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Effects of Garlic *Allium sativum* Extract on Lipid Oxidation, Microbiological, and Organoleptic Qualities in Hot-smoked Sardines, *Sardinella longiceps* During Frozen Storage

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Abstract
Fatty fishes such as Indian oil sardines (*Sardinella longiceps*) are suitable for smoking but it contains high amount of polyunsaturated fatty acids making it very prone to lipid oxidation. Furthermore, the nature of the product also makes it susceptible to microbial spoilage and proliferation of pathogenic bacteria which may cause foodborne diseases outbreaks. The banning of synthetic antioxidants and antibiotics due to its adverse effects on human health has necessitated the use of natural antioxidants and antimicrobial agents such as herbs and spices. This study undertakes to determine the effectiveness of garlic (*Allium sativum*) extract (GE) as natural antioxidant and antimicrobial agent in smoked sardines.
The inhibitory effect of garlic (*Allium sativum*) extract (GE) on lipid oxidation in hot-smoked sardines (*Sardinella longiceps*) during frozen storage (-18 ºC) for 30 days was assessed by monitoring the peroxide value (PV), free fatty acid (FFA) and acid values of the fish oil. The antimicrobial effect of GE was determined by Aerobic Plate count. Changes in the quality during storage period were likewise determined using Descriptive Sensory Score Sheets.
Results of the study revealed that the use of garlic extracts (GE) at 5 g, 10, and 15 g concentrations showed significant reduction ($p<0.05$) in peroxide values. However, there was no significant difference in acid and free fatty values between the control and Garlic Extract-treated smoked sardines.
No significant difference in aerobic plate count was likewise observed between control and garlic treated samples. The addition of garlic extracts (5 g, 10 g, and 15 g) was found to have no significant effect on most of the sensorial characteristics except for color and smoky odor which have been affected at the 30th day of frozen storage. Moreover, the acceptability of smoked sardines with 15g garlic extract was significantly low at 30th day of frozen storage.

Keywords: *Allium sativum, Sardinella longiceps,* Hot-smoked, Peroxide, Free fatty, Aerobic Plate Count, Organoleptic Qualities.

1. Introduction
Small pelagic, fatty species such as sardines (*Sardinella longiceps*) have historically dominated fishery landings in the Philippines [1]. In 2011, the Philippine Fisheries Profile (2011) [2] reported the commercial production of sardines (*Sardinella longiceps*) as 167,014.31 MT or 16.2% and 65, 893.11 MT or 5.8% at marine municipal level. Sardines, together with anchovies, are the most inexpensive source of animal protein available to Filipinos, and thus, they are potential raw materials for value-added seafood products [3]. This fishery is an economic engine providing thousands of jobs and generating revenue at the individual, municipal, and national levels. Sardines lipids with their high polyunsaturated fatty acid (PUFA) content are nutritionally important. It improves health and reducing the risk of acquiring diseases.
Smoked fish is fish that has been preserved by the application of smoke with the aid of salting, drying, and heat treatment. Smoking is a very old preservation method [4] and the most popular method of fish preservation widely used in many developing countries [5]. There are two types of smoking, the hot smoking which temperature ranges from 66 to 88 °C and the cold smoking which temperatures ranges from 32 to 43 °C [6]. The former will be applied in this study.

Fatty fishes such as Indian oil sardines (*Sardinella longiceps*) are suitable for smoking. However, the high fat content makes them unstable to oxidation leading to the development of fishy, rancid odors [7] and thus, reduces its nutritional health benefits and quality. The oxidation of fatty acids is one of the most fundamental reactions in food chemistry [8]. It involves a reaction between lipid and molecular oxygen [9].

The unsaturation of EPA and DHA in fatty acids undergoes rapid oxidative deterioration [10, 11]. Lipids are susceptible to oxidative processes in the presence of catalytic system such as light and heat, due to this catalytic system, high temperature is believed to accelerate the rate of lipid oxidation [12]. The process of smoking, combined with frozen storage, is believed to make fish more prone to oxidative rancidity. The high temperature during hot-smoking ranges from 66 °C to 88 °C contributes to the destabilization of fatty acids [13].

On the other hand, the increasing occurrence of foodborne disease outbreaks caused by foodborne pathogenic microorganisms raise awareness to the public about food security parameters [14]. New improved antibiotics are constantly being produced to cure bacterial diseases. However, antibiotics are losing effectiveness due to overuse of these synthetic antibiotics. Over usage of synthetic antibiotics causes bacteria to undergo mutation or acquire antibiotic resistance genes from other bacteria [15].

Foodborne pathogens and microbial contamination pose a difficult problem to health concerns. Furthermore, foodborne illness usually arises from improper handling, preparation or food storage [16, 17].

Over the years, there has been a constant increase in the search of alternative and efficient compounds for food preservation aimed at a partial or total replacement of antimicrobial chemical additives [18, 19]. However, there is a little data on antimicrobial activities and most medicinal plants [20].

The use of synthetic anti-oxidants such as Butylated Hydroxytoluence (BHT) and synthetic antibiotics has been widely used in most smoked-fish industries to retard rancidity of oil in fish and thus preventing the entry of pathogens. However, the use of these synthetic anti-oxidants has been banned in many countries because of their negative effects on the enzymes of the liver and lungs [21] and the use of synthetic antibiotics, if use regularly, will only creates a defensive mechanism for the pathogens.

This necessitated the need to use of natural anti-oxidants and antimicrobial agents such as herbs and spices to prevent rancidity in smoked fish [22] and the entry of pathogens to food. Two of the common spices that could be used as anti-oxidants and anti-bacterial for smoking are ginger and garlic. The antioxidant and antimicrobial ability of these two spices has been reported in various studies.

Garlic (*Allium sativum L.)* is one of the commonly use spices to enhance flavor in food. Apart from it, *Allium sativum L.* has a wide spectrum of actions which include antibacterial, antifungal, antioxidant and beneficial effects on the cardiovascular and immune system of human [21, 23]. The presence of allicin, a sulfur-containing compound found in garlic, that enables the antimicrobial effects.

This study aims to determine the effects of Garlic (*Allium sativum*) extract on lipid oxidation and microbial count on hot-smoked sardines (*Sardinella longiceps*) by evaluating its chemical, microbial, and organoleptic test.

2. Materials and Methods

2.1 Raw materials

A total of 800 pieces of sardines (*Sardinella longiceps*) with an approximate body weight of 20 g were purchased from General Santos City Fish Port. Fish samples were placed in styropor boxes and kept iced before processing. Samples were prepared on the same day at the Multipurpose Building and Laboratory, College of Fisheries, Mindanao State University-General Santos City. The garlic *(Allium sativum)* was purchased from the Local Public Market in General Santos City.

2.2 Preparation of garlic extract

One (1) kilo of fresh garlic cloves were peeled and finely chopped with sterile knife and blended with 30 ml of distilled water using an electric blender for 2 minutes. The blended garlic juice was separated using a clean cheese cloth and made to pass through a strainer to obtain the pure garlic extract.

2.3 Preparation of smoke sardines

Sardines were cleaned in running water and soaked in 15% saturated brine solution and different amount of garlic extract for 90 minutes and drained for 15 minutes. Treatment 1 was soaked in brine solution with 5 g garlic extract, treatment 2 in brine solution with 10 g garlic extract and treatment 3 in brine solution with 15 g garlic extract.

Smoking was patterned after the method described by [4]. Briefly, whole sardines were smoked inside a modified drum smokehouse for 30 to 60 minutes or more until it attains a golden brown color at a temperature of 66 to 88 °C. Wood shavings and chips from a tropical hardwood *(Gmelina arborea)* was used as source of smoke. Fish was turned every 30 minutes to obtain a uniform golden brown color. Then after, the smoked fishes were cooled and packed in plastic bags (polyethylene) and stored in the freezer until use for analysis.

Table 1: Amount of garlic extracts in different treatments

Treatments	Amount of garlic extract (g)
Control	0
I	5
II	10
III	15

2.4 Experimental design

Four treatments were assigned in Complete Randomized Design (CRD) with 3 replicates. A weekly aerobic plate count for the 1 month storage period was done and every after 15 days for peroxide, free fatty, acid value analysis and sensory evaluation.

2.5 Determination of peroxide values (PV)

The American Oil Chemists' Society (AOCS) [25] method was used in measuring peroxide values. The extracting solution consisted of acetic acid-chloroform solution (480 ml acetic acid and 320 ml chloroform) and 0.1 N commercially available sodium thiosulfate solution, liberating solution which is the saturated potassium iodide and 1% commercially available starch solution as indicator. Approximately 1.0 g oil mixed with 30 ml acetic acid-chloroform solution and swirl the flask to completely dissolve the oil. A 0.5 ml of saturated potassium iodide solution was added in the flask to liberate the chloroform layer with the addition of 30 ml distilled water. The sample was titrated with 0.1 N sodium thiosulfate solutions after the addition of 1ml starch as indicator.

$$\text{Peroxide value} = \frac{\text{(B-S) x N thiosulfate x 200}}{\text{Weight of sample}}$$

2.6 Determination of acid value and free fatty acid

The acid value and free fatty acid was determined by the American Oil Chemists' Society (A.O.C.S) method [26]. About 0.1 gram of oil sample was dissolved in 50 ml neutralized isopropanol. Five drops of phenolphthalein solution (1% in isopropanol) was added as an indicator. The solution was immediately kept in the dark for 5 minutes. Then after, the solution was titrated with a standard 0.1 N potassium hydroxide solution until the pink end point was reached.

$$\text{Acid Value} = \frac{\text{mL KOH x N x 56.1}}{\text{Wt. of sample in grams}}$$

$$\text{Free Fatty Acid, \%} = \frac{\text{mL KOH x N x MW (fatty acid)}}{\text{10 x Wt. of sample in grams}}$$

2.7 Microbiological analysis

Aerobic plate count was determined according to standard procedure described by U.S. Food and Drug Administration-Center for food Safety and Applied Nutrition [27]. Fish samples (25 g) were homogenized with 90 ml of sterile peptone water (1 g/l) in a laboratory homogenizer and serial dilutions was prepared (10^{-2} 10^{-3}, 10^{-4}) respectively. Then 0.1 ml of each dilution was spread out with the use of L-shape rod on duplicate plates of pre-poured and dried aerobic plate count agar. After 48 hours of incubation at 35 °C, colonies were counted and results were expressed as $\log_{10}$ CFU/g of fish sample. All preparations were done aseptically.

2.8 Sensory evaluation

A sensory panel consisting of ten (10) students assessed the raw samples for the sensorial qualities such as, color, texture, smokiness, saltiness, smoky odor, smoky flavor, garlic odor and garlic flavor on a 5-1 descriptive point scale with 5 as highest point and 1 as the lowest point. The sensory score sheet is provided in Appendix 5.Approximately 10 g thawed smoked sardines were placed in plastic cup and distributed to the sensory panelists.

2.9 Data analysis

All analyses were performed using SPSS version 22 for windows, using a randomized complete block design. Analysis of Variance was used in all data and Least Significant Differences at the 5% level of significance was used to determine differences in mean of all treatments. Microsoft Office Excel 2010 was used in the calculation of mean and standard deviation.

3. Results and Discussions

3.1 Fish oil content of smoked samples

The fat content of smoked sardine samples was 2.24±0.69%. At this level of fat, the fish may be considered as medium fatty. Pacific herring caught off in Pacific Ocean (west coast) has lipid content ranges from 1.8, 9.28-15% [28] while 4.03-7.54% in smoked Indian sardines [4].

3.2 Effects of garlic extract on lipid oxidation

The efficiency of garlic extract to inhibit lipid oxidation has been reported in various studies. Fresh garlic (FG), garlic powder (GP), and garlic oil (GO) fractions delayed the formation of lipid peroxides (PV) and thiobarbituric acid (TBA) when added to chicken sausage during storage at 3 °C. Kumolu-Johnson *et al.* (2013) [29] also reported the ability of fresh garlic to inhibit the increase of peroxide and TBA values on hot-smoked catfish during the 28 days of storage period.

3.2.1 Peroxide values

Based on Figure 1, the degree of lipid autoxidation in untreated samples were generally high (3.56±1.20 meq/kg of sample fish oil) than those of the garlic treated samples. Treatment 1 had a peroxide value (PV) of 1.6±1.07 meq/kg, treatment 2 with 0.98±0.01 meq/kg, and treatment 3 with 0.98±0.01 meq/kg, respectively. Results imply that the control (not treated with garlic extract) is more prone to oxidative rancidity development than the garlic treated samples. Further, statistical analysis revealed that there is significant difference among treatments at 5% significance level.

This finding was in agreement with the study conducted by Kumolu-Johnson *et al.* (2013) [29] when the antioxidant activity of fresh garlic was evaluated on hot-smoked catfish having a lower peroxide values than the untreated sample.

Accordingly, the acceptability limit for peroxide value of crude fish oil is between 7-8 meq/kg and not more than ≤ 5.0 meq/kg as maximum level for fish products [30].

3.2.2 Free fatty acid values

The free fatty acid (FFA) values of the lipid extracted from samples are shown in Figure 2. An FFA value of 4.95, 2.35, 4.23, and 2.35% in day 0; 2.83, 1.28, 1.16, and 1.28% in day 15; and 2.91, 2.56, 1.18, and 2.35%, at day 30, respectively, denote that the use of garlic extract has no significant effect on the FFA values or lipid autolysis in the fish oil extracted from smoked sardines.

3.2.3 Acid values

Acid value is generally associated with lipases activity originating from microorganisms or biological tissue (Pop, 2012) [31]. Like free fatty value, treatment of garlic extract was Found to have no significant effect on the acid values of the fish oil samples. The acceptable limit for acid value is reported to be 7-8 mg/KOH. Thus, this study showed that the acid values did not exceed the acceptable limit.

In general, the low peroxide values of the fish oil extracted from garlic (5 g, 10 g, and 15 g extract) treated samples indicate that the lipids may remain quite stable over an

extended period of storage at frozen temperature.
Colin *et al.* (2012) [32] explained that the antioxidant activity of garlic was because of S-Allylcysteine (SAC) which contains a thiol group responsible of its antioxidant capacity, this nucleophile can easily donate proton to an electrophilic species, thereby neutralizing them or making them less reactive. Further, SAC is known to scavenge superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite anion.

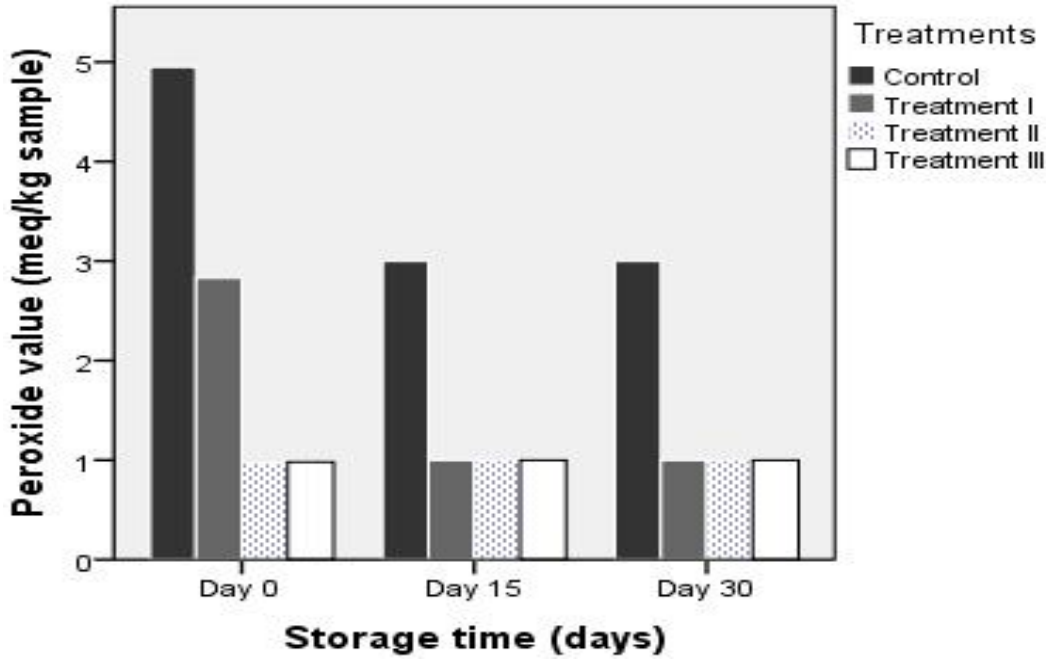

Fig 1: Effect of various garlic extract (GE) levels on Peroxide value in hot-smoked sardine from 0 to 30 days of frozen storage (-18 °C).

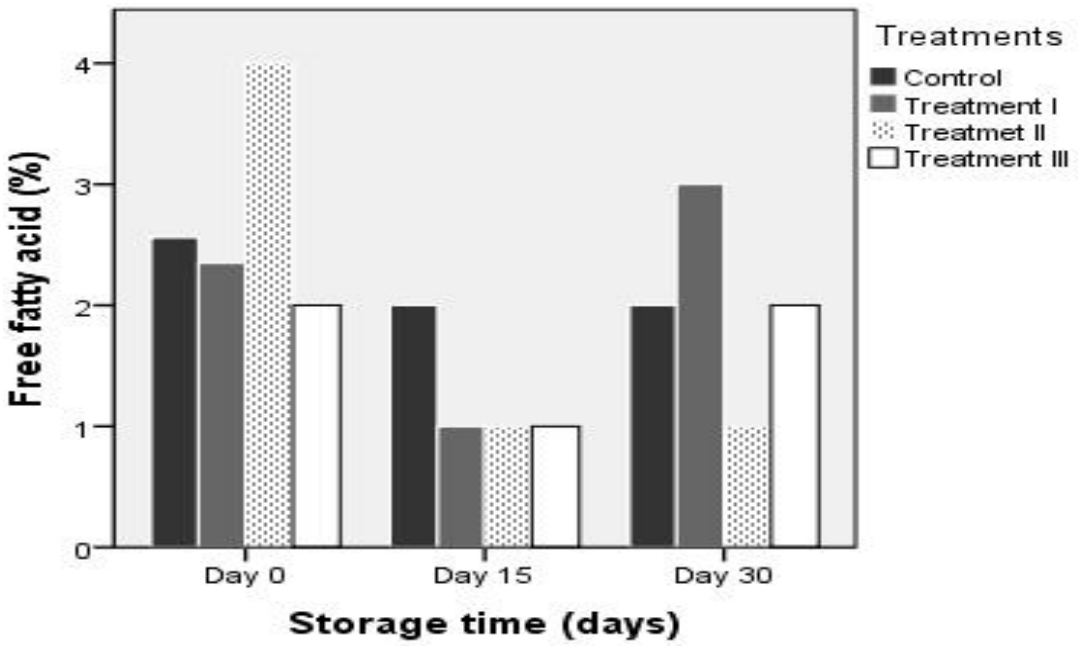

Fig 2: Effect of various garlic extract (GE) levels on Free fatty acid in hot-smoked sardine from 0 to 30 days of frozen storage (-18 °C).

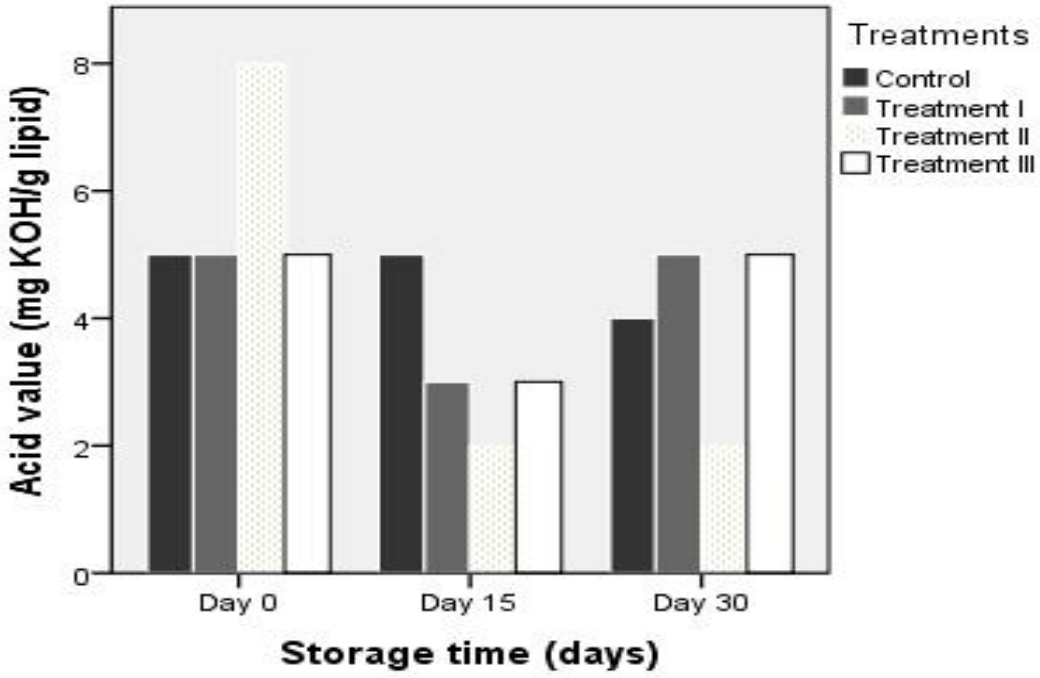

Fig 3: Effect of various garlic extract (GE) levels on Acid value in hot-smoked sardine from 0 to 30 days of frozen storage (-18 °C).

3.2.4 Effects of garlic extract on microbial load (cfu/g)
Data on the weekly mean aerobic plate counts in cfu/g of smoked sardine samples during the 30 days of frozen storage are shown in Figure 4. Results indicate that there is a significant reduction in the microbial counts of the sardine samples from Day 0 to Day 14. The initial microbial loads of the garlic-treated samples are generally lower than that of the control. Among garlic treated samples, Treatment 3 (15 g GE) yielded the lowest microbial count all through-out the period of frozen storage. At days 14, 21 and 30, statistical results revealed that there is no significant difference on the mean aerobic plate counts among treatments ($p<0.05$).

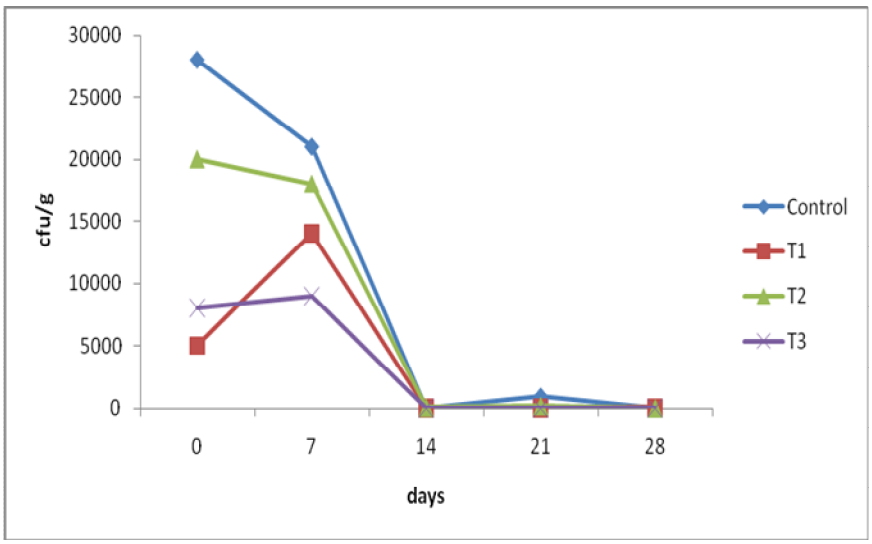

Fig 4: Effect of various garlic extract (GE) levels on microbial load in hot-smoked sardine from 0 to 30 days of frozen storage (-18°C).

The results are consistent with the findings of Kumolu-Johnson and Ndimele (2011) [22], when fresh garlic was likewise found to decrease microbial load in smoked catfish.
After 28 days, smoked sardine with 5 g, 10 g, and 15 g GE had continually decreased cfu/g count. This decrease significantly differ from the control at ($p<0.05$) level. It is however noted that even the untreated smoked fish samples had a decreased microbial count in the later period of frozen storage. This could be accounted to the acidity of the smoked sardines as shown by decreased acid values. Low pH generally held to have antagonistic effect to most spoilage and pathogenic bacteria. Kaba *et al.* (2013) [33] had the same observation in his study on the microbiological characteristics of canned-smoked whiting.

The result of microbiological analysis of all treatments further reveal that the mean aerobic plate counts are all below the minimum requirement for smoked fish which is $1.0x10^5$cfu/gas set forth by PNS/FDA (2010) [34].

3.2.5 Sensory Analysis

The organoleptic evaluation of food products to any food processing technology is very important in determining the consumer acceptability [35]. The hot-smoked sardine samples were evaluated for color, texture, flavor, which includes the intensity of smokiness, saltiness, and garlic flavor, odor intensity which includes the fishy odor, garlic odor, and smoky odor, and lastly overall acceptability. Analysis of variance (ANOVA) was used to determine the significant difference between the means. The results of sensorial analysis for hot-smoked sardine fish are presented in Table 2.

Table 2: Raw mean±SD scores of sensory characteristics of hot-smoked sardines (*Sardinella longiceps*) treated with varying concentrations of garlic (*Allium sativum*) extract (0 g, 5 g, 10 g, and 15 g).

Storage period (days)	Garlic extract conc. (g)	Color	Texture	Smokiness	Saltiness	Garlic flavor	Fish odor	Garlic odor	Smokey odor	Overall
0	0	2.7 ± 1.49	3.5 ± 1.35	4.0 ± 0.94	2.8 ± 0.78	2.4 ± 0.84	3.9 ± 1.28	3.0 ± 1.33	4.1 ± 1.10	6.7 ± 1.25
	5	3.5 ± 0.85	3.5 ± 1.35	4.6 ± 0.69	3.0 ± 1.05	2.4 ± 0.51	4.3 ± 0.94	2.6 ± 0.96	3.2 ± 1.03	6.6 ± 1.07
	10	2.6 ± 0.69	3.4 ± 1.26	4.1 ± 0.73	2.9 ± 0.87	2.2 ± 0.42	4.0 ± 1.05	2.8 ± 1.31	4.3 ± 1.15	6.7 ± 1.33
	15	3.1 ± 0.87	3.9 ± 1.10	3.5 ± 0.84	3.2 ± 0.63	2.4 ± 0.69	4.1 ± 1.44	2.5 ± 1.08	3.9 ± 0.73	7.2 ± 0.78
15	0	3.6 ± 0.84	3.5 ± 1.35	3.8 ± 0.91	3.5 ± 1.17	3.0 ± 0.94	3.3 ± 1.25	3.8 ± 1.22	4.1 ± 0.87	4.9 ± 1.10
	5	3.8 ± 1.03	3.5 ± 1.35	4.0 ± 0.94	3.3 ± 0.94	3.1 ± 1.28	2.4 ± 0.69	2.8 ± 1.22	3.2 ± 1.39	5.1 ± 2.02
	10	4.4 ± 0.84	3.4 ± 1.26	4.2 ± 0.91	3.3 ± 0.94	2.8 ± 1.13	3.5 ± 1.43	2.8 ± 1.13	3.2 ± 1.31	5.5 ± 2.36
	15	3.4 ± 0.84	3.9 ± 1.10	4.0 ± 1.05	3.3 ± 1.15	2.6 ± 0.84	3.7 ± 1.33	2.8 ± 1.13	4.1 ± 1.19	4.8 ± 2.39
30	0	3.5 ± 1.08**a**	4.1 ± 1.19	3.9 ± 1.10	3.2 ± 0.91	2.7 ± 1.05	3.1 ± 1.28	2.6 ± 0.96	3.1 ± 0.99**a**	6.6 ± 1.77**a**
	5	2.8 ± 0.42**bc**	3.3 ± 1.41	3.8 ± 0.63	2.8 ± 0.42	2.7 ± 0.94	3.1 ± 1.44	2.9 ± 1.19	3.9 ± 0.84$_b$	5.3 ± 1.41**a**
	10	5.0 ± 0**bd**	3.4 ± 1.26	4.3 ± 0.67	2.9 ± 0.87	2.6 ± 0.96	2.6 ± 0.69	2.4 ± 0.84	2.7 ± 0.82$_a$	5.3 ± 1.63**a**
	15	4.1 ± 0.87**ab**	3.4 ± 1.26	3.6 ± 0.69	2.6 ± 0.51	2.3 ± 0.48	2.6 ± 0.96	2.4 ± 0.84	3.8 ± 0.63**ab**	5.2 ± 1.22**b**

Means in the same row followed by the same bold subscript are not significantly different (p<0.05) when the effect of garlic extracts on sensory characteristics was evaluated in hot-smoked sardines (*Sardinella longiceps*) at 30 days of storage period.

In 0 day, Analysis of Variance (ANOVA) showed that there were no significant difference (p<0.05) among the treatments in all the sensorial parameters measured except for the intensity of smokiness. The sensory panel rating showed that the intensity of smokiness at 5 and 10g garlic extract concentration were higher, which has a mean score of 4.6 and 4.1 respectively as compared to control with the mean score of 4.0. This result agreed with the study conducted by Kumolu-Johnson and Ndimele (2011) [36], when organoleptic parameters was evaluated on the hot-smoked catfish, the results showed that the control samples with no ginger extract received a lower panel scores than the ginger paste-treated samples.

Sallam *et al.* 2004 [21] also found out that storage time has no significant effect on the intensity of garlic flavor.

In day 15, ANOVA results showed insignificant difference in all sensorial qualities. However, using Least Significance Difference (LSD) p<0.05 to compare means of all treatments in all the parameters, only 10 and 15 g garlic extract concentration was found significantly different when color was evaluated, which has a mean scores of 4.4 and 3.4 respectively, as against the control with no garlic extract concentration with a mean score of 3.6.

In day 30, statistics results showed a significant difference only on the color parameter, when it was evaluated in all treatments, a mean scores of 3.5, 2.8, 5.0, and 4.1 respectively. It was found out that the treatment 2 with 10 g garlic extract concentration got a high rating from the panels with roughly perfect score of 5.0. It can observe, that when overall acceptability was evaluated, only 15 g garlic extract concentration at 30 days of storage period was differ significantly from the rest of the treatments. The overall acceptability scores decreased while the storage period increased in all the samples.

When the effect of garlic extract on sensorial attributes was assessed with varying duration of storage, only 5 g and 10 g garlic extract have significantly affect the color at day 30 of storage period and 5 g for smoky odor. Overall acceptability was only significantly affected by 15 g garlic at day 30 storage period. This means that the effect of garlic extract on overall acceptability and taste panel could only be detected if 15 g garlic extract was used.

4. Conclusion

Crude extracts of garlic have been proven to inhibit lipid oxidation and reduce microbial load. Garlic extract offers a promising alternative to synthetic antioxidants and antibiotics. This study revealed that smoked sardines treated with different concentrations of garlic extracts (5, 10, and 15 g) generally had lower peroxide, free fatty and acid values than the control or untreated smoked sardine samples. On the other hand, smoked sardines treated with garlic extracts showed marked reductions in aerobic plate count (cfu/g) during the 30 days of frozen storage (-18 ^{0}C).

When the effect of garlic extract was evaluated on the sensorial characteristics of smoked sardines during frozen

storage (18 ^{0}C), only color, intensity of smoky odor, and overall acceptability were found to be significantly affected ($p<0.05$) by garlic extracts. Most of the sensorial characteristics were not significantly affected by the addition of garlic extract.

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