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Effect of pomegranate (*Punica granatum*) peel extract on lipid oxidation in sardine fish oil

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Abstract

This study was carried out to determine the prospective use of pomegranate (*Punica granatum*) peel extract (PPE) as an effective antioxidant in sardine fish oil. Total phenolic content and antioxidant activity of PPE was examined. Three different concentrations of PPE (1000, 1500 and 2000 ppm) were used to find out the appropriate concentration and also compared with the synthetic antioxidant butylated hydroxyanisole (BHA). The sardine (*Sardinella longiceps*) fish oil which was not treated with any antioxidant was maintained as control. The primary and secondary oxidation products formed in the sardine oil was determined by peroxide value and thiobarbituric acid analyses. The result found out that, concentration of 2000 ppm of PPE was more effective in slowdown of lipid oxidation in sardine fish oil. The antioxidant capacity of PPE at 2000 ppm was more likely as BHA at 200 ppm. It proves that PPE which was the byproduct of pomegranate juice can be used as effective natural antioxidant instead of synthetic antioxidants in retarding lipid oxidation of fish oil.

Keywords: Sardine fish oil, lipid oxidation, natural antioxidant, pomegranate peel extract

Introduction

Fish oil is a rich source of omega-3 polyunsaturated fatty acids (PUFA). Omega-3 fatty acids have a lot of health benefits such as reducing cardiovascular risk, serum cholesterol level and benefiting in infants brain development. Sardine fish (*Sardinella longiceps*) is one of the marine fishes which contain a considerable amount of omega-3 fatty acids. The requirement for high quality fish oils are expanding due to the development of nutraceutical markets and oil sardine (*Sardinella longiceps*) is one of the major fishery resources to produce fish body oil. The important omega-3 fatty acids are EPA (eicosapentaenoic acid, C₂₀:5n₃) and DHA (docosahexaenoic acid, C₂₂:6n₃). These are the long chain polyunsaturated fatty acids, considered to have a number of health benefits in patients. Some researchers [1, 2, 3, 4] investigated extensively about the beneficial effects of omega-3 PUFA on cardiovascular risk factors and an average intake of EPA and DHA in a range of 250-500 mg per day has been shown to decrease the mortality risk of coronary heart disease.

As it has a good source of omega-3 fatty acids, one of the major drawbacks is it has highly susceptible to oxidation, which in turn produce the toxic compounds such as peroxides or volatile compounds which are responsible for non-desirable off-flavour and rancidity of fish oil [5-7]. Therefore, optimum processing, storage and packaging of fish oils are essential to preserve omega-3 PUFA from oxidation. In industrial practices, synthetic antioxidants like butylatedhydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) have been used as food additives to prevent oxidation of fats and oils. Those chemicals are doubted for their safety and health risks associated with their use in food products [8, 9]. In the last decade, the use of natural antioxidants instead of synthetic compounds has acquired great importance and several studies regarding the efficiency of plant extracts on the stabilization of bulk fish oil has been developed [10]. Agricultural byproducts such as potato peel, apple peel, rice bran and grape seed can be used as natural antioxidants. Habeebullah [11] studied about the antioxidant activity of potato peel extracts in a fish-rapeseed oil mixture and in oil-in-water emulsions and Sekhon-loodu [12] investigated about antioxidant ability of fractionated apple peel phenolics to inhibit fish oil oxidation.

Pomegranate peel extract with an abundance of flavonoid and tannins has been shown to have a high antioxidant activity. Studies show that pomegranate juice has potent antioxidant activity (ability to scavenge free radicals), significantly higher than more commonly consumed fruit juices such as grape, cranberry, grapefruit, and orange [31, 32, 33]. Several antioxidant activities,

including radical scavenging ability, ferrous ion chelating and ferric ion reducing antioxidant power, were identified on *P. granatum* [34].

Literatures available for the antioxidant activity of PPE but less about the studies of effect of PPE on the lipid oxidation of fish oil [21]. The primary objectives of this study were, 1. To determine the influence or effect of PPE as a natural antioxidant on the lipid oxidation of sardine oil, 2. To analyse and compare the antioxidant potential of natural antioxidant PPE with synthetic antioxidant (BHA) in sardine fish oil.

Materials and Methods

Materials

Sardine (*Sardinella longiceps*) fishes were brought freshly from the landing center in mangaluru, Karnataka. Pomegranate (*Punica granatum*) peels were brought freshly from juice vendors in mangaluru, Karnataka. Butylated hydroxyanisole (BHA) was purchased from Merck specialities, Pvt. Ltd. (Mumbai, India).

Extraction of sardine fish oil

Sardine fishes were transferred to the laboratory in clean condition with crushed ice on it. It was then beheaded, gutted and washed. Sardine fillets were made in to small pieces and mixed with deionized water at the ratio of (1:1, v/v) and heated at 95 °C for 30 min and then centrifuged at 10000 rpm for 20 min. The resulting supernatant was transferred to a separatory funnel, and the oil was removed. The extracted oil was stored with nitrogen at -80°C until further analysis.

Pomegranate peel extraction

The pomegranate peels were brought to the laboratory in ice box and washed thoroughly to remove dirt if any. Peels were then cut into small pieces and dried in oven at 60 °C for 12-48 hr. After drying the peels were ground in the kitchen blender to make the fine power to pass through 1mm sieve. The extraction procedure was carried out according to the methods described by Iqbal [9] with slight modifications. About 25g of pomegranate peel powder and 150 mL of ethanol were mixed well. The mixture was then subjected to shaking for 12h at the speed of 190 rpm in an ambient temperature. The mixture was filtered and residue was re extracted with same solvent. The filtrates of the mixture were placed under a hood in the rotary evaporator to remove the residual ethanol under vacuum at 40°C. The extract was collected and stored at -20 °C in a sample container for further analysis.

Sample preparation

The pomegranate peel extract, which was consider to be rich in phenolic compounds were added to slightly preheated (at 40°C) sardine oil at concentrations of 1000, 1500 and 2000 ppm. Synthetic antioxidant (BHA) was added at its legal limit of 200 ppm to compare the efficiency of natural antioxidants. All sardine oil samples were mixed well with vortex mixer at room temperature for the uniform dispersion of PPE and BHA in fish oil. All the samples, which containing different concentrations of antioxidants (PPE and BHA) and sardine fish oil (control) were placed in brown coloured amber glass vials and stored at -20°C for 15 days. Analyses were performed in triplicate.

Analysis of antioxidant activity

Antioxidant activity of PPE and BHA were examined using ABTS free radical scavenging activity. The ABTS (2,2-

azinobis 3-ethylbenzothiazoline-6-sulfonic acid, diammonium salt) free radical scavenging activity was determined by ABTS radical cation decolorization assay [13]. The ABTS was dissolved in water to a 7mM concentration. The ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and kept in the dark at room temperature for 12-16 h before use. Prior to the analysis the ABTS⁺ solution was diluted with distilled water to adjust the abs 0.700 ± 0.02 at 734 nm. Exactly 30 µL of different concentration of PPE was added to 3.0 mL of diluted ABTS⁺ solution and the absorbance was read exactly 6 min after initial mixing. A control tube also prepared using sample solvents instead of extracts. The percentage of inhibition of ABTS⁺ was calculated using the following formula,

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100$$

Analysis of total phenolic content

The concentration of phenolics in the extracts was determined by following the method of Singh [14] and results were expressed as gallic acid equivalents. A 12 mg of PPE was dissolved in a 25 mL of 10% ethanol. The dissolved sample (0.2 mL) was mixed with 1.0 mL of 10 fold diluted Folin-Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution. The solution was kept at room temperature for 30 min and then the absorbance was measured at 765 nm using a VIS double beam spectrophotometer. The estimation of phenolic compounds in the extract was carried out in triplicate. The total phenolic content of the pomegranate peel extracts was calculated by using standard curve prepared from gallic acid at the concentration range of 20 to 100 µg/mL. The total phenolic content of the sample was expressed as mg/g of gallic acid equivalents.

Peroxide value analysis

The peroxide value was determined by AOCS method [15]. About 2 g of fish oil sample was taken in an iodine flask and 25mL of solvent, (chloroform: acetic acid in the ratio 1:2) was added with it and mixed well. One gram of potassium iodide was added to the content and shaken well to be mixed and allowed to stand in dark place for 30 min. About 35mL of distilled water was added to the content by washing the stopper and sides of the flask. The liberated iodine from the flask was titrated against 0.01N sodium thiosulfate solution using 1% starch as indicator with vigorous shaking till first complete disappearance of blue color. A blank was also done by taking 10mL of chloroform with solvent alone. Peroxide Value (PV) was expressed as milliequivalent of O₂ per kg of fat by using the following formula,

$$\text{Peroxide value (meq O}_2\text{/kg fat)} = \frac{(\text{S-B}) \cdot \text{N} \cdot 1000}{\text{W}}$$

Where:

S = Volume of titrated sample (mL)

B = Volume of titrated blank (mL)

N = Normality of sodium thiosulfate solution

W = Weight of oil (g)

Analysis of TBARS (Thiobarbituric acid-reactive substances)

The thiobarbituric acid-reactive substances (TBARS) assay

was performed by the method of Ozogul ^[16] with slight modifications. A fish oil sample of about 1 g was added with 2.5 ml of TBA solution. The mixture was heated in boiling water for 10 min to develop a pink colour and then it was cooled with running tap water and centrifuged at 10000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (malonaldehyde; MA) at a concentration ranging from 0 to 10 ppm and TBARS values were expressed as mg of MA equivalents/kg sample.

Statistical analysis

Experiments and analyses were conducted in triplicate. Data obtained were appraised using Statistical Package for Social Sciences (SPSS, version 21.0). The analysis of variance (ANOVA) was performed to determine the differences between treatments. The differences between means of parameters were compared using the Duncan's Multiple Comparison test at 5% level of significance.

Results and discussion

Total phenolic content of PPE

Major phenolic compounds present in the plant kingdom are flavonoids, tannins and phenolic acids said by Rababah ^[17]. Those phenolic contents in plants are associated with their antioxidant activities, probably due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers ^[18]. Pomegranate is a rich source of polyphenols. The polyphenols shows various biological activities, it helps in eliminating free radicals and retard oxidation ^[19]. The total phenolic content present in the PPE was estimated as 115.21 ± 1.32 mg of gallic acid equivalents per gram (mg GAE/g). The result was compared with the findings of Saad ^[20] and it was in agreement with their findings (100.4–181.0 mg GAE/g). The value found was less than the value 141.6 ± 3.42 (mg GAE/g) reported by Osman ^[21]. The pomegranate is one of the fruits with richest polyphenols. The fruit peel contain major amount of phenolic compounds like punicalagins, gallic acids, ellagic acids and anthocyanins ^[22]. Different parts of pomegranate fruits and extracting solvents produced different quantities of different phytochemicals leading to different magnitude of biological activity of the products ^[23]. The variation in the phenolic content might be due to extraction procedure followed and condition of the pomegranate peel used because the phenolic content are highly affected by environmental conditions ^[24].

Antioxidant activity of PPE (ABTS radical scavenging activity)

The antioxidant activity was found by determining the ABTS radical scavenging ability of PPE. Decrease in colour indicates reduction of ABTS radical ^[25] and the concentration of the extracts was directly proportional to the reduction in absorbance. Pomegranate *Punica granatum* fruits were a rich source of dietary antioxidants ^[26]. The result showed PPE (at the concentration of 1000 ppm) had the ABTS radical scavenging activity as 93.50 ± 0.03 % and BHA (at the concentration of 200 ppm) showed 90.13 ± 0.56 %. This result showed that the PPE at higher concentration had the similar ABTS radical scavenging activity as synthetic antioxidant BHA. The result obtained in the present study was similar with the result found by Shalini ^[27] and also in support with the result of El-Said ^[28] who found 90.94% of ABTS radical scavenging activity. The pomegranate fruit acts as

considerable source of natural antioxidant and is having good ABTS radical scavenging activity ^[29].

Changes in peroxide value (PV)

Primary oxidation products (hydro peroxides) were estimated by peroxide value (PV) analysis. Changes in the peroxide value of sardine fish oil treated with different concentration of PPE and BHA is presented in Fig. 1. The PV found on the initial day was 2.74 ± 0.57 meq/kg. PV of all the samples was significantly ($P < 0.05$) increasing with storage time till ninth day and after 9 days PV was decreasing with increased storage time. The sample taken as control which was not treated with any antioxidant had increased its PV at the maximum up to 17.51 ± 1.07 meq/kg at the ninth day and it was the highest value among all the samples. The PV value of the sample with BHA was increased to 14.38 ± 0.54 meq/kg at ninth day and then decreased to 11.38 ± 0.71 meq/kg at the day 15. The samples treated with PPE increased to 8.45 ± 0.17 , 11.74 ± 0.73 and 15.36 ± 0.17 meq/kg at the day 9 and decreased to 7.91 ± 1.14 , 9.77 ± 0.41 and 12.74 ± 0.54 meq/kg at the day 15 for 2000, 1500 and 1000 ppm respectively. There was a significant difference was observed between control and all the treatment samples with PPE and BHA ($P < 0.05$). The PV recorded in the sample treated with PPE at the concentration of 2000 ppm showed significantly ($P < 0.05$) lower value than the sample treated with 200 ppm of synthetic antioxidant BHA throughout the storage period. This result showed that the PPE at higher concentration had better retardation of the formation of hydro peroxide than synthetic antioxidant BHA. Pomegranate peel extract (PPE) contain the considerable amount of phenolic compound (115.21 ± 1.32 mg GAE/g) which has the ability to scavenge free radicals during the oxidation process is the reason of slow down the hydro peroxide formation in the treated samples compared to control and thus increase the shelf life of the product ^[19]. After reaching high level, the PV slightly decreased in all the samples, this trend could be due to the decomposition of hydro peroxide ^[23].

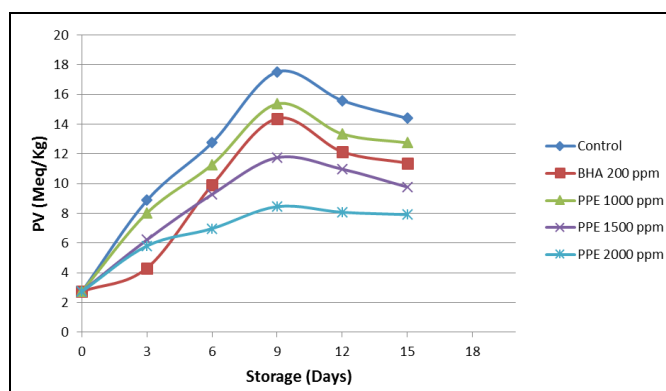


Fig 1: Changes in peroxide value (PV) (Meq/kg) of sardine fish oil samples treated with different concentrations of pomegranate peel extract (PPE) and butylated hydroxyanisole (BHA) during 15 days of storage.

Changes in thiobarbituric acid-reactive substances (TBARS)

Secondary oxidation products (aldehydes, ketones) were estimated by thiobarbituric acid-reactive substances (TBARS) analysis. Changes in the TBARS value of sardine fish oil treated with different concentration of PPE and BHA is presented in Fig. 2. The initial TBARS value was 3.77 ± 0.81 mg MA/kg of fat. The TBARS value increase in trend

throughout the storage period for 15 days. The control sample without any antioxidant showed significant ($P < 0.05$) increase in TBARS value than the samples treated with PPE and BHA. The highest TBARS value recorded was 8.91 ± 0.35 mg MA/kg for control sample at the day 15. The sample treated with PPE at 2000 ppm concentration had the TBARS value of 5.91 ± 0.17 mg MA/kg and BHA treated sample showed 5.94 ± 0.71 mg MA/kg at the day 15 and this result showed that there is no significant ($P > 0.05$) difference between the samples treated with 2000 ppm PPE and BHA. This proves that the pomegranate peel extracts at higher concentration have the potential to act as an effective natural antioxidant instead of synthetic antioxidants in arresting oxidation in sardine fish oil. The TBARS value increased to 6.84 ± 0.51 and 7.26 ± 0.61 mg MA/kg for sample treated with PPE at 1500 and 1000 ppm respectively at the day 15. The TBARS levels in sardine oil increased as the storage time increased [30]. The gradual increase in TBARS values for all the samples proves the conversion of primary oxidation products to secondary oxidation products. The low TBARS value of all the samples treated with PPE compared to the control could be due to the phenolic content present in the PPE and its strong antioxidant ability [21] because this phenolic contents includes polyphenols which involves in various bioactivities like eliminating free radicals and inhibiting oxidation [19].

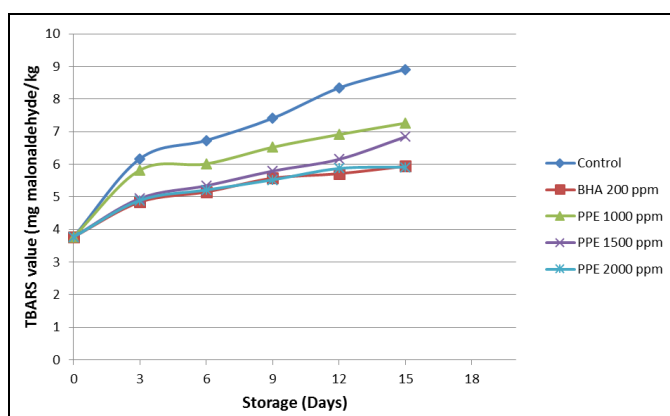


Fig 2: Changes in thiobarbituric acid-reactive substances (TBARS) (mg MA/kg) of sardine fish oil samples treated with different concentrations of pomegranate peel extract (PPE) and butylated hydroxyanisole (BHA) during 15 days of storage.

Conclusion

Sardine oil is a potential source of omega-3 fatty acids and it is highly susceptible to oxidation. In this study, PPE at the higher concentration (2000 ppm) helps in arresting oxidation procedure effectively when compared to lower concentration. PPE showed the potential equal to BHA in retarding the formation of secondary oxidation products. All three concentrations (1000, 1500 and 2000) showing effective result when compared to control but when compared to synthetic antioxidant (BHA) 2000 ppm PPE showed high potential than other two concentrations. This study clearly proves that the pomegranate peel extract (PPE) which was obtained as a byproduct from the pomegranate juice fabric can be used as an effective natural antioxidant in extending the shelf life of sardine fish oil.

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