Protective Effects of Green tea, Clove and Thyme against Mullet (*Mugil cephalus*) Lipid Hydrolysis and Oxidation: Role of Natural Antioxidants

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Abstract: Fish is one of the healthiest sections of food. Therefore, the enhancement of the shelf life of fish during freezing storage is important for their quality and toxicity. For these reasons, water extracts with concentration of 10% of three plants; namely green tea, clove and thyme were tested for their abilities to enhance period of shelf life of mullet (*Mugil cephalus*) during a storage period of six months. To fulfill of this requirement, both biochemical and microbiological examinations were done. From the biochemical point of view; peroxide value (PV), thiobarbituric acid (TBA) values and free fatty acid (FFA) were estimated. Microbiologically, total viable bacterial count (TVBC) and the count of *Staphylococcus* spp., lactose fermenting and non-lactose fermenting bacteria, *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp. were done. The three plants extracts showed an obvious antioxidant effects via reduction in PV and TBA. Also, this preservation system led to lowering FFA. At the same time TVBC in mullet tissue, gill and intestine showed a variable behavior during preservation. These include absence of bacterial growth after freezing for 1 and 4 months when the fish was treated with clove and green tea, respectively. The three plant extracts showed slight effect on the number of *Staphylococcus* spp., lactose fermenting and non-lactose fermenting bacteria and *Aeromonas* spp. Surprisingly, no *Pseudomonas* spp. or *Vibrio* spp. were detected during this study. Moreover, the in vitro study using 20 % green tea extract showed the highest effect against *Staphylococcus* sp. while clove extract (10%) and thyme extract (20%) showed higher effects against *Aeromonas* sp. than that of green tea. In conclusion: Green tea, clove and thyme extracts can be used to protect against bacterial growth which may participate in tissue lipids deterioration during fish preservation. The possible antioxidants effects of these extracts are also included.

Keywords: antibacterials, antioxidants, clove, green tea, lipid oxidation, *Mugil cephalus*, thyme.

1 Introduction

Fish is a well-known source of protein, vitamins, minerals and omega-3 fatty acids [1]. So, it is commonly consumed all over the world [2]. It is considered as an important food in coastal countries. Microbial spoilage is the cause of the annually loss of one-third of the world's food production [3]. Moisture content, polyunsaturated fatty acids and autolytic enzymes make fish a perishable product that spoiled even under refrigerated conditions [4], [5]. The latter spoilage may result from lipid oxidation, elevation of autolytic activities of the enzymes; including lipases that hydrolyze fats. These enzymes had been found in fish muscles and microorganisms' metabolic activities [6]. The bacteria which are found in the intestines of fish are relying on the consumed food [7]. These bacteria are, in general, characterized by Psychrophilic growing between 0°C and about 30°C with some strains growing as low as -75 °C. The same genera of bacteria mostly found in the freshly caught fish with different proportion. Gram negative genera of these bacteria include *Pseudomonas* sp., *Moraxella* sp., *Acinotobacter* sp., *Vibrio* sp., *Aeromonas* sp., *Flavobacterium* sp. and *Cytophaga* sp. On the other hand, the Gram positive genus includes *Micrococcus* sp. and Coryneform group, the prevalent floras of tropical fish. In general, aerobic spore forming bacilli may be isolated from fish that are caught close to land [8]. In addition, fish may carry water borne pathogens such as *Salmonella* sp., *Aeromonas hydrophilla*, *Shigella* sp., *Vibrio cholerae* and the hepatitis A virus if the water is polluted with sewage [7]. Food preservatives have been gaining much attention to extend the shelf life [2]. Some additives may be added as preservatives to enhance seafood quality. These include many of the synthetic antioxidants which have been used to delay lipid oxidation [9].

Due to the possible carcinogenic effect of synthetic preservatives, they are used under strict regulation [10] and the need for natural ones is urgent which is the case in this study.

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2 Materials and Methods

2.1 Plant materials and preparation of extracts

2.1.1 Plant materials

Dried clove buds (*Syzygium aromaticum*), dried thyme (*Thymus vulgaris*) and dried green tea leaves (*Camellia sinensis*) were purchased from a local market in Damietta, Egypt.

2.1.2 Preparation of the aqueous extracts

Preparation of green tea aqueous extract

Ten grams of dried ungrounded leaves of green tea were soaked in 100 ml boiling distilled water for 10 min. The extract was filtered from the leaves by mean of gauze [11].

Preparation of thyme aqueous extract

Thyme extract was prepared by pouring 100 ml of boiled distilled water over 10 g of ground plant at room temperature. The extraction was done for 30 min with continuous stirring. The extract was filtered through medical gauze [12].

Preparation of clove bud aqueous extract

10 g clove bud powder were added to 100 ml boiled distilled water and the mixture was left for 3 h at room temperature.The extract was,then, obtained by filtration through medical gauze [13].

2.2 Fish sample preparation

106 fresh mullet (*Mugil cephalus*) fish were purchased from a fish farm in Damietta in December 2014. The average weight of the fish is 500 gm. Then, these 106 fish were divided into 4 groups (25 fish each). The 1st group was untreated control that was dipped into distilled water, while the 2nd group was dipped into green tea extract (10%), the 3rd group was dipped into thyme extract (10%), and the 4th group was dipped into clove extract (10%). In each case the dipping solution was discarded after 1 hour. The treated fish samples were packaged, labeled and then stored inside a deep freezer at – 20 ºC. The remaining 6 fish constitute the fresh group. The treated groups were subjected to biochemical and microbiological assessments at the beginning of the experiment as fresh and then after 1st, 2nd, 4th and 6th months of freezing storage.

2.3 Biochemical analysis

2.3.1 Peroxide value (PV) determination

Peroxide value was determined in the lipid extract according to the method described by [14]. In this method, the fish tissue was dipped in diethyl ether for 24 hrs. After filtration, the diethyl ether layer was evaporated and the remaining fats were weighed. After that, 30 ml of a mixture of 2 volumes of chloroform and 3 volumes of glacial acetic acid were added to the weighed fat in a 250 ml conical flask. The substance was shaken to dissolve and 0.5 ml of saturated potassium iodide solution was added before shaking for exactly 1 min. After that, 30 ml of water followed by 5 ml of starch solution were added followed by titration with 0.01 M sodium thiosulphate with continuous and vigorous shaking until blue color development. Simultaneously a blank test was done under the same conditions and the PV was then calculated.

2.3.2 Malondialdehyde (MDA) estimation

10 gm of tissue were homogenized for 2 min in 34.25 ml of 4% cold perchloric acid. The homogenate was filtered with the Whatman No. 1 filter paper. 5 ml aliquots of the filtrate were transferred into separate tubes and mixed with 5 ml of thiobarbituric acid (TBA, 20 mmol/ L). The tubes were vortexed, incubated in boiling water bath for 1 h and then cooled for 10 min with cold tap water. The absorbance was determined at 532 nm against blank containing 5 ml of perchloric acid (4 %) and 5 ml of TBA reagent [15].

2.3.3 Estimation of free fatty acids (FFA) contents

It was determined in the extracted lipid layer by diethyl ether [16]. 50 ml of neutralized absolute ethanol (pre-heated to 70 ºC) were added to the extracted fat. The mixture of fat and alcohol was heated on a hot plate until it was almost boiled. A few drops of phenolphethaline were added to the mixture and then titrated with 0.1 N NaOH. The end point of the titration was determined when the pink color develops and persists for 30 seconds [17].

2.4 Microbiological analysis

2.4.1 Preparation of samples

10 gm of fish sample were dissected out and transferred to a sample flask containing 0.1% (w/v) sterile chilled peptone water. The flask was closed, shaken and allowed to stand [18]. 1 ml of the previous dilutions was transferred to 9 ml of sterile water to prepare different dilutions.

2.4.2 Determination of total viable bacterial count (TVBC)

Nutrient agar was used for such determination [19]. 1 ml of each dilution was pipetted into sterile Petri dishes and then nutrient agar media were poured onto the sample (after cooling to 45ºC), then they were mixed thoroughly and uniformly by alternate rotation. The poured agar let to solidify; the solidified Petri dishes were inverted and incubated for 24 - 48 hours at 37 º C [16]. The count of the growing bacteria was recorded in triplicate.

2.4.3 Detection of *Staphylococcus* spp.

Salt mannitol agar was used for quantitative detection of *Staphylococcus* spp. Pathogenic strains of staphylococci (*Staphylococcus aureus*) produced yellow colonies with yellow zones. Nonpathogenic *staphylococci* produced small red colonies with no color change to the surrounding medium [20]. Then, the incubation was done for 24 – 48 hours at 37 º C.

2.4.4 Detection of lactose-fermenting (coliform) and non-lactose fermenting organisms

MacConkey agar was used for quantitative detection of total coliforms [19]. Lactose-fermenting organisms (such as *Escherichia coli*) grow as pink colonies. Non-lactose fermenting organisms (such as *Proteus mirabilis* and *Salmonella typhimurium*) grow as colorless (clear) colonies [21]. Then, the incubation was done for 24 – 48 hours at 37 º C.

2.4.5 Detection of *Aeromonas* spp.

Starch ampicillin agar was used for quantitative detection of *Aeromonas* spp. Iodine solution was poured on the plates after incubation and those colonies that have clear zone surrounding them were considered as colonies of *Aeromonas* spp. [22]. Then, the incubation was done for 24 – 48 hours at 37 º C.

2.4.6 Detection of *Vibrio* spp.

Thiosulphate citrate bile salts sucrose agar (TCBS) was used for quantitative detection of *Vibrio* spp. After incubation, *Vibrio* spp. colonies appear as yellow–greenish-blue in color [23]. Then, the incubation was done for 24 – 48 hours at 37 ^{o} C.

2.4.7 Detection of *Pseudomonas* spp.

Medium B was used for quantitative detection of *Pseudomonas* spp. A light, bright green / yellow color diffusing into the agar, with a fluorescent zone surrounding the growth, indicates the presence of *Pseudomonas* spp. colonies [24]. Then, the incubation was done for 24 – 48 hours at 37 ^{o} C.

2.4.8 Identification of the bacterial isolates

The isolated bacteria were identified according to Bergey's Manual of Systematic Bacteriology [25], [26].

2.4.9 Antibacterial effects of green tea, clove and thyme by agar well diffusion method

It is widely used to evaluate the antimicrobial activity of plants extracts in vitro [27]. The agar plate surface is inoculated by spreading a volume of the isolated identified bacterial inoculum over the entire agar surface. The three bacterial isolates from fresh fish; namely, *Staphylococcus* sp., *Bacillus* sp. and *Aeromonas* sp. were used. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer, and then a volume of the three plants extracts (green tea, clove and thyme) with different concentration (10 %, 20 % and 30 %) was introduced into the well. Then, agar plates are incubated at 37o C for 24 hours. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the tested bacteria. Zones of inhibition were measured and the average zone diameter of each dilution was taken.

3 Results

3.1 Chemical analysis

3.1.1 Peroxide value (PV)

At the beginning of the storage, PVs was absent in all fresh fish samples. Then this value was increased up to 2nd month. Surprisingly, the latter value was decreased at the 4th month. Finally, an increase of PV at the 6th month in all treatments was observed except that of the control group. On the other hand, the mean PV of mullet stored with the three plant extracts were significantly decreased ($p < 0.05$) compared with that of the control ones except that of green tea at the 1st and the 2nd months which showed no significant decreases ($p > 0.05$) (Table 1).

3.1.2 Malondialdehyde (MDA)

The mean level of MDA of fresh fish samples was 0.12 ± 0.04 mg /Kg. Generally, the values of MDA of all groups were increased up to the 4th month and decreased during the remaining 2 months of the storage time. On the other hand, the mean value of MDA of mullet stored with plant extracts were significantly decreased ($p < 0.001$) when compared with that of the control one (Table 1).

3.1.3 Free fatty acids (FFA)

The mean FFA levels were determined as % of oleic acid. It was 0.33 ± 0.04% for the fresh samples. The latter value was increased during storage time in all groups up to the 2nd month; while it was decreased at the 4th month. When the mean values of FFA of green tea, clove and thyme treated groups were compared with that of the control group during storage time, significant decreases were shown during the first and the second months. After the 4th month, no significant changes were found ($p > 0.05$) (Table 1).

3.2 Microbiological analyses

3.2.1 Total viable bacterial count (TVBC)

The mean values of TVBC of the untreated fresh fish samples were 366 ± 55, 85 ± 21 and 703 ± 31 CFU/gm tissue wt in tissue, gill and intestine, respectively. The results of TVBC during the 6 months of mullet storage showed that in the case of tissue, the count increase till the 4th month and suddenly decrease at the 6th month. In the case of gill and intestine there was a fluctuation in TVBC. The results of TVBC during the 6 storage months showed that in the case of tissue, the highest number was recorded after 4 months when treated with thyme while the lowest number was recorded after 2 months when treated with clove. In the case of gill, there was a high number of TVBC after 2 months with thyme treatment while low number was recorded after 4 months with clove treatment. Finally, in case of intestine, the highest number was observed after 2 months with thyme treatment while no bacterial count was recorded after 1 and 4 months when treated with clove and green tea, respectively. The mean value of TVBC of mullet stored with plant extracts were significantly decreased ($p < 0.05$) compared with those which were stored without plant extracts in all cases except thyme at 2nd month in case of gill which showed a non-significant decrease ($p > 0.05$) (Table 2).

3.2.2 Detection of *Staphylococcus* spp.

When the mean levels of *Staphylococcus* spp. count of treated mullet were compared with those of mullet stored in distilled water (control); in the case of tissue there were significant decreases ($p < 0.05$) except after green tea treatment at the 1st and 6th months, clove at 4th and 6th months and thyme at 2nd and 6th months (Table 3). In case of gill, there were significant decreases ($p < 0.05$) except after clove treatment at the 4th month. In the case of intestine, there were significant decreases ($p < 0.05$) at the 1st and the 4th months only while there was non-significant difference at the 2nd and the 6th months as the *Staphylococcus* spp. was not detected in all cases (Table 3).

3.2.3 Detection of lactose-fermenting (coliform) and non-lactose fermenting organisms

Generall, all the three extracts completely inhibit the growth of both lactose and non- lactose fermenting organisms in tissues and intestine. Also in Gill, clove showed the latter phenomena but the other two only reduce such count. Also, all of them inhibit such growth at the 6th months of the storage period (Table 4).

3.2.4 Detection of *Aeromonas* spp.

Generally, green tea inhibits the growth of *Aeromonas* sp. in tissue, gill and intestine. Clove found to enhance the bacterial growth at 1st month in tissue and gill, but in case of intestine the enhancement was found at 4th month. Thyme also showed the same phenomena at 1st month in tissue, at 1st and 2nd months in gill and at 2nd month in case of intestine. Also, all of them inhibit the bacterial growth on the other cases (Table 5).

TABLE 1

CHANGES IN PV (MMOL OF O_2 / KG), MDA MG (MDA/KG) AND FFA (% OLEIC ACID) DURING FREEZING STORAGE TIME IN MULLET STORED IN DISTILLED WATER (CONTROL), GREEN TEA, CLOVE AND THYME.

Parameters	Group	Storage time (months)				
		0 (Fresh)	1	2	4	6
PV	Control	0.00±0.00	5.05 ± 0.80 [a]	103.5 ± 5.7 [a]	66.3 ± 9.9 [a]	64.8 ± 5.00 [a]
	Green tea	-	4.60 ± 0.20 [a]	102.1 ± 5.70 [a]	26.7 ± 6.55 [a, b]	37.4 ± 5.40 [a, b]
	Clove	-	3.20 ± 0.25 [a, b]	14.9 ± 1.42 [a, b, c]	14.0 ± 3.00 [a, b, c]	34.0 ± 3.00 [a, b]
	Thyme	-	2.93 ± 0.60 [a, b]	7.3 ± 0.45 [a, b, c]	6.2 ± 1.60 [a, b, c]	11.3 ± 20 [a, b, c, d]
MDA	Control	0.12 ± 0.04	0.30 ± 0.07 [a]	0.47 ± 0.06 [a]	0.52 ± 0.06 [a]	0.3 ± 0.03 [a]
	Green tea	-	0.16 ± 0.04 [b]	0.18 ± 0.02 [a, b]	0.26 ± 0.02 [a, b]	0.2 ± 0.02 [a, b]
	Clove	-	0.18 ± 0.03 [b]	0.25 ± 0.02 [a, b]	0.31 ± 0.04 [a, b, c]	0.15 ± 0.03 [b]
	Thyme	-	0.18 ± 0.04 [b]	0.2 ± 0.02 [a, b]	0.30 ± 0.04 [a, b]	0.17 ± 0.03 [a, b]
FFA	Control	0.33 ± 0.04	5.40 ± 0.20 [a]	10.01 ± 1.05 [a]	3.33 ± 0.63 [a]	3.6 ± 0.40 [a]
	Green tea	-	2.72 ± 0.30 [a, b]	6.57 ± 0.95 [a, b]	3.23 ± 0.04 [a]	3.5 ± 0.20 [a]
	Clove	-	0.65 ± 0.015 [b, c]	5.02 ± 0.40 [a, b, c]	3.24 ± 0.36 [a]	3.4 ± 0.08 [a]
	Thyme	-	1.70 ± 0.40 [a, b, c, d]	6.27 ± 0.20 [a, b, d]	2.94 ± 0.21 [a]	3.4 ± 0.09 [a]

The values are expressed as mean ± standard deviation, n=6. a = significant (P< 0.05) when compared with fresh, b = significant (P< 0.05) when compared with control at each month, c = significant (P< 0.05) when compared with green tea treated group at each month, d = significant (P< 0.05) when compared with clove at each month. Pv: peroxide value, MDA: malondialdehyde and FFA: free fatty acids.

TABLE 2

CHANGES IN TVBC (CFU/ gm TISSUE WT) DURING FREEZING STORAGE TIME IN MULLET (TISSUE, GILL AND INTESTINE) STORED IN DISTILLED WATER (CONTROL), GREEN TEA, CLOVE AND THYME.

TVBC	Group	Storage time (months)				
		0 (Fresh)	1	2	4	6
Tissue	Control	366 ± 55	803 ± 120 [a]	2100 ± 424 [a]	5833 ± 1041[a]	1483 ± 116 [a]
	Green tea	-	330 ± 70.7 [b]	60 ± 14 [b]	85 ± 21.2 [b]	150 ± 14 [b]
	Clove	-	207 ± 50 [b]	15 ± 7 [b]	70 ± 00 [b]	460 ± 28 [b, c]
	Thyme	-	250 ± 56.5 [b]	660 ± 65.6 [b, c, d]	1117 ± 102 [b, c, d]	145 ± 35 [b, d]
Gill	Control	85 ± 21	8933 ± 115 [a]	4500 ± 707 [a]	5487 ± 480 [a]	3533 ± 611 [a]
	Green tea	-	220 ± 14 [b]	155 ± 35 [b]	707 ± 99 [b]	567 ± 87 [b]
	Clove	-	475 ± 35 [b]	115 ± 49 [b]	50 ± 14 [b, c]	357 ± 49 [b, c]
	Thyme	-	860 ± 580 [a, b]	4293 ± 536 [c, d]	1085 ± 248 [a, b, c, d]	65 ± 21 [b, c, d]
Intestine	Control	703 ± 31	9000 ± 00 [a]	2590 ± 509 [a]	3270 ± 252 [a]	3910 ± 551 [a]
	Green tea	-	20 ± 00 [a, b]	40 ± 28 [b]	00 ± 00 [a, b]	225 ± 92 [b]
	Clove	-	00 ± 00 [a, b]	70 ± 00 [b]	200 ± 28 [a, b, c]	33 ± 6 [b, c]
	Thyme	-	73 ± 12 [a, b, c, d]	1423 ± 232 [a, b, c, d]	125 ± 35 [a, b, c]	247 ± 55 [b, d]

The values are expressed as mean ± standard deviation, n=3. a = significant (P< 0.05) when compared with fresh, b = significant (P< 0.05) when compared with control at each month, c = significant (P< 0.05) when compared with green tea treated group at each month, d = significant (P< 0.05) when compared with clove at each month, TVBC= total viable bacterial count

TABLE 3

CHANGES IN *STAPHYLOCOCCUS* SPP. COUNT (CFU/ gm TISSUE WT) DURING FREEZING STORAGE TIME IN MULLET (TISSUE, GILL AND INTESTINE) STORED IN DISTILLED WATER (CONTROL), GREEN TEA, CLOVE AND THYME.

Staphylococcus spp.	Group	Storage time (months)				
		0 (Fresh)	1	2	4	6
Tissue	Control	0 ± 0	25 ± 7 [a]	250 ± 98 [a]	0 ± 0	0 ± 0
	Green tea	-	15 ± 7 [a]	15 ± 7 [b]	0 ± 0	0 ± 0
	Clove	-	0 ± 0 [b, c]	0 ± 0 [b]	0 ± 0	0 ± 0
	Thyme	-	0 ± 0 [b, c]	200 ± 56 [a, c, d]	525 ± 219 [a, b, c, d]	0 ± 0
Gill	Control	110 ± 57	943 ± 210 [a]	500 ± 0 [a]	740 ± 28	20 ± 0
	Green tea	-	0 ± 0 [b]	113 ± 12 [b]	0 ± 0 [b]	0 ± 0 [a, b]
	Clove	-	0 ± 0 [b]	35 ± 7 [b, c]	10 ± 0	0 ± 0 [a, b]
	Thyme	-	50 ± 14 [b, c, d]	2266 ± 35 [a, b, c, d]	3567 ± 404 [a, b, c, d]	0 ± 0 [a, b]
Intestine	Control	45 ± 7.0	23 ± 6 [a]	0 ± 0 [a]	135 ± 21 [a]	0 ± 0 [a]
	Green tea	-	0 ± 0 [a, b]	0 ± 0 [a]	0 ± 0 [a, b]	0 ± 0 [a]
	Clove	-	0 ± 0 [a, b]	0 ± 0 [a]	0 ± 0 [a, b]	0 ± 0 [a]
	Thyme	-	0 ± 0 [a, b]	0 ± 0 [a]	0 ± 0 [a, b]	0 ± 0 [a]

The values are expressed as mean ± standard deviation, n=3. a = significant (P< 0.05) when compared with fresh, b = significant (P< 0.05) when compared with control at each month, c = significant (P< 0.05) when compared with green tea treated group at each month, d = significant (P< 0.05) when compared with clove at each month.

TABLE 4

CHANGES IN LACTOSE-FERMENTING (COLIFORM) AND NON- LACTOSE FERMENTING ORGANISMS COUNT (CFU/ gm TISSUE WT) DURING FREEZING STORAGE TIME IN MULLET (TISSUE, GILL AND INTESTINE) STORED IN DISTILLED WATER (CONTROL), GREEN TEA, CLOVE AND THYME.

Lactose and non- lactose fermenting organisms	Group	Storage time (months)				
		0 (Fresh)	1	2	4	6
Tissue	Control	0 ± 0	130 ± 28 [a]	55 ± 7 [a]	10 ± 0.7 [a]	0 ± 0
	Green tea	-	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0
	Clove	-	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0
	Thyme	-	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [b]	0 ± 0
Gill	Control	45 ± 7.0	125 ± 35 [a]	315 ± 50 [a]	17 ± 5.7 [a]	0 ± 0 [a]
	Green tea	-	15 ± 7 [b]	35 ± 7 [b]	0 ± 0 [a, b]	0 ± 0 [a]
	Clove	-	0 ± 0 [b]	0 ± 0 [b]	0 ± 0 [a, b]	0 ± 0 [a]
	Thyme	-	15 ± 7 [b]	265 ± 21 [a, c, d]	0 ± 0 [a, b]	0 ± 0 [a]
Intestine	Control	23 ± 5.7	75 ± 7 [a]	20 ± 0	40 ± 0 [a]	0 ± 0 [a]
	Green tea	-	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a]
	Clove	-	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a]
	Thyme	-	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a, b]	0 ± 0 [a]

The values are expressed as mean ± standard deviation, n=3. a = significant (P< 0.05) when compared with fresh, b = significant (P< 0.05) when compared with control at each month, c = significant (P< 0.05) when compared with green tea treated group at each month, d = significant (P< 0.05) when compared with clove at each month.

TABLE 5

TABLE 5 CHANGES IN *AEROMONAS* SPP. COUNT (CFU/ gm TISSUE WT) DURING FREEZING STORAGE TIME IN MULLET (TISSUE, GILL AND INTESTINE) STORED IN DISTILLED WATER (CONTROL), GREEN TEA, CLOVE AND THYME.

Aeromonas SPP.	Group	Storage time (months)				
		0 (Fresh)	1	2	4	6
Tissue	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Green tea	-	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Clove	-	235 ± 50 [a, b, c]	0 ± 0	0 ± 0	0 ± 0
	Thyme	-	20 ± 0 [d]	0 ± 0	0 ± 0	0 ± 0
Gill	Control	20 ± 0	35 ± 7	0 ± 0 [a]	70 ± 0 [a]	80 ± 26 [a]
	Green tea	-	0 ± 0	0 ± 0 [a]	0 ± 0 [a, b]	0 ± 0 [b]
	Clove	-	95 ± 35 [c]	0 ± 0 [a]	0 ± 0 [a, b]	0 ± 0 [b]
	Thyme	-	85 ± 35 [c]	35 ± 7 [a, b, c, d]	45 ± 7 [a, b, c, d]	0 ± 0 [b]
Intestine	Control	60 ± 0	330 ± 14 [a]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
	Green tea	-	0 ± 0 [a, b]	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
	Clove	-	30 ± 14 [b]	0 ± 0 [a]	45 ± 21 [b, c]	0 ± 0 [a]
	Thyme	-	200 ± 28 [a, b, c, d]	10 ± 0.7 [a, b, c, d]	0 ± 0 [a, d]	0 ± 0 [a]

The values are expressed as mean ± standard deviation, n=3. a = significant (P< 0.05) when compared with fresh, b = significant (P< 0.05) when compared with control at each month, c = significant (P< 0.05) when compared with green tea treated group at each month, d = significant (P< 0.05) when compared with clove at each month.

3.2.5 *Vibrio* spp. and *Pseudomonas* spp.

In the present study, the results showed that *Vibrio* spp. and *Pseudomonas* spp. were not found at all cases.

3.2.6 Identification of the tested isolated bacteria

Generally, the isolated bacteria were identified according to the cultural, morphological and physiological properties listed in Bergey's Manual of Systematic Bacteriology. According to the previous criteria, they were closely related to *Staphylococcus* sp., *Bacillus* sp. and *Aeromonas* sp.

3.2.7 Antibacterial effects of green tea, clove and thyme *in vitro* (Agar well diffusion)

The three plants extracts with different concentrations have antibacterial activities against *Staphylococcus* sp., *Bacillus* sp. and *Aeromonas* sp. as represented in (table 6 and figures 1- 3). When the three different concentrations of green tea were compared with each other, green tea (20 %) was found to be the best one against all tested isolates. There were no significant differences between all clove concentrations when compared with each other in case of *Staphylococcus* sp. and *Aeromonas* sp. strains, but in the case of *Bacillus* sp. the 20 % and 30 %concentrations gave the best results. In the case of thyme; the 30 % concentration gave the best result against *Staphylococcus* sp. Also, in case of *Aeromonas* sp. 10 % and 20 % concentrations gave the best results, but in the case of *Bacillus* sp. the effect of thyme was not determined.

TABLE 6

THE ANTIBACTERIAL ACTIVITIES OF GREEN TEA, CLOVE AND THYME EXTRACTS.

Plant	Concentration (%)	Inhibition zone (Cm)		
		Staphylococcus sp.	*Aeromonas* sp.	*Bacillus* sp.
Green tea	10	3.6 ± 0.26	1.9 ± 0.12	1.3 ± 0.08
	20	4.4 ± 0.7 [a]	2.9 ± 0.5 [a]	1.8 ± 0.05 [a]
	30	3.3 ± 0.15 [b]	2.5 ± 0.3 [a]	1.7 ± 0.16 [a]
Clove	10	2.8 ± 0.15	3.7 ± 0.23	1.5 ± 0.05
	20	3 ± 0.14	3.6 ± 0.35	1.8 ± 0.05 [e]
	30	2.8 ± 0.2	3.6 ± 0.35	1.9 ± 0.05 [e]
Thyme	10	1.3 ± 0.19	2.6 ± 0.24	ND
	20	1.8 ± 0.19	2.8 ± 0.18	ND
	30	2.5 ± 0.35 [c, d]	1.5 ± 0.14 [c, d]	ND

The values are expressed as mean ± standard deviation, n=6. a = significant (P ≤ 0.05) when compared with green tea (10 %), b = significant (P ≤ 0.05) when compared with green tea (20 %), c = significant (P ≤ 0.05) when compared with thyme (10 %), d = significant (P ≤ 0.05) when compared with thyme (20 %), e = significant (P ≤ 0.05) when compared with clove (10 %) and ND = not determined.

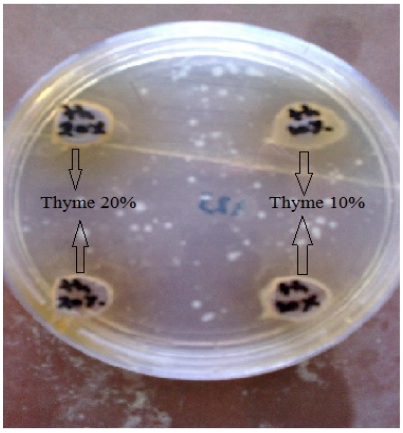

Fig. 1. Zones of inhibition produced by thyme (10% and 20%) against *Aeromonas* sp.

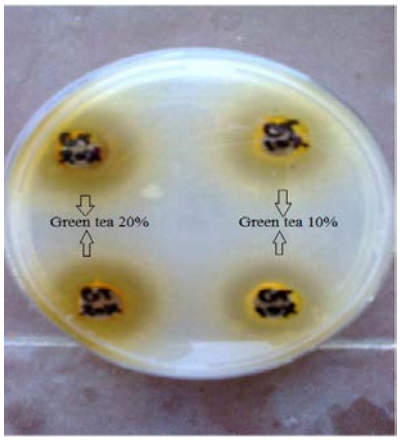

Fig. 2. Zones of inhibition produced by green tea (10% and 20%) against *Bacillus* sp.

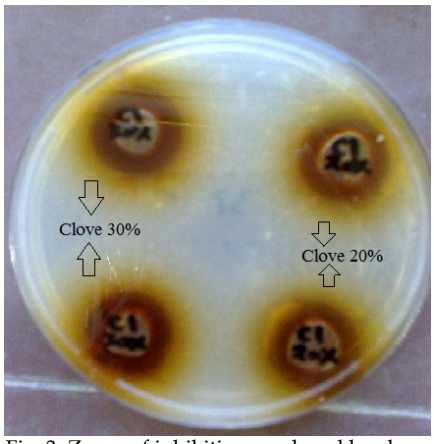

Fig. 3. Zones of inhibition produced by clove (20% and 30%) against *Bacillus* sp.

4 Discussion

Microbial activity is responsible for fish spoilage. Therefore, total viable bacterial count (TVBC) is an important parameter [3]. According to [28], 106 CFU/ gm is the maximum acceptable limit of total viable bacterial count in chilled fish and 107 in fresh fish [30]. Normally, the acceptable number of microorganisms on gills and in intestine was found to be between 103-109 CFU/g [29]. In the present study, the tissue, gill and intestine contain *Staphylococcus* spp., lactose and non-lactose fermenting organism and *Aeromonas* spp. in gill and intestine, even during storage. Surprisingly, all the three extracts completely inhibit the growth of both lactose and non-lactose fermenting organisms in tissues and in intestine and the clove added that in tissues.Also in gill, clove showed the latter phenomena and all of these extracts inhibit such growth at the 6th months of the storage period. These findings confirm the possible abilities of such extracts to mediate shelf period of preservation. These observations is in cotorary to that of [30] who observed that freezing only can causes a reduction in bacterial count and the number will continue, in most cases, to fall during storage. A similar decrease in the TVBC count of the control fish was found by [31].

The high prevalence of *Staphylococcus* in fish samples indicates the unhygienic handling of fish. Degradation of fish is accelerated by *Staphylococcus* spp. associated with aquatic environments as well as contamination during post-harvesting handling [32]. *Staphylococcus* spp. has pathogenic strains which could cause food poisoning. This is because the heat stable nature and gastrointestinal enzymes antagonists of *Staphylococcus* enterotoxin [33]. According to [29], 103 CFU/ gm is the maximum acceptable limit of *Staphylococcus aureus* in chilled fish.

National Academy of Science and Karim & Sanjee [34], [35] reported that Coliform group of bacteria in fish has been considered important in microbiological analysis on account of their significance as indicator organisms for determining the unhygienic conditions during catching, handling, processing and distribution.

Aeromonas spp. are widely distributed in aquatic environments [36] and considered arising food borne pathogens that are related with septicemia, gastroenteritis, enterocolitis and wound infection in humans [37].

Pseudomonads spp. is a good spoilage index in fishes [38] and a marker for fish deterioration; furthermore they have to be the specific spoilage organism (SSO) in ice-stored tropical freshwater fish [39]. *Vibrio* spp. has been frequently defined as opportunistic and potential pathogenic bacteria of the water bodies especially in warm climate zones [40]. According to [41], fresh and frozen fish should be free of *Vibrio*. Fortunately, *Pseudomonads* spp. and *Vibrio* spp. can't be detected in any sample.

Primary oxidation can be measured by peroxide value (PV) [42], [43]. PV is one of the most important factors responsible for fish spoilage during storage [44]. Boran, Karacam and Boran [45] suggested 8 meq/kg as the acceptable limit of PV of oils for human consumption. Other reviews found that PV of crude fish oil was between 3 and 20 meq O_2 /Kg [46].The values of PV were hugely elevated compared with the later reported value at 1st and 2nd months, especially for the non-treated control group. At 4th and 6th months, a slight decrease of PV was observed for the same group. The causative factor for the primary increase in PV may be due to the formation of hydroperoxide which is the primary product of lipid oxidation [47], [43]. The latter decrease in PV may depends on hydroperoxide degradation into secondary peroxidation products one to produce low molecular weight compounds; including aldehydes and ketones [48]. After treatment, the PV values showed significant decreases ($P< 0.05$) when compared with that of the control groups except those of green tea at the 1st and 2nd months. These results may be due to the presence of higher levels of phenolic compounds in the green tea extracts that partially inhibit the peroxide formation and fatty free radicals. The latter readicals react with or absorb oxygen in the autoxidation process, thus delaying the onset of the autoxidative process in fat [49]. Also, [50] showed that higher concentration of green tea may act as pro-oxidant, thus, participate in the excessive PV formation. This is actually the case in the present study. The re-reduction of PV after 4th and 6th months may be due to the consumption of phenolic compounds either via their antioxidative effect or for their auto-destruction with time. The higher reduction in the PV

levels after treatment of the fish with both clove and thyme during the time range of preservation may lead one to conclude that the parameter which participates in the antioxidants defense of clove and thyme may carry higher antioxidative activities compared with those of green tea.

Thiobarbituric acid (TBA) value is a commonly used indicator for secondary lipid oxidation that measure malondialdehyde (MDA) content directly. MDA is an oxidative secondary product that results from lipid hydroperoxides degradation [51], [49], [5], [43]. TBA values were increased in tissue homogenate of all groups during storage time up to the fourth month. At the 6th month in all groups the latter levels were decreased. This phenomena was also observed by [52], [49], [53].The increase in TBA value during storage time is due to the formation of the secondary lipid oxidation products; including, MDA. On the other hand, the final decrease of TBA may be due to the breakdown of the MDA via tertiary degradation [54]. The latter decrease may also be due to the fact that during storage period the protein molecules decompose to give smaller peptides, amino acids, etc. these molecules interact with MDA to give tertiary products [55]. The statistical analysis of TBA values showed that the values of the control were significantly increased ($P < 0.05$) than those of all treated groups. Again, the mechanism may involve the participation of the high amount of phenolic compounds of clove, thyme and green tea in the retardation of lipid peroxidation via their antioxidant effect [56], [57], [49], [53].

Free fatty acids (FFAs) are used for determination of lipid hydrolysis in tissue [58]. These acids may be produced via hydrolytic and/or enzymatic hydrolysis mechanisms [42], [59]. In this study, the levels of FFAs were rapidly increased for all groups at 1st and 2nd months of freezing storage. This may be due to decomposition of tissue fats; including phospholipids and triglycerides, by the effect of lipid hydrolyzing enzymes; namely, lipase and phospholipase. The release of these enzymes may involve liposomal degradation at the first stage of storage [44], [60], [61]. At 4th month there is a decrease in the value of FFAs. A possible bacterial involvement will also contribute for such disintegration in the present study.This decrease may be as a result of interaction between FFAs with the smaller peptides and other protein derivatives of protein degradation via proteolytic hydrolysis as was previously reported by [62], [63], [64]. The exact mechanism of this interaction was not yet been shown, but it is likely to be mediated through electrostatic, Vander Waals, hydrogen or hydrophobic forces rather than the covalent binding one [65]. Also, one can expect the involvement of oxidative process in such ligation process. Again, the reduction of FFAs values in all treated groups at 1st and 2nd months compared with that of the control may be attributed to the effect of phenolic compounds of used plant extracts [61], [49]. In our opinion, the mechanism may involve absence of liposome degradation, lipase elevation and then decrease in mullet tissue degradation via free radical dismutation by thyme, clove and green tea incorporation.

The protective effect of clove against bacterial growth and tissue deterioration may be mediated by eugenol, major phenolic component, which cause bacteria envelope damage [66]. Also, [67] reported that carvarcol and thymol (extracted from thyme) can dissolve in the cytoplasmic membrane by hydrophobic – hydrophobic nature interaction causing increase in membrane permeability for ATP, thus, causing membrane damage. The antimicrobial compounds in green tea are from catechins group. This group binds to bacterial lipids bilayer cell membrane causing its damage [68], [69]. This damage inhibits the ability of the bacteria to bind to host cells [70] and inhibit toxins secretions [71]. Green tea extract can inhibit the synthesis of bacterial fatty acids, a fuel of bacterial growth, and inhibit the production of toxic metabolites [72].These mechanisms illustrate why the 3 extracts showed antibacterial effect in vitro.

5 CONCLUSION

Bacterial contamination mediates fish's lipids deterioration. Simultaneously, the antioxidants contents of the extracts participate in such bacterial intoxication via preventing lipid rancidity elevation as was described by in vitro and in vivo investigation. Also, the possible involvement of the antioxidant and free radical mediated mechanisms of these extracts during fish storage was included.

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