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Original Article

Antimicrobial Action of Sambacus nigra, Symphytum officinale, Urtica dioica, and Mentha pulegium Plant Compounds against Antibiotic Resistant Streptococcus pneumoniae

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Antimicrobial Action, Sambacus Nigra, Symphytum Officinale, Urtica Dioica And Mmentha Pulegium.

ABSTRACT

Infections by *Streptococcus pneumoniae* are among the leading causes of illness and death in children below 5 years, people with underlying debilitating medical conditions, and the elderly worldwide. Over the past three decades, antimicrobial resistance in *S. pneumoniae* has drastically increased, with resistance reported in Penicillin, erythromycin, trimethoprim/sulfamethoxazole, vancomycin, tetracycline, chloramphenicol and ofloxacin. This phenomenon has necessitated a continuous search for alternative compounds against *S. pneumoniae*, such as Plants. The objective was to investigate antimicrobial natural compounds present in *S. nigra*, *S. officinale*, *U. dioica*, and *M. pulegium* against antibiotic-resistant *S. pneumoniae*. The study utilised cross-sectional design where *S. nigra leaves*, *S. officinale* roots, *U. dioica* leaves, and *M. pulegium* leaves were collected from Kisii highland forests. Phytochemicals were identified by gas chromatography (GC/MS). Microbiological testing of the resistant strain of *S. pneumoniae* was done at Jaramogi Oginga Odinga Teaching and Referral Hospital Laboratory (JOOTRHL). The MBC and MIC were determined using disk diffusion. Data was analysed using ANOVA ($P<0.05$). Results showed alkaloids, phenols, tannins, flavonoids, saponins, glycosides, steroids and terpenoids at different extraction solvent concentrations. *S. nigra*, *U. dioica*, *S. officinale* and *M. pulegium* phytochemical compounds showed significant differences at $0.825 < 3.01$. *S. nigra* and *U. dioica* showed considerable activity on resistant strains of *S. pneumoniae*. The MIC of *S. nigra* and *U. dioica* were 0.625 mg/L and 1.25 mg/L, while MBC was 0.3125 mg and 0.625 mg respectively. GC/MS analysis of phytochemical compounds on *S. nigra* and *U. dioica* showed a total of 8 NIST drug compounds. GC/MS study data; thymol, metharbital, Diethyltoluamide, Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1. alpha., 2. beta., 5. alpha.)-, aspirin, Piperonyl butoxide, triamterene and Glycine, N-benzoyl- are the most effective phytochemical

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compound in *S. nigra* and *U. dioica* against *S. pneumoniae.* The study concluded that *S. nigra* and *U. dioica* leaves pose active compounds that could be further used as alternatives in the treatment of antibiotic-resistant *S. pneumoniae*.

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INTRODUCTION

Streptococcus pneumoniae is a bacterium from the family of Streptococcaceae and a genius of *Streptococcus. S. pneumoniae* are gram-positive, lancet-shaped, capsulated with polysaccharides, non-sporing, non-motile facultative anaerobic bacteria (Cilloniz *et al*., 2016). *S. pneumoniae* has over 100 known serotypes, but only a few serotypes produce most pneumococcal infections (Petrovska, 2012).

Streptococcus pneumoniae can cause various illnesses, depending on which part of the body is infected (Cilloniz *et al*., 2016). *S. pneumoniae* causes pneumonia, which is an inflammation of the lungs; meningitis, which is an inflammation of the membranes that enclose the brain and spinal cord, osteomyelitis, septic arthritis, bacteremia, otitis media, bronchitis, conjunctivitis sometimes and sinusitis. Pneumonia is the most common, severe type of pneumococcal disease (Cilloniz *et al*., 2016). Pneumonia is a respiratory infection that affects the lungs (Grief, 2013). In affected individuals, the immune response to the invading pathogen fills the lung sacs with pus and causative pathogens (WHO, 2016). Consequently, the affected individual develops a breathing problem. There are more than 30 causes of pneumonia bacterial, viral, mycoplasma, and other pneumonia; Cilloniz *et al*., 2016). However, bacterial pneumonia is the most common type worldwide (Cilloniz *et al*., 2016). It is caused by various bacteria, with S. *pneumoniae* being the most common causative bacteria (Cilloniz *et al*., 2016).

S. pneumoniae has a number of virulence factors that enable it to cause pneumonia. They include the capsule, PLY protein, LPXTG-anchored surface proteins, pilus, lipoproteins, choline-binding proteins (CBPs), pneumococcal adherence and virulence factor A (PavA), glyceraldehyde-3-phosphate dehydrogenase, enolase, and the pneumococcal histidine triad protein (Pht) family group (Kadioglu *et al*., 2008; Mitchell and Mitchell, 2010). However, the capsule plays a bigger role in pathogenesis, including antiphagocytic, hinders the access of leukocytes to complement fixed on the underlying cell wall,

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promotes colonisation, and promotes antibiotic resistance (Geno *et al*., 2015).

It is estimated that 17 to 26% of children deaths below 5 years in Africa are a result of pneumonia (Onyango *et al.*, 2012). Clinical pneumonia among children below 5 years is a big health challenge in Kenya. Deaths arising from pneumonia cases are huge, making Kenya position 41 worldwide (WHO Reports, 2018). In Kenya, pneumonia deaths in children below five years old are ranked position two above HIV/AIDS (Kenya National Bureau of Statistics Economic Survey, 2017).

The complication of Drug Resistant *Streptococcus pneumoniae*, (DRSP) is mainly due to inefficient treatment of the infections with the currently available antibiotics. Since immemorial, plants have been used to treat infections (Petrovska, 2012). For example, turmeric and garlic have been used to treat bacterial pneumonia. Interestingly, no resistance reports against plant-based therapy have emerged. Consequently, researchers have constantly investigated natural compounds found in plants of medicinal value to identify active compounds against microbial pathogens. The identified natural compounds are synthesised to produce new antibacterial drugs (Yuan *et al*., 2016).

Main Objective

To determine the antimicrobial action of *Sambacus nigra*, *Symphytum officinale*, *Urtica dioica,* and *Mentha pulegium* plant compounds against antibiotic-resistant *Streptococcus pneumoniae.*

Specific Objectives

- To determine phytochemical compounds present in Sambacus nigra, Symphytum officinale, Urtica dioica, and Mentha pulegium
- To determine the antimicrobial susceptibility of crude extracts of *Sambacus nigra*, *Symphytum officinale*, *Urtica dioica*, and *Mentha pulegium* against antibiotic-resistant *S. pneumoniae*.
- To determine the MIC and MBC of crude extracts of *Sambacus nigra, Symphytum officinale, Urtica dioica,* and *Mentha pulegium* against antibiotic-resistant *S. pneumoniae.*
- To determine marker drug compounds in Sambacus nigra, Symphytum officinale, Urtica dioica, and Mentha pulegium responsible for antibacterial activity against antibiotic-resistant *S. pneumoniae.*

MATERIALS AND METHODS

Study Site

Sambacus nigra, Symphytum officinale, Urtica dioica, and *Mentha pulegium* leaves and roots were collected from forests within the Bonchari constituency in Kisii County. The forest lies within Kisii South Sub County (Bonchari Constituency) at about latitude 0° 41' 0 S and longitude 34° 46' 0 E. Suneka is the Sub-county's major urban and administrative Centre. The plants were analysed at Kisii University Science Research Laboratory, Jaramogi Oginga Odinga Teaching and Referral Hospital Microbiology Laboratory, and Kenya Government Chemistry Laboratory.

Study Design

The study design was cross-sectional. It was an analytical observation study designed to measure the outcome of the selected plants' extracts and the study organism that is, antibiotic resistant *Streptococcus pneumoniae*.

Test Organism

Antibiotics resistant *S. pneumoniae* resistant to Penicillin, amoxicillin/clavuxicillin, Sulphamethazole and Erythromycin isolates were obtained from Jaramogi Oginga Odinga Teaching and Referral Hospital laboratory, – Kisumu. The isolates were from patients archived and stored in a freezer at -80 ^{0}C.

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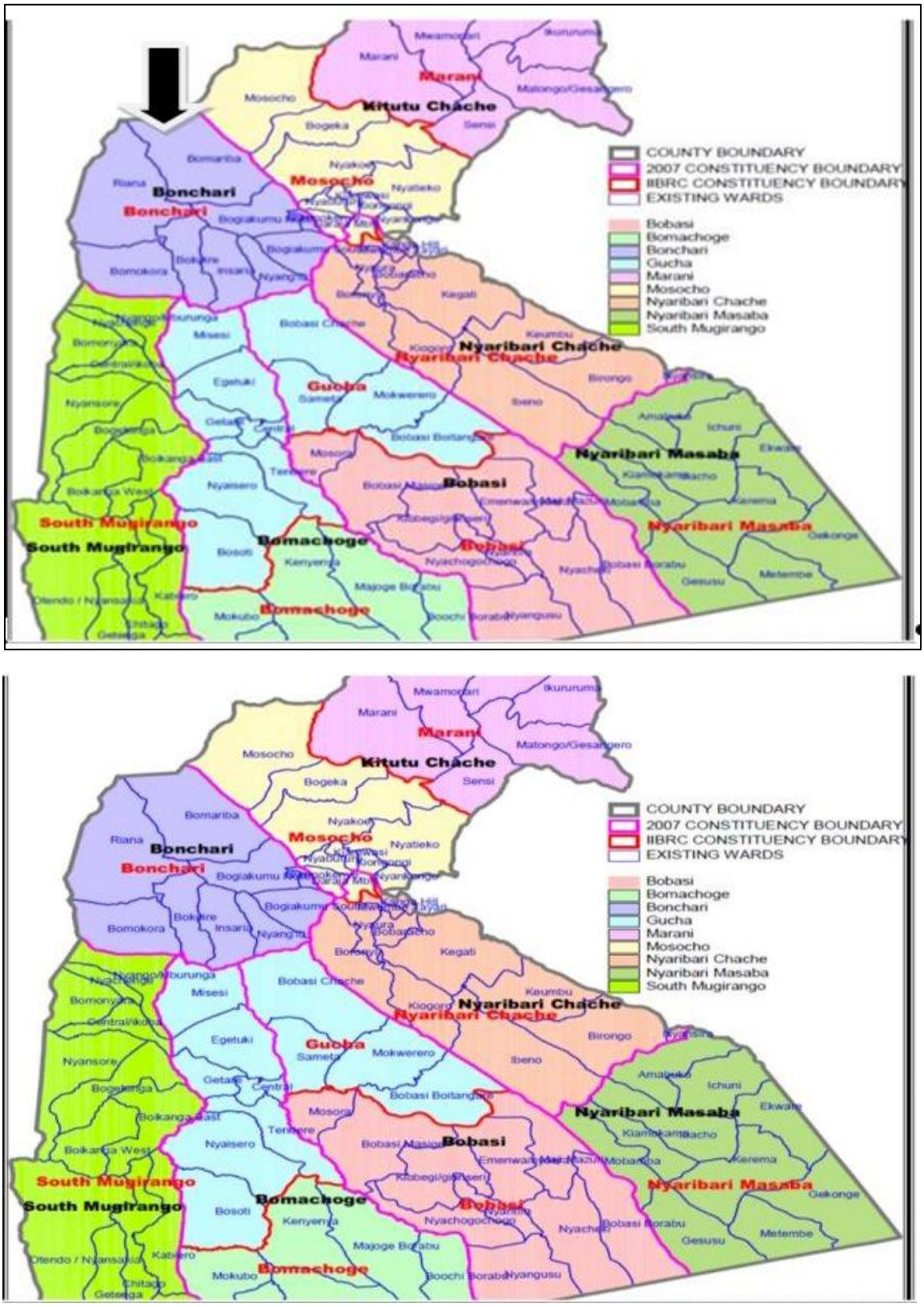
Figure 1: Geographical Map of Kisii County/Bonchari Constituency



Target Plants

The plant targets for this study were *Sambacus nigra*; *Urtica dioica*; *Symphytum officinale*; *Mentha pulegium*. The target parts for each plant were leaves for *S. nigra, U. dioica* and *M. pulegium* and roots for *S. officinale*.

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Sampling Criteria

Purposive sampling method was used for sampling. For the roots and leaves, the near edge parts were picked for the selected plants for the study. The plants were sourced randomly from the forest. The samples picked were those that lied within the middle and along the riverbeds in the forests.

Collection of Plant Samples

5 kilograms each of *Sambacus nigra, Symphytum officinale, Urtica dioica,* and *Mentha pulegium* leaves and roots were collected from forests of Riana, Bomokora, Bonyando, Bomorenda, and Bogiakumu wards within Bonchari constituency in Kisii County. Upon collection, the plant samples were washed and transported by road in sacks to Kisii University Science Research Laboratory.

Laboratory Procedures

Sample Preparation

Upon reaching the Kisii University Science Research Laboratory, samples were prepared as illustrated below.

Air Drying

Air drying was done by spreading the samples in a light-free environment at the research laboratory store using cartons and sacks for 1 week of 7 days for the leaves and 2 weeks of 14 days for the roots.

Grinding

The samples were grinded using the university grinder model YC112M2-2 made by Nakuru Tiger Machinery Company until fine powder was obtained.

Weighing

Weighing the samples was then done to identify the initial weight of each sample for the experiment. *U. dioica*, 480 g, *S. nigra*, 635 g, *S. officinale*, 210 g and *M. pulegium*, 289 g.

Extraction Methods

The samples were soaked using differential imparity to give the actual secondary metabolites present within a given sample in a solvent concentration. Soaking for each concentration was done for 48 hours. After soaking, each sample was concentrated using rotary evaporator model RE100-PRO at a reduced temperature of 45 ^{0}C and a pressure of 50 psi. Storage was then done in beakers, awaiting analysis of phytochemical compounds. The following were the solvents used at different concentrations.

1. Hexane extraction

This was used for the removal of straight-chain aliphatic hydrocarbons.

Dichloromethane in hexane, (1:1) extraction

This was used for the removal of the slightly polar secondary metabolites.

Dichloromethane

This was used for the removal of other slightly polar secondary metabolites.

Methanol extraction

This was used for the removal of more polar secondary metabolites.

5% Water in Methanol

Water has the ability to enter into the internal tissue cells of a given sample. Hence, its mixture with methanol enables it to remove the more polar secondary metabolites from the samples.

Qualitative Phytochemical Compounds Tested

1. Test for Alkaloid

Two millilitres of the plant extracts were mixed with 4 drops of Dragendorf's reagent in a test tube. Alkaloids give a Brown colour precipitate with this reagent.

Test for Phenol and Tannin

Two milliliters of 2% solution of $FeCl_3$ were mixed with the crude extract. The black or blue-green colour indicated the presence of tannins and phenols.

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Tests for Flavonoid

Alkaline reagent test: 2 ml of 2% sodium hydroxide solution was mixed with plant crude extract; the intensive yellow colour was formed, which turned colourless when 2 drops of diluted hydrochloric acid were added to the solution; this result indicated the presence of flavonoids.

Test for Saponins

5 ml of distilled water was added to the crude plant extract in a test tube and shaken vigorously. The foam formation indicated the presence of saponin.

Tests for Glycoside

Liebermann's test: 2 ml of acetic acid and 2 ml of chloroform mixed with entire plant crude extract. The mixture was then cooled, and added concentrated sulfuric acid; the green colour indicated the entity of a glycone steroidal part of glycosides. Salkowski's test: 2 ml concentrated sulfuric acid was added to the entire plant crude extract. A reddish-brown colour indicated the entity of the steroidal aglycone part of the glycoside.

Test for Steroid

2 ml of chloroform and 2 ml of concentrated sulfuric acid were mixed with the entire plant crude extract. The lower chloroform layer produced a red colour indicating steroids' presence. Another test was performed by mixing 2 ml each of acetic acid with concentrated sulfuric acid and crude extract with 2 ml of chloroform. The green colour indicated the entity of steroids.

Test for Terpenoid

2 ml of chloroform was mixed with the plant extract, evaporated on the water path, and then boiled with 2 ml of concentrated sulfuric acid. A grey colour produced indicated the entity of terpenoid.

Quantitative Phytochemical Compounds Tested

Quantitative testing of plant samples was done using a photometric machine of model Stat Fax 4700 reader. The machine linear measurement range was 0.0 to 3.0 Absorbance Units (A) of photometric accuracy of ± (1% of the reading + 0.005A), with a Tungsten lamp as a light source and a wavelength of 340 – 700 nm.

1. Determination of Total Phenolic Compounds

100 mg of the extract of the sample was weighed and dissolved in 100 ml of triple distilled water. 1 ml of this solution was transferred to a test tube, then 1.5 ml 20% of Na_2CO_3 solution and 0.5 ml 2N of the Folin Ciocalteu reagent and solution was added. The resulting volume was made up to 8 ml with distilled water and then shaken vigorously to mix. The setup was then stood 2 hours, after which the absorbance was taken at 700 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid.

Determination of Total Flavonoid

Flavonoids - aluminium complex formation was used as the determinant factor for flavonoids, which has an absorptivity maximum at 415 nm. 100 µl of the sample extracts in methanol (10 mg/ ml) was mixed with 100 µl of 20 % aluminium trichloride in methanol and a drop of acetic acid and then diluted with methanol to 5 ml. This solution was allowed to stand for 40 minutes and then read at 415 nm. Blank samples were prepared from 100 ml of sample extracts and a drop of acetic acid and then diluted to 5 ml with methanol. The standard rutin solution (0.5 mg/ ml) absorption in methanol was measured under the same conditions.

Determination of Total Alkaloid

100 mg of the sample was weighed into a 250 ml beaker, and 200 ml of 10% acetic acid in ethanol was added and covered, then allowed to stand for 4 hours. The mixture was then filtered, and the extract was concentrated in a water bath to 113 mls (one-quarter of the original volume). Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle, and the precipitate was collected and

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washed with dilute ammonium hydroxide and then filtered. The residue (which is the alkaloid) was then collected, dried, and weighed.

Determination of Total Tannin

100 mg of the sample was weighed into a 50 ml container. 50 ml of distilled water was added and shaken for 1 hour in a shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then, 5 ml of the filtered was pipetted into a test tube and mixed with 2 ml of 0.1 Molar $FeCl_3$ in 0. I N hydrochloric acid and 0.008 Molar potassium ferrocyanide. The absorbance was measured at 340 nm within 10 min.

Determination of Total Saponin

100 mg of the sample was put into a conical flask, and 100 mls of 20% aqueous ethanol was added. The samples were heated over a hot water bath for 4 hours with continuous stirring at 55 °C. The mixture was filtered, and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over a water bath at about 90 °C. The concentrate was transferred into a 250 ml separator funnel, and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered, while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath at 100 ^{0}C. After evaporation, the samples were dried in the oven at 55 ^{0}C and weighed; the saponin content was then calculated from the weight.

Determination of Terpenoid

100 mg of dried plant extract was soaked in 9 ml of ethanol for 24 hours. The extract was filtered, and the filtrate was extracted with 10 ml petroleum ether using a separating funnel. The ether extract was then separated into 20 mg pre-weighed glass vials and dried. Ether evaporated, and the yield in percentage of the total terpenoid contents was measured.

Determination of Glycoside

The estimation of glycoside in the various plant samples was done using the spectrophotometric method.

Determination of Steroid

Estimation of steroid was done by:

$$\%\, Steroid\ = \frac{AAbsorbance\ of\ sample\ x\ Gradient\ x\ Dilution\ factor}{WWeight\ of\ sample} x\ 10{,}000$$

GC/MS Processing Phytochemical Compounds

Phytochemicals were identified through the gas chromatography-mass spectrometry (GC/MS) process. The extracts were analysed using the Quadrupole 5977 instrument using MassHunter software for advanced mass spectrometry application. The GC/MS was conducted at the Kenya Government Chemistry Laboratory, Nairobi. The scan direction was high to low, with a background reading of low versus high retention time. Median low retention time S/N = 291 while high retention time S/N = 490. High noise level; median signal (noise level)/threshold=117.4. The plant samples were first re-extracted in dichloromethane solvent, the preferred solvent for the chromatography machine. Stock solutions were prepared from each extract as follows: 1 g of each extract was dissolved in 15 ml of polypropylene centrifuge tube, and then 5 ml of water was added and mixed using a vortex for 5 minutes to form a homogeneous mixture. 5 ml of acetonitrile was added to the mixture and vortexed for 5 minutes. The mixture was then centrifuged for 10 minutes at 5000 revolutions per minute. The obtained stock solution was subjected to GC/MS for analysis. A 0.5 ml of supernatant was added to an auto-sampler vial. 0.05 ml of the internal standard (1, 3-propanediol at 5.0 ml/ ml) was added. The mass spectrometer was set to operate in electron ionisation mode with an ionising energy of 70 eVolts at acquisition mass range from 45-700 a.m.u. The samples were then allowed to run within the GC/MS for 30 minutes each. The ultraviolet and spectrum of chromatographic peaks were then collected for

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analysis. This test was run in duplicate. Further identification was made by comparison of compound mass spectra with those stored in the National Institute of Standards and Technology (NIST) database.

Viability Check of Test Organism

Samples were transported from Kisii University Science Research Laboratory to Kisumu Jaramogi Oginga Odinga Teaching and Referral (JOOTRH) Laboratory, an ISO 15189:2012 accredited laboratory, by road at room temperature. The samples were packed in plastic polypots with a scooping spoon attached before transport. The samples were stored in a refrigerator at 2-8^0 centigrade within the microbiology laboratory during and after testing.

S. pneumoniae organisms were obtained from the organism bank/archive of the JOOTRH microbiology laboratory. The organisms were of patients' origin and showed resistance to commercially prepared Penicillin, Sulphamethazol, Erythromycin and amoxyclav (amoxicillin/clavuxicillin) drugs. The organisms were retrieved by sub-culturing them to CSBA under enhanced conditions of 5% CO_2 at 37^o centigrade for 20 hours. This was done to confirm the organisms' viability and potency and the hemolysis nature of *S. pneumoniae* on CSBA. The organisms were then sub-cultured to MHA under the same condition to prepare them for storage on TSB and day-to-day work activities and for Gram staining. Viability of *S. pneumoniae* ATCC 49619 used as control through the process was also set and stored in the same manner as the study organisms. Gram staining was done to verify the characteristics and morphology of *S. pneumoniae*. The study organisms showed gram-positive diplococci, and confirmation was done by catalase test, which showed a – catalase test negative, susceptibility test to optochin, which showed - optochin sensitivity of 21 mm and solubility to bile giving a positive test result - soluble.

Drug Sensitivity Testing

0.5 McFarland standard was used to standardise the concentration, where 20 cfu/ml was approximated using a 20 µL wire loop and put in a cryovial of 2 ml sterile normal saline and mixed. The resultant solution was then diluted while comparing visually with the 0.5 McFarland standard card until universal comparison match was achieved. The final solution was then stricken to the MHA plates using a sterile swab. Resistant *S. pneumoniae*, was then tested against the drugs of Penicillin, erythromycin, amoxyclav and sulphamethazole drug disks. This was done by setting the drug disk onto MHA, pre-swabbed by study organisms, and incubating at 37^0 centigrade for 20 hours in a 5% CO_2 jar. *S. pneumoniae* ATCC, which was the control organism, was also set in the same manner as the study organism with the same drugs under the same conditions. This was done to confirm the resistance of the study organism to the drugs mentioned above.

Antimicrobial Susceptibility Testing of Plant Extracts against S. pneumoniae

This was done using sterile normal saline. 5 mg of each plant extract was weighed using a weighing balance, Precisa XB120A and put on labelled cryovials. 50 microliters of sterile normal saline were pipetted using a thermoscientific LH96725 pipette and put into each cryovial. An equivalent of one 20 microlitre wire loop full of the study organism was added to each cryovial and mixed. The suspension was mixed thoroughly using a cryovial mixer for 15 minutes to form a homogeneous mixture of extract, study organism and sterile normal saline.

0.5 McFarland standard was used to standardise the concentration, where the mixer was serially diluted to match the 0.5 McFarland standard card colouration. This was compared visually. The final dilution was taken as the stock solution for the study. The final solution was then stricken to the MHA plates using sterile swabs.

Filter paper of 5 mm size was used in this study as a disk. The sterile filter paper was soaked in a suspension prepared above. MHA media plates were then swabbed with the study organism suspension only. Each filter paper was removed from the cryovial mixture using forceps and put in study organism MHA-prepared plates. This test

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was set in duplicate. Preparation was incubated for 20 hours at a 5% CO_2-enhanced jar at 37^0 centigrade, and the results were read for zone of inhibition in millimetres.

Minimum Inhibitory Concentration (MIC) Method

The study plant extract concentration was picked to be 5 mg. The *S. pneumoniae* organism colony concentration was picked to be 20 µL wire loop full. 2 mls of nutrient broth were used as the final volume. The mixing of the tube content was done for 15 seconds. The solution was compared visually with the 0.5 McFarlands standard against the McFarland visual comparison card and standardised to that of 0.5 McFarlands to form a stock solution concentration. Serial dilution was then done on stock concentration. This was then re-mixed to form a homogeneous mixture and labelled stock solution.

The researcher then prepared each plant extract in the study range of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Setting up of the experiment was done using the Westergren sterile tubes in a test tube rack. The setup was then done in triplicate. Both negative and positive controls were 5 mg plant extract/nutrient broth mixture and ATCC *Strep. Pneumoniae/*Penicillin/nutrient broth mixtures were also set in triplicate. Positive control was set by using the ATCC *S. pneumoniae* number 49619 in place of the study organism and Penicillin in place of plant extract in nutrient broth media.

All the tubes in the rack were then incubated at 37^0 centigrade for 20 hours at 5% CO_2 incubator. MIC was expressed in mg/L in this study. This was visually done on the tube, which showed no turbidity compared to the negative control.

Minimum Bacterial Concentration (MBC) Method

The tube with no turbidity was re-suspended to form a homogeneous mixture, then using a sterile wire loop of 20 µL, CSBA and MHA culture media plates were stricken using a sterile swab and set for sub-culture. The plates were then incubated at 37^0 centigrade for 20 hours at 5% CO_2 jar. This was done in duplicate, both on the CSBA media plate and the MHA media plate. Then, the tubes and agar plates were examined for growth visually.

Data Analysis and Management

The data included the plant extract compounds and compound derivatives, zone of inhibition diameter lengths, MIC, and MBC. The experiment was duplicated for the qualitative tests and triplicate for quantitative tests. The MIC of Penicillin was used as a positive control to ascertain resistance. The raw result data were tabulated. The tabulated result data were analysed using Excel and Analysis of variance (ANOVA) with a confidence interval of α=0.05. The experiment analysed data in tables, pie charts and bar graphs.

Ethical Consideration

Before carrying out the study, approval was sought from the University of Eastern Africa, Baraton (UEAB) Research Ethics Committee. Further, the approval to carry out the research was sought from the National Commission for Science, Technology, and Innovation (NACOSTI).

RESULTS

Phytochemical Profiles of Plant Crude Extracts

Qualitative Phytochemical Profiles of Plant Crude Extracts

S. nigra, *U. dioica*, *S. officinale* and *M. pulegium* have phytochemical compounds regardless of the solvent (alcoholic or aqueous solvent) used during extraction. The presence or absence of crude extract compounds depends on the solvent of extraction used while extracting the compounds from the plant samples, as shown in *Table 1*

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Table 1: Qualitative phytochemical profiles of plant crude extracts

		Alkaloid	Phenol	Tannin	Flavonoid	Saponin	Glycoside	Steroid	Terpenoid
1. *Sambacus nigra*	Hexane	+	-	-	+	-	+	+	+
	1:1 DCM/Hex	+	+	+	+	+	+	-	-
	DCM	+	+	+	+	+	+	-	+
	Methanol	+	-	-	+	-	+	-	+
	5% H_2O/Methanol	+	+	-	+	-	+	-	+
2. *Urtica dioica*	Hexane	+	+	+	+	-	+	+	+
	1:1 DCM/Hex	+	-	-	-	+	+	-	-
	DCM	+	+	+	-	-	+	-	-
	Methanol	+	+	+	+	-	+	+	-
	5% H_2O/Methanol	+	+	+	+	-	+	+	+
3. *Symphytum officinale*	Hexane	+	-	-	-	+	-	+	-
	1:1 DCM/Hex	+	+	+	+	+	-	+	-
	DCM	+	-	-	-	+	+	+	+
	Methanol	+	+	-	-	+	-	+	-
	5% H_2O/Methanol	+	-	-	-	+	-	-	-
4. *Mentha pulegium*	Hexane	+	+	+	+	+	+	-	-
	1:1 DCM/Hex	+	-	-	-	+	-	+	+
	DCM	+	-	-	-	-	+	-	-
	Methanol	+	+	+	+	+	+	-	-
	5% H_2O/Methanol	+	+	-	+	+	+	+	-

KEY: = - Absence; + Presence

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Quantitative Photometric Phytochemical Profiles of Plant Crude Extracts

From the table below, there were a total of eight (8) crude extract compounds available and measured. Terpenoids measured the lowest values while saponins measured highest values all through the four study plant samples as shown in *Table 2* below.

Table 2: Quantitative Phytochemical Profiles of Plant Crude Extracts

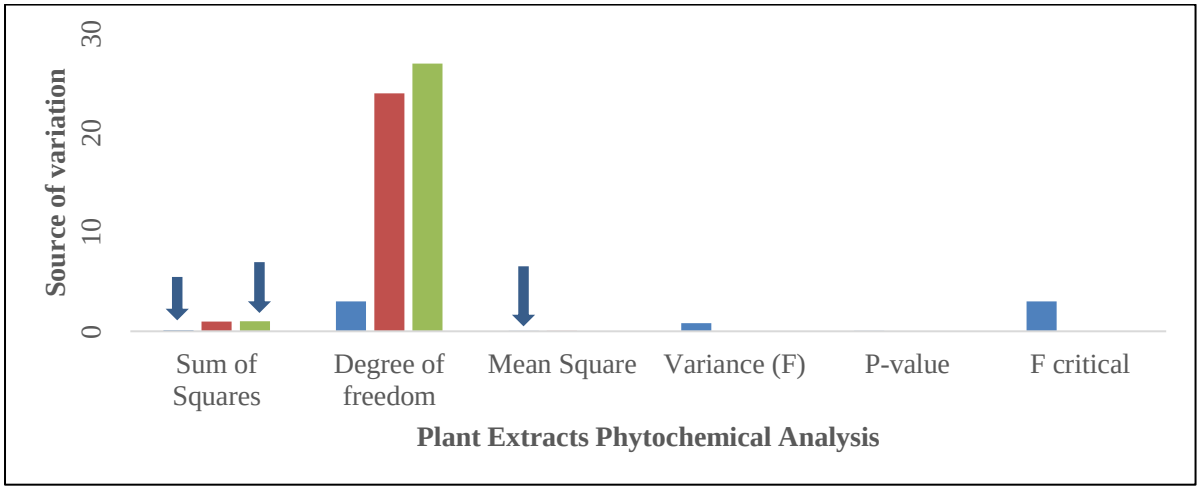
Phytochemical	*S. nigra*	*U. dioica*	*S. officinale*	*M. pulegium*
Alkaloids	0.241±0.83	0.254±0.23	0.287±0.35	0.304±0.66
Saponins	0.556±0.72	0.577±0.17	0.544±0.18	0.513±0.39
Tannins	0.021±2.79	0.024±4.17	0.021±4.76	0.020±5.00
Phenols	0.047±4.26	0.044±2.27	0.047±2.13	0.041±2.44
Flavonoids	0.024±2.47	0.016±6.25	0.017±5.58	0.018±5.56
Glycosides	0.060±1.67	0.063±1.59	0.058±1.72	0.059±1.69
Steroids	0.028±2.09	0.033±1.73	0.029±3.45	0.034±2.94
Terpenoids	0.006±0.00	0.047±1.29	0.041±3.7	0.043±2.33

Key: ± derived from %CV

Quantitative Phytochemical Profiles of Plant Crude Extracts Analysis

S. nigra had a mean of 0.14, *U. dioica* had a mean of 0.144, *S. officinale* had a mean of 0.143, and *M. pulegium* had a mean of 0.141. The source of variation was determined between and within groups. The sum squares for between and within groups were 0.967 and 0.099, respectively, with a total of 1.01. The degree of freedom between and within groups was 3 and 24, respectively, with a total of 27. The mean squares between and within groups were 0.033 and 0.04, respectively. The P-value, <0.05, variance, 0.825 and F critical of 3.009 (*Figure 2*).

Figure 2: Photometric quantitative plant extraction.



Antimicrobial Susceptibility Patterns of Plant Extracts against *Streptococcus pneumoniae*

Only two plant crude extracts showed significant activity against the study organism; these were *S. nigra* and *U. dioica* at 1:1 DCM/Hexane mixture and concentrated hexane solvents, respectively, as shown in *Table 3* below.

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Table 3: Antimicrobial susceptibility patterns of plant extracts against *Streptococcus pneumoniae*

Results Plant	Extraction Solvent	Results Length in mm (millimetres)	Interpretation
Sambacus nigra	1:1 DCM/Hexane	20	Sensitive
	Conc. Hexane	6	Resistant
	Conc. DCM	8	Resistant
	Methanol	6	Resistant
	5% Water/Methanol	7	Resistant
Symphytum officinale	1:1 DCM/Hexane	5	Resistant
	Conc. Hexane	5	Resistant
	Conc. DCM	8	Resistant
	Methanol	6	Resistant
	5% Water/Methanol	9	Resistant
Urtica dioica	1:1 DCM/Hexane	6	Resistant
	Conc. Hexane	36	Sensitive
	Conc. DCM	5	Resistant
	Methanol	9	Resistant
	5% Water/Methanol	6	Resistant
Mentha pulegium	1:1 DCM/Hexane	5	Resistant
	Conc. Hexane	5	Resistant
	Conc. DCM	5	Resistant
	Methanol	6	Resistant
	5% Water/Methanol	9	Resistant

Key: Disk diameter 5 mm; 18.4 < Resistant 18.5 > Sensitive

Source: (Chalo *et al*., 2017)

Minimum Inhibition Concentration and Minimum Bacterial Concentration of Plant Extracts against *Streptococcus pneumoniae*

The MIC for both plant samples is less than 300, as shown in Table 4 below, meaning the plant extract compounds are susceptible to the test organism. The MIC for *S. nigra* and *U. dioica* were 0.625 mg/L and 1.25 mg/L, respectively, while the MBC of *S. nigra* and *U. dioica* were 0.3125 mg/L and 0.625 mg/L respectively as shown in *Table 4* below.

Table 4: Minimum inhibition concentration and minimum bacterial concentration of plant extracts against *Streptococcus pneumoniae*

Stock Conc. (2.5 mg/ ml)	Minimum Inhibition Concentration, (MIC)		Minimum Bacterial Concentration, (MBC)		MBC/MIC Ratio	Interpretation
S. nigra	12.5%	0.625 mg/L	6.25%	0.3125 mg/L	**0.5**	Bactericidal
U. dioica	25%	1.25 mg/L	12.5%	0.625 mg/L	**0.5**	Bactericidal

Key: MBC/MIC ratio: <4 bactericidal and >4 bacteriostatic,

Source: (Mogan *et al*., 2020).

Marker Drug Compounds

NIST Drug Library on blank dichloromethane

There was a total of 13 compounds found on NIST on blank dichloromethane. Dronabinol was the only drug and toxic compound found in the blank dichloromethane solvent (*Table 5*).

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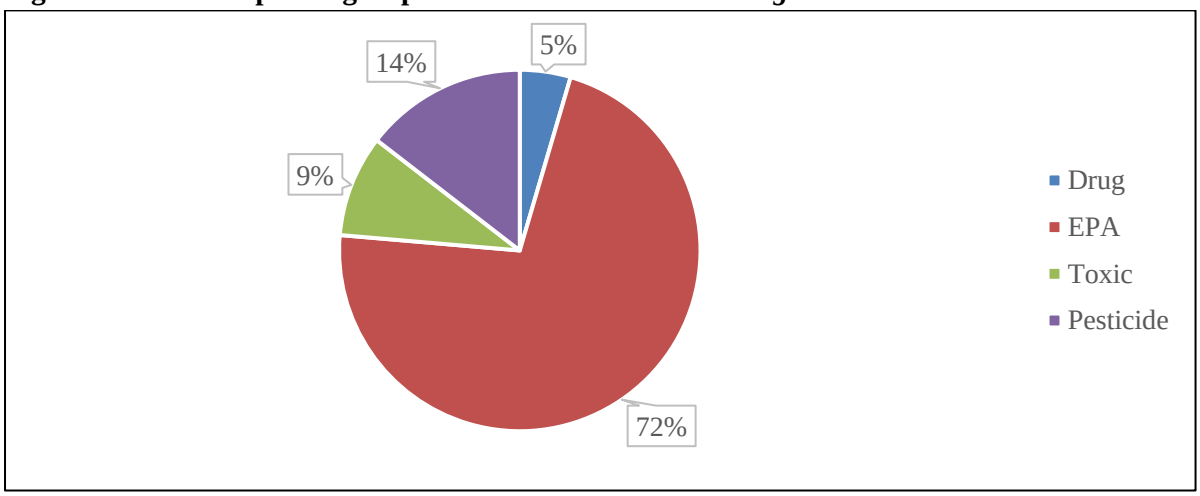
Table 5: NIST Drug Library on blank dichloromethane

Peak#	R. Time, (Minutes)	Name	ID#
		NIST Drug Library	
1	24.0712	Dronabinol	1972-08-3
		NIST EPA LIBRARY	
1	4.1255	Methylene Chloride	75-09-2
2	5.3687	Benzene, chloro-	108-90-7
3	6.0233	1,3-Benzenediol, 4-ethyl-	2896-60-8
4	7.8633	Acetonitrile	75-05-8
5	8.0162	1,3-Dioxalane	646-06-0
6	8.8621	Propene	115-07-1
7	16.6343	Dibutyl phthalate	84-74-2
8	17.8402	Acetic anhydride	108-24-7
9	18.4674	2-Propenoic acid, butyl ester	141-32-2
10	26.3132	Propane, 2-methoxy-2-methyl-	1634-04-4
11	27.2983	Propane, 2-nitro-	79-46-9
		NIST Tox Library	
1	24.0712	Dronabinol	1972-08-3
		NIST Pest3 Library	
1	16.6343	Di-n-butyl phthalate	84-74-2
Total	**30**	**13**	**N/A**

NIST Drug Library for Sambacus nigra

From the raw data (see appendix), there were a total of 110 phytochemical compounds identified. Thymol was recorded in drugs, pesticide and toxic NIST libraries, while aspirin and triamterene were present in the drug and toxic NIST libraries, and piperonyl butocide was present as a drug and pesticide. Glycine, N-benzoyl- was only present in the drugs library. There were 13 phytochemical compounds in the blank dichloromethane; only dibutyl phthalate and di-n-butyl phthalate compounds were present in the NIST library. These were removed since they were part of the blank phytochemical compounds. From the results obtained for *S. nigra* GC-MS NIST compound database, a total of 5 were drugs, 72 were EPA, 10 were toxic, and 16 were pesticide compounds (*Figure 3*).

Figure 3: NIST compound groups in numbers in *Sambacus nigra*



NIST Drug Library for Urtica dioica

From the study, a total of 77 phytochemical compounds were identified: 4 were drugs, 54 were EPA, 6 were toxic, and 13 were pesticide compounds (Figure 4). Thymol was recorded in drugs, pesticide and toxic NIST libraries, while metharbital was only present in drug and toxic

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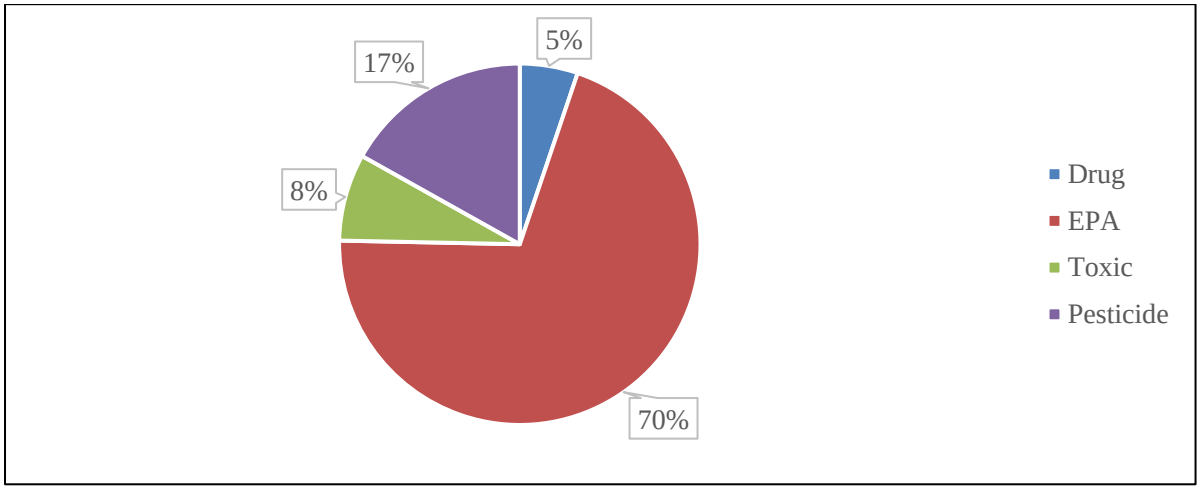
NIST libraries. Diethyltoluamide and cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1. alpha., 2. beta., 5. alpha)- were only present in the drugs library. Benzophenone was both toxic and a pesticide. There were 13 phytochemical compounds in the blank dichloromethane; only one, propobane, 2-methoxy-2-methyl- compound was present in the NIST library. This was removed since it was part of the blank phytochemical compound (*Figure 4*).

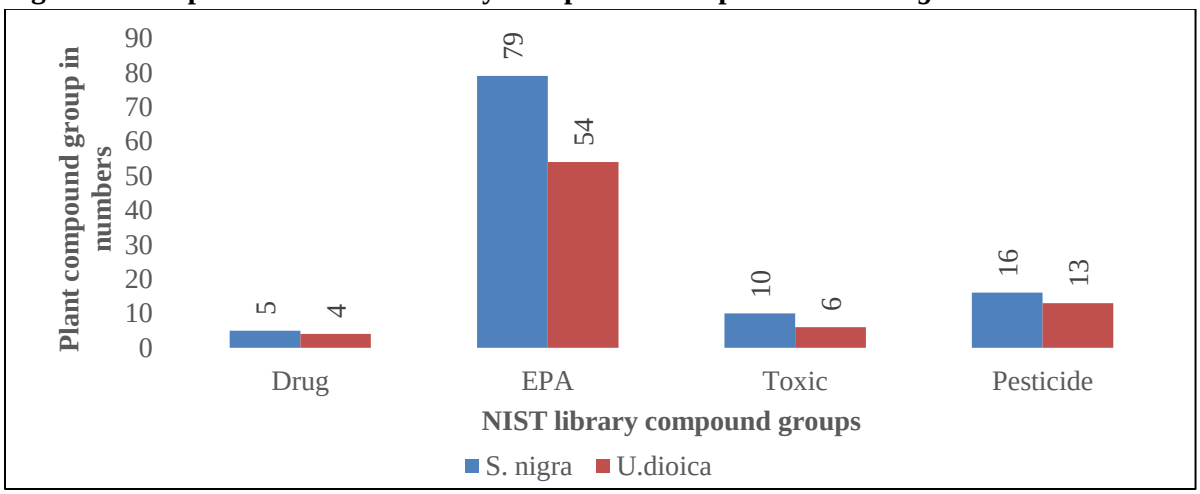
Figure 4: NIST compound groups in numbers in *Urtica dioica.*



Comparison of NIST Compound Groups between S. nigra and U. dioica

Sambacus nigra compounds were more than *Urtica dioica* plant extract leaves phytochemical compounds in all the NIST phytochemical compound analysis standards groups (*Figure 5*).

Figure 5: Comparison of NIST Library Compound Groups between *S. nigra* and *U. dioica.*



DISCUSSION

Qualitative tests for phytochemical bioactive constituents demonstrated that *Sambacus nigra, Urtica dioica, Mentha pulegium* leaves and *Symphytum officinale* roots extracts were rich in auxiliary metabolites. All plant extracts exhibited nearness of alkaloids, saponins, flavonoids, steroids, tannins, glycosides, phenols and terpenoids. This study's higher number of phytochemicals in organic extracts most likely clarified their comparatively better antimicrobial potential and their use in folk medicine in the treatment of pneumonia. The result from the quantitative method means were found not to significantly differ from each other at $p<0.05$. These results are consistent with the qualitative and quantitative analysis of phytochemicals study done by Ajiboye *et al.* (2013) on *Senecio biafrae*

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leaves and another study by Khanal (2021) on qualitative and quantitative phytochemical screening of *Azadirachta indica Juss*. Plant parts. The quantitative screening of *S. nigra*, *U. dioica*, *S. officinale* and *M. pulegium* crude extracts phytochemical compounds showed a significant difference at 0.825 < 3.01, that is, study variance less than F critical value rejecting the null hypothesis. Saponin and alkaloids measured the highest values all through the four study plants. These two crude compound extracts could be the ones significant in the efficacy of the antibiotic-resistant *S. pneumoniae*. The results obtained here are in agreement with those obtained by Ugbaja *et al.* (2017) on the comparative phytochemical and nutritional composition of *Tichosanthes cucumerina* (L.) and Some *Solanum lycopersicum* (L.) cultivars which showed that flavonoid, glycosides, alkaloids, lycopene, tannin, oxalate, and quercetin were higher in *T. cucumerina* than *S. lycopersicum*.

S. nigra and *U. dioica* leaves antibiotic patterns against drug-resistant *S. pneumoniae* (DRSP) are active. A positive result found among *S. nigra* and *U. dioica* zone of inhibition values signifies the biocide and antibiotic effects of their compounds against antibiotic-resistant *S. pneumoniae*. The experiment confirmed the efficacy of *S. nigra* and *U. dioica* plant extracts as natural antimicrobials. The results, therefore, suggest the possibility of employing them as drugs for the treatment of antibiotic resistant *S. pneumoniae*. This study is the first on *Streptococcus pneumoniae* resistant strain; however, the result obtained from this study is similar to those obtained by Chalo *et al*. (2017) on antimicrobial activity, toxicity, and phytochemical screening of selected medicinal plants and Grace *et al.* (2019) on GC-MS analysis of bioactive compounds and evaluation of the antimicrobial activity of the extract of *Daedalea elegans*. *Symphytum officinale* and *Mentha pulegium* extracts were further studied by making a suspension of the antibiotic-resistant *Streptococcus pneumoniae* and extract of the *Mentha pulegium* and *Symphytum officinale* extracts each and then sub-cultured on CSBA for 20 hours at 5% CO_2 enhanced jar incubator at 37^0 centigrade. The results showed no hemolysis, which confirms that the plant extracts could not penetrate the study organism's cell wall, leading to non-hemolysis. This, therefore, confirms that the plant extracts produce the typha genes that prevent it from hemolysis. This is in line with the study of antibiotics and bacterial resistance in the 21st Century study by Fair and Tor (2014), which showed that *Streptococcus pneumoniae* is an aerotolerant, anaerobic, opportunistic pathogen with a polysaccharide capsule that makes it naturally resistant to phagocytes hence resistance to *Mentha pulegium* and *Symphytum officinale* plant extracts.

The 0.5 MBC/MIC ratio is <4, which shows that the *S. nigra* and *U. dioica* have bactericidal effects against antibiotic resistant *S. pneumoniae.* A positive correlation was found between MIC and MBC values for the two plant extracts. The higher the hydrophobicity of the cell surface, the higher the susceptibility to biocides, suggesting that surface characteristics of bacterial cells influence resistance to these plants' compounds. This result shows that the two plants are potential antimicrobial sources due to their low MBC/MIC ratio. There is minimal literature on DRSP. However, the result is consistent with the antibacterial and anticancer activity of ethnomedicinal plants by Lall *et al*. (2018), which showed that *Mundulea sericea* was able to inhibit *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and *Propionibacterium acnes* with minimum inhibitory concentrations (MIC) of 125, 31.25 and 7.9 µg/ ml respectively. Though resistance of *Streptococcus pneumoniae* is not as prevalent as in some other gram-positive pathogens, the pathologies associated with *Streptococcus pneumoniae* infection make the prospects of increased resistance worth particular consideration, as shown by the results of the study on the *Mentha pulegium* and *Symphytum officinale* extracts.

Thymol drug marker compound was found in both plants, therefore significant in the sensitivity against the antibiotic-resistant *S. pneumoniae*. Thymol is used in the treatment of expectorant

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and antibacterial, mainly in the treatment of the upper respiratory system. Asprin is used in antiplatelet therapy in the treatment and prevention of cardiovascular diseases, acute coronary syndrome, and thrombotic stroke, among others. The use of aspirin can reduce mortality and recurrence of cardiovascular diseases. Glycine, N-benzoyl- is an enzyme involved in mitochondrial activities in the process of detoxification, while triamterene is frequently used in combination with hydrochlorothiazide to treat hypertension. Piperonyl butoxide does not have any antimicrobial activity on its own. However, it is a synergist used to enhance the efficacy of other pesticides. Metharbital is used in the treatment of insomnia**,** Diethyltoluamide is used as an insect repellent but does not kill insects, while Cyclohexanol,5-methyl-2-(1-methyl ethyl)-, (1. alpha., 2. beta., 5. alpha.)- has no major use known in drug preparation, but it is categorised as environmentally protected compound under NIST library. These drug compounds are significant in the susceptibility of the plant compounds against antibiotic-resistant *S. pneumoniae*. This result is similar to the study of thymol and thyme essential oil usage in traditional medicine but varies slightly with GC-MS studies done on *Daedalea elegans* (Grace et al., 2019).

CONCLUSIONS

Sambacus nigra, Urtica dioica, Mentha pulegium leaves and roots of *Symphytum officinale* contain alkaloids, Saponins, phenols, tannins, flavonoids, glycosides, steroids and terpenoids compounds. *Sambacus nigra* and *Urtica dioica* leaves are susceptible to antibiotic drug-resistant *Streptococcus pneumoniae,* while *Symphytum officinale* roots and *Mentha pulegium* leaves are not susceptible to antibiotic resistant *Streptococcus pneumoniae.* The minimum inhibition concentration and minimum bacterial concentration of *Sambacus nigra* and *Urtica dioica* are 0.625 and 1.25 mg/L and 0.3125 and 0.625 mg/L respectively. Thymol, aspirin, triamterene, Piperonyl butoxide, Glycine, N-benzoyl- metharbital, Diethyltoluamide and Cyclohexanol, 5-methyl-2-(1-methyl ethyl)-, (1. alpha., 2. beta., 5. alpha.)- marker drug compounds in *Sambacus nigra and Urtica dioica* are responsible for bactericidal effect on antibiotic resistant *Streptococcus pneumoniae*.

Recommendations

Sambacus nigra and *Urtica dioica* leaves to be used for treatment of antibiotic resistance *Streptococcus pneumoniae* and pesticide in pest control after toxicity study. There is need to research on other parts of the plants apart from leaves and roots to exploit the more opportunities exhibited by these plants. *Sambacus nigra* and *Urtica dioica* have medicinal compounds; however, further study on toxicity level needs to be done to be able to develop safe drug(s). There is a need to do more research on the marker drugs to determine their mode of action and target sites on the antibiotic resistant *Streptococcus pneumoniae* strain.

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