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Effect of bitter leaf (*Vernonia amygdalina*) Extract on microbial stability of smoked-dried African catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract
The paper evaluates the stability of microbes on of smoked-dried African catfish (*Clarias gariepinus*) treated with different concentrations of bitter leaf *(Vernonia amygdalina)* extract. The experimental treatments were the control, 0.5%, 1%, 1.5% (w/v) bitter leaf extract solutions. A total of fifty two fish of average mean weight of 30.98±1.32g were gutted, washed and randomly assigned to the treatments. Thereafter the fish were soaked in the treatments for 30 minutes and later smoked-dried for 12 hours. After smoking the fish were stored in cartons and placed on laboratory table for 21 days. Six bacterial species namely, *Bacillus subtilis, Corynebacteria sp, Proteus mirabilis, Streptococcus faecalis, Staphylococcus albus and Staphylococcus aureus* were observed in the study. There was no increase in the microbial loads after 21 days of storage in samples treated with 0.5% 1.0% and 1.5% solutions of the bitter leaf extract, but there was significant increase in control from 27×10^3cfu to 40×10^3cfu. The sample treated with 1.5% solution of the bitter leaf extract exhibited the highest antibacterial effect and gave the best result. Therefore, *V. amygdalina* extract solution could be used in protecting stored smoked-dried catfish from bacterial spoilage, thus limiting economic loss and possible health risk to consumers.

Keywords: bitter leaf, extract, microbial stability, *Clarias gariepinus*, smoked-dried

1. Introduction
Fish is highly perishable because it provides favourable medium for the growth of microorganisms after death [1]. An estimate of 40% postharvest losses of total fish landings have been reported in Nigeria [2]. Fish spoilage in Nigeria is influenced to a large extent by high ambient temperatures, considerable distances from landing sites to the points of utilization and inadequate infrastructure for postharvest processing and handing [3]. Thus, it is imperative to process and preserve some of the fish caught in the period of abundance, so as to ensure an all year round supply. This will invariably reduce postharvest losses, increase the shelf-life of fish, and guarantee a sustainable supply of fish during off season with concomitant increase in the profit of the fishermen [4]. Proper preservation starts from the moment fish is harvested until it reaches the consumer's table (Oluborode *et al*., 2010).
Vernonia amygdalina commonly called bitter leaf is the most widely cultivated species of the genus *Vernonia* which has about 1,000 species of shrubs [6]. It belongs to the family *Astaraceae*. It is vegetatively cultivated by stem cutting and popular in most of West African countries including Nigeria, Cameroon, Gabon and Congo Democratic Republic. It was named after an English Botanist William Vernon. It is also referred to as ironweed. Bitter leaf is called Omjunso in East Africa especially Tanzania, Onugbo in Igbo-Eastern Nigeria and Orugbo among the Itsekiri and Urhobo tribes in Nigeria, Ewuro (Yoruba), Etidot (Ibibio), Ityuna (Tiv), Oriwo (Edo), Shiwaka (Hausa).
The leaves are eaten, after crushing and washing thoroughly to remove the bitterness [7]. All parts of the plant are pharmacologically useful. Both the roots and leaves are used in phyto-medicine to treat fever, hiccups, kidney disease and stomach discomfort, among others [8]. Antihelmitic and antimalarial properties [9] as well as antitumorigenic properties [10], have also been reported for extracts from the plant. The aqueous extract of the leaves has been found to inhibit the growth of the gram +ve bacterium *Staphylococcus aureus* and gram –ve bacterium *Escherichia coli* [11]. There has not been any documented report on the effect of bitter leaf

(*Vernonia amygdalina)* extract on microbial stability of smoked-dried *Clarias gariepinus.* Therefore, this research was designed to assess the possibility of improving the microbial stability of smoked-dried *Clarias gariepinus* using bitter leaf (*Vernonia amygdalina*) extract.

2. Material and Methods

Study area

The study was conducted in fish processing unit, Department of Fisheries, Faculty of Agriculture, University of Maiduguri, Borno State. The department is located at latitude 11º8'N and longitude 13º3'E and the state has two distinct seasons, that is rainy season with annual rain fall of about 600mm from July to October and a hot dry season from March to July. The dry season is preceded by a period of harmattan (November to February) with very low temperature (Encarta 2009). The state occupied a land area of 70,898km^2 (27,3745sq mil) making it the second largest state in terms of surface area after Niger state with a population of 4,151,193m (Census 2006) and with high ambient temperature.

Procurement of the bitter leaf

Fresh leaves of *V. amygdalina* were collected from Shoakari village, Jere local government Maiduguri, Borno state, Nigeria. The fresh leaves were air dried under shade for two days and they were grounded into fine powder using pestle and mortar, a solution was prepared by adding separately specific quantity (5g, 10g and 15g) of the bitter leave powder extracts to 1000ml of distilled water to form three treatments of 0.5%, 1% and 1.5% concentration respectively and were allowed to stay for 24 hours. No extract was added to the control treatment.

Procurement of fish samples

A total of 7000g of fresh African catfish (*Clarias gariepinus*) was procured from Gamboru fish market in Maiduguri Metropolis Council, Borno State which ranges in length from 23.95 – 26.65cm. The fish were collected in the morning in cold flask and transported to the fish processing unit. The processing and smoking of the fish were carried out in this unit. The fish were killed by striking the spinal cord, gutting using sharp knife by cutting laterally from the end of the gill cover through the belly portion to the anus. The weight of the fish were noted after gutting. Thereafter they were thoroughly washed with clean tap water to keep them in hygienic state.

Experimental design

The fish were randomly assigned to four experimental treatments. The treatments were divided based on the concentration of the extract in the solution to 0%, 0.5%, 1% and 1.5%. Each treatment was replicated thrice with 500g weight fish. The fish were soaked into the aqueous solution of the bitter leaf extract for 30 minutes. Thereafter, the fish were placed on wire mesh and allow to drain under shed. Light was set for the smoking kiln to glow for ten minutes. Then the fish were arranged based on their treatments and replications in the smoking kiln consisting of three racks and hard wood was used for ignition. The smoking process takes 12 hours and the fish were weight at the first 30min and after each 1 hour for all the smoking period until a minimum weight was obtained and the temperature was regulated between 45-85ºC. After the smoking the smoked-dried fish were allowed to cool and packed in different cartons based on their treatments and then transferred to a cool dry place save from any contamination in the laboratory for storage. Some of the samples were taken for microbial analysis at seven days interval for a period of 21 days.

Total bacterial count

The total bacterial count was carried out using tenth fold serial dilution with normal saline. 9ml each of the normal saline was dispensed in a sterilized test tube and 1gram of the sample was weighed and transferred into a sterile universal bottle and 9ml of normal saline was added and shake well. Then 1ml was taken using sterile tests to make 10 fold serial dilution, then 0.1ml was transferred from the 3rd tube to a sterile dried nutrient agar and spreaded, it was later incubated at 37ºC for 24 hours. The colony were counted using colony counter and result was recorded and presented as colony forming unit (cfu).

Identification of microorganism

The isolate were identified by conventional methods starting with grams staining briefly, using a sterile wire loop a drop of distilled water was put on the center of grease-free slide and a portion of colony was picked and emulsified into drop of sample and allow to air dry before fixing to gram stain, crystal violet was then applied for 3 minutes. It was then replaced with a gram's iodine for one munities, priori to rinsing with water and application of 95% alcohol until no colour appeared on the flow. Slides were then rinsed with water and safranin was applied for 1-2 minutes. This was folled by rinsing and air drying and then observed microscopically under x 100 emersion oil objective.

Data analyses

All the data were subjected to the one way analysis of variance and a significance test for difference among sample variance using the Least Significance Difference (LSD) in the mean comparison of means at $p<0.05$ level of significance with aid of statistix – version 9.0.

3. Results

Table 1 shows the weight loss of *C. gariepinus* during smoking period of twelve hours at temperature of 85ºC for the first hour and the remaining eleven hours maintained the temperature of heat at 45-85ºC for smoking trail.

Table 1: Difference in weight lost after twelve hours of smoking Clarias gariepinus treated with a solution of bitter leaf extract.

Bitter leaf extract concentration level (%)	Initial of fish weight(g)	Final weight(g)	Weight loss (g)	Percentage weight loss
0.0	500	170	330	66%
0.5	500	175	325	65%
1.0	500	180	320	64%
1.5	500	200	300	60%

Table 2 shows the microbial population in fresh fish sample and sample smoked with bitter leaf (*V. amygdalina*) extract at the level of 0%, 0.5%, 1% and 1.5% concentration. The highest value was recorded in the control ($25x10^3$) followed by ($23x10^3$) in sample smoked with 0.5% concentration of the extract. Least value was recorded in sample smoked with 1.5% concentration of the extract with value ($9x10^3$).

Table 2: Microbial population (cfu/g) in fresh Clarias gariepinus sample

Fresh fish sample	Total viable count (cfu/g)
Clarias gariepinus	76×10^3

Table 3: Microbial population (cfu/g) in fish smoked with different concentration of bitter leaf extract

Bitter leaf concentration (%)	Total viable count (cfu/g)
0.0	25×10^3
0.5	23×10^3
1.0	10×10^3
1.5	9×10^3

Table 3 shows change in microbial loads of smoked dried fish treated with bitter leaf extract solution of different levels of concentration during storage at room temperature for the period of twenty one (21) days. After one week (7) days of storage, the population of bacterial decrease in all the treated samples except in control which increase from (27×10^3) to (37×10^3). After (21) days of storage, decrease in microbial loads was recorded in all levels of concentration except for the sample treated with 0% extract concentration which increase from (38×10^3) to (40×10^3). *Clarias gariepinus* treated with 1.5% solution of the bitter leaf extract was recorded the lowest in microbial loads (7×10^3) after twenty one (21) days of storage.

Table 3: Changes in Microbial loads (populations cfu/g) of smoke fish after smoking with different concentration of bitter leaf extract stored at room temperatures.

Bitter leaf Concentration level (%)	1 day	7 days	14 days	21 days
0.0	27×10^3	37×10^3	38×10^3	40×10^3
0.5	25×10^3	23×10^3	16×10^3	12×10^3
1.0	26×10^3	20×10^3	13×10^3	10×10^3
1.5	25×10^3	24×10^3	9×10^3	7×10^3

Table 4 shows the percentage of bacteria isolated in fish smoked with different concentration of bitter leaf extract. Five bacteria species were isolated, *Bacillus subtilis, Corynebacteria specie, Streptococcus faecolis, Staphylococcus albus and Staphylococcus aureus.* Highest percentage of bacteria isolated was recorded in sample smoked with 0% and 0.5% concentration of the bitter leaf extract with same value of 28% and lowest percentage was recorded in sample treated with 1% and 1.5% of the extract with same value of 22%.

Table 4: Bacterial isolation and identification in fish smoked with bitter leaf extract at different concentration level for 21 days

Bacterial isolation	Bitter leaf extract Concentration (%)	No. of species	Percentage (%)
Bacillus subtilis *Corynebacteria specie* *Streptococcus faecalis* *Staphylococcus albus* *Staphylococcus aureus*	0.0	5	27.78
Bacillus subtilis *Corynebacteria specie* *Proteus mirabilis* *Staphylococcus albus* *Staphylococcus aureus*	0.5	5	27.78
Bacillus subtilis *Staphylococcus albus* *Corynebacteria specie* *Staphylococcus aureus*	1.0	4	22.22
Bacillus subtilis *Corynebacteria specie* *Staphylococcus albus* *Staphylococcus aureus*	1.5	4	22.22
Total		18	100

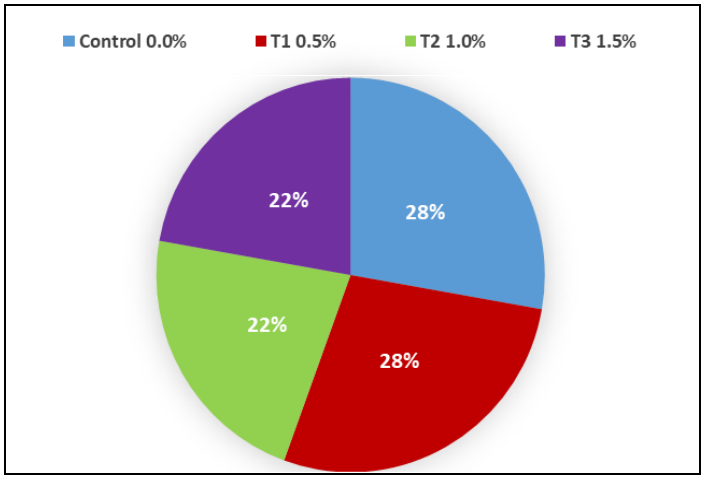

Fig 1: Percentage of bacteria isolated and identified in fish smoked with bitter leaf extract at different concentration level for 21 days

4. Discussion

The result of the weight characteristics of *C. gariepinus* treated with different solution of bitter leaf extract before and after smoking for 12 hours was presented in (Table 1). The percentage of average weight loss 63.75% was recorded which is similar to the percentage of average weight loss 63.34% obtained by [12] in their research work on the nutritional and storage quality of catfish (*C. gariepinus*) smoked with *Anthonotha macrophylla.*

From the total samples of smoked fish examined, the result shows bacteria contaminants in all the samples. The different types of bacteria isolated and identified were *Bacillus subtilis, Corynebacteria specie, Proteus mirabilis, Streptococcus faecalis, Staphylococcus albus and Staphylococcus aureus*. These micro-organisms may have contaminated the smoked fish through human handling, air and soil. The presence of this micro-organisms in smoked fish samples might be due to increase in moisture content of the product during storage and also increase in temperature which favours the growth of this organisms [13].

During handling of fish, the natural flora of the fish environment will be contaminated with organisms associated with man, such as members of *Enterobacteriaceae* and *Staphylococcus aureus* as which grow well at 30-70ºC the occurrence of *Staphylococcus* in the smoked dried fish samples was in accordance with [14] when he stated that this organisms is one of the most commonest micro-organisms associated with smoked fish and this bacteria (*Staphylococcus albus*) in fish sample according to [15] might have been through handling. The prevalence if *Staphylococcus albus* is not public health significance because it has been incriminated with food-borne intoxication and infection. Its ability to produce enzymes and toxins is responsible for the effect [16]. This organisms must have contaminated the smoked fish through human handlers. The presence of *Bacillus specie* may also result in food borne illness.

Bacterial population was high in the control 25×10^3 followed by 23×10^3 in sample smoked with 0.5% concentration of the extract. Least value was recorded in sample smoked with 1.5% concentration of the extract with value 9×10^3. In the changes in microbial loads of smoked fish products during storage at room temperature for the period of twenty one (21) days, the result agreed with work of [17] who reported that D-limo-nene abundant in Orange peel is known as an antimicrobial agent.

After one week (7) days of storage, the population of bacterial decrease in all the treated samples except in control which increase from 27×10^3 to 37×10^3. After (21) days of storage, decrease in microbial loads was recorded in all levels of concentration except for the sample treated with 0% extract concentration which increase from 38×10^3 to 40×10^3. *Clarias gariepinus* treated with 1.5% solution of the bitter leaf extract was recorded the lowest in microbial loads 7×10^3 after twenty one (21) days of storage.

5. Conclusion

In conclusion, there was positive significant influence of bitter leaf extract on the microbial stability of smoked-dried *Clarias gariepinus* making it naturally suitable and microbial stable. Six bacterial species namely, *Bacillus subtilis, Corynebacteria sp, Proteus mirabilis, Streptococcus faecalis, Staphylococcus albus and Staphylococcus aureus* were observed in the study. The sample treated with 1.5% solution of the bitter leaf extract exhibited the highest antibacterial effect and gave the best result. Therefore, bitter leaf (*Vernonia amygdalina*) extract solution could be used because of it anti-bacterial properties, therefore protecting stored smoked-dried catfish from bacterial spoilage, thus limiting economic loss and possible health risk to consumers.

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