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Biopreservative Potential of the Spices; Piper Guineense (Uziza), Xylopia Aethiopica (Uda) and Tetrapleura Tetraptera (Oshorisho) in Fresh Fruit Juices

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ABSTRACT

This study demonstrates the antimicrobial activity of three spices, *Piper guineense* (Uziza), *Xylopia aethiopica* (Uda), *Tetrapleura tetraptera* (oshorisho) and their potential use as food biopreservatives. Essential oils of the spices seed were obtained and used in the agar-diffusion and tube dilution assays to determine their minimum inhibition (MIC) and bactericidal concentrations (MBC) against a common fruit juice spoilage organism (Bacillus cereus). MIC values of 50mg/ml (Oshorisho) and 25mg/ml (Uziza and Uda) were obtained. Similarly, all spice extracts were bactericidal at a concentration of 100mg/ml. The spice extracts were utilized in the fortification of fresh fruit juice. Fortification of orange, pineapple and watermelon juices with extracts of the spices caused a reduction in the bacterial and fungi load of the juices over a period of 7 days in comparison to unpreserved controls. Orange juice preserved with uda had a fungal load of 1.9 x10^6 and bacterial load of 8.0 x10^6 compared to control with loads of 2.3 x10^7 and 1.1 x10^7, respectively. Watermelon juice preserved with oshorisho had a fungal load of 1.2 x10^6 and bacterial load of 1.1 x10^7 compared to control with loads of 2.1 x10^7 and 8.8 x10^7, respectively while pineapple juice preserved with uziza had a fungal load of 1.2 x10^7 and bacterial load of 1.6 x10^7 compared to control with loads of 2.7 x10^7 and 2.5 x10^8, respectively. These results show that the spice extracts have potential for use as food preservatives for extending the shelf life of freshly prepared fruit juices. This is important as the demand for fresh foods free from chemical preservatives but microbiologically safe is on the increase.

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Introduction

The greatest threat to quality and safety of food comes from microbial spoilage which can negatively impact on the organoleptic quality of foods and cause foodborne infections/intoxications [1]. Food preservation traditionally has been undertaken using heat treatments, fermentations and chemical additives. However, some challenges are associated with the use of chemical preservatives in foods including cardiovascular diseases, change in nutritional compositions of foods and certain cancers etc. Thus, there is need for the use of alternative naturally produced antimicrobial agents to replace chemical preservatives for shelf-life extension of foods. Overall, there is growing consumer demand for non-thermally processed foods free from chemical preservatives but microbiologically safe which are perceived to be healthier. This poses a challenge for the control of potential microbial agents especially spore formers such as *Bacillus* sp. in ready to eat foods such as fresh-squeezed fruit juices which is preferably consumed in the non-thermally treated state. More so, the increasing occurrence of food-borne disease outbreaks from this class of minimally preserved ready-to-eat foods/beverages raises considerable public health concern [2, 3]. Reported cases of food borne disease outbreaks in some countries have been linked to unpasteurized fruit juices, necessitating the search for alternative antimicrobial compounds from plant sources [4, 5]. The causal agents of microbiological spoilage in fruits and derivatives can be bacteria, as well as yeasts and molds. The latter are considered the main spoilage agents due to the low pH of most fruits and juices. Nevertheless, some bacteria such as *Erwinia* spp., *Enterobacter* spp., *Alicyclobacillus* spp., *Propionibacterium cyclohexanicum*, *Pseudomonas* spp., and lactic acid bacteria have been reported as deteriorative in cut fruit and juices [6, 7]. Certain common molds such as *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp., *Alternaria* spp., *Cladosporium* spp., *Paecilomyces* spp., and Botrytis spp. have been shown to be involved in the spoilage of fresh fruits and some processed fruit derivatives including the thermally processed juices [8]. These organisms can impact negatively on the organoleptic quality of fruit juices and can even

cause various types of food poisoning.

In recent times, there is an increased interest in the use of plant extracts and antioxidants in microbial inhibition and possibly food preservation [9, 10]. This is due to their strong biological activity which has been found to exceed those of many synthetic agents which can be deleterious to health [11]. Spices are a large group of natural ingredients which include dried seeds, fruits, roots, rhizomes, barks and flowers plants which are used in very small quantities as food additives to improve colour, flavour, aroma and other organoleptic qualities of food and also serve in food preservation [12]. The bulk of the major components of spice materials consist of carbohydrate, proteins, minerals and phytochemicals. The phytochemicals present in spices such as alkaloids, steroids, tannins, flavonoids and phenolic compounds are responsible for their flavouring, colouring, preservative and health promoting characteristics [13]. Essential oils (EOs), usually obtained from several parts of plants contain volatile aromatic compounds which are predominantly secondary metabolites [14]. They have potent antibacterial and antifungal activity and have also been known to have antioxidant properties and cancer suppressive activity against human malignant (cancer) cell lines [15-19].

Various authors have reported the antimicrobial activity of local spices including *Piper guineense* (Uziza), *Xylopia aethiopica* (Uda) and *Tetrapleura tetrapetra* (Oshorisho) which feature prominently in the foods prepared in West Africa. Uziza (*Piper guineense*), also known as Ashanti pepper, belongs to piperacea family, like all true pepper seeds. They are prolate spheroids, small and smooth in appearance and generally bear a reddish tinge. They are native to tropical regions of Central and Western Africa and are cultivated in Nigeria where the leaves are used as a flavouring for stews [20]. They contain large amounts of beta – caryophyllene which is an anti – inflammatory agent and significant proportions (10%) of myristicin, elemicin, safrole and dillapoil which are believed to have preservative and antioxidant properties [20]. Uda (*Xylopia aethiopica*) also known as Guinea pepper belongs to the annoneceae family. It is an ever green aromatic dicotyledenous plant commonly found in West Africa, South Africa and Brazil. The fruits are cylindrically thick, about 5cm long and are green when unripe, reddish when ripe and black when dried [21]. The fruits contain 3-9 seeds which are used as substitute for pepper. Chemical composition of the seed consists of flavonoids, alkaloids, cineol, phytosterols, tannins, saponins, glycosides, carbohydrates, terpenes, paradol, cryptone, limonene, myrtenol etc. and have been shown to have antioxidizing, laxative and antimicrobial properties [22]. *Tetrapleura tetrapetra* (Oshorisho) belongs to the genera *Tetrapleura* and of the family *Minosaceae*. The plant is a perennial tree, about 30m high, found in the West Central and East Africa. The fruits are green when tender, reddish brown when fully ripened and contains some brownish black seeds. The fruit has four longitudinal wings like fleshy ridges of about 10cm broad. It contains essential oils, saponosides, triterpene, caumarins, tannins, steroids, triterpene glycosides and is utilized in the preparation of pepper soups for mothers after labour to prevent postpartum contraction [23,24]. These spices have shown promise in the inhibition of microbial growth. Okoronkwo and Echeme (2012) reported that extracts of *T. tetraptera* effectively inhibited the growth of *E. coli*, *S. aureus* and *Proteus mirabilis* with resultant zones of inhibition of 5.00 – 18.00 mm for the leaves, stem, bark and roots etc [25]. indicating that extracts of *T. tetraptera* can be bacteriostatic/bacteriocidal against these test pathogens. Ebana et al., (2016) reported a higher value of 21.30±1.20mm against *E. coli* while the least inhibition of 10.00±0.08mm was observed upon treatment of *P. aeruginosa* with aqueous extracts of *T. tetraptera*. A more recent study by Oguoma et al. (2015) [27] on the antimicrobial activity of *T. tetraptera* on *E. coli*, *S. aureus*, *Shigella* spp and *Salmonella typhi* showed zones of inhibition of 21.00 mm for *E. coli* which is consistent with previous reports [26,27]. Several studies of the antimicrobial activities of *P. Guineense* leaves and seeds showed the efficacy of this plant extract to inhibit potentially pathogenic microbial species. Furthermore, Ebana et al., (2016) reported zones of inhibitions of 37.50 and 30.00 mm with the aqueous and petroleum extracts and a minimum inhibitory concentration of ≥80 mg/ml was observed for *P. guineense* seeds. Also, they recorded antifungal activity of the extracts against *Aspergillus*, *Mucor* and *Rhizopus* species [26]. However, none of the extracts had complete inhibition against all the fungal isolates. This is in tandem with an earlier study by Okoronkwo and Echeme (2012) [25] which recorded no inhibition against Fusarium oxysporum, Penicillium chrysogenum and Mucor species using aqueous and ethanolic extracts of the leaves, stem, bark and roots of *T. tetraptera*. A lot of research has reported on the antimicrobial activity of the essential oil of *X. aethiopica*. It has been shown that the essential oil as well as the crude extracts (both alcoholic and aqueous) have antimicrobial properties against a wide range of Gram positive and Gram-negative bacteria as well as Candida albicans [22, 28, 29]. Fleischer et al., (2008) described various degrees of inhibitory activity against the test organisms including *S. aureus*, *B. subtilis* and *C. albicans* [30].

A lot of the studies on the antimicrobial activity of the selected local spices against food spoilage organisms were conducted in-vitro and may not take into account the potential roles food matrix may play on the activity of the extracts and survivability of microbial species. Thus, this research work was aimed at evaluating the efficacy of these spices in elongating the shelf life of food items by infusing their essential oils into fresh fruit juices. This will provide a veritable alternative to the use of chemical preservatives in fresh juice while maintaining microbial safety.

Materials and Methods

Sample Collection

The seeds of *Piper guineense* (uziza), fruits of *Xylopia aethiopica* (uda) and *Tetrapleura tetrapetra* (Oshorisho) were purchased in their dried state from Ekeonunwa market Owerri, Imo State, Nigeria. They were cleaned of stalks, unwholesome seeds, fruits, and extraneous materials. The selected seeds and fruits were washed in distilled water to remove dust and then spread on trays and oven dried at 65°C for 24h. The cleaned dried spices were ground into powder using a laboratory homogenizer and stored in air tight containers prior to extraction.

Extraction of Essential Oils

30g of each sample was weighed out and wrapped in Whatman No. 2 filter paper. The essential oils were extracted with the aid of a Soxhlet extractor. The resulting extract was concentrated using a Cole-Parmer diagonal rotary evaporator and heating mantle to form a slurry which was then transferred into amber bottles and stored in the refrigerator until required for analysis. Stock solutions of the crude extracts were prepared by diluting with 20% dimethyl sulphoxide (DMSO) and serial dilution as appropriate to obtain various concentrations of the working solution.

Preparation of Test Isolates

The test isolate utilised for this study is *Bacillus cereus* isolated from spoilt fruit juice by streaking on nutrient agar. Its identity was confirmed by Gram staining and biochemical tests and interpreted

using the identification schemes of the 8th Edition of Bergey's manual of Determinative Bacteriology as described by Harrigan, (1998) [31]. The concentration of the organism was adjusted to the MacFarlands standard by diluting with phosphate buffered saline and measuring to an optical density (OD) value of 0.1 at 600nm wavelength using a UV/Vis spectrophotometer (Jenway 6300).

Antimicrobial Assay of Essential Oils

The antimicrobial activity of the essential oils was determined by the agar-well diffusion assay using Muller-Hinton agar and incubation at 37^{O}C for 24 hours. The average diameter of inhibition zones formed was measured with the aid of a calliper. The Minimum Inhibitory Concentration (MIC) of extracts was determined by spectroscopic methods after infusing different concentrations of the extracts ranging from 6.25 to 100mg/ml into Muller Hinton broth inoculated with the test organism as previously described by Anumudu et al., (2020) [32]. The Minimum Bactericidal Concentration (MBC) was determined by subculturing tubes that showed growth inhibition onto fresh nutrient agar as described by Fasoyiro and Adegoke (2007) [33] and Nwachukwu et al. (2009) [34]. The MIC was reported as the broth containing the lowest concentration of the bacteriocin extract, which showed no growth (was able to inhibit microbial growth) while the MBC is the lowest concentration of the bacteriocin extract that caused cell death.

Preservation of Fruit Juices with Spice Extracts

Fruit juices from pineapple, watermelon and oranges were prepared from whole fruits by squeezing out the juice and filtering through a muslin cloth. This was pasteurized at 63^{O}C for 30 minutes. 9mls of each juice was transferred into six (6) sterile test tubes and corked. To three of these test tubes, 1ml of the undiluted crude spice extract was added. The remaining three test tubes were corked without addition of extracts to serve as negative control. This was repeated for all crude extracts (uziza for pineapple juice, uda for orange juice and oshorisho for watermelon juice) and stored for a period of seven (7) days. This experiment was designed in this manner to ensure that different replicate of the same sample was taken on two days interval for culturing to determine microbial load without having to open and close the sample over the period of analysis, reducing risks of environmental contamination.

Determination of Total Heterotrophic Bacterial and Fungal Count of Fruit Juices

The microbial load of the fruit juices at onset before preservation with the crude extract for each juice was recorded on Day 1. On subsequent days of storage, the total heterotrophic bacterial and fungal count was determined by culturing using the spread plate method onto nutrient agar (for bacteria) and potato dextrose agar (for fungi) after serial dilution (Day 3,5 and 7). Concisely, 9mls of sterile phosphate buffered saline was placed in five test tubes, 1ml of the fruit juice was introduced into the first tube and serial dilution of the extract is undertaken to reduce the concentration. This is repeated for both the preserved and unpreserved samples for all juices.

Aliquots (100µl) from each test tube were inoculated onto agar plates in duplicates using a micropipette. The inoculum is spread evenly over the surface of the agar plate (nutrient agar for total viable heterotrophic bacteria count and potato dextrose agar for total viable heterotrophic fungal count) using a sterile L-shaped spreader. The plates were then incubated at 37°C for 24 hours in an incubator for bacteria on nutrient agar and at 25°C for 72 hours for fungi on potato dextrose agar supplemented with chloramphenicol to prevent bacteria growth. After incubation, resultant colonies on the different plates were counted using a colony counter. Mean of the counts from the duplicate plates were obtained and multiplied with the reciprocal of appropriate dilution factor to obtain the colony forming unit (cfu) per milliliter of the sample. This was repeated in two days intervals (days 3, 5 and 7) for all fruit juices.

Results and Discussion

Antimicrobial Activity of Extracts against Bacillus cereus

The well-in-agar assay showed potent inhibition of B. cereus by all the spices as shown in Table 1.

Table 1: Antimicrobial screening of the crude spice extracts

Conc (mg/ml)	Oshorisho	Uziza	Uziza	Chloramphenicol
100	37mm	44mm	22mm	40mm
50	34mm	25mm	-	34mm
25	18mm	21mm	-	36mm
12.5	-	17mm	-	26mm
6.25	-	-	-	-

The result obtained from the antimicrobial screening of the crude spice extracts (Table 1) shows that the inhibitory effects of the extracts was dependent on concentration. Uziza had the largest zone of inhibition (44mm), followed by Oshorisho (37mm) and Uda (22mm), all at a concentration of 100mg/ml. Furthermore, at 50mg/ml and 25mg/ml concentrations oshorisho and uziza gave significant zones of inhibition. The lowest activity was recorded by the uda extracts which produced a zone of inhibition of 22mm for the 100mg/ml dilution and no inhibition zone for the lower dilutions. The Uziza extract outperforms the highest zone of inhibition recorded by the antibiotic chloramphenicol (40mm). This may be related to the rising resistance of *Bacillus* sp. to chloramphenicol due to the proliferation of resistance genes in the *Bacillus cereus* group [35]. Diverse group of microorganisms are present in fruit surfaces during harvest and contaminates fresh fruit juices during postharvest processing [36]. Many microorganisms especially the acid tolerant bacteria and fungi (moulds, yeasts) use them as substrate for their growth and cause spoilage in the juices which includes cloudiness, development of off-flavours, gas formation, colour change and texture defects thus there is need for their effective control [37, 38]. The findings from this study is in consonance to the findings of Ekwenye and Chigozie, (2010) who reported a zone of inhibition of 26.40mm with ethanolic and aqueous extracts of *T. Tetraptera* (oshorisho) against S. aureus and the study of Oguoma et al. (2015) which recorded a zone of inhibition of *T. Tetraptera* against *E.coli*. The zones of inhibition obtained for *P. guineense* (uziza) is similar to those reported by Ebana et al., (2016) which was recorded as (37.50 and 30.00mm for the aqueous and petroleum extracts of uziza against the test organism [39,27,26]. These zones of inhibition demonstrates the ability of low concentrations of spice extracts to effectively inhibit the growth and proliferation of the test organism. Furthermore, the tube dilution assay gave MIC values of 50mg/ml (Oshorisho) and 25mg/ml (Uziza and Uda) as presented in Table 2.

Table 2: MIC test of crude spice extracts on the test organism

Conc (mg/ml)	Oshorisho	Uziza	Uziza	Chloramphenicol
100	Clear	Clear	Clear	Clear
50	Clear	Clear	Clear	Turbid
25	Turbid	Clear	Clear	Turbid
12.5	Turbid	Turbid	Turbid	Turbid
6.25	Turbid	Turbid	Turbid	Turbid
MIC	50 mg/ml	25 mg/ml	25 mg/ml	100 mg/ml

The MIC results showed potent inhibition of the test organism by all the extracts at different concentrations. The lowest MIC value was recorded by the uziza and uda extracts (25mg/ml). This outperforms the MIC value obtained from the positive control chloramphenicol (100mg/ml). The extracts of oshorisho gave higher MIC values of 50mg/ml, but still lower than that of chloramphenicol. The MIC result obtained for *P. Guineense* (Uziza) and *T. Tetraptera* (oshorisho) is much lower than the 80mg/ml reported by Ebana et al., (2016) possibly because they used the aqueous extract of the uziza seeds [26]. This result indicates that the spice extracts contain potent bioactive compounds which actively inhibited the proliferation of the test organism, justifying their use in folk medicine for the control of microbial spoilage in different foods. Of note is that the inhibitory effect of the extracts are concentration dependent. However, the MIC assay only indicates an inhibition of growth of the test organism. The ability of the spice extracts to effectively kill the test bacteria is demonstrated in the minimum bactericidal concentration assay. All spice extracts where bactericidal at a concentration of 100mg/ml after sub-culturing to sterile nutrient agar plates as shown in Table 3. This result shows comparable antimicrobial activity of the spice extracts with conventional antibiotics in-vitro.

Table 3: MBC test of crude spice extracts on the test organism

Conc (mg/ml)	Oshorisho	Uziza	Uziza	Chloramphenicol
100	No growth	No growth	No Growth	No growth
50	Growth	Growth	Growth	-
25	-	Growth	Growth	-
MBC	100 mg/ml	100 µm/ml	100 mg/ml	100 mg/ml

Preservative Potential of the Crude Extracts in Fruit Juices

Total heterotrophic bacteria count

The spice extracts were utilized to evaluate shelf life extension of fresh fruit juice. Fresh fruit juice of orange, pineapple and watermelon preserved with the spice extracts of uda, uziza and oshorisho showed a significant reduction in the bacterial load respectively in comparison to unpreserved controls over a period of 7 days. The results are shown in Table 4.

Table 4: Total heterotrophic bacteria count (THBC)

Day	Bacteria population (CFU/ml) in stored juice					
	Pineapple	Pineapple + Uziza	Orange	Orange + Uda	Watermelon	Watermelon + oshorisho
Day 1	1.5×10^6	-	3.4×10^6	-	4.9×10^6	-
Day 3	3.2×10^6	4.7×10^5	5.7×10^6	6.7×10^5	5.8×10^6	6.5×10^5
Day 5	1.6×10^7	2.5×10^6	9.8×10^6	1.0×10^6	8.4×10^7	4.0×10^6
Day 7	2.5×10^8	1.6×10^7	1.1×10^7	8.0×10^6	8.8×10^7	1.1×10^7

Extracts of uda, uziza and oshorisho completely inhibited bacterial growth on orange, watermelon and pineapple juices respectively after 24 hours of storage. Fresh orange juice preserved with uda and stored for 7 days showed a bacteria population of 8.0×10^6 as against the unpreserved sample of 1.1×10^7. Pineapple juice preserved with uziza showed a bacterial load of 1.6×10^7 after 7 days storage as against the unpreserved sample of 2.5×10^8. Also for watermelon juice preserved with oshorisho, the bacterial load after 7 days of storage was 1.1×10^7 against the unpreserved sample of 8.8×10^7. This resulting reduction in the microbial load of the fortified juices (pineapple, orange and watermelon) by uziza, uda and oshorisho extracts is in consonance with previously published work by Friedman et al (2004) who observed a reduction in the microbial load of apple juice inoculated with *E. coli* and *Salmonella enterica*. Figure 1. represents a line chart of bacterial load with time [40].

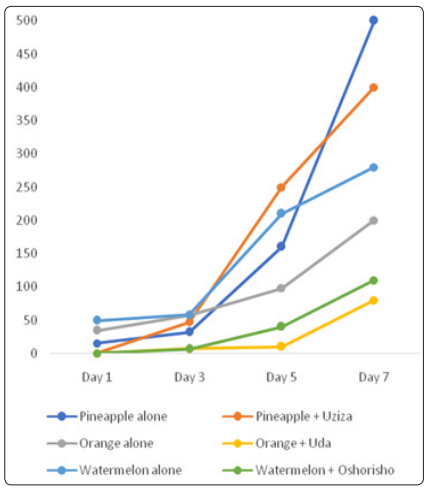

Figure 1: Total Heterotrophic Bacteria count (THBC) in days (10^6 CFU/ml)

Total Heterotrophic Fungal Count

Fortification of the fresh juices with spice extracts brought about a reduction in the fungal load of the juices over time in comparison to the unfortified control as shown in Table 5.

Table 5: Total heterotrophic fungi count (THFC)

Day	Bacteria population (CFU/ml) in stored juice					
	Pineapple	Pineapple + Uziza	Orange	Orange + Uda	Watermelon	Watermelon + oshorisho
Day 1	5.1×10^5	-	1.8×10^4	-	1.0×10^3	-
Day 3	1.6×10^6	1.9×10^6	6.4×10^5	3.0×10^5	8.7×10^5	2.5×10^5
Day 5	7.1×10^6	1.8×10^7	5.5×10^6	5.3×10^5	5.9×10^6	6.8×10^5
Day 7	2.7×10^7	1.2×10^7	2.3×10^7	1.9×10^6	2.1×10^7	1.2×10^6

Extracts of uda, uziza and oshorisho completely inhibited fungal growth on orange, watermelon and pineapple juices respectively after 24 hours of storage. Fresh orange juice preserved with uda and stored for 7 days showed a fungal load of 1.9×10^6 as against the unpreserved sample of 2.3×10^7. Pineapple juice preserved with uziza showed a fungal load of 1.2×10^7 after 7 days storage as against the unpreserved sample of 2.7×10^7. Also for watermelon juice preserved with oshorisho, the fungal load after 7 days of storage was 1.2×10^6 against the unpreserved sample of of 2.1×10^7. This resulting reduction in the fungal load of the preserved juices (pineapple, orange and watermelon) by uziza, uda and oshorisho extracts corresponds to the report of Tserennadmid et al. (2011) who studied the anti-yeast activity of some essential oils (clary sage, juniper, lemon and marjoram). Figure 2. below represents a line chart of microbial load with time [41].

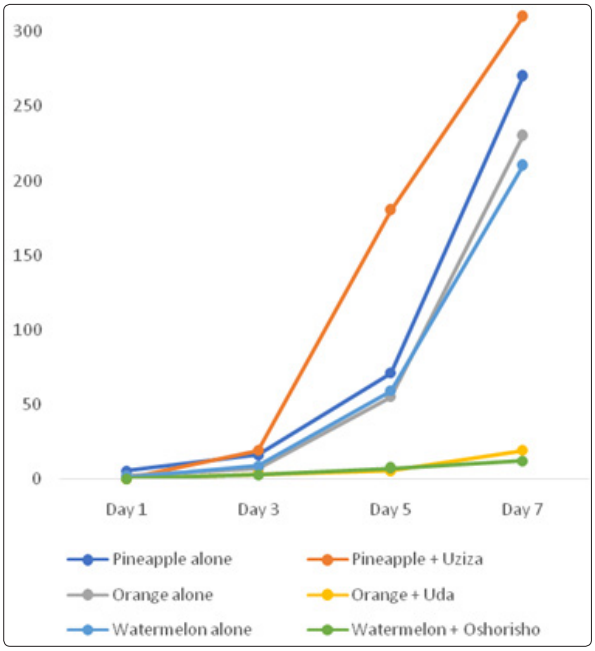

Figure 2: Total Heterotrophic Fungi count (THFC) in days (10^6 CFU/ml)

Conclusion

This work demonstrated the antimicrobial activity of crude extracts of *P. guineense* (Uziza), Uda (*Xylopia aethiopica*) and *T. tetraptera* (oshorisho) against the test organism (Bacillus sp). The MIC values obtained for the spice extracts showed potent inhibition of the test organism by all the extracts with the lowest MIC value recorded by the uziza and uda extracts (25mg/ml), outperforming the positive control chloramphenicol which had an MIC value of (100mg/ml). Furthermore, MBC assay shows that the spice extracts is bactericidal at a concentration of 100mg/ml. The extracts of uziza, uda and oshorisho brought about a reduction in the bacterial and fungi load of pineapple, orange and watermelon juices preserved with these spices respectively in comparison to unpreserved controls over a period of 7 days. This shows the great potential of these spices to be utilized in fruit juice preservation for shelf life elongation.

Recommendation

It is recommended that further research should be carried out to isolate, purify and identify the active ingredients responsible for the antimicrobial properties of these spices. Also, the organoleptic impact/attributes of the spices in fruit juices should be examined.

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