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EFFICACY OF FEW INDIAN SPICES AS ANTIOXIDANTS IN STABILISATION OF FISH OIL UNDER ACCELERATED CONDITION

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ABSTRACT

Antioxidant activities of five common spices - fennel, black-pepper, ginger, cinnamon, celery seeds and BHT, a synthetic antioxidant were evaluated on total phenol content, inhibition to peroxidation, metal chelating activity. Potential to reduce lipid peroxidation was determined in terms of peroxide, thiobarbituric acid, *p*-anisidine and conjugated diene-triene value, post addition in tilapia fish oil under accelerated condition. Maximum phenolic concentration was recorded in ginger followed by fennel and inhibition to peroxidation was highest in fennel followed by pepper. Fennel could remarkably control peroxide formation and decreased the amount of malonaldehyde accumulation in oil with longer thermal exposure. Though pepper and cinnamon also could reduce the TBARS substances, negative correlation of peroxide and *p*-anisidine value was recorded only in fennel. Fennel resulted as most efficient antioxidant, followed by pepper, both better than BHT. Celery seeds recorded high metal chelating activity but turned out to be ineffective in controlling oxidation.

KEY WORDS

Antioxidant, Lipid peroxidation, Phenolic compounds, Metal chelating activity and Malonaldehyde.

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INTRODUCTION

Fish oil is the lipid fraction extracted from fish and fish byproducts. Generally, fish oils are more complex than animal fats or vegetable oils due to its long - chain unsaturated fatty acids[1]. Fish oils are unique in their composition of variety of fatty acids and in their degree of unsaturation[2]. These oils are rich in long-chain polyunsaturated fatty acids (LC-PUFA), ω-3 and ω-6 fatty acids namely eicosapentaenoic (EPA) and docosahexaenoic (DHA), which have been reported to induce several human health benefits[3], and play a role in preventing

heart disease and also have anti-inflammatory and anti-thrombosis effects[4]. They also reduce some risk factors associated with arteriosclerosis[5]. Both ω-3 and ω-6 polyunsaturated fatty acids are considered essential and must be obtained through diet[6], since they cannot be synthesized in the human body. High content of polyunsaturated fatty acids makes it more susceptible to attack by oxygen leading to oxidative spoilage. The oxidation process not only changes the flavour and odour, but also deteriorates the overall quality of the oil and reduces its shelf life[7]. Oxygen free radicals / reactive oxygen species (ROS) are sources of threat for decreasing the self stability of lipids as well as causing chronic diseases in biological systems. Lipid oxidation decreases nutritional properties of food and involves the loss of essential fatty acids and vitamins and leads to generation of potentially toxic compounds such as the malonaldehyde (MDA) and cholesterol oxidation products (COPs)[8].

In order to prevent such lipid oxidation, many substances have been investigated as potential antioxidant. Synthetic antioxidants, such as butylated -hydroxyanisole (BHA), butylatedhydroxytoluene (BHT), ter-butyl hydroquinone (TBHQ) are commonly used as food additives[9], since they are effective and less expensive than natural antioxidants[10]. Due to some toxic effects and health concerns[11] the uses of natural antioxidant from plant sources are being recommended. Besides medicinal plants, fruits, vegetables, dietary fibre, trace elements, and vitamins, spices contribute significantly to the dietary antioxidants and exhibit multifold biological activities like preventing various diseases[12].

The aim of the study is to determine the efficacy of some common Indian spices as natural antioxidants and their potential to increase the stability of extracted fish oil under accelerated oxidative stress. The comparative effectiveness of the spices was determined against a synthetic antioxidant BHT.

MATERIALS AND METHODOLOGY

Chemicals

2-Thiobarbituric acid was purchased from Lobachemie (India). Ammonium thiocyanate and ferric chloride, choloroform, methanol, isooctane all other solvents and reagents were procured from Merck (India). All chemicals used were of analytical grade.

Spices

Fennel seeds (*Foeniculum vulgare)*, black pepper (*Piper nigrum)*, dry ginger (*Zingiber officinale)*, cinnamon (*Cinnamomum verum*), and celery seed (*Apium graveolens*) were bought from local market in south Kolkata and grinded and sieved at125 micron.

Procurement of Fish Sample

Total amount of 1.5 Kg of Tilapia fish (each having an average weight of 300 gram, length 6.5 inches and age of 3 months) was procured from Chowbaga bheri located in east Kolkata wetland. Only the muscle portion of fishes were taken, and minced in a blender.

Sample Preparation and Extraction of Fish Oil

The fish oil was extracted from minced fish muscle samples following Bligh Dyer method[13] (1959). Extraction from blended fish muscle was done with 1:1 methanolic chloroform and then filtered through Whatman no.1 filter paper. The organic layer were separated through separatory funnel and dried with anhydrous sodium sulphate. The solvent was removed at low temperature (40^oC).

Weight of lipid = (weight of container + extracted lipid) - (weight of container)

Lipid content (%) = amount of lipid extracted (g)/weight of original sample (g) X 100

The obtained fish oil was divided into 3*(6+1) = 21 samples. An amount of 10 grams of fish oil was taken in each conical and 100 mg five different spices (fennel seeds, black pepper, dry ginger, cinnamon, and celery seed) were added in five batches of conical, a synthetic antioxidant BHT in another batch, each of three sets. Another batch of three conicals was fish oil with no added spice and this was considered as control. The 3 sets of (5+1+1) batches of samples were subjected to heating in hot plate (100 ^0C) for 2 mins, 6 mins and 10 mins respectively. These heat treated oil samples were further used to evaluate oxidative stability.

Estimation of antioxidant activity in spices

Sample preparation for spices

An amount of 5 gm of spice sample was extracted with 50 ml chloroform. It was further treated with activated charcoal to decolourize, then centrifuged and filtered with Whatman 1 filter paper. This solution was used for execution of all the antioxidant tests mentioned which was performed following the stated standard protocol.

Determination of Total Phenolic Compounds

Total phenolic compound was determined using Folin-Ciocalteu reagent using the modified method of Wolfe et al. (2003)[14]. A sample solution of 0.2 ml was pipetted in glass tube and 1 ml of Folin-Ciocalteu reagent, 0.8 ml of sodium carbonate (7.5%) was added to it. The mixture was stored at room temperature for 30 min and absorbance was recorded at 765 nm. Total phenolic compounds were calculated using a standard curve prepared with dilutions of gallic acid. Gallic acid (0 - 800 mg/L) was used to produce standard calibration curve. The total phenolic content was expressed in mg of gallic acid equivalents (GAE) / g of extract.

Antioxidant Activity in a Linoleic Acid System

Antioxidant activity was evaluated by the thiocyanate method[15]. Sample was added to 0.5 ml of chloroform, linoleic acid emulsion (2.5 ml 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0) in a test tube and stored in darkness, at 37 ^{o} C, to accelerate oxidation. The linoleic acid emulsion was prepared by mixing an equivalent weight of linoleic acid and Tween 20 in phosphate buffer (0.2 M, pH 7.0). The peroxide value was determined by reading the absorption at 500 nm with a spectrophotometer, after colour development with $FeCl_2$ and thiocyanate at various intervals during incubation. The peroxidation of linoleic acid was calculated as

$$\textbf{Peroxidation (\%)} = (A^1/ A^0) \text{ X } 100$$

Where A^0 = the absorption of the control reaction and

A^1 = the absorption in the presence of sample.

Metal Chelating Activity

The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis *et al*. (1994)[16]. Extracts were added in different concentrations (0.2 to 1 mg mL^{-1}) to a solution of 1mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 1mM Ferrozine (0.1 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. All test and analyses were done in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below.

$$\textbf{PI} = \frac{A_{(control)} - A_{(sample\ or\ standard)}}{A_{(control)}} \text{ X } 100$$

$A_{(control)}$ = Absorbance of control reaction

$A_{(sample\ or\ standard)}$ = Absorbance of sample or standard

Estimation of Oxidative Stability of Heat Treated Oil

Peroxide Value

Peroxide value was calculated using IUPAC method 2.501. In a test tube, 0.2 grams of oil sample were taken and saturated solution of potassium iodide was added. A mixture of glacial acetic acid and chloroform (20 ml) was added to the mixture. This was then warmed in water bath for 30 seconds. The content was then poured into a flask containing 20 ml of 5% potassium iodide solution. To it was added 25 ml of distilled water and 1 ml of starch solution. This was then titrated against 0.01N sodium thiosulphate solution. Peroxide Value was calculated as:

$$\textbf{Peroxide Value} = \frac{\text{Volume (ml) X Strength of sodium thiosulphate X 1000}}{\text{Weight of the oil sample}}$$

Thiobarbituric Acid Number

Thiobarbituric acid value was calculated using IUPAC method 2.531. In a test tube, 200 mg of oil sample was taken and 5 ml of thiobarbituric acid reagent was added. The mixture was stoppered and warmed in water bath at 95 ^{O}C for 120 minutes. It was then cooled and the absorbance was measured (A_s) at 530 nm in a 10 mm cell against water. A reagent blank absorbance (A_b) was also carried out.

$$\textbf{Thiobarbituric acid number} = 50 \text{ X } (A_s - A_b)/ \text{ weight of the sample}$$

***Para*-Anisidine Values**

Para-anisidine value was calculated using IUPAC method 2.504. In a 25 ml volumetric flask, 0.2 grams of oil was taken and diluted with isooctane. The absorbance (A_1) of the solution was measured at 350 nm against a blank isooctane. Sample solution (5 ml) was mixed with 1 ml of *p*–anisidine solution. After 10 minutes, the absorbance of this solution was measured (A_2).

$$P\text{–anisidine values} = 25 \times [1.2 \times (A_2 - A_1)] / \text{weight of the oil sample}$$

Conjugated Dienes-Triene Value

These values were calculated using IUPAC method 2.505. In a 100 ml conical flask 100 mg of oil sample was added to 75 ml purified isooctane. The flask was warmed to completely dissolve the sample, cooled to room temperature and allowed to stand for 15 minutes. The absorbance was measured at 233 nm. Conjugated dienes value was calculated as:

$$\text{Conjugated dienes value} = A_0 / \text{weight of the sample} \times \text{cell length in centimeters}$$

Absorbance measurements were repeated at 268 nm for CT determination.

Statistical Analysis

The experiment was performed in triplicate and data from three different experiments were subjected to analysis of variance (ANOVA) ($P < 0.05$). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 16.0 for windows, SPSS Inc.).

RESULTS AND DISCUSSION

Estimation of Antioxidant Activity of Spices

Total Phenol Content

Phenolic compounds are considered to be important plant materials because of their inhibitory effect on autoxidation of oils [17] and their radical scavenging ability[18]. Therefore, it is important to determine the total phenolic compound in the spices. It is explicit (Figure No.1) that highest phenolic concentration was recorded with ginger followed by fennel. These two values are remarkably higher than the synthetic antioxidant BHT. Cinnamon and celery have similar phenol content which is more than pepper and BHT respectively.

It is reported that rhizome of ginger contains over twenty phenolic compounds of which the important ones are gingerols[19], shogaol and diarylheptanoids[20] whereas twenty-nine phenolic compounds were isolated from the root bark of fresh (Yunnan) ginger[21]. Most of these compounds were found to inhibit lipid peroxidation. Forty-two phenolic substances were identified in fennel and the major ones are 3-O-caffeoylquinic acid, chlorogenic acid, 4-O-caffeoylquinic acid, eriocitrin, rutin, miquelianin, 1,3-O-dicaffeoylquinic acid, 1,5-O-dicaffeoylquinic acid, 1,4-O-dicaffeoylquinic acid and rosmarinic acid[22]. The major phenolic compounds present in cinnamon are cinnamaldehyde, cinnamic acid, cinnamyl alcohol and coumarin along with caffeic, ferulic, *p*-coumaric, protocatechuic and vanillic acids[23]. Celery belonging to a part of the Umbelliferae plant family is rich in phenolics like hydroxycoumarins (apigravin, celerin, osthenol), isoquercitrin, and umbelliferone[24]. Piperine is the active phenolic compound present in black pepper extract[25]. The phenolics in black pepper are a mixture of phenolic acid glycoside and flavonol glycosides which on hydrolysis yields nine phenolic acids comprising hydroxyl cinnamic acid and hydroxyl benzoic acid along with significant quantities of quercetin and kaempferol[26]. It should be mentioned that the total phenolic content measured by the Folin–Ciocalteu procedure does not give a full picture of the quality or quantity of the phenolic constituents in the extracts[27].

Antioxidant Activity in Linoleic Acid Emulsion

Linoleic acid being a polyunsaturated fatty acid easily undergoes auto-oxidation in air leading to occurrence of coupled double bonds, and subsequently secondary products, such as aldehydes, ketones, and alcohols as a result of series of reaction[28]. Spices being natural antioxidants are expected to inhibit the peroxidation. The graph (Figure No.2) expresses the extent of peroxidation in linoleic acid system with addition of spices. The oxidation order is expressed as fennel < pepper < ginger < cinnamon < celery < BHT. This indicates that highest oxidation in linoleic acid system occurred on adding BHT whereas the least peroxidation was observed in case of fennel. It implies that fennel followed by pepper could control the percentage of peroxidation to a greater extent

than the respective spices. The antioxidant activity order of the spices is actually the reverse of the stated oxidation order.

Metal Chelating Activity

Ferrozine can quantitatively form complexes with Fe^{2+}. However, in presence of other chelating agents this complex formation is disrupted. This results in the decrease in red colour intensity of the solution. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The ferrous ions possess the ability to move single electrons by virtue of which it triggers propagation of many radical reactions, even with relatively non-reactive radicals[29]. Main strategy to avoid the reactive oxygen species generation is through chelating metal ions. Figure No.3 exhibits highest metal chelating activity for celery followed by cinnamon whereas BHT scored the least. The order is as follows: celery > cinnamon > fennel > pepper > ginger > BHT.

Peroxide Value

Peroxide value measures the degree of oxidative rancidity of oil and the amount of oxidized substance formed during lipid oxidation[30]. The significantly increasing peroxide value (P>0.05) in case of both spiced and non-spiced control (Figure No.4) indicate higher rate of oxidation which leads to greater accumulation of peroxide. The non-spiced control had suffered maximum oxidation throughout whereas fennel could successfully maintain a low peroxide concentration in all the heating conditions. It recorded the lowest concentration of 0.174 in 2 minutes heating and recorded a value of 0.504 post 10 minutes heating. Though pepper recorded a peroxide value of 0.282 initially after 2 minutes heating, could finally control the rate of peroxidation more efficiently than fennel during 10 minutes heating and recorded the least value of 0.301. Ginger was more effective in controlling peroxidation for only 2 minutes heating, whereas did not appear to be useful for a longer duration of heating for 10 minutes. It should be noted that the increase in peroxide concentration on 2 to 10 minutes heating has increased 189.6 % in presence of fennel and 6.7 % in case of pepper whereas 445.3% in control and 882.2 % in case of ginger. This infers that ginger though effective in initial stage of oxidation does not function effectively for later stages of oxidation or at higher temperature.

Thiobarbituric Acid Value

Thiobarbituric acid value measures the rate of oxidative rancidity by the formation of oxidized lipids specially malonaldehyde which is a non-volatile aldehyde[31]. The TBA values (Figure No.5) delineate a gradual and significant increase (P>0.05) in malonaldehyde concentration in fish oil containing celery, BHT, ginger and the control with increasing heating time. Oil with celery and control recorded remarkably higher TBARS concentration throughout with maximum value of 3.928 on 10 minutes heating of control. Ginger showed a peculiar trend of decreasing the TBA value to a very low value of 0.063 initially just after 2 minutes of heating but again started increasing with longer heating duration. In spite of this increasing trend of ginger on longer oxidation, the final value recorded after 10 minutes was actually less than the initial value. Most interesting observation was noted on addition of fennel, pepper and cinnamon. The values decreased significantly (P>0.05) on longer heating exposure and the comparable minimum value was recorded by pepper (0.02) and fennel (0.0267) on 10 minutes of heating. These observations indicate an effective oxidation controlling activity of fennel (96.9 %), pepper (97.7 %) followed by cinnamon (58.5 %) in which the flavour molecules have either actually scavenged free radicals or have reacted with TBARS to decrease their concentration in the oil[32]. On the contrary the antioxidants present in celery were unable to inhibit the accumulation of malonaldehyde. The synthetic antioxidant BHT though displayed an increase in TBARS concentration with longer heating span, could maintain an overall value which is less than the initial value recorded before heating. In long run ginger and celery turned out as less efficient antioxidant than the synthetic counterpart.

***Para* – Anisidine Values**

Para-anisidine value reflects the magnitude of aldehydic secondary oxidation products and rate of formation of the non volatile carbonyl compound products[33]. Figure No.6 represents the *p*-anisidine value of the spiced fish oil as well as non-spiced

control which had undergone induced oxidative stress. It clearly exhibits significant increase (P>0.05) in *p*-anisidine values in all the cases except fennel with longer oxidative stress. Though all the spices have undergone secondary oxidation the control had suffered maximum oxidation which scored a value of 468.8735 on 10 minutes of heating. An interesting finding recorded in this experiment was that though in the later phase control underwent a steep rise in the graph, the *p*-anisidine value of fennel and cinnamon was higher than control initially after 2 minutes of heating. Both fennel and cinnamon contain carbonyl compounds like fenchone, anisaldehyde and cinnamaldehyde[22,23] respectively which might increase the concentration of the aldehyde in the oil and the values recorded might not be due to aldehydes formed during secondary oxidation. It is interesting to note that fish oil containing fennel showed a decreasing trend with increase in heating time. This may be due to the fact that fennel contain more of volatile compounds which on prolonged heating have reduced the amount of carbonyls actually present in the beginning. Pepper had throughout maintained low values and remarkably controlled the secondary oxidation of the fish oil. From the *p*-anisidine values it might be concluded that celery seeds were found to be effective in the initial stages of oxidation but on prolonged heating failed to control the secondary oxidation and recorded a value as high as 110.376. This might be due to the presence of majorly coumarin, 42.3% of different volatile compounds like phthalides (sedanolide, apiole) and other organic aldehydes which increase the *p*-anisidine value[34]. However BHT could control the oxidation moderately at all the stages of oxidation.

Conjugated Dienes and Trienes Value

The formation of conjugated dienes and trienes as a result of oxidation requires presence of unsaturated fatty acids with at least two double bonds and with more than two double bonds respectively in the lipid samples. The UV absorbance measurement at 233 nm and 268 nm indicates the formation of conjugated dienes and trienes. CD and CT values monitor and evaluate the oxidative stability and effectiveness of different antioxidants in oil[35]. Both Figure No.7 and 8 explicitly exhibits the significant increase (P>0.05) in CD and CT value in all the cases. Both the curves are almost similar specially the order of CD and CT post 10 minutes heating is pepper < fennel < ginger < BHT < cinnamon < celery seed < control and fennel < pepper < ginger < BHT < cinnamon < celery seed < control respectively. Maximum values both in CD and CT had been recorded by control which are 155.7 and 98.99 respectively. The values indicate comparable antioxidant effectiveness both for fennel and pepper. It should be mentioned that fennel did not show enough efficiency at an initial level after 2 minutes of heating, whereas cinnamon though appeared to be effective in imparting oxidative stability for shorter span of thermal exposure could not control oxidation for a longer oxidative stress. Percentage of increase in CD and CT value from 2 minutes 10 minutes heating is 418.7 and 397.4 for fennel and 594.3 and 755.5 for pepper respectively. This clearly indicates a higher efficiency of fennel in controlling oxidation.

CORRELATION STUDY

Correlation between various parameters of lipid oxidation was determined. Table No.1 represents the correlation values of peroxide value - TBA value, TBA - *p*-anisidine value and peroxide value - *p*-anisidine value. All the spices as well as control have shown very high correlation. Fennel, pepper and cinnamon have exhibited a very high negative correlation (-0.96334, -0.98588 and -0.88172 respectively) between peroxide and TBA value due to the depletion of TBARS substances with increase in oxidation as manifested from increasing peroxide values. Since the *p*-anisidine values of all the spices (except fennel) have increased with oxidative stress, the TBA values of pepper and cinnamon have negatively (-0.91942, -0.89251 respectively) correlated with *p*-anisidine value. Here fennel have positively correlated (0.901796) since both *p*-anisidine value and TBA has decreased with prolonged heating. Since peroxide value has increased in all the cases, all the spices except fennel have undergone high positive correlation while decreasing value of *p*-anisidine in fennel recorded a high negative correlation (-0.98468). The negative

correlation indicates effective control of formation of malonaldehyde by fennel. These negative correlations values of spices especially fennel emphasize the efficiency of the spices as effective antioxidants.

Table No.1: Over all correlation of the values for individual spices

S.No	Spices	TBA : peroxide value	*p*-anisidine: TBA	*p*-anisidine: peroxide value
1	Fennel	-0.96334	0.901796	-0.98468
2	Pepper	-0.98588	-0.91942	0.840591
3	Ginger	0.982099	0.999883	0.9791
4	Cinnamon	-0.88172	-0.89251	0.999727
5	Celery seed	0.992428	0.999771	0.994831
6	BHT	0.842892	0.94986	0.968875
7	Control	0.990903	0.983833	0.998985

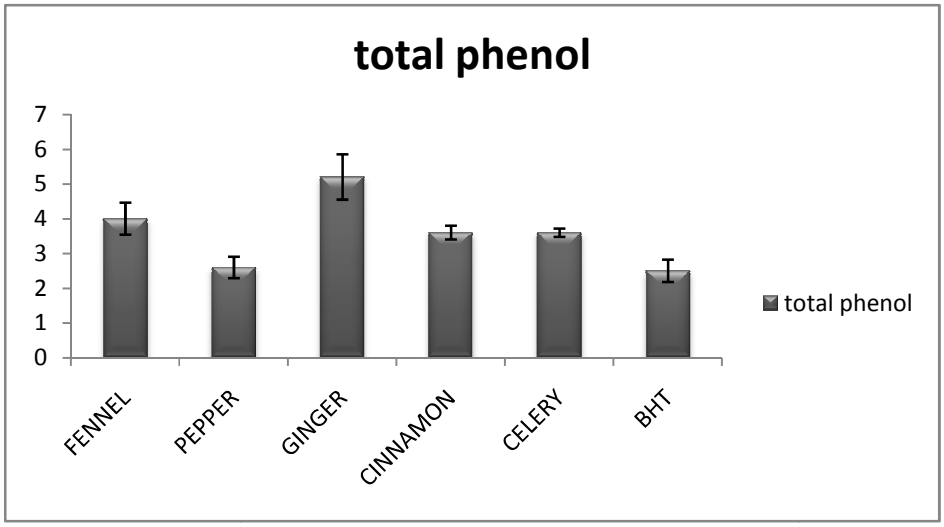


Figure No.1: Total phenol content of spices (GAE; mg gallic acid /g of extract)

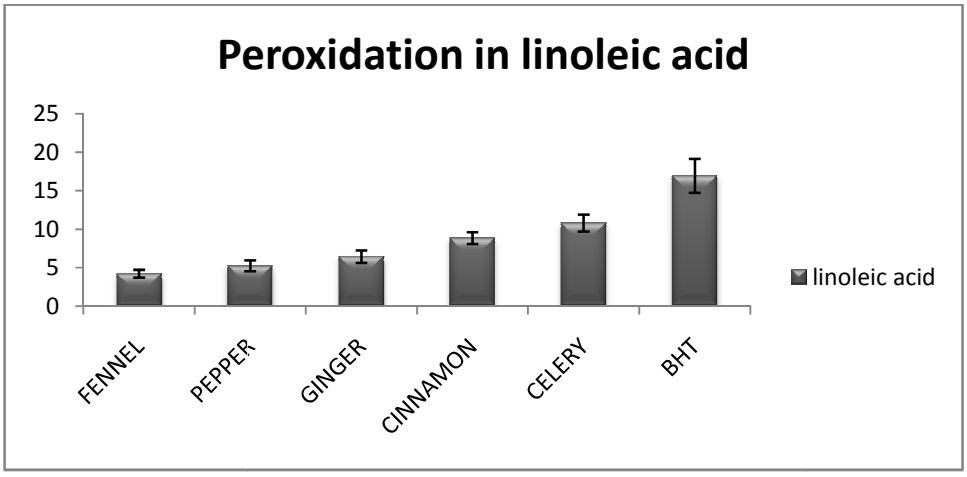


Figure No.2: Percentage of oxidation in linoleic acid system on addition of spices

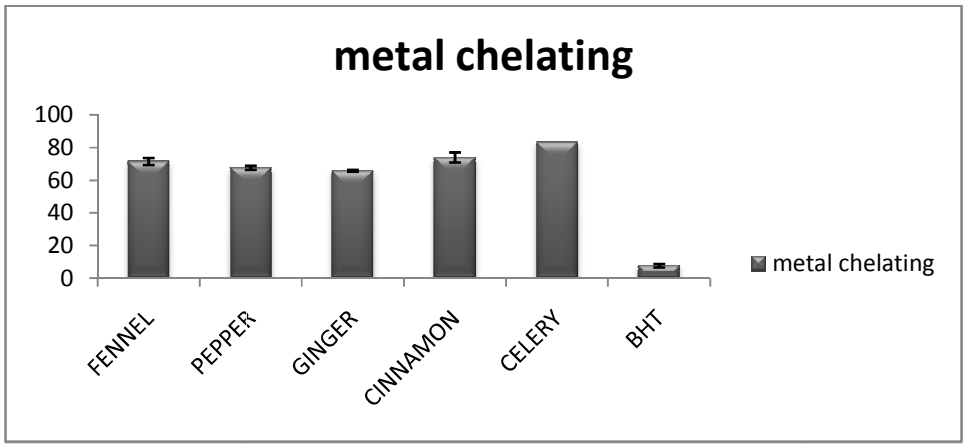
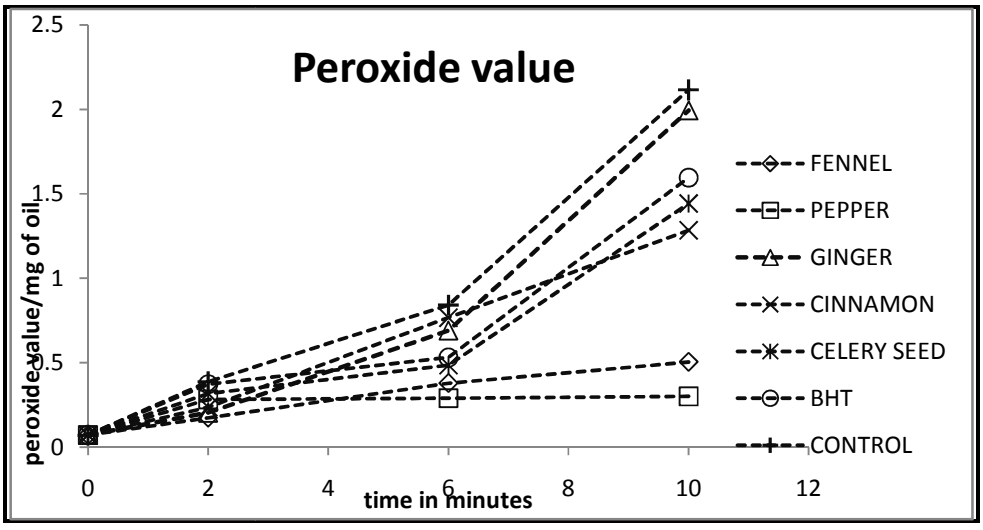


Figure No.3: Metal chelating activities of the spices



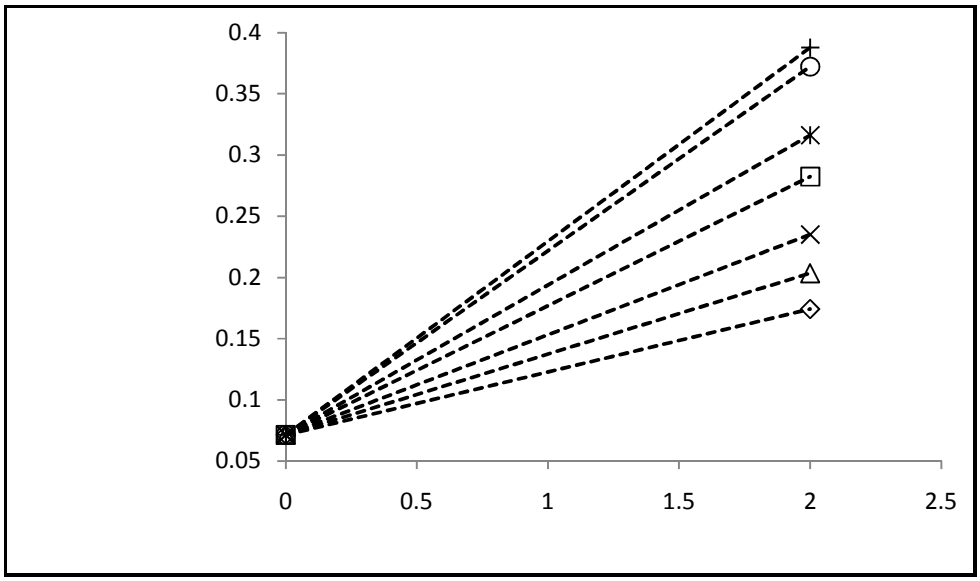


Figure No.4: Peroxide value of spiced and non-spiced fish oil after heating for 2 minutes, 6 minutes and 10 minutes

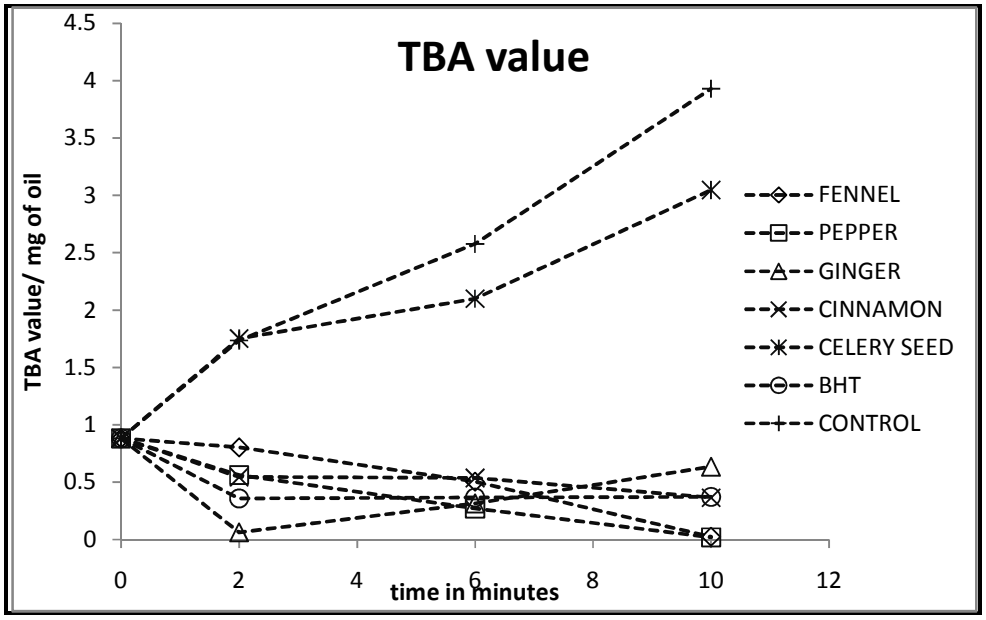


Figure No.5: Thiobaruitaric acid value of spiced and non-spiced fish oil after heating for 2 minutes, 6 minutes and 10 minutes

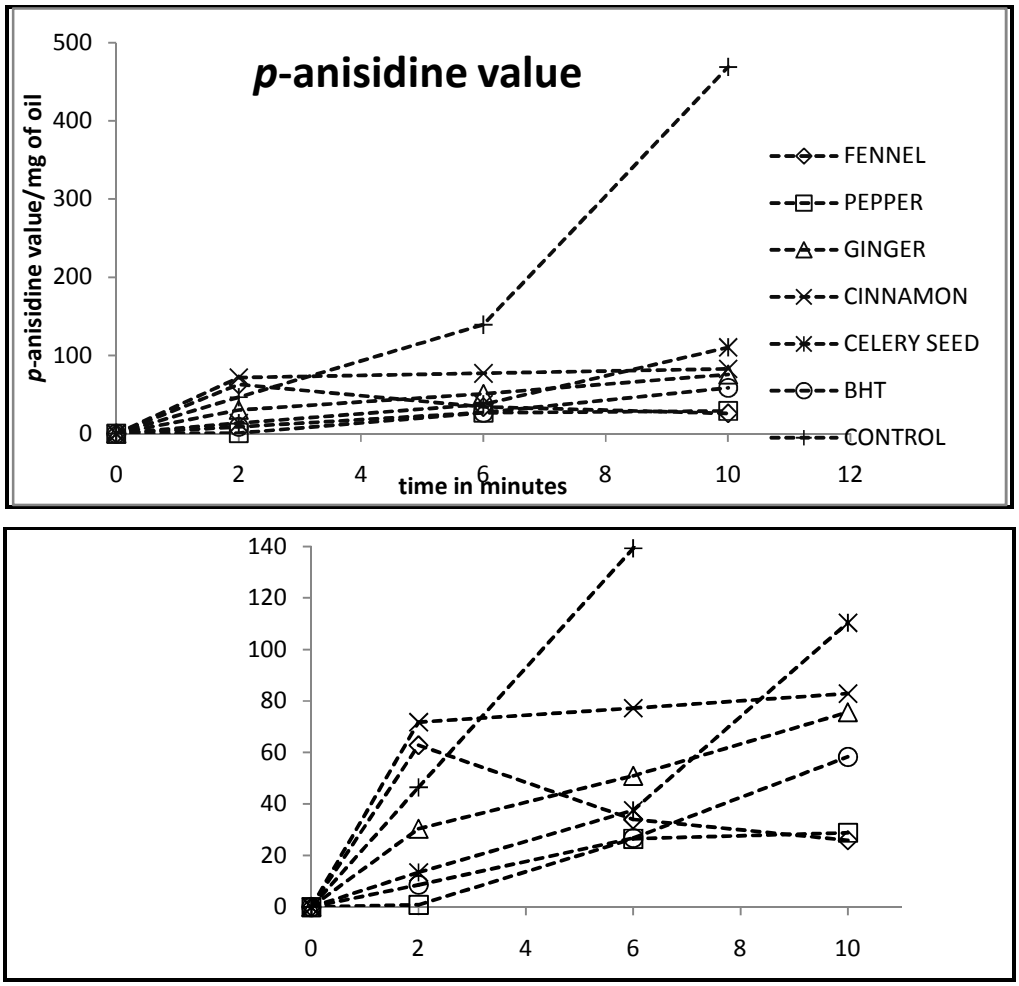
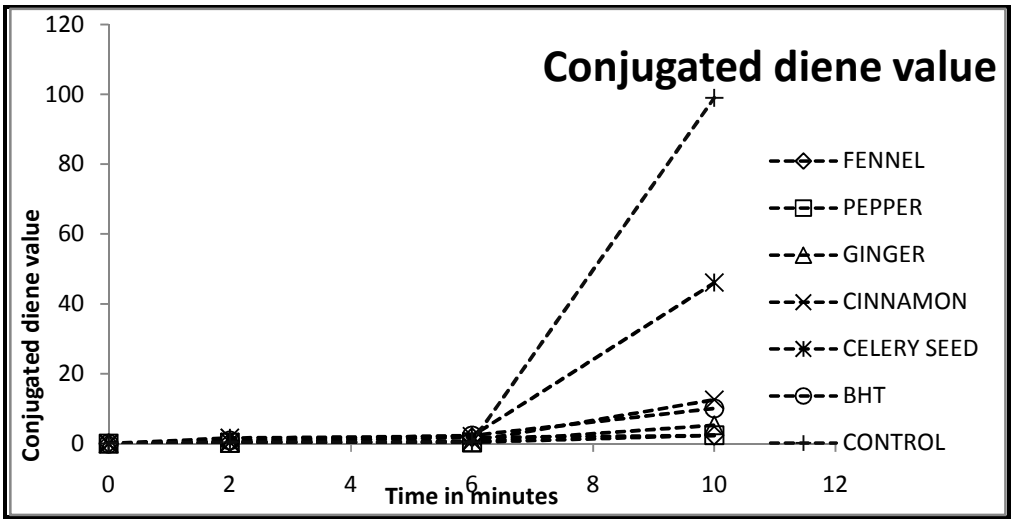


Figure No.6: *P*-Anisidine value of spiced and non-spiced fish oil after heating for 2 minutes, 6 minutes and 10 minutes



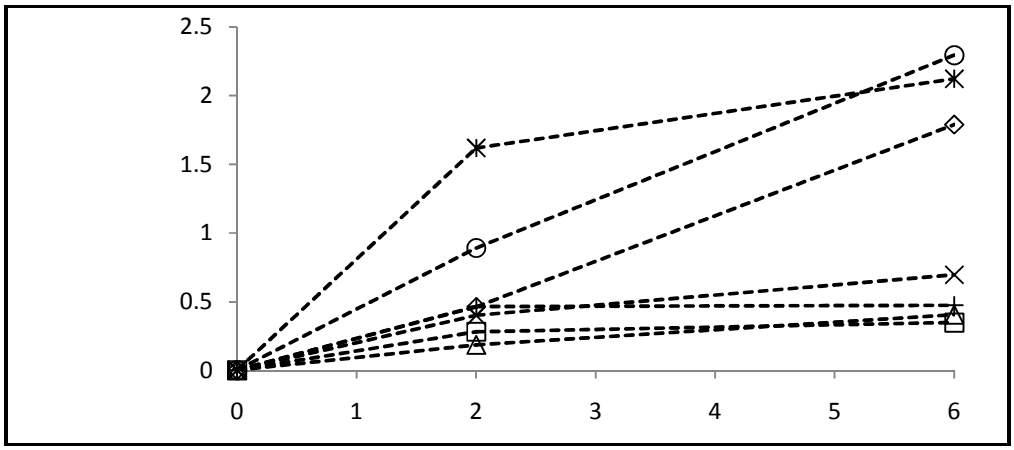


Figure No.7: Conjugated diene value of spiced and non-spiced fish oil after heating for 2 minutes, 6 minutes and 10 minutes

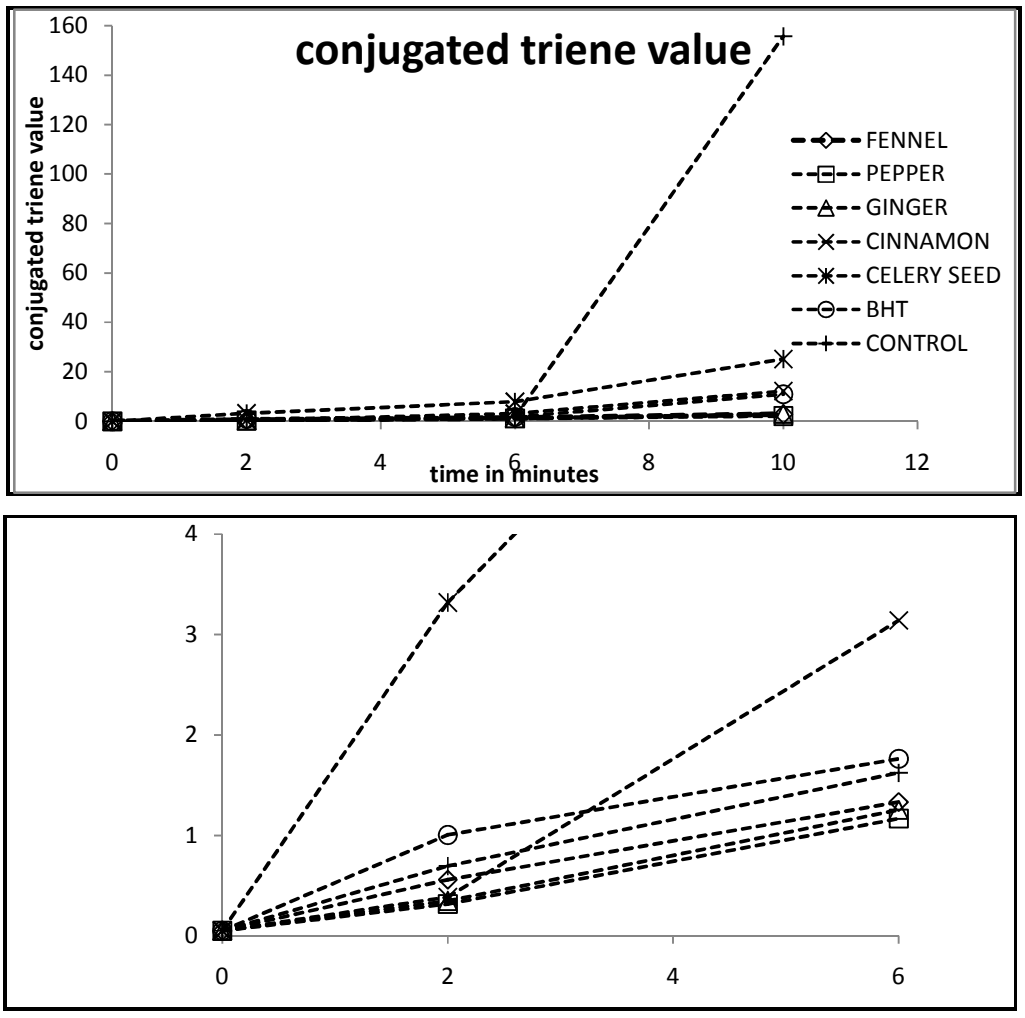


Figure No.8: Conjugated triene value of spiced and non-spiced fish oil after heating for 2 minutes, 6 minutes and 10 minutes

CONCLUSION

This study has provided us with some interesting and important findings. It clearly indicates the high antioxidant activity of the spices fennel and pepper followed by cinnamon. These spices turned out to be more effective than commonly used synthetic antioxidant BHT. The interesting finding was that fennel pepper and cinnamon could reduce the malonaldehyde and other TBARS accumulation in fish oil thereby reducing the potential health risk of this aldehyde. Moreover the decreasing *p*-anisidine value along with conjugated diene triene value of fennel mixed fish oil indicates enrichment of fish oil with antioxidants which are beneficial to human body. It also recommends the use of fennel as a potential antioxidant which can be used in preservation/ storage of fish oil. The future scope of the study is in further analyzing the biological activity of enriched fish oil as well as understanding spices in terms of flavour molecules.

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