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Phytochemical and Antimicrobial Screening of Ethanol Extracts of *Zingiber officinale*, *Allium sativum* and *Syzygium aromaticum* against Some Food Associated Bacteria and Fungi

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Abstract
The *in vitro* antimicrobial activities of ethanol extracts of ginger (*Zingiber officinale*), garlic (*Allium sativum*) and clove (*Syzygium aromaticum*) were evaluated against some bacteria (*Staphylococcus aureus* and *Escherichia coli*) and moulds (*Aspergillus niger* and *Rhizopus stolonifer*) isolated from food samples by agar well diffusion method and poisoned food technique respectively. The phytochemical screening revealed that the three extracts contained carbohydrates, flavonoids and triterpenes in addition to other bioactive components such as glycosides, saponins, steroids, tannins and alkaloids which varied among the extracts, however, anthraquinones are absent in all the extracts. The extracts showed inhibitory effects against the test organisms with zones of inhibition ranging from 10mm to 23mm at concentration range of 62.5 to 500mg/ml. The minimum inhibitory concentration (MIC) value of clove was 3.90mg/ml, ginger 62.5mg/ml and garlic 125mg/ml on both bacteria. The minimum bactericidal concentration (MBC) values were recorded for cloves at 15.62mg/ml while garlic and ginger had no MBC values. Percentage mycelial growth inhibitions ranging from 2.5% to 100% were recorded against the test fungi. Based on these findings, it may be suggested that these plant materials may be used as natural antimicrobial additives to improve the quality and shelf-life of foods.
Keywords: phytochemical screening, antimicrobial activities, *Zingiber officinale, Allium sativum, Syzygium aromaticum,* food, bacteria, moulds.

INTRODUCTION

Spices can be defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste and quality of foods. Spices include leaves (coriander, mint), buds (clove), bulbs (garlic, onion), fruits (red chilli, black pepper), stem (cinnamon), rhizomes (ginger) and other plant parts (Arora and Kaur, 1999). Many plant derived products such as spices, fruit preparations, vegetable preparations or extracts have been used for centuries for the preservation and extension of the shelf life of foods (Chattopadhay and Bhattacharyya, 2007). *Zingiber officinale* has been found to posses the ability to neutralize the excess gastric acid produced by the bacterium *Helicobacter pylori* which leads to formation of ulcers (Arora and Kaur, 1999). It has been shown that ginger extract and its pungent compounds demonstrated greater antibacterial activity against a variety of bacteria including *H. pylori., E. coli, P. aeruginosa* and *S. aureus* although mixed results is attributed to different ginger preparations and varying strengths (Arora and Kaur, 1999).

Both *in vitro* and *in vivo* studies have demonstrated that fresh garlic has antimicrobial activities including antibacterial, antiviral, antifungal and antiprotozoan activities. Particular activity against *B. subtilis, E. coli, P. mirabilis, S. typhi,* methicillin-resistant *S .aureus, S. enteritidis* and *V. cholera* have been noted. It is reported that garlic is more effective against pathogenic yeast than Nystatin especially *C. albicans* (Arora and Kaur, 1999).

The presence of cloves and cinnamon when added to bakery items function as mould inhibitors in addition to adding flavour and aroma to baked products. They are also effective against Streptococci, Staphylococci and Pneumococci bacteria. The essence of clove oil is included in toothpastes and its antiseptic power is three times superior to that of phenol.

MATERIALS AND METHODS

Plant samples

The plant samples were purchased from Sabon Gari market in Zaria, Kaduna state and were identified in the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The voucher specimens (numbers; *Zingiber officinale* - 2261, *Allium sativum* - 2196 and *Syzygium aromaticum* – 2199) have been deposited.

Test organisms

Test organisms (*Escherichia coli, Staphylococcus aureus, Aspegillus niger* and *Rhizopus stolonifer*) used were isolated from various food samples; breads, cakes, pastries, buns and meat collected from the local market of Samaru, Zaria. Briefly, 1 gram of each food sample was added to 9ml of 0.1% peptone water to make homogenate of food sample. Serial dilution with 0.1% peptone water was done to the 10^{-3} dilution and 0.1ml was plated out on selective media; mannitol salt agar for *S. aureus*, eosin methylene blue agar for *E. coli* and potato dextrose agar for *A. niger* and *R. stolonifer*. The plates for bacteria were incubated at 37^0C for 24 hours, while those for fungi were incubated at room temperature for 5-7 days (Samson *et. al.*, 2004).

Typical bacterial colonies were characterized using standard microbiological procedure as described by McFadden, (2000) while fungal isolates were identified using an Atlas for fungal morphology and growth on culture media and under the microscope using wet mount technique (Samson *et. al.*,2004). The isolates were stored in refrigerator on slants for further use.

Preparation of Extracts

Dried ginger roots and clove were thoroughly washed with distilled water to remove dirt, and then air-dried to a constant weight for 21 days.

The dried ginger and clove were pulverized to fine powder using a clean mortar and pestle and 200 grams of each powdered plant was extracted with 500 ml of 99% ethanol using a separating funnel plugged with cotton wool around the tip of funnel leading to the tap (Percolation method). The tap was opened and allowed to drain solution into a clean conical flask after standing undisturbed (Trease and Evans, 1996). The garlic was also washed thoroughly with distilled water and the outer covering was peeled off. The garlic was then blended into a paste and 200 grams of the paste was extracted with 500ml of 99% ethanol using the same procedure as described for ginger and clove. The various filtrates were placed in evaporating dishes and heated to dryness over a water bath. The extracts were transferred into bottles and kept in desiccator for future use. Percentage yield of the extracts were 11.35%, 17.41% and 29.5% for ginger, clove and garlic respectively.

Phytochemical Screening

Test for Carbohydrates

Few drops of Molisch's reagent were added to a little quantity of extract in a test tube and a small quantity of concentrated sulphuric acid was allowed to run down the side of the test tube to form a purple to violet colour at the interface indicates the presence of carbohydrates (Trease and Evans; 1983).

Test for Glycosides (Kella-Killiani Test)

Extract was dissolved in glacial acetic acid containing traces of ferric chloride. The test tube was held at an angle and 45.1ml of concentrated sulphuric acid was added down the slide. Purple ring colour at the interface indicates cardiac glycosides (Trease and Evans, 1983).

Test for Anthraquinones (Borntrager's Test)

A small portion of the extract was shaken with 10ml of benzene and filtered 5ml of 10% ammonia solution was added to the filtrate and stirred. The production of a pink – red or violet colour indicates the presence of free anthraquinones (Trease and Evans, 1983).

Test for Saponins (Frothing Test)

A small quantity of the extract was dissolved in 10ml of distilled water. This was then shaken vigorously for 30 seconds and was allowed to stand for 30 minutes. A honey comb for more than 30 minutes indicates saponins.

Test for steroids/Triterpenes (Lieberman-Burchard's Test)

Equal volume of acetic anhydride was added to the extract 1ml of concentrated sulphuric acid was added down side of the tube. The colour change was observed immediately and later. Red, pink or purple colour indicates the presence of triterpenes, while blue or blue-green indicates steroids (Trease and Evans, 1983).

Test for Flavonoids (NaOH Test)

Few drops of NaOH were added to 5ml of extract, a yellow colouration shows the presence of flavonoids (Trease and Evans, 1983).

Test for Tannins (Ferric Chloride Test)

About 0.5ml of extract was dissolved in 10ml of distilled water, then filtered few drops of ferric chloride solution was added to the filtrate formation of a blue-black precipitate indicates hydrolysable tannins and green precipitate indicates the presence of condensed tannins (Trease and Evan 1983).

Test for Alkaloids (Tannic acid test)

Few drops of tannic acid were added to a sample of the extract. Black precipitate indicates the presence of alkaloids (Sofowora 1982).

Bioassay

Agar well diffusion method was used to determine the antibacterial activity. Briefly, the test organisms from growth on nutrient agar incubated at 37°C for 18hr were suspended in saline solution (0.85% NaCl) and adjusted to match a turbidity of 0.5 (10^8 cells/ml) McFarland standard. The standardized suspension was used to inoculate the surfaces of Mueller Hinton agar plates (90mm in diameter) using sterile cotton swab. Six millimeter diameter wells were punched using cork borer in agar and filled with the desired concentrations (62.5mg/ml, 125mg/ml, 250mg/ml and 500mg/ml) of the extracts (Aliyu *et al.,* 2009). About 0.1ml of distilled water and equal volume of chemical preservative, acetic acid was transferred into the fifth and sixth wells to serve as negative and positive controls respectively (Rios *et. al.,* 1988). The plates were allowed to stand for 5 hours at room temperature for extract to diffuse into the agar and then incubated at 37°C over night. Antibacterial activities were evaluated by measuring inhibition zone diameters. The entire test was conducted in duplicate.

Antifungal Assay

The antifungal activity of the plant extracts was determined using poisoned food technique. The fungal isolates were inoculated on PDA plates and incubated at 25°C for 7 days to obtain actively growing colonies of moulds. Aliquots of each of the plant extracts were mixed with 15ml of cooled (45°C) molten PDA medium and allowed to solidify at room temperature for thirty minutes to attain the desired concentrations (62.5mg/ml, 125mg/ml, 250mg/ml and 500mg/ml). Using 6mm diameter cork borer, a mycelial disc was cut out from the periphery of 7 days old cultures and aseptically inoculated onto agar plates containing the plant extracts. PDA plates with 0.1ml acetic acid were used as negative control (Georgii and Korting, 1991; McCutcheon *et. al.,* 1994). The inoculated plates were incubated at room temperature and colony diameter was measured and recorded after 7 days. Percent mycelial growth inhibition was calculated using the formula:

$$\% \text{ Mycelial Growth inhibition} = \frac{\text{Mean diameter of Inhibition of colony in control - colony in extract}}{\text{Mean diameter of Colony in control}} \times 100\%$$

Determination of MIC

The minimum inhibitory concentration (MIC) was determined according to the Clinical and Laboratory Standards Institute (CLSI, 2006). Each extract was separately dissolved in sterile distilled water and 2ml of sterile Mueller Hinton broth was transferred into a set 5 of tubes and 2ml of each concentration (62.5mg/ml, 125mg/ml, 250mg/ml and 500mg/ml) of the extracts was added to obtain final concentrations of 31.25mg/ml, 62.5mg/ml, 125mg/ml, and 250mg/ml respectively. Each test organism was inoculated into the labeled tube except the control; the tubes were incubated at 37°C for 18 hours. The MIC was taken as the lowest concentration that prevented visible growth.

Determination of MBC

The minimum bactericidal concentration was determined according to the Clinical and Laboratory Standards Institute (2006). From the test tubes used in the determination of MIC, the tubes that showed no visible growth were sub cultured onto freshly prepared Mueller Hinton agar and incubated at 37°C for 48. The least concentration at which the organisms did not recover and grow was taken as the minimum bactericidal concentration (MBC).

Results

The ginger extract obtained was a brown-black, oily with spicy-sweet smelling, that of garlic was a golden-yellow, gummy residue with pungent offensive smell while the clove extract was a black, oily residue with strong aromatic smell.

Result of the preliminary phytochemical screening of ethanolic extracts of ginger, garlic and clove is presented in Table 1. Carbohydrates, triterpenes, flavonoids, tannins, glycosides, saponins and alkaloids were present in ginger extract and steroids were absent, carbohydrates, triterpenes, flavonoids, saponins, alkaloids and steroids were found in garlic extract while tannins and glycosides were absent. The ethanolic extract of clove showed the presence of carbohydrates, triterpenes, flavonoids, tannins and glycosides and showed absence of saponins, alkaloids and steroids. However, anthraquinones were absent in all the three extracts.

Table 1: Phytochemical Constituents of ethanol extracts of ginger, garlic and clove

Constituents	Ginger	Garlic	Clove
Carbohydrates	+	+	+
Glycosides	+	-	+
Anthraquinones	-	-	-
Saponins	+	+	-
Steroids	-	+	-
Triterpenes	+	+	+
Flavonoids	+	+	+
Tannins	+	-	+
Alkaloid	+	+	-

Key: + = present, - = absent

Antibacterial assay of ethanol extract of ginger revealed that the extract possessed antibacterial activity against the test organisms. The largest zone of inhibition was observed against *S. aureus* (11mm) followed by *E. coli* (10mm) at 500mg/ml. The observed antibacterial activities of the extract are presented in Table 2.

Table 2: Diameter of zones of inhibition (mm) by ginger extract against the bacteria

Test organisms	Zones of inhibition (mm)/concentration (mg/ml)				Control	
	500	250	125	62.5	Positive	negative
Escherichia coli	10	8	8	8	25	8
Staphylococcus aureus	11	10	8	8	27	8

Table 3 shows the zones of inhibition by garlic extract on test bacteria. Zones of inhibition were only observed at 500mg/ml (10mm and10mm) for both test bacteria, and no inhibition zones were recorded at 250mg/ml, 125mg/ml and 62.5mg/ml on both test bacteria.

Table 3: Diameter of zones of inhibition (mm) by garlic extract against the bacteria

Test organisms	Zones of inhibition (mm)/concentration (mg/ml)				Control	
	500	250	125	62.5	Positive	negative
Escherichia coli	10	8	8	8	36	8
Staphylococcus aureus	10	8	8	8	26	8

The results of the antibacterial activity of clove extract are presented in Table 4. The bacteria were inhibited by the extract at various concentrations with zones of inhibition ranging from 10mm to 23mm.

Table 4: Diameter of zones of inhibition (mm) by clove extract against the bacteria

Test organisms	Zones of inhibition (mm)/concentration (mg/ml)				Control	
	500	250	125	62.5	Positive	Negative
Escherichia coli	23	16	15	10	30	8
Staphylococcus aureus	20	16	14	8	28	8

Results of the MIC and MBC are presented in Table 5. The MICs of the ginger and garlic extracts against *S. aureus* and *E. coli* were 62.5mg/ml and 250mg/ml respectively while they had MIC values of 3.9mg/ml and 7.8mg/ml for the clove extracts respectively. Higher MBC of 15.62mg/ml was observed against both *E. coli* and *S. aureus* only for the clove extract.

Table 5: MIC and MBC ($mgml^{-1}$) of Ginger, Garlic and Clove extracts against the test bacteria

Test organism	MIC($mgml^{-1}$)			MBC($mgml^{-1}$)		
	Ginger	Garlic	Clove	Ginger	Garlic	Clove
Escherichia coli	62.5	250	7.8	-	-	15.62
Staphylococcus aureus	62.5	250	3.9	-	-	15.62

Table 6 shows the antifungal activity of the extracts as indicated by the percentage mycelial growth inhibition on *Rhizopus stolonifer*. The garlic extract showed the least percentage inhibition (2.5%) followed by ginger (11.5%), while clove had the highest percentage inhibition of 100% at 500mg/ml.

Table 6: Antifungal activity of the extracts on *Rhizopus stolonifer*

Extract	% mycelial growth inhibition (%)/concentration (mg/ml)			
	500	250	125	62.5
Ginger	11.5	0.0	0.0	0.0
Garlic	2.5	0.0	0.0	0.0
Clove	100	77 .0	23.0	0.0

The antifungal activity of the extracts as indicated by the percentage mycelial growth inhibition against *Aspergillus niger* is presented in table 7. Percentage mycelia growth inhibitions ranging from 15% to 100% were seen at concentration range of 250mg/ml to 500mg/ml.

Table 7: Antifungal activity of the extracts on *Aspergillus niger*

Extract	% mycelial growth inhibition (%)/concentration (mg/ml)			
	500	250	125	62.5
Ginger	17.0	10.0	0.0	0.0
Garlic	28.0	15.0	0.0	0.0
Clove	100	100	55.0	20.0

DISCUSSION

Phytochemical screening of the ethanol extracts of ginger, garlic and clove showed the presence of tannins, flavonoids, triterpenes, glycosides, alkaloids, steroids, saponins, tannins and carbohydrates while anthraquinones are absent in all the three extracts.

The clove extract showed the highest antimicrobial activity against the bacteria and the fungi. The activity of this plant extract may be due to the presence of high tannin content (10-19%) which is a complex phenolic compound that can bind to proteins and carbohydrates resulting in reduction in digestibility of these macromolecules and thus inhibiting microbial growth (Bulter, 1989; Gupta *et. al.,* 2008; Nwogu *et. al.,* 2008).

Ginger extract showed activity against *E. coli*, *S. aureus*, *R. stolonifer* and *A. niger*. The antimicrobial activity of this extract is generally low, however, it has been reported that ginger extract and its pungent compounds demonstrated greater antibacterial activity against a variety of bacteria including *Helicobacter pylori, E. coli, P. aeruginosa* and *S. aureus,* although mixed result is attributed to different ginger preparations and varying strengths. Presence of alkaloids and other bioactive compounds such as glycosides in which non-toxic glycosides have been found to be more present in ginger extract which can be hydrolyzed to release phenolics that are toxic to microbial pathogens could be responsible for antimicrobial activity (Aboaba and Efuwape, 2001).

Alkaloids found in garlic are basic naturally occurring products generally found primarily in many plants in the form of salts with organic acids and they are toxic to microorganisms (Cheeke, 1989). Garlic also contains flavonoids which cause cell damage, contain free radical scavengers and have anticancer activities. Allicin and other sulphur containing compounds which have been found to be fungistatic against *Aspergillus* spp such as *A. flavus, A. fumigatus, A. terrus* have been found in garlic (Harris *et. al.,* 2001). It has been reported that garlic extracts can inhibit the growth of bacteria, fungi, viruses in culture media and food systems and it has been shown to possess insecticidal, antiparasitic and antitumor properties (Kumar and Berwal, 1987). The low activity shown by ginger and garlic extracts may be due to the fact that the active components in the extracts may not have been activity *in vitro* against the test organisms, or that the various concentrations used may not have been high enough to cause antimicrobial effects.

High MIC and MBC values are indicative of low activity while low MIC and MBC values are indicative of high activity. In this study, clove extract had low MIC and MBC values suggesting that the plant has high antibacterial activity. However, the MIC values for ginger and garlic on the test bacteria were higher when compared to clove and both extracts had no MBC values.

The superior potency exhibited by the control (Acetic acid) when compared with the extracts on the test organisms may be due to the fact that extracts of plant origin are a mixture of various plant constituents, some of which can interfere with antibacterial activity and are subjected to degradation and decomposition on storage (El-Mahmood and Ammy, 2007).

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