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Structural elucidation, mechanism of action, antibacterial proficiency and synergistic forte of purified *Euphorbia hirta* whole plant extract against multi-drug resistant otitis media infection

[Elucidación estructural, mecanismo de acción, competencia antibacteriana y fuerza sinérgica del extracto de planta entera de *Euphorbia hirta* purificado contra la infección por otitis media resistente a múltiples fármacos]

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Abstract: This study investigated the antibacterial potential of *Euphorbia hirta* whole plant extracts, honey and conventional antibiotics and their synergistic effects against selected multidrug resistant and typed bacterial strains associated with otitis media. *E. hirta* whole plant extract was purified using column chromatography technique. The antibacterial assays of extracts were done using standard microbiological procedures. Protein, sodium and potassium ion leakage of the synergistic mixtures was determined using flame-photometry. At 100 mg/ml, acetone extracts presented highest inhibition against *S. aureus* (NCTC 6571) with 32 ± 0.83 mm zone of inhibition. The fractional inhibitory concentration indices displayed higher synergism in combination of plant extract, honey and ciprofloxacin against *P. mirabilis* at 0.02 compared to drug combination synergy standard (≤ 0.5). This work revealed augmentation of ciprofloxacin potency when combined with purified *E. hirta* acetone extract and honey and implies their high potential in the treatment of multidrug resistant infection of otitis media.

Keywords: Acetone extract; Antibacterial; Fractional inhibitory concentration index; Honey; Multidrug resistance.

Resumen: Este estudio investigó el potencial antibacteriano de extractos de plantas enteras de *Euphorbia hirta*, miel y antibióticos convencionales y sus efectos sinérgicos contra cepas bacterianas seleccionadas multirresistentes y tipificadas asociadas con la otitis media. El extracto de la planta entera de *E. hirta* se purificó usando la técnica de cromatografía en columna. Los ensayos antibacterianos de extractos se realizaron utilizando procedimientos microbiológicos estándar. La fuga de iones de proteínas, sodio y potasio de las mezclas sinérgicas se determinó mediante fotometría de llama. A 100 mg/ml, los extractos de acetona presentaron la mayor inhibición contra *S. aureus* (NCTC 6571) con una zona de inhibición de 32 ± 0,83 mm. Los índices de concentración inhibitoria fraccional mostraron un mayor sinergismo en combinación de extracto de planta, miel y ciprofloxacina contra *P. mirabilis* a 0,02 en comparación con el estándar de sinergia de combinación de fármacos (≤ 0,5). Este trabajo reveló un aumento de la potencia de la ciprofloxacina cuando se combina con extracto de acetona purificado de *E. hirta* y miel e implica su alto potencial en el tratamiento de infecciones de otitis media resistentes a múltiples fármacos.

Palabras clave: Extracto de acetona; Antibacteriano; Índice de concentración inhibitoria fraccional; Miel; Resistencia a múltiples fármacos.

INTRODUCTION

Otitis media (OM) an inflammation of the middle ear region, is one of the leading causes of healthcare visits, it affects all age group but prevalent in infants and children below the age of 10 (Dickson, 2014; Ashik, 2016). This is usually as a result of immune system, shorter/horizontal eustachian tube, which permits easier access to organisms from the naso-pharynx into the middle ear (Ilechukwu *et al*., 2017), and forced feeding (Basnet *et al*., 2017). OM also can be due to manifold etiology like nasopharyngeal, sinusitis or oropharyngeal infections (Ibekwe & Nwaorgu, 2011). Common symptoms include ear discharge, deafness, itching, pain and sometimes fever (Ibekwe & Nwaorgu, 2011). Infection often spreads from middle-ear to neighboring structures such as mastoid air cells, facial nerve, labyrinth, lateral sinus, meninges and brain with complications like neurological sequalae and brain damage (Ibekwe & Nwaorgu, 2011).

The common bacterial causative organisms of OM are *Streptococcus pyogenes*, *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella species* or mixed infections/organisms (Ibekwe & Nwaorgu, 2011; Nwokoye *et al*., 2012). Treatment is usually by topical or systemic antibiotic therapy (Mittal *et al*., 2018). The high rate at which bacteria develop resistance to common antibiotics has become a major problem in treating OM (Deshpande *et al*., 2014). This led to the increase in the search for alternative medicine (medicinal plants, nanotechnology) with varied chemical structures with novel mechanisms of action, against emerging and pre-existing diseases (Deshpande *et al*., 2014).

Medicinal plants are of major interest because of their usefulness in treating infectious diseases traditionally worldwide (Onyeka *et al*., 2018). They have diverse secondary metabolites as called phytochemicals, such as tannins, alkaloids, terpenoids, flavonoids, quinines and phenols (Gupta & Gupta, 2019). *Euphorbia hirta* is an herb that belongs to the Euphorbiaceae family, the plant is small in size, hairy, and its common name is asthma weed (Adjeroh *et al*., 2015). The Igbo, Hausa and Yoruba ethnic groups of Niger call it OgbunaIzu, Kadanya, Emi-ile respectively (Adjeroh *et al*., 2015), and this can grow up between 40-50 cm. The stem of the plant is red, covered with yellow thick hairs (Patel & Patel, 2014). Researchers have reported its efficacy in bronchitis treatment, skin diseases, earache, eye and ear infections, bronchial disease, asthma, kidney stones, cough, also as sedative, antipyretic, analgesic, and as anti-inflammatory agent (Patel & Patel, 2014; Igoli *et al.,* 2016; Jakhar & Dahiya, 2017; Gupta & Gupta, 2019).

Honey is a natural product that is traditionally used in the treatment of diabetics, inflammation, burns, wound dressings, bacterial diarrhea and gastroenteritis in children and infants, cold and a flu symptom like coughs, sore throats, and congestion (Adeoye-Isijola *et al*., 2017). Reports show its effectiveness as eardrops in treating ear infection (Ramalivhana *et al*., 2014; Henatsch *et al*., 2015; Adeoye-Isijola *et al*., 2017 and Henatsch *et al*., 2017). The low pH concentration, hydrogen peroxide, and the high sugar are known bacterial inhibitory constituents of honey (Sabaa & Zaat, 2012). Also, methylglyoxal and antimicrobial peptide bee defensin-1 were reported to be important antibacterial compound (Sabaa & Zaat, 2012).

Combination therapy is an alternative therapeutic method which combines two or more antimicrobial agents together to combat resistance (Omoya & Ajayi, 2016). The need to reduce cost on disease treatment, increase efficacy of drugs, lower doses and toxicity of drugs had led to this new treatment method. The combination of plant and honey has been presented in literature (Tariq *et al*., 2014; Sheas *et al*., 2019). Studies revealed that medicinal plants and honey mixture can combat multidrug resistance of bacterial isolates and shows potential to being a source of antibiotic resistance modifying compounds (Tariq *et al*., 2014; Oluyege *et al*., 2019).

However, there is lack of information on the synergistic antibacterial effects of *E. hirta* and honey on multiple antibiotic resistant pathogens associated with OM. Hence, this research focused on the comparative evaluation of antibacterial effects of purified acetone extract of *E. hirta* whole plant and honey on multiple antibiotic resistant bacteria implicated in OM, their synergistic effect in combination and their potentiating effects when combined with conventional antibiotics.

MATERIALS AND METHODS

Collection of bacterial isolates

Clinical bacterial isolates used were locally isolated from ear swab samples of patients with otitis media and were obtained from University of Medical Sciences Teaching Hospital, Akure, Nigeria and typed isolates from Federal Institute of Industrial Research, Oshodi. The clinical bacterial isolates were *S. aureus*, *E. coli*, *Klebsiella. pneumoniae*, *Proteus*

mirabilis, *Pseudomonas aeruginosa*, and *S. pyogenes*, while the typed bacterial isolates were *S. aureus* (NCTC 6571), *S. pyogenes* (ATCC 12384), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13883) and *P. aeruginosa* (ATCC 10145) which served as reference strains (positive control). The confirmation of organism was done using morphological identification and biochemical tests. The stock cultures were maintained at 4°C on slopes of Nutrient agar (Hi-media) and sub-cultured for 24 h before use (AOAC, 2012).

Collection of honey samples

Pure honey was obtained from Nectar Honey store in Akure, Ondo state, Nigeria. The honey was sterilized using suction pressure with 0.45 µmillipore membrane filter. Filtrate was streaked aseptically on nutrient agar plates and incubated at 37ºC for 24 h to test sterility. The honey sample was diluted using 5% dimethyl sulfoxide (DMSO) at different concentrations (25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL, 200 mg/mL and 400 mg/mL) and stored in the refrigerator at 4ºC prior use.

Collection of the whole E. hirta plant

Fresh *E. hirta* whole plant was collected at environs of Federal University of Technology Akure (FUTA), plant identification and authentication was done at the Crop, Soil and Pest Management Department, FUTA.

Preparation of plant and Extraction of acetone extracts

Euphorbia hirta collected was rinsed in distilled water, air-dried and pulverized. The crude extract was obtained by soaking 100 g of pulverized plant in 500 mL of acetone solvent and allowed to stand overnight inside a shaking water bath at 40°C. A clean muslin cloth and Whatman No. 1 filter paper was used to filter the mixture. A rotary evaporator (RE-52A Union Laboratories, England) was used to remove the solvent. Dry weights of the extracts were measured and reported.

The percentage extract yield was estimated as $\frac{\text{Dry weight ofplant extract}}{\text{Weight of pulverized plant material}}$ x 100%

Reconstitution of extracts

Crude acetone extract of *E. hirta* (20 mg) was dissolved in 0.1 mL of 5% DMSO to prepare a standard solution of 200 mg/mL. Each concentration 100, 50, 25, 12.5, 6.25, 3.085 and 1.03 mg/mL were prepared from the standard solution by double dilution method.

Fractionation of plant extract

Euphorbia hirta acetone extract was chromatographed on silica gel (60 - 120 mesh size) matrix packed into a glass column, and successively eluted with 100% petroleum ether, 100% chloroform, 100% ethyl-acetate and 100% methanol. The sample was mixed with a small quantity of gel to form powder, this was poured carefully on top of the silica gel already packed in the glass column. Then, it was covered with glass wool which prevented splattering of the eluant on the extract. The solvent system was gently poured on the sample by the side wall of the inside column through a glass funnel. The column tap was opened gently, this allowed eluant to flow at a rate of 30 drops per minute. The eluted fractions were collected in 100 mL conical flask. (Igwe *et al*., 2015; Oseni *et al.,* 2019).

Thin layer chromatography

Thin layer chromatography (TLC) was used to examine the fractions. The method adopted was Harborne *et al.* (1971). Each fraction was spotted on aluminum plates pre-coated with silica gel (60 F254), then eluted using Petroleum ether/water (5:5), chloroform, ethyl acetate in a small TLC tank. The samples were spotted 0.5 cm from the margin and slanted in the TLC tank. The distance moved by the sample and the distance moved by the solvent were measured and recorded. The Resolution front (Rf) was determined by the ratio of the distance moved by the sample and the solvent.

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The stock purified extracts were prepared by pooling fractions of same solvents together and dissolving in 5% DMSO and stored at 4ºC as prior to use.

Quantitative and qualitative phytochemical constituents of E. hirta extracts and honey

The plant extract was investigated to determine the presence and quantity of phytochemical constituents such as tannins, phenols, alkaloids, glycosides,

anthroquinones, saponins and flavonoids using specific reagents according to AOAC (2012).

Standardization of Inoculum

The CLSI (2014) procedure was adopted for the standardization of bacterial inoculums before carrying out antibiotic sensitivity. The McFarland's standard of (1%v/v) sulphuric acid solution which was prepared with (1% w/v) solution of barium chloride (BaCl and $2H_2O$) served as the inoculum standard.

Antibiotics sensitivity for bacterial isolates

The Disk diffusion method of Omoya and Ajayi (2016) was adopted for antibiotic susceptibility testing and interpreted using the CLSI 2014 guidelines. Standardized inoculum was used, antibiotic disc was placed aseptically on the surface of the molten Mueller-Hinton agar and allowed to stand for 30 min to pre-diffuse. The gram negative disc contains CN- Gentamycin 10 µg, S-Streptomycin 30 µg, PEF- Pefloxacin 10 µg, OFL-Tarivid 10 µg, SXT-Septrin 30 µg, CH-Chloramphenicol 30 µg, SP- Sparfloxacin 10 µg, CPX- Ciprofloxacin 10 µg, AM- Amoxacillin 30 µg, AU- Augumentin 30 µg, while the gram positive disc contains CN-Gentamycin 10 µg, S-Streptomycin 30 µg, PEF- Pefloxacin 10 µg, SXT-Septrin 30 µg, CPX- Ciprofloxacin 10 µg, AM-Amoxicillin 30 µg, APX-Ampiclox 30 µg, E-Erythromycin 10 µg, Z-Zinnclof 20 µg, R-Rocephin 25 µg; manufactured by Maxicare Medical Lab. The inhibition zones were measured and recorded, the results were interpreted using standard charts by CLSI, 2014.

Antibacterial Assay of acetone extract of E. hirta and honey on bacterial isolates

Antibacterial activity of acetone extract of *E. hirta* and honey against test bacteria was carried out using agar-well diffusion method of Abegunde *et al*. (2018). Standardized inoculums were streaked on Mueller Hinton agar plates with sterilized cotton swabs and allowed to set for 15 min. A sterile borer was used to bore wells of 6 mm diameter and 3 mm depth in the solidified agar. A 1ml aliquot of the crude extract of *E. hirta*, purified *E. hirta* extracts (100 mg/mL) and honey (400 mg/mL) were dispensed into the wells aseptically and allowed to stand for 15 min for samples to pre-diffuse. A 1 mL of ciprofloxacin (0.1 mg/mL) was used as positive control while distilled water as a negative control. The plates stood upright for an hour to allow diffusion of the solutions into the medium and was incubated at 37ºC for 24 h. Zone of inhibition surrounding the wells were measured and recorded in millimetre (mm).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of E. hirta whole plant extract, honey and ciprofloxacin on test bacterial isolates

The Broth dilution method of Adegoke *et al*. (2010) was used to determine MIC. Prepared stock solutions of purified *E. hirta* whole plant acetone extracts and honey was used, 1 mL each of the extracts and honey stock concentration of 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg was allotted in different test tubes containing sterile broth. The experiment control were sterile tubes that contained ciprofloxacin, and distilled water respectively. 1 mL of standardized inoculum was transferred each into all the tubes and mixed thoroughly. The tubes were incubated at 37°C for 24 h and the bacterial growth were observed. The MIC was the lowest concentration of extract with no visible bacterial growth or no turbidity (CLSI, 2014). A 0.1 mL of bacterial suspension from the MIC tubes was streaked on solidified Mueller Hinton agar plates and incubated at 37°C for 24 h. After incubation, the minimum bactericidal concentration (MBC) was recorded as the concentration at which no visible growth (Abegunde *et al*., 2018).

Synergistic effect of E. hirta whole plant extract, honey and ciprofloxacin mixture on bacterial isolates.

The checkerboard method

The checker board method described by Shah and Williamson (2019) with modification was used to detect the synergism in the combinations. The extract, honey, antibiotics were combined by incorporating them into sterilized molten nutrient agar. After set, the plates were inoculated with standardized inoculums and incubated for 24 h at 37°C, the minimum inhibitory concentration (MIC) values were estimated. The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic, honey and extract combination that did not permit visible growth of the test organisms on the plates.

The FIC value for each agent was estimated using the following formula:

FIC (antibiotics) = MIC of antibiotics in combination/MIC of antibiotic alone.

FIC (extract) = MIC of extract in combination/MIC of extract alone
FIC (honey) = MIC of extract in combination/MIC of honey alone
The interactions between the antibiotics, honey and the extracts were assessed in terms of the FIC indices calculated using the formulas:
FIC Index (plant extract/honey and antibiotics) = FIC (plant extract/honey) + FIC (antibiotics)
FIC Index (plant extract and honey) = FIC (plant extract) + FIC (honey)
FIC Index (plant extract, honey and antibiotics) =FIC (plant extract) + FIC (honey) + FIC (antibiotics)
As described by Omoya and Ajayi, 2016, the antibacterial activity of the mixture indicates synergism if FICI $\leq$ 0.5, additive if FICI > 0.5 and $\leq$ 1, indifference if FICI > 1 and $\leq$ 2 and antagonism if FICI >2.

The time-kill method
The kill time method, using the broth macrodilution technique as described by Shah and Williamson (2019) with modifications was used to assay the antibacterial effect of the combinations. The extract, honey and antibiotics combination that showed synergistic interaction in the checkerboard assay were dispensed into 50 ml of nutrient broth at the minimum inhibitory concentration. The controls for the experiment were broth, nutrient broth mixed with extract, honey and the antibiotic singly for each experiment. The test and control flasks were inoculated with each bacterial isolate to a final inoculum density of approximately 10^5cfu mL^{-1}. Aliquots (100 µL) of the negative control flasks were serially diluted in normal saline and plated on nutrient agar in order to determine the zero hour counts immediately. The test flasks were incubated at 37ºC for 24 h with shaking at 120 rpm. Samples from the test and control flasks were serially diluted in normal saline and plated (100 µL) on nutrient agar in triplicates. The numbers of colonies were estimated and the mean colony counts (cfu mL^{-1}) for each plate was determined and expressed as $\log_{10}$.

Determination of bacterial cell leakages after treatment with E. hirta extract, honey and antibiotics.
Potassium (K^+) and sodium (Na^+) ions leakage
The Hugo and Russel (1997) method was adopted for determining the sodium/potassium ions leakages from susceptible microorganism. The cells 18 h old nutrient broth culture was washed twice in normal saline then centrifuged before use. A 0.5 mL of the cell suspension of each organism was added to 4.5 mL of the prepared concentration of the synergistic combinations. After centrifugation at 6000 rpm at interval of 20 min, the supernatant solutions obtained were assayed for potassium/sodium ion with the use of a flame-photometer. Each supernatant reading was done in triplicates.

Protein ion leakage
Test bacterial cells picked from an 18 h old nutrient broth culture was washed separately in 0.9% w/v physiological saline. Washed suspension of standardized inoculum was treated with minimum inhibitory concentration of the *E. hirta* acetone extract, honey and ciprofloxacin for 20 min. Each suspension was then centrifuged at 7000 rpm, the supernatant collected was analyzed for the presence of protein using Bradford method by Abegunde *et al*. (2018). In assaying for protein, 0.4 mL Bradford reagent was added to 1.6 mL of sample (0.2 mL supernatant plus 1.4 mL sterile distilled water) to make up a total volume of 2 mL. After 5 min Optical density (OD) of the resulting solution was thereafter taking at 595 nm.

Gas Chromatography – Mass Spectrometry (GC-MS) elucidation of bioactive compounds in E. hirta acetone extract
The bioactive constituent in the *E. hirta* extract was investigated using (GC-MS) according to Abegunde *et al*. (2018). A Varian 3800 gas chromatograph equipped with an Agilent MS capillary column (30 m × 0.25 mm i.d.) that was connected to a Varian 4000 mass spectrometer operating in the EI mode (70 eV; m/z 1 – 1000; source temperature 230ºC and a quadruple temperature 150°C) was used for the GC-MS analysis. Initially the temperature of the column was maintained at 200°C for 2 min, later increased to 300°C, at 4°C/min, and maintained for 20 min at 300°C. Nitrogen was the carrier gas and its flow rate was 1.0 mL/min. The temperature of the inlet was maintained at 300°C with a split ratio of 50:1. A sample volume of 1µL in chloroform was injected using a split mode, with the split ratio of 50:1. The mass spectrometer was set to scan in the range of m/z 1-1000 with electron impact (EI) mode of ionization. The analysis of the sample was carried out, the runtime was 70min, and a computer search on NIST Ver.2.1 MS data library was done and comparing the spectrum obtained through GC - MS compounds present in the samples were identified. The sample and replicates were injected continuously as one batch, in a random order to distinguish technical

from biological variations. Additionally, the prepared pooled samples were used as quality controls, which were injected at regular intervals throughout the analytical run to provide a set of data from which the repeatability can be assessed.

Statistical analysis

Statistical comparison of the quantitative phytochemical composition, antibiotic susceptibility pattern, antibacterial effect of plant extract and MIC data were carried out in triplicate and data obtained were expressed as mean ± SEM using two-way analysis of variance. Means comparison was done using Duncan's new multiple range test with level of significance set at $p \leq 0.05$. SPSS software, version 20.0 was used for statistical analysis. The time-kill kinetics data was analyzed using Microsoft excel 2016.

RESULTS

Percentage yield of E. hirta crude acetone extract

The acetone extract showed extraction yield of 19% (Figure No. 1).

Phytochemical constituent of the crude whole E. hirta plant extracts and honey

Flavonoids, terpenoids saponins, glycosides, and steroids were present in the samples, tannin, terpenoids and phenol was present in only the plant while phlobatanins and alkaloids were absent in both the plant extracts and honey. Saponin (68.63 ± 0.37) and phenols (35.3 ± 0.50) were more abundant in acetone extract while glycoside was the most abundant in honey with 6.97 ± 0.009. (Table No. 1 and Table No. 2).

The proximate composition of honey sample

The honey sample used in this study has a high carbohydrate content of 77.950 ± 0.010, moisture content of 9.454 ± 0.004, energy value of 1541.523 kj/g but has no crude fibre (Table No. 3).

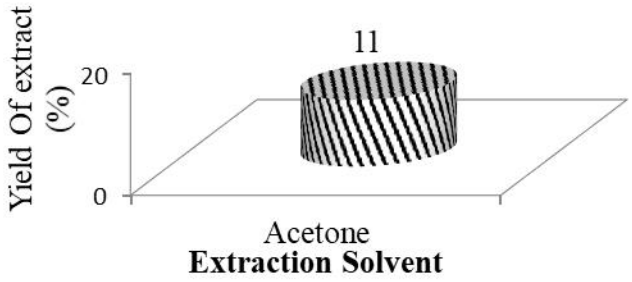

Figure No. 1
Percentage yield (%) of acetone extract of whole *Euphorbia hirta* plant

Table No. 1
Qualitative phytochemical constituent of Honey and acetone extract of whole *Euphorbia hirta* plant

Solvent	Phytochemical constituents											
	Saponin	Glycoside	Tannin	Phlobatanin	Flavonoid	Steroid	Terpenoid	Alkaloid	Phenol	Keiller's	Salkowski	Legals
Acetone	+	+	+	-	+	+	+	-	+	+	+	+
Honey	+	+	-	-	+	+	-	-	-	+	-	+

Key: + Present, - Negative

Table No. 2
Quantitative constituents of phytochemical of honey and acetone extract of whole *Euphorbia hirta* plant

Solvents	Phytochemical constituents						
	Saponins	Flavonoids	Tepernoids	Steroids	Tannins	Phenols	Glycosides
Acetone	68.63±0.37^{c}	2.98±0.34^{c}	30.64±0.17^{c}	3.04±0.03^{e}	1.74±0.06^{d}	35.03±0.50^{e}	2.84±0.09^{d}
Honey	1.00±0.37^{a}	0.41±0.34^{a}	0.00±0.17^{a}	1.07±0.03^{b}	0.00±0.06^{a}	0.00±0.50^{a}	6.97±0.09^{b}

Data are represented as mean ± SE (standard error)
Values with the same superscript letters along the same column are not significantly different ($p \leq 0.05$)

Table No. 3
Proximate components of honey

Parameters	Proximate value (%)
Ash	1.181±0.001
Moisture	9.454±0.004
Crude fat	1.116±0.002
Crude fibre	0.000±0.000
Protein	10.299±0.000
Carbohydrate	77.950±0.010

Energy value is 1541.525 KJ/g
Data are represented as mean ± standard error (n=3)

Chromatographic purification of E. hirta whole plant extracts

Eight fractions were separated, and using chloroform as mobile phase solvent, the retention factor ranged between 0.1833 – 0.9533 (Table No. 4).

Table No. 4
Retention factor for acetone extract of whole *Euphorbia hirta* plant

Fraction	Sample font	Solvent Font	R_f value	Solvent for mobile phase
A	1.1	6.0	0.1833	Chloroform
B	1.8	6.0	0.3000	Chloroform
C	2.9	6.0	0.4533	Chloroform
D	2.3	6.0	0.5300	Chloroform
E	3.7	6.0	0.6167	Chloroform
F	4.3	6.0	0.7167	Chloroform
G	5.7	6.0	0.9500	Chloroform
H	5.9	6.0	0.9833	Chloroform

Antibiotic resistance patterns of bacterial isolates

Generally, all the test isolates were resistant to at least 5 of the 10 antibiotics present on the conventional antibiotic disc (Tables Nº 5 and Nº 6).

Antibacterial effect of whole E. hirta plant extracts and honey against bacteria

The crude and purified extract showed highest inhibitory effect against *S. aureus* ATCC 6571 with zone of 25 ± 0.63 mm and 30 ± 0.37 mm respectively. Honey showed highest inhibitory effect against *E. coli* ATCC 13883 with zone of 18.5 ± 0.37 mm. Plate 1shows the antibacterial effect of extract of *E. hirta* extract on *S. aureus* with zone of 12 ± 0.43 mm recorded in the purified extract, while crude showed no zone of inhibition (Figures Nº 2 and Nº 3).

Minimum inhibitory concentration of E. hirta acetone extract, honey and ciprofloxacin singly and in combination

The MIC of acetone extract on the test isolates ranged from 1.56 mg/mL - 50 mg/mL, for honey it ranged from 50 mg/mL - 200 mg/mL and for ciprofloxacin it ranged from 0.0125 mg/mL - 0.1 mg/mL. For the mixture of acetone extract and honey the MIC on the bacterial isolates ranged from 1.56 mg/mL – 25 mg/mL, for the combination of acetone extract and ciprofloxacin ranged from 0.025 mg/mL - 0.1 mg/mL, for honey and ciprofloxacin ranged from 0.0125 mg/mL - 0.1 mg/mL and for honey ciprofloxacin and acetone extract ranged from 0.0065 mg/mL - 0.1 mg/mL (Table Nº 7).

Minimum bactericidal concentration of E. hirta acetone extract, honey and ciprofloxacin singly and in combination

The MBC of acetone extract ranged from 25 mg/mL - 100 mg/mL, for honey ranged from 100 mg/mL - 400 mg/mL, and for ciprofloxacin ranged from 0.025 mg/mL - 0.1 mg/mL. The MBC for the combination of acetone extract and honey ranged from 25 mg/mL - 100 mg/mL, for the combination of honey and ciprofloxacin ranged from 0.005 mg/mL - 0.1 mg/mL and for the combination of acetone extract and ciprofloxacin and the combination of acetone extract, honey and ciprofloxacin, the MIC ranged from 0.025mg/mL - 0.1 mg/mL (Table Nº 8).

Table No. 5
Antibiotic susceptibility pattern of selected multidrug resistant Gram-negative bacterial isolate

Isolates	CN	PEF	OFL	S	SXT	CH	SP	CPX	AM	AU
	Zones of Inhibition (mm)									
E. coli	0.00 ± 0.06^{a}	12.00 ± 0.12^{b}	10.0 ± 0.15^{c}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.30 ± 0.09^{a}	9.00 ± 0.12^{b}	0.00 ± 0.08^{c}	10.00 ± 0.03^{a}
P. mirabilis	12.00 ± 0.06^{b}	14.37 ± 0.12^{a}	8.00 ± 0.15^{a}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 0.09^{c}	8.03 ± 0.12^{a}	0.00 ± 0.08^{a}	0.00 ± 0.03^{a}
K. pneumoniae	0.00 ± 0.06^{a}	0.00 ± 0.12^{a}	0.00 ± 0.15^{a}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.09^{a}	8.37 ± 0.12^{a}	10.30 ± 0.08^{b}	0.00 ± 0.03^{a}
P. aeruginosa	0.00 ± 0.06^{a}	0.00 ± 0.12^{a}	0.00 ± 0.15^{a}	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.09^{a}	0.00 ± 0.12^{a}	0.00 ± 0.08^{a}	0.00 ± 0.03^{a}

KEY: CN - Gentamycin 10 μg, S- Streptomycin 30 μg, PEF- Pefloxacin 10 μg, OFL- Tarivid 10 μg, SXT-Septrin 30 μg, CH- Chloramphenicol 30 μg, SP - Sparfloxacin 10 μg, CPX - Ciprofloxacin 10 μg, AM - Amoxicillin 30 μg, AU - Augumentin 30 μg
Data are represented as mean ± SE (standard error)
Each value is a mean of three (3) replicates
Values with the same superscript letters along the same column are not significantly different ($p \leq 0.05$)

Table No. 6
Antibiotic susceptibility pattern of selected multidrug resistant Gram-positive bacterial isolate

Isolates	CN	PEF	S	SXT	CPX	AM	APX	E	Z	R
	Zones of inhibition(mm)									
S. pyogenes	0.00 ± 0.00	8.37 ± 0.12[b]	0.00 ± 0.00	0.00 ± 0.00	10.37 ± 0.12[b]	0.00 ± 0.00	9.00 ± 0.15[b]	11.00 ± 0.03[b]	0.00 ± 0.00	0.00 ± 0.00
S. aureus	0.00 ± 0.00	0.00 ± 0.12[a]	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.12[a]	0.00 ± 0.00	0.00 ± 0.15[a]	0.00 ± 0.03[a]	0.00 ± 0.00	0.00 ± 0.00

KEY: CN - Gentamycin 10 µg, S - Streptomycin 30 µg, PEF - Pefloxacin 10 µg, SXT-Septrin 30 µg, CPX - Ciprofloxacin 10 µg, AM - Amoxicillin 30 µg, APX - Ampiclox 30 µg, E - Erythromycin 10 µg, Z - Zinnclof 20 µg, R - Rocephin 25 µg
Data are represented as mean ± SE (standard error). Values with the same superscript letters along the same column are not significantly different ($p \leq 0.05$). Each value is a mean of three (3) replicates

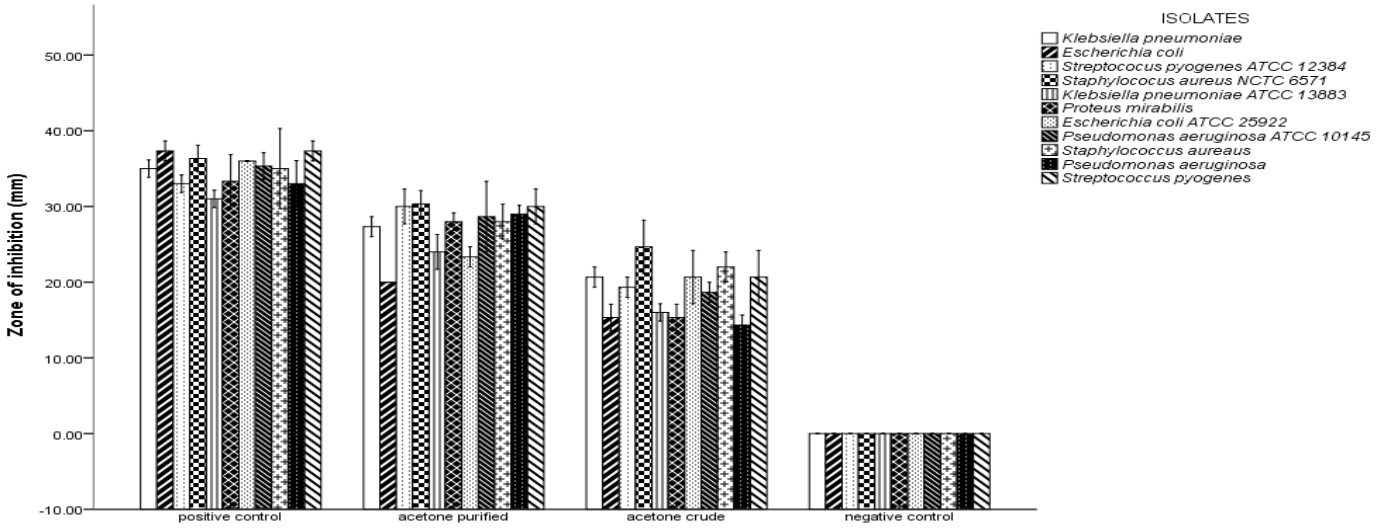

Figure No. 2
Antibacterial effect of *Euphorbia hirta* acetone extract (100 mg/mL) on bacterial isolates
Key: Positive control=ciprofloxacin (0.1 mg/mL), Negative control=Distilled water

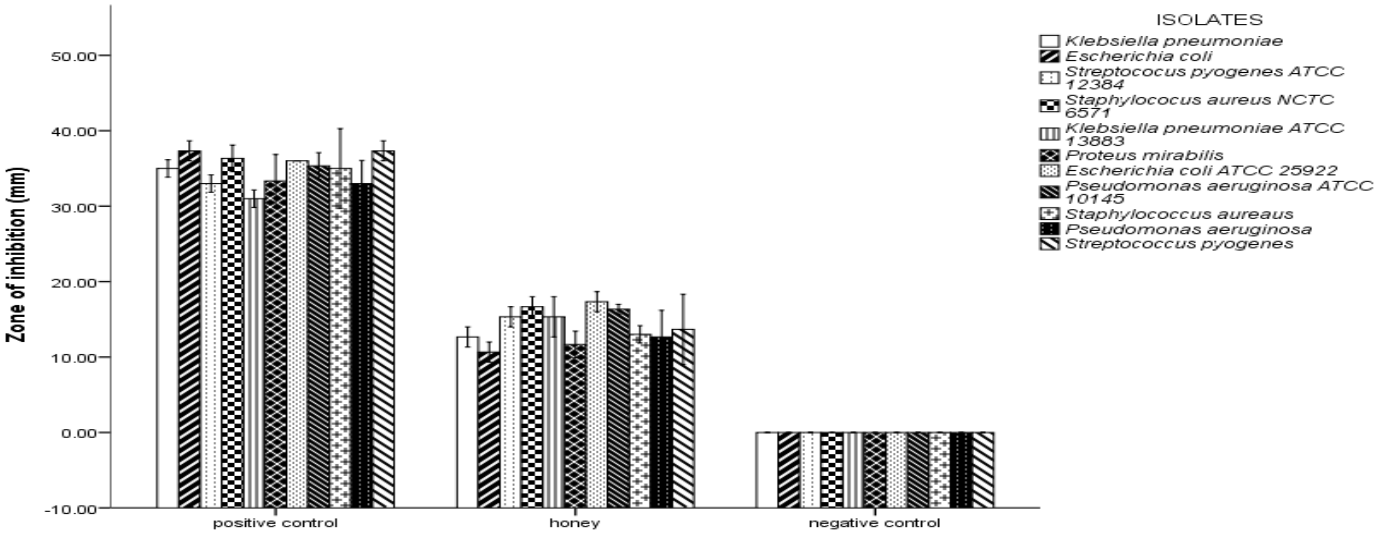

Figure No. 3
Antibacterial effect of honey (400 mg/mL) on bacterial isolates
Key: Positive control=ciprofloxacin (0.1 mg/mL), Negative control=Distilled water

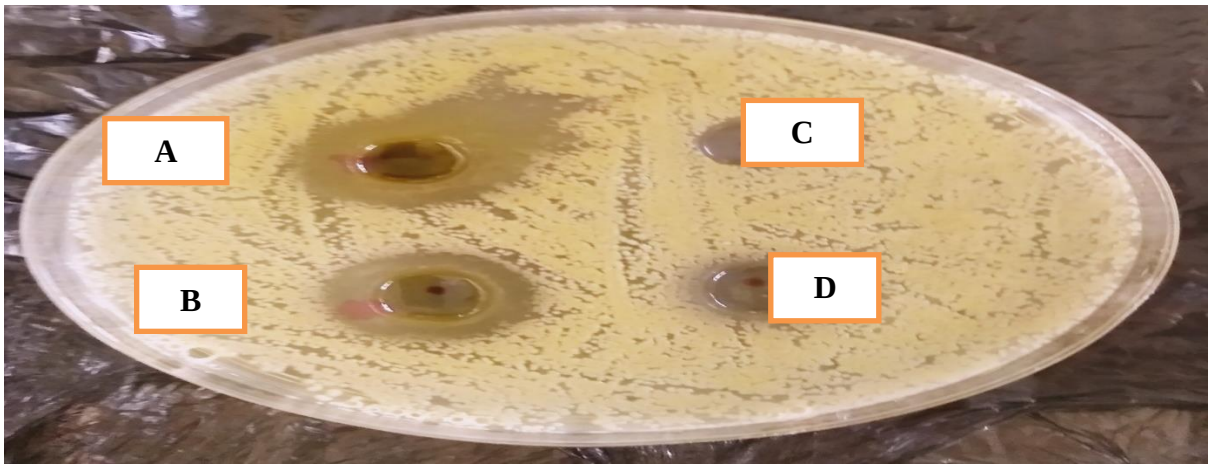

Plate No. 2
Zone of inhibition of purified and crude *Euphorbia hirta* hot water extract against *E. coli*
Key: A = ciprofloxacin, B = purified *Euphorbia hirta* acetone extract, C = distilled water, D = mcrude acetone extract

Table No. 7
Minimum inhibitory concentration (mg/mL) of *Euphorbia hirta* extracts, honey and ciprofloxacin against bacterial isolates

Test isolate	E	H	C	E + H	E + C	H + C	E+H+ C
P. aeruginosa	50.000	100.00	0.1000	12.500	0.1000	0.1000	0.1000
K. pneumoniae	25.000	50.000	0.0250	6.2500	0.0500	0.0500	0.0250
S. aureus	6.2500	100.00	0.0250	6.2500	0.0500	0.0500	0.0250
P. mirabilis	6.2500	100.00	0.0250	1.5600	0.0250	0.0250	0.00650
S. pyogenes	25.000	100.00	0.0250	12.500	0.0500	0.0500	0.0250
E. coli	50.000	200.00	0.1000	25.000	0.1000	0.1000	0.1000
***P. aeruginosa* ATCC 10145**	25.000	50.000	0.0500	12.500	0.0500	0.0500	0.0500
***K. pneumoniae* ATCC 13883**	1.5600	50.000	0.0250	1.5600	0.0500	0.0125	0.0250
***S. aureus* NCTC 6571**	12.500	100.00	0.0250	6.2500	0.0250	0.0500	0.0250
***S. pyogenes* ATCC 12384**	3.1250	200.00	0.0125	1.5600	0.0250	0.0125	0.0125
***E. coli* ATCC 25922**	6.2500	50.000	0.0100	6.2500	0.1000	0.1000	0.0500

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin

Table No. 8
Minimum bactericidal concentration (mg/mL) of *Euphorbia hirta* acetone extract, honey and ciprofloxacin against the bacterial isolates

Test Isolates	E	H	C	E+H	E+C	H+C	E+H+C
P. aeruginosa	100.00	400.00	0.1000	50.000	0.1000	0.1000	0.1000
K. pneumoniae	50.000	100.00	0.0500	50.000	0.0500	0.0500	0.0500
S. aureus	25.000	100.00	0.0500	50.000	0.0500	0.0500	0.0500
P. mirabilis	50.000	200.00	0.0500	25.000	0.0250	0.0500	0.0500
S. pyogenes	100.00	200.00	0.1000	50.000	0.1000	0.0500	0.1000
E. coli	100.00	400.00	0.1000	100.00	0.1000	0.1000	0.1000
P. aeruginosa ATCC 10145	50.000	100.00	0.0500	50.000	0.0500	0.1000	0.0500
K. pneumoniae ATCC 13883	50.000	100.00	0.0500	25.000	0.0500	0.0500	0.0500
S. aureus NCTC 6571	25.000	100.00	0.0500	50.000	0.0500	0.1000	0.0500
S. pyogenes ATCC 12384	50.000	200.00	0.0250	25.000	0.0250	0.0500	0.0250
E. coli ATCC 25922	50.000	400.00	0.1000	50.000	0.1000	0.1000	0.1000

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin

Synergistic interaction of the combination of acetone extract of E. hirta, honey and ciprofloxacin against selected multiple antibiotic resistant bacteria

Synergistic interaction was shown against *P. aeruginosa, K. pneumoniae, and P. mirabilis* with FIC indices of 0.375, 0.0375, and 0.266 respectively (Table No. 9).

Synergistic interaction was shown against *K. pneumoniae* ATCC 13883 with FIC index of 0.500 (Table No. 10). No synergistic interaction was observed (Table No. 11). Synergistic interaction was shown against *P. mirabilis* and *E. coli* ATCC 25922 with FIC index of 0.211 and 0.5 respectively (Table No. 12).

Table No. 9
Fractional inhibitory concentration (FIC) index for *Euphorbia hirta* acetone extract and honey against the bacterial isolates

Isolates	FIC E	FIC H	∑FIC	Remark
P. aeruginosa	0.25	0.13	0.47	Synergistic
K. pneumoniae	0.25	0.13	0.47	Synergistic
S. aureus	1.00	0.06	1.06	Indifferent
P. mirabilis	0.03	0.02	0.05	Synergistic
S. pyogenes	0.50	0.13	0.63	Additive
E. coli	0.50	0.13	0.63	Additive
P. aeruginosa ATCC 10145	0.50	0.03	0.53	Additive
K. pneumonia ATCC 13883	1.00	0.03	1.03	Indifferent
S. aureus NCTC 6571	0.50	0.06	0.56	Additive
S. pyogenes ATCC 12384	0.50	0.01	0.51	Additive
E. coli ATCC 25922	1.00	0.13	1.13	Indifferent

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin, FIC = Fractional inhibitory concentration

Table No. 10
Fractional inhibitory concentration index of honey and ciprofloxacin against the bacterial isolates

Isolates	FIC H	FIC C	∑FIC	Remark
P. aeruginosa	0.00010	1.000	1.00	Additive
K. pneumoniae	0.00010	2.000	2.00	Indifferent
S. aureus	0.00050	2.000	2.00	Indifferent
P. mirabilis	0.00030	1.000	1.00	Additive
S. pyogenes	0.00050	2.000	2.00	Indifferent
E. coli	0.00050	1.000	1.00	Additive
P. aeruginosa ATCC 10145	0.00010	1.000	1.00	Additive
K. pneumoniae ATCC 13883	0.00250	0.500	0.50	Synergistic
S. aureus NCTC 6571	0.00010	2.000	2.00	Indifferent
S. pyogenes ATCC 12384	0.000065	1.000	1.00	Additive
E. coli ATCC 25922	0.00200	1.000	1.00	Additive

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin, FIC = Fractional inhibitory concentration

Table No. 11
Fractional inhibitory concentration index for *Euphorbia hirta* whole plant acetone extract and ciprofloxacin against bacterial isolates from ear swab samples of otitis media

Isolates	FIC E	FIC C	∑FIC	Remark
P. aeruginosa	0.002	1.000	1.00	Additive
K. pneumonia	0.002	2.000	2.00	Indifferent
S. aureus	0.004	2.000	2.00	Indifferent
P. mirabilis	0.004	1.000	1.00	Additive
S. pyogenes	0.002	2.000	2.00	Indifferent
E. coli	0.002	1.000	1.00	Additive
P. aeruginosa ATCC 10145	0.002	1.000	1.00	Additive
K. pneumoniae ATCC 13883	0.031	2.000	2.03	Indifferent
S. aureus NCTC 6571	0.002	1.000	1.00	Additive
S. pyogenes ATCC 12384	0.008	2.000	2.00	Indifferent
E. coli ATCC 25922	0.016	1.000	1.00	Additive

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin, FIC = Fractional inhibitory concentration

Table No. 12
Fractional inhibitory concentration index for whole *Euphorbia hirta* plant acetone extract, honey and ciprofloxacin against bacterial isolates from ear swab samples of otitis media

Isolates	FIC E	FIC H	FIC CIP	∑FIC	Remark
P. aeruginosa	0.0020	0.00100	1.000	1.00	Additive
K. pneumonia	0.0001	0.00005	1.000	1.00	Additive
S. aureus	0.00400	0.00025	1.000	1.00	Additive
P. mirabilis	0.00104	0.00006	0.026	0.02	Synergistic
S. pyogenes	0.00100	0.00025	1.000	1.00	Additive
E. coli	0.00200	0.00050	1.000	1.00	Additive
P. aeruginosa ATCC 10145	0.00200	0.00010	1.000	1.00	Additive
K. pneumoniae ATCC 13883	0.01603	0.00050	1.000	1.01	Additive
S. aureus NCTC 6571	0.00200	0.00025	1.000	1.00	Additive
S. pyogenes ATCC 12384	0.00400	0.00006	1.000	1.00	Additive
E. coli ATCC 25922	0.00800	0.00100	0.500	0.50	Synergistic

Key: E = Acetone extract, H = Honey, C = Ciprofloxacin, FIC = Fractional inhibitory concentration

The time-kill of bacterial isolates by combination of whole Euphorbia hirta plant acetone extract, honey and ciprofloxacin

The percentage of the *P. aeruginosa* killed by the extract and honey combination after 24 h of contact was to 34.5% (2.9 – 1.7 log cfu/mL reduction in cell count), for *P. mirabilis* 72.4% (2.9 - 1.7 log cfu/mL), for *E. coli* ATCC 25922 was reduced to 72.4% (3.0 - 0.7 log cfu/mL), for *K. pneumoniae* ATCC 13883 was 43.8% (2.9 - 1.5 log cfu/mL) and for *K. pnuemoniae* was 44.8% (2.9 - 1.56 log cfu/mL) (Figures No. 4 - No. 8).

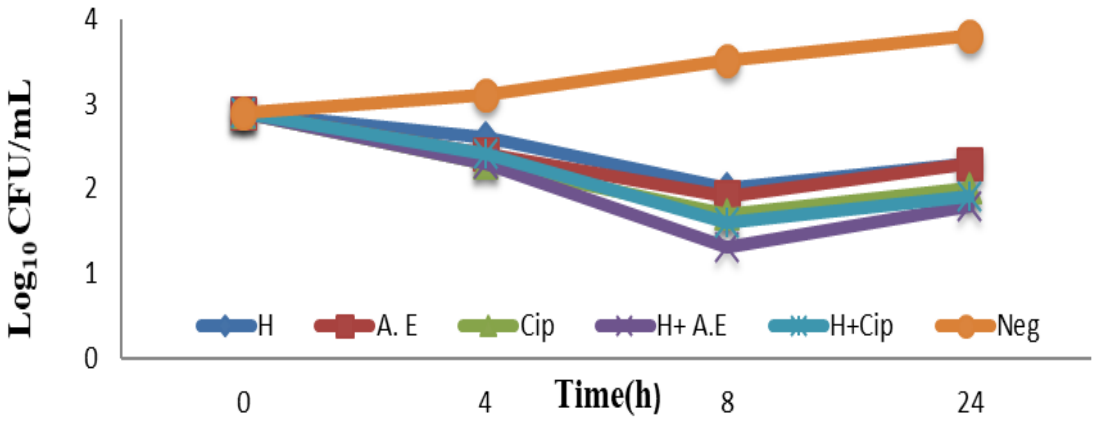

Figure No. 4
Time-kill kinetics of the bactericidal effect of acetone extract, honey and ciprofloxacin combination on *P. aeruginosa*. Key: H = Honey, A. E = Acetone extract, Cip = Ciprofloxacin, H+A.E = Honey + Acetone extract, H + Cip = Honey + Ciprofloxacin, Neg = Untreated

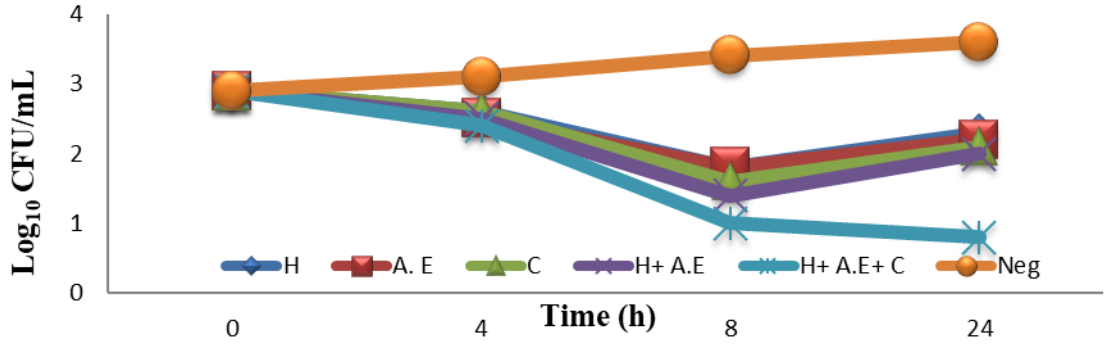

Figure No. 5
Time-kill kinetics of the bactericidal effect of acetone extract, honey and ciprofloxacin combination on *P. mirabilis*. Key: H = Honey, A.E = Acetone extract, C = Ciprofloxacin, H+A.E = Honey+ Acetone extract, H+E+C = Honey + Ciprofloxacin, Neg = Untreated

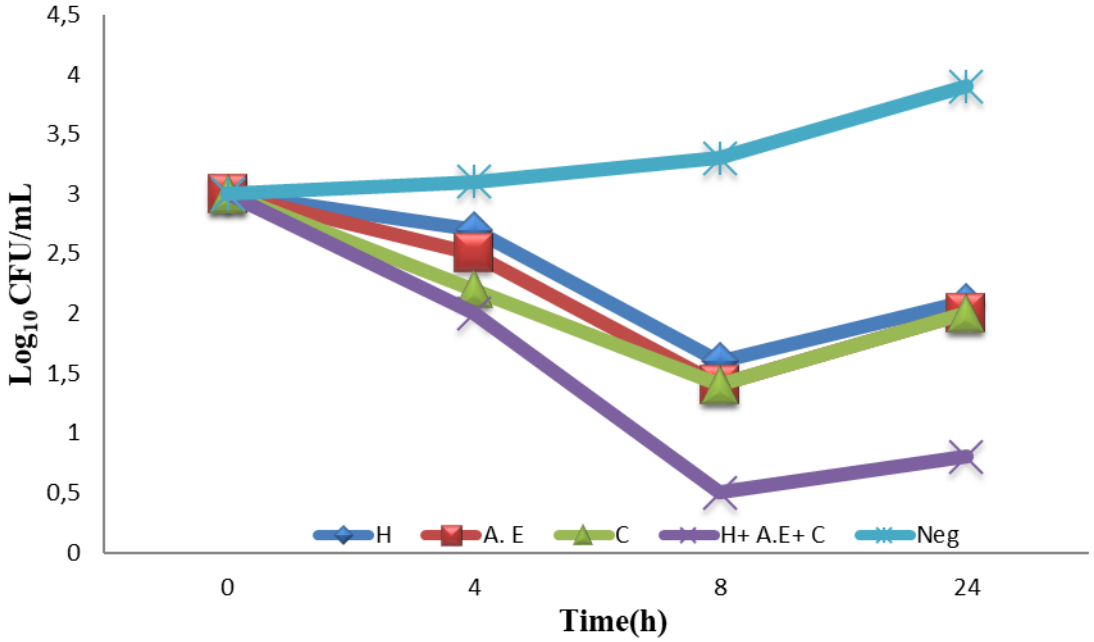

Figure No. 6
Time-kill kinetics of the bactericidal effect of acetone extract, honey and ciprofloxacin combination on *E. coli* ATCC 25922. Key: H = Honey, A.E = Acetone extract, C = Ciprofloxacin, H+A.E+C = Honey + Ciprofloxacin, Neg = Untreated

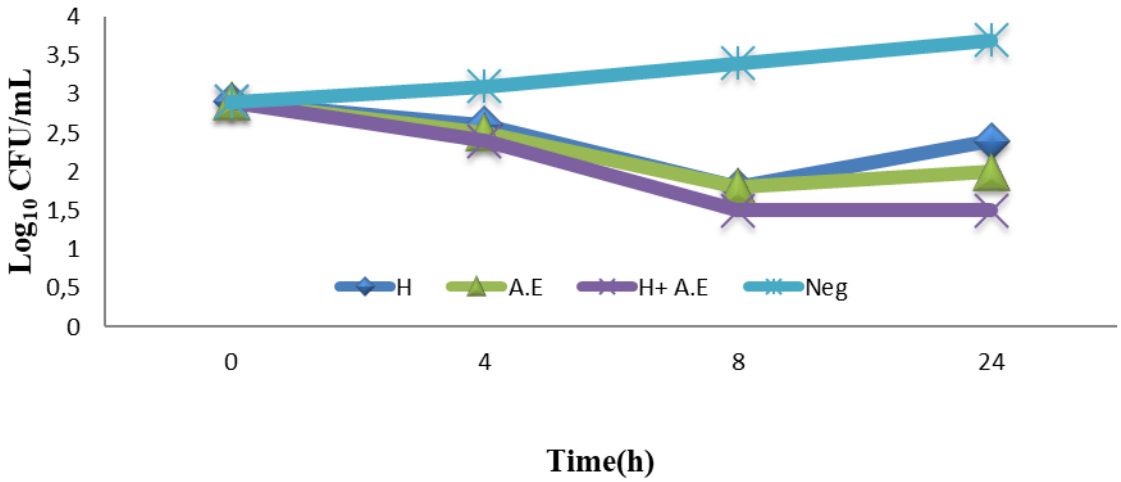

Figure No. 7
Time-kill kinetics of the bactericidal effect of honey and ciprofloxacin combination on *K. pneumoniae* ATCC 13883. Key: H = Honey, C = Ciprofloxacin, H+C = Honey + Ciprofloxacin, Neg = Untreated

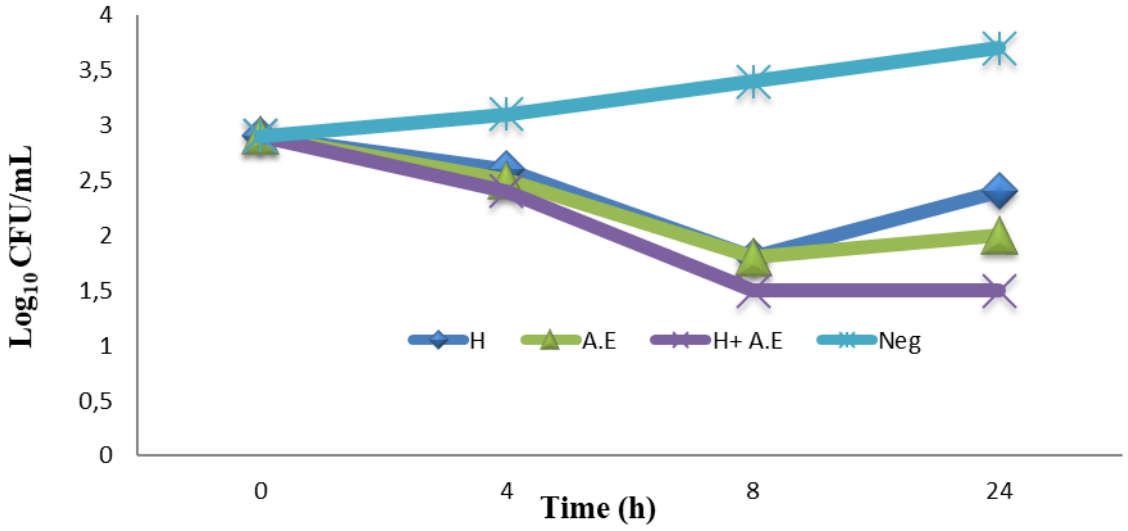

Figure No. 8
Time-kill kinetics of the bactericidal effect of acetone extract and honey combination on *K. pneumoniae*
Key: H = Honey, A.E = Acetone extract, H+A.E = Honey + Acetone extract, Neg = Untreated

Comparative potassium ion, sodium ion and protein leakage from bacterial isolates

The highest amount of potassium ion leaked was from *P. mirabilis* cells with 86.96 cmol/g from synergistic combination of acetone extract, honey and ciprofloxacin. The highest amount of sodium ion leaked was in *P. mirabilis* cells with 274.14 cmol/g from the synergistic combination of acetone extract, honey and ciprofloxacin. The highest amount of protein leaked was in *P. mirabilis* cells with 32.93 cmol/g from the synergistic combination of acetone extract, honey and ciprofloxacin and the least amount of protein leaked was in *E. coli* ATCC 25922 cells with 21.26 cmol/g from the synergistic combination of acetone extract, honey and ciprofloxacin (Tables No 13 – No. 15).

Table No. 13
Comparative potassium ion leakage (cmol/g) of synergistic *Euphorbia hirta* whole plant acetone extract, honey and ciprofloxacin combination

Isolate 1D	Control	Acetone extract	Honey	Ciprofloxacin	Acetone +honey	Acetone + ciprofloxacin	Honey + ciprofloxacin	Acetone + honey + Ciprofloxacin
P. aeruginosa	2.00	48.50	42.10	18.80	84.40	ND	ND	ND
K. pneumoniae	3.00	32.30	31.60	8.25	84.40	ND	ND	ND
P. mirabilis	2.45	49.35	43.10	11.11	74.17	ND	ND	86.96
K. pneumoniae ATCC 13883	2.36	39.30	41.25	10.10	ND	ND	81.84	ND
E. coli ATCC 25922	3.33	49.50	37.50	8.51	ND	ND	ND	69.06

Key: ND = Not determined, Control = Distilled water + isolate

Table No. 14
Comparative sodium ion leakage (cmol/g) of synergistic *Euphorbia hirta* whole plan acetone extract, honey and ciprofloxacin combination

Isolate 1D	Control	Acetone extract	Honey	Ciprofloxacin	Acetone + honey	Acetone + ciprofloxacin	Honey + ciprofloxacin	Acetone + honey + ciprofloxacin
P. aeruginosa	1.00	48.63	53.30	26.05	117.45	ND	ND	ND
K. pneumoniae	6.00	24.50	61.90	10.25	252.00	ND	ND	ND
P. mirabilis	5.35	19.00	21.02	6.90	21.75	ND	ND	274.15
K. pneumoniae ATCC 13883	4.55	30.50	30.40	12.27	ND	ND	60.80	ND
E. coli ATCC 25922	3.85	20.90	12.00	8.18	ND	ND	ND	39.15

Key: ND = Not determined, Control = Distilled water + isolate

Table No. 15
Comparative protein leakage of synergistic *Euphorbia hirta* whole plant acetone extract, honey and ciprofloxacin combination

Isolate	Combination	Amount (mg/mL)
Pseudomonas aeruginosa	Acetone extract + honey	28.83
Klebsiella pneumoniae	Acetone extract + honey	21.67
Proteus mirabilis	Acetone extract + honey	23.48
Proteus mirabilis	Acetone extract + honey + ciprofloxacin	32.93
Klebsiella pneumoniae ATCC 13883	Honey + ciprofloxacin	22.84
Escherichia coli ATCC 25922	Acetone extract + honey + ciprofloxacin	21.26
Negative control	Bradford reagent + normal saline	5.40

Key: Negative control = bradford reagent + normal saline

The bioactive compounds in purified acetone extract of E. hirta whole plant

Eighteen (18) peaks indicating the presence of eighteen constituents were shown from the GC-MS spectrometry chromatogram elucidation of compounds in purified acetone extract of *E. hirta* whole plant (Figure No. 9). On comparison of the constituent mass spectra to National Institute Standard and Technology (NIST) 14.0 library; Trimethylamine borane, pantolactone, Butane, 2,2-dimethyl-, Butane, 2,2,3-trimethyl-, Tridecane, Pentane, 2,2,3,4-tetramethyl-, Decane, 2,3,5,8-tetramethyl-, Undecane, 2-Pentadecanone, 6,10,14-trimethyl-, Oxirane, tetradecyl-, Heptadecane, 2,6,10,15-tetramethyl-, n-hexadecanoic acid, hexadecane, longifolenaldehyde, myristaldehyde, tetratriacontane, eicosane, squalene were present in the purified acetone extract, the most prevailing compound was tetratriacontane with a retention time and peak area of 48.03 and 46.75 respectively

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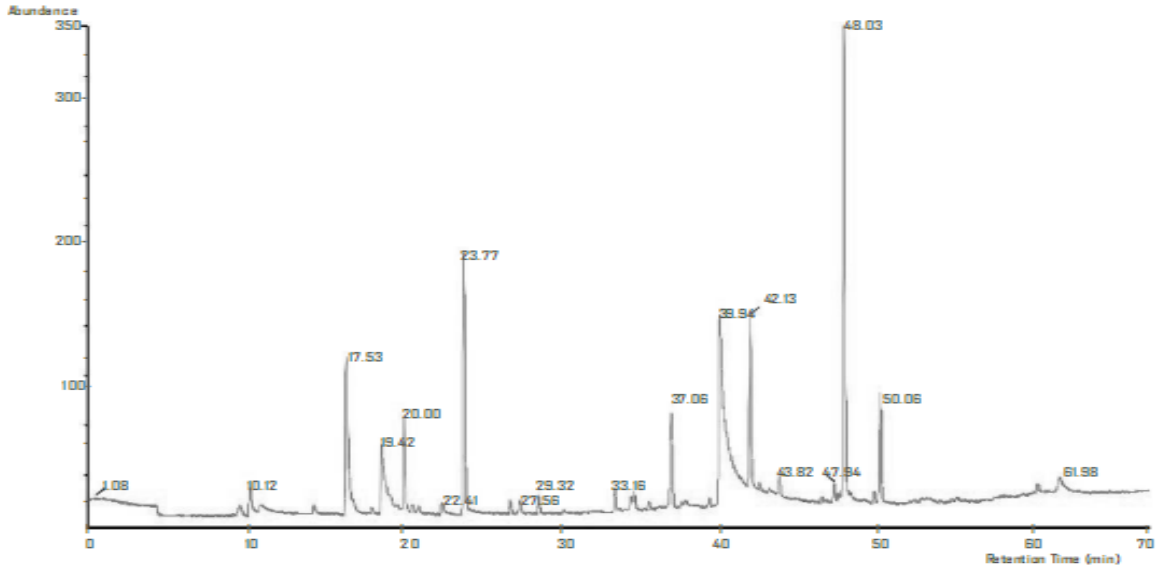

Figure No. 9
GC-MS chromatogram of purified acetone extract of whole *Euphorbia hirta* plant

DISCUSSION

Sporadic usage of antibiotics has led to increased rate of bacterial resistance to conventional antibiotics and hence amplified rate of complications associated to OM (Mallick *et al*., 2018).

The result of multiple antibiotic sensitivity revealed that comparatively ciprofloxacin fared well against all test MDR isolates compare to the other antibiotics. This result is similar to that of Rubena *et al*. (2018) in which bacterial isolates associated with Chronic Suppurative Otitis Media (CSOM) showed highest antibiotic sensitivity to ciprofloxacin (64.7%) in Mulago, Uganda. This study recorded an alarming high rate of multiple antibiotic resistance pattern in the test isolates. This is in line with reports by Muluye *et al*. (2013), where 192 (94.1%) of the tested otitis media isolates had multiple antibiotic resistant pattern and Seid *et al*. (2013) that showed 22.2% of otitis media isolates were resistant to eight antibiotics. The lack of professional supervision in prescribing antibiotics, indiscriminate over the counter sales of antibiotics, incomplete dosage and an over-zealous craving to treat all infection (Seid *et al.*, 2013) are factors that could have contributed to this pattern of high-level drug resistance.

Plant extracts showed the presence of phyto-constituents such as phenolics, tannins, flavonoids, saponins and terpenoids. This result is similar with reports by Onyeka *et al*. (2018) and Gupta and Gupta (2019) that both reported presence of phenolics, tannins, flavonoids, saponins and terpenoids in *E. hirta*. The result of the phytochemical screening of honey revealed that constituents such as saponins, steroids, glycosides, and flavonoids were present. This corroborates the report by Azza (2015) that showed the presence of phenolics and saponins in honey sample. The proximate screening of honey sample revealed the composition of its physico-chemical parameters such as moisture content, ash content, carbohydrate content, protein and fat content. Odeyemi *et al.* (2013) and Ajao *et al.* (2013) reported similar result for the composition of physico-chemical parameters of the screened honey samples in Osun and Kwara respectively.

Our studies showed that purified and crude *E. hirta* extract showed great inhibitory activity against the test bacterial isolates. The antibacterial efficacy of *E. hirta* plant was also reported by Gupta and Gupta (2019), with zones of inhibition of 24 ± 1.08 mm and 16 ± 0.30 against *B. subtilis* and *S. aureus* respectively in Malaysia, and Bhat *et al*. (2019), recorded a zone of 18mm against *S. aureus* (MTCC96) in India. The antibacterial effect is hypothetically due to the presence of plant secondary metabolites such as terpenoids, tannins, flavonoids and steroids which have been shown to possess antibacterial, anti-inflammatory, antifungal, antioxidant and antiviral activities as a result of their

probable effect on intracellular redox status (Rahman *et al*., 2013; Asha *et al*., 2014; Igoli *et al.*, 2016; Karanga *et al*., 2017; Abegunde *et al*., 2018; Onyeka *et al*., 2018). The purified *E. hirta* extracts showed inhibitory effect higher that of crude extracts on isolates. According to Abegunde *et al*. (2018) and Abdelkhalek *et al*. (2018) purified extracts exhibited better inhibitory effect on test bacteria compared to the crude extracts in Nigeria (Osun) and Egypt respectively. This may be because inert impure substances are presence in the crude extracts that may impede its inhibitory potential (Adegoke *et al*., 2010).

Honey inhibited the growth of all isolates tested in this study, this is similar to the findings of Akinnibosun and Itedjere (2013), that honey had significant antibacterial activity against bacteria. The inhibitory effect of honey may be because of its high sugar concentration, hydrogen peroxide generation, low pH, osmotic effect and some unidentified compounds present in it (Vishnu *et al*., 2012; Akinnibosun & Itedjere, 2013; Azza, 2015). Generally, the antibacterial effectiveness of the extract and Honey increased with a corresponding increase in concentration.

The acetone extract of *E. hirta* showed great inhibitory effect against multidrug resistant *S. aureus* with MIC (6.25 mg/mL). The acetone extract, honey and ciprofloxacin used in this study showed varied MIC and MBC values in bacterial isolates when used singly and in combination, which indicates the antibacterial potential of the drugs. Drugs with higher antimicrobial effects showed lower MIC and MBC values; hence the combination of purified acetone extract, honey and ciprofloxacin showed best antibacterial activity, showing the least concentration that inhibited (MIC) and was cidal (MBC) to the tested isolates. Akinnibosun and Itedjere (2013) stated in a similar research that combination of plant and honey has higher antimicrobial effect compared to when used singly. The MIC and MBC values gives a prediction of the effectiveness of antibacterial agents *in-vivo*.

The combination of *E. hirta* whole plant acetone extract and honey showed synergistic effects in *P. aeruginosa* (0.47), *K. pneumoniae* (0.47) and *P. mirabilis* (0.05) isolates. Other researchers have shown synergistic inhibitory potential of plant extract and honey against bacterial infections. Khalil *et al*. (2012) reported the synergy between honey and *Occimum basilicum* on bacterial isolates in Pakistan. Nwankwo *et al*. (2015) showed the synergistic antibacterial effect of *Citrus aurantifolia* (Lime) and honey against some bacterial strains isolated from sputum in Abia. The combination of the *E. hirta* whole plant acetone extract and honey showed no antagonistic effects against other test bacterial isolates, which depicts that the combination showed better antibacterial activity against test isolates; and can be an alternative in the treatment of multidrug resistance cases of OM.

This study also revealed low fractional inhibitory concentration index value on *P. mirablis* (0.02) when *E. hirta* whole plant acetone extract and honey was mixed with ciprofloxacin. This correlate with Shah and Williamson (2019) who showed low fractional inhibitory index value when aqueous garlic extract was combined with antibiotics against resistant strain of *E. coli* in India. The synergistic effects of *E. hirta* whole plant acetone extract, honey and ciprofloxacin shown in this study reveals high level of ciprofloxacin potentiation against multidrug resistant *P. mirablis* isolate. *Euphorbia hirta* whole plant acetone extract and honey could be used with ciprofloxacin eardrops and tablets usually prescribed in severe cases of OM. This also corroborates the traditional use of herbs and honey with antibiotics in the treatment of diseases in Nigeria (Onyeka *et al.*, 2018).

No combination had synergistic effect (> 2 $\log_{10}$ cfu/mL reduction from the most active single agent from the kill assay; as oppose to the report by Shah & Williamson (2019) that showed synergistic effect in the combination of garlic and cefotaxime against extended spectrum beta-lactamase *E. coli* isolate with a 3 $\log_{10}$ cfu/mL reduction from cefotaxime. Even though the synergism detected using checkerboard method was not established by the time kill curve in this study, the lack of antagonistic effect as shown in the *E. hirta* whole plant acetone extract and honey mixture is an inspiring outcome which suggests that purified acetone extracts and honey has potential to be effective as combination or mono therapy, also they may contain a myriad of bioactive constituents that can enhance the activity of ciprofloxacin against drug resistant bacteria isolates associated with OM. We combined *E. hirta* whole plant acetone extract, honey and ciprofloxacin in this study to treat the MDR test isolates, the treatment's time kill assay showed bactericidal activity against *P. mirabilis* isolate after 24 h. This is analogous with report by Abegunde *et al*. (2018) and Shah & Williamson (2019), in Nigeria and India respectively. This bactericidal action recorded in this study shows *E. hirta* acetone extract,

honey when combined with ciprofloxacin can kill thebacterial cells.

The *E. hirta* acetone extract and honey combination showed considerable leakage of potassium ion, sodium ion and protein, which predicts cell membrane leakage by the antibacterial compounds present in the acetone extract and honey, this is in agreement with the study of Abegunde *et al*. (2018), that the leakage of potassium ion, sodium ion and protein are as a result of membrane disruption action of plant extract in Osun. Potassium and sodium ion are needed for maintenance of stable pH and membrane potential of cell, so the efflux of this ions out of the cell can affect proper cell functioning and cause cell death (Abegunde *et al*., 2018). Presence of pytoconstituents could aid the synergy in this study. Phytochemicals such as polyphenols have been revealed to exercise their antibacterial action through membrane perturbations; an important factor for bactericidal activities (Abegunde *et al*., 2018). The synergism observed between the *E. hirta* acetone extract and honey, and potentiating effect when used with ciprofloxacin is a significant finding demonstrating this combination may be used as an alternative therapy in the treatment of multidrug resistance cases of OM.

The GC-MS analysis of acetone extract of *E. hirta* whole plant showed tetratriacontane as the most prevalent compound in this study, tetratriacontane has been reported to have anti-lipid peroxidation action (Salehi *et al*., 2019). We also found squalene in our plant extract, the antioxidant, antitumor and immune-stimulant activities of this compound was reported by Sivasubramanian & Brindha (2013). N-hexadecanoic acid, is another compound present in the test plant extract, it is reported to exhibit antioxidant and antimicrobial activity (Sermakkani & Thangapandian, 2012). We suspect the presence of these bioactive constituents may have contributed to bactericidal efficacy exhibited by our *E. hirta* whole plant acetone extract, therefore, can be useful in production of potent drugs with a broad spectrum of activity.

CONCLUSION

Antibiotic resistance is associated with the bacterial causal agents of otitis media. The purified extract of *E. hirta* L. whole plant in combination with honey has bactericidal effect on the test isolates at low concentration, by causing leakages of protein, potassium and sodium ions in the cells; furthermore, purified *E. hirta* whole plant acetone extract and honey also enhanced the efficacy of ciprofloxacin in the treatment of multidrug resistance cases of otitis media. Purified *E. hirta* whole plant acetone extract and honey can be considered singly/combination as an alternative therapy to combat the rising occurrence of antibiotics resistance. The toxicological effect of the products (singly and in combination) used in this study should be considered in further researches.

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