

Full Length Research Paper

Antimicrobial activity of *Xylopi*a *aethi*o*pica*, *Aframomum melegueta* and *Piper guineense* ethanolic extracts and the potential of using *Xylopi*a *aethi*o*pica* to preserve fresh orange juice

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Accepted 28 March, 2013

Antimicrobial activity of ethanolic extracts of *Xylopi*a *aethi*o*pica*, *Aframomum melegueta* and *Piper guineense* fruits were assayed against fourteen (14) microorganisms commonly associated with food poisoning and/or food spoilage. The microorganisms were *Bacillus subtilis* IAM1069, *Bacillus cereus* IFO 13494, *Staphylococcus aureus* FDA 209p, *Escherichia coli* NRIC 1023, *Salmonella typhimurium* IFO12529, *Lactobacillus plantarum* IAM 1041, *Pediococcus acidilactici*-M, *Leuconostoc mesenteroides*-M, *Lactobacillus casei* TISTR390, *Saccharomyces cerevisiae* OC-2, *Hansenula anomala* IFO 0140 (p), *Pichia memb.*IFO 0128, *Penicillium funiculosum* NBRC 6345 and *Candida* species. All the plant extracts exhibited selective antimicrobial activities on the test organisms. *X. aethi*o*pica* extract exhibited the highest antimicrobial activity on the organisms with a minimum inhibitory concentration (MIC) of 50 ppm on *Bacillus* species and *S. aureus*. *S. cerevisiae* (MIC = 300 ppm), *P. funiculosum* NBRC 6345 and *L. mesenteroides* (MIC = 500 ppm) were also susceptible to *X. aethi*o*pica* fruit extract but the MIC values for the other tested microorganisms were higher than 1000 ppm. This was followed by *A. melegueta* fruit extract with MIC of 100 ppm for *B. cereus* and *S. aureus*. Although *P. guineense* fruit extract inhibited the growth of *B. cereus* and *S. aureus* (MIC = 300 ppm); and *B. subtilis* (MIC = 1000), the MIC for the other microorganisms were higher than 5000 ppm. On the whole, all the plant extracts exhibited the least antimicrobial activities on *Lactobacilli* and fungi species. *X. aethi*o*pica* fruit extract was used to preserve fresh orange juice. The ability of 100 and 1000 ppm extract to preserve the orange juice was significantly greater ($p < 0.05$) than 50 ppm. The microbial concentration in orange juice containing 100 ppm of *X. aethi*o*pica* extract was 4 cfu/mL after 28 days of storage at room temperature.

Key words: Food spoilage, food poisoning, microorganisms, spices, ethanolic extract, natural preservatives, orange juice.

INTRODUCTION

Food poisoning and food borne infections are very common all over the world, especially in tropical countries with elevated temperature and humidity that favour micro-

bial growth (Adebajo, 1993). In most developing countries, food preservation is done mostly by the conventional methods such as drying and salting since refrigera-

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tion and freezing facilities are expensive, electricity supply is very unstable and some remote areas do not have electricity supply at all. Although, there are many chemical food preservatives used by man, most of them have adverse side effects on human health. The use of plant materials traditionally used as food spices, condiments and or as medicine would be more beneficial to human health than the use of synthetic chemical food preservatives. In many tropical countries, these medicinal plants and spices are abundant and easily accessible. In Nigeria, for example, it has been estimated that over 40% of known plants serve as food whereas about 30% serve as spices and medicinal plants (Nwobegu, 2002). The spices are used to give aroma and flavour to food and at the same time they can serve as food preservatives because they possess active ingredients which are either microbistatic or microbicidal (Adegoke and Sagua, 1993; Okeke et al., 2001; Okigbo et al., 2005; White, 2006; Okigbo and Igwe, 2007). Among these medicinal plants are *Xylopi aethiopica* (Negro pepper), *Aframomum melegueta* (Aligator pepper) and *Piper guineense*.

X. aethiopica is a medicinal plant of great repute in West Africa and contains a variety of complex chemical compounds (Adegoke et al., 2003). These active ingredients are extracted in various forms such as crude aqueous or organic extracts or in the form of essential oils. The medical importance of *X. aethiopica* has been extensively reported (Fleischer, 2003; Okigbo et al., 2005; Adewoyin et al., 2006; White, 2006; Okigbo and Igwe, 2007). The fresh and dried fruits, leaf, stem bark and root bark essential oils were reported to have various degrees of activity against some Gram positive and negative bacteria (Fleischer, 2008). *X. aethiopica* (Annonaceae) is widely distributed in the West African rainforest from Senegal to Sudan in Eastern Africa, and down to Angola in Southern Africa (Irvine, 1961; Burkhill, 1985). Almost every part of the plant is used in traditional medicine for managing various ailments including skin infections, candidiasis, dyspepsia, cough and fever (Irvine, 1961; Burkhill, 1985; Mishana et al., 2000).

A. melegueta (Aligator pepper) is another plant popularly used as a food spice and as a traditional medicine for treating various ailments in Nigeria and other parts of the world. There are a lot of scientific work on the activities of *A. melegueta* such as antinociceptive (Oloke, 1992; Okigbo and Ogbonnaya, 2006; Umukoro and Ashorobi, 2007), antifungal activity (Ejehi et al., 1997; Adejumo and Langenkämper, 2011), for controlling insect pests (Ewete et al., 1996; Ejehi et al., 1997; Oparaeke et al., 2005; Ukeha et al., 2009) and as a flavouring agent in food (Ajaiyeoba and Ekundayo, 1999).

P. guineense commonly referred to as African black pepper or Ashanti pepper belongs to the family Piperaceae. It is known with different vernacular names in Nigeria which include 'Uziza' in Igbo, and 'lyere' in Yoruba. *P. guineense* has culinary, medicinal, cosmetic and insecticidal uses (Dalziel, 1955; Okwute, 1992). The leaves are

considered aperitive, carminative and eupeptic. They are also used for the treatment of cough and bronchitis, (Martins et al., 1998) intestinal diseases and rheumatism (Sumathykutty et al., 1999; Saganuwana, 2009; Ogbole et al., 2010). The seeds and leaves are used as spices in various African dishes. It has also been used as an insect repellent (Adewoyin et al., 2006). The plant is utilized in different forms, such as whole herbs, powders, extracts and vapours, for a variety of purposes (Martins et al., 1998).

The microorganisms that are mostly involved in food poisoning and spoilage are mainly bacteria and fungi. The Gram negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*, as well as Gram positive bacteria such as *Staphylococcus aureus* are associated with food poisoning and food borne infections. *Bacillus subtilis* and *Bacillus cereus* are known to produce exoenzymes that hydrolyze food materials causing food spoilage and they are also involved in certain types of food poisoning (Pelczar et al., 1993). The lactobacilli are fermentative group of bacteria that are known to be associated with most food materials including dairy products. Although, their activities are required in some cases as probiotics, they need to be controlled in some food materials where they may cause food spoilage by fermenting food materials to lactic acid and other metabolites which may not be desirable in some food products. Another group of microorganisms that are involved in food spoilage are the fungi. Both filamentous and unicellular fungi have been reported to cause food spoilage and/or food poisoning (Pitt and Hocking, 2009).

Majority of the work on African spices have concentrated on their medicinal values with the aim of using them for disease treatment and insect control. There are very few reports on the use of the extract of these plants as natural food preservatives. The aim of this study was to determine the antimicrobial activities of ethanolic extracts of the fruits of *X. aethiopica*, *A. melegueta* and *P. guineense* on fourteen (14) food spoilage and pathogenic microorganisms with the aim of using them as natural food preservatives.

MATERIALS AND METHODS

Procurement of plant materials

X. aethiopica (Dunal) A. Rich (Negro pepper), *A. melegueta* (Roscoe) K. Schum (Aligator pepper) and *P. guineense* Schumacher and Thonn (African black pepper) fruits were bought from Ogbete main market in Enugu, Enugu state, Nigeria. They were authenticated in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka by Prof. M. Nwosu.

Preparation of plant extracts

The fruits were dried at ambient temperature for five days and milled into powder. Then, 5 to 20 g of each sample was put into a 300 ml Erlenmeyer flask and ten times volume of 50% ethanol (50

Table 1. Percentage ethanolic extract yields of various spices.

Spice	Amount used (g)	Amount of extract (g)	Yield (%)
<i>Aframomum melegueta</i>	20	1.5	7.5
<i>Xylopiya aethiopica</i>	5	1.0	20
<i>Piper guineense</i>	20	2.7	13.5

to 200 mL) was added into each flask. The flasks were kept at room temperature with intermittent shaking for three days. Each sample was filtered through No.5C Whatman filter paper. The filtrate was concentrated to about 1/3 the original volume using a rotary evaporator at 40°C. The filtrate was transferred into Petri dishes and dried completely using a drier at 40°C. The amount of extract obtained from each spice was expressed as a percentage of the quantity of powder used for the extraction (Table 1).

Reconstitution of the extract

Each plant extract was reconstituted by dissolving an appropriate quantity in 50% ethanol to give a final concentration of 10% weight per volume. The reconstituted extracts were filter sterilized using 0.45 µm membrane filter. The sterilized extracts were then transferred into Petri dishes and various volumes of sterile agar medium were poured onto the extract to give a final concentration of 50 to 5000 ppm.

Preparation of trypton agar medium

The medium consisted of trypton (1.7 g); peptone (0.3 g); glucose (0.28 g); KH₂PO₄ (0.25 g); NaCl, (0.5 g) and agar powder (1.5 g). All were dissolved in 100 ml of distilled water and the pH was adjusted to 6.0 using tartaric acid. They were then autoclaved at 121°C for 15 min.

Test microorganisms

Pathogenic bacteria, lactic acid bacteria and fungi that are usually associated with food spoilage and/or food poisoning were used. The pathogenic bacteria included *B. subtilis* IAM1069, *B. cereus* IFO 13494, *S. aureus* FDA 209p *E. coli* NRIC 1023 and *S. typhimurium* IFO12529. The lactic acid bacteria were *Lactobacillus plantarum* IAM 1041, *Pediococcus acidilactici*-M, *Leuconostoc mesenteroides*-M, and *Lactobacillus casei* TISTR390, while the fungi species were *Saccharomyces cerevisiae* OC-2, *Hansenula anomala* IFO 0140 (p), *Pichia memb.* IFO 0128, *Penicillium funiculosum* NBRC 6345 and *Candida* species. All the strains were obtained from culture stock of Asama Chemicals Co. Ltd, Tokyo, Japan.

Determination of minimum inhibitory concentration (MIC)

Each microorganism was pre-cultured and diluted with medium to give a concentration of about 10⁶ CFU/ml. 10 µL of the diluted cell culture was inoculated into the medium using micro-planter, incubated at 30°C for 72 h and the presence or absence of colonies was recorded. The minimum extract concentration that inhibited the growth of the microorganism was taken to be the minimum inhibitory concentration. The results were confirmed by repeating the experiments two more times.

Preservation of orange juice with ethanolic extract of *X. aethiopica* fruits

Moderately ripped orange fruits were bought from new market in

Enugu, Enugu State, Nigeria. The orange fruits were carefully and thoroughly washed with tap water and aseptically peeled. The juice was squeezed out using an electric juice extractor and filtered through a sterile muslin cloth. The filtered juice was dispensed in 100 mL aliquots into four 250 mL conical flasks. Then, the ethanolic extract of *X. aethiopica* was added into each flask at the concentrations of 0 (control), 50, 100 or 1000 ppm. Each flask was swirled to mix and the content dispensed in 25 mL aliquots into 30 mL bottles and capped. Two sets of four bottles were prepared from each level of the extract concentration. A set was pasteurized at 60°C for 20 min while another set was not pasteurized. All were stored at room temperature (28 ± 2°C) and samples were taken weekly to determine microbial growth by the pour plate method using agar medium composed of sodium chloride (1%), polypeptone (1%), yeast extract (1%) and bacteria agar powder (2%). The whole experiments were done three times and the results are the average of the triplicates.

RESULTS

Extract yields from the spice powders

The percentage extract yields of the three different spices are summarized in Table 1. The yield varied among the spices with *X. aethiopica* giving the highest extract yield of 20%, followed by *P. guineense* with 13.5%, while *A. melegueta* gave the lowest yield of 7.5%.

Antimicrobial activities of the spice extracts on pathogenic bacteria

The effects of the three spice extracts on five test pathogenic bacteria are summarized in Figure 1. All the extracts exhibited selective antimicrobial activity on all the organisms. The three extracts showed higher inhibitory effect on the growth of *B. subtilis*, *B. cereus* and *S. aureus* with the minimum inhibitory concentration ranging from 50 to 1000 ppm. On the other hand, *E. coli* and *S. typhimurium* were less sensitive to the extracts and their MIC values were all above 5000 ppm. *X. aethiopica* extract showed the highest growth inhibitory activity on the test organisms, with MIC of 50 ppm for *B. subtilis*, *B. cereus* and *S. aureus*. *A. melegueta* also had a high growth inhibitory effect on the test pathogenic bacteria with a minimum inhibitory concentration of 100 ppm for *B. cereus* and *S. aureus* and 300 ppm for *B. subtilis*. Out of the three spices, *P. guineense* exhibited the least growth inhibitory effect on the pathogenic bacteria tested with minimum inhibitory concentrations that ranged from 300 to 1000 ppm on *B. subtilis*, *B. cereus* and *S. aureus*.

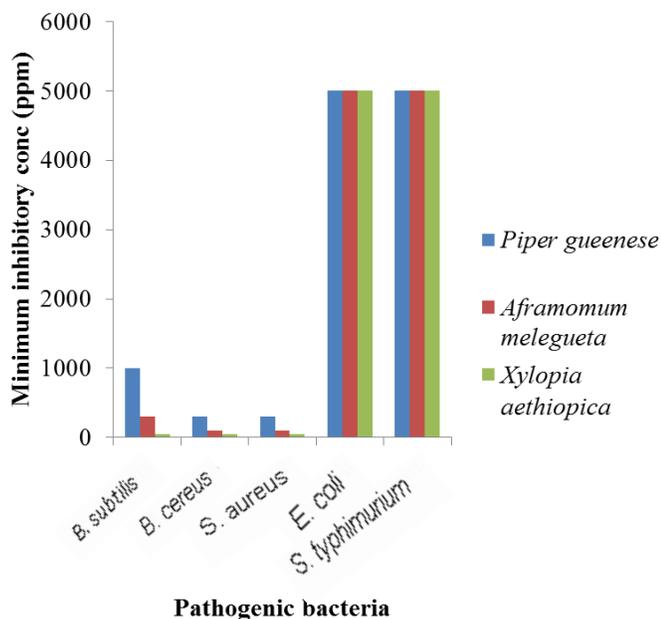


Figure 1. Inhibitory effects of spice fruit extracts on some pathogenic bacteria.

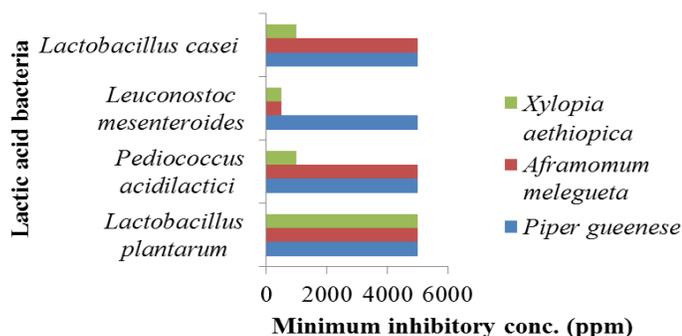


Figure 2. Effects of some spice fruit extracts on the growth of some lactic acid bacteria.

Antimicrobial activities of the spice extracts on lactic acid bacteria

The effects of the spice extracts on the growth of some lactic acid bacteria are shown in Figure 2. *P. acidilactici*-M, *L. mesenteroides*-M and *L. casei* were very sensitive to *X. aethiopica* extracts with a minimum inhibitory concentration of 500 to 1000 ppm. *L. mesenteroides*-M was the most sensitive of the lactic acid bacteria tested with an MIC of 500 ppm for *X. aethiopica* and *A. melegueta*. On the other hand, *L. plantarum*, *P. acidilactici* and *L. casei* were less sensitive with MIC values higher than 5000 ppm for *A. melegueta* and *P. guineense* extracts.

Effects of the spice extracts on the growth of fungi

The effects of the spice extracts on the growth of fungi

are shown in Figure 3. *S. cerevisiae* and *P. funiculosum* were the most sensitive to *X. aethiopica* extract with MIC values of 300 and 500 ppm, respectively. *P. membranae* was sensitive only to *A. melegueta* but relatively resistant to *X. aethiopica* and *P. guineense*. *H. anomala* and *Candida* species were the least sensitive to all the extracts.

Potential of using *X. aethiopica* fruit extract to preserve orange juice

Since *X. aethiopica* was the most active of the three spices against all the groups of microorganisms tested, its potential application as food preservative was evaluated, using orange juice as an example. Microbial growth in pasteurized orange juice containing varying concentrations of *X. aethiopica* extract is shown in Table 2. The three different extract concentrations inhibited the growth of microorganisms in the orange juice but the preservative effect of the extract was concentration dependent. However, the preservative abilities of 100 and 1000 ppm (the microbial concentrations in the juice) were not statistically different ($p > 0.5$). The use of 100 ppm for the preservation of orange juice is therefore recommended. In the case of unpasteurized orange juice, microbial growth commenced almost immediately irrespective of the extract concentration, reaching above 10^3 cells/mL within 24 h.

DISCUSSION

The results of this work have shown that all the three spices tested inhibited the growth of all the 14 microorganisms associated with food poisoning and/or food spoilage, though the sensitivities of the microorganisms varied. Antimicrobial activities of these spices, especially *X. aethiopica* on some microorganisms have also been reported (Fleischer, 2008). However, most of the works on them have focused on their medicinal values. The present work seems to be the first comparative study on their antimicrobial activities against a wide range of microorganisms. On the whole, Gram negative bacteria were more resistant to the spice extracts than the Gram positive bacteria. This result was in agreement with that of Agatemor (2009) and Nwinyi et al. (2009) who reported that Gram negative bacteria are more resistant to antibacterial agents than the Gram positive species. This may be due to the differences in the cell wall composition and structure, especially the polysaccharide and protein outer membrane in the cell wall of the Gram negative bacteria which limit diffusion of antimicrobial agents into the cell. The antimicrobial activities of these spices can be attributed to the contents of active ingredients such as mono- and sesquiterpene hydrocarbons in *X. aethiopica* (Karioti et al., 2004), β -pinene, β -caryophyllene, β -elemene, cyclogermacrene and α -humulene in *P. guineense* (Parmar et al., 1997; Martins et al., 1998).

This work has demonstrated that *X. aethiopica* extract can be used to preserve orange juice. This is the first

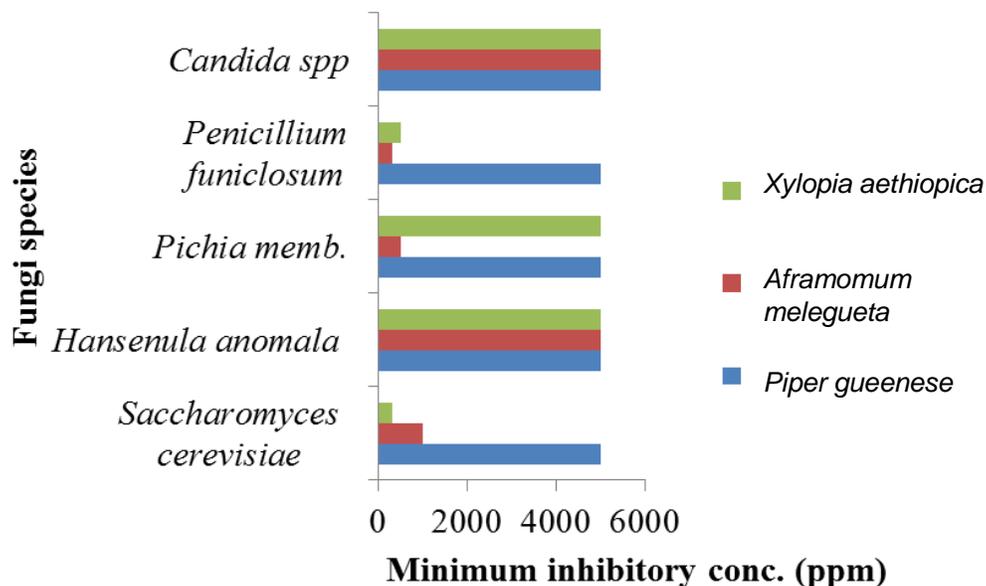


Figure 3. Inhibitory effects of some spice fruit extracts on some fungi species.

Table 2. Effect of addition of *X. aethiopica* fruit extract on microbial growth in orange juice during storage at room temperature.

Extract concentration (ppm)	Incubation period (days)	Cell concentration (cfu/ mL)
0	0	-
	7	330
	14	810
	21	6×10^4
	28	2.5×10^6
50	0	-
	7	10 ± 2.4
	14	8 ± 1.92
	21	7 ± 1.68
	28	10 ± 2.00
100	0	-
	7	4 ± 1.3
	14	3 ± 1.0
	21	2 ± 0.8
	28	3 ± 1.2
1000	0	-
	7	2 ± 0.8
	14	1 ± 0.5
	21	2 ± 0.35
	28	2 ± 0.45

Various concentrations of *X. aethiopica* ethanolic extract were added to freshly squeezed orange juice in 30 mL bottles. They were pasteurized at 60°C for 20 min and stored at room temperature ($28 \pm 2^\circ\text{C}$). Samples were taken weekly to determine microbial growth by the pour plate method.

report on the use of *X. aethiopica* fruit extract to preserve orange juice. Even after about one month of storage, the microbial counts in the juice containing 100 ppm was within the range regarded as safe by the FAO. The shelf life of the orange juice can still be extended by combining the spice extract with other safe treatments such as carbonation and high pressure bottling. What can be considered a limitation to the use of *X. aethiopica* extract to preserve food is that it has a strong aroma which may be objectionable for some people in certain food products. Nevertheless, some people like the aroma, hence its wide use as a spice. It is necessary to investigate whether the antimicrobial compounds in the fruits are responsible for the strong aroma. If not, development of a method for extracting the antimicrobial agents that are free of the aroma, or a method of removing or masking the strong aroma can broaden the scope of its applications as a food preservative.

A major advantage of using *X. aethiopica* as a food preservative is that foods preserved by this spice may qualify as a functional food since it has many health benefits such as anti-tumour, anti-asthmatic, anti-inflammatory, antimicrobial (Okigbo et al., 2005; White, 2006; Okigbo and Igwe, 2007), hypotensive and coronary vasodilatory effects (Fleischer, 2003). On the whole, these natural preservatives, especially spices that have very long history of use in many parts of the world, are preferred to chemical preservatives which often have undesirable side effects on the consumers.

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