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Antimicrobial effect of aqueous extracts of *Garcinia kola, Cymbopogan citratus and Bryophyllium pinnatum* against sputum bacterial isolates from human subjects

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Abstract

Introduction: *Garcinia kola, Cymbopogan citratus* and *Bryophyllium pinnatum* are multipurpose plants used in diverse ways for medicinal and nutritional purposes across regions of the world, with their benefits yet to be fully exploited.

Aim: This study investigated the antimicrobial effects of aqueous extracts of these plants against bacterial sputum isolates of consenting cough patients.

Method: Isolates were identified based on colonial, morphological, gram staining, relevant biochemical and molecular characterization. Aqueous extracts of individual plants, binary, and ternary combinations were used for antimicrobial studies on four isolated bacterial species. The response of the bacterial isolates to aqueous extracts of the seeds of *G. kola* and leaves of *B. pinnatum and C. citratus* were determined using lactate dehydrogenase method and mean inhibitory concentrations (MIC) determined.

Results: *Streptococcus* specie infection had the highest prevalence of 90% followed by *Klebsiella pneumonia* (85%), *Staphylococcus aureus* (80%), and *E. coli* (30%) among the cough patients. Co-bacterial infection was seen in 95% of the cases**.** Responses of the bacterial isolates to the plants' aqueous extracts were dose-dependent. Dehydrogenase activities of the bacterial isolates were significantly and progressively inhibited by the aqueous extracts of the individual, binary and ternary combinations of the plants. However, MIC was significantly lowest in the ternary combination compared to the individual and binary extract formulations.

Conclusion: The study revealed that individual, binary, and ternary combinations of the plant extracts have significant antibacterial properties, with ternary combination of 40% *G. kola*, 30% *B. pinnatum* and 30% *C. citratus* demonstrating the highest potency. The ternary combination of the extracts could potentially be exploited for treatment of bacterial-induced cough. However, more research is needed to further evaluate the plants' safety and effectiveness for clinical use in humans.

Keywords: Lemon grass; Bitter kola; Miracle leaf; Medicinal plants; Lactate Dehydrogenase; Antimicrobial profiles

1. Introduction

Medicinal plants have found uses globally in the treatment of diseases, and are commonly considered for use because of their lower cost, ready availability, and are perceived to be with minimal side effects compared to the more synthetic drugs. Also the use of conventional antimicrobials has recently experienced disturbing increase in antimicrobial

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resistance [1,2], thus necessitating the search for novel classes of antimicrobial substances from natural sources such as medicinal plants with high potency. The growing challenge of antimicrobial resistance to conventional antibiotics has made it expedient for use of combination treatment strategies, and researches into polyherbal therapies. This provides opportunities for synergism among the drug agents' thereby achieving effective treatment while preventing or minimizing development of resistance by pathogenic microorganisms. Meanwhile, folkloric medicine has always been based on polyherbal therapy. With the advancement of science, the plants used for traditional medicine have been demonstrated to contain a wide range of substances useful in the treatment of an array of sicknesses and diseases [3,4]. These herbal remedies are usually applied orally or topically to effect treatment of different disorders.

Lemon grass (*Cymbopogan citratus),* bitter kola *(Garcinia kola*), and miracle leaf (*Bryophyllium pinnatum)* are plants widely distributed in tropical and subtropical regions of the world. Different species are cultivated in Asian and Africa countries, with geographical diversity influencing their medicinal importance [5,6]. They are commonly used as herbal remedies for various medical conditions. Variations in their phytochemical compositions, as well as differences in the herbal formulations contribute to the differences in their medicinal importance.

Lemon grass is an herb used in folk medicine for the treatment of body pains, infections, colds or influenza, cough, elevated blood pressure, epilepsy, dysentery, and knee achy symptoms [7-10]. It has been demonstrated to possess many biological activities such as antibacterial, antifungal, antidiarrhoeal, anti-inflammatory, antimutagenic, antihepatotoxic, antimutagenicity potential, cardioprotective, antirheumatic and antimalarial effects [11-15].

Bitter kola plant is a canopy tree that belongs to a large genus, *Garnicia* consisting of more than 250 species. Both the seeds and stem bark of bitter kola plant have found use in folklore remedies for treatment of headache, laryngitis, bronchitis, malaria, gonorrhea, cough, diabetes, various types of inflammation and liver cirrhosis [16-18]. The plant has also been reported to be a natural antioxidant and has anti-inflammatory properties, resulting to ameliorated renal-toxicity via the activation of antioxidative pathways, and also mitigated genotoxicity in mice [19]. Many chemical substances such as tannin, saponins, alkaloids, phenols, anthocyanin, and cardiac glycoside have been isolated from the plant [20,21].

Another medicinal plant of interest is *Bryophyllium pinnatum.* It is commonly known as miracle leaf or life plant, and is a widely accepted herbal remedy. It is a crassulescent herb distributed widely but grows primarily in the rain forest areas of tropical Africa, India, China, and Australia. It has an astringent sour taste, and has hot potency. Miracle leaf plant has found several applications in folk medicine as well as contemporary medicine. The plant parts are frequently applied for the cure of burns, rheumatoid arthritis, antiseptic, blisters, cough, insect bites, psychiatric disorder, abdominal discomforts, and as a neuroprotective remedy [22,23]. It is well-known for its anti-inflammatory, wound healing, hypoglycaemic, anti-diabetic, anticancer, analgesic and hemostatic qualities [24,25]. Leaf extracts are useful for the treatment of jaundice, hypertension, renal stones and diabetes. Slightly heated leaves are applied on superficial skin infections and also used for the post-natal management of placental dropping in Southeast Nigeria. Hence, it was suggested to act as a tocolytic agent to prevent premature labour [26,27]. A recent study also supported the use of *B. pinnatum* as a new, well-tolerated therapeutic approach for dysmenorrhea [28]. The leaves of *B. pinnatum* contain bryophylin, malate, potassium, ascorbic acid, and citric acid. It is also a rich source of macro and micronutrients such as vitamins, calcium, phosphorous, ascorbic acid and inulin; all of which have beneficial medicinal properties [29].

Although, many studies have been carried out on these abundantly available medicinal plants, such studies mainly evaluated the individual plant extracts. Thus, the present study assessed the antimicrobial properties of single and polyherbal aqueous formulations of lemon grass (*Cymbopogan Citratus), bitter kola (Garcinia kola*), and miracle leaf (*Bryophyllium pinnatum)* against clinical cough caused by bacterial infections. The study also evaluated the possible synergic interactions from different combinations of the extracts in the treatment of bacteria-induced cough.

2. Material and Methods

2.1. Plant samples collection and preparation

Fresh healthy leaves of *B. pinnatum* and *C. citratus* and seeds of *G. kola* used for the study were sourced from Owerri, Nigeria and authenticated by a plant taxonomist, Mr. Francis Iwueze of the Department of Forestry and Wildlife Technology, Federal University of Technology Owerri (FUTO), Imo State and given voucher numbers FUTO/FWT/ERB/2021/59, FUTO/FWT/ERB/2022/66, and FUTO/FWT/ERB/2023/102 respectively. The samples were washed under running tap water, air-dried at room temperature, and pulverized into fine particulate forms using industrial-grade grinding machine. The individual plant samples, their binary (50:50%) and ternary (40% *G. kola*, 30% *C. citrates* and 30% *B. pinnatum*) combinations were prepared, and subsequently stored in labeled compact containers

at room temperature. Aqueous extraction of the plant samples followed standardized preparation method [30]. Four hundred and fifty grams of the ground plant sample was dissolved in distilled water in a 2.5 L volumetric flask and the solution made up to mark and subjected to boiling for a duration of 30 minutes, followed by decanting, filtration, and freeze-drying.

2.2. Sputum sample collection and identification of bacterial isolates

Fresh sputum samples were obtained from willing and consenting adult patients attending the Federal University Teaching Hospital, Owerri, Imo State through aseptic collection procedure. Blood agar and nutrient agar were used to prepare pure cultures of different strains after sub-culturing. Streak-plate technique was used for isolation of bacteria [31]. Gram staining, motility test, and biochemical tests such as catalase, coagulase, methyl red, Voges-proskaeur, indole, citrate, and sugar fermentation tests were carried out for bacterial identification [32]. Confirmatory molecular identification of the isolates was done through polymerase chain reaction and agarose gel electrophoresis techniques [33].

2.3. Antimicrobial assay using inhibition of lactate dehydrogenase method

Antimicrobial studies were carried out using inhibition of total dehydrogenase activity as measure of toxicity as described by Nweke *et al* [34]. The toxicity of the plants' aqueous extracts compared to ciprofloxacin on the microbial isolates were determined in 2 ml volumes containing varying concentrations of respective test materials in requisite volumes of distilled water, nutrient broth, 0.1% 2,3,5-triphenyltetrazolium chloride (TTC) and isolate. The toxicity of the compounds was determined for all the plants' aqueous extracts (1000-5000 µg/ml), and ciprofloxacin (200 –500 µg/ml).

Antimicrobial properties of aqueous extracts of the plants were carried out for the 3 individual plants, 3 binary and 1 ternary combinations of all the extracts, and standard anti-cough drug on 4 bacterial isolates using standard zone of inhibition method [35].

2.4. Statistical analysis

Data obtained from the study were presented as mean ± standard deviation, and were analyzed using one-way analysis of variance (ANOVA) and Turkey post-HOC test with the aid of GraphPad Prism Version 5.0. Statistical level of significance was taken at $p<0.05$.

3. Results

Results of the biochemical characterization of the isolated bacterial species as presented on Table 1 demonstrates that the prevalent organisms in the sputum samples were *Streptococcus* specie, *K. pneumonia*, *S. aureus*, and *E. coli*. The identities of the bacteria were confirmed through sequencing, their query length, percentage similarity, and Genbank blast identity. The query length, percentage similarity, and Genbank blast identity of the respective isolates were 1838, 100%, MH194190.1 (*E. coli*), 1460, 99%, MZ474084.1 (*S. aureus*), 1347, 98%, MF578786.1 *(Streptococcus* specie) and 1404, 100%, KT26177.1 (*K. pneumonia)*(Table 2-5).

Table 3 presents the relative frequency of the bacterial strains and species that grew in the human cough sputum samples. *Streptococcus* specie infection had the highest prevalence (90%) followed by *K. pneumonia* (85%), *S. aureus* (80%), and*E. coli* (30%). Co-bacterial infection was seen in 95% of the cases.

Figures 1-4 show the inhibitory effect of aqueous extracts of single, binary and ternary combinations of *C. citratus, B. pinnatum* and *G. kola* against the four different organisms isolated from the sputum samples. Figure 1 shows that: *C. citratus* demonstrated inhibitory effects ranging from 36.39% to 47.27% against *K. pneumoniae* at concentrations of 1000, 2000, 3000, and 5000 µg/ml. *B. pinnatum* exhibited lower inhibitory effects, ranging from 16.25% to 24.68%, while *G. kola* showed the highest antibacterial properties with inhibitory effects ranging from 40.99% to 50.3% at the same concentrations against *K. pneumoniae*. The binary combination of *C. citratus* and *B. pinnatum* demonstrated inhibitory effects ranging from 54.05% to 96.16%, while the combination of *B. pinnatum* and *G. kola* exhibited relatively higher inhibitory effects (51.31% to 76.64%) compared to the combination of *C. citratus* and *G. kola* (51.75% to 61.78%) at the same concentrations. Ternary combinations of the extracts showed higher inhibition zones compared to single and binary combinations at respective extract concentrations. In comparison the standard antibiotic, ciprofloxacin demonstrated 100% inhibition of *K. pneumoniae* at concentrations of 400 and 500 µg/ml, indicating significantly higher antimicrobial efficiency compared to the three plant extracts.

Figure 2 presented the inhibitory effects of various combinations of the three plant extracts on *S. aureus*. *C. citratus* demonstrated inhibitory effects ranging from 26.61% to 40.33% at concentrations of 1000µg/ml to 5000µg/ml, respectively, while *B. pinnatum* and *G. kola* exhibited inhibitory effects ranging from 15.46% to 36.87% and 27.1% to 34.9% at concentrations of 1000µg/ml to 5000µg/ml, respectively. The binary combination of *C. citratus* and *B. pinnatum* demonstrated inhibitory effects ranging from 18.74% to 52.18%, while that of*C. citratus* and *G. kola r*anged from 40.85% to 60.53%, and combination of *B. pinnatum* and *G. kola* was 40.85% to 45.31% at the same concentration ranges. Ternary combinations of the extracts demonstrated higher inhibition zones of 80.37% and 96.30% at concentrations of 2000µg/ml and 3000µg/ml, respectively, against *S. aureus* compared to the binary and single preparations of the plant extracts. *Staphylococcus aureus* showed inhibitions of 44.31% and 50.0% by ciprofloxacin at concentrations of 400µg/ml and 500µg/ml, respectively.

In figure 3, the inhibitory effects of aqueous extracts of single, binary, and ternary combinations of *C. citratus*, *B. pinnatum*, and *G. kola* against *Escherichia coli* isolated from cough sputum samples were presented. The single extracts of *C. citratus* demonstrated moderate inhibition ranging from 17.05% to 26.1% at concentrations of 1000µg/ml to 5000µg/ml respectively. *B. pinnatum* showed higher inhibitory effects while *G. kola* exhibited highest inhibitions at 42.08% to 46.49% amongst the single extracts treatment. Binary combinations of *C. citratus* and *B. pinnatum* demonstrated inhibition rates of 50.59%, 65.93%, 80.28%, and 95.45% at concentrations of 1000µg/ml, 2000µg/ml, 3000µg/ml, and 5000µg/ml respectively, while that of *C. citratus* and *G. kola* were 38.67%, 42.42%, 48.79%, and 53.95% andcombination of *B. pinnatum* and *G. kola* exhibited inhibition rates of 32.75%, 36.85%, 41.05%, and 49.91% at same concentration ranges. The ternary combination generated an impressive inhibition zone of 95.01% against *E. coli*, which was significantly higher than the observed Ciprofloxacin inhibition rates of 49.55% and 51.95% at concentrations of 400µg/ml and 500µg/ml respectively.

Streptococcus sp isolated from the cough sputum samples exhibited sensitivity ranging from 29.02 to 37.02% against single extracts of *C. citratus* at concentrations of 1000 to 5000 µg/ml respectively (Figure 4). *B. pinnatum* demonstrated slightly higher inhibitory effects at 30.73% to 40.69%, while *G. kola* showed lowest effect (12.88 to 26.09%) at same concentration ranges. Binary combinations of *C. citratus* and *B. pinnatum, C. citratus* and *G. kola,* and *B. pinnatum* and *G. kola* exhibited inhibition rates ranging from 35.15 to 98.11%, 42.69 to 55.56% and 53.54 to 68.35% respectively at similar concentration ranges. The ternary **c**ombination showed high sensitivity of 90.89% significantly higher than the binary and individual combinations. *Streptococcus sp* showed susceptibility rates of 38.10% and 33.65% to ciprofloxacin at concentrations of 400µg/ml and 500µg/ml respectively, significantly lower than the sensitivity to the ternary mixture.

Amongst the individual extracts, *G. kola* had the lowest minimum inhibitory concentration (IC_{50}), followed by *C. citratus* and *B. pinnatum*, across the respective bacterial strains (Table 7). Generally, the ternary combination demonstrated lower IC_{50} values across all tested organisms compared to both single and binary extract combinations. However, ciprofloxacin, the antibiotic used for comparison, consistently exhibited the lowest IC_{50} values against all the test organisms. Correlation analysis of the inhibitory effects of the extracts against the various isolated organisms showed that the ternary and binary extract combinations of *C. citratus* and *B. pinnatum* had correlation coefficient (R^2) and p-values comparable with those of ciprofloxacin.

Table 1 Biochemical test results of bacterial isolates from cough sputum samples

S/N	Biochemical test on bacteria isolates pure culture							Decision
	Gram reaction	Catalase	Coagulase	Indole test	Citrate test	Methyl red test	vogues proskauer	
1	+	-	ND	ND	ND	ND	ND	*Streptococcus* sp
2	-	+	+	-	+	+	-	*E. coli*
3	+	+	+	-	+	-	+	*Staphylococcus aureus*
4	-	+	ND	-	+	-	+	*Klebsiella pneumonia*

Legend: + = present; - = absent; ND = not determined

Table 2 Sequence analysis of *Escherichia coli* isolate using Genbank

Sequences Obtained	**Query Length**	**% Similarity**	**Genbank**	**Blast Id**
5_27-F_B09_06 >TGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTG TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAGACATAAAAGGTGGC TTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTC CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAA GAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAA AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAA GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCC CCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAA TCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGAC AAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAA CAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGC GTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA CACCACGAGAGTTTGTAACACCC	1838	100%	MH194190.1	Escherichia coli strain UCCB 108

Table 3 Sequence analysis of *Staphylococcus sp* isolate using Genbank

Sequences Obtained	**Query Length**	**% Similarity**	**Genbank**	**Blast Id**
5_23-C_B09_01 >TGGCGTGCGGCGTGCTATACATGCAGTCGAGCGAACAGATGAGAAGCTTG CTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACC TATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATT TTGAACCGCATGGTTCGATAGTGAAAGACGGTTTCGGCTGTCACTTATAG ATGGACCCGCCCCGTATTAGCTAATTGGTAAGGTAATTGGTTACCACAGC GAACATACGTAACCGACCAGAAAGGGGGGAGCGGGATGTTGAAACTGATC CCGGCCCTCATTCGTATTGGAGGCGGCGGTAAAAATCTTCCCAATGGGAG AAAGCCTGACGGACCTAAGCCGTGGGACTGGTTGAGGCATTGAACCCAAA ATCTCAGGAAGCAAGCTAAAAACACCTGGCACCACCTGTCTCTTTGTAGC CGAAGGGAAAAATACAATCACACCAACGTCATGCAATCCGCGGTTTTGAT ACGCTGGTGGCGACGTTATGAGTAATTCGTGGGCGTCAACCGCTGGTAGG CGCTTCTCAATCTGATGAGAACCCCTTGCTCACGCATGCACCGTGTGAGA GCTGATGCACTAGCTGGAAGACTACGAGACAGAAATCCCCTATGAATCAG CACTCATCGTTACGTATGGACTACAAAGTATCTAATCCTGTTGATGCCAG CTTTGAAACATCAGCGTCAATGCTGATCGATCAACTGGCATCACAACTGT GTACTCATATCTAGCGCATTTATGCTAGTGTTGGAGATTCACGTCCCCTT TTTGGCAGCACAGATCCCTAATCCTACGACTGCAGTACGAGCCTGTTGTA ACTCAAGAGATTGACACCGACCGCCACCGTTGACCATGTAGTCAGATCAC CTAGCCAAAACCTTACAGATCT	1460	99%	MZ475084.1	Staphylococcus sp strain LP 159

Table 4 Sequence analysis of *Streptococcus pneumoniae* isolate using Genbank

Sequences Obtained	**Query Length**	**% Similarity**	**Genbank**	**Blast Id**
>CATGAAGGAGGAGCTTGCTATCTCTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTGGTAGCGGGGGA TAACTATTGGAAACGATAGCTAATACCGCATAACAGTAGATGTTGCATGACATTTGCTTAAAAGGTGCAATTGCATCACT ACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGG GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAA AGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGAGAAGAACGAGTGTGAG AGTGGAAAGTTCACACTGTGACGGTATCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG TCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTCTGAAGTTAAAGGCTGTGGCTTA ACCATAGTACGCTTTGGAAACTGTTTAACTTGAGTGCAAGAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCTTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGACCCTTTCCGGGGTTTAGTGCCGCAG CTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTCTGACCGCTCTAGAGATAGA GTTTTCCTTCGGGACAGAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA ACGAGCGCAACCCCTATTGTTAGTTGCCATCATTCAGTTGGGCACTCTAGCGAGACTGCCGGTAATAAACCGGAGGAAGG TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAACGAGTCGCAAG CCGGTGACGGCAAGCTAATCTCTTAAAGCCAGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATC GCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAC	1347	98%	MF578786.1	Streptococcus pneumoniae strain K12

Table 5 Sequence analysis of *Klebsiella pneumonia* isolate using Genbank

Sequences Obtained	**Query Length**	**% Similarity**	**Genbank**	**Blast Id**
>GCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA TGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCAT GCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAG AGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC GCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTA AGGTTAATAACCTTGCCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCT CAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGC AAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGG AGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGG ATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGA AGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGC GACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA ATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG TGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCT	1404	100%	KT261777.1	Klebsiella pneumoniae strain K13

Table 6 Prevalence of bacterial isolates in sputum samples

	Streptococcus sp	***E. coli***	***Staphylococcus aureus***	***Klebsiella pneumonia***
1	+	-	+	+
2	+	+	+	+
3	+	-	+	+
4	+	+	+	+

5	+	+	+	+
6	+	-	+	+
7	+	+	+	+
8	+	-	+	+
9	+	+	+	+
10	+	+	+	+
11	+	-	+	+
12	+	-	-	+
13	+	-	-	+
14	+	-	+	+
15	+	-	+	+
16	+	-	+	+
17	-	-	+	-
18	-	-	-	-
19	+	-	+	+
20	+	-	-	-
% occurrence	90	30	80	85

Legend: + = present; - = absent.

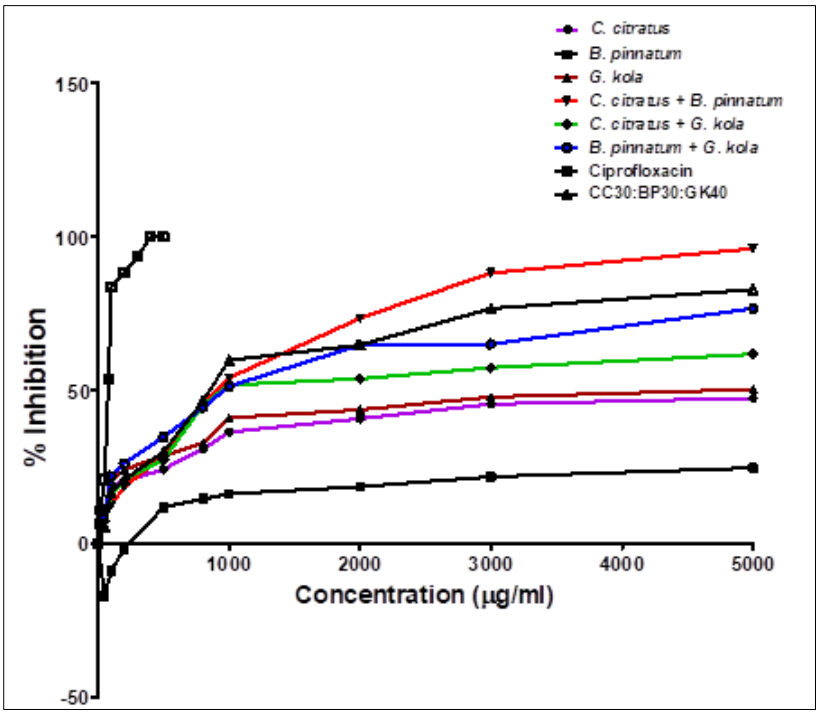

Figure 1: Inhibitory effect of aqueous extracts of single, binary and ternary (30:30:40) combinations of *C. citratus* (CC), *B. pinnatum* (BP), and *G. kola* (GK) against *Klebsiella pneumonia* isolated from human cough sputum samples

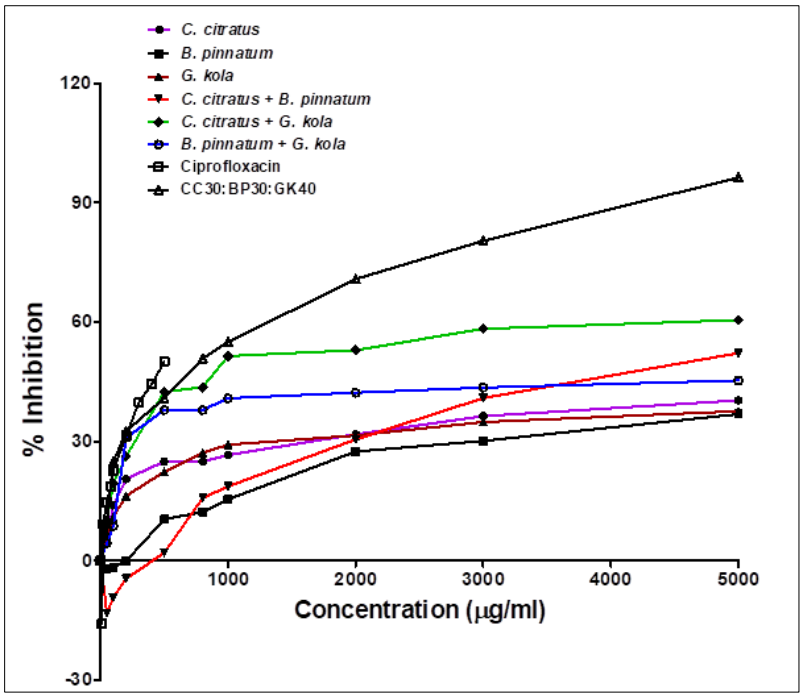

Figure 2: Inhibitory effect of aqueous extracts of single, binary and ternary (30:30:40) combinations of *C. citratus* (CC), *B. pinnatum* (BP), and *G. kola* (GK)against *Staphylococcus aureus* isolated from human cough sputum samples

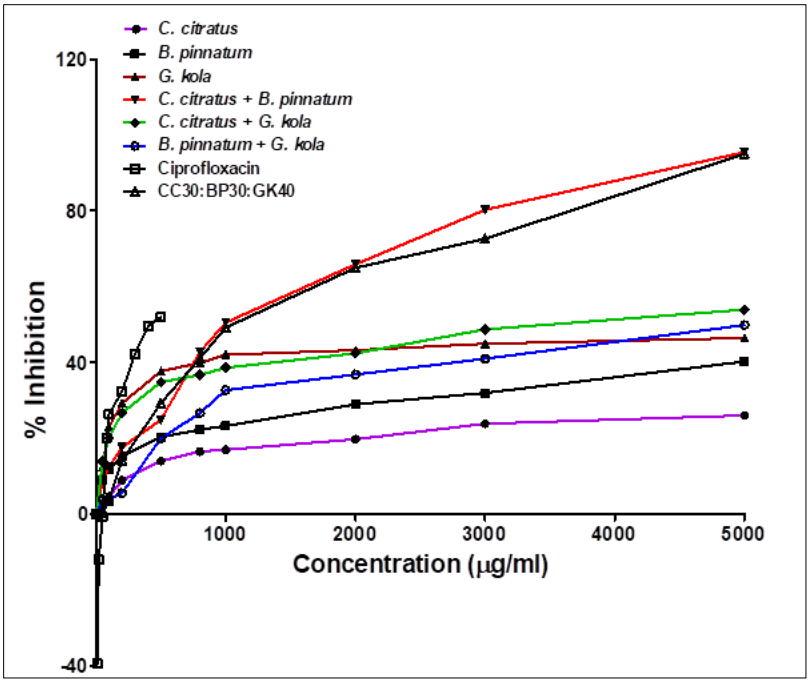

Figure 3: Inhibitory effect of aqueous extracts of single, binary and ternary (30:30:40) combinations of *C. citratus* (CC), *B. pinnatum* (BP), and *G. kola* (GK) against *Escherichia coli* isolated from human cough sputum samples

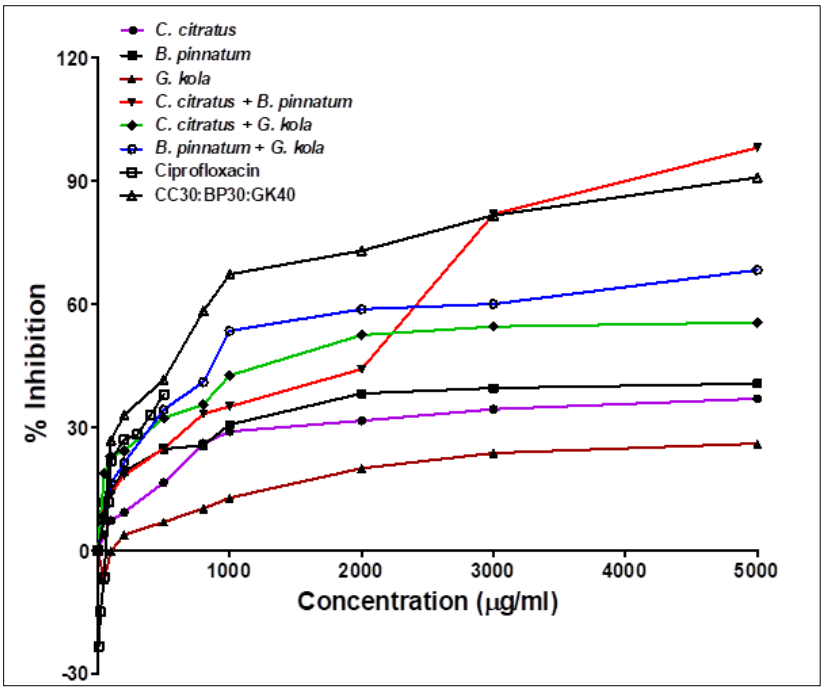

Figure 4 Inhibitory effect of aqueous extracts of single, binary and ternary (30:30:40) combinations of *C. citratus* (CC), *B. pinnatum* (BP), and *G. kola* (GK) against *Streptococcus sp* isolated from human cough sputum samples.

Table 7 Median inhibitory concentrations (IC_{50}) and R square values of effect of aqueous extracts of *C. citratus, B. pinnatum* and *G. kola* against bacterial isolates of cough sputum samples

Extract	***Klebsiella pneumonia***		***Staphylococcus aureus***		***Escherichia coli***		***Streptococcus sp.***	
	IC_{50}	**R^2 (P value)**	**IC_{50}**	**R^2 (P value)**	**IC_{50}**	**R^2 (P value)**	**IC_{50}**	**R^2 (P value)**
Ciprofloxacin	73.84	0.7224 (0.0018)	74.43	0.8160 (0.0003)	42.75	0.7233 (0.0018)	59.77	0.7248 (0.0018)
C. citrates	820.60	0.6723 (0.0037)	447.80	0.6624 (0.0042)	561.00	0.7206 (0.0019)	578.00	0.6745 (0.0036)
B. pinnatum	190.40	0.5726 (0.0113)	1625.00	0.8672 (<0.0001)	1804.00	0.7863 (0.0006)	815.60	0.6470 (0.0050)
G. kola	514.80	0.6255 (0.0064)	414.10	0.6346 (0.0058)	99.02	0.4377 (0.0372)	964.00	0.7934(0.0005)
C. citratus + B. pinnatum	162.00	0.8414 (0.0002)	783.00	0.8817 (<0.0001)	209.00	0.8777 (<0.0001)	110.00	0.9438 (<0.0001)
C. citratus + G. kola	517.40	0.6411 (0.0054)	287.00	0.5778 (0.0107)	547.30	0.6406 (0.0054)	1010.00	0.6485 (0.0049)
B. pinnatum + G. kola	960.20	0.7434 (0.0013)	76.40	0.4292 (0.0398)	891.30	0.7796 (0.0007)	607.70	0.6884 (0.0030)
CC + BP + GK	91.60	0.7559 (0.0011)	145.00	0.8089 (0.0004)	141.00	0.8630 (0.0001)	64.00	0.7000 (0.0025)

CC + BP + GK = ternary combination of *C. citratus, B. pinnatum* and *G. kola* extracts.

4. Discussion

Cough is more often, a reflex and repetitive action that helps clear the throat and airways of mucus, irritants, or foreign particles. It is characterized by violent release of air from the lungs following opening of the glottis, and usually accompanied by a distinctive sound. While coughing could be one of the lung's defenses, it is often a symptom of a problem that needs medical attention, especially when it becomes sustained. Cough is a common symptom that affects people of all ages and demographics globally. It is often caused by allergic reactions, irritants, medications, and asthma, but commonly by respiratory infections by bacteria and viruses [36-38]. It is one of the most common health problems and among the highest respiratory ailments for which patients seek attention. Medical evaluation of cough becomes urgently necessary when it is accompanied by symptoms such as fever, shortness of breath, bloody mucus, hoarse voice, vomiting, weight loss, leg swelling and difficulty swallowing.

In Nigeria, cough is prevalently due to asthma, pulmonary tuberculosis, air pollution, high rates of respiratory infections, and a lack of access to proper healthcare in some regions [39,40]. Untreated cough could affect vital organs such as the lungs, abdomen, heart, and central nervous system presenting as trivial or severe symptoms that can lead to complications such as pneumonia, bronchitis, or exacerbation of underlying conditions like asthma. It can also disrupt sleep, impair daily activities, and decrease quality of life [41,42].

There are many currently known cough remedies. The approach may be dependent on the type of cough, which may be dry, thickly or non-productive, wet, chesty or productive cough. The treatment of cough can be according to the symptoms or presentation of the cough. Knowing the cause of the cough will also aid effective management. The cost of treating cough varies depending on the underlying cause, severity, and accessibility to healthcare services. Expenses due to cough may be huge, and may include doctor consultations, diagnostic tests, medication, and, in severe cases, hospitalization [41,42]. Challenges associated with managing cough include misdiagnosis, especially in areas with limited healthcare resources, antibiotic resistance due to over-prescription, and addressing underlying health disparities that contribute to the prevalence and severity of cough in certain populations. Additionally, ensuring access to affordable and effective treatments poses a challenge, particularly in low-income settings [41,42]. Thus, the present study evaluated the potential use of readily available forkloric plants in the treatment of bacterial infection-induced cough.

Bacterial infections have long been identified as one of the common causes of cough. The relative frequency of the bacterial species that were implicated in causation of cough in this study is 90% for *Streptococcus sp*, followed by *Klebsiella pneumonia* (85%), *Staphylococcus aureus* (80%), and *E. coli* (30%). Co-bacterial infection was seen and had the highest prevalence rate at 95% of the cases. Studies have also implicated respiratory viruses such as influenza virus, rhinovirus, coronavirus (including SARS-CoV-2), adenovirus, and syncytial virus as frequent causes of cough [43]. Bacterial organisms such as *Mycoplasma pneumonia*, *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, and *Heamophilus influenza* have also been implicated. In other cases, fungal organisms such as *Aspergillus* and parasites such as *Ascaris lumbricoides* and *Strongyloides stercoralis* have been identified as causative organisms too. Thus, the etiology of cough is an important consideration in the treatment regimen of individuals with cough. The observed high co-infection prevalence in the causation of cough in the present study may be related to immunosuppression and poor sanitary condition that could be associated with poor income countries. Of note is the co-infection by different bacteria species, which may substantiate the requirement for multiple drug therapy and our proposed polyherbal therapy for effective remedy.

The result of this study showed that the aqueous extracts of *C. citratus, B. pinnatum* and *G. kola* demonstrated varying antimicrobial action against the isolates of *S. aureus, E. coli, Streptococcus sp, and K. pneumonia* tested. The degree of susceptibility of the bacteria to the aqueous extracts varied with the concentration of the plant extract. Antimicrobial activities were observed mostly at concentrations of 3000 to 5000 µg/ml.

The highest antimicrobial activity was exhibited by the extracts at the concentration of 5000 µg/ml. *G. kola* demonstrated higher activity than the other two extracts, in that at concentration of 5000 µg/ml it showed 50.3 and 46.49% inhibitory effects against *K. pneumonia* and *E. coli,* while *C. citratus* showed 40.33% inhibition against *S. aureus*. *B. pinnatum* showed a closely related percentage of inhibition against *Streptococcus sp* (40.69%) and *E. coli* (40.27%) at 5000 µg/ml of aqueous extracts of the individual plants. The least antimicrobial activity of 24.68% at 5000 µg/ml was demonstrated by *B. pinnatum* against *K. pneumonia*. A previous study demonstrated that ethanol-based extract of *G. kola* was highly effective against *S. aureus* [44]. The observed difference could be as a result of the method of extraction where ethanol-based extraction is believed to achieve higher extraction yield [45]. The finding of present study is in agreement with another study [46], where the sensitivity of *K. pneumonia* to methanol extract of *G. kola* was reported to be concentration dependent. Similarly, extracts of *C. citratus, B. pinnatum* and *G. kola* have previously been

demonstrated to possess antimicrobial potency against wide range of bacteria such as *Salmonella typhi, Staphylococcus aureus, Escherichia coli,* and *H. pylori,* as well as fungal organisms [20,47-51]. The antimicrobial activities of these plants were associated with their phytochemical and phenolic components such as citral, geraniol, garcinoic acid, citronellal, garcinial flavanoids, kolaviron, triterpenoid, steroids, alkaloids, flavonoids, tannins, and saponins [50]. It has been suggested that these compounds exhibit bacteriostatic and bacteriocidal properties by their interference with microbial cell membrane, metabolic enzyme activities, nuclear processes, inhibition of bacterial adhesion, biofilm formation, and other vital cell processes, ultimately resulting to bacterial cell growth retardation and death.

The combination of drugs or plant extracts that function synergistically has been in use for the treatment of diverse infections such as the commonly used artimisinin-based combination therapies for the treatment of malaria. The combination of *C. citratus* with Perilla essential oils have been reported to synergistically increase antimicrobial activity [52]. Also the combination of extracts of *G. kola* and honey has been recommended as a better option than their individual use for the treatment of infections caused by bacteria such as *P. aeruginosa*, *E. coli, S. aureus, Salmonella, K. pneumoniae and B. subtilis* [53]. These reports may explain why the binary formulations of *C. citratus, B. pinnatum* and *G. kola* extracts exhibited higher dose-dependent antimicrobial activity with observed stronger inhibitory effects when compared to the individual plant extracts. Similar binary combinations in previous studies also demonstrated better outcome further collaborating synergistic interactions of bioactive compounds in the extracts [54].

Previous phytochemical analyses of *G. kola, C. citratus,* and *B. pinnatum* demonstrated the presence of some secondary metabolites which are believed to be responsible for the antimicrobial activities of the plant. Alkaloids, flavonoids, tannins, triterpenoid, and steroids have been reported previously to be present in the plants and to possess antimicrobial activities [44,51,55-57]. Similarly, benzophenones and flavonones which have been successfully extracted from *G. kola* have been associated with antimicrobial activity. It is also suggested that alkaloids in these plants possess the ability to intercalate with DNA, disturb the activity of enzymes (esterase, DNA, RNA- polymerase or cell respiration known to inhibit gram positive and gram-negative, acid fast bacteria. Steroidal alkaloids such as solanine and tomatine have the ability to interfere with the stability of the bacterial cell membrane by forming a complex with phospholipid bilayer of the cell membrane ultimately creating a pore on cell membrane, leading to cell lysis and death. Some other alkaloids have been reported to interact with protein biosynthesis in the ribosome, bind to DNA thus inhibiting CDR1 protein, and induce fungal apoptosis [58].

Flavonoids were also present in the plants, and are believed to contribute to their antimicrobial property. Flavonoids are believed to inhibit nucleic acid synthesis in the bacterial cell wall and energy metabolism (caused by NADH-cytochrome C reductase or ATP synthase inhibition) as well as interruption of cell wall and cell membrane synthesis [59,60]. Similarly, tannins which are polyphenols suppress bacterial cell proliferation by blocking important enzymes of microbial metabolism [61]. While proanthocyanidins possess several mode of action such as destabilization of cell membrane, inhibition of extracellular microbial enzymes either directly or deprivation of substrate needed for growth, gallotannins have strong affinity for iron leading to inactivation of membrane bound proteins. Coumarins enhance reduction in cell respiration and terpenes cause cell membrane disruption. The presence of these phytochemicals and the highlighted properties strongly support the antimicrobial activity of the studied plants against the pathogens associated with cough causation in the present study.

The polyherbal extracts combination of the plants generally demonstrated higher antimicrobial activity than the individual and binary combinations with peak percent inhibitory values of 96.30%, 95.61%, 90.89% and 82.85%, against *S. aureus, E. coli, Streptoccocous sp* and *K. pneumonia* respectively at the highest extract combination concentration of 5000 µg/ml. The results strongly indicate that the ternary combination is a better natural antimicrobial remedy than the individual or binary combinations of the plants. This observation also suggests a greater antimicrobial synergism among the three different plant extracts and goes to corroborate the current use or advocacy of combination therapies for the treatment of most infectious diseases. Furthermore, the antibacterial effect of the ternary formulation is significantly comparable to the bacterial inhibitory effects of ciprofloxacin, compared to the single and binary formulations. Synergism in polyherbal formulations provide a direction for formulation of effective antibiotics. A report by Mussarat et al., [56] demonstrated that the mixture of highly used three individual plants *Mentha piperita, Camellia sinensis* and *Elettaria cardamom-mum*, was more effective at the lowest concentration against common gastrointestinal pathogen, piperia than the individual plants. The team also demonstrated that a mixture of three plants; *Terminalia chebula*, seeds of *Cumin cyminum* and *Foeniculum vulgare* at equal ratio showed higher significant inhibition against tested pathogens when compared to individual plants' extracts.

The three extracts in the present study inhibited the proliferation of the isolates of *S. aureus, E. coli, Streptococcus sp, and K. pneumonia* tested with variable minimum inhibitory concentrations (MIC). According to our results, the smallest value obtained in respect to the ternary combination of the three herbal plants was 64 against *Streptococcus specie*. It

was also observed that this combination gave low values across all the isolates. The smallest value obtained from the binary mixtures was 76.40 which was observed for the binary mixture of *B. pinnatum* and *G. kola.* The lowest value obtained when *C. citratus* and *B. pinnatum* was combined was recorded against *Streptococcus specie* was 110, while the lowest for *C. citratus and G. kola* was 287 against *Staphylococcus Sp.* The smallest MIC value obtained with C. citratus alone was 578 against *Staphylococcus Sp.* While with *B. pinnatum* it was 447 against *K. pneumonia,* and 99.02 for *G. kola* against *E. coli*. The antibacterial activities of these plants have been proven by literatures, and this work showed that the activities of the three polyherbal plant could exceed that of the individual plants, and the combination of these herbal plants is a potential good alternative as anticough.

5. Conclusion

This study revealed that individual, binary, and ternary extract combinations of lemon grass (*C. citratus), bitter kola (G. kola*), and miracle leaf (*B. pinnatum)* have significant antibacterial properties, with ternary combination of 40% *G. kola*, 30% *B. pinnatum* and 30% *C. citratus* demonstrating the highest potency. The study suggests possible synergic interactions from different combinations of the extracts in the treatment of bacterial-induced cough.

Dehydrogenase activities of the bacterial isolates were significantly and progressively inhibited by the aqueous extracts of the individual, binary and ternary combinations of the plants respectively. The MIC significantly varied for individual plants, binary, and ternary extract combinations against test microorganisms. The MIC was lowest in the ternary combination compared to the individual and binary extract formulations. The ternary combination of the extracts could potentially be exploited for treatment of bacterial-induced cough. However, more study will be needed to further evaluate the plants' safety and effectiveness for clinical use in humans. Furthermore, the antibacterial effect of the ternary formulation is significantly comparable to sensitivity to ciprofloxacin at 500 mg than to the single and binary formulations. While the ternary combination of the plant extracts showed remarkable inhibition against the isolated bacteria, ciprofloxacin exhibited superior antimicrobial efficacy compared to individual or combined plant extracts. However, the plant extracts may be associated with lesser toxicity and side effects making them safer for use compared to the standard antibiotic. This will need to be further evaluated.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

Authors' contributions

This work was carried out in collaboration with all authors. Authors CUI, NAO, CNP, FNU and JEO designed the study, wrote the protocol, and performed the statistical analysis and interpretation of study data. Authors NAO and JEO did the literature searches, while NAO wrote the first draft of the manuscript and incorporated all corrections from co-authors. Author CUI, CNP and FNU critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

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This study was self-sponsored.

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