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Ebenebe I Ngozi
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

Ikegbunam N Moses
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

Maduagwu N Ukamaka
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

Esimone C Okechukwu
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

Correspondence
Ikegbunam N Moses
Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria

The antimicrobial screening and preservative efficacy of essential oils from the dried fruits of *Piper guineense* and *Xylopia aethiopica* in contaminated herbal preparation

Ebenebe I Ngozi, Ikegbunam N Moses, Maduagwu N Ukamaka and Esimone C Okechukwu

Abstract
Piper guineense and *Xylopia aethiopica* are medicinal plants traditionally used as spices and seasoning in Nigerian food. Antimicrobial activity of the essential oils from *Piper guineense* and *Xylopia aethiopica* were evaluated using Agar well diffusion method. Ampicillin trihydrate and Clotrimazole were used as a positive control for the test bacteria and fungi respectively, and 1% v/v DMSO was also employed as a negative control for all the test microorganisms. The MIC, MBC and MFC of the essential oils were also evaluated against *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, E. coli, Salmonella typhi, Klebsiella pneumonia, Aspergillus niger,* and *Candida albicans* using agar dilution method. The combined antimicrobial activity of the essential oils from *Piper guineense* and *Xylopia aethiopica* were determined using checker board technique. The preservative efficacy of the essential oils were evaluated at 1%, 3%, 5%, by combining the essential oil at each of this concentration in a continuous variation method. The combined ratios of each concentration were introduced into five different contaminated herbal samples respectively. Methyl- and propyl- paraben (2:1), and Sodium benzoate were respectively used as control preservative at 1%, 3%, 5%. Result of the antimicrobial screening test shows that essential oil from *Piper guineense* were effective against all the test microorganisms, while the essential oil from *Xylopia aethiopica* was not effective against *E. coli* and *K. pneumonia*. The combined antimicrobial effect of the Essential oil (EO's) against test microorganisms were synergistic in most of the combined ratios used. The preservative effect of the EO's reveals that their actions were concentration dependent, with the highest concentration of 5% being the most efficient. M-and p-paraben and Sodium benzoate were not as effective as they should with the contaminated herbal product at all the concentrations except with the enumeration of *E. coli* and *Enterobacter sakazakii* where they are excellently efficient as a preservative. Herbal medicines, if unpreserved, readily become contaminated with microorganisms leading to spoilage. Therefore, the combination of these two essential oils from the dried fruits of *P. guineense* and *X. aethiopica* may have a potential for further study as an alternative in the preservation of herbal preparations.

Keywords: Antimicrobial screening, essential oils, dried fruits, *Piper guineense* and *Xylopia aethiopica*

Introduction
All higher plants contain secondary metabolites, and they include compounds produced in response to stress or those that centre on the defence of the plants or for protection against adaptation to extrinsic abiotic factors or a combination of these functions (Watermann, 1992) [26]. The metabolites are compounds responsible for the plants odour, colour, taste and physiolocical actions. Examples of these metabolites include saponins, tannins, anthraquinones etc (Wink. 2003) [29].

Essential oil is a concentrated hydrophobic liquid containing volatile aromatic compounds from plants. Oil is essential in the sense that it carries a distinctive scent or essence of the plant (Wikipedia, 2012) [27]. Essential oil from different parts of a named plant may have a totally different scent and characteristic. Health professionals are more interested in the medicinal properties of essential oils, they are known to possess antimicrobial activity which has been evaluated mainly in liquid medium (Shigeharu *et al*., 2001) [23].

Piper guineense is generally known as *Iyere*, *ata-iyere* (Yoruba), *masoro* (Hausa), *ahanhi akpoko* (Benin), *Uziza* (Igbo), *etinkene* (Efik), *uririe* (Urhobo) in Nigeria (Gill, 1992) [9]. *P. guineense* belongs to the family piperaceae.

The essential oil of *Piper guineese* has shown to exhibit no antibacterial activity against *E. coli, Serratia, Salmonella typhi, Klebsiella* spp.,*Citrobacter, Pseudomonas aeruginosa*, using agar diffusion disc impregnated method (Olonisakin, *et al.,* 2006) [19]. The chemical constituents of the essential oil from the fruits of *Piper guineense* was elucidated using gas chromatography- mass spectrophotometry techniques. The major component identified were D-Limonene (7.7%), carene (5.4%), (Is)-(-1)-β-pinene (43.9%), caryophylene (6.9%), 1,6,10-dedecetrien-z-ol, 3,7,11-trimetyl (2.9%) (Olonisakin, *et al.,* 2006) [19]. Also, 19.4% was found to be present as sesquiterpene hydrocarbons with β-sesquiphellandrene, 2.6%, caryophyllene, 6.9%, and epi-bicydosesquiphellandrene as the major components of sesquiterpene hydrocarbons (Olonisakin, *et al.,* 2006) [19]. The essential oils have been reported to be rich in β-Pinene and β-caryophyllene, α-humulene, bycyclogermacrene and β-elemene, (Martins, *et al.*, 1998) [13].

Xylopia aethiopica is the botanical name for *"Uda"* which is the local Igbo name. Other local names include *eeru* (Yoruba), *unien* (Benin), *atta* (Efik), *Kenya* (Boki), *kimba* (Hausa), *kimbare* (Fulani) (Gill, 1992) [9]. It belongs to the family *Annonaceae.* The plant contains high amounts of copper, manganese, and zinc. Its dried fruits are used as spice and a traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough and fever (Mshana, *et al.*, 2000) [14]. The essential oil from *X. aethiopica* has been well characterized with β- trans-ocimene, linalool, α- farnesene, α- pinene, β- phellandrene, β- pinene, mytrenol,, and 3-ethylphenol as the major volatile constituents (Tapu, *et al.,* 1999) [24].

Research has shown that essential oils can act as natural preservatives, their compounds are used to reduce microbial and chemical spoilage (Pessoa, *et al.,* 2002) [21]. Preservatives are added to pharmaceutical preparations when it is needed to combat the effects of contaminating microorganisms, which may be inherent in the ingredients or introduced during use by the patient (Esimone, *et al.,* 2002) [6]. For many years, it is known that traditional medicine practitioners use many substances of synthetic origin as preservatives (Esimone *et. al.,* 2002) [6]. These synthetic preservatives can destroy the stability of the herbal preparation (Willams *et al.,* 2012). It is therefore necessary to find an alternative preservative which is of natural origin that will not only be acceptable to the herbalist but also protect the integrity of the product and is safe for the consumer. The aim of this study was to determine the antimicrobial activity of the essential oils from dried fruits of *Piper guineense* and *X. aethiopica* against the selected organisms, the combined antimicrobial effect of the essential oils using the Checker board technique and their preservative efficacy in contaminated herbal preparations.

Materials and Methods

Materials

Plant material

Dried fruit of *Piper guineense* and *Xylopia aethiopica* were purchased from the local markets at Awka and Igbo-ukwu, Anambra state, Nigeria. They were identified by Dr. S. I. Okeke of the Department of Biology, School of Science Laboratory and Technology, Federal Polytechnic Oko, Anambra state.

Test microorganisms

The Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and Gram- negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi and Klebsiella pneumonia*) as well as Fungi (*Candida albicans and Aspergillus niger*) used in this investigation were obtained from the stocks in the Department of Pharmaceutical Microbiology and Biotechnology laboratory, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra state.

Culture media

The culture media used include Nutrient Agar and Nutrient Broth (Merck, Germany) for the enumeration of bacteria, Sabouraud Dextrose Agar and Sabouraud Dextrose Broth (Merck, Germany) for the enumeration of fungi, Chromocult Agar, (Merck, Germany) for the enumeration of Coliforms, *Escherichia coli* and *Enterobacter sakazakii*, Muller Hinton agar (Merck, Germany) were used according to manufacturers' specification.

Reagents and Antibiotic standard

The reagent used include Dimethylsulphuoxide (DMSO) (Kermel chemical company, U.K.), Tween 80, Sodium benzoate, methyl paraben and propyl paraben (Evans Medical Ltd., Liverpool). The antibiotic used are Ampicillin Trihydrate BP (WHO Center for chemical reference substance, Stockholm, Sweden) and Clotrimazole USP (USP reference standard, Rockville, MD).

Collection of Herbal samples

Eleven different liquid herbal medicines were purchased from different traditional medicine practitioners in Enugu and five of them that were found to be contaminated were used in this study. Table 1 shows the product data of the five different contaminated herbal samples used in this study.

Table 1: Product data of the contaminated herbal preparations used in this study

Sample No	Name of Sample (s)	Therapeutic claims	Route of administration
1	Redeemers Aloe Vera	Effective against typhoid, *S. aureus*, malaria, *E. coli*, syphilis, Chlamydia, barrenness, gonorrhea, herpes, menses problem, internal heat	Oral
2	Annek herbal tonic	For treatment and prevent- ion of gonorrhoea,syphilis *S. aureus*, malaria, Candidiasis, typhoid fever, watery low sperm count, viral hepatitis A&B, Rheumatism	Oral
3	Restoration liquid	Treatment of *St. aureus,* gonorrhoea,syphilis, hepatitis Candidiasis, typhoid fever,	Oral
4	Elcoyn-DS herbal Mixture	High fever, typhoid, Rheumatism Arthritis, venereal diseases.	Oral
5	Evacuation liquid Action	Effective against Diabetes, *S. Aureus* typhoid, cough, piles, Candidiasis, worms,STDs.	Oral

Methods

Extraction of the Essential oils

A 2000 g quantity each of the dried fruits of *Piper guineense* and *Xylopia aethiopica* was pulverized separately and subjected to hydrodistillation for three hours using a Clevenger apparatus.

Antimicrobial Sensitivity Test

Preliminary Sensitivity tests

Antimicrobial sensitivity testing was determined by using the agar well diffusion method to detect the presence of anti-bacterial and anti-fungal activities of the essential oils from the dried fruits of *Piper guineense* and *Xylopia aethiopica* (Perez *et al.,* 1990) [20]. A 250 µl of each of the essential oils were diluted separately, five-fold serially in 1ml of DMSO to obtain different concentrations of the essential oils (250 µl/ml, 50 µl/ml, 10 µl/ml, 2 µl/ml, and 0.4 µl/ml). A 0.1ml of standardized broth cultures of bacteria and fungi were seeded into a molten Nutrient agar and Sabouraud dextrose agar respectively, and allow to solidify. A total of 3 wells were made for each of the dilutions using a sterile cork borer of 10 mm. Approximately 0.1 ml of the diluted essential oils were introduced into each of the well respectively. A positive control well was filled up with 0.1 ml of 20 µg/ml w/v Ampicillin trihydrate BP reference standard and 0.1ml of 30 µg/ml w/v Clotrimazole USP reference standard for the test bacteria and fungi respectively, while 0.1 ml of 1% v/v of DMSO was used as a negative control for all the test microorganisms. The setup was allowed to stand for 1 hour for pre- diffusion before incubation at 25 ^{0}C (for fungi) and 37 ^{0}C (for bacteria) for 24hours. At the end of the incubation period, the antimicrobial activity of the essential oils was evaluated by measuring the inhibition zone diameter (IZD) against the test microorganisms. The values for inhibition zone are presented as standard error of the mean of the triplicate experiments.

Determination of the Minimum Inhibitory Concentration (MIC)

Agar plate dilution method was used to determine the Minimum Inhibitory Concentration (MIC) of the essential oils. Five-fold serial dilutions of the essential oil(s) from *P. guineense* and *X. aethiopica* respectively were made using DMSO as the solvent, as described above. A double strength Műeller Hinton agar was prepared according to the manufacturer's specification and, 19 ml of the medium, as described by European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2000) [7] was dispensed into McCartney bottles before sterilization at 121 ^{0}C for 15 minutes. The media were allowed to cool down to 45 ^{0}C and poured into a sterile Petri dish with a 0.1 ml of a standardized broth culture of the test microorganisms. Thereafter, 1 ml of the different dilutions of the essential oils was introduced into different sterile Petri dish that was setup for the experiment. The sterile Petri dishes was rotated to ensure an even distribution. The concentration of the essential oils in different plates was 250 µl/ml, 50 µl/ml, 10 µl/ml, 2 µl/ml, and 0.4 µl/ml.The inoculated plates were allowed to stand until the inocula have been completely absorbed by the medium. The Petri dishes were then incubated in an inverted position at 25 ^{0}C (for fungi) and 37 ^{0}C (for bacteria) for 24 hours before taking the results. The MIC was obtained as the least concentration that inhibited the growth of the test microorganisms divided by the dilution factor.

Determination of the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

Minimum Bactericidal Concentration (MBC) or Minimum Fungicidal Concentration (MFC) can be considered as an extension of the MIC procedure (Okore, 2009) [18]. The MBC and MFC were derived from the agar dilution method of determining MIC. Thereafter, all the agar plates showing no growth in the MIC test were returned into the incubator in an inverted position for another 24 hours (bacteria) and 48hours (fungi) at appropriate temperature. After the incubation period, the Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were obtained as the lowest dilution showing no growth divided by the dilution factor.

Determination of the Combined Antimicrobial efficacy of the Essential oils using checkerboard technique

The checkerboard technique was used (Schelz, *et al.,* 2006) [22] to obtain the fractional inhibitory concentration (FIC) index of EO combinations. Essential oils from *P. guineense* and *X. aethiopica* were measured separately by diluting the oils in DMSO, each containing twice the MIC of the oil. The essential oil from *P. guineense* and *X. aethiopica* were then combined using a continuous variation model. Each combination ratio was diluted two- fold serially up to 10 dilutions in sterile test tubes in duplicate using an equal volume of a double strength Nutrient broth. Thereafter, standardized 0.1 ml of 18 hour broth culture of the test microorganisms were inoculated into the dilutions (Okore, 2009; Gutierrez, *et al*., 2009) [18, 10]. The test tubes were covered with sterile cotton wool and were incubated at 37^{o}C (for bacteria) and 25^{o}C (for fungi) for 24 hours. After the incubation period, the test tubes were checked for turbidity. For the combined ratios the dilution showing no turbidity (visibly clear) was taken as the MIC in combination and the dilution showing no turbidity in the EO's that was not combined was taken as the independent MIC.

The FIC_{index} was calculated as:

The FIC of each essential oil = MIC in the combination divided by the independent MIC of the EO.

Therefore; FIC_{Index} (A) = MIC A in B and FIC_{Index} (B) = MIC B in A

$$\frac{\text{MIC A}}{\text{MIC B}}$$

Hence, $\sum FIC_{Index} = FIC (A) + FIC (B)$

Where MIC A in B and MIC B in A represent minimal concentrations of the essential oil A and B having inhibitory effect when acting together, while MIC A and MIC B stands for the respective MICs of the oils. Thus, the FIC index was interpreted as synergism if its value is less than 1.0, Additivity if its value is equal to 1.0, Indifference if its value is more than 1.0, and Antagonism if its value is more than 2.0.

Determination of the Preservative Efficacy of the Essential oils from the dried fruits *P. guineense* and *X. aethiopica*

The preservative efficacy of the essential oils was determined by combining the two essential oils at different concentrations of 1%, 3% and 5% respectively, using a continuous variation model. Different concentrations (1%, 3%, and 5%) of the essential oils were obtained using 2% Tween80 as diluents and as an emulsifier. A 5 ml aliquot of each of the contaminated herbal medicines was dispensed separately into sterile McCartney bottles using 5ml sterile syringe.

Thereafter, the combined ratios of the different concentrations of the EO's were introduced into the individual herbal medicines and stored at ambient temperature, usually the type that the product will meet during storage. A combination of M-and P-paraben (2:1) at concentration of 1%, 3%, 5%, and Sodium benzoate at the same concentrations were used as positive control preservatives respectively in the herbal medicines, while the unpreserved herbal samples served as a negative control. At time intervals of day 0, 1, 3, 7, 14, 21, and 28, the viable counts of the herbal samples were determined on Nutrient agar (for viable bacteria count), Chromocult agar (for coliforms count and *E. coli* count) and Sabouraud dextrose agar (for fungi count), using pour plate method. The plates were incubated at 37 ^{0}C (for bacteria) and 25 ^{0}C (for fungi) for 24 hours in an inverted position. After the incubation period, the viable counts in each of the plates were taken and the results recorded.

Results and Discussions

Extraction of the Essential oil

Percentage yield of the Essential oil

The percentage yield (v/w) of the essential oil from the dried fruits *Piper guineense* (Uziza) and *Xylopia aethiopica* (Uda) were 0.75% and 0.55% respectively. Essential oil from the dried fruits of *P. guineense* and *X. Aethiopica* were amber and pale yellow in colour respectively, with a characteristically strong and pleasant odour. Fleischer, *et al.,* (2008) [8], reported that the percentage yield of essential oil from the dried fruit of *Xylopia aethiopica* (Uda) was 3.33% v/w while Olonisakin, *et al.,* (2006) [19] reported that the percentage yield of essential oil from the dried fruit of *Piper guineense* was about 0.83% v/w. The poor or low yield of the essential oils reveals that geographical locations may have affected the yield or it may be that the fruits were over air- dried (Kelly, and Valtacho, 2004) before it was bought for the research work. Another possible cause of the low yield might be attributed to the harvesting time, is either the fruits were immature or over matured before it was harvested (Aflatuni, 2005) [1].

Antimicrobial Sensitivity test

Antimicrobial Screening of essential oil from the dried fruits of *Piper guineense*

Table 2 showed the result of the antimicrobial activity of the essential oil from the dried fruit of *Piper guineense* at different dilutions. The result shows that the essential oil from the dried fruit of *Piper guineense* had various degree of activity against all the test microorganisms. It was found that among the gram-positive bacteria, *Bacillus subtilis* was the most sensitive, followed by *Staphylococcus aureus.* For the gram-negative bacteria, *Klebsiella pneumonia* was found to be the most sensitive, followed by *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Escherichia coli,* which showed the least sensitivity. *Aspergillus niger* was the most sensitive among the fungi, followed by *Candida albicans.*

Table 2: Antimicrobial activities of the Essential oil from *P. guineense* against 8 different microorganisms.

Test microorganisms/Concn	250µl/ml	50µl/ml	10µl/ml	2µl/ml	0.4µl/ml	Amp.20µg/ml	Clot.30µg/ml
Aspergillus niger	13.67±0.66	9.33±0.34	7.00±0.58	5.33±0.62	1.67±0.34	-	21.33± 0.88
Candida albicans	8.00±0.58	5.00±0.58	3.67±0.34	3.00±0.58	2.33±0.34	-	25.33± 0.17
Escherichia coli	5.33±0.34	NI	NI	NI	NI	12.33± 1.45	-
Staphylococcus aureus	10.33±0.34	8.33±0.91	6.00±0.41	5.67±0.66	2.33±0.34	13.33± 0.88	-
Pseudomonas aeruginosa	8.67±0.88	7.00±0.41	6.33±0.34	5.33±0.34	4.33±0.41	9.33± 0.34	-
Bacillus substilis	10.67±0.34	8.33±0.88	4.67±0.34	NI	NI	9.33± 0.34	-
Salmonella typhi	12.33±0.34	10.67±0.66	8.00±0.58	7.00±0.58	6.33±0.34	9.33± 0.88	-
Klebsiella pneumonia	15.67±0.34	10.67±0.88	6.33±0.66	4.00±0.58	2.67±0.66	17.00± 0.58	-

Key: concn= concentration, NI= No inhibition, - = Not tested, Amp. = Ampicillin, Clot. = Clotrimazole. Values for inhibition zone are presented as mean ± S.D. of three triplicate

Antimicrobial Screening of essential oil from the dried fruits of *Xylopia aethiopica*

Table 3 shows the result of the antimicrobial activity of the essential oil from the dried fruit of *Xylopia aethiopica* at different dilutions. The result shows that the essential oil from the dried fruit of *Xylopia aethiopica* had various degree of activity against the test microorganisms. Among the gram-positive bacteria, *Staphylococcus aureus* was the most sensitive followed by *Bacillus subtilis* confirming previous report (Fleischer, *et al.,* 2008) [8], and among gram-negative bacteria, *Salmonella typhi* was found to be the most sensitive, followed by *Pseudomonas aeruginosa*, while *E. coli* and *K. pneumonia* was not sensitive to the essential oil from the dried fruit of *Xylopia aethiopica*, confirming previous report of the insensitivity of *E. coli* to the essential oil (Fleischer, *et.al.,* 2008; Iwu, 1993; Boakye-Yiadom, *et al.,* 1977; Okigbo, *et al.,* 2005) [8, 11, 4, 15]. This suggests that the essential oils from the dried fruit of *X. aethiopica* will not be useful in the treatment of diseases caused by *E. coli and K. pneumonia*. The result from Table 2 and 3 also revealed the antimicrobial activity of the reference antibiotic standard (Ampicillin and Clotrimazole) against the test microorganisms. These results showed that the reference antibiotic competed well with the essential oils in their activity against Gram-positive and Gram-negative bacteria. Among the fungi, the reference antibiotic standard used exhibited a higher activity against the fungi more than the essential oils. The DMSO did not show any activity against all the test micro-organism indicating that DMSO at 1% does not exert any antimicrobial activity.

Table 3: Antimicrobial activities of the Essential oil from *X. aethiopica* against 8 different microorganisms

Test microorganisms/Concentration	250µl/ml	50µl/ml	10µl/ml	2µl/ml	0.4µl/ml	Amp.20µg/ml	Clot.30µg/ml
Aspergillus niger	9.67± 0.88	7.00± 1.16	3.33± 0.34	2.33± 0.34	2.00± 0.00	-	21.33± 0.88
Candida albicans	10.67± 0.66	9.00± 0.58	6.33± 0.91	5.00± 0.00	2.67± 0.34	-	25.33± 0.17
Escherichia coli	NI	NI	NI	NI	*NI*	12.33± 1.45	-
Staphylococcus aureus	10.67± 1.49	9.00± 0.58	7.33± 0.34	5.33± 0.34	3.33± 0.62	13.33± 0.88	-
Pseudomonas aeruginosa	10.33± 0.88	8.00± 0.58	7.00± 0.00	6.33± 0.34	4.33± 0.34	9.33± 0.34	-
Bacillus substilis	9.00± 0.58	7.33± 0.34	3.33± 0.88	NI	NI	9.33± 0.34	-
Salmonella typhi	12.33± 0.88	8.33± 0.66	6.67± 0.34	4.33± 0.34	NI	9.33± 0.88	-
Klebsiella pneumonia	NI	NI	NI	NI	NI	17.00± 0.58	-

Key: concn= concentration, NI= No inhibition, - = Not tested, Amp. = Ampicillin, Clot. = Clotrimazole. Values for inhibition zone are presented as mean ± S.D. of three triplicates.

The MIC, MBC and MFC of the Essential Oil from *P. guineense*

Table 4 shows the MIC, MBC and MFC results of the essential oil from the dried fruit of *P. guineense*. It reveals that the MIC of the gram-positive bacteria is the same, with 2.5µl/ml as the value. The MIC of the gram-negative bacteria ranges from 0.5 to <12.5 µl/ml, while that of fungi ranges from 2.5 to 12.5 µl/ml. These suggest that the essential oil from the dried fruit of *P. guineense* has a better inhibitory effect against the gram-negative bacteria, followed by gram-positive bacteria and then fungi. The MBC values ranges from 2.5 to <12.5 µl/ml for gram-negative bacteria, while that of gram-positive bacteria is the same, with 12.5 µl/ml as the value and MFC value for the fungi ranges from 2.5 to <12.5 µl/ml. The result reveals that the essential oil at a higher concentration than the MIC will become lethal to the test microorganisms. Thus it could be inferred that the essential oil have biocidal (bactericidal and fungicidal) activities.

Table 4: The Minimum Inhibitory Concentrations, Minimum Bactericidal Concentrations and Minimum Fungicidal Concentrations of the Essential Oil from *Piper guineense.*

Test organism(s)	MIC (µl/ml)	MBC(µl/ml)	MFC(µl/ml)	Amp(µg/ml)	Clot.(µg/ml)
Aspergillus niger	2.5	Nt	2.5	Nt	0.012
Candida albicans	12.5	Nt	<12.5	Nt	0.0024
Escherichia coli	<12.5	<12.5	Nt	0.008	Nt
Staphylococcus aureus	2.5	12.5	Nt	0.0016	Nt
Pseudomonas aeruginosa	12.5	<12.5	Nt	0.04	Nt
Bacillus substilis	2.5	12.5	Nt	0.008	Nt
Salmonella typhi	0.5	2.5	Nt	0.2	Nt
Klebsiella pneumonia	0.5	2.5	Nt	0.04	Nt

Key: Nt = Not tested

The MIC, MBC and MFC of the Essential oil from *X. Aethiopica*

Table 5 shows the MIC, MBC and MFC results of the essential oil from the dried fruits of *X. aethiopica.* The result reveals that the MIC of the gram-positive bacteria has the same value as 2.5 µl/ml for the test microorganisms, while that of gram-negative bacteria and fungi is the same with values ranging from 2.5 to12.5 µl/ml. The result suggests that the essential oil from the dried fruit of *X. aethiopica* have a better inhibitory effect against gram-positive bacteria than gram-negative bacteria and fungi. The result equally shows that the MBC value for gram-positive bacteria is the same, with 12.5 µl/ml as the value and the gram-negative bacteria ranges from 12.5 to <12.5 µl/ml, while the MFC value for fungi ranges from 12.5 to <12.5 µl/ml. The result reveals that the essential oil at a higher concentration than the MIC will become lethal to the test microorganisms. Thus it could be inferred that the essential oil have biocidal (bactericidal and fungicidal) activities. The MBC and MFC result from the same table, reveals that both essential oils have the same level of bactericidal and fungicidal activities against gram-negative bacteria and fungi respectively. The MIC results from Tables 4 to 5 reveal that the essential oil from the dried fruit of *P. guineense* has a better inhibitory effect against gram-negative bacteria when compared with the effect of the essential oil from the dried fruit of *X. aethiopica* against test microorganisms, while the two essential oils exhibit the same level of inhibitory against fungi. The results from Table 4 to 5 also reveal that the MBC of the gram-positive bacteria from both essential oil is the same. Hence, it's an indication that they both exert the same level of bactericidal action against the gram-positive bacteria. The MBC and MFC value for gram-negative bacteria and fungi from the essential oil of the dried fruit of *X. aethiopica* is higher than the MBC and MFC value for gram-negative bacteria and fungi from the essential oil of the dried fruit of *P. guineense*. This reveals that the essential oil from the dried fruit of *P. guineense* have a better inhibitory and biocidal activities against test microorganisms than essential oil from the dried fruit of *X. aethiopica*.

Table 5: The Minimum Inhibitory Concentrations, Minimum Bactericidal Concentrations and Minimum Fungicidal Concentrations of the Essential Oil from *Xylopia aethiopica*

Test microorganism(s)	MIC (µl/ml)	MBC(µl/ml)	MFC(µl/ml)	Amp(µg/ml)	Clot.(µg/ml)
Aspergillus niger	12.5	Nt	<12.5	Nt	0.012
Candida albicans	2.5	Nt	12.5	Nt	0.0024
Escherichia coli	<12.5	<12.5	Nt	0.008	Nt
Staphylococcus aureus	2.5	12.5	Nt	0.0016	Nt
Pseudomonas aeruginosa	2.5	12.5	Nt	0.04	Nt
Bacillus substilis	2.5	12.5	Nt	0.008	Nt
Salmonella typhi	2.5	12.5	Nt	0.2	Nt
Klebsiella pneumonia	<12.5	<12.5	Nt	0.04	Nt

Key: Nt means Not tested

The Combined Antimicrobial Effect of the Essential Oils from Dried Fruits of *P. guineense* and *X. aethiopica*

Tables 6 to 11 show the combined antimicrobial effect of the Essential oil from dried fruits of *P. guineense* and *X. aethiopica*. The combined antimicrobial effect of the essential oils reveals synergy at different combination ratios in all the test microorganisms used, except with *Aspergillus niger* at ratio 0.9:0.1, *P. aeruginosa* at ratio 0.1:0.9 and 0.3:0.7, and *Salmonella typhi* at ratio 0.9:0.1 showing indifference, Additivity, Additivity and indifference respectively, and *B. subtilis* at ratio 0.1: 0.9, showing Additivity. This indicates that the combined antimicrobial effect of the essential oil is not only a function of the nature of the Essential oils involved, but also that of their combined ratios.

Table 6: Combined Antimicrobial Effect of the Essential Oils from *P. guineense* and *X. aethiopica* against *Aspergillus niger*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	6.25	0	0	0	- -
0.1	0.9	0.125	5.625	0.05	0.9	0.95	Synergism
0.3	0.7	0.188	2.188	0.075	0.35	0.425	Synergism
0.5	0.5	0.156	0.781	0.063	0.125	0.188	Synergism
0.7	0.3	0.438	0.938	0.15	0.175	0.325	Synergism
0.9	0.1	2.25	1.25	0.9	0.2	1.1	Indifference
1	0	2.5	0	1	0	1	--

Table 7: Combined Antimicrobial Effects of the Essential Oils from *P. guineense* and *X. aethiopica* against *Candida albicans*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	1.25	0	1	1	- -
0.1	0.9	0.313	0.563	0.05	0.45	0.5	Synergism
0.3	0.7	0.938	0.438	0.15	0.35	0.5	Synergism
0.5	0.5	0.391	0.078	0.063	0.062	0.125	Synergism
0.7	0.3	1.094	0.094	0.175	0.075	0.25	Synergism
0.9	0.1	2.813	0.063	0.45	0.05	0.5	Synergism
1	0	6.25	0	1	0	1	--

Table 8: Combined Antimicrobial Effect of the Essential Oils from *P. guineense* and *X. aethiopica* against *Staphylococcus aureus*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	2.5	0	1	1	--
0.1	0.9	0.125	1.125	0.05	0.45	0.5	Synergism
0.3	0.7	0.188	0.438	0.075	0.175	0.25	Synergism
0.5	0.5	0.156	0.156	0.062	0.062	0.125	Synergism
0.7	0.3	0.438	0.188	0.175	0.075	0.25	Synergism
0.9	0.1	1.125	0.125	0.45	0.05	0.5	Synergism
1	0	2.5	0	1	0	1	--

Table 9: Combined Antimicrobial Effect of the Essential Oils from *P. guineense* and *X. aethiopica* against *Pseudomonas aeruginosa*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	2.5	0	1	1	- -
0.1	0.9	1.25	2.25	0.1	0.9	1	Additivity
0.3	0.7	3.75	1.75	0.3	0.7	1	Additivity
0.5	0.5	3.125	0.625	0.25	0.25	0.5	Synergism
0.7	0.3	2.188	0.188	0.175	0.075	0.25	Synergism
0.9	0.1	1.406	0.031	0.112	0.012	0.125	Synergism
1	0	12.5	0	1	0	1	- -

Table 10: Combined Antimicrobial Effect of the Essential Oils from *P. guineense* and *X. aethiopica* against *Bacillus subtilis*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	25	0	1	1	--
0.1	0.9	0.25	2.25	0.1	0.9	1	Additivity
0.3	0.7	0.375	0.875	0.15	0.35	0.5	Synergism
0.5	0.5	0.313	0.313	0.125	0.125	0.25	Synergism
0.7	0.3	0.875	0.375	0.35	0.15	0.5	Synergism
0.9	0.1	1.125	0.125	0.45	0.05	0.5	Synergism
1	0	2.5	0	1	0	1	--

Table 11: Combined antimicrobial effect of Essential oil from *P. guineense* and *X. aethiopica* against *Salmonella typhi*

Combination by continous variation		MIC'S in the combination (µl/ml)		FICs in the combination		Sum (FIC index)	Result
A(Uziza)	B (Uda)	A	B	A	B		
5µl/ml	25µl/ml						
0	1	0	2.5	0	1	1	--
0.1	0.9	0.025	1.125	0.1	0.45	0.55	Synergism
0.3	0.7	0.038	0.438	0.15	0.175	0.325	Synergism
0.5	0.5	0.063	0.313	0.252	0.125	0.375	Synergism
0.7	0.3	0.175	0.375	0.7	0.15	0.85	Synergism
0.9	0.1	0.45	0.25	1.8	0.1	1.9	Indifference
1	0	0.25	0	1	0	1	--

The Preservative Efficacy of the Essential Oils from the Dried Fruits of *P. guineense* and *X. aethiopica*

For oral liquid product, the USP (1990) requires that for a preservative to be effective in the product examined, the concentration of the viable bacteria must be reduced at or not more than the 0.1% of the initial concentrations by 14th day, while the concentrations of viable yeast and molds must remain at or below the initial concentration during the first 14th day; and the concentration of each test microorganisms must remain at or below the designated levels during the remainder of the 28 days test period.

The viable count of bacteria, fungi, and enumerated *E. coli*, *Entrobacter sakazakii*, and the total coliforms count in the five different herbal samples both the unpreserved and preserved with the essential oil (using continuous variation model), M-and P-Paraben (2:1), and Sodium benzoate at 1% was undertaken. At 1%, the result shows that the essential oil were able to reduce the increasing microbial load in the herbal samples up to the 7th day in most of the samples but at the 14th day and 28th day there was an increase in the microbial growth higher than the growth in the initial day. This reveals that at 1%, the essential oil did not obey the 1st and 2nd criteria of a good preservative in the contaminated herbal sample but were bactericidal against *E. coli* and *E. sakazakii*; sodium benzoate was not able to fulfill the 1st and 2nd criteria in most of the samples but was effective against *E. coli* and *E. sakazakii*, while M-and P-Paraben (2:1) was effective against coliforms, *E. coli and E. sakazakii* in some the samples.

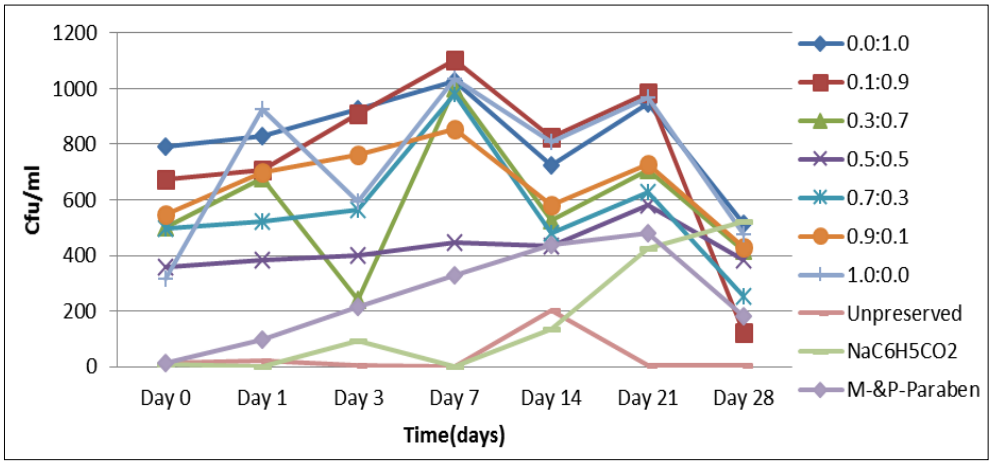

Fig 1: Viable counts of bacteria in sample 1 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 1%

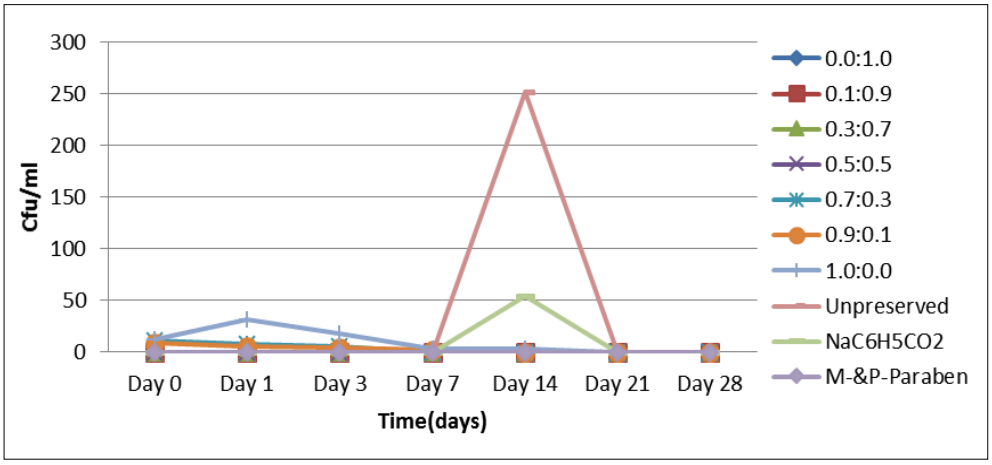

Fig 2: Enumeration of *E. coli* in sample 2 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 1%

The viable count of bacteria, fungi, and enumerated *E. coli*, *Entrobacter sakazakii*, and the total coliforms counts in the five different herbal samples both the unpreserved and preserved with the essential oil (using continuous variation model), M-and P-Paraben (2:1), and Sodium benzoate at 3% was done. The result shows that the essential oil both in single and in combination was able to reduce the increasing microbial load in the herbal samples fairly, though it was not

able to fulfill the criteria of a good preservative. The Sodium benzoate was able to reduce the increasing microbial load in the herbal samples but did not fulfill the 1st and 2nd criteria though it was effective against *E. coli* and *E. sakazakii*. M- and P-Paraben was able to fulfill the criteria in a very few of the sample at 3%, but their effectiveness against coliforms, *E. coli* and *E. sakazakii* was excellent.

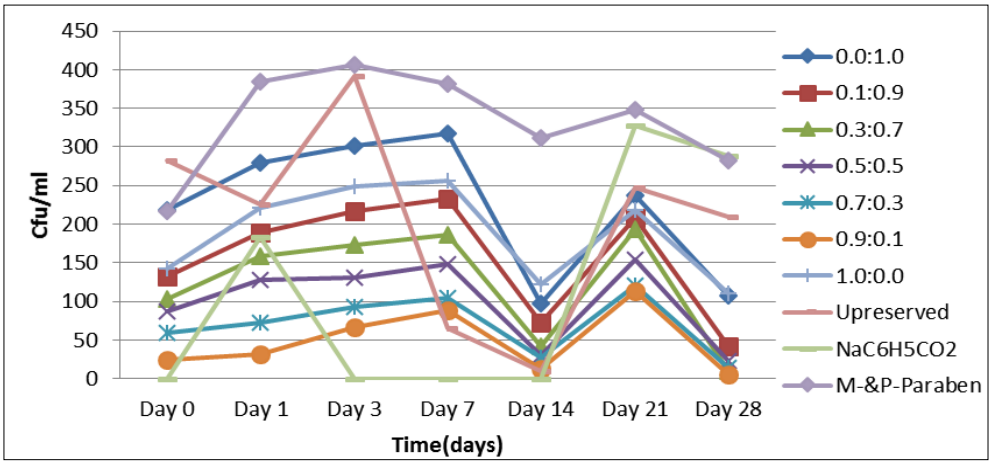

Fig 3: Viable counts of fungi in sample 2 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 3%

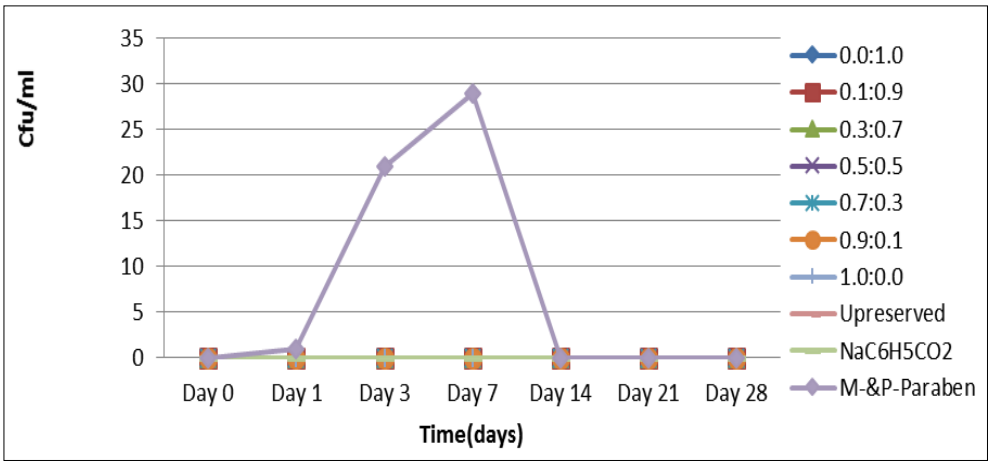

Fig 4: Enumeration of *E. coli* in sample 1 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 3%

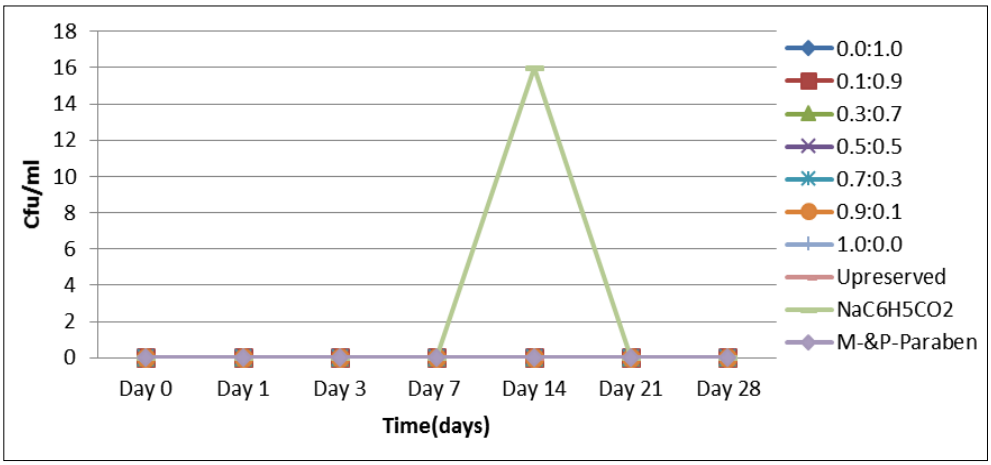

Fig 5: Enumeration of *E. coli* in sample 5 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 3%

The viable count of bacteria, fungi, and enumerated *E. coli*, *Entrobacter sakazakii*, and the total coliforms counts in the five different herbal samples both the unpreserved and preserved with the essential oil (using continuous variation model), M-and P-Paraben (2:1), and Sodium benzoate at 5% was done. The result shows that there is a drastic reduction in the bioload of the contaminated herbal sample preserved with the essential oils, M-and P-Paraben and Sodium benzoate. The essential oils at 5% was able to fulfill the criteria of a good preservative, M-and P-Paraben also fulfilled the criteria in most of the samples, while sodium benzoate was still finding it difficult to fulfill the 1st and 2nd criteria but was still effective against *E. coli* and *E. sakazakii.* The combined effect of the essential oil from the dried fruits *P. guineense* and *X. aethiopica* was effective in suppressing the survival of the microbial contaminant to some extent but was not able to

work as a good preservative at 1% and 3%. The combined effect of m- and p- paraben competed favorably with the effect of sodium benzoate in suppressing the survival of the microbial contaminants. Sodium benzoate was able to suppress the survival of the fungi more than the bacteria. Sodium benzoate, as well as m- and p- paraben is known to possess antibacterial and antifungal activities, hence they are use as preservatives but in this study they were not able to meet this requirement.

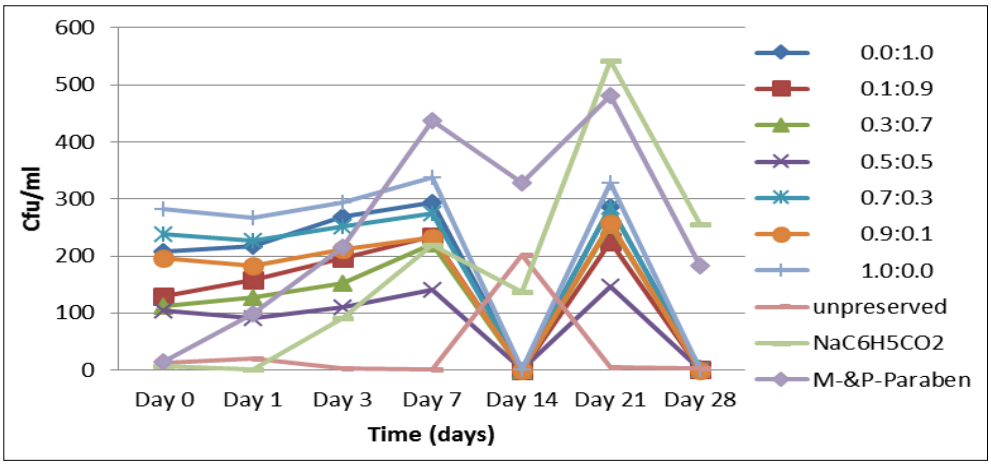

Fig 6: Viable counts of bacteria in sample 1 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 5%

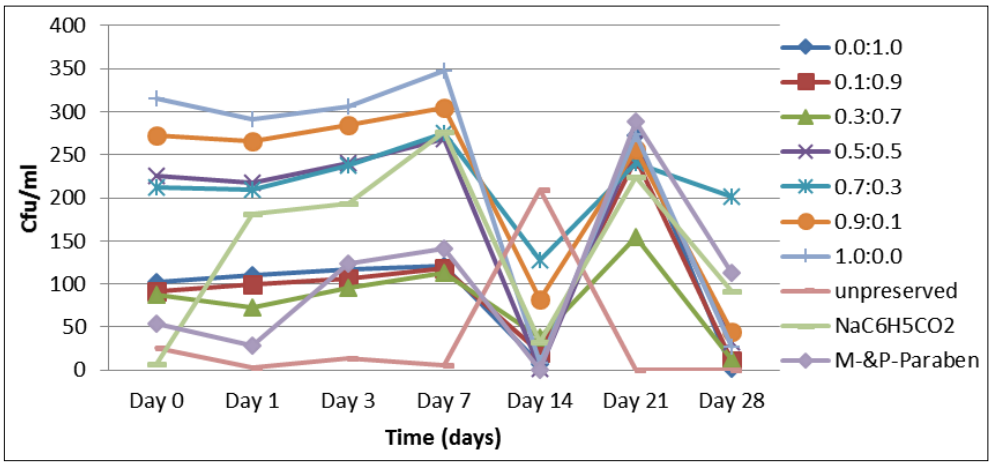

Fig 7: Viable counts of bacteria in sample 4 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 5%

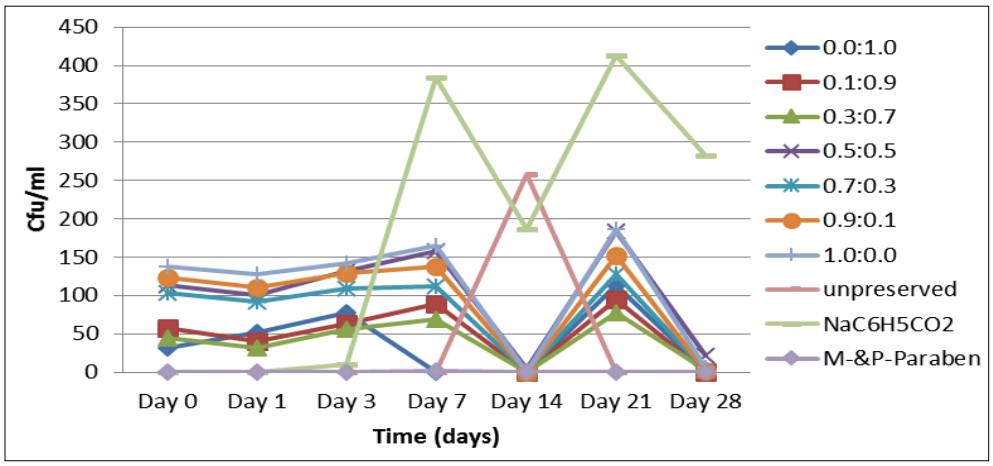

Fig 8: Coliforms count in sample 4 unpreserved and preserved with the essential oils, m-and p-paraben and sodium benzoate at 5%

The results reveals that the preservative efficacy of the essential oils and the conventional preservative system were both concentrations dependent. Also, the different concentrations used for the preservatives, were bactericidal against *E. coli* and *Enterobacter sakazakii* on most of the herbal samples. The effect of these preservative in herbal sample did not meet the requirement for a good preservative system despite the high concentrations used in this study, this relatively poor performance of these preservative especially the synthetic ones, might be an indication that the microbial contaminants in the herbal samples contain resistance genes that made the conventional preservative not to be able to fulfill the criteria of a preservative. Growth of the bacteria, fungi and Coliforms in the unpreserved herbal products did not maintain a steady trend. It is evident that these herbal preparations proved a suitable environment for the survival or

growth of microorganisms. However, these contaminated liquid herbal preparations were also able to be preserved to varying degree by all these preservative systems used. It has shown that the combination of the essential oils of *Piper guineense* and *Xylopia aethiopica* are synergistic at 5%, like the combination of methyl paraben and propyl paraben, which are popular in preserving oral pharmaceuticals (Beveridge, 1992) [3]. The essential oils from *P. guineense* and *X. aethiopica* were able to prevent the emergence of a putrefactive odour, which is an indication of microbial proliferation during the period of study but M- and P-Paraben, and sodium benzoate were not able to do it as perfectly as the essential oil.

Conclusion

Most of the antimicrobial activity of EOs appears to derive from oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols (Bassolé, and Juliani, 2012). The present study shows that both the essential oils from *P.guineense* and *X. aethiopica* have antimicrobial activity, but when compared, the essential oils from the dried fruits of *P. guineense* had more activity against the test microorganisms. The combined antimicrobial effect of the essential oils from the dried fruits of *P. guineense* and *X. aethiopica* showed they are synergistic. The combination of the two essential oils showed its preservative effect at the highest concentration of 5%.

Therefore, the combination of these two essential oils from the dried fruits of *P. guineense* and *X. aethiopica* may have a potential for further study as an alternative in the preservation of herbal preparations. However, further application and challenge assessment studies are required to extensively quantify the preservative effect of these two essential oils. In this regards, further *in vivo* studies on the different types and concentrations of essential oils need to be assessed to evaluate a safe concentration of the essential oil to be used in preserving herbal medicines.

Recommendations

Pre-formulation handling by collectors and herb-sellers are likely to contribute to the contamination of the raw material which affected the bioburden of the finished products. The increasing microbial count in the herbal products explains the poor performance of the preservative in the products. Hence it is strongly recommended that steps should be taken to decontaminate the raw material before the preparations. Enlightening Traditional medicine practitioners on the need to observe simple hygienic conditions such as maintenance of clean environment, proper washing, drying and storage of plant part before use, procurement of good water supply is also necessary. Pre-boiling and proper storage of such water will go a long way at improving the microbial quality. While research is still going on the effective preservative of plant or natural origin, Traditional medicine practitioners are advised to include conventional preservatives such as paraben at the recommended concentrations in their products.

References

1. Aflatuni A. The yield and essential oil content of mint (*Mentha spp.*) in Northern Ostrobothnia. Academic Dissertation presented with the assent of the Faculty of science, University of Oulu, Finland, 2005, 11-41.
2. Bassolé IHN, Juliani HR. Essential Oils in Combination and Their Antimicrobial Properties. Mol. 2012; 17:3989-4006.
3. Beveridge EG. In: Hugo WB, Russel A. D. (Eds.) Pharmaceutical Microbiology Blackwell Scientific Publication, 5th Edn., Oxford, 1992, 355-373.
4. Boakye-Yiadom K, Fiagbe NIY, Ayim JSK. Antimicrobial properties of some West African medicinal plants and Antimicrobial activity of Xylopic and other diterpenes from the fruits of *Xylopia aethiopica* (Annonaceae) Lloydia. Afr. J of Trad., Compl. and Alter, Med. 1977; 40:543- 545.
5. Cheesbrough M. District Laboratory Practice in Tropical Countries. 2nd Edition. Cambridge University Press, UK, 2000, 178-187.
6. Esimone CO, Ezugwu CO, Okide GB, Nwodo NJ. Preservative effect of *Aframomum melegueta* oil on promethazine HCl syrup. Plant prod. Res. J. 2002; 7(2):25-30.
7. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infection Disease (ESCMID). 2000; 6:509-515.
8. Fleischer TC, Mensah MLK, Mensah AY, Komlaga G, Gbedema SY, Skaltsa H. Antimicrobial activity of essential oils of *Xylopia aethiopica. Afr.* J of Trad. Compl., and Alt. Med. 2008; 5(4):391- 393.
9. Gill LS. Ethnomedicinal uses of plants in Nigeria, First Edition. Uniben press, university of Benin, Benin city, Edo state, Nigeria, 1992, 193-248.
10. Gutierrez J, Barry-Ryan C, Bourke P. Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. School of Food Science and Environmental Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Ireland, 2009, 1-40.
11. Iwu MR. Handbook of African medicinal plants. CRC Press, Boca Raton, FL, 1993, 423-431.
12. Kelly MB, Valtacho DZ. Factors affecting yields of Essential oil quality of *Ocimum sanctum* L. and *Ocimum basilicum* L. cultivars. J Amer. Soc. Hort. Sci. 2004; 129(6):789-794.
13. Martins AP, Salgueiro L, Vila R, Tomi F, Canigueral S, Casanova J, *et al*. Essential oils from four *Piper* Species. Phytochem. 1998; 49(7):2019-2023.
14. Mshana NR, Abbiw DK, Adde- Mensah I, Adjanouhun E, Ahyi MRA, Ekpere JA, Enow-Orodc *et al*. Traditional medicine and pharmacopoeia, contribution to the revision of ethno- botanical and Floristic studies in Ghana. OAU/ STRC Technical Report, 2000, 67.
15. NCCLS-National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing: eleventh informational supplement. Document M100-S11. National Committee for Clinical Laboratory Standard, Wayne, PA, USA, 2003.
16. NCCLS-National Committee for Clinical Laboratory Standards. Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA, USA, 1997.
17. Okigbo RN, Mbajiuka CS, Njoku CO. Antimicrobial potential of (Uda) *Xylopia aethiopica* and *Ocimum gratissimum* L. on some pathogens of Man. Intern. J Mol. Med. Advance Sci. 2005; 1(14):392- 397.
18. Okore Vincent C. Principles of pharmaceutical microbiology. Nigeria, Ephrata Publishers No. 2 Orba Rd, Nsukka Enugu State, Nigeria. 2nd edition, 2009; 80, 90, 94 – 95, 105.

19. Olonisakin O, Oladimeji MO, Lajide L. Chemical Composition and Antibacterial Activity of Steam Distilled oil of Ashanti Pepper (*Piper guineese*) Fruits. J of Appl. Sci. 2006; 6(11):2520-2522.
20. Perez C, Pauli M, Bazerque P. An antibiotic assay by agar-well diffusion method. Acta Bio. et Med. Experi. 1990; 15:113-115.
21. Pessoa LM, Morais SM, Bevilaqua CML, Luciano JHS. Antihelmintic activity of essential oil of *Ocimum gratissimum* L. andeugenol against Haemonchus contortus. Vet. Parastiol. 2002; 109(1-2):59-63.
22. Schelz Z, Molnar J, Hohmann J. Antimicrobial and plasmid activities of essential oils. Fitoter. 2006; 77:279-285.
23. Shigeharu I, Toshio T, Hideyo Y. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J of Antimicrob. chemother. 2001; 47(5):565- 573.
24. Tapu AO, Hofmann T, Schieberle P. Identification of the key aroma compounds in dried fruit of *X. aethiopica*. In J Janick (ed), perspective on new crops and new uses. ASHA Press, Alexandria, VA, 1999, 474-478.
25. United States Pharmacopocia. 20th Edition, 1990, 873-882.
26. Waterman PG. Roles of secondary metabolites in plants. Ciba found symp. 1992; 171:258-269.
27. Wikipedia, 2012. http:// en.wikipedia.org/wiki/File: *Piper guineense.* Last updated 6 Mar. 2012. Retrieved on 11 May, 2012 at 12:00 pm.
28. William AH, Christian AA, Ivan CSB. Application of essential oils as a preservative to improve the shelf life of Nile Tilapia (*Oreochromis niloticus*). Vitae, Revista De La Facultad De Quimica Farmaćeutics. 2012; 19(1):34-40.
29. Wink M. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phyto chem. 2003; 64:3-19.