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## Microbial Evaluation of *Carica papaya* Leaf Extract Pre-Treated Smoke cured Grass Eater (*Distichodus rostratus* Gunther 1864)

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### **ABSTRACT**

The sensory and microbial evaluation of *Carica papaya* leaf extract pre-treated smoke cured *Distichodus rostratus* (Grass eater) was studied. Twelve (12) fish samples weighing between 0.80kg-1.80kg were weighed eviscerated and washed properly with tap water. These were randomly divided into 4 groups of 3 fish each (T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> & T<sub>4</sub>). T<sub>1</sub> was immersed into 3% Brine only. T<sub>2</sub> was immersed in a mixture of 3% brine and 2.5% *C. papaya* leaf extract. T<sub>3</sub> was immersed into 3% Brine and 5% *C. papaya* leaf extract. T<sub>4</sub> was immersed into 3% Brine and 8% *C. papaya* leaf extract. These mixtures were thoroughly mixed and allowed to stand for three hours. Soaked samples were poured out on muslin clothing for draining and cured with wood smoke on a brick kiln for four hours at a temperature range of 85°C-95°C. The cured samples were left overnight to cool and were wrapped in sterile polythene bags and kept in a refrigerator for microbial analysis. The samples were subjected to microbial analysis, culturing, incubating, colony count and statistical analysis. Increasing concentrations of water extract of *C. papaya* leaf inhibited microbial growth at all dilutions (highest T<sub>1</sub>{control} was  $2.6 \times 10^7$ cfu/ml while T<sub>2</sub>=  $2.2 \times 10^5$ cfu/ml, T<sub>3</sub>=  $1.5 \times 10^5$ cfu/ml and T<sub>4</sub>=  $1.24 \times 10^4$ cfu/ml, decreased in that order). Probable organisms in T<sub>1</sub> were *Staphylococcus aureus*. T<sub>2</sub> and T<sub>3</sub> had *Proteus* sp. T<sub>4</sub> had *Klebsiella* sp. Biochemical characterisation of pure culture isolates confirmed the presence of these organisms. The fungi found were *Aspergillus* (T<sub>1</sub>), *Penicillium* (T<sub>2</sub> & T<sub>3</sub>) and *Mucor* (T<sub>4</sub>). These results show that usage of *C. papaya* in processing and preservation of fish will improve the shelf life of stored fish.

**Keywords:** Microbial; Grass-eater; Evaluation; leaf-extract; Smoke-cured; *Carica-papaya*; Shelf life.

### **INTRODUCTION**

Estimates show that about a million people worldwide rely on fish as their primary source of animal

protein (FAO, 2000). Fresh fish is perishable. Various methods have been devised to prolong its' shelf

life. Smoke curing is the predominant means of fish preservation in the third world. Unfavourable environmental conditions shorten the shelf life of smoke cured fish (FDA, 2001). Vital nutrients, chemical spoilage, microbial spoilage and consequent economic losses are associated with this problem (Gram et al, 2004). This results in scarcity of protein and fish mongers and Artisanal fishermen do not reap the full benefits of their effort (Osifo, 2004 and Feldhusan 2000).

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*C. papaya*, a member of the family Caricaceae is palatable feedstuff whose leaves, fruits and byproducts are used to feed domestic animals (Ecocrop, 2009). Extracts of different parts of the plant have antibiotic properties (Baskaran et al., 2002, Fakeye et al., 2007, Ayoola and Adeyeye, 2010, and Ahmed et al, 2010). The enzyme, Papain from *C. papaya* is still in use as a meat tenderizer of which the leaves contain 2% of the enzyme. Latex from the plant is known to heal wounds while the leaves are used in dressing wounds. The plant does not contain cyanogenic glycosides

Deleterious results have been obtained from use of synthetic materials to check microbial and chemical spoilage of fish (Aouny, 2006). Due to this problem, attention has been turned to spices and other plant materials that are user friendly and medicinal too in the processing and preservation of fish and meat products (Svirivasan et al., 2001, Sandeep, 2013, Hubbard 2014 and UNICAF, 2014). Several of these including *Carica papaya*, make the long list that serve as food, medicine, etc. and grow well in the West African sub region.

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and is thus safe for inclusion into edible products (Imaga et al., 2010). Extracts of dried leaves of the plant have demonstrated antioxidant properties (Ona, 2002 and Ekeke et al., 2005).

*Distichodus rostratus* (Grass eater) is commonly landed by artisanal fishermen and it is a common fish commodity on the table of fish mongers in fish markets (Idodo Umeh, 2003) and therefore deserves to be well preserved since they are caught in large quantities. Literature search does not show that *C. papaya* leaves have been used in fish

preservation. The aim of this work is to evaluate the inhibitory potentials of aqueous leaf extracts of *C.papaya* on the microbial load of smoke cured pre-treated fish in an attempt to extend its shelf life

## MATERIALS AND METHODS

### Sample Collection

12 Specimens of *D. rostratus* of mean weight  $1120 \pm 0.45\text{g}$  were purchased from Yenagoa, Bayelsa State and transported to the fish processing laboratory of Imo State Polytechnic, Umuagwo, Ohaji, South eastern Nigeria, in a 20L ice chest filled with crushed ice but without direct contact to avoid microbial cross contamination.

### Sample Preparation

Fish were sorted into four groups ( $T_1$ ,  $T_2$ ,  $T_3$  and  $T_4$ ), weighed, degutted, washed and weighed again to obtain the dress weight. The extracts were made by soaking 250g, 500g and 800g of squashed *C. Papaya* leaves in 10L of water to which 300g of salt had been added for 4hours. These were soaked for one hour as follows:  $T_1$  + 3% brine,  $T_2$  + 3% brine + 2.5% *C.papaya* extract,  $T_3$  + 3% brine + 5% *C. papaya* extract and  $T_4$  + 3% brine + 8% *C.papaya* extract.

### Fish Processing

The extract pre-treated samples were then drained on muslin and cured with wood smoke for 4hours with turning at intervals to prevent charring and promote even curing, according to the methods of Agbabiaka (2002). Cured fish was left under ambience for one day. Fish was weighed to obtain dry weight before being wrapped in sterile polythene bags and storage in refrigerator until analysis.

### Biological Evaluation

The weight loss was computed as follows:

Dressed weight = Carcass weight - weight of offals

Total weight loss = Carcass weight - weight after smoking

%weight loss =  $(\text{Total weight loss} / \text{Carcass of fish}) \times 100$ .

### Microbiology

The procedures described in Agbabiaka *et al.*, (2016) were adopted.

### Media Preparation

Nutrient Agar (NA), Eosin Methylene Blue (EMB) Agar and Potato Dextrose Agar (PDA) were used for the media preparation according to Cheesebrough (2000). 28 g of Nutrient Agar (NA), 31.2g of

Potato Dextrose Agar (PDA), and 17.28 g of Eosin Methylene Blue (EMB) Agar were measured out according to the manufacturer's direction. Thereafter, the three measured media were dispersed into 3 conical flasks and 1 litre of distilled water was added respectively and shook vigorously for proper mixing. The media were autoclaved for 15 minutes at 120°C and cooled at room temperature respectively. After that, 20 ml of each of the media were poured into 12 plates, i.e., quadruplet per agar sample. Other instruments such as wire loop, petri dishes, pipette, and beakers were sterilized. In the preparation of Potato Dextrose Agar (PDA), broad spectrum antibiotic (Chloramphenicol) was added to prevent the growth of bacteria.

#### **Serial Dilution**

Serial dilutions of each homogenate were prepared as described by Cheesbrough (2000). Ten (10) fold serial dilution was made for each fish sample, 5 test-tubes were filed with 9ml of peptone water, 1g of the sample was dissolved and later transferred with syringe into assigned test tube (making it 10 ml) and thoroughly mixed; further sequential dilution were made by taking 1 ml from each of the 10 ml

mixture into other test tubes respectively.

#### **Culturing, Incubating, Colony Count and Identification**

These methods were carried out according to Cheesbrough (2000). After the serial dilution, 1 millilitre of each sample taken from 2nd and 3rd ( $10^{-2}$  and  $10^{-3}$ ) test tubes were transferred to petri- dishes that have been appropriately labelled. The spread plate method was used for culture. 2-3 drops of the diluents sample were dropped in each of the media and a bent glass rod dipped into ethanol and sterilized in an open flame was used to distribute the dropped samples in the media evenly and was repeated for other samples. The plates for bacterial count were kept on laboratory bench and allowed for 24 hours, while that of fungi and coliform were kept for 48 hours at room temperature. Thereafter, the bacterial count was done and the colonies that appeared as clusters in each plate was counted and recorded. Similar counts were done on fungi and coliform. The numbers were counted and recorded; identification was carried out using standard methods with biochemical tests such as Gram Staining Techniques, Catalase Test, Motility

Test, Indole Test, Citrate Test, Methyl-Red and Vogues Proskaur according to Cheesebrough [2000].

### Microscopic Examination of Microbes

The traditional method in the microscopic examination of bacteria in the laboratory is the grams staining method. The description of the staining method was extracted from Cheesebrough [2000], while the method of the microscopic study of fungi was conducted according to Harrigan and Mclance [1990].

### Procedure of the Gram Staining

The Gram stain is basically four step involving water rinses after each step. The smear was air dried and gently heat fixed. Flood the slide

with crystal violet (30 seconds) and wash with tap water. Flood with Grams iodine (brown) for 30 seconds and wash with tap water. Carefully decolorize with 70% ethanol for 10-15 seconds until the thinnest parts of the smear are colourless. Wash with tap water. Flood with Safranin (red) for 30 seconds and wash with tap water. Thereafter, place it at the draining rack for drying before viewing under microscope. Representative colonies of the microorganisms were gram-stained, purified and stored in nutrient agar slants at 4 °C.

### STATISTICAL ANALYSIS

Analysis of variance (ANOVA) test and DMRT for mean separation were used (Duncan) were used.

## RESULTS AND DISCUSSION

**TABLE 1: Weight characteristics of smoked cured fish, *Distichodus rostratus* pre-treated with leaf extracts**

Samples	Live weight	Dressed weight	Weight after smoking	Total weight loss	% weight loss
T <sub>1</sub>	800	600	317	483	60.3
T <sub>2</sub>	1500	1200	453	1047	69.8
T <sub>3</sub>	1400	1200	352	1048	74.8
T <sub>4</sub>	1800	1500	661	1139	63.2
<b>Mean weight loss = 67.0%</b>					

**KEYS:**

T<sub>1</sub> = Fish sample treated with 3% brine

T<sub>2</sub> = Fish sample treated with 2.5% *Carica papaya* leaf extract and 3% brine.

T<sub>3</sub> = Fish sample treated with 5% *Carica papaya* leaf extract and 3% brine

T<sub>4</sub> = Fish samples treated with 8% *Carica papaya* leaf extract mix with 3% brine.

**TABLE 2: Total bacterial heterotrophic fungi plate count on *Carica papaya* leaf extract pre-treated smoke cured fish**

Samples	Dilution	TVC on NA	TVC on MAC	Isolated conc on NA	Isolated conc on MAC	TFC on PDA
T <sub>1</sub>	1 x 10 <sup>2</sup>	TNTC	52	-	5.2 x 10 <sup>5</sup>	4.8 x 10 <sup>3</sup>
	1 x 10 <sup>4</sup>	260	32	2.6 x 10 <sup>7</sup>	3.2 x 10 <sup>4</sup>	
T <sub>2</sub>	1 x 10 <sup>2</sup>	TNTC	20	-	2.0 x 10 <sup>4</sup>	4.0 x 10 <sup>3</sup>
	1 x 10 <sup>4</sup>	220	-	2.2 x 10 <sup>5</sup>	-	-
T <sub>3</sub>	1 x 10 <sup>2</sup>	TNTC	12	-	1.2 x 10 <sup>4</sup>	3.0 x 10 <sup>3</sup>
	1 x 10 <sup>4</sup>	150	-	1.5 x 10 <sup>5</sup>	-	-
T <sub>4</sub>	1 x 10 <sup>2</sup>	TNTC	1	-	1.0 x 10 <sup>4</sup>	1.0 x 10 <sup>3</sup>
	1 x 10 <sup>4</sup>	124	-	1.24 x 10 <sup>4</sup>	-	-

**KEYS:**

TVC = Total Viable Count

TFC = Total Fungal Count

NA = Nutrient Agar

MAC = MacConkey Agar

TNTC = Too Numerous To Count

- = No Growth

**TABLE 3: Colonial morphology of bacterial pure culture from *C. papaya* leaf extract pre-treated smoke cured fish**

Samples	Media	Dilution	Shape	Elevation	Size	Chromogenese	Probable organism
T <sub>1</sub>	NA	10 <sup>-2</sup>	Round	Flat	Small	Pink	<i>Staphylococcus sp</i>
T <sub>1</sub>	NA	10 <sup>-4</sup>	Round	Flat	Small	Pink	
T <sub>1</sub>	MAC	10 <sup>-2</sup>	Circular	Flat	Punctiform	Yellow	
T <sub>1</sub>	MAC	10 <sup>-4</sup>	Irregular	Flat	Punctiform	Yellow	

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T <sub>2</sub>	NA	10 <sup>-2</sup>	Round	Viscid	Moderate	Cream	<i>Proteus sp</i>
T <sub>2</sub>	NA	10 <sup>-4</sup>	Round	Viscid	Moderate	Cream	
T <sub>2</sub>	MAC	10 <sup>-2</sup>	Irregular	Flat	Moderate	Yellow	
T <sub>2</sub>	MAC	10 <sup>-4</sup>	Irregular	Flat	Moderate	Yellow	
T <sub>3</sub>	NA	10 <sup>-2</sup>	Irregular	Convex	Large	Pink	<i>Proteus sp</i>
T <sub>3</sub>	NA	10 <sup>-4</sup>	Round	Convex	Large	Cream	
T <sub>3</sub>	MAC	10 <sup>-2</sup>	Circular	Flat	Moderate	Cream	
T <sub>3</sub>	MAC	10 <sup>-4</sup>	Round	Flat	Moderate	Cream	
T <sub>4</sub>	NA	10 <sup>-2</sup>	Circular	Viscid	Small	Cream	<i>Klebsiella sp</i>
T <sub>4</sub>	NA	10 <sup>-4</sup>	Circular	Viscid	Small	Cream	
T <sub>4</sub>	MAC	10 <sup>-2</sup>	Round	Flat	Punctiform	Cream	
T <sub>4</sub>	MAC	10 <sup>-4</sup>	Round	Flat	Punctiform	Cream	

**TABLE 4:** Characteristics of bacteria isolate from leaf extract pre-treated smoke cured fish. (*Distichodusrostratus*)

Samples	Cell morphology	Gram reaction	Coagulase test	Catalase test	Motility test	Citrate test	Indole test	Identified organisms
T <sub>1</sub>	COCCI		+	+				<i>Staphylococcus</i>
T <sub>2</sub>	ROD	+	-	+	+	-	+	<i>Proteus sp</i>
T <sub>3</sub>	ROD	+	-	+	+	-	+	<i>Proteus sp</i>
T <sub>4</sub>	ROD	+	-	+	-	+	-	<i>Klebsiella sp</i>

**KEYS:**

+ = Positive

- = Negative

**TABLE 5:** Macroscopic / microscopic morphology of fungi isolates

Samples	Macroscopy	Microscopy	Probable fungal
T <sub>1</sub>	Powdery, dark brown, flatly spread on the surface of the medium with brown reverse	Septate and branched typhae and conidia in chains	<i>Aspergillus sp</i>
T <sub>2</sub>	Grey colonies that were large white border. Reverse was white	Long conidiophores consisting of broom like conidia in chains	<i>Penicillium sp</i>
T <sub>3</sub>	Grey colonies that were large with	Long conidiophores	<i>Penicillium sp</i>

	border. Reverse was white	consisting of broom like conidia in chains	
T <sub>4</sub>	Grey to black and thick abundant cottony, mycelium and white reverse.	Now separate with sporangium containing black sporangiophores. Columella separated by septum and without rhizoids	<i>Mucor sp</i>

The results of the weight characteristics of Carica papaya leaf extract pre-treated smoke cured *Distichodus rostratus* is presented in Table 1. The range of the weight loss is 60.2% - 74.8%. The mean weight loss is 67.0% and this range is in agreement with the 65.00% recommended by Cardinal *et al.*, (2001).

The results of total bacterial count are summarized on Table 2. The highest microbial count recorded in T<sub>1</sub> was  $2.6 \times 10^7$ cfu/ml while the lowest were  $2.2 \times 10^5$ cfu/ml,  $1.5 \times 10^5$ cfu/ml and  $1.24 \times 10^4$ cfu/ml recorded in samples T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub> respectively. It is interesting to note that the high microbial count in control (T<sub>1</sub>) is in excess of  $10^6$ cfu/ml, which if exceeded in microbiology; product may be declared unfit for human consumption (Cheesebrough 2000). Results in leaf extract pre-treated smoke cured fish at increasing concentrations (T<sub>2</sub>-T<sub>4</sub>) proved to be progressively potent in their bactericidal activity. The

counts recorded were of the order of  $\leq 10^5$  which is the normal limit of plate count in microbiology for food fit for consumption (ICSMF, 1986 and PHLS 1992). The higher microbial count in sample T<sub>1</sub> (control) may be as a result of the salt pre-treatment on it without any added treatment. Adam and Moss (1999) noted that most organisms are salt tolerant. There is evidence to prove that smoke curing may not be as efficient as gas and electric oven in moisture removal hence permitting some degree of increased water activity that may aid microbial growth (Eyo, 2001). Hygroscopy is a renowned attribute of salt. While these factors may aid intrinsic and extrinsic facilitators of microbial establishment and survival in untreated fish (T<sub>1</sub>), inhibition may be promoted with addition and increasing concentrations of leaf extract. In corroboration of this supposition, lowest microbial count in sample T<sub>2</sub> and T<sub>3</sub>, especially T<sub>4</sub> can be attributed to the presence of anti -



microbial substances in the leaf extract of *C. papaya*. Aravind *et al.*, (2013)) reported that *C. papaya* contains proteolytic enzymes that have anti-viral, anti-bacterial and anti-fungal properties. This report supports the findings in this experiment.

The probable organisms' colonial morphology of bacterial pure culture is shown on Table 3. The identified organisms were *Staphylococcus* sp (T<sub>1</sub>), *Proteus* sp (T<sub>2</sub>) and (T<sub>3</sub>) and *klebsiella* sp (T<sub>4</sub>). This result is in agreement with the finding of Orhue *et al* (2013), which proved that the water extract of *C. papaya* leaf terminated such bacteria as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus* sp. The mentioned organisms were not found in any of the treated samples. Table 4 is the summary of the result of the cell morphology and biochemical characteristics of the bacteria isolates. The organism found in T<sub>1</sub> was *Staphylococcus* sp. The organisms found in T<sub>2</sub> and T<sub>3</sub> was *Proteus* sp. The organisms found in T<sub>4</sub> were *Klebsiella* sp. The result of biochemical and cell morphology characteristics of bacteria isolate confirm that T<sub>1</sub> was 'cocci,' T<sub>2</sub> and T<sub>3</sub> are 'rod,' and T<sub>4</sub> is also 'rod.' The identified organisms are therefore

*Staphylococcus* sp (T<sub>1</sub>), *Proteus* sp (T<sub>2</sub>) and (T<sub>3</sub>), *Klebsiella* sp (T<sub>4</sub>). *Staphylococcus* sp. is a gram positive bacteria while *Proteus* and *Klebsiella* species are gram negative bacteria present almost everywhere and are opportunistic pathogens, often nosocomial (Ryan and Ray, 2004). It may therefore be transmitted through equipment and handling by man in the laboratory onto the experimental samples.

The macroscopic and microscopic result of fungal isolates is represented on Table 5. The identified species of fungi on *C. papaya* leaf extract pre – treated smoke cured fish are *Aspergillus* sp (T<sub>1</sub>), *Penicillium* sp (T<sub>2</sub>) and (T<sub>3</sub>), *Mucor* sp (T<sub>4</sub>). The presence of *Aspergillus* sp is as a result of their aerobic nature and is found in almost all oxygen – rich environment where they commonly grow as moulds on the surface of a substrate, as a result of the high oxygen tension (Kirk *et al.*, 2008). The presence of *Penicillium* sp on sample T<sub>2</sub> and T<sub>3</sub> could be as a result of their spores in the environment, they are found in plants and most surfaces and are important in the production of drugs (Pitt, 1979). *Mucor* sp. Grows in every moist surface and survives

high temperature (International microbiology association, 2011).

## CONCLUSION

The results obtained in the microbial evaluation of *C. papaya* prove that a host of bacteria present in numerous numbers on fish smoked cured by the various available methods (Abidemi-Iromini *et al.*, 2011 ) are absent in *C. papaya* extract pre- treated smoke cured *D. rostratus*. This presents a new future in fresh fish handling and processing which leaves man with the option of taking full advantage of the bactericidal potency of *C. papaya* prolonging the shelf life and also putting the anti-oxidative properties of this plant to full use in preventing oxidative rancidity of smoke cured fish.

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