Formulation and in vitro antibacterial screening of a bi-herbal syrup against some selected respiratory tract pathogens

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Submitted: 25th April, 2023; Accepted: 4th June, 2023; Published: 30th June, 2023
DOI: https://doi.org/10.54117/jcbr.v3i3.5
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Abstract

Introduction: Respiratory tract infection remains one of the most significant cause of death and hospital visit globally, with high burden in developing countries. The aim of the study was to formulate and carry out *in vitro* antibacterial screening of bi-herbal syrup against some selected respiratory tract pathogens.

Methods: 75% ethanol extracts of *Citrus aurantifolia* (CA) whole fruits and *Garcinia kola* (GK) seeds were made by cold maceration. Antibacterial activity and optimum minimum bactericidal concentrations (OMBC) of each extract (singly and combined) were determined using agar well diffusion and pour plate methods respectively. Formulations of the bi-herbal syrup were compounded by agitation without heat application method and re-evaluated for antibacterial activities.

Results: The percentage yield were 12.68 and 7.09 for CA and GK respectively. The extracts were tested against *Streptococcus pyogene*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. The MIC of the extracts varied from one pathogen to another. Clinical *Staphylococcus aureus* was highly resistant to GK, to the extent that the MIC value was indeterminate. However, CA exhibited more potent antibacterial activity with MBC ranging from 4.76- 9.53mg/mL than GK (MBC: 28.60 to 34.76 mg/mL). An optimal minimum bactericidal concentration of 9.523mg/mL (0.9523 % w/v) of *Citrus aurantifolia* whole fruits extract exhibited a total clearance of all the isolates tested, while 34.76 mg/mL (3.476% w/v) of Garcinia *kola* seeds extract did the same .When tested singly, the average zone of inhibition of the bacteria showed that the OMBC of CA tends to increase in diameter with increase in percentage concentration against the isolates while that of GK varied. The highest efficacy of the combined OMBC was at 85% ≡ 37.64mg/mg. At this concentration, a total kill on all the isolates tested was observed. An assessment of all the formulations with this same concentration and varying excipients concentration, showed that the average zone of inhibitions against all the isolates were higher than the standards used as positive controls at P-value of 0.05.

Conclusion: A combination of *Citrus aurantifolia* and *Garicia kola* extracts have shown *in vitro* antibacterial activities and therapeutic potential effects *in vivo* which was retained when the extracts were formulated into a bi-herbal syrup

Key words: Formulation, *In vitro* antibacterial screening, bi-herbal syrup

Introduction

Respiratory tract infection (RTI) accounts for one of the debilitating illnesses with significant mortality and morbidity in both adult and children, as various indices have pointed it as one of the major commonest diseases encountered in clinical and community medicine. This could be attributed to the large surface area (70 m^2) of the lungs presented to the atmosphere. Significantly, RTI associated diseases place a high burden on healthcare personnel and infrastructure. According to Umoh *et al*., [(1)] who conducted a study in a Hospital in South East Nigeria, documented that the overall mortality associated with RTI was 8.7%. High percentage of death from RTI were attributed to pulmonary TB (50%), followed by pleural disease (25%), pneumonia (12.5%) while acute exacerbation of chronic obstructive pulmonary disease (COPD) (6.25%) was the least recorded. Clinical manifestations of these diseases include common cold, sinusitis, pharyngitis, epiglottitis and laryngotracheitis, bronchitis, bronchiolitis and pneumonia. From various microbiological studies, the etiologic agents implicated in respiratory tract infections include *Streptococcus pyogene*, *Staphylococcus aureus, Streptococcus pneumoniae* and other bacteria, fungi and viruses [(2,3 and 4,)]. At early stage, respiratory tract infections are treated with cough syrups; however, conventional relevant antimicrobial agents may be prescribed. However, the use of these orthodox agents have encountered problems ranging from antimicrobial resistance, drug unavailability, affordability, contradiction and/ or precautionary use in pediatrics and drug addiction. As a result, *Citrus aurantifolia* and *Garcinia kola* components have often been used in ethnomedicinal practice in the treatment of some respiratory tract infections manifested as cough and the use of these plants in the management of some respiratory tract infections has been supported by a lot of research works [(5, 6, 7, 8, 9, 10, and 11)]. Studies have shown that 80% of the world's population, especially in developing countries, depends on traditional/herbal medicine [(12 and 13)]. Therefore, pharmaceutical approaches have become absolutely necessary to harness these crude drugs into pharmaceutically acceptable dosage forms, which would guarantee safety, efficacy, potency, stability, consistency and public trust. This is achievable through proper scientific evaluation, standardization, quality control and good manufacturing practice. Hence, this study, aim to evaluate the whole fruit of *Citrus aurantifolia* (Rutaceae) and seeds of *Garcinia kola* (Clusiaceae) [singly and in combination] for antibacterial activity, formulate the extracts into bi-herbal syrup and determine the antibacterial activity of the formulated products against respiratory tract bacteria [*Streptococcus pyogene, Streptococcus pneumoniae* and *Staphlococcus aureus* (both typed and clinical)]. Until now, no such formulation has been prepared and tested against the listed organisms. The bi- herbal syrup, if potent, may therefore, be a remedy for some respiratory tract infections whose etiologic organisms may be due to *Streptococcus* species, and *Staphylococcus* species and other bacterial pathogens.

Materials and Methods

The Plant Collection and Identification

Whole fruits of *Citrus aurantifolia* (lime), Rutaceae and seeds of *Garcinia kola* (bitter kola), Gutiferae were purchased from Kaduna Central Market Kaduna, Nigeria and were authenticated at the Department Herbarium of the Department of Biological Science, Faculty of Life Sciences
ABU, Zaria.

Extraction, Concentration and Determination of Percentage Yield of Fresh Plant Materials

Citrus aurantifolia (lime)

A modification of the method used by Azwanida [14] was used. The fruits of *Citrus aurantifolia* (lime) were cleaned and washed with distilled water and allowed to drain off.

A 100 g of the washed fruits were grinded with a grinding machine (Corona Landersycia S.A) and transferred into a macerating tank containing 250 mL of 75% ethanol. The tank was stirred intermittently for 72 h and the macerated mixture filtered through a Whatman filter paper 1 into a conical flask .The filtrate was transferred into a weighed porcelain dish and concentrated to constant weight using automated water bath (Huaia Angel Medical Instruments Co. Ltd China) set at 60ºC and the weight of the porcelain and the dried extract were taken using digital weighing machine (Top balance digital, U.S.A. Ohaus, PA313-model).

The percentage yield of the fresh plant materials were determined by extracts weight method. The dried extract was recovered and transferred into a dispensing bottle and stored in a desiccator for further use. The same method was applied to *Garcinia kola* seeds .More extracts were made as the need arose.

Preparation, Identification and Confirmation of the selected Pathogens

Clinical isolates of *Staphylococcus aureus, Streptococcus pyrogene,* and *Streptococcus pneumonia*e were obtained from Medical Microbiology Laboratory, Barau Dikko Teaching Hospital Kaduna whereas typed/standard organisms – *Staphylococcus aureus VT 000326 IVL*™ (Sigma Aldrich Germany), *Streptococcus pneumoniae ATCC 6305* ™ (Himedia Lab, India) and *Streptococcus pyogene ATCC 12384*™ (Thermo fisher USA) were procured

Preparation of Streptococcus pyogene and Streptococcus pneumoniae on blood agar base

A 8.6 g of blood agar base (Oxoid Ltd., Basingstoke, Hampshire, England) was weighed, dissolved into 200 mL of distilled water, heated to boiling point to dissolve the medium completely and sterilized by autoclaving (Adelphi MFG Co Ltd, Portland autoclave) at 15 psi 121°C for 15 minutes and cooled to 45^0C. Sheep blood {14 mL (7 % v/v)} was aseptically added and mixed thoroughly. Thereafter, 20 mL of the admixture was aseptically poured into Petri dishes using 20 mL sterile syringe and allowed to solidify.

Two of the plates were inoculated with *S. pneumoniae ATCC 6305* ™ and two others were inoculated with *S. pyogene ATCC 12384*™ by streak plate method. Clinical samples suspected to be *S. pneumoniae* and *S. pyogene* were also streaked on the supplemented blood agar base and all incubated at 5% CO_2 35°C.

Preparation of clinical typed/standard Staphlococcus aureus

Nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England) was prepared according to manufacturer's instructions and respiratory tract samples were collected on the nutrient agar slants. Mannitol salt agar (Oxoid Ltd., Basingstoke, Hampshire, England) was prepared according to manufacturer's instructions and inoculated with the isolates from the slants and incubated at 37 °C for 24 hours [15]. Colonies with distinct features of small golden yellow was sub-cultured on nutrient agar to obtain pure colonies.

Identification and confirmation of the selected pathogens

***Streptococcus pyogene*:** The *S. pyogene* prepared on the blood agar base supplemented with sheep blood, and incubated under

anaerobic conditions- 5 % CO_2 35 °C, was observed after 24 hours of incubation for dome-shaped colonies with a smooth or moist surface and clear margins.

The colonies that displayed a white-greyish colour, a diameter of ≥ 0.5 mm and were surrounded by a zone of β–hemolysis that is often two to four times as large as the colony diameter were selected [16]. Gram staining and catalase tests were also carried out on the isolates. The clinical isolates and *S. pyogene ATCC 12384* were confirmed on Microgen Strep Identification Kit according to the manufacturer's instruction and confirmed as *Streptococcus pyogenes* .The confirmed organisms were properly stored in a refrigerator (NAPCO Model 630 Portland, Oregon, USA) at 4 ℃, sub-culturing at interval of 4-5 days on blood agar base supplemented with 5 % sheep blood.

Streptococcus pneumoniae: The *S. pneumoniae* prepared on a blood agar base supplemented with 7 % v/v sheep blood, and incubated under anaerobic conditions -5 % CO_2 35 °C, was observed after 24 hours of incubation for α-hemolysis, lanceolate diplococci shape. Colonies of *S. pneumoniae* on blood agar plate were small (0.5 mm), round, translucent or mucoid with α-hemolysis, a green discoloration of the agar around the colonies. Gram staining (Gram positive) and catalase tests (catalase negative) [17] were also carried out.

The clinical isolates and *S. pneumoniae* ATCC *6305* were confirmed as *Streptococcus pneumoniae* on Microgen Strep. Identification Kit according to the manufacturer's instruction. The confirmed organisms were properly labelled and stored at 4 ℃, sub-culturing at interval of 4-5 days on slant 5 % sheep blood supplement.

Staphylococcus aureus

Morphological investigation such as Grams reaction, shape and arrangement of cells and biochemical ability to ferment mannitol, catalase and coagulase production tests were carried out.

The clinical isolates and standard *Staphylococcus aureus VT 000326 IVL™*, were confirmed as *Staphylococcus aureus* on Microgen Staph Identification Kit according to the manufacturer's instruction .The identified *Staphylococcus aureus* was properly labelled and was sub-cultured into slant nutrient agar and stored at 4 °C

Preparation of 0.5 McFarland Standard Suspensions of the Identified Organisms

The primary aim of standard inoculums preparation is to achieve a high level of viable pure biomass in a suitable physiological state for use as an inoculum. A 0.5 McFarland standard (an average of 1.5 x 10^8 colony forming unit per ml) of *Staphylococcus aureus* was prepared according to methods described by Carolyn *et al* [18] and Elin *et al* [19] whereas *S. pyogene* and *S. pneumoniae* was prepared by the modifications of the method described by Pankuch *et al* [20] and Andrews [21] respectively and was used as inoculum population.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *C. aurantifolia* fruits and *G. kola* seed extracts

MIC is defined as the lowest concentration of antimicrobial agent that is able to inhibit visible microbial growth, whereas, MBC is the minimum concentration that produced bactericidal /killing effects on the bacteria.

A modification of the methods as described by Adeshina *et al* [22] and Ehinmidu and Ibrahim [23] were adopted to determine MIC and MBC. The dried extracts were weighed and different concentrations of the different extracts were made by dissolving each weighed extract in 1 mL using 75% ethanol as solvent (the concentration of the ethanol will drop to 3.75% in 20 mL molten agar). Graded concentrations in % w/v of *C. aurantifolia* extract were made: 800 mg/mL, 750 mg/mL, 700 mg/mL, 650 mg/mL, 600 mg/mL, 550 mg/mL, 500 mg/mL, 450 mg/mL, 400 mg/mL, 350 mg/mL, 300

mg/mL, 200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL whereas 400 mg/mL, 450 mg/mL, 500 mg/mL, 550 mg/mL, 600 mg/mL, 650 mg/mL, 700 mg/mL, 750 mg/mL, 800 mg/mL, 850 mg/mL, 900 mg/mL, 950 mg/mL and 1000 mg/mL concentrations were made for *G. kola* extract.

A mL of each of the above graded concentrations was respectively mixed with 19 mL molten Muller Hinton agar prepared, such that the resultant admixture of molten agar and extract is 20 mL. The extract-agar admixtures were respectively inoculated with 20 µL of 0.5 McFarland standard suspension of a given test organism, poured into a sterile plate and evenly mixed. The inocula were allowed to diffuse into the test agar plates for 30 min. The test agar plates were then incubated at appropriate conditions (*Staphlococcus aureus* at 37°C for 24 h, *Streptococcus pyogene and Streptococcus pneumoniae* at 37 °C, 5 % CO_2 for 24-48 h and the lowest concentration of the extract in the test agar plates that showed no growth was considered as MIC of the extract against the test organism. The test procedure was carried out in triplicate and the average taken. The concentrations of the extract in the test agar plates showing no visible growth were selected.

Direct weighing of different masses (6.67, 5.71, 4.76, 3.81, 2.86, 1.90 mg/mL for *S. pyogene*; 5.34, 4.76, 4.27, 3.80, 3.33, 2.86 mg/mL for *S. pneumonia* and 10.48, 9.52, 8.57, 7.62, 6.67, 5.71 mg/mL for *S. aureus*) of the *Citrus orantifalia* extract was used to carry out this procedure, by dissolving each weighed extract in 1 mL of 75% ethanol, after which 1 mL 3% v/v Tween 80 was added in a 20 mL bottle. Thereafter, sterile 18 mL Muller Hinton agar was prepared in another 20 mL bottles and added to make up the total resultant extract-tween 80- agar admixture of 20 mL. The admixtures were inoculated with 20µ1 of a given organism and poured into a sterile plates. The final volume of agar-extract-3% Tween 80 admixture was 20.02 mL. These plates were then incubated at appropriate respective conditions and they were examined for presence or absence of growth. The plate that yielded less than six colonies were selected and the extract concentration in the plate was taken as the minimum bactericidal concentration according to Ehinmidu and Ibrahim [23]. This procedures were carried out in triplicate and average result recorded. The same procedure was repeated for *G. kola*

Determination of Combined Antibacterial Activities of the Extracts based on Optimum Minimum Bactericidal Concentration (OMBC) of each of the Extracts

Optimum minimum bactericidal concentration (OMBC) of the extract is the minimum bactericidal concentration (MBC) that is able to produce effective bactericidal actions against all the tested microorganisms. This is the upper limit range of MBC (Citrus MBC range is 4.76 to 9.523 mg/mL while G. kola is 30.48 – 34.76 mg/mL). This implies that OMBC is expected to produce similar bactericidal actions both in combined forms and in formulations so that the product-bi-herbal syrup may be used for its antibacterial property against respiratory tract pathogens. The combined antibacterial screening would indicate possibilities of synergism, antagonism or additive effects between the two extracts.

In this study, 9.523 mg/mL *Citrus aurantifolia* whole fruits extract was considered as optimum minimum bactericidal concentration (OMBC) whereas 34.76 mg/mL extract of *Garcinia kola* seeds extract was considered as OMBC except in clinical *Staphylococcus aureus*, where all the concentrations could not exhibit bactericidal action even at 100% concentration.

Determination of Antibacterial Activities of various Combination Pattern between the Extracts of Citrus aurantifolia whole fruits and Garcinia kola seeds using their respective OMBC

The optimum minimum bactericidal concentration (OMBC) (9.523mg/mL for *Citrus aurantifolia* and 34.76 mg/mL *Garcinia kola* seeds) of the extracts were calculated and combined based on a given fractional percentage contribution of each of the respective plant extract's OMBC as shown in the Tables 1 and 2 below: 25, 50, 75, 100 and 120 fractional percentages of OMBC of *Citrus aurantifolia* extract were respectively combined with 25, 50, 75, 100 and 120 fractional percentages of OMBC of *Garcinia kola* extracts and their combined zones of inhibition and minimum bactericidal concentrations determined (Table 3).

Table 1: Fractional Percentage of OMBC (9.523 mg/mL) of *Citrus aurantifolia* whole Fruits Extract

25% x OMBC	50% x OMBC	75% x OMBC	100% x OMBC	120% OMBC
0.25 x 9.523 mg/mL	0.50 x 9.523 mg/mL	0.75 x 9.523 mg/mL	1.0 x 9.523 mg/mL	1.2 x 9.523 mg/mL
2.381 mg/mL	4.762 mg/mL	7.142 mg/mL	9.523 mg/mL	11.428 mg/mL
23.81/10mL	47.62 mg/10mL	71.42 mg/10mL	95.23 mg/10mL	114.28 mg/10mL

Table 2: Fractional Percentage of OMBC (34.76 mg/mL) *Garcinia kola* Seeds Extracts

25% x OMBC	50 % x OMBC	75% x OMBC	100% x OMBC	120% x OMBC
0.25 x 34.76 mg/mL	0.50 x 34.76 mg/mL	0.75 x 34.76 mg/mL	1 x 34.76 mg /mL	1.2 x 34.76 mg/mL
8.69 mg/mL	17.38 mg/mL	26.07 mg/mL	34.76 mg/mL	41.71 mg/mL
86.90 mg/10mL	173.38 mg/10mL	260.70/10mL	347.60 mg/10mL	417.10mg/10mL

Table 3: Combined *Citrus aurantifolia* whole Fruits (CA) and *Garcinia kola* Seeds (GK) OMBCs in 10ml Preparations

25% x OMBC	50% x OMBC	75% x OMBC	100% x OMBC	120% x OMBC
23.81 mg CA + 86.90 mg GK	47.62 mg CA + 173.38 mg Gk	71.42 mg CA + 260.70 mg Gk	95.23 mg CA + 347.60 mg GK	114.28 mg CA + 417.10 mg GK

Determination of the Zone of Inhibition of the combined fractional percentages of OMBCs

The zone of inhibition of the combined fractional percentages of OMBCs against all the test isolates were determined using a modification of method as described by Balouiri *et al.*,[24]

A unit of OMBCs (100% OMBCs of each plant extract) was selected as optimum combination pattern and combined MBC determined

Determination of the Minimum Bactericidal Concentration of the OMBC Combination Pattern

A modification of the same methods as described by Adeshina *et al* [22] and Ehinmidu and Ibrahim [23] were also used to determine the new MBC of the selected/optimum combined MBCs of the two plant extracts. Different fractional percentage concentrations: 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 of the selected combination pattern were made. Each 1 mL (containing appropriate quantities of CA and Gk) of each fractional percentage concentration (as calculated in the Table 3 below) was respectively admixed with 20 ml sterilized Muller-Hinton agar, and inoculated with 20 mL appropriate organism. The admixture was poured into a sterile plate and allowed to set before incubating it at appropriate conditions. Stock solutions of CA and GK were prepared such that 1 mL that would be transferred to 20 ml agar plate would contain a given quantity of CA and GK as shown in Table 4). The culture observed for growth after 24 hours and the number of colonies counted. The same procedure was carried out in triplicate.

Table 4: Fractional Percentages of the Selected /Optimum Combined Portions

	CA Extract		**GK Extract**	
F/%	Fractional percentage of 9.523 mg/mL	Conc.in 1 mL CA + GK admix. stock (mg/mL)	Fractional percentage of 34.76 mg/mL	Conc.in 1 mL CA + GK admix. stock (mg/mL)
55	0.55 x 9.523 = 5.24	110.04	0.55 x 34.76 = 19.12	401.52
60	0.60 x 9.523 = 5.71	119.91	0.60 x 34.76 = 20.86	438.06
65	0.65 x 9.523 = 6.19	129.99	0.65 x 34.76 = 22.59	474.39
70	0.70 x 9.523 = 6.67	140.07	0.70x34.76=24.33	510.93
75	0.75 x 9.523 = 7.14	149.94	0.75x34.76=26.07	547.47
80	0.80 x 9.523 = 7.61	160.02	0.80x34.76=27.81	584.01
85	0.85 x 9.523 = 8.09	170.10	0.85x34.76=29.55	620.55
90	0.90 x 9.523 = 8.57	179.97	0.90x34.76=31.28	656.88
95	0.95 x 9.523 = 9.05	190.05	0.95x34.76=33.02	693.42
100	1 x 9.523 = 9.523	199.98	1.0x34.76=34.76	729.96

Key: Admix. = admixture, CA= *Citrus aurantifolia* extract,GK= *Garcinia kola* extract, F/% = fractional percentage combinations.

Determination of Quantity of Active Ingredients (*Citrus aurantifolia* (CA) extract, and *Garcinia kola* (GK) extracts) to be used in the Formulation of the Bi-herbal Syrup

The final combination quantities of CA and GK extracts used as active ingredients in the formulation of the bi-herbal syrup were worked out as follows:

A combination pattern of 85% of the combined OMBCs exhibited optimum bactericidal actions on all the respiratory tract bacterial pathogens, killing all the isolates .However, a positive adjustment of 2.5% was made so as to accommodate possibility of loses, hence the optimum combination pattern was shifted to 87.5 % .This 87.5% of OMBC was a bit above 85% which is the highest concentration that would bring about optimum bactericidal activities against all the tested organisms

Mathematically:

87.5% of Selected OMBC of CA=0.875 x 9.525mg/mL =8.33mg/mL

87.5 % of Selected OMBC of GK = .875 x 34.76mg/mL=30.42mg/mL

These figures implied that, in combining CA and GK extracts, a lesser concentration of CA extract (8.33mg/mL) than the OMBC of CA extract (9.525mg/mL) was needed in the formulation of the bi-herbal syrup whereas a lesser concentration of GK extract (30.42 mg/mL) than the OMBC of GK extract (34.76mg/mL) was required to bring about bactericidal effects across all the tested organisms in the bi-herbal syrup formulation. These smaller concentrations, in addition to the high possibilities of bacterial resistance of a single extract, justified the need for combining the extracts. These decreased concentrations resulted from the additive effects of the combining CA extract with that of GK extract.

Syrup Formulation

This was carried out using the method described by Kaushik [25] with some modifications. Formulation of 25 mL volume bi-herbal syrup was carried out by making calculations for the appropriate quantities of *Citrus aurantifolia* (CA) and *Garcinia kola* (GK) extracts needed. Allowances/calculations were also made such that if 1ml of the syrup was transferred to 20 mL molten Muller-Hinton agar, the resulting concentrations in mg/mL of CA and GK extracts would be 8.33mg/mL and 30.42 mg/mL respectively.

This was calculated using the formula: $C_1V_1 = C_2V_2$

C_1 = Concentration of the extract in the formulated bi-herbal syrup

V_1 = 1 mL = Volume of the formulated bi-herbal syrup to be transferred to 20 mL molten agar.

C_2 = final concentration of the extract in 21 mL admixture (20 mL molten agar +1 mL of the syrup)

V_2 = 21 mL = 20 mL molten agar +1 mL syrup in inoculated plate.

Calculating for quantity of Citrus aurantifolia (CA) extract needed in 25mL bi-herbal syrup

$C_1 V_1 = C_2 V_2$

C_1=Concentration of the CA extract in the formulated bi-herbal syrup

V_1= 1mL= Volume of the formulated bi-herbal syrup to be transferred to 20mL molten agar.

C_2= 8.33mg/mL= final concentration of CA extract in 21ml admixture (20 mLmolten agar +1ml of the syrup)

V_2= 21 mL= 20 mL molten agar + 1ml syrup

Therefore, $C_1 = C_2\ V_2/V_1$=8.33mg/mL x 21/1=174.93mg/mL.

Therefore, 25mL bi-herbal syrup would contain (174.93mg/mL x 25) of CA = 4373mg=4.373g/25mL

Calculating for quantity of Garcinia kola (GK) extract needed in 25mL bi-herbal syrup

$C_1 V_1 = C_2 V_2$

C_1= Concentration of GK extract in the formulated bi-herbal syrup

V_1= 1ml= Volume of the formulated bi-herbal syrup to be transferred to 20mL molten agar.

C_2=30.42 mg/mL = final concentration of the GK extract in 21mL admixture (20ml molten agar +1ml of the syrup)

V_2= 21mL =f inal volume (20mL molten agar + 1mL syrup)

Therefore, $C_1 = C_2\ V_2/V_1$=30.42 mg/mL x 21/1=638.82 mg/mL.

Therefore, 25mL bi-herbal syrup would contain (638.82x 25) mg of GK= 15970.50 mg=15.97g/25mL

Bi-herbal Syrup Compounding Formula

Table 5: Derived Bi-herbal Syrup Formula

Formulations	Ingredients

	CA(g)	Gk (g)	Sucrose BP %w/v	NaCMC %w/v	Glycerin to 25mL
F_0	4.37	15.97	0	0.0	to 25mL
F_1	4.37	15.97	10	0.4	to 25mL
F_2	4.37	15.97	30	0.6	to 25mL
F_3	4.37	15.97	40	0.8	to 25mL
F_4	4.37	15.97	60	1.0	to 25mL

Keys: F= Formulations, CA=*Citrus aurantifolia* extract, GK=*Garcinia kola* seed extract, NaCMC= Sodium Carboxymethylcellulose.

Compounding of Different Formulations of the Bi-herbal Syrup

The different graded formulations were compounded separately:

Preparation of formulation$_1$ (25ml) using agitation without heat method

The method of Kaushik [25] was used with some modifications. A *4.37g Citrus aurantifolia* (CA) extract and 15.97 g of *Garcinia kola* extract (Gk) were respectively weighed into a mortar and both were dispersed with 5ml 75% ethanol. A 2.5 g of sterilized sucrose BP was weighed, dissolved in 5ml of sterilized distilled water and transferred into the mortar and mixed properly with pestle. A one milliliter (1ml) of the prepared stock solution of sodium carboxylmethylcellulose (NaCMC) was added and mixed very well

Glycerin was added to the mortar as required so that final volume of the syrup became 25ml The resulting mixture was homogenized with mortar and pestle, dispensed through sterilized sieve cloth into a sterilized dispensing bottle and labeled. The same procedures were repeated for F_0, F_2, F_3 and F_4 using their respective amount of excipients as shown in the Table 5 above.

Determination of the Antibacterial Activities (Zone of Inhibition) of the Compounded Bi-Herbal Syrup

The zones of inhibition (ZI) and minimum bactericidal concentration (MBC) of the compounded bi-herbal syrup were re-evaluated to determine whether the extracts would retain their antibacterial activity in a formulated (product) form. Agar well diffusion method was used in the determination of ZI of both the herbal syrup and the two positive antibiotics controls (gentamycin 10 µg and levofloxacin 20 µg discs) (Oxoid, UK) as described by Balouiri *et al*., [24]

Statistical Analysis

Using SPSS version 20, statistical tools such as mean, percentage and two ways ANOVA were employed for analysis of the results were they are appropriate.

Results

Extract Yield

The percentage yield of 75 % ethanol extract of *Citrus aurantifolia* (CA) whole fruit was 12.68 % whereas *Garcinia kola* (GK) seed yielded 7.09 %, using a cold maceration method.

Minimum Inhibitory Concentration (MIC) of the Plant Extracts

The minimum inhibitory concentration (MIC) of CA extracts against *S. pyogene* (both typed and clinical samples) was 4.76 mg/mL; clinical isolates of *Streptococcus pneumoniae* and *Staphylococcus aureus* expressed more resistance with an MIC of 7.14 mg/mL and 9.53 mg/mL respectively than the typed culture ATCC 6305 and *VT* 000326 with 4.76 mg/mL respectively (Table 6). Comparative assessment of tables 6 and 7 showed that both

extracts had varied MICs as a result of their varied sensitivity. Clinical *Staphylococcus aureus* was resistant to GK extract to the extent that the MIC value was indeterminate. *Citrus aurantifolia* extracts exhibited more potent antibacterial activity as MICs across all the selected organisms ranged from 4.76 - 9.53 mg/mL than *Garcinia kola* extract which MICs had ranged from 28.60 to 33.33 mg/mL.

Table 6: Minimum Inhibitory Concentration (MIC) *Citrus aurantifolia* Extract

Organism	Concentration (mg/mL)/Av. Colonies										MIC
	0.30	**0.60**	**1.12**	**2.38**	**4.76**	**7.14**	**9.52**	**14.29**	**21.43**	**23.81**	**(mg/mL)**
S. pyogene ATCC12384™	TMC	TMC	34	15	NG	NG	NG	NG	NG	NG	4.76
S. pyogene (Clinical)	TMC	TMC	45	25	3	NG	NG	NG	NG	NG	4.76
S. pneumoniae ATCC6305	TMC	TMC	LG	10	NG	NG	NG	NG	NG	NG	4.76
S. pneumoniae (clinical)	TMC	TMC	30	15	12	NG	NG	NG	NG	NG	7.14
S. aureus VT 000326	LG	LG	LG	LG	45	NG	NG	NG	NG	NG	4.76
S. aureus (Clinical)	TMC	TMC	LG	27	16	10	NG	NG	NG	NG	9.53

Keys: NG= No growth, TMC = Too many to count, LG=light growth, Av =Average

The MIC of GK extracts against *S. pyogene* (both typed and clinical isolates) was 28.6mg/mL. The clinical and typed culture ATCC 6305 isolates of *Streptococcus pneumoniae* had MIC of 31.1mg/mL and 33.33mg/mL respectively. The standard culture of *Staphlococcus aureus VT* 000326 had also 31.1 mg/mL MIC while the clinical one expressed resistant profile even higher than 42.9mg/mL (Table 7).

Table 7: Minimum Inhibitory Concentration (MIC) of *Garcinia kola* extracts

Organism	Concentration (mg/mL)/Av. Colonies										MIC
	19.04	**21.40**	**23.80**	**26.20**	**28.60**	**31.00**	**33.33**	**35.70**	**38.10**	**42.90**	**(mg/mL)**
S. pyogene ATCC12384™	TMC	TMC	40	18	5	NG	NG	NG	NG	NG	28.60
S. pyogene (Clinical)	TMC	TMC	45	25	12	NG	NG	NG	NG	NG	28.60
S. pneumoniae ATCC 6305	TMC	TMC	TMC	475	277	29	4	NG	NG	NG	33.33
S. pneumoniae (clinical)	TMC	TMC	LG	90	34	4	NG	NG	NG	NG	31.10
S. aureus VT 000326	TMC	TMC	TMC	LG	89	NG	NG	NG	NG	NG	31.10
S. aureus (clinical)	TMC	TMC	TMC	TMC	TMC	TMC	TMC	TMC	TMC	TMC	NBE

Keys: NG= No growth, TMC =Too much to count, LG= light growth, Av.no = average number, NBE=No Bactericidal Effects

Minimum Bactericidal Concentration (MBC) of the Plants Extracts

Table 8 showed that typed cultures (*S. pyogene* ATCC12384™ and *S. pneumoniae* ATCC 6305) were more susceptible to CA whole fruit extracts with MBC of 4.76 mg/mL while clinical isolates

exhibited more resistance especially clinical *S. aureus* 9.523 mg/mL MBC. Comparative assessment of Tables 8 and 9 showed that *Citrus aurantifolia* has higher antibacterial activity compared to *Garcinia kol*a.

Table 8: Minimum Bactericidal Concentration (MBC) of *Citrus aurantifolia* Whole Fruit Extracts

Organism	Concentration (mg/mL)/Av. Colonies						MBC (mg/mL)
	6.67	**5.71**	**4.76**	**3.81**	**2.86**	**1.90**	
S. pyogene ATCC12384™	NG	NG	1	20	30	70	4.76
S. pyogene (Clinical)	NG	2	7	45	80	120	5.71
	5.34	**4.76**	**4.27**	**3.80**	**3.33**	**2.86**	
S. pneumoniae ATCC 6305	NG	NG	7	7	17	20	4.76
S. pneumoniae (Clinical)	NG	7	8	8	25	35	5.34
	10.48	**9.52**	**8.57**	**7.62**	**6.67**	**5.71**	
S. aureus VT 000326	NG	NG	NG	3	48	83	7.62
S. aureus (Clinical)	NG	NG	7	8	12	55	9.523

Keys: NG= No growth, TMC = Too many to count, Av=Average.

The MBC of GK extract ranged from 30.48 – 34.76 mg/mL (Table 9). *S. aureus VT* 000326 had the least MBC followed by *S. pyogene* (both clinical and typed) while *S. pneumoniae* had the highest MBC 34.76mg/mL. *Garcinia kol*a extract had no bactericidal effect against clinical *S. aureus* even at 34.76mg/mL MBC.

Table 9: Minimum Bactericidal Concentration (MBC) of *Garcinia kol*a extract

Organism	Concentration (mg/mL)/Av. Colonies								MBC (mg/mL)
	32.85	**30.95**	**28.60**	**26.20**	**23.80**	**21.40**	**19.05**	**16.67**	
S. pyogene ATCC12384™	NG	12	23	30	LG	TMC	TMC	TMC	32.85
S. pyogene (Clinical)	NG	7	12	LG	TMC	TMC	TMC	TMC	32.85
	36.66	**35.71**	**34.76**	**33.81**	**32.85**	**31.90**	**30.95**	**30.00**	
S. pneumoniae ATCC 6305	NG	NG	NG	7	10	17	33	68	34.76
S. pneumoniae (Clinical)	NG	NG	NG	23	70	80	97	102	34.76
	34.76	**33.33**	**32.38**	**31.43**	**30.48**	**29.52**	**28.57**	**23.81**	
S. aureus VT 000326	NG	NG	NG	NG	NG	13	23	27	30.48
S. aureus (Clinical)	NBE	NBE	NBE	NBE	NBE	NBE	NBE	NBE	NBE

Keys: NG = No Growth (sensitive), LG = little growth, TMC = Too much to count (resistant) Av. = Average, *S = Streptococcus*, NBE= No Bactericidal Effect (resistant)

Optimum Minimum Bactericidal Concentration (OMBC)

From Tables 8, an OMBC of 9.523mg/mL (0.9523 % w/v) of 75% ethanol extract of *Citrus aurantifolia* whole fruits exhibited total clearance of all the isolates tested, whereas, 34.76 mg/mL (3.476% w/v) ethanol extract of *Garcinia kola* seeds (Tables 9) did the same for most of the bacterial tested.

When tested singly, the average zone of inhibition of the bacteria showed that the OMBC of *Citrus aurantifolia* whole fruits extract tends to increase in diameter with increase in percentage while that of *Garcinia kola* seeds extract varies with increase in concentration. When the extracts were combined, there was a progressive increase in activity. More so, when compared with the zones tested singly, the result showed synergistic activities of the extracts.

However, at 50% OMBC, all the isolates were resistant except *S. pyogene*. A unit (100%: (9.523 mg/mL of CA and 34.76 mg/mL GK extracts)) of OMBC of CA and GK gave 22.7 mm and 25.2 mm diameter respectively whereas their composite average zone of inhibition stood at 26.7 mm diameter across all the tested organisms.

Table 10: Determination of the Zone of Inhibition of *Citrus aurantifolia* Whole Fruits and *Garcinia kola* Seeds OMBC (Singly and Combined)

Bacteria	25% OMBC (mm)			50% OMBC (mm)			75% OMBC (mm)			100% OMBC (mm)			120% OMBC (mm)		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
S. pyogene ATCC12384	15	18	20	20	18	25	25	18	30	24	28	25	25	22	35
S. pyogene (Clinical)	12	14	18	18	14	20	22	16	26	21	25	25	23	20	28
S. pneumoniae ATCC 6305™	10	18	25	16	25	6	20	22	20	24	28	26	25	22	35
S. pneumoniae (Clinical)	9	15	23	15	24	9	18	18	20	22	26	24	24	23	30
S. aureus VT000326	12	18	22	18	25	6	24	25	25	22	20	35	20	20	25
S. aureus (Clinical)	6	20	20	16	20	6	16	20	24	20	24	25	22	25	30
CAZI	10.7	17.2	21.3	17.2	21.0	12.0	20.8	19.8	24.2	22.7	25.2	26.7	23.2	22.0	30.5

Keys: CAZI = Composite average zone of inhibition, A = *Citrus aurantifolia* extract, B = *Garcinia kola* extract, C = *Citrus aurantifolia* extract + *Garcinia kola* extract

Evaluation of the Minimum Bactericidal Concentration of the Combined OMBC

Table 11 showed that the highest efficacy of the combined OMBCs was at 85% ≡ 37.64mg/mg (CA+GK). At this concentration, it is believed that 85% of 9.523mg/mL *Citrus aurantifolia* extract and 85% of 34.76 mg/mL of *Garcinia kola* extract which is approximately 37.64mg/mg will effect a total kill on all the isolates tested, even against clinical isolates of *S. aureus* which susceptibility was recoded to be indeterminate due to high level of resistance.

Table 11: MBCs of Combined OMBC of 55% to 100 %

Organisms	Percentage Concentration of the Selected OMBCs	New MBCs

	(CA = 9.523 mg/mL and GK = 34.76 mg/mL)										(mg/mL)
	55	60	65	70	75	80	85	90	95	100	
S. pyogene ATCC12384™	180	160	96	15	NG	NG	NG	NG	NG	NG	75% ≡ 33.21 mg/mL
S. pyogene (clinical)	220	190	87	24	NG	NG	NG	NG	NG	NG	75% ≡ 33.21 mg/mL
S. pneumoniae ATCC 6305™	123	43	NG	NG	NG	NG	NG	NG	NG	NG	65% ≡ 28.78 mg/mL
S. pneumoniae (clinical)	228	189	76	NG	NG	NG	NG	NG	NG	NG	70% ≡ 31.0 mg/mL
S. aureus VT 000326	45	NG	NG	NG	NG	NG	NG	NG	NG	NG	60% ≡ 26.57 mg/mL
S. aureus (clinical)	TMC	TMC	LG	230	190	79	NG	NG	NG	NG	85% ≡ 37.64 mg/mg

Key: TMC = Too many to count, NG = No growth, LG = light growth, CA = *Citrus aurantifolia* extract, GK = *Garcinia kola* extract, Av. No. = Average Number

Table 12 showed 5 different formulations having the same concentration of active ingredients [i.e. 8.33mg/mL (87.5% 9.525mg/mL) of CA combined with 30.42mg/mL (87.5% of 34.76mg/mL) of GK] with varying excipients concentrations. At these concentrations, the average zone of inhibitions against all the isolates were higher than the standards - gentamicin10µg and levofloxacin 20µg discs used as positive control. This implied that at this concentrations, the bi-herbal syrup would have a better potential therapeutic outcome when compared to gentamycin and levofloxacin.

Table 12: Zone of Growth Inhibition of Different Formulations and Positive Controls, at Compounding

Formulations & Controls	Organisms and AZI (mm)						Average ZI (mm)
	S. pyogene		*S. pneumoniae*		*S. aureus*		
	ATCC12384™	Clinical	*ATCC 6305™*	Clinical	*VT 000326*	Clinical	
F_0	30.33	28.33	31.33	28.33	34.67	32.33	30.89
F_1	28.33	27.00	36.00	32.00	33.00	32.67	31.50
F_2	30.33	28.66	34.00	33.67	30.67	26.33	30.58
F_3	33.00	29.33	28.33	27.00	31.33	30.67	29.94
F_4	40.00	35.33	30.33	28.00	32.00	28.00	32.28
Gent. 10µg	28.00	26.67	22.00	20.33	26.00	28.33	25.22
Levo. 20µg	35.66	30.67	27.00	27.23	25.33	22.00	28.22

Keys: F = Formulations, AZI = Average zone of growth inhibition, Gent = Gentamycin disc, Levo = Levofloxacin disc

Table 13 showed that formulations F_0 – F_3 retained their MBCs as they recorded no or less than 6 colonies whereas F_4 could nor retain MBC only in clinical *S. aureus* as more than 6 colonies were recorded in clinical *S. aureus*.

Table 13: Formulations MBC Retention, 0 Day of Compounding

Organisms	F_0	F_1	F_2	F_3	F_4
S. pyogene ATCC12384™	NG	NG	NG	NG	NG
Clinical *S. pyogene*	NG	NG	NG	NG	2 Colonies
S. pneumoniae ATCC 6305™	NG	NG	NG	NG	NG
*S. pneumoniae (*clinical)	NG	NG	NG	NG	NG
S. aureus VT 000326	NG	NG	NG	NG	NG
Clinical *S. aureus*	NG	NG	NG	3 Colonies	15

Key: F= Formulations, NG= No Growth.

Figure 1 below showed that the P-values decreased as excipients concentration increased; became significant between F3 and F4. This implied that increase in excipients (sucrose and NaCMC) concentrations may be connected with increased antibacterial activity in terms of zone of growth inhibition. Also, P -values were significant between treatments (Fs) and controls; indicating a significantly more antibacterial performance of the formulation (F_4) than the positive controls

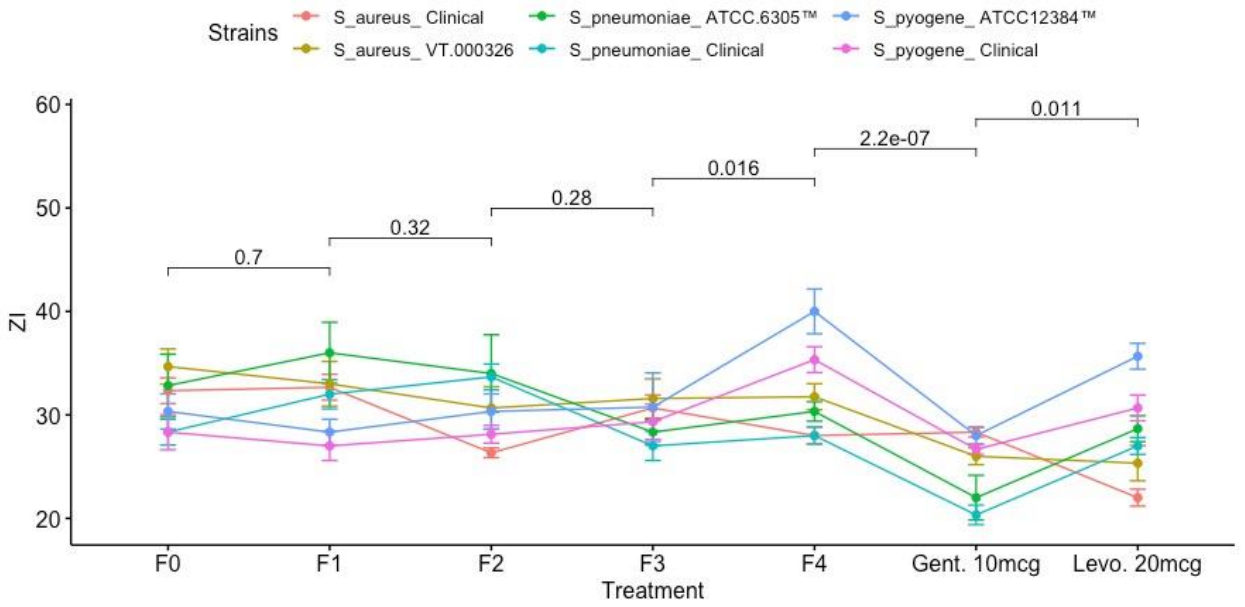


Fig 1: Zone of Inhibitions of Different Formulations and Positive Controls, at Zero Day of Compounding

Discussion

This study observed a percentage yield of 12.68% and 7.09% for 75% ethanol extract of *Citrus aurantifolia* whole fruit and *Garcinia kola* fresh seeds respectively. The percentage yield of 3.97 % reported by Ugwuowo *et al*., [26] on Garcinia kola seed oil was far less than our finding; also that of Ordu *et al*., [27] (8.5%) extracted by cold maceration using about 2.5 liters of n-hexane was also slightly higher than ours while the submission of Jack *et al*., [28] (7.5%) on dried seed ethanol extract of

Garcinia kola agree with this study. The variation in percentage yield might be attributed to the method, solvent and filtration process employed during extraction [29].

Literatures had documented percentage yields on the peels of *citrus aurantifolia* fruits [30, 31 and 32] but none has reported total yield on the fresh whole fruits. The differences in the yield observed in this study and others might be attributed to the extraction procedures/specifications, which varied so much as compared to existing reports.

On minimum inhibitory concentration (MIC), different studies have shown that extracts of G. kola had MIC that ranged between 0.008 to 25 mg/mL against different bacteria isolates including *S. aureus* [9, 10], but our observation was higher, ranging between 28.60 to 33.33 mg/m. This could be attributed to the nature and resistant status of the organisms used in the different studies. The assessment of *Citrus aurantifolia* fruits extracts MIC against a number of clinical isolates including *S. aureus* in previous studies ranged from 32 mg/mL - 128 g/ml [5] while this study recorded MIC ranged from 4.76 to 9.52mg/mL far lesser than others. This low concentration with effective antibacterial activity observed in this study might be attribute to possible variation of their chemical (bioactive) constituents.

Further evaluation showed that the minimum bactericidal concentration (MBC) of *Garcinia kola* extract in this study ranged from 30.48 – 34.76mg/mL (Table 9) depending on the resistant status. However, *Garcinia kol*a extract had no bactericidal effect against clinical *S. aureus* even at 34.76 mg/mL. This is contrary to the report of Amalu *et al*., [33] who observed that *S. aureus* was more susceptible with high zone of inhibition to ethanol extract of *Garcinia kola* than *E. coli* and *Candida albicans*. The study conducted by Idowu *et al.*, [34] also acknowledged that ethanol extracts of *G. kola* seed with MBC range of 12.5 to 50 mg/mL had antibacterial activity against pathogen isolated from respiratory tract such as *Streptococcus pyogenes* and *Klebsiella pneumonia*. The study according to Nas *et al* [35] further showed that *G. kola* extract had activity against methicillin resistant *Staphylococcus aureus* (MRSA). The MBC of *Citrus aurantifolia* whole fruits ranged from 4.76 to 9.52mg/mL (Table 8). Ibukun *et al.*, [5] reported MBC of *C. aurantifolia* fruit juice (not whole fruit) that ranged between 32 to 512 mg/mL depending on isolates and extracting solvent whereas Ugwu *et al* [36] reported MBC range of 3.13 to 12.5 mg/mL on fruit juice of *C. aurantifolia* for all the organisms tested.

Evaluation of the efficacy of the two plants combined showed that *C. aurantifolia* had more activity than *G. kola* as lower MIC and MBC were observed with *C. aurantifolia.* The variations observed might be due to type and resistant status of the isolates, plant components, extracting solvents and procedures.

This study reported that an OMBC of 9.52 mg/mL (0.9523 % w/v) of 75% ethanol extract of *Citrus aurantifolia* whole fruits would exhibit total clearance of all the isolates tested, whereas, 34.76 mg/mL (3.476% w/v) ethanol extract of *Garcinia kola* seeds (Tables 9) would do same for most of the bacterial tested. These findings concurred with the report of Pao-Chuan *et al* [37] who documented an increased antimicrobial effects of various combinations at certain ratio. However, literatures had no previous reports on the combined antibacterial activities of *C. aurantifolia* and *G. kola*. In this study the combination of the extracts was based on fractional percentages of each plant extract OMBC. There was increased activity with increasing concentration. However, at 50% OMBC, all the isolates were resistant except *S. pyogene* (Table 10). This implied that additive/synergistic or antagonistic effects could be achieved at certain ratios of OMBC of each extract; depending on the type of organisms.

At a combined state of 29.546 mg/mL *Garcinia kola* and 8.094 mg /ml *Citrus aurantifolia*, the combined extract exhibited a total kill on all the isolates tested, even against clinical isolates of *S. aureus,* which susceptibility was observed to be indeterminate due to high level of resistance.
Literature review showed that there was no report on a bi-herbal syrup formulation from *Garcinia kola* seed and *Citrus aurantifolia* whole fruit extracts with the aim of evaluating the antibacterial activities of the syrup. Despite the presence of excipients in all the formulations, the zones of inhibitions ranged from 27 – 40 mm (Table 12) .Table 13 showed that formulations $F_0 – F_3$ retained their MBCs as they recorded no or less than 6 colonies whereas F_4 could not retain its MBC only in clinical *S. aureus* as more than 6 colonies were recorded in clinical *S. aureus*. This retention failure in F_4 may be as a result of intrinsic bacterial resistance and matrix protection of the organisms as excipient concentrations increased. At the end of the study we observed that the formulation had more activity than the control (Figure 1). The optimum minimum inhibitory concentration (OMIC) and optimum minimum bactericidal concentrations (OMBC) of 75% ethanol extract of *Citrus aurantifolia* stood at same strength of 9.523mg/mL across all the tested organisms whereas the OMIC and OMBC of 75% extract of *Garcinia kola* across five tested organisms- *S. pyogene ATCC12384*™, *S. pyogene* (clinical), *S. pneumoniae ATCC 6305*, *S. pneumoniae* (clinical), *S. aureus VT 000326* stood at a concentration of 33.33mg/mL and 34.76mg /ml respectively. However, the MIC and MBC of 75% ethanol extract of *G. kola* against the clinical *S. aureus* were indeterminate even at 100% w/v. Assessment of combined activity of the extracts using 8.33mg/mL CA and 30.42mg/mL GK extracts in the bi-herbal syrup produced an optimum *in vitro* antibacterial performance in terms of ZI and MBC. The excipients (sucrose, sodium carboxylmethylcellulose and glycerin) were microbiologically compactible with the extracts as the formulations did not affect the antimicrobial activities of the extracts except in F_4. The 75 % ethanol extracts of *Citrus aurantifolia* and *Garcinia kola* OMBC, formulated into bi-herbal syrup may be used for the treatment of bacterial respiratory tract infections subject to further investigations.

Conclusion and recommendations

This study established that *Citrus aurantifolia* whole fruit and *Garcinia kola* fresh seed extracts have activity against respiratory tract pathogens and could be formulated into a syrup which exhibited the same effective strength as designed. Therefore, further studies on the plants component could also be carried out against fungi and viruses of respiratory tract origin.

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