

Thymus vulgaris L. as a Natural Antioxidant in Cooked Fillet of Trout (*Oncorhynchus mykiss*)

M. Azizkhani^{1*}, N. Abdi²

1. Department of Food Hygiene, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran

2. Department of Food Sciences, Faculty of Agriculture, Khazar University of Higher Education, Mahmoudabad, Iran

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Background: Due to high content of unsaturated fatty acids, trout is susceptible to oxidative spoilage. In this study, the effects of *Thymus vulgaris* Essential Oil (EO) and extract on oxidative stability of cooked rainbow trout fillet during four months frozen storage period were investigated.

Methods: Three groups of fish fillets were treated with thyme EO and three other groups were treated with thyme extract and cooked by frying, oven, and steam. Fat hydrolysis was evaluated by measuring Free Fatty Acid (FFA) value and oxidation products were measured via Peroxide Value (PV) as well as Thiobarbituric Acid (TBA) value. Sensory analysis was evaluated by the overall acceptability using a 9-point hedonic scale. Statistical analyses were performed in SPSS, Inc, Chicago, IL software.

Results: Main components of *T. vulgaris* were thymol (60.54%), α -terpinen (9.47%), p-cymene (8.54%) and carvacrol (3.33%). The amount of FFA in oven baked samples (4.51–4.75% oleic acid) and steamed fillets (4.83–5.20% oleic acid) was significantly ($p < 0.05$) higher than control and fried fillets. PV values showed an increase in all groups, especially fried fillets with the highest amount of PV ($p < 0.05$). TBA values in the treated groups were significantly lower than control fillets ($p < 0.05$). At day 0, steamed samples containing EO and extract showed lower scores of overall acceptability (8.64 ± 0.31 and 8.64 ± 0.64 , respectively) compared to fillets cooked by frying and oven. However, at the end of the four months storage period, both treated and control groups had the lowest sensory scores with no significant difference ($p > 0.05$).

Conclusion: Both thyme EO and extract effectively retarded the oxidation during frozen storage. However, the samples treated with thyme extract showed slower formation of free fatty acid, hydroperoxide and malonaldehyde than those of EO-treated or control samples. It is recommended to apply thyme EO and extract in producing ready-to-eat fish products.

Introduction

Trout, as a freshwater fish, has been one of the most widely cultured species all over the world due to its fast

growth rate, easy cultivation, and high feed efficiency ratio. In Iran, a type of trout species, named “rainbow”

*Corresponding author
E-mail: m.azizkhani@ausmt.ac.ir

trout (*Oncorhynchus mykiss*), is extensively cultured (Rezaei and Hosseini, 2008). Ready-to-eat products such as cooked types of fish fillet are commonly stored and marketed in the frozen state. However, sea products may undergo chemical and microbial spoilage during frozen storage limiting the shelf-life of the product. Fish meat is more susceptible to oxidative degradation than the other meat products, since it contains high level of unsaturated fatty acids (Vandamme et al., 2015). Deterioration of flavor, odor, color, and production of toxic compounds can result from oxidation of lipids. As an example, malonaldehyde (MA), the secondary product of lipid peroxidation, is thought to be toxic, carcinogenic, and also mutagenic (Ayala et al., 2014). Although, cooking enhances desirable flavors and tastes of fish flesh, inactivates enzymes and destroys microorganisms and can lead to undesirable changes, e.g. lipid oxidation, protein denaturation, as well as color alteration (Ozyurt, 2013; Vandamme et al., 2015). It is also known that cutting, mincing, and cooking affect the lipid stability of meat and meat products. Processing operations lead to increase product surface area, disrupt and expose phospholipid fractions of subcellular membranes and intramuscular fat to pro-oxidants, introduce interstitial air and generally accelerate oxidative reactions (McBride et al., 2007).

Applying antioxidant is one of the methods that retards or prevents lipid oxidation, preserves the quality and extends the shelf-life of food products. Because of the possible toxicity of synthetic preservatives that are used as antioxidants, interest in natural antioxidants has been increased in recent decades, both on industry and consumer side (Ozyurt, 2013). A number of studies in relation to the potential of herbs and spices as natural antioxidants have been reported (Dorman et al., 2003; Exarchou et al., 2002; Ozcan, 2003; Tsai et al., 2005). Among the plants reported to have antioxidative activity, garden thyme (*Thymus vulgaris* L.) as Essential Oil (EO) or as an extract is used in some food products. The high antioxidant capacity of garden thyme is due to the phenolic compounds, carvacrol, thymol, and the main non-phenolic constituents such as linalool and p-cymene (Atti-Santos et al., 2004; Goodner et al., 2006).

Some researchers have investigated the effects of thyme EO on organoleptic characteristics and nutritional value of raw rainbow trout fillets during chilled storage, and found that thyme EO addition helped prolonging the shelf-life of raw trout fillets, gave acceptable sensory quality and limited microbiological growth during chilled storage (Chamanara et al., 2012; Kykkidou et al., 2009). However, if antioxidant activities of thyme can remain stable during cooking of trout fillet or not, is still unknown. As heat treatment and subsequent storage of the cooked fish containing high levels of unsaturated lipids enhance the formation of oxidized off flavors, food

industry has an interest in new approaches that allow seafood products to be processed with less oxidative deterioration. The aim of this study was to investigate lipid stability of rainbow trout fillets treated with garden thyme (*T. vulgaris*) EO and extract cooked by three methods of cooking (frying, oven baking, and steaming) during four months frozen storage.

Materials and methods

EO and extract of thyme

T. vulgaris EO and extract were obtained from Barij Essence Pharmaceutical Company, Kashan, Iran. The EO and extract were stored in airtight dark glass vials at 4 °C.

Gas chromatography (GC)/Mass spectroscopy (MS)

Components of the thyme EO was analyzed by GC apparatus (Thermo Quest 2000, Finnigan, UK). The chromatograph was equipped with a DB5 capillary column (30 m×0.25 mm ID×0.25 mm film thickness) and the data were obtained under the following conditions: initial temperature 50 °C, program rate 2.5 °C, final temperature 265 °C, and injector temperature 250 °C. The carrier gas was helium and the split ratio was 120. The EOs were also analyzed by GC/MS (Thermo Quest, USA) using the same capillary column and analytical conditions indicated earlier. MS system was run in the electron ionization mode, using ionization energy of 70 eV. All components were identified based on the comparison of their relative retention time as well as mass spectra with those of standards. Alkanes were applied as reference points in calculation of relative retention indices.

Total phenolics assay of the extract

The total phenolic content of thyme extract was determined based on Shetty et al. (1995) with small modification. Briefly, 1 ml homogenized extract was transferred into a test tube and mixed with 1 ml 95% ethanol and 5 ml distilled water. Then, 0.5 ml 50% (v/v) folin-ciocalteu reagent was added to all samples and mixed thoroughly. After 5 min, 1 ml 5% Na₂CO₃ was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. Then, total phenolic contents were calculated using the absorbance values and were reported as microgram equivalents of gallic acid per gram of the sample. Standard curves were prepared using various concentrations of gallic acid in water.

Preparation of samples

Fresh rainbow trout (*O. mykiss*), between 500 and 700 g in weight, were purchased from local markets,

Mahmoudabad, Iran. Having been transferred in ice to the laboratory in less than 2 h, fish samples were beheaded, gutted, washed, and then filleted. Three groups of fish were treated with thyme extract and three other ones were treated with thyme EO. Three groups without thyme (neither EO nor extract) were also cooked by the same methods considered as control. The EO and extract were applied to fish as described by Akhtar et al. (1998). In brief, fillets were immediately immersed in EO (1 ml/L) and extract (20 ml/L) in distilled water for 2 min and cooked by different cooking methods. Sample fillets were deep-fried for 4 min in sunflower oil at 180 °C, oven baked for 22 min at 200 °C, and steamed for 10 min at 200 °C. One sample of each cooked groups (with and without thyme) was analyzed immediately, and then the other samples were stored in a deep freezer (-18 °C) inside the refrigerator bags separately for four months period in which samples were analyzed monthly. All experimental groups were assessed in triplicate.

Lipid extraction

The extraction of lipid was based on the method described by Mortensen et al. (2002). About 10 g of each sample was transferred to a 500 ml plastic centrifuge tube and 200 ml of chloroform:methanol (7:3) was added. Each sample was homogenized using a homogenizer (Heidolph, DiAx 600, Kelheim, Germany), 50 ml 1 mM CaCl₂ was added to the suspension that was shaken for 10 s. The mixture was then centrifuged at 1400 g at 20 °C for 30 min and the supernatant was transferred to a 500 ml separation funnel to collect the chloroform (lower) layer. The upper layer and the pellet were combined and mixed with 150 ml chloroform. The mixture was homogenized, and then centrifuged as mentioned above. This procedure was repeated once more. The three separated chloroform layers were combined and poured into a 1000 ml conical flask, and chloroform was evaporated at 60 °C using a Buchi vacuum Rotavapor R-215 (Büchi Labortechnik AG, Flawil, Switzerland). The remaining oil was then flushed with nitrogen and used for future analysis.

Determination of Free Fatty Acid (FFA)

FFA contents, expressed as percentage of oleic acid, was determined by the acidimetric titration of the blight and dyer extract after adding ethanol and using phenolphthalein as an indicator, following AOCS (1994).

Determination of Peroxide Value (PV)

Lipid samples (0.02 g) were weighed into a 25 ml volumetric flask and 15 ml chloroform:methanol (7:3) was added. To each sample were added 0.2 ml 1% ferrous

chloride and 0.2 ml 4 M ammonium thiocyanate, and then the final volume was made up to 25 ml using chloroform:methanol (7:3). Samples were mixed and kept under dimmed light for 5 min for absorbance determination at 505 nm. The result was expressed as milliequivalents of oxygen per kilogram of lipid. A sample blank and a reagent blank were also measured. All measurements were carried out in triplicate and under dimmed light. A standard curve was determined under the same conditions using ammonium ferric sulfate as the standard (Ye et al., 2009).

Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS was measured using a method described previously by Kristensen et al. (2001). The Thiobarbituric Acid (TBA) reagent was prepared immediately before use by mixing equal volumes of freshly prepared 0.025 M TBA (brought into solution by neutralizing with NaOH) and 2 M H₃PO₄/2 M citric acid. The combination of citric acid and phosphoric acid was used as both acidulants and metal chelators. Then, 18 ml TBA reagent was added into 6 g sample, and the resulting mixture was homogenized using an Ultra Turrax (Heidolph, DiAx 600, Germany) for 2 min until the mixture appeared to be homogeneous. An aliquot (6 ml) of the suspension was transferred to a pyrex tube to which 3.5 ml chloroform was added, followed by gentle mixing for 5 min. The mixture was centrifuged at 6000 g for 15 min at room temperature. The aqueous layer was transferred to another test tube, which was placed in a water bath at 100 °C for 10 min, followed by cooling with ice. Orange-red cyclohexanone supernatant was decanted, and then its absorbance at 532 nm was measured by spectrophotometer (BSA 3000 Chemistry Analyzer, SFRI, Saint Jean d'Ilac, France). The results were expressed as milligram of MA per kilogram of fish flesh.

Sensory analyses

Six experienced panelists (MS students in food science and technology course at Khazar University of Higher Education, Mahmoudabad, Iran) performed sensory analyses. Six trout fillets were reheated in a conventional oven at 75 °C for 3 min before presenting to the panelists. For sensory analysis, the panelists evaluated the overall acceptability using a 9-point hedonic scale (1, dislike extremely to 9, like extremely).

Statistical analyses

Statistical analyses were performed in SPSS, Inc, Chicago, IL software version 16.0 using different tests

including one-way ANOVA, Tukey, Kruskal–Wallis, and Mann–Whitney U test.

Results

GC/MS analysis identified 42 components for thyme EO, representing more than 99% v/v oil's content. Its main components were thymol (60.54%), α -terpinen (9.47%), p-cymene (8.54%), and carvacrol (3.33%). The total phenolic content in garden thyme extract was 21.5 ± 0.62 μg equivalents of gallic acid per gram.

During the whole storage period, the amount of FFA in oven baked samples (4.51–4.75% oleic acid) and steamed fillets (4.83–5.20% oleic acid) was significantly ($p < 0.05$) higher than control and fried fillets (Fig. 1). As illustrated in Fig. 2, there was difference between the PV of some samples in different months. Totally, PV values showed an increase in all groups, especially fried fillets with the highest amount of PV ($p < 0.05$). Changes in TBA values

of thyme treated and cooked trout fillets during frozen storage are shown in Fig. 3. TBA values in the treated groups were significantly lower than control fillets ($p < 0.05$).

Table 1 shows the changes in the overall acceptability values of cooked trout fillets with or without thyme. According to Table 1, at day 0, steamed samples containing EO and extract showed lower scores of overall acceptability (8.64 ± 0.31 and 8.64 ± 0.64 , respectively) compared to fillets cooked by frying and oven. During the first three months of storage, there was a statistically significant difference between treated samples (either with EO or extract) and control in all cooking methods ($p < 0.05$). However, at the end of the 4-month storage period, both treated and control groups had the lowest sensory scores with no significant difference ($p > 0.05$). At the 4th month of storage, the lowest overall sensory acceptability was obtained for steam cooked samples (control: 2, EO: 2.3, and extract: 2).

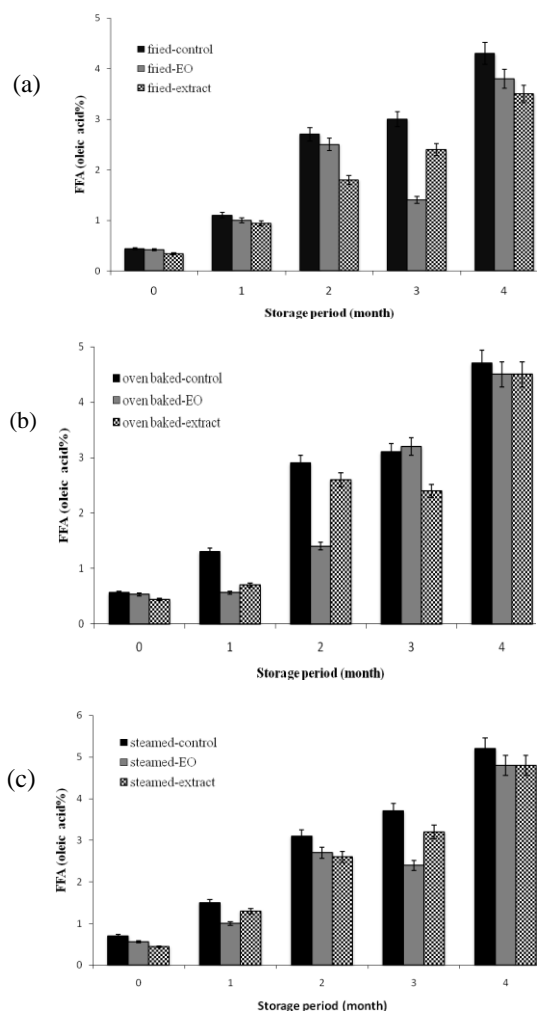


Fig. 1: FFA values (of oleic acid%) of thyme treated and control fillets groups cooked by frying (a), oven (b), and steam (c) during different storage period

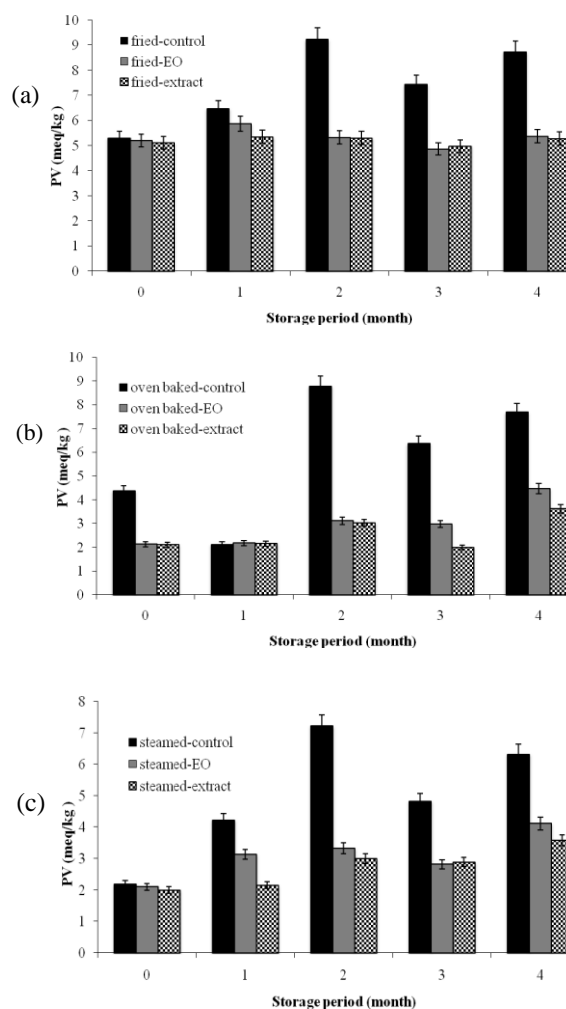


Fig. 2: PV values (meq/kg) of thyme treated and control fillets groups cooked by frying (a), oven (b), and steam (c) during different storage period

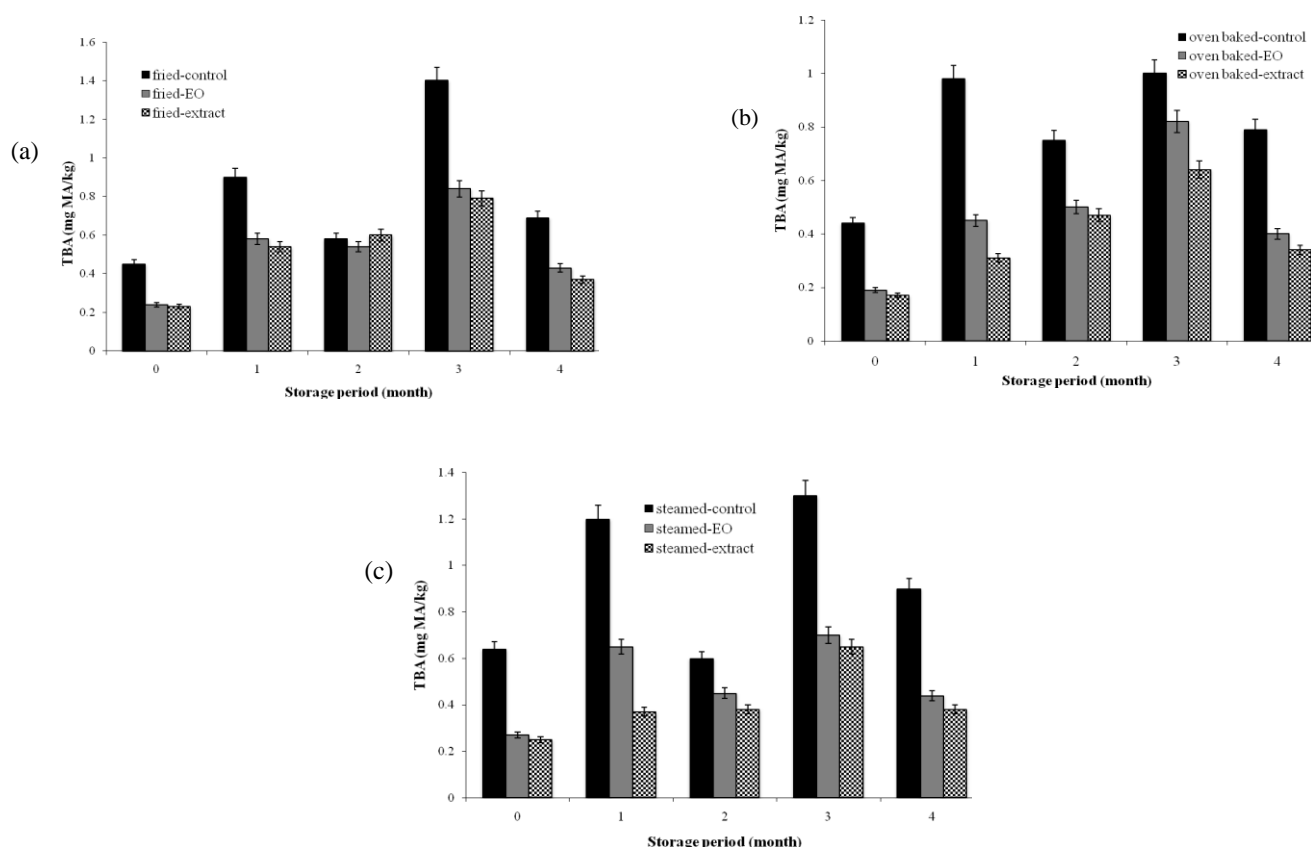


Fig. 3: TBA values (mg MA/kg) of thyme treated and control fillets groups cooked by frying (a), oven (b), and steam (c) during different storage period

Table 1: Overall acceptability scores of cooked trout fillets treated with thyme treated and control fillets during frozen storage

Test groups	Storage period (month)				
	0	1	2	3	4
Fried-control	9±0.00 ^{ab}	8.28±0.43 ^{ab}	7.56±0.28 ^{ab}	5.80±0.14 ^{ab}	5.43±0.72 ^{ab}
Fried-EO	8.82±0.24 ^a	8.46±0.75 ^a	8.1±0.61 ^a	6.45±0.54 ^{ab}	5.95±0.11 ^{ab}
Fried-extract	8.64±0.83 ^a	8.46±0.27 ^a	8.1±0.23 ^a	6.22±0.32 ^{ab}	5.80±0.81 ^{ab}
Oven baked-control	8.82±0.35 ^a	7.92±0.16 ^b	7.2±0.75 ^b	5.80±0.17 ^{ac}	5.43±0.22 ^{ac}
Oven baked-EO	8.82±0.19 ^a	7.74±0.56 ^b	7.74±0.66 ^a	6.22±0.68 ^{ab}	5.80±0.57 ^{ab}
Oven baked-extract	8.64±0.52 ^a	7.56±0.23 ^b	7.56±0.33 ^{ab}	5.95±0.34 ^{ac}	5.43±0.39 ^{ac}
Steamed-control	8.46±0.46 ^b	7.74±0.37 ^b	6.61±0.25 ^c	4.14±0.57 ^{bc}	3.6±0.33 ^{bc}
Steamed-EO	8.64±0.31 ^a	7.38±0.53 ^{cb}	7.01±0.50 ^b	4.5±0.32 ^{bc}	4.14±0.21 ^{bc}
Steamed-extract	8.64±0.64 ^a	7.38±0.46 ^{cb}	6.82±0.62 ^b	4.14±0.26 ^{bc}	3.6±0.42 ^{bc}

Different letters (a-c) in the same row indicate significant differences ($p < 0.05$) during storage periods

Discussion

In the present study, the main components of thyme EO were terpenes, terpenoids, as well as the other aromatic and aliphatic constituents. The results also showed that EO contained oxygenated monoterpenes and hydrocarbons monoterpenes. Our results confirmed earlier reports that major volatile constituents obtained from the aerial parts of *T. vulgaris* were thymol, carvacrol, p-cymene, -terpinene, and -caryophyllene (Bahreininejad et al., 2013; Rahimmalek and Goli, 2013).

According to this work, oven baked and steamed fillet samples showed an increase in their FFA contents. In a study conducted by Bakar et al. (2008), lipid quality in

cooked and chill-reheated fillets of mackerel has been investigated. The researchers reported that steaming, grilling, frying, and microwave cooking increased the FFA content of the fish samples. In our study, FFA contents showed fluctuations in the EO-treated-fried, EO-treated-steamed, and also extract-treated-oven baked groups during period of frozen storage. As, the lipolytic enzymes in fish tissue are active even at -20°C , so an increase in the level of hydrolysis of triglycerides and FFA formation can be expected during frozen storage (Hui, 2007). Also, decrease in FFA contents may be due to formation of complexes between FFA as well as the sarcoplasmic and myofibrillar muscle proteins (Reddy et

al., 1992). During heat treatment of food materials, lipid oxidation occurs and so, oxidation products are formed (Eder et al., 2003). In this study, the same effect has been observed by all three methods of cooking. Similarly, Tokur (2007) reported that PV for rainbow trout cooked by frying, oven baking, and grilling were increased. Also, Bakar et al. (2008) stated that the initial PV (1.78 meq/kg) of the samples was increased to 2.65, 2.80, and 3.12 meq/kg in fried, grilled, and steamed mackerel, respectively. In the present investigation, a considerable increase in the hydroperoxide formation was observed in all samples during the four months frozen storage (-18 °C) period. However, the samples treated with thyme EO or thyme extract showed lower PV in comparison to the untreated samples. In a similar study, hydroperoxide formation in sardine fish (*Sardina pilchardus*) chops treated with rosemary extract during the frozen storage was evaluated by Serdaroglu and Felekoglu (2005). They showed that PV in samples with rosemary extract was significantly lower than control group during storage at -20 °C. It can be concluded from our work that treatments with the thyme EO and extract decreased hydroperoxide formation rate in cooked trout fillets during frozen storage which is in accordance with studies mentioned before. Progressing oxidation of lipid, the hydroxyperoxides will be degraded into the secondary oxidation products with unpleasant sensory effects which may also have harmful effects on body health due to their neurotoxic, mutagenic, and cytotoxic actions (Long and Picklo, 2010; Uchida, 2000). Formation of secondary oxidation products (as mg MA/kg) from degradation of hydroxyperoxides in sardine samples was investigated by Serdaroglu and Felekoglu (2005). They reported that control samples were more rancid than the ones treated with rosemary extract during the storage time at -20 °C. It was found that initial TBA value (0.22 MA/kg) of rainbow trout muscle cooked by oven baking increased to 5.78 mg MA/kg and similarly, that heating such as frying, grilling, and smoking led to increase of TBA value (Tokur, 2007). Also, according to another survey, a raise in the initial TBA value (0.54 mg MA/kg) of raw muscles to 2.98, 2.80, 3.13, and 2.65 mg MA/kg have been revealed after applying several cooking methods, microwave, grilling, and frying, respectively (Bakar et al., 2008). Effects of hydroalcoholic and water extracts of nettle leaf on the chemical properties of super chilled minced meat of the common kilka fish (*Clupeonella cultriventris caspia*) was evaluated by Ahmadi et al. (2014) who showed that TBA values were gradually increased in all samples during 28 days storage. They stated that extract of nettle significantly increased the shelf life of minced kilka from 2 to 8 days. The results of the above studies are mainly in accordance with our work; however, in the present research TBA values of

steamed samples (both treatment and control groups) were higher than the samples cooked by other methods. TBA values in all groups in this study increased to the end of the third month but then started to decrease. The decreasing of TBA could be resulted from the formation of lipid oxidation products and the interaction of the present MA with the other compounds, myofibrillar protein in particular (Melton, 1983). Totally, it could be concluded that treating trout fillets with thyme (either EO or extract) exerted considerable effects in preventing MA formation in comparison to control group.

From the results of the sensory analysis, fried and steamed fillets had the highest and lowest score in the overall acceptability, respectively. Dreeling et al. (2000) assessed the effect of several cooking methods, such as grilling, frying, griddling, roasting, and deep fat frying on quality of low-fat beef burgers. They found that griddled burgers had the highest scores for overall acceptability. Similarly, Tokur et al. (2006) found that sensory scores of cooked carp finger (*Cyprinus carpio L.*) at 180 °C for 30 s, were decreased during a five-month frozen storage period but they were still within acceptable range. In the present study, there was not any significant difference between sensory scores of EO treated and extract treated fillets at the beginning of the study (just after cooking) and during the frozen storage period. However, overall acceptability score of thyme treated samples were higher than the control. The acceptability of sea products during storage in frozen condition depends on the changes in their sensory characteristics. The data obtained from this work also showed that fried and oven baked fillets of trout could be stored at -18 °C for three months while maintaining their acceptable quality in terms of sensory analysis of the samples, but steamed samples retained their sensory acceptability for about two months storage at freeze temperatures.

Conclusion

The results of the present study showed that treating fish fillets with thyme EO or extract before cooking reduced the rate of lipid oxidation. It can be concluded that the thyme EO and extract retarded lipid oxidation of cooked trout fillets during frozen storage. The acceptability scores of all cooked groups were decreased during the four months frozen storage. However, it was found that the oven baked and fried fillets samples remained quite acceptable during the first three months period of the frozen storage. Also, it is recommended to apply thyme EO and extract in producing ready-to-eat fish products.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

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