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Research Article

Protection against Oxidation of Omega-3 Fatty Acids with Natural Antioxidants in Clove (*Szygium aromaticum*) Water Extracts during Storage of Sun dried Sardines (*Ratrineobola argentea*)

Davis Chaula[1*], Charlotte Jacobsen[2], Henry Laswai[1], Bernard Chove[1], Anders Dalsgaard[3], Robinson Mdegela[4] and Grethe Hyldig[2]

[1]Department of Food Technology, Nutrition and Consumer Sciences, Sokoine University of Agriculture, Morogoro, Tanzania

[2]National Food Institute, Division for Food Technology, Technical University of Denmark, Søltofts Plads, Lyngby, Denmark

[3]Faculty of Health and Medical Sciences, Section of Food Safety and Zoonoses, Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg C, Denmark

[4]College of veterinary and medical sciences, Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, Morogoro, Tanzania

***Corresponding Author:** Davis Chaula, Department of Food Technology, Nutrition and Consumer Sciences, Sokoine University of Agriculture, P.O. Box 3006, Chuo Kikuu, Morogoro, Tanzania, Tel: +255 759 725 607/+255 714 035 198; E-mail: chaula@sua.ac.tz

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Abstract

Omega-3 fatty acids are a family of polyunsaturated fatty acids (PUFAs) with beneficial health effects to humans if consumed in required amounts. Fatty fish species are known to be rich in marine-based omega-3 fatty acids, eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and docosapentaenoic acid (DPA, C22:5n-3). Sardines (*Rastrineobola argentea*) from Lake Victoria are a good source of health promoting omega-3 fatty acids. Open sun drying is a common and traditional sardine processing and preservation method. Due to their chemical instability omega-3 fatty acids in sun dried sardines are prone to lipid oxidation during processing and subsequent storage. This study investigated the use of clove (*Syzygium aromaticum*) water extracts as natural antioxidants to protect omega-3 fatty acids against oxidative damage during storage of sun dried sardines. Lipid oxidation was assessed by peroxide value, volatile secondary oxidation products and fatty acid profiles. The antioxidant capacity of extracts was evaluated by total phenolic content, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and iron (Fe^{2+}) chelating ability. Clove extracts resulted into significantly higher retention of total

PUFAs, DHA, EPA and DPA at the end of 30 days storage period and lower concentrations of secondary lipid oxidation products. This is an evidence of their enhanced oxidative stability in the real food system due to presence of natural clove antioxidants.

Keywords: Omega-3 fatty acids; Lipid oxidation; Natural antioxidants; Sardines; Lake Victoria

1. Introduction

Omega-3 fatty acids are a family of polyunsaturated fatty acids (PUFAs) that have a double bond on the third carbon from the omega (ω) position of the carbon chain [1]. Omega-3 fatty acids attract great attention because of their beneficial health effects [2-4]. Studies have shown that omega-3 fatty acids may play a role in prevention of cardiovascular diseases, reducing inflammation, illnesses such as high blood pressure, atherosclerosis, thrombogenesis, cancer, skin diseases and have been found to be necessary for the brain development in fetuses [5, 6, 7]. There are several sources of omega-3 fatty acids including fishes. In particular, fatty fish species are known to be rich in marine-based omega-3 fatty acids, eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, C22:6n-3) and docosapentaenoic acid (DPA, C22:5n-3).

Rastrineobola argentea (sardines, popular as *dagaa* in Tanzania) is one of the three leading commercial fish species of Lake Victoria, together with the Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*). The freshwater fatty *dagaa* are a richer source of omega-3 PUFAs than *Lates niloticus* and *Oreochromis niloticus* [8, 9]. Sardines have high crude protein content (47.9-58.8%), rich in iron (8.18-10.91 mg/100 g), zinc (4.07-10.25 mg/100 g) and calcium (1556.4-1866.5 mg/100 g) [10]. The stock of sardines, given by its biomass and catches, has increased steadily in the last ten years, being the largest fishery by volume with contribution of about 72% of the total catch weight in the Lake. Its annual biomass in the Lake is estimated at over 1.3 million tonnes. The rapid growth and short life cycle of sardines allow their utilization at an annual level of 70% without overexploitation [11, 12].

Despite its economic and nutritional value sardines are perceived negatively and considered as an inferior food for poor and pro-poor communities. This may be attributed to poor handling and processing technologies along the sardine value chain. The low cost, low-technology and weather dependant open sun drying is the dominant method used for processing and preservation of sardines. The method result into oxidized products [9] with characteristic off flavours, which discourage consumption of dry *dagaa* and limit product diversification. Chemical indicators show that lipid oxidation reactions are pronounced in sun dried *dagaa* with production of secondary oxidation products beyond acceptable levels [9]. Furthermore, changes in lipid contents associated with significant decrease in omega-3 fatty acids in sun dried *dagaa* occur during storage.

The drawback in protection of omega-3 fatty acid during processing and storage of omega-3 rich products like sardines is that PUFAs are readily oxidized in the presence of oxygen, heat, light, and metal ions [13-15]. Oxidation

of PUFAs, such as EPA and DHA not only results in nutritional loss and offensive odours due to formation of volatile secondary oxidation products, but is also a safety concern [16, 17]. Commercially available synthetic antioxidants like butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) are commonly used to hinder lipid oxidation in food systems. Conversely, there are studies questioning their application in foods due to their potential carcinogenic effects and toxicity [18-20]. Due to safety concerns and increased consumer interest in natural products, efforts are directed to replacing synthetic antioxidants with ones of natural origin.

Clove is among spices with potential to protect PUFAs against oxidation because they contain anti-oxidative compounds (e.g. polyphenols and flavonoids) that may exert anti-oxidative effect by different mechanisms like scavenging of free radicals, singlet oxygen quenching, oxygen scavenging, metal chelation and inhibition of oxidizing enzymes [21, 22]. The major phenolic compounds in clove are phenolic acids such as flavonol glucosides, phenolic volatile oils and tannins, recovery of which is highly dependent on extraction conditions, type of solvent and extraction method [22-24]. Previous studies have shown that successful recovery of phenolic compounds from plant matrices by aqueous extraction depend on factors such as temperature, extraction time and solvent to solid ratio [25]. Elsewhere, the clove water extract has been found to contain substantial amounts of phenolic compounds and powerful antioxidant activity in linoleic acid emulsion [26]. Clove water extracts are reported to result in improved retention of nutritionally valued long chain PUFAs DHA, EPA and DPA during open sun and oven drying of sardines [27, 28]. The aim of the current study was to evaluate the efficacy of clove (*Szygium aromaticum*) water extracts in protecting omega-3 fatty acids during extended storage of sun dried sardines.

2. Materials and Methods

2.1 Materials

Fresh whole *dagaa* (20 Kg) were collected directly from fishermen at Kijiweni landing site at the shore of Lake Victoria, Tanzania. The *dagaa* were placed in ice in insulated boxes and immediately transported to the National Fish Quality Control Laboratory, Nyegezi, Mwanza for experiment. Dry clove *(Szygium aromaticum*) buds were obtained from a local market in Zanzibar, transported at ambient temperature to Mwanza and kept at 5 to 10°C in a refrigerator.

2.2 Preparation of clove extracts

For water extraction, 5, 10 and 20 g grounded powder (to pass through a 250 µm sieve) of clove buds were mixed with 1 L boiling water with continuous stirring to make 5, 10 and 20 gL^{-1} concentrations of clove extracts. The mixtures were boiled for 15 min and subsequently cooled to 0 to 5°C in a refrigerator thereafter gravity filtered to remove the particles present.

2.3 Preparation of sun dried *dagaa*

For each concentration of clove extract, 1 kg of *dagaa* (wet basis) was blanched in boiling water for 10 sec. Blanched *dagaa* were soaked in cooled clove extracts (1:1 w/w) for 40 min. at room temperature. After that, the fish

were removed from the extracts, spread on wire mesh and sun-dried on raised platform as traditionally done by the local fish processors. *Dagaa* samples without clove pre-treatment were prepared in a similar way and used as control. All samples were packed in polyethylene bags each containing 200 g fish and stored in temperature controlled room at 25°C for 30 days. Analyses were done at the start (day 0) and the end of storage experiment (day 30). The room temperature was monitored by using Tinytag temperature data logger (Gemini data loggers Ltd, West Sussex, UK). For each treatment 100 g portion of whole fish was made into mince using a mixer (Moulinex Moulinette S type 643 02 210, Hamburg, Germany) for analysis.

2.4 Methods

2.4.1 Dry matter content and lipid extraction: The dry matter content for fish and clove powder samples was determined by weighing after drying a sample of approximately 2 g of homogeneous fish mince and powder at 105°C for 18 h according to the AOAC [29] and results expressed as a percentage dry matter. Lipids were extracted following the Bligh and Dyer method [30] with modifications according to Iverson et al. The sample (5 g of fish mince) was homogenized in chloroform, methanol, and water mixture (1:1:0.8 v/v) at the speed of 15000 rpm (226 g) for 90 sec using an Ultra Turrax homogenizer (T25 Homogenizer, Staufen, German). The homogenate was centrifuged at 2800 rpm (1595 g) at 18°C for 10 min using a centrifuge (Sigma 4K15, Osterode am Harz, German) to obtain the extract (Chloroform phase). The lipid content was determined by gravimetry after evaporation of chloroform and expressed as percentage of the dried fish sample.

2.4.2 Primary and secondary lipid oxidation products: Peroxide values (PV) of the lipid extracts were determined according to the method of Shantha et al. [31] based on the formation of an iron-thiocyanate complex. The colored complex was measured by spectrophotometer (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD) at 500 nm. The analysis was done in duplicate, and the results were expressed in millequivalent peroxides/Kg oil (meq O_2/Kg oil). The volatile compounds from fish mincewere collected using the dynamic headspace technique. The procedure was carried out using 1 g of fish mince in which 30 mg of internal standard, 4-methyl-1-pentanol were added and mixed with 15 mL of distilled water. The volatiles were collected in Tenax GR tubes at 37°C by purging with nitrogen for 30 min at 150 mL/min. The tubes were flushed with nitrogen at 50 mL/min for 20 min to remove water.

The trapped volatiles were desorbed from the Tenax tubes by heat (200°C) using an automatic thermal desorber (ATD-400, PerkinElmer, Norwalk, CT), cryofocused on a cold trap (−30°C), released again at 220°C, and led to a GC an Agilent 5890IIA model (Palo Alto, CA, USA) equipped with a HP 5972 mass selective detector. Separation was done on a DB1701 column (30 m × ID 0.25 mm × 0.5 µm film thickness, (J&W Scientific, Folsom, CA). The carrier gas used was helium at flow rate of 1.3 mL/min. The oven temperature was rising by 2.0°C/min from initial temperature of 45 to 80°C followed by an increase of 3.0°C/min to 150°C and finally increased by 12.0°C/min to 240°C. The individual compounds were identified by MS-library searches and addition of the internal standard. Quantification was done through calibration curve made by adding the standard directly on the Tenax tubes as

described by Hartvigsen et al. [32]. For the quantification, a stock solution of 19 volatiles was prepared and a calibration curve was conducted in a range from 0 to 1.2 mg/g. The analysis was carried out in triplicate.

2.4.3 Free fatty acids and fatty acid composition (Fatty Acid Methyl Esters, FAME): Free fatty acids (FFAs) content was determined by acidometric titration of the lipid extract using NaOH (0.1 M). The FFAs content was calculated as oleic acid according to the AOCS [33] and the results were reported as % oleic acid. The fatty acid composition of the oil phase was determined after fatty acid methylation and analysis by GC-FID according to the American Oil Chemists' Society (AOCS) official method; Ce 1i-07 [34] with some modification as follows. Approximately 1 g of extract was weighed in a methylation glass tube and evaporated to dryness under a gentle stream of nitrogen. Thereafter, 100 µL of internal standard solution (2% w/v C23:0 in heptane), 200 µL of heptane including 0.01% w/v butylated hydoxy toluene (BHT) as antioxidant, 100 µL of toluene and 1 mL of boron trifluoride in methanol (BF3-MeOH) was added.

Samples were mixed and methylated in the microwave oven (Microwave 3000 SOLV, Anton Paar) for 10 min at 100°C and power of 500Wand then cooled down for 5 min. Then, 1 mL of saturated salt water (NaCl) and 0.7 mL of heptane with BHT were added. After the separation of heptane, the upper phase of the sample (around 0.7 mL) was transferred into vials. Samples were analyzed by gas chromatography system (HP-5890 A, Agilent Technologies, Santa Clara, CA, USA). Fatty acid methyl esters were separated and detected by the GC column Agilent DB-wax (10 m × 100 µm × 0.1 µm), from Agilent Technologies (CA, USA). The carrier gas was helium with a flow rate of 0.38 mL/min and an inlet pressure of 51psi. The oven temperature program for separation was from 160 to 200°C, then from 200 to 220°C and from 220 to 240°C at 10.6°C /min. All analyses were done in duplicate. The result of each fatty acid was expressed as g fatty acid/100 g lipid.

2.4.4 Antioxidant Activity of Clove Water Extracts:

2.4.4.1 Total phenolic content: The total phenolic compounds of the extracts were determined using Folin–Ciocalteu reagent by a procedure described by Farvin and Jacobsen [35] in which gallic acid was used as a standard. The standard curve was prepared in distilled water at a concentration range of 0-125 µg/mL. The original extracts were diluted with water as necessary to fit within the standard curve. The absorbance was read at 725 nm using UV-vis spectrophotometer and results reported in µg gallic acid equivalent (µg GAE)/mL of clove water extracts. All measurements were performed in duplicate.

2.4.4.2 Free radical scavenging ability: The free radical scavenging activities of clove water extracts were measured by utilizing the stable radical, 1,1-diphenyl-2-picryl-hydrazil (DPPH) as described by Yang et al. [36]. The solutions of prepared extracts were diluted with water (1:1 v/v). Diluted solutions (100 µL) were added to the microplate and mixed with 100 µL of 0.1 mM DPPH in ethanol (96%). The mixtures were shaken vigorously and maintained for 30 min at ambient temperature in the dark. The absorbance of mixtures and the control (100 µL

DPPH solution + 100 µL BHT) was measured at 517 nm against a reagent blank by using a UV-V is spectrophotometer. The scavenging activity was calculated as inhibition percent by using the following equation:

$$\text{Inhibition } (\%) = \left(1 - \frac{A_S - A_0}{A_b}\right) \times 100 \quad (1)$$

Where A_s is the absorbance of DPPH after reaction with antioxidant, A_0 is the absorbance of antioxidant and ethanol (blank) and A_b is the absorbance of water and DPPH (blind).

2.4.4.3 Iron (Fe^{2+}) chelating ability: The ferrous ion chelating activity of clove extracts was measured as described by Farvin et al. [37] with 20 µL of 0.5 mM ferrous chloride and 20 µL of 2.5 mM ferrozin being mixed with 100 µL of clove extracts. The mixture was allowed to equilibrate in the darkness at room temperature for 10 min before measuring the absorbance. The decrease in the absorbance at 562 nm of the iron (II)-ferrozin complex was measured. EDTA was used as the positive control and the ability of the extracts to chelate Fe^{2+} was calculated using the equation:

$$Fe^{2+}chelatingactivity = \left(\frac{A_{blank} - (A_{sample} - A_{blind})}{A_{blank}}\right) \times 100 \quad (2)$$

A_{blank} is the absorbance of blank (only iron chloride and Ferrozin), A_{sample} is the absorbance of sample and A_{blind} is the absorbance of blind (only antioxidant).

2.5 Statistical analysis

Data were analyzed using IBM SPSS (SPSS for Windows Version 20.0, 2013, IBM, Bethesda, MD, USA). Data were reported as mean ± standard deviation. Differences between means were determined using one-way analysis of variance (one-way ANOVA) with Tukey's HSD post hoc test, according to the equal variance of different groups. A p-value <0.05 was considered statistically significant.

3. Results and Discussion

3.1 Antioxidant activity of clove and seaweed water extracts

The clove (*Szygium aromaticum*) water extracts had total phenolic content in the range 18.18 to 28.75 µgGAE/mL (Table 1). The 20 gL^{-1} extract had significantly higher total phenolic content than 5 and 10 gL^{-1}. This observation concurs with previous studies which showed that recovery of phenolic compounds from plant matrices by aqueous extraction depends on temperature, extraction time and solvent to solid ratio [25]. The DPPH of extracts was in the range 93 to 95% inhibition. The lack of linear relationship between total phenolic content and DPPH of clove extracts suggests that compounds other than phenolics (e.g. flavonoids) might have contribution to free radical scavenging activity of clove extracts. The DPPH decreased from 95.59 to 94.34% when the amounts of clove extracted in one litre of hot water was increased from 10 to 20 g. This could be due to decrease in extraction efficiency of clove phenolics in boiling water at concentration above 10 g/L as reported in another study [28].

Elsewhere, the clove water extract has been found to contain substantial amounts of phenolic compounds and powerful antioxidant activity in linoleic acid emulsion [26].

Extracts (g/L)	**Total phenolic content (µgGAE/mL)**	**DPPH scavenging activity (% inhibition)**	**Fe^{2+} ion chelating activity (%)**
CL 5	$18.18^{a} \pm 1.29$	$93.33^{u} \pm 0.21$	$14.74^{n} \pm 4.60$
CL 10	$25.94^{b} \pm 2.62$	$95.59^{v} \pm 1.44$	$20.87^{p} \pm 5.98$
CL 20	$28.75^{c} \pm 1.35$	$94.34^{w} \pm 0.38$	$22.24^{q} \pm 4.08$

CL: Clove, GAE: gallic acid, 5, 10 and 20: grams of seaweed or clove extracted in 1 L of boiling water. Means marked with different letters in a column statistically significant ($p<0.05$), n=3

Table 1: Antioxidant capacity of different doses of clove water extracts.

The Fe^{2+} chelating activity increased (though not linearly) with the amount of clove powder extracted. The dependence of iron chelating capacity on concentration of clove water extracts and type of solvent used was reported by Gulcin et al. [26]. Essential oils of clove have been tested in omega-6 and omega-3 fatty acids enriched food supplements and found to have high iron-chelating properties and higher hydrogen donating power than the standard antioxidants BHT and α-tocopherol.

3.2 Fat, free fatty acids and dry matter content

The dry matter content of clove was 86.40%, whereas the mean dry matter content of clove pre-treated sardines was 92.19% and there was no significant difference between pre-treated and control samples (Table 2). For all samples the fat content decreased significantly after 30 days of storage at 25°C. The substantial decrease in fat content of fish during processing and storage has been associated with oxidative changes in fish lipids.

Owaga et al. previously reported a significant difference ($P<0.05$) in the total fat content of fresh sardine fish (14.8% dry weight basis [dwb]) and sun-dried sardine fish (13.9% dwb) during the sun-drying process which may result from rapid lipid oxidation. Elsewhere, another study reported 82.64 and 56.68% decrease in lipid content during the 21 days of chilled and frozen storage of fish (*Mystus seenghala*) muscles respectively.

Sample	**DM (%)**	**Fat content (%)**		**Free fatty acids (%)**		**PV (meq.O_2/Kg oil)**	
		Day 0	**Day 30**	**Day 0**	**Day 30**	**Day 0**	**Day 30**
POO	$92.27^{x} \pm 0.64$	$17.30^{a} \pm 0.19$	$16.18^{d} \pm 0.28$	$9.50^{g} \pm 0.96$	$12.57^{k} \pm 1.15$	$20.32^{p} \pm 0.26$	$11.81^{t} \pm 1.38$
CL 5	$91.63^{x} \pm 0.92$	$17.65^{b} \pm 0.13$	$16.76^{e} \pm 0.73$	$12.84^{h} \pm 2.06$	$15.49^{l} \pm 0.35$	$11.54^{q} \pm 0.11$	$6.29^{u} \pm 0.67$
CL 10	$92.61^{x} \pm 0.48$	$17.52^{b} \pm 0.43$	$16.72^{e} \pm 0.77$	$11.27^{i} \pm 2.01$	$13.73^{m} \pm 1.49$	$8.56^{r} \pm 1.97$	$5.74^{v} \pm 0.41$

CL 20	$92.34^{x} \pm 0.25$	$16.28^{c} \pm 0.10$	$16.05^{f} \pm 0.05$	$12.08^{j} \pm 0.20$	$14.68^{n} \pm 0.58$	$8.28^{s} \pm 0.28$	$5.01^{w} \pm 0.14$

POO: Control sample; CL: Clove; GAE: gallic acid; 5, 10 and 20: grams of clove powder extracted in 1 L of boiling water. Means marked with different letters in a column statistically significant, n=3.

Table 2: Dry matter (DM), fat, free fatty acid and hydroperoxide (PV) content of sun dried sardines stored at 25°C for 30 days.

Free fatty acid levels increased by 32.32% in the control sample and by 20.64, 21. 83 and 21.52% in samples treated with 5, 10 and 20 g/L clove extracts, respectively at the end of storage experiment. Increased levels of free fatty acids suggest activity of lipase and phospoholipase found in fish or which could be produced by certain microorganisms therein contributing to lipolytic breakdown of fish lipids. Lower percentage increase of free fatty acids in clove treated samples than the control sample is indicative of antimicrobial activity of clove that might have suppressed lipolytic enzymes producing microorganisms in treated samples. For ages clove has been known to have strong antimicrobial activity [38].

3.3 Primary and secondary lipid oxidation products

Both, at the start and end of storage experiment the control sample had significantly higher levels of hyroperoxides (indicated by PV) than the clove pre-treated samples (Table 3). The PV among clove treated samples decreased as the amount of clove extracted in 1 L of water increased which shows the dependence of antioxidant activity on concentration of clove extracts. Concentrations of volatile secondary oxidation products after 30 days of storage are presented in Table 3. The control samples had significantly higher concentrations of individual volatile compounds than pre-treated samples, indicating that oxidation of PUFAs was more pronounced in untreated sardines than in clove treated samples. From Table 3 it can be seen that concentrations of compounds such as 1-penten-3-ol, t-2-penten-1-ol, Hexanal and 1-octene-3-ol in clove treated samples were approximately 50% less than in the control (untreated). This observation suggests that phenolic compounds in clove extracts were still active as anti-oxidants throughout the storage period. The compounds 1-penten-3-ol, 4-heptanal and t, t-2, 4-heptadienal are recognized as decomposition products of EPA and DHA [39].

Compound	**POO**	**CL 5**	**CL 10**	**CL 20**
4-heptenal	$86.19^{a} \pm 13.22$	$62.97^{b} \pm 0.88$	$72.50^{c} \pm 3.99$	$49.83^{d} \pm 1.80$
2-heptanal	$34.55^{e} \pm 10.97$	$6.66^{f} \pm 0.54$	$5.35^{g} \pm 0.04$	ND
2-methyl furan	ND	ND	ND	ND
2,4-heptadienal	$23.69^{h} \pm 1.16$	$22.78^{i} \pm 0.44$	$17.01^{j} \pm 2.37$	$11.51^{k} \pm 0.74$
2-pentanal	$87.99^{l} \pm 22.34$	$53.34^{m} \pm 3.45$	$48.25^{n} \pm 5.86$	$28.99^{o} \pm 1.28$
1-penten-3-ol	$1403.41^{p} \pm 292.00$	$641.01^{q} \pm 64.82$	$875.85^{r} \pm 80.89$	$509.17^{s} \pm 14.53$
Benzaldehyde	213.50 ± 25.49	147.17 ± 5.97	273.68 ± 12.32	150.02 ± 3.61

Butanal	269.50^{t} ± 74.33	132.42^{u} ± 9.84	205.53^{v} ± 13.02	138.78^{w} ± 8.92
2-methyl butanal	366.00^{x} ± 12.19	280.53^{y} ± 28.41	543.29^{z} ± 38.96	399.47^{ab} ± 8.49
t-2-penten-1-ol	838.89^{ac} ± 62.68	339.68^{ad} ± 2.35	429.17^{ae} ± 23.98	258.95^{af} ± 3.85
Hexanal	1827.77^{ag} ± 373.51	725.64^{ah} ± 2.97	917.40^{aj} ± 54.68	536.73^{ak} ± 8.91
Pentanal	893.97^{al} ± 27.19	509.12^{am} ± 34.47	682.98^{an} ± 37.35	495.52^{ap} ± 21.23
1-octene-3-ol	642.77^{aq} ± 60.98	258.24^{ar} ± 6.65	299.35^{as} ± 19.06	209.03^{at} ± 7.59
3-methyl butanal	1041.67^{au} ± 293.11	737.71^{av} ± 72.07	1321.13^{aw} ± 67.42	997.02^{ax} ± 24.94
Heptanal	508.62^{ay} ± 97.63	228.47^{az} ± 6.11	231.78^{bc} ± 16.34	179.00^{bd} ± 8.21

Values are expressed in mean ± standard deviation (n=3). CL: clove, 5, 10 and 20: grams of clove extracted in 1 L of boiling water, ND: not detected. POO: Control sample. Means marked with different letters in a row are statistically significant ($p<0.05$).

Table 3: Concentration of volatile compounds (ng/g fish mince) in sun dried sardines pre-treated with extracts and stored at 25°C for 30 days.

Lower concentrations of such compounds in pre-treated samples imply that the nutritionally valued PUFAs DHA and EPA were protected against oxidative damage during storage. Clove water extracts have been reported to retard lipid oxidation during sun-dry processing of sardines resulting into lower concentrations of volatile compounds in dry sardines [27]. Phenolic compounds are known to exert anti-oxidative effect by different mechanisms such as scavenging of free radicals, singlet oxygen quenching, oxygen scavenging, metal chelation and inhibition of oxidizing enzymes [21, 22]. In literature, it has been reported that use of the whole spices and herbs or their extracts with strong antioxidant activity [40], can control lipid oxidation in muscle food such as mullet fish, frozen chub mackerel and smoked rainbow trout [41].

3.4 Polyunsaturated fatty acids

After 30 days of storage the retention of total PUFAs was significantly higher in clove pre-treated samples than the control (Table 4). The total PUFAs ranged from 29.58 to 30.61% in clove pre-treated sardines and it was 25.95% in the control (untreated). Correspondingly, pre-treated samples retained higher amounts of total omega-3 fatty acids (21.36-22.18%) than the control sample which had 17.31%. The same trend was observed for contents of individual nutritionally valued omega-3 PUFAs, DHA (13.22-13.38%), DPA (1.95-2.11%) and EPA (5.21-5.72%) in pre-treated against 8.71, 1.37 and 3.62% respectively in the control. In previous study, the total PUFAs in untreated sun dried sardines decreased from 29.29 to 11.49% and DHA was beyond detectable levels after 21 days of storage at ambient temperatures.

PUFAs	**POO**	**CL5**	**CL10**	**CL20**
16:2 (n-4)	0.51 ± 0.004	0.65 ± 0.02	0.58 ± 0.02	0.62 ± 0.01

16:3 (n-4)	2.52 ± 0.02	2.41 ± 0.08	2.40 ± 0.04	2.32 ± 0.09
18:3 (n-4)	2.84 ± 0.04	3.65 ± 0.02	3.64 ± 0.02	3.65 ± 0.17
Total (n-4)	**5.87^{a} ± 1.27**	**6.71^{b} ± 1.53**	**6.63^{b} ± 1.54**	**6.58^{b} ± 1.52**
18:3 (n-3)	0.33 ± 0.04	0.45 ± 0.02	0.42 ± 0.01	0.44 ± 0.04
18:4 (n-3)	2.84 ± 0.01	0.05 ± 0.001	ND	ND
20:3 (n-3)	0.43 ± 0.01	0.28 ± 0.03	0.54 ± 0.02	0.57 ± 0.02
20:5 (n-3)EPA	3.62 ± 0.01	5.72 ± 0.10	5.21 ± 0.03	5.69 ± 0.03
22:5 (n-3)DPA	1.37 ± 0.03	2.11 ± 0.01	1.95 ± 0.03	2.10 ± 0.03
22:6 (n-3)DHA	8.71 ± 0.01	13.22 ± 0.02	12.25 ± 0.02	13.38 ± 0.04
Total (n-3)	**17.31^{c} ± 3.76**	**21.83^{d} ± 4.84**	**20.36^{e} ± 4.51**	**22.18^{f} ± 4.93**
18:2 (n-6)	0.12 ± 0.01	0.13 ± 0.02	0.16 ± 0.02	0.18 ± 0.02
18:3 (n-6)	0.34 ± 0.01	0.33 ± 0.001	0.32 ± 0.02	0.38 ± 0.08
20:2 (n-6)	0.21 ± 0.01	0.25 ± 0.01	0.24 ± 0.001	0.25 ± 0.01
20:4 (n-6)	2.11 ± 0.04	1.36 ± 0.07	2.68 ± 0.01	2.82 ± 0.04
Total (n-6)	**2.77^{g} ± 0.95**	**2.07^{h} ± 0.57**	**3.41^{i} ± 1.23**	**3.64^{j} ± 1.28**
Total PUFAs	**25.95^{k} ± 2.36**	**30.61^{l} ± 3.67**	**30.41^{m} ± 3.38**	**29.58^{n} ± 3.95**

*Fish lipid was extracted from whole fish. Values are expressed in mean ± standard deviation (n=3). PUFAs, polyunsaturated fatty acids; CL; sample treated with clove, 5, 10 and 20: grams of clove powder extracted in 1 L of boiling water, POO: Control sample, ND, not detected; Means marked with different letters in the same row are statistically significant ($p<0.05$).

Table 4: Polyunsaturated fatty acid content (% fatty acid of total fatty acids)* in sun dried sardine pre treated with clove water extracts and stored at 25°C for 30 days.

Higher proportion of DHA, DPA and EPA in lipid fractions of clove treated sardine after 30 days of storage is an evidence of their enhanced oxidative stability in the presence of natural antioxidants in clove extracts. Of the three clove extract concentrations, 20 g/L is the best as it resulted in retention of higher amounts of both total omega-3 and omega-6 fatty acids. Antioxidant activity of clove water extracts is attributed to the presence of phenolic compounds with strong hydrogen donating ability, metal chelating ability and free radicals scavenging activity. Use of spices like clove as a natural antioxidant to protect lipids against oxidation in meat and fish oil has been demonstrated. Protective effect of clove water extracts against oxidation of polyunsaturated omega-3 fatty acids during oven and sun drying of sardines has been reported [27, 28]. Clove oil and clove water extracts are also known to have as strong peroxidation inhibitory effect as ethanol extract in linoleic acid emulsion [26]. Nevertheless, the major phenolic compounds in clove are phenolic acids such as flavonol glucosides, phenolic volatile oils and tannins, recovery of which is highly dependent on extraction conditions, type of solvent used and extraction method [22-24].

4. Conclusion

The present study evaluated the protective effect of natural antioxidants in clove water extracts against oxidation of omega-3 fatty acids in real food system (sun dried sardines) after 30 days storage at 25°C. Generally, clove treated sardines retained significantly higher amounts of total polyunsaturated fatty acids at the end of storage experiment. The pre-treatment also resulted into lower amounts of volatile secondary lipid oxidation products. It was found that the chemically unstable omega-3 fatty acids DHA, EPA and DPA were retained in products, an evidence of their enhanced oxidative stability in presence of natural clove antioxidants in extracts. The small size and weight of sardine fish might have been a key to successful protection of omega-3 fatty acids. These findings would be of interest during incorporation of sardines into other food product formulation at industrial scale for product diversification. Nevertheless, for product diversification further investigation on possible alterations in products' sensory acceptability are needed.

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