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ASSESSMENT OF THE MICROBIAL STABILITY OF PRESERVED AND UNPRESERVED BI-HERBAL FORMULATED SYRUP OF *CITRUS AURANTIFOLIA* WHOLE FRUITS AND *GARCINIA KOLA* SEED EXTRACTS

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ABSTRACT

Liquid herbal products are highly prone to microbial deterioration due to high content of organic matter. The aim of this study was to carry out 90 days microbial stability study of a bi- herbal syrup formulated from extracts of *Citrus aurantifolia* (CA) whole fruits and *Garcinia kola* (GK) seeds. The bi-herbal syrup was formulated based on combined extracts minimum bactericidal concentrations (MBC): 8.33mg/mL GA, 30.42mg/mL GK and excipients and compounded by agitation without heat method. Both the preserved and unpreserved samples were challenged with determined population of organisms and counts taken at 7th, 14th and 28th day after inoculation. The zones of growth inhibition (ZGI) and MBC retention of the syrup were determined by Agar well diffusion and Agar–extract–inoculum pour plate methods respectively and the same procedure repeated 60th and 90th days of compounding.There was a slight decline (8.88 %) in the average percentage change of antibacterial activities (ZGI) of the optimum formulation (F_3) in a period of 90 days. Only formulation 3 recorded five colonies at the 90 days of compounding. The microbial challenged of the preserved and unpreserved syrup yielded no growth on the 7th, 14th and 28th days, after inoculation. Preservative has no significant contribution to the microbial stability of the formulation, as the unpreserved bi-herbal syrups were much stable within 90 days of stability study and in case of inadvertent introduction of microbes, both preserved and unpreserved syrup would resist microbial growth.

Keywords: Bacteria, Fungi, Microbial stability, Preserved and unpreserved bi-herbal syrup

INTRODUCTION

Assessment of the microbial degradation/contamination potential of drugs or any formulation accounts for one of the priority factor of concern in drug manufacturing. This important factor ensures the stability of the pharmaceutical active ingredient, drug efficacy sustenance and contamination prevention, as unstable contaminated drugs will tend to infect consumers by increasing ill patient microbial load, contribute to resistance, infect patients with biotoxins and increase degradation of bioactive constitutes of the drug (Nyirenda *et al*., 2020). This inadvertently will influence product recall, loss of credibility, reliability and trust on the company by consumers. According to WHO, the pharmaceutical stability of any drug is defined as the ability of a pharmaceutical product to maintain its chemical, physical, microbiological, and biopharmaceutical properties within specified limits throughout its shelf life (González-González *et al*., 2022). Drug formulation shelf life justifies how safe and effective the formulation could be despite exposure to various environmental factors such as temperature, humidity, and light (Romera, 2022).

Most locally formulated herbal drugs in Nigeria, sold mainly in open markets has no certification of their formulated shelf life and microbial load. Sadly, liquid herbal products are highly prone to both physical deterioration and microbial contamination due to high content of organic matter, physical and chemical complexities. This is worrisome, as 88% of the world's population, especially in developing countries, depends on traditional/herbal medicines (WHO, 2022). For this to be overcome, the bioactive ingredient of any formulation or its preservative should be able to preserve it and these herbs should be subjected to good manufacturing process and quality control measures to ensure quality, efficacy, safety, consistency and stability. The microbial stability of preserved and unpreserved bi-herbal formulated syrup of 75% ethanolic extracts of *Citrus aurantifolia* whole fruit (CA) and *Garcinia kola* seed (GK) was assessed for 90 days. These two plants have been reported in various studies to contain bioactive components in the management of some respiratory tract infections especially cough (Tauchen *et al*., 2023; Maňourová *et al*., 2023; FDA 2023; Hussein *et al*., 2019; Jesumirhewe *et al*., 2019). According a Balekundri and Mannur (2020) a pharmaceutical syrup is a liquid preparation of medicinal substances in a concentrated aqueous solution of sugar (may contain approximately 85% sucrose), polyols which serve as solubilizing agent (such as glycerin or sorbitols), antimicrobial agents, preservatives, viscosity enhancers and flavouring agents. To ensure that these formulated herbal medicines are still effective at the point of consumption, preservation of liquid herbal products is imperative as microbes may be inadvertently introduced during production processes and during product use (Balekundri and Mannur 2020). This study therefore assessed the 90 days' microbial stability of a bi- herbal syrup formulated from extracts of *Citrus aurantifolia* (CA) whole fruits and *Garcinia kola* (GK) seeds.

MATERIALS AND METHODS

Plant components authentication and extraction

The plant materials – 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, Ahmadu Bello University, Zaria.

Extraction was done by a modification of cold maceration method described by Olayemi and Olaleye (1999) using 75% ethanol (India) as an extracting solvent.

Isolation, identification and confirmation of selected microorganisms

Isolation, identification of Staphylococcus epidermis

Staphylococcus epidermis was isolated from the production environment of the bi-herbal syrup. (Laboratory staff skin) using nutrient agar prepared, in dishes and slants, according to manufacturer's instructions. Morphological investigation such as Grams reaction, shape and arrangement of cells and biochemical ability to ferment maltose, sucrose, catalase (catalase positive) and coagulase (coagulase negative) production were carried out (Bannerman, 2003). The isolate was sub-cultured into slant nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England) and stored at 4°C.

Confirmation of the bacterial organism

The bacterial isolates *Staphylococcus epidermis* and standard organisms -*Staphylococcus aureus VT 000326 IVL*™, (Sigma Aldrich Germany) were confirmed on Microgen Staph Identification Kit whereas *Streptococcus pneumoniae ATCC 6305* ™ (Himedia lab.,India) and *Streptococcus pyogene ATCC 12384*™ (Thermo fisher USA) were confirmed on Microgen Strep. Identification Kit; all according to the manufacturer's instruction. The confirmed organisms were properly stored 4°C and sub cultured weekly Muller-Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, England)

Isolation, identification and confirmation of Aspergillus niger (environmental isolates) and Aspergillus niger ATCC 16404 (Sigma Aldrich Germany)

Slices of wet bread were exposed at different positions at the compounding laboratory until the appearance of black fungal growth on the exposed slices of wet bread.

Sabouraud dextrose agar (India) slants were prepared according to manufacturer's instructions and the black fungi isolates from the wet slices breads were collected on Sabouraud dextrose agar slants. Macro- morphological characteristics of the fungal isolates were observed and recorded. Taxonomic guide as described by Thipeswamy *et al*., (2012); Hakeem and Bhatnagar, (2014) and Cheesbrough, (2018) were used to identify the fungal isolates. The identified *Aspergillus niger* isolates pure cultures were maintained on agar slants and stored in a refrigerator. The same procedure of re-identification procedures was repeated for *Aspergillus niger* ATCC 16404.

Formulation of Bi-Herbal Syrup

The bi-herbal syrup was formulated based on a combination of 8.33mg/mL (CA) and 30.42 mg /mL (GK) extracts which produced *in vitro* bactericidal effects across all the selected respiratory pathogens such as *Staphylococcus aureus VT 000326 IVL*™, *Streptococcus pneumoniae ATCC 6305* ™ and *Streptococcus pyogene ATCC 12384*™

The bi-herbal syrup also contained glycerin (solubilizing agent) and graded concentrations of sucrose BP (sweetener) and sodium carboxymethyl cellulose (NaCMC) (viscosity enhancer).

Both preserved (preserved with a combination of methyl-4-hydroxybenzoate 0.16 % w/v and sodium benzoate 0.15 % w/v) and unpreserved samples were challenged with determined population of standard organisms - *Aspergillus niger* ATCC 16404 and *Staphylococcus aureus VT 000326 IVL*™) and with environment isolates-*Staphlococuus epidermitis and Aspergillus niger* isolated from production environment and observed for growth (Nerkar and Gade 2023). Different formulations of the bi-herbal syrup were made as shown in Table 1 below:

Table 1: Bi-herbal Syrup Compounding 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

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

Compounding of Different Formulations of the Bi-herbal Syrup

Preparation of formulation F_1 (25mL) using agitation without heat method (prototype).

The amount of extracts 4.37g *Citrus aurantifolia* (CA) and 15.97g of *Garcinia kola* (Gk) were calculated such that when 1mL of the bi-herbal syrup was transferred to 20 mL molten Muller-Hinton agar, the resulting concentrations of CA and GK would be 8.33mg/mL CA and 30.42 mg /mL GK. This concentration was earlier calculated as the MBC that is capable of clearing *Staphylococcus aureus, Streptococcus pneumoniae* and *Streptococcus pyogene* tested. A 4.37g CA and 15.97g Gk were respectively weighed into a mortar and both were dispersed with 2mL 75 % ethanol. 2.50g (10%w/v) of sterilized sucrose BP was weighed, dissolved in 5mL of sterilized distilled water and transferred into mortar and mixed properly with pestle (Amala,*et al*., 2021). Appropriate volume (1mL) of a prepared stock solution of NaCMC (India) was added and mixed very well. Glycerin (India) was added to the mortar as required so that final volume of the syrup became 25mL. The resulting mixture was homogenized, dispensed through 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 ingredients as shown in Table 1.

Determination of antibacterial activities of the compounded bi-herbal syrup

The zones of growth inhibition and minimum bactericidal concentration retention of the compounded bi-herbal syrup were evaluated to determine whether the extracts would retain their antibacterial activities in a formulated (product) form.

Determination of average zone of growth inhibition (ZGI) of the compounded bi-herbal syrup

Employing agar well diffusion method, 20mL of a prepared molten Muller-Hinton agar was dispensed into each 20mL sterilized bottles, autoclaved at conditions of 15 PSI (121°C) for 15 minutes and allowed to cool to about 45º C. A set of three bottles of molten agar was each inoculated with 0.1mL of 0.5 McFarland suspension of the same bacterial organism, each bottle shaken and poured into a sterilized Petri dish and allowed to solidify. A set of 5 holes, each 6mm hole were bored on the solidified agar plate using a sterilized cork borer and each hole sealed with molten agar and 0.2mL of the compounded bi-herbal syrup was pipetted into the hole and allowed to stay for 30 minutes for proper diffusion of the herbal syrup.

A positive controls- gentamycin disc 10 mcg and levofloxacin 20mcg were placed on the inoculated agar to serve as a positive control and then incubated at the appropriate conditions (37°C for 24-48 h for *Staphylococcus aureus*, 37°C, 5% CO_2, 24-48 hours for *Streptococcus pneumoniae* and *Streptococcus pyogene*)

The syrup diffused into the agar to bring about antibacterial effects. The experiment was carried out in triplicate, the zones of clear inhibition were measured, recorded and average taken (Zazharskyi *et al*., 2019).

Determination of minimum bactericidal concentration retention of the bi-herbal syrup

Minimum bactericidal concentration (MBC) retention was determined by a modification of the methods as described by Adeshina *et al.,* (2010) and Ehinmidu and Ibrahim (2004). The combined extracts, at a ratio of 8.33mg/mL CA: 30.42 mg /mL GK, exhibited bactericidal effects against all the tested organisms. One mL of each of these compounded bi-herbal syrup formulations was respectively mixed with each 20 mL bottle of prepared Muller Hinton agar and shaken. (1mL of the compounded bi-herbal syrup contains 174.8mg/mL of CA and 638.8mg/mL of GK such that when 1mL of the syrup is transferred to 20 mL of the molten agar, the final concentrations of CA and GK extracts would be equal to 8.33mg/mL and 30.42mg/mL respectively). The syrup -agar admixtures were respectively inoculated with 20µl 0.5 McFarland suspension of test organism, poured into a sterile plate, evenly mixed. The inocula were allowed to diffuse into the test agar plates for 30 min.

The test agar plates were then incubated at the same respective appropriate conditions. The test procedures were carried out in triplicate and the average taken. At the end of incubation periods the plates were visibly observed for growth and the number of colonies counted using colony counter to ascertain the retention of bactericidal effects of the combined extracts in the formulated (product) form.

Determination of antibacterial activity (microbial stability) of the unpreserved bi-herbal syrup 60 and 90 days of compounding

Optimum formulation (F_3) was selected based on antimicrobial performance and organoleptic properties such as palatability (taste). Determination of antibacterial activity (microbial stability) of the selected formulation (F_3) using ZGI and ability to retain MBC was repeated and recorded at the end of 60 and 90 days of compounding.

Determination of microbial stability of both the preserved and unpreserved bi-herbal syrup- employing total viable count of the microbial challenged bi-herbal syrup

The total viable counts of samples of unpreserved and persevered bi-herbal cough syrups were determined. The syrup was preserved by a combination of preservatives (methyl-4-hydroxybenzoate 0.16 %w/v and sodium benzoate 0.15 % w/v) targeting both bacteria and fungi contaminant (Khanfar *et al* 2009). The typical allowed concentrations in oral formulations for methyl-4-hydroxybenzoate and sodium benzoate ranges from 0.015 to 0.2% w/v and 0.002 to 0.5% w/v respectively (Rowe *et al*; 2009). A modified method of pharmaceutical products preservative efficacy test of Choudhary, (2008) was used to determine the preservative efficacy of the combined preservatives in the bi-herbal syrup. Cultures of *Staphylococcus aureus VT000326* ^{TM}, *Staphylococcus epidermis*, *Aspergillus niger* (environmental isolate) and *Aspergillus niger* ATCC 16404 were used as challenge organisms.

Determination of inocula populations

The *Staphylococcus* species were sub-cultured in a nutrient agar slants; incubated at 30 ºC for 24 hours whereas *Aspergillus niger* were sub-cultured in Sabouraud Dextrose agar; incubated between 20-25 ºC for 7 days. Sterile normal saline solution containing 0.1% w/v peptone solution was prepared. The two bacteria were separately harvested into separate test tubes and diluted using 10mL of the normal saline to bring the count to about $1x10^8$cfu/mL. Similarly, the fungi- *Aspergillus niger* were harvested using 10 mL a normal saline containing 0.05% Tween 80 and adjusted to a count of $1x10^8$ cfu/mL using the normal saline. The number of colony forming units per mL in each suspension was determined using serial dilution method. Ten-fold dilutions, each of the four organisms, using sterile normal saline containing 0.1% w/v peptone for the two bacteria and sterile normal saline containing 0.05% Tween for *Aspergillus niger* were prepared. Using a pour plate method, the counts of each of the dilutions were determined. Nutrient agar was used for bacterial spp and incubated at 30 °C for 2 days whereas Sabouraud Dextrose agar was prepared for fungal spp and incubated at 20 to 25 °C for period of 4 days (Black, 2020; Udofa *et al*., 2022). This was done in duplicate, colony forming units were counted using colony counter and tabulated.

From Tables 2, 3, 4 and 5, estimated logs of 10^6 cfu/mL for *Staphylococcus epidermis*, *Staphylococcus aureus VT000326*^{TM} and *Aspergillus niger* (both environmental isolates and typed culture) i.e. serial dilutions number 3, 1, 3 and 3 above respectively were used to challenge the bi-herbal syrup.

Table 2: Serial dilutions and population count of *Staphylococcus epidermis* suspension

Serial dilutions	No of counts (cfu/mL)	No of counts (cfu/mL)	Average no of counts (cfu/mL)	Estimated log_{10}
1 $\equiv 10^{0}$	VHG	VHG	VHG	8
$2 \equiv 10^{-1}$	VHG	VHG	VHG	7
$3 \equiv 10^{-2}$	VHG	VHG	VHG	6
$4 \equiv 10^{-3}$	6.34×10^{5}	5.56×10^{5}	5.95×10^{5}	5
$5 \equiv 10^{-4}$	4.99×10^{4}	3.22×10^{4}	4.11×10^{4}	4
$6 \equiv 10^{-5}$	9.21×10^{3}	2.67×10^{4}	1.80×10^{4}	4
$7 \equiv 10^{-6}$	9.55×10^{2}	4.00×10^{3}	2.48×10^{3}	3
$8 \equiv 10^{-7}$	8.4×10^{1}	9.9×10^{2}	5.79×10^{2}	2
$9 \equiv 10^{-8}$	4.5×10^{1}	7.8×10^{1}	8.4×10^{1}	1
$10 \equiv 10^{-9}$	-	-	-	-

Key: VHG= very heavy growth

Table 3: Serial dilutions and population count of *Staphylococcus aureus VT000326* ™ suspensions

Serial dilutions	No of counts (cfu/mL)	No of counts (cfu/mL)	Average no of counts (cfu/mL)	Estimated log_{10}
1 $\equiv 10^{0}$	3.5×10^{5}	8.44×10^{6}	4.40×10^{6}	6
$2 \equiv 10^{-1}$	6.77×10^{4}	4.57×10^{4}	5.67×10^{4}	4
$3 \equiv 10^{-2}$	2.22×10^{2}	5.65×10^{3}	5.87×10^{3}	3
$4 \equiv 10^{-3}$	9.8×10^{2}	8.65×10^{2}	1.85×10^{3}	3
$5 \equiv 10^{-4}$	5.5×10^{1}	7.8×10^{2}	8.35×10^{2}	2
$6 \equiv 10^{-5}$	4.7×10^{1}	8.7×10^{2}	4.58×10^{2}	2
$7 \equiv 10^{-6}$	2.0×10^{1}	9.0×10^{1}	5.5×10^{1}	1
$8 \equiv 10^{-7}$	9.0×10^{0}	8.0×10^{0}	6.5×10^{0}	0
$9 \equiv 10^{-8}$	4.0×10^{0}	7.0×10^{0}	5.5×10^{0}	0
$10 \equiv 10^{-9}$	-	-	-	-

Table 4: Serial dilutions and population count of *Aspergillus niger* (EI) suspensions

Serial Dilutions	No of counts (cfu/mL)	No of counts (cfu/mL)	Average no of counts (cfu/mL)	Estimated log_{10}
1 $\equiv 10^{0}$	VHG	VHG	VHG	8
$2 \equiv 10^{-1}$	VHG	VHG	VHG	7
$3 \equiv 10^{-2}$	7.56×10^{6}	5.98×10^{6}	6.77×10^{6}	6
$4 \equiv 10^{-3}$	3.45×10^{5}	5.98×10^{5}	7.71×10^{5}	5
$5 \equiv 10^{-4}$	7.40×10^{4}	4.60×10^{4}	6.00×10^{4}	4
$6 \equiv 10^{-5}$	1.46×10^{3}	8.12×10^{3}	4.79×10^{3}	3
$7 \equiv 10^{-6}$	6.04×10^{2}	5.88×10^{2}	5.96×10^{2}	2
$8 \equiv 10^{-7}$	3.40×10^{1}	4.40×10^{1}	3.90×10^{1}	1
$9 \equiv 10^{-8}$	1.90×10^{1}	3.10×10^{1}	2.50×10^{1}	1
$10 \equiv 10^{-9}$	-	-	-	-

Key: VHG= very heavy growth, cfu = colony forming unit, EI= Environmental isolate

Table 5: Serial dilutions and population count of *Aspergillus niger* ATCC16404 suspensions

Serial dilutions	No of counts (cfu/mL)	No of counts (cfu/mL)	Average no of counts (cfu/mL)	Estimated log_{10}
1 $\equiv 10^{0}$	VHG	VHG	VHG	8
$2 \equiv 10^{-1}$	VHG	VHG	VHG	7
$3 \equiv 10^{-2}$	8.35×10^{6}	5.89×10^{6}	7.12×10^{6}	6
$4 \equiv 10^{-3}$	4.55×10^{5}	5.67×10^{5}	5.06×10^{5}	5
$5 \equiv 10^{-4}$	7.50×10^{4}	5.85×10^{4}	6.68×10^{4}	4
$6 \equiv 10^{-5}$	2.66×10^{3}	8.33×10^{3}	4.79×10^{3}	3
$7 \equiv 10^{-6}$	6.24×10^{2}	5.65×10^{2}	5.50×10^{2}	2
$8 \equiv 10^{-7}$	4.20×10^{1}	4.70×10^{1}	4.45×10^{1}	1
$9 \equiv 10^{-8}$	1.85×10^{1}	2.44×10^{1}	2.15×10^{1}	1
$10 \equiv 10^{-9}$	-	-	-	-

Key: VHG= very heavy growth, cfu = colony forming unit.

Formulation$_3$ (F_3) bi-herbal syrup was prepared and dispensed in 10mL sterilized bottles. Twenty-four bottles were preserved with the combined preservatives whereas another 24 bottles of the syrup were left unpreserved. The bottles of the bi-herbal syrup were divided in a set of four bottles. Each set of four bottles was inoculated with a given pre-determined

population of micro-organism suspensions as stated above. Both the preserved and unpreserved products were inoculated with 1mL of the inoculum suspensions and shaken and incubated between 20 to 30 ºC (ambient temperature) for a period of 7, 14, and 28 days.

At the end of 7th, 14th and 28th after 1st day of inoculation, the bottles of syrup were observed for changes; 4 samples of each of the preserved and unpreserved bottles of the bi-herbal syrup were selected for both bacterial and fungal count using a pour plate method: A 0.2mL of the preserved bi-herbal syrup previously inoculated with *Staphylococcus epidermis* was transferred separately to each of three molten nutrient agar bottles and shaken. Each of the three bottles was poured into separate petri dish and incubated at 37 ºC for 48 hours and observed for growth. The same procedure was carried out using the unpreserved syrup. The same procedure was repeated with bottles of bi-herbal syrup (both preserved and unpreserved bottles) previously inoculated with *Staphylococcus aureus VT000326* ™ and *Aspergillus niger* except that Sabouraud Dextrose agar (SDA) medium was used for the fungi and incubation carried out between 20 to 28 ºC for 6 days. At the end of the incubation periods of both bacterial and fungi, the bottles of both the preserved and unpreserved bi-herbal syrup were observed for changes, growth and counts taken.

RESULTS AND DISCUSSION

Antibacterial activities of the bi-herbal syrup

Zone of growth inhibition of different formulations and positive controls, at zero day compounding

Table 6 showed that Formulation 3 (F3) had the least average zone of inhibition (AZI) compared to other formulations prepared The AZI of F3 was still observed to be higher than those exhibited by standard conventional drugs gentamicin (10µg) and levofloxacin (20µg) disc used as positive controls. Different formulations contained different graded concentrations of excipients- sucrose, sodium carboxymethylcellulose and glycerin as contained in Table 1. In formulated dosage form (syrup), the different formulations exhibited slight changes in average zones of growth inhibition (ZGI) across the tested organisms (ranged from 29 -32 mm). Clinical isolates as compared to typed/standard isolates, exhibited slightly lower zones of growth inhibition as a result of innate resistant statues of the clinical isolates. The zones of growth inhibition showed that the organisms (clinical and typed/standard isolates) were susceptible to the formulations as well as positive controls (table 6). *S.pyogene* and *S .pneumoniae* are said to be susceptible to an antibacterial agent if the agent produces zone of growth inhibition ≥ 17mm whereas *S .aureus* is considered susceptible to an agent if the agent produces zone of growth inhibition ≥ 19 mm. (CLSI 2018)

Table 6: Zone of growth inhibition of different formulations and controls, at zero day of compounding Organisms and AZI (mm)

Formulations & controls	*S. pyogene*		*S. pneumoniae*		*S. aureus*		Average ZI (mm)
	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	28.50	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	28.67	27.00	25.33	22.00	28.22

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

Zone of growth inhibition of formulation3 and positive controls for 60 and 90 days after compounding

Samples of formulation3 (F3) were selected as the optimum formulation based on antibacterial performance and palatability, hence, it was selected for further microbial stability studies at 60 and 90 days of compounding (Tables 7 and 8). At 60 and 90 days of compounding, F3 (AZI) still exhibited a higher zone of inhibition compared to gentamicin but slightly less than levofloxacin (Table 7 and 8).

At 60 days and 90 days of compounding, formulation 3, exhibited slight decreases in the zone of growth inhibition across the organisms represented with -6.14 % and -8.88 % respectively (Table 7 and 8, equqtions 1 and 2). Even at this slight decrease, the stability of the product was insignificantly affected as the organisms were still very susceptible to the formulation (CLSI 2018)

This slight negative change (8.88%) suggested that the bi-herbal syrup was stable within a period of 90 days' studies. However, the formulation may need a chemical stabilizer or antioxidant such as sodium metabisulphite to extend the shelf life (Rowe *et al*., 2009).

Table 7: Zone of Growth Inhibition at 60 Days of Compounding

Formulation & Controls	Organisms and ZI (mm)						Average ZI (mm)
	S. pyogene		*S. pneumonia*		*S. aureus*		
	ATCC12384™	Clinical	*ATCC 6305*™	Clinical	*VT 000326*	Clinical	
F_3	30.33	30.76	25.11	25.89	26.67	30.00	28.13
Gent.10mcg	26.00	24.11	20.45	22.27	26.45	22.27	23.59
Levo.20mcg	31.70	29.44	30.31	27.22	26.89	19.23	27.47

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

Table 8: Zone of Growth Inhibition at 90 Days of Compounding

Formulation & Controls	Organisms and ZI (mm)						Average ZI (mm)
	S. pyogene		*S. pneumonia*		*S. aureus*		
	ATCC12384™	**Clinical**	***ATCC 6305™***	**Clinical**	***VT 000326***	**Clinical**	
F_3	29.30	30.11	23.23	24.44	28.67	27.90	27.28
Gent.10 µg	26.00	26.23	24.21	20.00	26.75	25.78	24.82
Levo.20 µg	31.45	28.42	26.65	26.00	26.89	19.20	27.84

Key F= Formulations, AZI= Average Zone of growth inhibition, Gent= Gentamycin disc, Levo = Levofloxacin disc

Assessment of Differences in Zone of Growth Inhibition of Formulation 3 at 60 and 90 days of Compounding Compared to zero day of Compounding

$\frac{A_2-A_1\times100\%}{A_1} = \frac{28.13-29.94\times100\%}{29.94} = -6.05$ (1)

A_2 = Average zone of inhibition at 60 days

A_1 = Average zone of inhibition at zero day

$\frac{B_2-A_1\times100\%}{A_1} = \frac{27.28-29.94\times100\%}{29.94} = -8.88$ (2)

B_2 = Average zone of inhibition at 90 days, A_1 = Average zone of inhibition at zero day

Retention of Minimum Bactericidal Concentration (MBC) of the Combined Extracts in the Product Form, at zero, 60 and 90 days of Compounding

Table 9 showed that none of the pathogens tested grew on different samples 1, 2, 3, 4 and 5 of formulation3, at zero day of compounding, whereas at 60 days of compounding 5 colonies of *S. aureusVT 000326* grew on sample 4 (Table 10). At 90 days of compounding (Tables 11) there were 2 colonies of *S.pyogene ATCC12384*™ and 5 colonies of *S. aureus VT* growth observed in sample 5 of the formulation3.

Table 9: Retention of MBC in Formulation3, at zero Day of Compounding

Organisms	Formulation$_3$				
	S_1 (NC)	**S_2 (NC)**	**S_3 (NC)**	**S_4 (NC)**	**S_5 (NC)**
S. pyogene ATCC12384™	NG	NG	NG	NG	NG
S. pneumoniae ATCC 6305™	NG	NG	NG	NG	NG
S. aureus VT 000326	NG	NG	NG	NG	NG

Key: F= Formulations, NG= No Growth, NC = number of colonies

Table 10: Retention of MBC in Formulation3, at 60 days of Compounding

Organisms	Formulation$_3$				
	S_1 (NC)	**S_2 (NC)**	**S_3 (NC)**	**S_4 (NC)**	**S_5 (NC)**
S.pyogene ATCC12384™	NG	NG	NG	NG	NG
S.pneumoniae ATCC 6305™	NG	NG	NG	NG	NG
S.aureus VT 000326	NG	NG	NG	5	NG

Key: F= Formulations, NG= No Growth, NC = number of colonies

Table 11: Retention of MBC in Formulation3, at 90 days of Compounding

Organisms	Formulation$_3$				
	S_1 (NC)	**S_2 (NC)**	**S_3 (NC)**	**S_4 (NC)**	**S_5 (NC)**
S.pyogene ATCC12384™	NG	NG	NG	NG	2
S.pneumoniae ATCC 6305™	NG	NG	NG	NG	NG
S.aureus VT 000326	NG	NG	NG	NG	5

Key: F= Formulations, NG= No Growth, NC = number of colonies

Total Viable Count of the Microbial Challenged Preserved and Unpreserved Bi-herbal Syrup

Half samples of the optimum formulation (F_3) were preserved by a combination of methyl-4-hydroxybenzoate (0.16 w/v %) and benzyl benzoate (0.15w/v %) whereas a half portion were left unpreserved. Each of the preserved and unpreserved samples inoculated with one of the following viable population of the challenge organisms: *Staphylococcus epidermis,* (1.0×10^6 cfu/mL), *Staphylococcus aureus VT000326* ™ (4.40×10^6 cfu/mL), *Aspergillus niger* (EI) (7.12×10^6 cfu/mL*)* and *Aspergillus niger* ATCC 16404 (6.77×10^6 cfu/mL) as shown in tables 2, 3, 4 and 5 respectively, subjected to viable count (pour plate method) on the 7th, 14th and 28th day after the day of inoculation yielded absence of growth.(Table 12)

This showed that the syrup would be stable in case of unintentional introduction of microbes during production or use.

Table 12: Total viable count of the challenged preserved and unpreserved bi-herbal syrup 7th, 14th and 28th days after the day of inoculation

Challenge Organisms	Sample1 (cfu/mL)			Sample2 (cfu/mL)			Sample3 (cfu/mL)		
	7th	14th	28th	7th	14th	28th	7th	14th	28th
Staphylococcus epidermis	NG	NG	NG	NG	NG	NG	NG	NG	NG
Staphylococcus aureusVT000326	NG	NG	NG	NG	NG	NG	NG	NG	NG
Aspergillus niger(EI)	NG	NG	NG	NG	NG	NG	NG	NG	NG
Aspergillus niger ATCC 16404	NG	NG	NG	NG	NG	NG	NG	NG	NG

Keys: EI = Environmental isolate, NG = No growth

DISCUSSION

The investigation was aimed at assessing the ability of the bi-herbal syrup to maintain stability by retaining antibacterial parameters such as average zones of growth inhibition and retention of MBC for a period of 90 days of compounding and ability of the samples of the microbial challenged bi-herbal syrup to resist both bacterial and fungal growth for period of 28 days.

The bi-herbal cough syrup was formulated based on a combined extracts of *Citrus aurantifolia* whole fruits and *Garcinia kola* seed at concentration ratio of 8.33mg/mL: 30.42 mg /mL which was the minimum concentration that exhibited bactericidal actions against all the tested organisms- *Staphylococcus aureus VT 000326 IVL*™, *Streptococcus pneumoniae ATCC 6305* ™ and *Streptococcus pyogene ATCC 12384*™.

At zero day of compounding, the formulations showed slight variations in the average zone of growth inhibitions and ranged from 29.94 to 32.28 mm in diameter across the selected organisms. These average zone of growth inhibitions were slightly higher than that produced by the positive controls (gentamycin disc- 25.22mm and levofloxacin disc- 28.22 (Table 6).

Clinical isolates as compared to typed/standard isolates, exhibited slightly lower zone of growth inhibition, probably, as a result of innate resistant statues of the clinical isolates. The zones of growth inhibition showed that the organisms (clinical and typed/standard isolates) were susceptible to the formulations as well as positive controls (Table 6). *S. pyogene* and *S. pneumoniae* are said to be susceptible to an antibacterial agent if the agent produces zone of growth inhibition ≥ 17mm whereas *S. aureus* is considered susceptible to an agent if the agent produces zone of growth inhibition ≥ 19 mm (CLSI 2018).

Formulation 3, exhibited slight decreases in the zone of growth inhibition across the organisms represented with -6.14 % and -8.89 % (Table 7 and 8, equations 1 and 2) at 60 and 90 days respectively. The formulation may need a chemical stabilizer or antioxidant such as sodium metabisulphite to extend the shelf life (Rowe *et al.,* 2009). Literatures have not documented use of zone of growth inhibition measurement, at interval of times, as a yardstick for stability monitoring of herbal syrup, intended for antibacterial activity.

Considering the retention of minimum bactericidal concentration (MBC) of the combined extracts in the product form, within 90 days' stability study, the prototype formulation (F_3) recorded a maximum of no growth, five colonies and five colonies growth at zero, 60 and 90 days of compounding respectively (tables 8, 9 and 10). According to modified method as described by Ehinmidu and Ibrahim (2004) MBC is retained when the number of colonies is less than six colonies. Here stability of the herbal syrup was assessed based on the ability of the syrup to retain MBC. It should be recalled that the syrup was formulated in such a way that when 1 mL of the syrup was transferred to 19 mL of molten agar, the resulting concentration is the MBC for the selected bacterial organisms. No documented study has followed this line of research thought, hence basis for comparison is non- existing.

Both preserved and unpreserved samples of the product (bi – herbal syrup) could not support both bacterial and fungal growth at 7th, 14th and 28th day after inoculation with a predetermined viable population of both bacterial and fungal isolates (Table 11) Limits or acceptance criteria for effective preservative: The preservative is effective in the product examined if: the concentration of the viable bacteria is not more than 0.1 % of the initial concentration at the end of the 14th day of the challenge test. The concentrations of the viable yeasts or molds remains at or below the initial concentration by the end of first 14th day. The concentrations of the test organisms remain at or below these designated levels at the end of the 28th day (Choudhary, 2008). This inability of the syrup to support microbial growth indicates that, in case of inadvertent introduction of microbes during production or use, the product would still be stable (Ratajczak *et al*., 2015)**.** From the result so far, it indicated that the combined extracts have intrinsic antibacterial and antifungal constituents at bactericidal and fungicidal concentrations and therefore could not allow microbial growth. This suggest that the formulated product (bi-herbal syrup) may not need extra preservatives to extend the shelf life.

The stability study of formulated herbal syrups containing aqueous root extract of Nauclea

latifolia, after storage at different temperatures for 30 days showed no changes in colour, odour, taste and no growth of organisms (Olayemi *et al*., 2020 and Alka- Saxena *et al*., 2020) This is in congruence /conformity with the microbial stability of this study which recorded no growth from both preserved and unpreserved samples of the syrup challenged with selected organisms. This inability of the syrup to support the growth of microbes must be connected to antimicrobial constituents of the plants extracts; at lethal concentrations. However, recent studies conducted by Shakeel *et al* (2015) and Devkar *et al.,* (2021) focused on physiochemical parameters of herbal cough syrup rather than microbial stability determination using microbial challenge tests

CONCLUSION AND RECOMMENDATIONS

Considering average ZGI and retention of MBC, samples of the unpreserved bi-herbal syrup formulated with a combined extracts of *Citrus aurantifolia* whole fruits and *Garcinia kola* seed and excipients were much stable within 90 days of stability study. Furthermore, both the samples of the preserved (preserved with a combination of methyl-4-hydroxybenzoate (0.20 w/v %) and benzyl benzoate (0.15w/v %) and unpreserved bi-herbal syrup challenged with both bacterial and fungal microbes yielded absence of growth on the 7th, 14th and 28th day after the day of inoculation, hence the product (bi-herbal syrup) would be stable in case of inadvertent introduction of microbes during production or on

use. This study, therefore, recommend monitoring of the microbiological stability parameters – zone of growth inhibition and ability to retain MBC for a period of 730 days. In addition, monitoring of organoleptic properties as well as physiochemical properties of the bi-herbal syrup under varied prevailing conditions is recommended; to determine the overall stability and the product shelf life.

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