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Effect of *Bambusa tuldoides cv. ventricosa* leaf extracted with fermented steep liquors of maize and sorghum on some pathogenic organisms

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ABSTRACT

Aims: *Bambusa tuldoides cv. ventricosa (bamboo)* extract is used locally in the treatment of different types of fever in Africa. The extraction by cooking of fresh bamboo leaves using fermented steep liquor of *ogi* either made from maize or sorghum has been used traditionally in the treatment of Typhoid fever in the South-Western Nigeria. This work is designed to evaluate the antibacterial activity of the different fermented cereal (*Zea mays* and *Sorghum bicolor*) grains steep liquors as the extraction medium for the bamboo leaf in comparison with the ethanol extraction using agar well diffusion.
Methodology and results: The extracted leaves (dried and fresh) from different fermented cereal steep liquors [maize (white and yellow) and sorghum (white and red)] media were screened for inhibition of some pathogenic species such as *Staphylococcus aureus*, *Salmonella typhi*, *Streptococcus faecalis* and *Escherichia coli.* The extracts were then tested at different concentrations for bacteriostatic activity towards these clinically-important species. The fresh leaf extracts from the fermented maize and sorghum liquors at a concentration of 20 g/100 mL showed antibacterial activities for all the tested bacteria, while the ethanolic leaf extract did not show any antibacterial activity for both *S. typhi* and *S. faecalis.* Higher concentration (20 g/ 40 mL) produced higher antibacterial activity in all the leaf extracts from the fermented maize and sorghum liquors.
Conclusion, significance and impact study: Our data provide support for the traditional use of fermented maize and sorghum grill steep liquor extract of *Bambusa tuldoides cv. ventricosa* in treating the symptoms associated with the test organisms, in which the yellow maize liquor displayed greater antibacterial activity in comparison with the others.

Keywords: Antibacterial, *Bambusa tuldoides* extract, fermented liquors, maize, sorghum

INTRODUCTION

Bamboo leaf has been used in Asia for herbal medicine and as food. In China, the most common species is *Bambusa vulgaris* whereas the most common species in West Africa is *Bambusa tuldoides.* It has been established that various extracts made from different parts of *Bambusa vulgaris* have multiple biological activities including antioxidants, antibacterial, and scavenging oxygen radicals which protect humans from cardiovascular diseases and cancer (Lu *et al*., 2005; Lu *et al*., 2006; Zhang *et al*., 2007). The effective components of bamboo leaves, shavings, and shoots include flavonoids, phenolic acids, polysaccharides, antraquinones, coumarins, special amino acids, and peptides (Zhang *et al*., 2004; 2007). Among these, phenolic compounds, antraquinones, and coumarins are of relatively strong anti-bacterial and bactericidal functionalities. Bamboo leaves have excellent anti-fatigue, anti-hyperlipidemic, and antihypertensive activities (Zhang *et al*., 2006). There are many bamboo leaf formulations for febrile conditions which were designed to treat any acute feverish disease that did not resolve in a few days, and sometimes caused a drying of the fluids (particularly of the stomach) as well as affecting other internal organs (Yang, 2002). Typical symptoms are fever, irritability, and insomnia. The formula of *Zhuye Shigao Tang* (bamboo leaf and gypsum combination) has been adopted in modern treatment of some chronic ailments, including diabetes (Huang and Wang, 1993).

Fermentation is particularly common in the tropics where high temperature and high humidity, coupled with insanitary conditions promote food spoilage. Under these conditions, lactic acid fermentation inhibits spoilage and pathogenic microorganisms by a combination of factors which include the production of organic acids, hydrogen

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peroxides, and antibiotics-like substances (Cooker *et al*., 1987). Lactic acid fermentation also improves the organoleptic properties of food by producing a variety of flavour of the existing food and possible increase of its nutritional value (Chavan and Kadam, 1989).

The growing resistance of microorganisms to antimicrobial agents is a major concern in the treatment of infectious diseases. Therefore, continuous efforts are being made to develop antimicrobial agents from local sources for better chemotherapeutic effects. The objective of this research was therefore to determine the efficacy of extracts of bamboo leaves using fermented steep liquors of maize and sorghum grill as the solvent on pathogenic microorganisms *Staphylococcus aureus*, *Salmonella typhi*, *Streptococcus faecalis*, *and Escherichia coli.* Also to study the effects of heating on the antimicrobial activity of the extracted leaf and provide scientific explanation for the use of this cheap and affordable traditional means of treating feverish symptoms caused by some pathogenic bacteria.

MATERIALS AND METHODS

Plant material

Source, collection and identification

Fresh green leaves of *Bambusa tuldoides* were collected from the Adekunle Ajasin University campus reservation, Ondo state, south-west Nigeria and authenticated at the Department of Plant Science and Biotechnology at the University. Some of the collected plant materials were air-dried for 14 days while others were used fresh.

Antibacterial activity

Microbial strains

The microorganisms used in this study were *Staphylococcus aureus* (isolated from skin), *Escherichia coli* (isolated from faecal origin), *Salmonella typhi* (collected from the Nigeria Institute of Medical Research, Yaba, Lagos), and *Streptococcus faecalis* (collected from the Federal University of Technology, Akure).The bacterial strains were cultured in nutrient broth (BHI) (Difco, MI, USA) under aerobic condition at 37 ˚C for 24 h and sub-culturing was done twice weekly. Suspensions of the test organisms were prepared by picking colonies from appropriately incubated agar cultures to sterile broth, to match a McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/mL).

Preparation of test organisms for seedling

Two millilitres of all the test microorganisms were added aseptically to the sterilized nutrient agar medium at a temperature of 50 °C, swirled gently for uniform distribution of the microorganism, allowed to gel in a sterilized Petri-dish, and incubated for 18 h for viability. The different microorganisms were then seeded into the respective media, i.e., Mueller Hinton agar for *Salmonella typhi*, MacConkey agar for *Escherichia coli*, and nutrient agar for both *Streptococcus faecalis*and *Staphylococcus aureus* at 45 °C in sterilized plates and allowed to gel. The agar was seeded with the fresh pathogens and then incubated for 5 h for proper diffusion of the microorganisms.

Preparation of steep liquors

Steep liquors were obtained from different types of Pap (Yoruba name: *Ogi*). Clean and healthy maize and sorghum grains were bought from the local market. One kilogram of each grain type was weighed and washed thoroughly with clean tap water and placed in sterile covered plastic buckets containing 3 L of sterile distilled water and steeped for 72 h at 28±2 °C. After steeping, the water was decanted and the grains were wet-milled separately using clean stainless steel blender. The resulting pastes were sieved using different sterile muslin cloths. The filtrates were collected into different sterile containers and allowed to settle for 3 days during which natural fermentation took place (Adebolu *et al*., 2007).

Lactic acid bacterial counts of fermented steep liquors

The total lactic acid bacterial count of the various fermented steep liquor was done by transferring 1.0 mL of various liquor diluents of 10^6 from serial dilution to a sterilize Petri-dish using pour plate method with MRS agar. After inoculation, the plates were observed for bacteria and the number of colonies counted using a colony counter and recorded to calculate the amount of bacteria present in each sample in CFU/mL.

Identification of fermented steep liquors bacteria

Colonies were picked aseptically from the plate count and sub-cultured to get pure cultures of each distinct colony. The pure colonies were morphologically characterized and gram stained to ascertained how arsenic they were and the organisms were confirmed by using characterized biochemically using the API 50LB kit and 50LB media for Lactobacilli (Biomérieux Product, France).

Total sugar content of fermented steep liquors

One-tenth mL of 4% phenol was added to 2 mL of the filtered ethanolic sample from different fermented steep liquors and allowed to stand for 10 min after which 2.5 mL concentrated sulphuric acid (H_2SO_4) was added. The optical density was taken at a wavelength of 490 nm using a UV spectrophotometer (WPA, Linton Cambridge, UK, type 5104D). The blank was prepared by adding 1.0 mL of 4% phenol to 2.5 mL concentrated sulphuric acid (Chow and Landhausser, 2004).

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Titratable acidity of fermented steep liquors

Twenty millilitres of fermented steep liquors was put in conical flasks and two drops of phenolphthalein were added and titrated against 0.1M of sodium hydroxide using AOAC (2000) procedure. The formula used is given below with lactic acid as the reference acid:-

$$TTA = \frac{V \times N \times 100 \times 0.833}{v \times 1000}$$

TTA – Total Titratable Acidity
V – Volume of 0.1M sodium hydroxide used
N – Normality or Molarity of sodium hydroxide used and
v – volume of the sample aliquot used.

Preparation of extract

Extraction of fresh bamboo leaves with fermented maize and sorghum liquors

Twenty grams of fresh bamboo leaves was added to 100 mL of the fermented steep liquor of the various cereals and homogenised with a blender. This was divided into two groups, A and B. Group A was heated at 70 °C for 20 min and allowed to cool before the filtrates were kept in labelled containers while group B, the uncooked set of mixtures, was left to stand for 20 min, filtered, and the extracts were kept in labelled containers. The whole process was repeated using 20 g of the leaves to 40 mL of the fermented steep liquors of the different cereals.

Extraction of fresh bamboo leaves using ethanol

Ethanolic extracts of fresh bamboo leaves were obtained by homogenising 20 g of fresh bamboo leaves with 100 mL of ethanol (70% v/v) in a blender. Each extract was filtered using filter paper, labelled, and dried by allowing standing on the work bench for 1 h and kept in the refrigerator. The process was repeated using 20 g of the leaves to 40 mL of ethanol.

Extraction of fresh bamboo leaves using sterile distilled water

Extracts of bamboo leaves in distilled water were made by homogenising 20 g of fresh bamboo leaves in 100 mL of sterile distilled water. The extracts were filtered using filter paper, labelled, and kept in the refrigerator. The process was repeated using 20 g of the leaves to 40 mL of sterile distilled water.

Extraction of dried bamboo leaves using steep liquor

The same procedure as for the fresh leaves was repeated for air-dried leaves for two weeks.

Antimicrobial assay of the extracts

Antimicrobial activity of the constituents of bamboo leaf extracts was carried out using the agar-well diffusion method (Omemu and Faniran, 2011) on Mueller-Hinton agar. Sterilized cork borer was used to punch wells of 5 mm in diameter on the agar about 3 cm apart and 0.2 mL of each of the extracts were introduced aseptically into the wells which filled them respectively to capacity. The culture was incubated at 37 °C for 24 h. After incubation, zones of inhibition were measured using ruler graduated in millimetres.

RESULTS

Nine microorganisms were isolated from the different steep liquors as shown in Table 1. They comprised three genera of bacteria which consist of five *Lactobacillus* spp., one *Staphylococcus* spp., and three *Micrococcus* spp. *Lactobacillus* and *Micrococcus* were common in all the steep liquors while *Staphylococcus* spp. were not common in any of the samples as shown in Table 1.

Table 1: Morphology characterization of bacterial isolates from different steep liquors.

Isolate from steep liquor*	Shape	Elevation	Colour	Opacity	Surface	Edge
Staphylococcus spp.	Cocci	Flat	Creamy	Transparent	Smooth	Entire
Micrococcus spp.	Cocci	Flat	Creamy	Opaque	Smooth	Entire
Lactobacillus spp.	Rod	Convex	Creamy	Opaque	Smooth	Entire
Lactobacillus spp.	Rod	Convex	Creamy	Opaque	Smooth	Entire
Lactobacillus spp.	Rod	Convex	Creamy	Translucent	Smooth	Entire
Lactobacillus spp.	Rod	Raised	Creamy	Opaque	Smooth	Entire
Lactobacillus spp.	Rod	Raised	Creamy	Translucent	Smooth	Entire

* The isolates were identified using API 50 LB

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Table 2: Occurrence of predominant genus bacteria in the different steep liquors.

Samples steep liquor	*Staphylococcus* spp.	*Micrococcus* spp.	*Lactobacillus* spp.
White maize	+	+	+
Yellow maize	_	+	+
Red sorghum	_	+	+
White sorghum	+	+	+

Key: (+) means present in the fermented steep liquor
(-) means not present in the fermented steep liquor

Table 3: Total sugar (TS) content of the different fermented steep liquors of maize and sorghum varieties.

Time (Day)	White maize (mg/ml)	Yellow maize (mg/ml)	Red sorghum (mg/ml)	White sorghum (mg/ml)
Day 1	0.301±0.004^{c}	0.321±0.004^{d}	0.252±0.004^{b}	0.246±0.004^{a}
Day 2	0.392±0.004 ^{c}	0.443±0.004 ^{d}	0.352±0.004^{b}	0.339±0.004 ^{a}
Day 3	0.561±0.004 ^{c}	0.603±0.004 ^{d}	0.501±0.004^{b}	0.496±0.004 ^{a}
Day 4	0.882±0.004 ^{d}	0.796±0.004 ^{c}	0.756±0.004^{b}	0.752±0.004 ^{a}
Day 5	0.910±0.004 ^{d}	0.900±0.004 ^{c}	0.878±0.004^{b}	0.862±0.004 ^{a}
Day 6	1.001±0.004 ^{d}	0.989±0.004 ^{c}	0.976±0.004^{b}	0.971±0.004 ^{a}
Day 7	0.921±0.004 ^{d}	0.916±0.004^{c}	0.902±0.004^{b}	0.898±0.004 ^{a}

SEM , Standard error of the mean. a, b = Values in the same column with different superscripts differ significantly ($p<0.05$).

In the different cereal fermented steep liquors, there was a general significant increase in the total sugar content from the first day (0.246 mg/mL) to the sixth day (1.001 mg/mL) after which there was a general decrease (Table 3). Yellow maize steep liquor had higher total sugar content on each of the first six days (0.321, 0.443, 0.603, 0.796, 0.900, and 0.989 mg/mL, respectively) in comparison with other used cereals (white maize, red sorghum and white sorghum) (Table 2). Figure 1 shows that there was a significant increase in the total bacterial counts from day 0 (3.00×10^6 CFU/mL) to the seventh day (107×10^6 CFU/mL) for all the cereal steep liquors. There was a general significant increase in the total titratable acidity (TTA) which is a function of the total lactic acid content of fermenting steep liquors as the fermentation days increased. Yellow maize steep liquor had the lowest total titratable acidity (5.00%) on day 0, while red sorghum steep liquor had the highest value (10.00%) rising to between 15.00% and 16.50% on the 5th day (Figure 2).

Figure 3a and 3b compared the antibacterial activity of dried bamboo leaves extracted with ethanol and distilled water at different concentrations of 20 g/100 mL and 20g/40mL.The ethanolic extracts were concentration-dependent in its antibacterial activities against the tested organisms. The aqueous extracts of the dried leaves also showed to be concentration-dependent in its antibacterial activities for all the isolates. The aqueous extract of dried leaves showed antibacterial activity for *Salmonella typhi* isolate only.

The antibacterial activities of the different steep liquors were shown in Figure 4. Generally, there were significantly high antibacterial activities from each of the isolates. The steep liquor extracts showed the highest antibacterial activities range from 16.8 mm to 18.2 mm for *Staphylococcus aureus* and *Escherichia coli* which were keenly followed by that for *Salmonella typhi* (15.4 mm to 16.6 mm) while the least activities were shown for *Streptococcus faecalis* (13.6 mm to 14.6 mm). *S. aureus* gave the highest zone of inhibition when treated with *B. tuldoides cv. ventricosa* white maize extract at a concentration of 20 g/100 mL (w/v).

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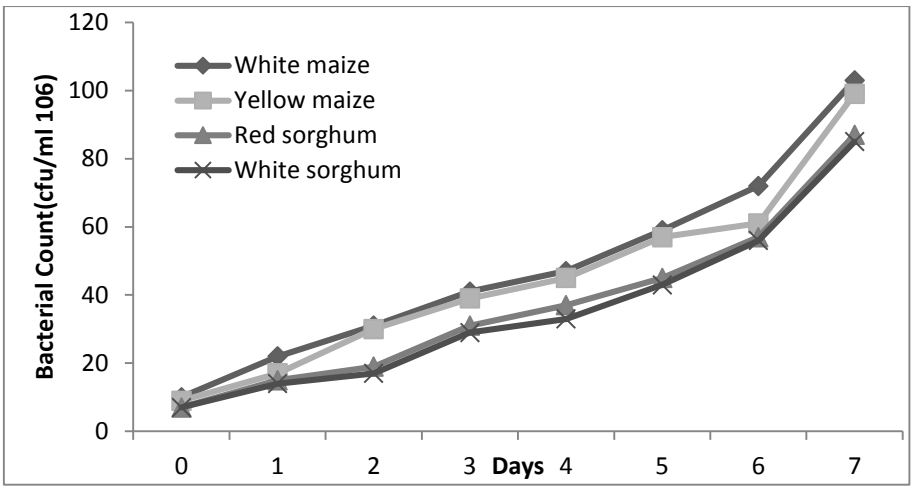

Figure 1: Bacterial counts of different steep liquors.

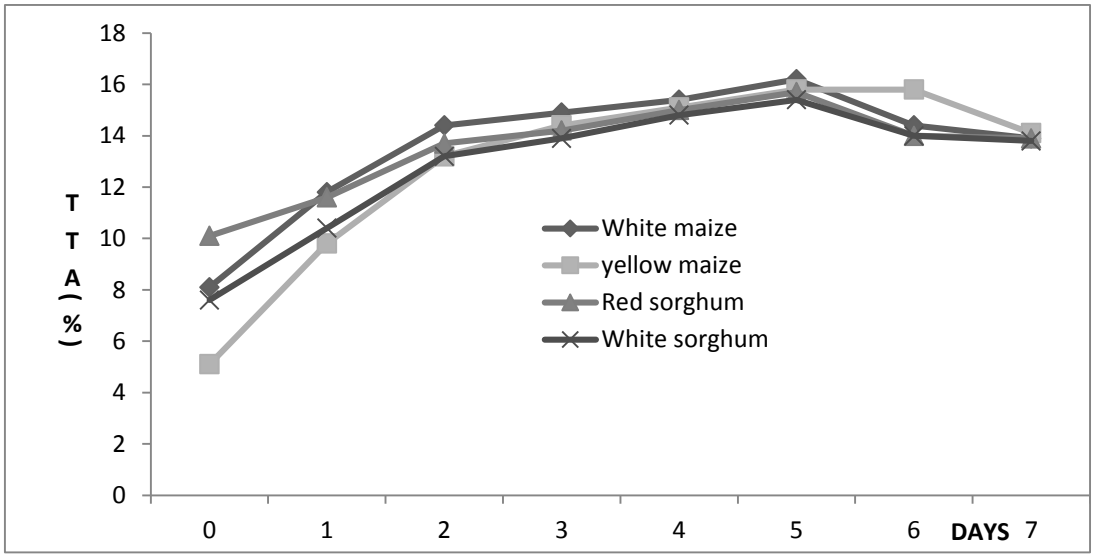

Figure 2: Change in total titratable acidity (TTA) of different steep liquors.

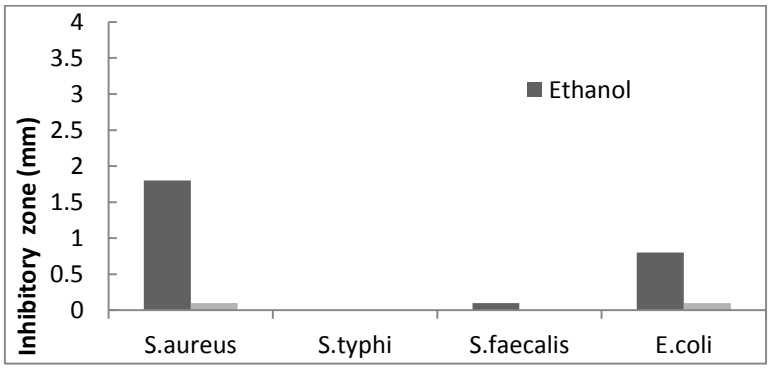

Figure 3a: Antibacterial activity (mm) extracts from dried bamboo leaves with ethanol and distilled water at 20g/100mL.

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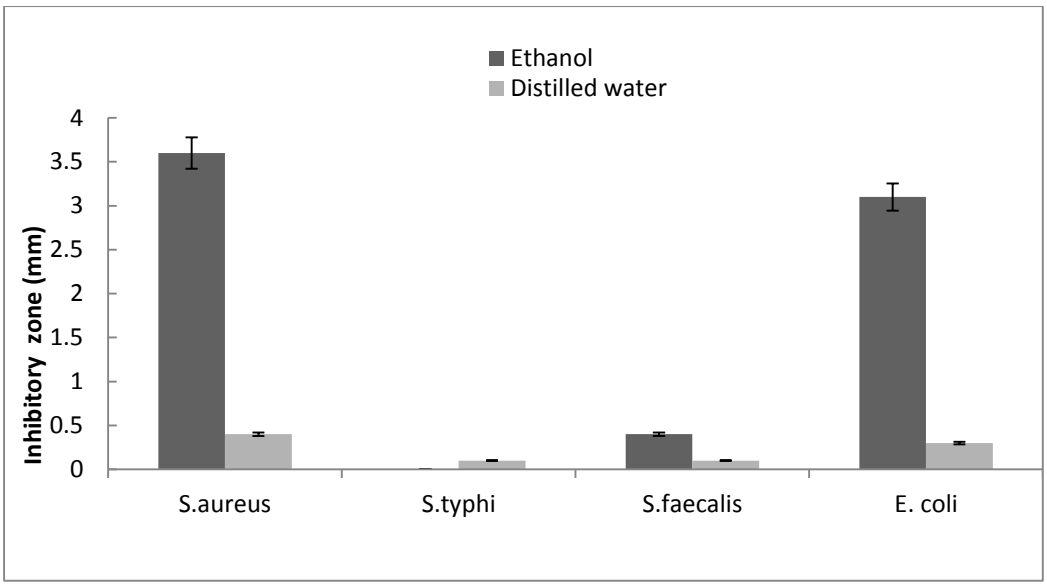

Figure 3b: Antibacterial activity (mm) of extracts from DRIED bamboo leaves with ethanol and distilled water at 20 g/40 mL.

At the same concentration for white maize extract of *B. tuldoides* cv. *ventricosa*, *E. coli* was next in inhibition followed by *S. typhi* and lastly *S. faecalis* with the least zone of inhibition. Yellow maize fermented extract of *B. tuldoides* cv. *ventricosa* gave the highest antibacterial activity with *S. aureus* keenly followed by *S. typhi*. The zone of inhibition of *S. faecalis* and *E. coli* showed a high standard deviation from that of *S. aureus* (Figure 4a). For red and white sorghum fermented extract of *B. tuldoides* cv. *ventricosa* at the same concentration of 20 g/100 mL, *S. aureus* gave the highest zone of inhibition followed by *E. coli*, *S. typhi* and *S. faecalis*, respectively. At the 20g /100 mL (w/v) concentration of the different fermented liquors using dried *B. tuldoides cv. ventricosa* leaves, the maize (both white and yellow) extracts were found to give the highest antibacterial activities against all the tested organisms than their sorghum counterparts (Figure 4a). Figure 5a showed the antibacterial activities of extracts from fresh *B. tuldoides* cv. *ventricosa* leaves without heating, while Figure 5b shows the activities of the heated fresh leaves extracts with the different fermented liquors as the solvents at 20g/100mL (w/v). In Figure 5a, the maize and sorghum extracts showed the highest sensitivity to *S. aureus* followed by *E. coli, S. faecalis*, and *S. typhi*, respectively. Figure 5a reveals that both white and yellow maize extracts of *B. tuldoides* cv. *Ventricosa* showed significant difference in their antibacterial activities compared with the sorghum extracts. The heated fresh extracts of *B. tuldoides* cv. *ventricosa* of all the fermented cereals gave a higher antibacterial activity for *S. aureus* and *E. coli* than for *S. typhi* at the concentration of 20 g/100 mL (w/v) (Figure 5b).

There was no significant difference in the use of heat on antibacterial activity of either maize or sorghum fermented liquor extracts on each of the tested microorganisms. The unheated extracts generally showed higher antibacterial activities (1.3 mm for *S. typhi* to 7.2 mm for *S. aureus*) than the heated extracts (0.4 mm for *S. typhi* to 3.4 mm for *S. aureus*).

With the use of the fermented liquors as solvents for fresh bamboo leaves, *Salmonella typhi* showed the lowest sensitivity at a concentration of 20 g/100 mL (w/v). However, *S. typhi* sensitivity was higher for maize steep liquor than sorghum steep liquor. Generally, the fermented steep liquor extracts showed higher antibacterial activities than the ethanolic extracts since the ethanolic extracts were only macerated for a few minutes and effect was as a result of the ethanol hence acting as a control for chemical extraction. The maize steep liquors extracts showed better antibacterial activities than the sorghum counterparts fermented liquor extracts. The results also indicated that white maize fermented liquors showed a higher activity than the yellow maize ones both with dried and fresh leaves.

Figure 3 showed that the susceptibility of the controlled extract of ethanol and water of the fresh leaves are concentration-dependent. At the concentration of 20 g/40 mL of the solvent, the susceptibility was more pronounced than at the concentration of 20 g/100 mL of both solvents. The zone of inhibition of the different fermented steep liquor extracts of the dried leaves at a high concentration of 20 g/40 mL of liquors revealed a very high susceptibility to the different pathogenic organisms tested.

Since the fresh leaves were used for extraction locally, the effect of heat on the extracts at a low concentration of 20 g/100 mL was examined. Heating was found to reduce the growth inhibition of the different pathogenic organisms by almost half.

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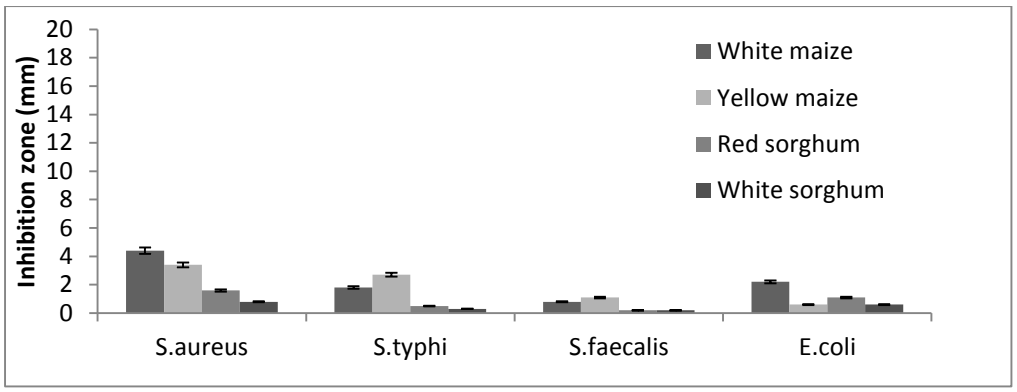

Figure 4a: Antibacterial activity (mm) of extracts from dried bamboo leaves with different steep liquors at 20g/100mL.

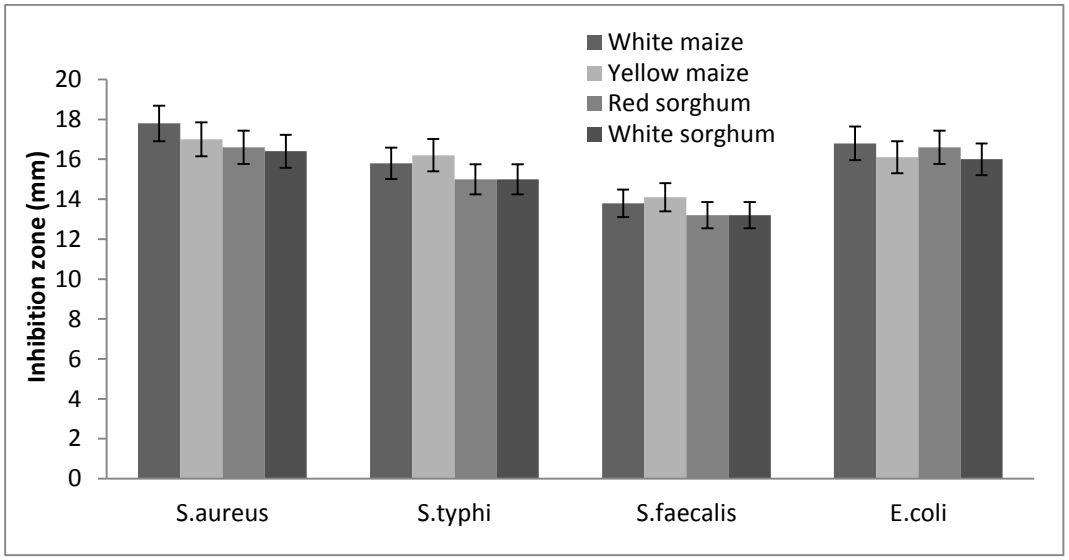

Figure 4b: Antibacterial activity (mm) of extracts from dried bamboo leaves with different steep liquors at 20g/40mL.

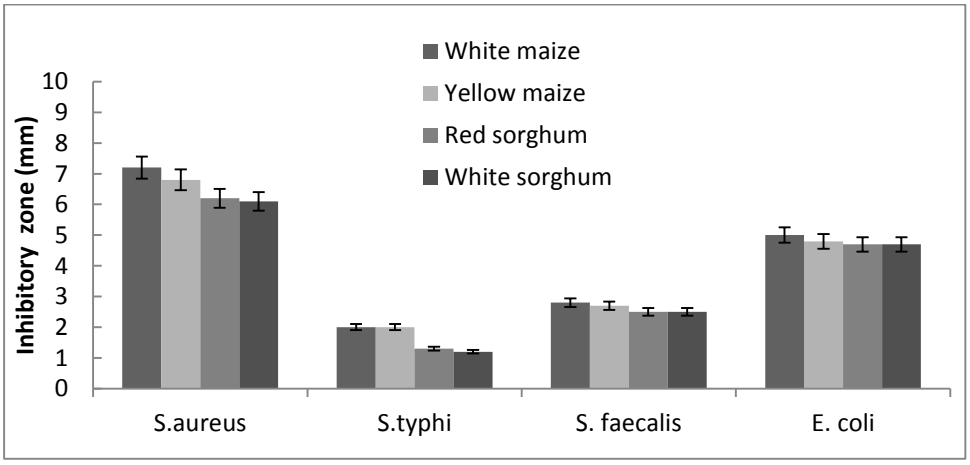

Figure 5a: Antibacterial activity (mm) of extracts from fresh bamboo leaves with different steep liquors at 20 g/100 mL.

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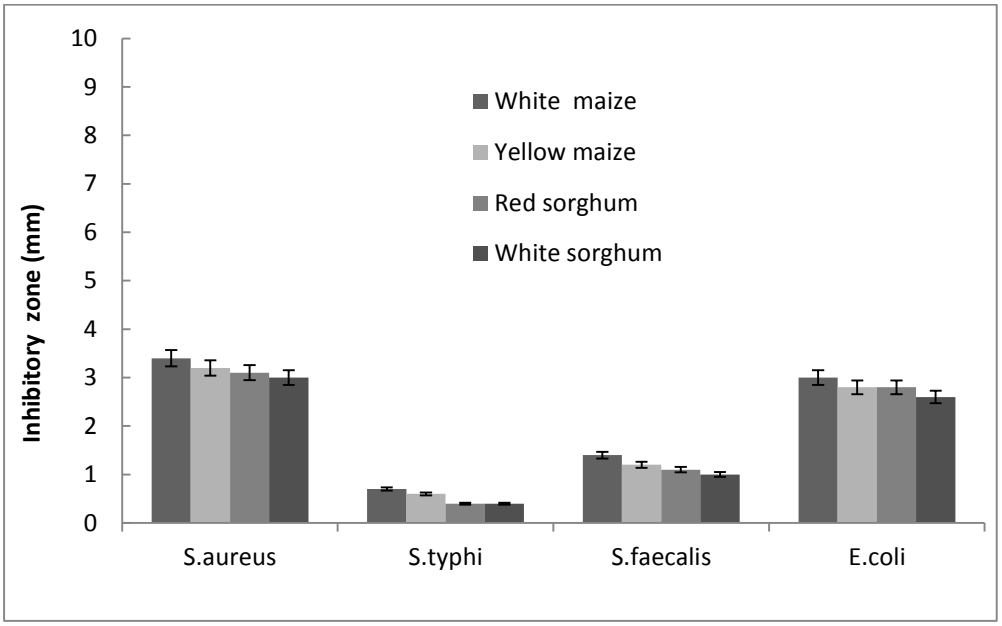

Figure 5b: Antibacterial activity (mm) of extracts from heating fresh bamboo leaves with different steep liquors at 20 g /100 mL.

DISCUSSIONS

The microorganisms identified in the fermented steep liquors were the *Lactobacillus* strains which were in accordance with those earlier identified by Omemu and Faniran (2011) from other cereal fermented products. These include *Lactobacillus acidophilus*, *L. casei*, *L. fermentum*, *L. lactis*, and *L. plantarum*.

The lactic acid bacterial count of the steep liquors generally increases with the days of fermentation. The trend of the total lactic acid bacterial counts was in agreement with the report of Omemu and Faniran (2011) in their Ogi production; for this study also the white corn has the highest microbial count and followed by the white sorghum. This provides support for the use of the steep liquors since the bacteria involved are probiotic as confirmed by Omemu and Faniran (2011). This is in agreement with the findings of Chavan and Kadam (1989) that fermentation of cereals increased nutrient availabilities. Adebolu *et al*. (2007) showed that fermented steep liquor (*Ogi*) is composed mainly of lactic acid bacteria and bioactive components which had inhibitory activity on pathogenic organisms. Components such as bacteriocins which are antimicrobial compounds with inhibitory effects towards sensitive microorganisms, which act by destroying the bacterial membrane, are the major components of the *Lactobacillus* species of the fermented steep liquors (Ogunbanwo *et al*., 2003). The presence of the Lactobacilli was reported by Lindgren and Dobrogosz (1990), Brink *et al.* (1994), and recently by Omemu and Faniran (2011), to produce various compounds such as organic acids, diacetyl alcohol, and hydrogen peroxide during lactic acid fermentation which has inhibitory properties. These were actually isolated from the liquors. Other bioactive component that may likely be present in the liquor is lactic acid which is normally determined as titratable acidity and expressed as the % of lactic acid content. Since the total titratable acidity observed ranged from 13-22%, this points to the fact that lactic acid was actually present in the liquors.

In addition, the low pH of the liquors (Table 2) could also be partly responsible for the inhibition because most bacteria do not grow at low pH except for few such as the lactic acid bacteria. This is in agreement with the findings of Nout *et al*. (1988) on the antimicrobial properties of fermented sorghum-based porridge from Lesotho against *Salmonella typhimirium* (Mensah *et al*., 1990) and on fermented maize dough against a variety of diarrhoeal pathogens in Ghana. This also agrees with the findings of Mbugua (1988) on fermented products such as *Uji* and *Motoho* against *Shigellaboydii, Salmonella typhi*, *and Escherichia coli.*

In an earlier study (Antony *et al*., 1998), authors concluded that inhibition of *Salmonella typhimirium* and *Escherichia coli* was more effective after a longer period of fermentation of finger millet flour, suggesting that metabolites produced by the fermenting microbes may have played a role in this effect. The unfermented millet sample also inhibited the pathogens on prolonged incubation for 48 h. This also confirms that the fermenting microbes from maize and sorghum, which are mainly probiotic, play a significant role in the antimicrobial properties of the extract (Omemu and Faniran, 2011).

Another factor that could produce this antimicrobial activity is the reduction in pH that occurs during fermentation, according to Mensah *et al*. (1991) since

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most enteropathogens are highly specific in their pH requirements. For example, *E. coli*, *Shigellae* and *Salmonellae* can survive a minimum pH of about 4.5 and a maximum between 8.0 and 9.0 (Jay, 1986). The total titratable acidity of the steep liquors increased for the first five days and decreased on the sixth and seventh day of fermentation. This increase was the major contribution of the reduction in pH (Figure 2).

The increased antimicrobial activities of the dried leaves extract from fermented steep liquors can be attributed to the process of drying which either removes or concentrates some components of the leaves, which is in line with the findings of Zhang (2002) and Yang (2002) that bamboo leaves have antimicrobial properties. Zhang *et al*. (2006) demonstrated that the phenolic compounds, antraquinones, and coumarins of the bamboo leaves are all of relatively strong antibacterial and bactericidal functionalities. From Figure 5a and 5b, it can be deduced that the effect of heat on the process of extraction reduced the activity of the extract on the tested organisms.

It can be inferred from this study that fresh *B. tuldoides* cv*. ventricosa* leaves macerated in fermented maize liquor gave the highest antibacterial activity and shows more sensitive to *S. aureus* amongst all the organisms tested.

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