alan, 2005). Treatment of fungal infections has been less successful because eukaryotic fungal cells are much more similar to human cells than are in bacteria (Prescott *et al*., 2008). More than 200,000 out of the 300,000 plants species so far identified are said to be in the tropical countries of Africa. A special feature of higher plants is their capacity to produce a large number of organic chemicals of high structural diversity called secondary metabolites (Castello *et al*., 2002). *Phyllanthus amarus* commonly called 'carry me seed' or 'wind breaker' in English, 'Oyomokiso Aman Ke Edem' in Efik; 'Eyin Olobe' in Yoruba; 'Geeron tsutsaaye' in Hausa and 'Ngwu' or 'Ite Kwonwa nazu' in Igbo belongs to the family Euphorbiaceae (Akinjogunla *et al*., 2009). *Phyllanthus amarus*, an erect annual herb of not more than one and half feet tall, has small leaves, yellow flowers and contains active constituents such as lignin, flavonoids and phenyl propanoids (Etukudo, 2003). *Nymphaea lotus*, also called 'Tiger Lotus', belongs to Nymphaeaceae family. This plant prefers clear and slightly acidic water and is localized to Central Europe, North Africa and West Africa. It is a perennial plant that grows up to 45 cm in height; it is an herbaceous aquatic plant, whose leaves floats or submerges in water (Abu-Zaida *et al*., 2008). Many bioactive and pharmacologically important compounds have been obtained from *N. lotus* (Siddhanta *et al*., 1997). The aim of this study was to evaluate the antimicrobial activities of *N. lotus* and *P. amarus* on organisms associated with middle ear infections (otitis media)

Corresponding Author: E mail Address / Phone No: papajyde2000@yahoo.com/ 08064069404

MATERIAL AND METHODS

Collection of Isolates

Three hundred and eighteen (318) multidrug resistant organisms, comprising 225 bacteria and 93 fungi, isolated from patients with otitis media were obtained in the Department of Microbiology, University of Uyo. The identities of the bacterial and fungal isolates were further confirmed using conventional methods.

Sources of Medicinal Plants

The leaves of *P. amarus* (Fig 1) and *N. lotus* (Fig 2) obtained in Akwa Ibom State were identified and authenticated by a Taxonomist at the Department of Botany and Ecological Studies. The plants were later transferred to Pharmacognosy and Natural Medicine Laboratory, University of Uyo, for processing. The plants were washed under running tap water, distilled water and air-dried at room temperature for one month. The dried plant parts were pulverized using mortar and pestle into fine powder. The powdered *N. lotus* and *P. amarus* (3 kg) each were extracted by Soxhlet Apparatus using 75 % ethanol**.** The filtrate was evaporated using a rotary evaporator attached to a vacuum pump. After complete evaporation, the extract was weighed and preserved at 4 ºC. The graded concentrations (20 $mgml^{-1}$, 40 $mgml^{-1}$ and 80 $mgml^{-1}$) of the extracts were prepared using 100 ml of Dimethyl sulphoxide and shaken vigorously to obtain a homogenous mixture.

Phytochemical Screening

The phytochemical constituents of the plant extracts were analyzed according to the methods described by Sofowora (1993); Trease and Evans (1996).

Alkaloidal Partitioning

The methods of Harborne (1998) and Trease and Evans (1996) were used for the alkaloidal partitioning. The two different ethanolic extracts (*P. amarus* and *N. lotus*) were dissolved separately in 5 % HCl and partitioned successively with 50 ml of chloroform ($CHCl_3$) with the aid of separating funnel to give aqueous fraction and chloroform fraction, respectively. The aqueous fraction was then made alkaline with ammonium hydroxide (NH_4OH) solution of pH 12 and subsequently partitioned with chloroform ($CHCl_3$) to give alkaloidal fraction.

Thin Layer Chromatography of Alkaloidal fraction

Chloroform and methanol (9:1) was the solvent system used. The alkaloidal fraction was drawn with the aid of clean capillary tube and applied as a spot on a stationary phase about 1 cm from the bottom of the TLC plate. The spot was then allowed to dry in air at ambient temperature for 30 min. The spotted plates were inserted into the chromatographic tanks which were covered for development. At the end of the chromatographic development, the plates were then removed from the chromatographic tank and the solvent front marked. The separated spots on the TLC plates were visualized under daylight (visible light) and with ultra-violet **(**UV) light type A4Q9 (max 366 nm) after spraying with Dragendorff's spray. Orange colour revealed the presence of alkaloid.

Antimicrobial Activity of Crude Ethanolic Extracts of *N. lotus* (ELENL) and *P. amarus* (ELEPA)

The decoctions and concoctions of ethanolic extracts were tested for antimicrobial activity by disc diffusion method (Somchit *et al*., 2004). Mueller – Hinton Agar (MHA) / Sabouraud Dextrose Agar was sterilized, cooled to 45 – 50 ^oC and then poured into sterilized Petri dishes. Sterile filter paper discs (6 mm diameter) were impregnated with decoction of each ethanolic extracts solution of graded concentrations (20 $mgml^{-1}$, 40 $mgml^{-1}$ and 80 $mgml^{-1}$) and carefully placed on to agar plates which had previously been inoculated with the bacteria / fungi isolated using sterilized forceps. Also sterile filter paper discs were impregnated with the two extracts concoction (*N. lotus* and *P. amarus*) of graded concentrations (20 $mgml^{-1}$, 40 $mgml^{-1}$ and 80 $mgml^{-1}$) prepared in 1:1 by volume and carefully placed onto agar plates which had previously been inoculated with the bacteria / fungi isolated using sterilized forceps. The plates were then incubated at 37 ^oC for 24 hr for bacteria and 25 ^oC for 72 hr for fungi. Each ethanolic extract concentration was replicated thrice and the mean zone of inhibition diameter (in millimeters) was determined in each case. The sensitivities of the organisms to the extracts were classified by the diameters of the inhibitory zones as follows: ≤ 8.5 mm (fairly sensitive), 8.6-12.5 mm (moderately sensitive) and ≥ 12.6 mm (highly sensitive).

Antimicrobial Activity of Alkaloidal Fractions of *N. lotus* and *P. amarus* Extracts

The alkaloidal fractions of *N. lotus* and *P. amarus* extracts were tested for antimicrobial activity by disc diffusion method (Somchit *et al*., 2004). Mueller–Hinton Agar / Sabouraud Dextrose Agar were poured into sterilized Petri dishes. The dry alkaloidal fraction was dissolved in sterile DMSO, thereafter sterile filter paper discs (6 mm) diameter were impregnated with alkaloidal fractions (20 $mgml^{-1}$ and 40 $mgml^{-1}$) and carefully placed on to agar plates which had previously been inoculated with the bacteria / fungi isolated using sterilized forceps. The plates were then

incubated at 37 ^{o}C for 24 hr for bacterial isolates and 25 ^{o}C for 72 hr for fungal isolates. Each alkaloidal fraction concentration was replicated thrice and the mean inhibition zone diameter (in millimeters) was determined. The sensitivities of the organisms to the extracts were classified by the diameters of the inhibitory zones as follows: ≤ 8.5 mm (fairly sensitive), 8.6-12.5 mm (moderately sensitive) and ≥ 12.6 mm (highly sensitive).

Fig 1*: Phyllanthus amarus*

Fig 2*: Nymphaea lotus*

RESULTS AND DISCUSSION

Antibacterial activities of ELEPA (decoction) and ELENL (decoction) showed a wide range of activity on the bacteria isolated from otitis media (Tables 1 and 2). The inhibitory zones of the ELEPA for *M. catarrhalis*, *H. influenzae*, *S. pneumoniae* and *S. pyogenes* for the different concentrations ranged from 10.2±0.5mm to 18.3±0.8mm, 8.2±0.1mm to 14.1± 1.5 mm, 9.0±0.5mm to 16.5±0.8mm, 7.0±1.3mm to 12.2±1.7mm, respectively (Table 1). Range of values in (mm ± S.D) obtained for *E. coli* was 8.0±0.5 to 18.0±1.5; *P. mirabilis* was 9.4±0.2 to 16.0±1.5 and *Alcaligenes* spp. was 9.2±0.5 to 15.8±0.2. The results also showed that 134 (59.6%), 153 (68.0%) and 168 (74.7%) of the bacterial isolates were sensitive to the ELEPA (decoction) at 20 mgml^{-1}, 40 mgml^{-1} and 80 mgml^{-1} concentrations, respectively (Table 1). *Corynebacterium* spp and *E. faecalis* were highly sensitive to the different concentrations of ELENL (decoction), while the crude extracts showed moderate activity against *C. freundii* and *P. mirabilis*. The results also showed that 121 (53.7%), 136 (60.4%) and 154 (68.4%) of the bacteria were sensitive to ELENL (decoction) at 20 mgml^{-1}, 40 mgml^{-1} and 80 mgml^{-1} concentrations, respectively **(**Table 2). The disc containing 80 mgml^{-1} of ELENL (decoction) showed maximum activity against all the *E. faecalis* and *E. coli* with the highest zones of inhibition (Mean ± SD) of 18.3±1.5mm and 17.9±1.5mm, respectively (Table 2).

Equal ratio by volume of ELEPA and ELENL (concoction) exhibited stronger antibacterial activity with relatively higher inhibitory zones against the organisms tested compared to decoction of ELEPA and ELENL. Thus, indicating the synergistic effects of the concoction. Only 146 (64.9%), 167 (74.2%) and 175 (77.8%) of the bacteria were sensitive to the concoction of ELEPA and ELENL at 20 mgml^{-1}, 40 mgml^{-1} and 80 mgml^{-1} concentrations, respectively (Table 3). Among the Gram-negative bacteria, lowest inhibitory zone (7.7±1.0 mm) was obtained in *S. marcescens*, while the highest inhibitory zone (18.9±1.7 mm) was obtained in *Enterobacter* spp. Among the Gram-positive bacteria, the lowest and highest inhibitory zone (mm ± SD) was obtained in *B. substilis* and CoN-*Staphylococcus* spp having 8.0±1.0 mm and 17.2±0.1 mm, respectively**.**

The 20 mgml^{-1}, 40 mgml^{-1} and 80 mgml^{-1} concentrations of ELEPA (decoction) showed 47.3%, 50.5% and 57.0% antifungal activity on the fungal isolates, respectively. Only 54 (58.1%) and 58 (62.4%) of the fungi were sensitive to the ELENL (decoction) at 40 mgml^{-1} and 80 mgml^{-1} concentrations, respectively, while 62 (66.7%) and 69 (74.2%) of the fungi were sensitive to concoction of ELEPA and ELENL at 40 mgml^{-1} and 80 mgml^{-1} concentrations, respectively. The results also showed that *A. flavus* and *Candida* spp. were more sensitive to different concentrations of the decoction and concoction of ELEPA and ELENL than the other fungal isolates (Table 4).

The sensitivity test results indicated that the alkaloidal fractions obtained from ELEPA and ELENL showed varying degree of activities against the bacterial isolates (Figs 3 and 4). Based on the inhibition profiles, the alkaloidal fractions obtained from ELEPA with concentration of 40 mgml^{-1} were more effective on *C. albicans* and *A. flavus*, while among the six fungi tested, the alkaloidal fractions obtained from ELENL was most effective and evinced moderate inhibitory activity against *A. flavus* (62.5%) and *Candida* spp. (61.5%) at concentrations of 20 mgml^{-1} and 40 mgml^{-1} (Figs 5 and 6)**.** The results of preliminary phytochemical analysis of the ELEPA and ELENL revealed the presence of phyto-constituents such as alkaloids, saponins, tannins, flavonoids, anthraquinones, terpenes, deoxy-sugar, phenolic and cardiac glycosides (Table 5). The presence of alkaloids was confirmed on thin layer chromatography (TLC) plates which showed orange spot for alkaloids when sprayed with Dragendorff's reagent. Two major

alkaloidal components (R_1 0.5, 0.3) were detected in ELENL, while only one alkaloidal component of 0.65 retention factors were detected in ELEPA (Plates 1 and 2)

Table 1: Antibacterial Activities of ELEPA (Decoction) on Bacterial Isolates from Otitis Media

Bacterial spp	No. Tested	Sensitivity 20 mg/ml No. (%)	40 mg/ml No. (%)	80 mg/ml No. (%)	Lowest Mean ±SD Inhibition	Highest Mean ±SD Inhibition
Gram Positive Bacteria						
S. pneumoniae	23	14(60.9)	16(69.6)	18(78.3)	9.0±0.5[b]	16.5±0.8[b]
S. pyogenes	12	7(58.3)	7(58.3)	9(75.0)	7.0±1.3[a]	12.2±1.7[a]
S. aureus	31	21(67.7)	23(74.2)	23(74.2)	9.2±1.5[b]	16.3±0.5[b]
CoN *Staphylococcus* spp	25	17(68.0)	18(72.0)	20(80.0)	9.2±1.2[b]	16.0±0.5[b]
E. faecalis	4	3(75.0)	3(75.0)	3(75.0)	10.0±0.8[b]	17.6±1.1[b]
Corynebacterium spp	6	4(66.7)	4(66.7)	5(83.3)	7.1±0.2[a]	11.1±1.0[a]
B. substilis	4	1(25.0)	2(50.0)	3(75.0)	7.5±1.3[a]	12.0±1.2[a]
Total	105	67(63.8)	73(69.5)	81(77.1)	---	---
Gram Negative Bacteria						
M. catarrhalis	10	6(60.0)	6(60.0)	7(70.0)	10.2±0.5[b]	18.3±0.8[b]
H. influenzae	12	6(50.0)	8(66.7)	8(66.7)	8.2±1.0[a]	14.1±1.5[b]
P. aeruginosa	23	12(52.2)	15(65.2)	15(65.2)	7.0±1.3[a]	11.9±1.1[a]
E. coli	15	9(60.0)	10(66.7)	10(66.7)	8.0±0.5[a]	18.0±1.5[b]
P. mirabilis	12	8(66.7)	8(66.7)	10(83.3)	9.4±1.2[b]	16.0±1.5[b]
P. vulgaris	9	5(55.6)	5(55.6)	6(66.7)	7.0±0.9[a]	12.0±0.7[a]
K. pneumoniae	13	7(53.8)	10(76.9)	10(76.9)	8.2±0.5[a]	14.2±1.0[b]
Enterobacter spp	5	4(80.0)	4(80.0)	5(100.0)	9.9±0.5[b]	16.8±1.2[b]
S. marcescens	6	3(50.0)	3(50.0)	4(66.7)	7.6±1.6[a]	13.0±0.5[a]
M. morganii	5	2(40.0)	4(80.0)	4(80.0)	7.2±1.5[a]	12.2±1.5[a]
C. freundii	5	3(60.0)	3(60.0)	4(80.0)	8.8±1.6[a]	15.1±2.0[b]
Alcaligenes spp	5	2(40.0)	4(80.0)	4(100.0)	9.2±0.8[b]	15.8±1.2
Total	120	67(55.8)	80(66.7)	87(72.5)	---	---

Values in parenthesis are percentages; Each inhibitory zone included 6 mm diameter of the disc., SD: Standard Deviation. Mean within the column followed by the different superscript letters are significant as determined by Duncan's multiple range test ($P < 0.05$); CoN: Coagulase Negative.

Table 2: Antibacterial Activities of ELENL (Decoction) on Bacterial Isolates from Otitis Media

Bacterial spp	No. Tested	Sensitivity 20mg/ml No. (%)	40mg/ml No. (%)	80mg/ml No. (%)	Lowest Mean ±SD Inhibition	Highest Mean ±SD Inhibition
Gram Positive Bacteria						
S. pneumoniae	23	11(47.8)	11(47.8)	12(52.2)	7.4±1.0[a]	11.0±1.0[a]
S. pyogenes	12	7(58.3)	7(58.3)	9(75.0)	8.9±0.5[a]	12.2±1.0[a]
S. aureus	31	16(51.6)	19(61.3)	19(61.3)	9.0±1.2[b]	14.8±1.5[b]
CoN *Staphylococcus* spp	25	13(52.0)	15(60.0)	17(68.0)	8.6±2.0[a]	16.0±0.5[b]
E. faecalis	4	3(75.0)	4(100.0)	4(100.0)	10.8±2.0[b]	18.3±1.5[b]
Corynebacterium spp	6	5(83.3)	5(83.3)	5(83.3)	10.1±0.5[b]	15.1±2.0[b]
B. substilis	4	2(50.0)	3(75.0)	3(75.0)	7.8±2.0[a]	10.0±1.0[a]
Total	105	57(54.3)	64(60.9)	69(65.7)	---	---
Gram Negative Bacteria						
M. catarrhalis	10	5(50.0)	6(60.0)	8(80.0)	10.2±1.5[b]	16.2±1.5[b]
H. influenzae	12	6(50.0)	6(50.0)	7(58.3)	7.8±1.1[a]	10.4±2.0[a]
P. aeruginosa	23	11(47.8)	13(56.5)	13(56.5)	7.5±1.0[a]	12.2±1.5[a]
E. coli	15	10(66.7	11(73.3)	11(73.3)	9.6±0.5[b]	17.2±1.0[c]
P. mirabilis	12	8(66.7)	8(66.7)	10(83.3)	9.0±2.0[b]	15.1±0.5[b]
P. vulgaris	9	5(55.6)	7(77.8)	7(77.8)	8.8±1.6[a]	14.4±1.0[b]
K. pneumoniae	13	5(38.5)	7(53.8)	9(69.2)	7.4±0.8[a]	14.2±1.0[b]
Enterobacter spp	5	4(80.0)	4(80.0)	5(100.0)	10.9±0.5[b]	17.9±1.5[c]
S. marcescens	6	3(50.0)	3(50.0)	4(66.7)	7.9±1.0[a]	14.2±1.2[b]
M. morganii	5	2(40.0)	2(40.0)	3(60.0)	7.5±0.5[a]	13.0±1.0[a]
C. freundii	5	3(60.0)	3(60.0)	3(60.0)	7.5±1.0[a]	12.0±2.0[a]
Alcaligenes spp	5	2(40.0)	2(40.0)	3(60.0)	8.4±1.5[a]	14.0±1.2[b]
Total	120	64(53.3)	72(60.0)	85(70.8)	---	---

Values in parenthesis are percentages; Each inhibitory zone included 6 mm diameter of the disc., SD: Standard Deviation. Mean within the column followed by the different superscript letters are significant as determined by Duncan's multiple range test ($P < 0.05$); CoN: Coagulase Negative.

Table 3: Antibacterial Activities of ELENL and ELENL (Concoction) on Bacterial Isolates from Otitis Media

Bacterial spp	No. Tested	Sensitivity 20 mg/ml No. (%)	40 mg/ml No. (%)	80 mg/ml No. (%)	Lowest Mean ±SD Inhibition	Highest Mean ±SD Inhibition
Gram Positive Bacteria						
S. pneumoniae	23	14(60.9)	16(69.6)	18(78.3)	8.9±0.7[a]	16.0±0.8[b]
S. pyogenes	12	7(58.3)	8(66.7)	9(75.0)	8.5±1.5[a]	14.0±1.0[b]
S. aureus	31	21(67.7)	23(74.2)	23(74.2)	9.5±1.0[b]	16.9±0.5[b]
CoN *Staphylococcus* spp	25	18(72.0)	18(72.0)	20(80.0)	9.0±1.2[b]	17.2±1.0[c]
E. faecalis	4	3(75.0)	4(100.0)	4(100.0)	9.7±1.1[b]	18.7±2.2[c]
Corynebacterium spp	6	5(83.3)	5(83.3)	5(83.3)	10.4±0.7[b]	15.2±2.0[b]
B. substilis	4	2(50.0)	3(75.0)	3(75.0)	8.0±1.0[a]	12.5±2.0[a]
Total	105	70(66.7)	77(73.3)	82(78.1)	---	---
Gram Negative Bacteria						
M. catarrhalis	10	7(70.0)	7(70.0)	8(80.0)	10.5±0.5[b]	18.5±1.5[c]
H. influenzae	12	6(50.0)	9(75.0)	9(75.0)	8.4±2.0[a]	14.1±2.0[b]
P. aeruginosa	23	13(56.5)	16(69.6)	16(69.6)	7.5±2.0[a]	13.0±1.5
E. coli	15	12(80.0)	12(80.0)	12(80.0)	10.2±1.5[b]	18.4±1.4[c]
P. mirabilis	12	9(75.0)	9(75.0)	10(83.3)	9.4±0.5[b]	17.4±2.0[c]
P. vulgaris	9	5(55.6)	7(77.8)	7(77.8)	8.0±1.0[a]	15.1±1.0[b]
K. pneumoniae	13	8(61.5)	10(76.9)	10(76.9)	8.4±2.0[a]	14.7±0.5[b]
Enterobacter spp	5	4(80.0)	4(80.0)	5(100.0)	10.5±1.2[b]	18.9±1.7[c]
S. marcescens	6	3(50.0)	4(66.7)	4(66.7)	7.7±1.0[a]	14.0±0.5[b]
M. morganii	5	3(60.0)	4(80.0)	4(80.0)	8.0±1.2[a]	13.5±1.1[a]
C. freundii	5	3(60.0)	4(80.0)	4(80.0)	9.1±1.0[b]	16.0±1.0[b]
Alcaligenes spp	5	3(60.0)	4(80.0)	4(80.0)	8.6±1.0[a]	16.0±2.0[b]
Total	120	76(63.3)	90(75.0)	93(77.5)	---	---

Values in parenthesis are percentages; Each inhibitory zone included 6 mm diameter of the disc., SD: Standard Deviation. Mean within the column followed by the different superscript letters are significant as determined by Duncan's multiple range test ($P < 0.05$); CoN: Coagulase Negative.

Table 4: Antifungal Activities of ELENL and ELENL on Fungal Isolates from Otitis Media

Source	Fungal spp	No. Tested	Sensitivity 20mg/ml No. (%)	40mg/ml No. (%)	80mg/ml No. (%)	Lowest Mean ±SD Inhibition	Highest Mean ±SD Inhibition
P. amarus	*C. albicans*	18	8(44.4)	10(55.6)	10(55.6)	8.2±0.5[a]	11.2±0.2[a]
	A. flavus	8	4(50.0)	5(62.5)	5(62.5)	9.3±0.8[b]	13.9±0.1[a]
	A. niger	24	12(50.0)	12(50.0)	14(58.3)	7.7±1.5[a]	13.0±0.5[a]
	C. neoformans	18	8(44.4)	8(44.4)	10(55.6)	8.0±1.0[a]	11.8±1.4[a]
	Fusarium spp	12	5(41.7)	5(41.7)	5(41.7)	7.6±2.0[a]	10.2±2.0[a]
	Candida spp	13	7(53.8)	7(53.8)	9(69.2)	9.5±1.5[b]	14.0±0.5[b]
	Total	93	44(47.3)	47(50.5)	53(57.0)	---	---
N. lotus							
	C. albicans	18	9(50.0)	9(50.0)	10(55.6)	7.6±0.5[a]	11.7±1.5[a]
	A. flavus	8	5(62.5)	5(62.5)	6(75.0)	9.6±1.2[b]	14.0±1.0[b]
	A. niger	24	14(58.3)	16(66.7)	16(66.7)	8.0±2.0[a]	12.8±1.2[a]
	C. neoformans	18	8(44.4)	10(55.6)	11(61.1)	7.9±0.5[a]	12.2±2.0[a]
	Fusarium spp	12	5(41.7)	6(50.0)	6(50.0)	8.0±1.0[a]	13.5±1.1[a]
	Candida spp	13	8(61.5)	8(61.5)	9(69.2)	9.1±1.0[b]	13.8±0.5[a]
	Total	93	49(52.7)	54(58.1)	58(62.4)	---	---
P. amarus							
+	*C. albicans*	18	11(61.1)	11(61.1)	14(77.8)	8.7±2.0[a]	13.0±0.2[a]
N. lotus	*A. flavus*	8	6(75.0)	6(75.0)	8(100.0)	10.1±0.2[b]	14.8±0.7[b]
	A. niger	24	16(66.7)	16(66.7)	17(70.8)	8.5±1.5[a]	13.9±1.5[a]
	C. neoformans	18	10(55.6)	12(66.7)	12(66.7)	8.5±0.5[a]	12.8±0.1[a]
	Fusarium spp	12	6(50.0)	7(58.3)	7(58.3)	8.0±2.2[a]	13.7±0.1[a]
	Candida spp	13	9(69.2)	10(76.9)	11(84.6)	10.5±0.5[b]	14.5±1.5[b]
	Total	93	58(62.4)	62 (66.7)	69(74.2)	---	---

Values in parenthesis are percentages; Each inhibitory zone included 6 mm diameter of the disc., SD: Standard Deviation. Mean within the column followed by the different superscript letters are significant as determined by Duncan's multiple range test ($P < 0.05$)

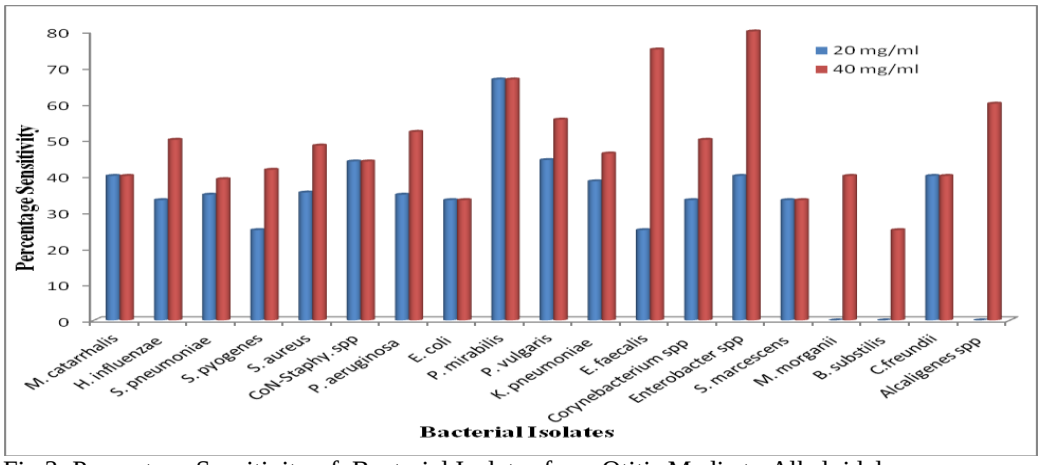

Fig 3: Percentage Sensitivity of Bacterial Isolates from Otitis Media to Alkaloidal Fraction from ELEPA

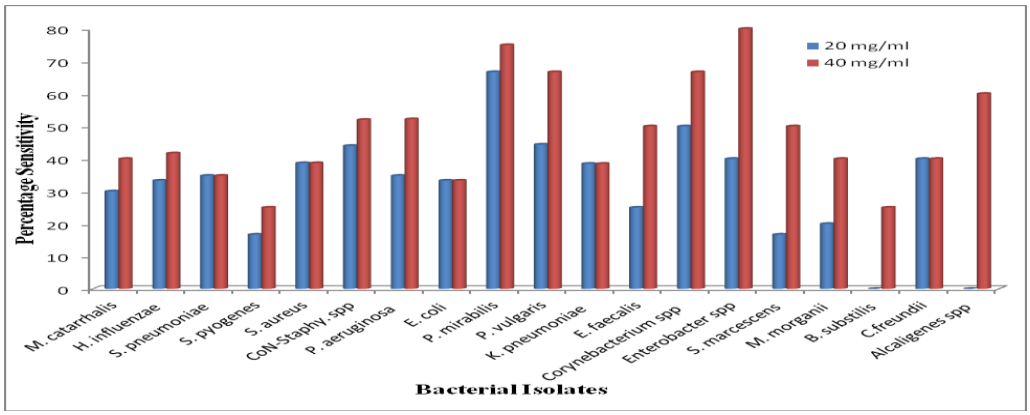

Fig 4: Percentage Sensitivity of Bacterial Isolates from Otitis Media to Alkaloidal Fraction from ELENL

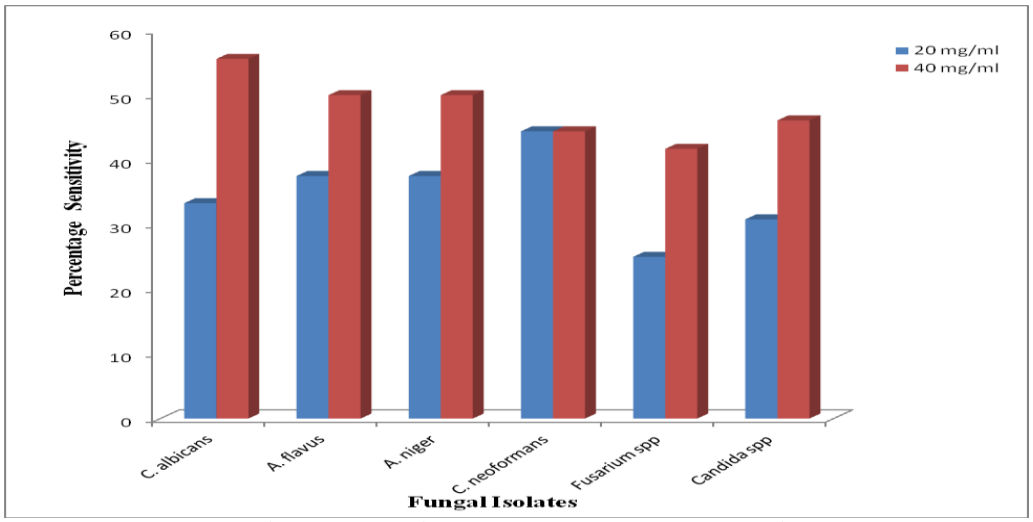

Fig 5: Percentage Sensitivity of Fungal Isolates from Otitis Media to Alkaloidal Fraction from ELEPA

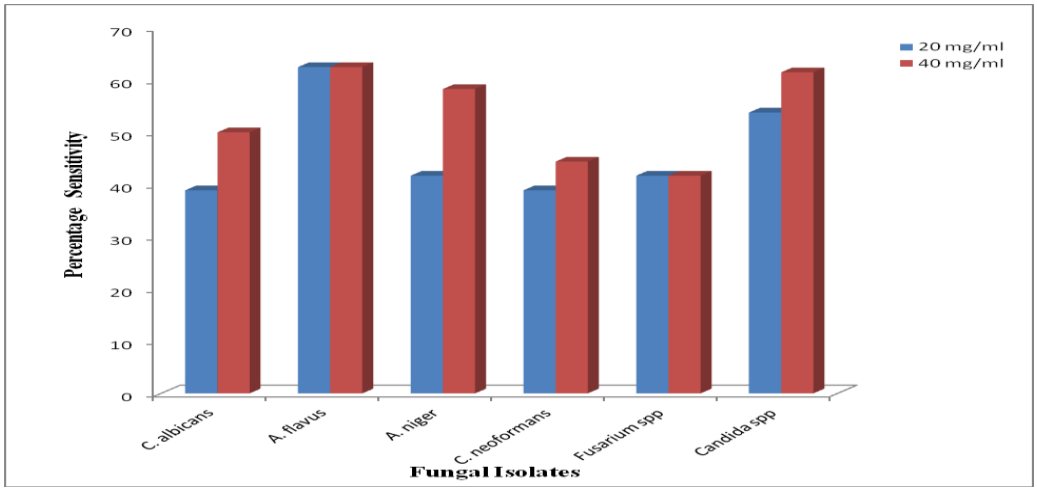

Fig 6: Percentage Sensitivity of Fungal Isolates from Otitis Media to Alkaloidal Fraction from ELENL

Table 5: Phytochemical Tests of the Crude Ethanolic leaf Extracts of *P. amarus* and *Nymphaea lotus*

Plant Constituents	*Phyllanthus amarus*	*Nymphaea lotus*
Alkaloids	+	+
Flavonoids	+	+
Terpenes	+	ND
Saponins	+	+
Tannins	+	+
Anthraquinones	+	+
Cardiac glycoside	+	+
Deoxy-sugar	+	+
Phlobatanins	ND	ND
Phenolics	+	+

Keys: + : Positive; ND: Not Detected

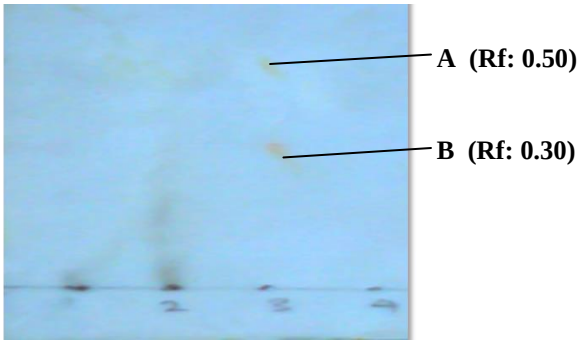

Plate 1: Thin layer chromatography (TLC) showing Alkaloidal spots of ELENL
A: Alkaloidal spot A (Rf: 0.50) B: Alkaloidal spot B (Rf: 0.30)

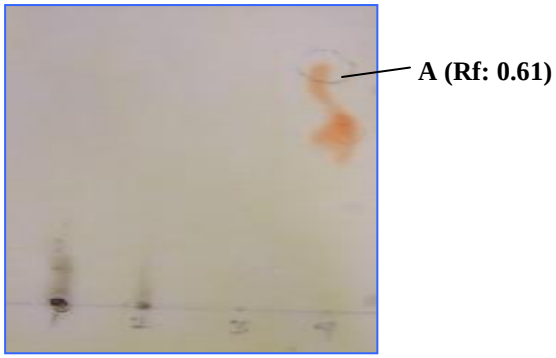

Plate 2: Thin layer chromatography showing Alkaloidal spot of ELEPA
A: Alkaloidal spot A (Rf: 0.61)

DISCUSSION

The relative amount of phytochemical substances from plant extraction depends on the solubility of the phytochemical in the solvent used for extraction (Olowosulu and Ibrahim 2006). Ethanol is generally able to dissolve multivariable types of compounds; polar and non-polar, simple and complex chemical structures (Cowan, 1999). Preliminary phytochemical tests of ELEPA revealed the presence of anthraquinones, cardiac glycosides, saponins, tannins, alkaloids, flavonoids and these are in conformity with the earlier reports of Olufemi and Debiri, (2008). ELEPA and ELENL tested were active against Gram positive bacteria, Gram-negative bacteria and fungi, suggesting a broader spectrum activity for these extracts. The Gram negative bacteria isolated from the otitis media samples were more susceptible to the decoction of ELEPA and ELENL and their concoction than the Gram positive bacteria and this disagrees with Ravikumar *et al*. (2010). The degree of susceptibilities of the Gram positive and Gram negative bacteria may be as a result of the physical and chemical compositions of their cell walls. The antimicrobial effects of these plant extracts could be due to the presence of the secondary metabolites which exert antimicrobial activity through different mechanisms (Ebana *et al*., 1993). Many bioactive and pharmacologically important compounds have been obtained from *Nymphaea* spp and used in medicine (Siddhanta *et al*., 1997). Hence, the presence of the secondary metabolites such as anthraquinones, cardiac glycosides, saponins, tannins, alkaloids, flavonoids and phenolics in ELENL may be responsible for its potential use as a drug against pathogenic organisms. The results showed that ELENL exhibited inhibitory activities against the tested organisms with different degrees as demonstrated by measuring the diameters of inhibition zones and these results are in conformity with the results obtained by Abu-Zaida *et al*. (2008). The ability of the ELENL to inhibit the growth of *S. aureus, K. pneumoniae. C. albicans* and *Fusarium* spp indicated that these organisms do not possess a mechanism that inactivates the active ingredients in the extracts or other mechanisms which include exclusion of the substance from the cell and modification of the target site of the substance. The findings also pointed out that the higher the concentrations of the extracts, the higher the bacterial and fungal spp. sensitivities to the extracts as showed by the increased size of the bacterial and fungal growth inhibitory zones and this is in conformity with Okigbo and Ogbonnanya (2006) and Eyob *et al*. (2008). The presence of alkaloids in the ELEPA and ELENL in this research is in agreement with Akinjogunla *et al*. (2009). The alkaloidal fractions obtained from ELEPA and ELENL showed antimicrobial activities on *M. catarrhalis, C. albicans* and *Fusarium* spp at 20 $mgml^{-1}$ and 40 $mgml^{-1}$ concentrations and the antimicrobial activity of alkaloidal fractions is in conformity with Kabir *et al*. (2005).

Conclusively, the significant antimicrobial activities of ELEPA and ELENL and their retention factors by the fractions have validated their use as antimicrobial agent in Nigerian ethno-medicine. Also, there is a need to consider the use of these potent ethanolic extracts of *P. amarus* and *N. lotus*, judging by the antimicrobial activity for formulation of synthetic drugs against otitis media caused by both bacteria and fungi.

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