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ASSESSMENT OF THE ANTIMICROBIAL EFFECTS OF SOME SPICES ON POTENTIAL FOOD BORNE PATHOGENS ISOLATED FROM DETERIORATED SOY-BEAN CAKE

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ABSTRACT
The growth of potential pathogenic microorganisms in ready to eat foods like soy-bean cake has been reported to shorten its shelf-life and cause food borne illnesses. Four commonly used spices in Nigeria were evaluated for their antimicrobial effects against potential food borne pathogens in an effort to discover natural preservatives. Five potential food borne pathogens (Salmonella spp; Escherichia coli; Enterobacter spp; Staphylococcus aureus and Mucor spp) were isolated from spoiled soy-bean cake and identified using standard microbiological methods. Extracts of Ginger (Zingiber officinale), Pepper (Capsicum annum), Turmeric (Curcuma longa l.) and Cinnamon (Cinnamomum verum) prepared at various concentrations were tested against the food borne pathogens using agar well diffusion method. The results revealed that these spices had antimicrobial effects on the food pathogens. The diameter zone of inhibition of Cinnamon extract was found to range from 7.2±0.1mm – 16.3±1.9mm while that of Ginger extract ranged from 6.3±0.3mm – 11.7±0.9mm. Zone of inhibition of Turmeric extract was from 7.3±0.7mm – 11.7±0.7mm and that of Chilli Pepper extract ranged from 7.3±0.7mm – 13.7±0.7mm. The finding also indicated significant difference in the treatment effect of all the four spices on the tests organisms. The analysis shows that the treatment has significant effect on Salmonella spp and Escherichia coli with the calculated F-value of 3.776 and 6.032 respectively and with the calculated P-value of .041 and .010 respectively. However, the treatment has no significant effect on Enterobacter spp, Staphylococcus aureus and Mucor spp because all their calculated P-values of 0.965; 0.083 and 0.155 respectively were greater than 0.05. These spices could be considered as potential source of natural food preservatives. Direct testing of the spices on the Soy bean cake is therefore recommended.
Keywords: Antimicrobial; spice; pathogen; spoiled; cake

INTRODUCTION

Soy-bean cake (Awara in Hausa) like other ready to eat foods are organic substances which are consumed for nutritional purposes. The growth of spoilage organisms will shorten the shelf life of the Soy-bean cake which has been reported to be rich in protein and essential elements (Idris and Dabo, 2016; Efiosa *et al*., 2017). This in turn would have financial implications for food manufacturers (Amit *et al*., 2017). Hence, the preservation of food is very important in order to avoid the huge financial losses occurring due to deteriorative changes brought by microbial, chemical, and physical process.

Several health hazards associated with artificial preservatives such as hypersensitivity, allergy, asthma, hyperactivity, neurological damage and cancer have been reported. Extracts of basil, clove, neem and rosemary are promising alternatives to their artificial counterparts (Anand and Sati, 2013). Spices and their essential oil can contain many different bioactive compounds present in variable amounts. Basically the bioactive constituents of spices can be divided into volatile and non-volatile compounds (Gottardi *et al*. 2012).

Many natural food ingredients which are traditionally added to foods to achieve a desired flavor also have the potential to control microbial growth. This is known to be true for vegetable extracts, mustard, onion, garlic, horse radish, spices and herbs etc. (Jay, 2005; Adams and Moss, 2009; Ukwuru and Uzodinma, 2010 and Adedeji and Omowaye, 2013).

Ihekoronye and Ngoddy (1985) had earlier asserted that an even greater increase in the amount of food available for human consumption could be realized by using appropriate food preservation methods. Bukar and Magashi (2013) suggested the application of waxes and plant aqueous extracts to preserve seasonally available fruits and vegetables which could provide a cheap and economically viable method of food preservation that can be adopted by farmers in Nigeria. The evaluation of plant constituents for antimicrobial and preservative activities on food products is part of the ongoing search for natural food preservatives (Bukar and Sani, 2018). The development and preservation of new food products like Awara from soy bean which has been reported to be rich in protein and essential elements can help immensely in the reduction of hunger, malnutrition and improve food security conditions.

The results of the investigation carried out by Yusha'u *et al.* (2017) and Bristone *et al.* (2018) revealed that the Awara samples examined were contaminated which render the food unsafe for human consumption. The study by Yusha'u *et al.* (2017) revealed the presence of *Staphylococcus aureus* 17(85%), *Escherichia coli* 16(80%), *Enterobacter aerogenes* 16(80%), *Shigella* spp. 10(50%), *Salmonella* spp. 9(45%) and *Vibrio* spp. 7(35%) which can cause food poisoning, typhoid, cholera etc. According to Idris and Dabo (2016) pathogenic bacteria isolated from awara samples vended in Kano metropolis include *Escherichia coli* 13(50.00%), *Staphylococcus aureus* 8(30.77%) and *Salmonella typhi* 5(19.23%). It was against this background that this study was undertaken to assess the antimicrobial effects of spice extract on food borne pathogens isolated from spoiled soy bean cake.

The objectives of the research were: to isolate and identify bacteria and fungi associated with spoiled soy bean cake as well as to test the antimicrobial effect of the spices on the isolated bacteria and fungi.

MATERIALS AND METHODS

Sample collection

Samples of dry Ginger (*Zingiber officinale*), Pepper (*Capsicum annum*), Turmeric (*Curcuma longa l.*) and Cinnamon *(Cinnamomum verum)* also called *Ceylon cinnamon* were purchased from Kano metropolitan markets and taken to the Herbarium at the Department of plant Biology Bayero University Kano for authentication. The voucher numbers for Soy-bean, Ginger, Turmeric and Cinnamon are BUKHAN 88, BUKHAN 296, BUKHAN 188 and BUKHAN 119 respectively. Samples of Awara were purchased from Kabuga Market in Kano which were aseptically taken in sterile plastic containers and left overnight at Post Graduate Hostel of Bayero University Kano.

Preparation of extracts of Spices (Ginger, Pepper, Turmeric and Cinnamon)

The spices were washed and oven dried at 55^{o}C for 24 hours. The dried spices were ground into fine powder in a mill and sieved. Ten grams (10g) each of the powder were extracted with 100ml of 99% ethanol overnight at room temperature. The extracts were filtered using No.1 Whatman filter paper to remove residue and then evaporated in a water bath at 40^{o}C. The extracts obtained after evaporation of ethanol were used as natural antioxidant (Zia-urRahman *et al.,* 2003)

Preparation of Sample Homogenate

Twenty-five gram (25g) of solid Awara was weighed into a sterile blender jar. Two hundred and twenty-five (225ml) of distilled water was added and blended for two (2) minutes at low speed at 8000rpm as described by Food Safety and Standards Authority of India (Fssai, 2012).

Isolation and Identification of bacteria associated with spoiled Soy bean cake

The homogenized awara samples were streaked on Salmonella Shigella Agar (SSA), Manitol Salt Agar (MSA) and Eosin Methylene Blue Agar (EMBA) for 24 hours at 37^{o}C for isolation of some bacteria. Cultural and morphological identification were carried out according to the methods described by Eze *et al. (*2014). The isolated colonies were gram stained to differentiate bacteria into gram positive and negative (Vaughan *et al.,*1994). Finally, biochemical characterization of isolates using protocols described by Cheesbrough (2002) was done. Pure cultures of the different organisms identified were sub-cultured and preserved on agar slants at refrigeration temperature (4°C) (Eze *et al.,* 2014).

Isolation and Identification of Fungi

The plates of Potato Dextrose agar (PDA) streaked with homogenized Awara samples were incubated at room temperature (25°C) for 5 days. The method described by Ibrahim and Rahma. (2009) was adopted for identification of the fungi using Lacto phenol cotton blue. The identification was achieved by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the mycelium from the fungal cultures was removed and placed in a drop of a mounting stain. The mycelium was spread very well on the slide with the aid of the needle.

A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted and observed with x10 and x40 objective lenses respectively. The species encountered were identified in accordance with Cheesbrough (2000) and Ellis *et al.* (2007).

Preparation of extract concentrations

Stock solution (800mg/2ml) of each spice extract was prepared by dissolving 0.8g in 2ml Dimethylsulphoxide (DMSO). Double serial dilution was carried out by adding 1ml of DMSO to each serial dilution. Six concentrations were prepared from the stock solution such that 400mg/ml, 200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml were obtained. The last four concentrations were used for the bioassay. The concentrations were increased up to 400mg/ml during the determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal/fungicidal concentration MBC/MFC (Bukar and Sani, 2018).

Standardization of the inoculum

One milliliter of concentrated Sulfuric acid (H_2SO_4) was added to 99ml sterile distilled water. One percent (1%) w/v of Barium Chloride was prepared by dissolving 1g Barium Chloride in 100ml distilled water and 0.6ml of the solution added to 99.4ml of H_2SO_4 solution. This was shaken thoroughly to ensure proper mixing. Ten milliliter of the turbid solution was transferred to a test tube and kept for comparison with test organisms' suspension in a similar test tube. The remaining solution was stored in a dark bottle for future use.

Inoculum were prepared from the cultures maintained on a slant of nutrient agar (bacteria) and potato dextrose agar (PDA) for fungal isolates. A loopful of overnight nutrient broth culture was added to 4ml physiological saline continuously until the density of bacterial suspension (turbidity) was matched with the turbidity of the 0.5 McFarland standard (1.5 x 10^8 cfu/ml) as described by Cheesbrough (2002). A loopful of fungal spores from an overgrown plate was taken and shaken thoroughly in 10ml of 20% Tween 80 solution. The solution was dropped in a hematocytometer and the spores were counted and multiplied by 10^4 to arrive at 1.70 x 10^6 cfu/ml (Dubey and Maheshwari, 2012).

Antimicrobial Testing of the extracts

Agar well diffusion technique was employed for the antibacterial and antifungal bioassay. The assessment of antibacterial and antifungal activity was based on measurement of the diameter of the inhibition zone formed around the well.

Antimicrobial susceptibility was determined by pouring the molten sterilized Muller Hilton Agar and Potato Dextrose Agar in sterile petri dishes. Loopful of the standardized test organisms were streaked on the surface of the media and wells of about 6mm diameter were punched on the solidified agar using sterile cork borer. The wells were filled with the different concentrations of each extract of spice using sterile syringe. The set up were incubated for 24hours at 37^{o}C (Bukar and Sani, 2018).

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of the active concentrations were determined using the tube dilution technique described by Bukar and Sani (2018). Appropriate test concentrations were prepared and added to sterile capped test tubes of Mueller Hinton Broth (bacteria) and Potato Dextrose Agar (fungal isolates) to cover the range of dilutions chosen in duplicates. After overnight incubation at 37^{o}C, the lowest concentration of the extract at which no turbidity was observed was recorded as the Minimum Inhibitory Concentration.

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

Sterile Mueller-Hinton agar and potato dextrose agar plates were inoculated with samples from the MIC tubes that show no visible bacterial and fungal growth. The lowest concentration in which no growth occurred on the medium was considered as the MBC/MFC (Bukar and Sani, 2018).

RESULTS AND DISCUSSION

Table 1: Percentage occurrence of Bacteria and Fungi isolated from spoiled Soy bean cake

Isolate	Sample(Frequency)	Percentage (%)
Salmonella spp	15 (01)	4.3
Escherichia coli	15 (05)	21.8
Enterobacter spp	15 (02)	8.7
Staphylococcus aureus	15 (06)	26.1
Mucor spp	15 (09)	39.1
Total	15 (23)	100

Table 2: The effect of Cinnamon extract on the test organisms

Concentration	Zone of inhibition of Organisms (mm)				
(mg/ml)	*Salmonella* spp	*E.coli*	*Enterobacter* spp	*Staph.aureus*	*Mucor* spp
12.5	7.3±1.3	11.7±2.8	6.0±0.0	6.0±0.0	11.7±1.5
25	11.0±2.9	12.0±4.6	9.0±0.6	7.2±0.1	12.0±1.2
50	13.3±1.2	14.0±1.5	9.0±1.0	10.0±0.1	12.7±1.2
100	16.3±1.9	14.7±3.8	12.0±1.2	10.9±1.9	15.7±2.2
MIC	200	100	100	200	200
MBC/MFC	400	200	200	400	400

Note: Diameter of the well = 06mm

Table 3: The effect of Ginger extract on the test organisms

Concentration	Zone of inhibition of Organisms (mm)				
(mg/ml)	*Salmonella* spp	*E.coli*	*Enterobacter* spp	*Staph.aureus*	*Mucor* spp
12.5	6.3±0.3	6.0±0.0	7.3±1.3	6.0±0.0	10.3±2.6
25	6.7±0.7	6.0±0.0	7.3±1.3	6.0±0.0	10.0±2.1
50	8.0±1.2	8.3±1.2	9.3±3.3	6.0±0.0	12.7±1.2
100	11.7±2.2	10.3±2.6	11.0±1.0	6.0±0.0	11.7±0.9
MIC	400	200	200	400	200
MBC/MFC	>400	400	400	400	400

Note: Diameter of the well = 06mm

Table 4: The effect of Turmeric extract on the test organisms

Concentration	Zone of inhibition of Organisms (mm)				
(mg/ml)	*Salmonella* spp	*E.coli*	*Enterobacter* spp	*Staph.aureus*	*Mucor* spp
12.5	8.3±2.3	8.3±2.3	9.0±3.0	6.0±0.0	12.3±1.2
25	8.3±2.3	9.0±2.1	8.7±2.7	6.0±0.0	11.7±2.4
50	7.3±0.7	9.7±2.0	8.7±1.8	6.0±0.0	9.0±2.1
100	9.7±0.9	11.7±0.7	8.7±1.3	6.0±0.0	8.7±1.3
MIC	400	400	400	400	200
MBC/MFC	>400	>400	>400	>400	400

Note: Diameter of the well = 06mm

Table 5: The effect of Chilli pepper extract on the test organisms

Concentration	Zone of inhibition of Organisms (mm)				
(mg/ml)	*Salmonella* spp	*E.coli*	*Enterobacter* spp	*Staph.aureus*	*Mucor* spp
12.5	7.3±1.3	6.0±0.0	7.7±1.7	6.0±0.0	9.3±0.7
25	7.7±1.7	6.0±0.0	9.0±3.0	6.0±0.0	10.0±2.1
50	8.0±2.0	7.3±0.7	9.3±3.3	8.1±0.5	10.0±2.1
100	9.0±3.0	12.0±0.0	11.0±2.1	9.1±0.1	13.7±0.7
MIC	200	50	100	200	200
MBC/MFC	400	200	200	400	400

Note: Diameter of the well = 06mm

Table 6: The difference in the effect of spice extracts on the test organisms

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	*Salmonella* spp	51.073	3	17.024	3.776	.041
	Escherichia coli	74.662	3	24.887	6.032	.010
	Enterobacter spp	.742	3	.247	.089	.965
	Staphylococcus aureus	22.935	3	7.645	2.828	.083
	Mucor spp	22.472	3	7.491	2.091	.155

A total of 23 isolates belonging to five genera/species were obtained from a total of fifteen samples (Table 1). *Mucor* spp was the most predominately encountered potential pathogen with the highest percentage of occurrence (39.1%) while *Salmonella* spp had the least percentage of occurrence (4.3%). The isolation of these food potential pathogenic organisms is in line with the findings of Yusha'u *et al.* (2017) who isolated *Enterobacter aerogenes; Escherichia coli; Salmonella* spp; *Shigella* spp; *Staphylococcus aureus* and *Vibrio* spp from Soy bean cake (Awara). Their presence especially *Mucor* spp might be connected with the storage of the samples for one day which led to microbial colonization and its subsequent deterioration.

The results of the antimicrobial assay are presented in tables 2 - 5. The activities of the extracts on the test organisms increases with the increase in the concentration of the extracts except with turmeric extract which showed decrease in activity as the concentration increases. The results in these Tables 2 - 5 also revealed that the extracts differed in their activity on the test organisms. This finding confirms the report of Jay (2005; Adams and Moss (2009); Ukwuru and Uzodinma (2010) and Adedeji and Omowaye (2013) who stated that many natural food ingredients which are traditionally added to foods to achieve a desired flavor also have the potential to control microbial growth. Also that this is known to be true for vegetable extracts, mustard, onion, garlic, horse radish, spices and herbs etc.

The diameter zone of inhibition of Cinnamon extract was found to range from 7.2±0.1mm – 16.3±1.9mm while that of Ginger extract ranged from 6.3±0.3mm – 11.7±0.9mm. Zone of inhibition of Turmeric extract was from 7.3±0.7mm – 11.7±0.7mm and that of Chilli Pepper extract ranged from 7.3±0.7mm – 13.7±0.7mm. This shows that Cinnamon extract was more active followed by Chilli pepper. Ginger and Turmeric extracts showed least activity on the test organisms.

The minimum inhibitory concentration of Cinnamon extract ranged from 100 – 200mg/ml (Table 2) and has its highest effects on *Salmonella* spp producing 16.3±1.9mm diameter zone of inhibition. The lowest diameter zone of inhibition of Cinnamon was 7.2±0.1mm. Ginger extract had the MIC range of 200 – 400mg/ml with no activity on *Staphylococcus aureus,* but had more effect on *Salmonella* spp and *Mucor* spp with 11.7±2.2mm and 11.7±0.9mm highest zones of inhibition respectively (Table 3). Turmeric extract also had no effect on *Staphylococcus aureus* and its MIC ranges from 200 – 400mg/ml (table 4), its highest activity was recorded on *E. coli* with highest zone of inhibition of 11.7±0.7mm and 7.3±0.7mm as its lowest diameter of activity. MIC range of Chilli pepper as shown in table 5 runs from 50 – 200mg/ml with highest effect on *Mucor* spp (13.7±0.7mm) and its lowest activity was found on *E. coli* with 7.3±0.7mm zone of inhibition.

The results showed that *Salmonella* spp was more sensitive to the four spice extracts while *Staphylococcus aureus* was the most resistant of all the test organisms. This part of the findings agrees with the report of Idris and Dabo (2016) who found out that *Staphylococcus aureus* was more resistant than the other pathogenic bacteria isolated from soy bean cake vended within Kano metropolis.

The activities of the spices extract against the test organisms were found at various concentrations which support the submission of Gottardi *et al*. (2012) who reported that spices and their essential oil can contain many different bioactive compounds present in variable amounts. The MIC, MBC and MFC were higher probably because the phyto- constituents of the spices used in the research are volatile and time dependent. This scenario could be linked to the report of Gottardi *et al*. (2012) who opined that basically the bioactive constituents of spices can be divided into volatile and non-volatile compounds.

Table 6 revealed the difference in the effect of Cinnamon Bark, Ginger, Turmeric and Chilli Pepper on the test organisms ($P < 0.05$). The analysis shows that the treatment has significant effect on *Salmonella* spp and *Escherichia coli* with the calculated F-value of 3.776 and 6.032 respectively and with the calculated P-value of .041 and .010 respectively. However, the treatment has no significant effect on *Enterobacter* spp, *Staphylococcus aureus* and *Mucor* spp because all their calculated P-values of 0.965; 0.083 and 0.155 respectively were greater than 0.05.

CONCLUSION AND RECOMMENDATION

Five food borne pathogens were isolated from spoiled Soy bean cake. They included *Salmonella* spp, *Escherichia coli, Enterobacter* spp, *Staphylococcus aureus* and *Mucor* spp. The crude extract of the spices had activity on the test organisms at various concentrations. Based on this finding the spices can be considered as potential source of natural food preservatives. Direct testing of the spices on the Soy bean cake is therefore recommended.

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