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Original article

Effectiveness of rutin and its lipophilic ester in improving oxidative stability of sardine oil containing trace water

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Summary Poor oxidative stability exhibited by n-3 polyunsaturated fatty acid rich sardine oil is a major challenge for its utilisation in industry. Considering the fact that water is always present in bulk oil in trace amounts during storage, an effort was made to understand and compare the effectiveness of rutin and its corresponding lipophilic ester in enhancing oxidative stability of refined sardine oil containing trace water (0.16% w/w). Peroxide value, conjugated diene value, p-anisidine value and thiobarbituric acid reactive substances (TBARS) value were determined during 20 days storage. Rutin fatty ester showed 50% reduction in primary oxidation and 42.46% reduction in secondary oxidation, whereas rutin showed 20.6% and 20.43% reduction in primary and secondary oxidation, respectively, by the end of 20 days storage. Thus, it is clearly established that rutin fatty ester is more effective than hydrophilic rutin in sardine oil containing trace water, which contradicts the polar paradox theory.

Keywords Oxidative stability, rutin, rutin fatty ester, sardine oil, trace water.

Introduction

Lipid oxidation during transportation and storage is a major problem encountered in the utilisation of n-3 polyunsaturated fatty acid (PUFA) rich oils in food and pharmaceutical industry. Among all the methods employed for preventing oxidation in oils, addition of antioxidants is considered to be most effective and economical. Recently, there is a shift in focus towards the application of natural antioxidants with high antioxidant prowess in edible oil industry. These molecules are also found to confer several beneficial properties such as antimicrobial, antitumor and antimutagenic activities (Brewer, 2011). Flavonoids are interesting group of polyphenolic compounds derived from plants, possessing a broad spectrum of such biological activities (Ma *et al*., 2015). They are the preferred ones for protection of lipid based food due to their ability to react with peroxy radicals to form a resonance stabilised flavonoid radical (Ross & Kasum, 2002). However, the applicability of these compounds in oils and fats is limited due to their low solubility in lipophilic media. Though 'polar paradox theory' hypothesises the efficiency of hydrophilic antioxidant in bulk oil (Zhong & Shahidi, 2012a), a more comprehensive assessment in many recent studies has reported findings that contradict this theory (Lue *et al*., 2010a, b; Viskupicova *et al*., 2010; Sorensen *et al*., 2011; Zhong & Shahidi, 2012b; Aladedunye *et al*., 2015; Chen *et al*., 2016). This was further confirmed from our previous results on the antioxidant activity of six hydrophilic phenolic compounds which indicated only a maximum of 25–30% reduction in primary oxidation of sardine oil (Vaisali *et al*., 2016). Though aglycon and methoxylated flavonoids can be implemented in such environments, their occurrence in nature is rare.

Consequently, lipophilisation by enzymatic incorporation of hydrophobic groups to flavonoids, resulting in novel compounds with increased solubility in oil became a field of great interest. The acylation of antioxidant to its lipophilic derivative tends to alter the biological activities. While some studies report reduction in the activity upon lipophilisation (Salem *et al*., 2010), several reports are available which indicate the opposite effect (Lue *et al*., 2010a; Aladedunye *et al*., 2015; Chen *et al*., 2016). This could suggest that not all antioxidants behave according to this theory, and the antioxidant activity in bulk oil systems is more complicated than previously assumed.

Bulk oil is a heterogeneous system with wide variation in the fatty acid composition along with the presence of minor components (Vaisali *et al*., 2015), which in turn increases the complexity of oxidation process. It was proposed that the initial oxidation in bulk oil

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occurs at the interface of micellar/colloidal structures present in oil due to the presence of minor components (Chaiyasit *et al*., 2007; Shahidi & Zhong, 2011; Budilarto & Kamal-Eldin, 2015a) such as phospholipids, free fatty acids, lipid hydroperoxides and trace water. Among them, water plays a pivotal role in the formation of these structures and the progress of oxidation. Additionally, insights into water accumulation and its ability to enhance micelle formation during oxidation in bulk oils have been demonstrated (Chen *et al*., 2011; Park *et al*., 2014; Budilarto & Kamal-Eldin, 2015b). This makes it hard to anticipate the antioxidant capacity of a compound under study, in bulk oil during the course of oxidation.

Indian oil sardine (*Sardinella longiceps*) is a chief pelagic fishery resource of India and ranks as a very valuable commercial fish owing to its food value and industrial use. Oil sardines are one of the richest and cheapest sources of n-3 polyunsaturated fatty acids (PUFA) such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) (Charanyaa *et al*., 2017). The major challenge in effective utilisation of this oil in food and pharmaceutical industry is their poor oxidative stability which in turn is attributed to their high degree of unsaturation. Research on the application of lipophilised antioxidants is mostly dedicated to *in vitro* antioxidant assays, model systems and very few vegetable oils. As these systems fail to duplicate the complex oxidation mechanisms found in fish oils during storage, the utility of *in vitro* antioxidant assays and data generated by model systems is limited. Moreover, reports are rather scarce on the effectiveness of lipophilised antioxidants in marine oils. The available literature on the application of lipophilic antioxidants compares the efficiency of these compounds against hydrophilic counterparts. To the best of our knowledge, no reports are available that explain the reason for higher efficiency of lipophilic antioxidants in oil. Similarly, no reports indicate the significance of the inherent trace water on altering the antioxidant efficiency. Hence, this work endeavours to understand and compare the effectiveness of rutin with its corresponding lipophilic ester in reducing oxidation in complex marine oil containing trace water.

Materials and methods

Materials

Crude sardine oil was purchased from Mukka seafood industry, Mangalore, and was refined by a methodology defined in our laboratory. The physicochemical properties of the oil were determined. The trace water content was measured by Karl Fischer titration method using Metrohm Karl Fischer titrator. The refined sardine oil was stored at −20 °C for subsequent studies. Immobilised *Candida antartica* lipase was purchased from Sigma-Aldrich, India. Rutin, p-Anisidine and 1,1,3,3-tetramethoxypropane (malondialdehyde) were procured from Sigma-Aldrich (India). Potassium iodide, sodium thiosulphate, thiobarbituric acid and trichloroacetic acid were of analytical grade and purchased from Loba Chemie (India).

Methodology

Preparation and purification of lipophilic rutin fatty ester

Ester derivative of rutin was prepared by esterification with decanoic acid in acetone, catalysed by immobilised *Candida antartica* lipase as described by Vaisali *et al*. (2017). After the reaction, enzyme molecules were separated from the mixture by filtration. To obtain a relatively pure rutin fatty ester from the mixture containing unreacted rutin and fatty acid along with lipophilic rutin, a two-stage solvent extraction was employed. Initially, the reaction solvent was removed by evaporation under vacuum in rotary evaporator. The mixture was then washed consecutively for three times with 1:1 hexane:water (v/v) at room temperature. The hexane phase was removed after centrifugation at 2500 ***g*** for 5 mins, and the subsequent water phases were pooled. Next, liquid–liquid extraction using ethyl acetate: water (1:2 v/v) was performed. The purity of the product was analysed using HPLC-ESI-MS system by a method described by Vaisali *et al*. (2017). Ethyl acetate was then allowed to evaporate under vacuum by rotary evaporator. The purified fatty ester was stored in refrigeration at 4 °C for further use.

Oxidative stability studies

The studies on effectiveness of rutin and its ester on improving the oxidative stability of sardine oil with trace water were performed as described in our previous study (Vaisali *et al*., 2016) for a period of 20 days. Prior to storage experiments, water content of oil was adjusted to maintain same trace water content in all test samples (0.16%) so that it does not exceed the acceptable range of water content (0.20%) in food grade fish oils (Bimbo, 1998). A calculated quantity of sardine oil was then added to vials containing antioxidants to achieve a final concentration of 100 ppm antioxidants in the mixture. The samples were homogenised for 15 min. The vials were stored at 37 °C and in constant contact with atmospheric air for a period of 20 days. Respective controls were maintained without any antioxidant addition. All the oxidation experiments were triplicated. The measurement of oxidation was determined by analysing the samples for peroxide value, p-Anisidine value, conjugated diene and thiobarbituric acid reactive substance (TBARS) value.

Peroxide value
The lipid hydroperoxide level in oil was measured based on the standard AOCS method (2009). The peroxide value in terms of mill equivalents of peroxide per 1000 g of oil was calculated as follows:

$$\text{Peroxide value} = \frac{(\text{sample titre} - \text{blank titre}) * \text{Molarity of thisulfate} * 1000}{\text{mass of test in } \boldsymbol{g}}$$

p-Anisidine value
The measurement of p-Anisidine value was made in accordance with AOCS (2009) and calculated as follows:

$$\text{Anisidine value} = \frac{25 * (1.2\text{As} - \text{Ab})}{\text{mass of test in } \boldsymbol{g}}$$

Ab—absorbance of oil dissolved in isooctane, As—absorbance of oil dissolved in isooctane after reaction with p-Anisidine.

Conjugated diene (CD) analysis
The CD value of sardine oil was measured based on the method described by Hopia *et al.* (1996). The absorbance of oil dissolved in isooctane was measured at 234 nm. The increase in absorbance was correlated to progress of oxidation in oil.

Thiobarbituric acid reactive substance (TBARS) value
The TBARS value of sardine oil was measured during the course of storage based on the method described in our previous study (Vaisali *et al.*, 2016). A standard graph was plotted using 1,1,3,3-tetramethoxypropane (malondialdehyde).

Statistical significance

All oxidation experiments were done in triplicates, and the analyses for oxidation products were duplicated. The data were compared by one-way analysis of variance (ANOVA) using MiniTab 17 software Pennsylvania, USA. Significance was declared for $P < 0.05$.

Results and discussion

Purification of rutin fatty ester

To analyse the applicability of rutin fatty ester in hydrophobic medium, relatively pure form of this compound is a necessity. During the initial stages of purification, the immobilised enzyme catalyst was removed by filtration followed by the evaporation of acetone under vacuum through rotary evaporator. To remove the unreacted fatty acid from the reaction mixture, the samples were subjected to liquid extraction at room temperature using hexane/water (1:1 v/v). Thus, the unreacted hydrophobic fatty acid extracted into the nonpolar organic phase. Though the same procedure could be utilised for removal of residual rutin, a report by Lue *et al.* (2010b) indicated the inefficient separation of rutin fatty ester into the organic phase. The separation of unreacted rutin and its lipophilic ester poses many challenges unlike the separation of hydrophobic fatty acid from the mixture. This is due to the close difference in polarity of the two compounds and also due to the poor solubility of rutin in majority of the solvents (Razak & Annuar, 2015). Hence, ethyl acetate was chosen based on its intermediate polarity and tested for separating unreacted rutin. The HPLC chromatogram of sample before and after subjecting to ethyl acetate: water (1:2 v/v) extraction is shown in Fig. 1. It can be noted that the concentration of rutin eluting at 2.8 min reduced effectively, while the rutin fatty ester concentration was found to improve with the removal of unreacted substrates.

To understand the effectiveness of rutin fatty ester as antioxidant, studying their activity against radicals such as DPPH (2,2-diphenyl-1-picryl hydrazyl) would provide information on their antioxidant capacity. However, the radical scavenging ability tested by *in vitro* methods cannot be directly correlated to their activity in oil (Aladedunye *et al.*, 2015; Chen *et al.*, 2016; Vaisali *et al.*, 2016). Further, reports also indicate that there is no loss of scavenging activity on esterification with medium chain fatty acids (Viskupicova *et al.*, 2010). Hence, the effectiveness of rutin fatty ester was directly tested in sardine oil and compared with the effectiveness of native rutin.

Oxidative stability studies

The bulk sardine oil used in the current study was a heterogeneous system with higher number of long chain fatty acids (Charanyaa *et al.*, 2017). On further analysis of sardine oil, it was identified that it possessed minor components such as surface active compounds and a variable trace water content of 0.09% to 0.12% (data not shown). Literature suggests the self-assembling of these compounds in the presence of trace water, to form variety of physical structures that further acts micro/nano reactors of oxidation (Chaiyasit *et al.*, 2007). As trace water in bulk oil is one of the significant parameters in the formation of these structures, the effectiveness of antioxidant has to be tested in sardine oil containing trace water. Hence, the water content was adjusted to a constant value prior to storage experiments.

Peroxide value

Lipid hydroperoxides are important intermediates that decompose to form free radicals (Vaisali *et al.*, 2016).

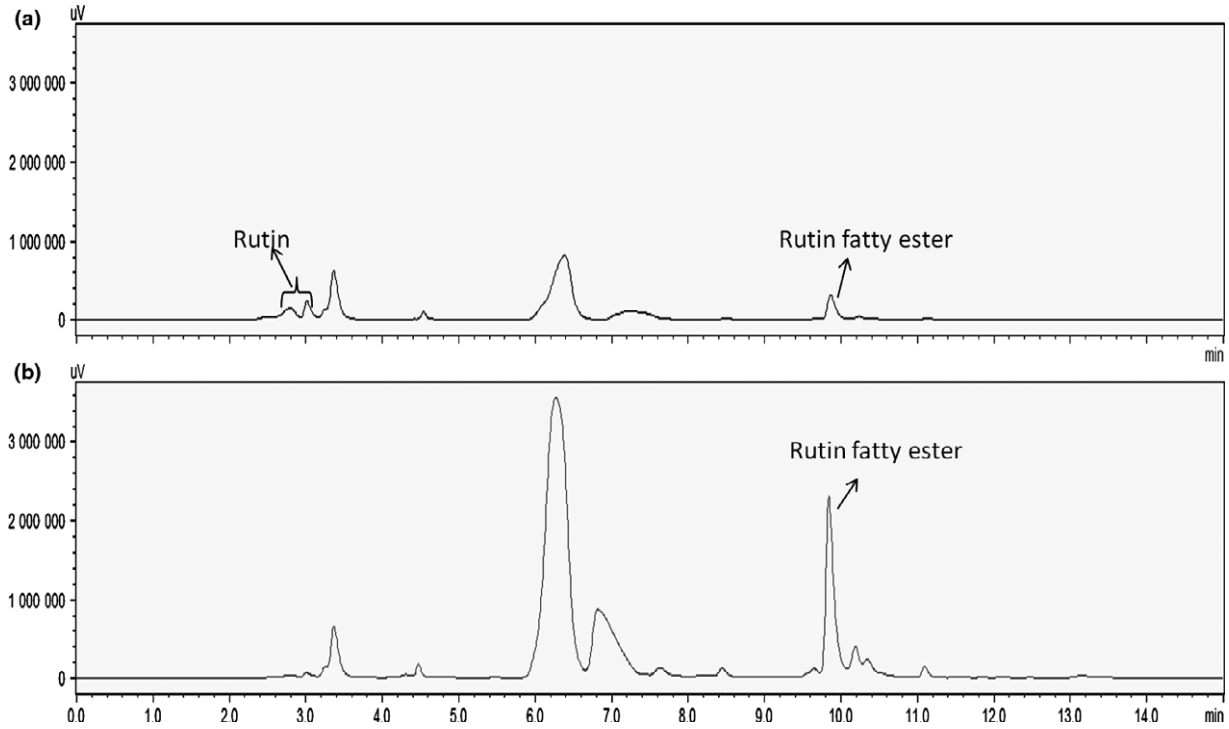

Figure 1 HPLC chromatogram of rutin and rutin fatty ester (a) before purification and (b) after purification using ethyl acetate:water.

These are the products of primary oxidation and they are measured in terms of peroxide value based on their ability to oxidise potassium iodide. Primary oxidation of sardine oil containing trace water, in the presence of rutin and lipophilic rutin fatty ester was monitored for a period of 20 days and depicted in Fig. 2. The peroxide value increased at higher rate after 2nd day of storage indicating the induction period of sardine oil. Thus, the peroxide value drastically increased from 12 meq kg^{-1} to 30.23 meq kg^{-1} from 2nd day of storage to 9th day, respectively (Fig. 2). Fortification with rutin and rutin fatty ester resulted in the latter showing higher performance with 50.08% reduction than the former with 20.63% reduction in oxidation at the end of 20 days (Table 1). In the presence of lipophilic rutin ester, the peroxide value reduced significantly ($P < 0.05$) in comparison with control. This indicated that rutin fatty ester effectively reduced oxidation. This is consistent with the results obtained by Sorensen *et al.* (2012), when the efficiency of rutin was compared to rutin laurate and rutin palmitate. The lipophilic ester showed significant reduction ($P < 0.05$) in primary oxidation with a maximum of 17.76 meq kg^{-1} in comparison with a control value of 35.59 meq kg^{-1} of peroxide value at the end of 20 days storage period. As the presence of trace water enhances the formation of micellar structures (Budilarto & Kamal-Eldin, 2015b), it can be concluded that the lipophilic nature of rutin fatty ester allows it to effectively associate at the interface of these structures, thus improving oxidative stability. However, its polar counterpart failed to show antioxidant activity during initial days of storage, after which it showed moderate antioxidant activity.

Over the years, many studies were performed to establish a relationship between polarity of antioxidant and their effectiveness in lipids (Sorensen *et al.*, 2011; Zhu *et al.*, 2014; Aladedunye *et al.*, 2015; Laguerre *et al.*, 2015; Ma *et al.*, 2015; Almeida *et al.*, 2016; Chen *et al.*, 2016; Costa *et al.*, 2016a,b; Silva *et al.*, 2017). It has been widely established that polar antioxidants are effective than nonpolar antioxidants in bulk oils (Frankel *et al.*, 1994; Zhong & Shahidi, 2012b). This phenomenon was reported to be due to the ability of polar antioxidants to associate at the air–oil interface, which dominates the oxidation in bulk oil (Frankel *et al.*, 1994). However, it was noted in the present study that the antioxidant activity of hydrophillic rutin was lower in comparison with lipophilic rutin ester. According to Chaiyasit *et al.* (2007), the polar lipids and minor components in oil, along with amphiphilic oxidation products self-assemble to form a variety of physical structures and the presence of water alters the structure and characteristic of these association colloids. It is widely accepted that the occurrence of trace amount of water in refined oils is inevitable (Kim *et al.*, 2014; Kittipongpittaya *et al.*, 2016; Upadhyay &

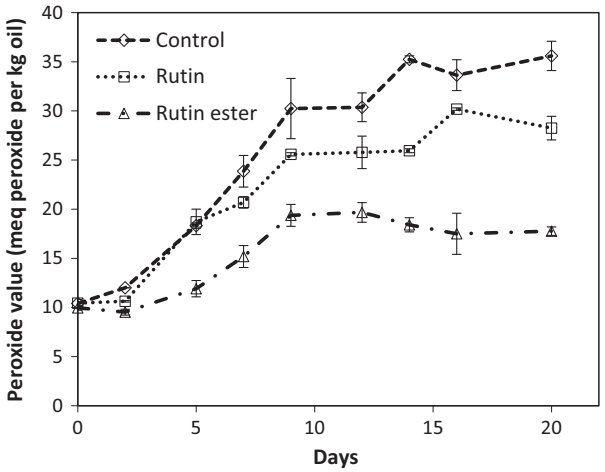

Figure 2 Peroxide value of sardine oil containing trace water fortified with native rutin and rutin fatty ester.

Table 1 Effectiveness of rutin and rutin fatty ester on improving oxidative stability of sardine oil containing trace water at the end of 20 days storage period

	% Decrease in oxidation at the end of 20 days storage period*	
Oxidation parameter	**Rutin**	**Rutin fatty ester**
Peroxide value	20.63 ± 3.3^{a}	50.08 ± 1.2^{b}
Conjugated diene value	27.61 ± 6.2^{a}	39.48 ± 2.4^{b}
p-Anisidine value	$21.25 \pm 1.1_{a}$	46.76 ± 9.9^{b}
Thiobarbituric acid reactive substances (TBARS) value	20.43 ± 8.5^{a}	42.46 ± 8.6^{b}

[a,b]values with different letters in the same row were significantly different ($P < 0.05$).

*Percentage decrease was calculated by considering control without antioxidants to be 100% oxidised.

Mishra, 2016). This trace water has been reported to affect oxidative stability of oils in two ways. They either trigger oxidation by acting as substrates (Kim *et al*., 2014), or by enhancing micelle formation that acts as site of oxidation (Park *et al*., 2014).

The presence of water in sardine oil could result in the partitioning of polar rutin into water phase of micelles, leading to a decrease in its activity. Further it has been suggested that micellar structures collapses after sufficient oxidation (Budilarto & Kamal-Eldin, 2015a). This could explain the improvement in rutin activity after a particular period of storage. Thus when micellar structures collapses, partitioning of rutin into water phase is reduced. On the other hand, this partioning effect was not found in rutin fatty ester due to its lipophilic nature. Similar results were noted by Kittipongpittaya *et al*. (2016), when the presence of water failed to affect the activity of hydrophobic tocopherol during oxidation of stripped corn oil. Thus, lipid oxidation in sardine with trace water seemed to proceed from colloidal formation followed by primary and secondary oxidation, which is consistent with the work by Park *et al*. (2014), when corn oil oxidation was studied in the presence of moisture.

Conjugated diene (CD) analysis

During initial lipid oxidation, hydrogen is abstracted from fatty acid and results in a shift in double bond. This results in the formation of conjugated dienes and trienes. Antioxidant activity of rutin and its lipophilic ester was further tested in sardine oil containing trace water, by analysing their ability to reduce the conjugated diene (CD) formation, which are isomeric derivatives of unsaturated lipid hydroperoxides. The CD analysis of sardine oil showed that rutin ester was better suited for significant improvement ($P < 0.05$) in oxidative stability (Fig. 3), whereas rutin showed only moderate antioxidant activity in oil with a CD value of 0.522 in comparison with the CD value of 0.574 and 0.426 for control and sample with rutin fatty ester, respectively, at the end of 20 days of storage (Fig. 3). This is in accordance with the results obtained by Zhong & Shahidi (2012b), as lipophilic derivatives of ascorbic acid, gallic acid and epigallocatechin gallate (EGCG) showed higher reduction in CD value of stripped corn oil than their corresponding polar counterparts. Another similar study on the effect of tocopherol on corn oil oxidation indicated that 200 ppm of lipophilic tocopherol was effective in increasing the oxidative stability in the presence of trace water (Jung *et al*., 2016).

In case of samples with rutin fatty ester, a drastic reduction in absorbance was noted after 2nd day of storage (Fig. 3). However, control and samples fortified with rutin failed to show the same profile. Rutin fatty ester is a relatively hydrophobic compound, due to which it gets dispersed in bulk oil effectively. Additionally, the presence of both hydrophillic and hydrophobic groups in rutin fatty ester increases its ability to associate at the interface of the microenvironments, whereas rutin associates at the inner core of the water phase in the micellar structure as suggested by Laguerre *et al*. (2015). Thus, rutin fatty ester has higher ability to interact with conjugated dienes than rutin due to the hydrophobic nature of diene molecules. This could explain the reduction in the absorbance in samples containing rutin ester as it prevents further formation of conjugated dienes. As in peroxide value, this further proves that oxidation in bulk sardine oil containing trace water is influenced by these association colloids. Hence, this additionally confirms

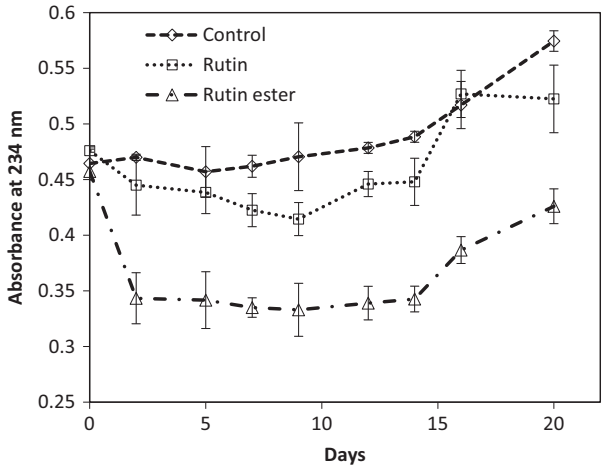

Figure 3 Conjugated diene analysis of sardine oil containing trace water fortified with native rutin and rutin fatty ester.

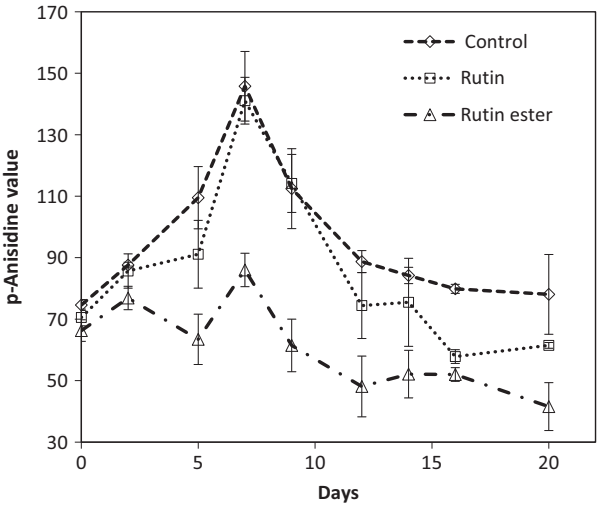

Figure 4 p-Anisidine value of sardine oil containing trace water fortified with native rutin and rutin fatty ester.

that lipophilic rutin ester was effective in reducing oxidation influenced by these colloidal/micellar structures.

p-Anisidine value

The hydroperoxides formed during the primary oxidation are usually decomposed to form an array of secondary oxidation products, the most prominent compounds being those with carbonyl groups. These aldehydes are analysed based on their reaction with the amine group of p-anisidine, resulting in a Schiffs base that shows specific absorption at 350 nm (Wang *et al.*, 2011). Changes in the p-Anisidine value of sardine oil with trace water in the presence of rutin and its nonpolar counterpart were compared with oil samples devoid of antioxidants and are presented in Fig. 4. It was noted that a more lipophilic antioxidant was required to improve oxidative stability of sardine oil containing trace water.

In agreement with our results on primary oxidation, it was noted that rutin fatty ester showed consistently significant reduction ($P < 0.05$) in secondary oxidation than hydrophilic rutin. This is similar to the findings by Zhu *et al.* (2014), who noted a better antioxidant activity of acylated epigallocatechin gallate (EGCG) than native EGCG in sunflower oil due to the homogenous dispersion of lipophilic antioxidant. A similar conclusion was derived by Zhong & Shahidi (2012a), when lipophilic derivative of EGCG showed better antioxidant activity than hydrophillic EGCG in corn oil. In case of effectiveness of polar rutin, it was noted that it failed to show significant reduction ($P > 0.05$) till 12th day of storage (Fig. 4) after which it showed slight antioxidant activity, showing a reduction of 21.25% at the end of 20 days storage (Table 1).

Thiobarbituric acid reactive substances (TBARS) value

To confirm the effectiveness of the antioxidants, another measurement of secondary oxidation was carried out based on the reaction between thiobarbituric acid and malondialdehyde-like substances in oil. From the results of TBARS value, it was noted that native rutin showed mild antioxidant activity throughout the storage period, whereas rutin ester performed with better efficiency showing maximum reduction in TBARS value ($P < 0.05$) with 42.46% reduction at the end of 20 days storage (Table 1). Thus at the peak of oxidation, control showed a maximum TBARS value of 42.79, while sample with rutin and its ester showed a TBARS value of 37.32 and 18.01, respectively (Fig. 5).

Additionally, samples with rutin ester showed only a minor increase in TBARS value throughout the course of oxidation ($P < 0.05$), denoting a possible delay of secondary oxidation by lipophilic rutin ester. As it has been established that oxidation in bulk oil containing minor components is driven by the presence of physical structures (Chaiyasit *et al.*, 2007), it can be concluded that rutin fatty ester has effectively reduced the progress of oxidation by efficient interaction with colloidal/micellar structures during primary oxidation itself, thus delaying secondary oxidation. As the TBARS value is an indication of secondary oxidation of unsaturated lipids (Decker *et al.*, 2010), it can be concluded that the lipophilic nature of rutin ester provided better protection against the oxidation of hydrophobic PUFA lipids in sardine oil.

Considering the overall oxidation results from the current study, it can be seen that the results obtained contradicts the polar paradox theory, which suggests

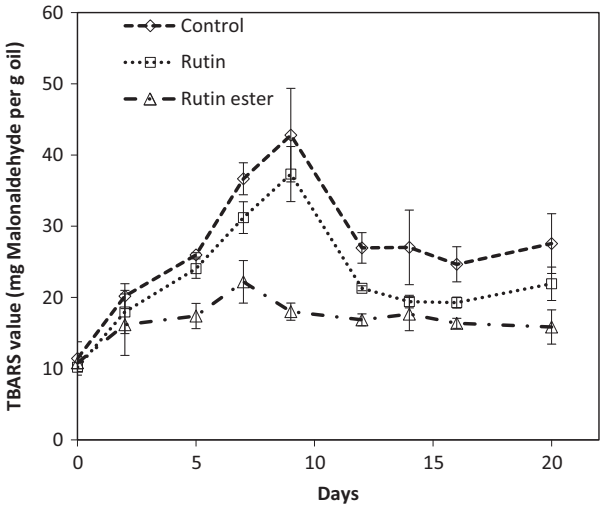

Figure 5 Thiobarbituric acid reactive substances (TBARS) value of sardine oil containing trace water fortified with native rutin and rutin fatty ester.

that hydrophilic antioxidants work well in bulk oil. However, due to the complexity of sardine oil and the presence of trace water, formation of association colloids is inevitable. Many recent studies suggest that these association colloids act as micro/nano reactors of oxidation as it provides oil–water interface for oxidation. Hence, the progress of oxidation in bulk oils containing these structures proceeds at the oil–water interface similar to the oxidation in emulsions. As a result, a more lipophilic antioxidant is required to improve oxidative stability.

Conclusions

Rutin fatty ester was purified by a simple two-stage solvent extraction process. This alternative method to preparative chromatography can be potentially used to produce large quantities of rutin fatty ester for analysing their widespread applications. Incorporation of a fatty acid chain in rutin, significantly improved the performance of rutin in sardine oil under the current storage conditions. Rutin fatty ester was found to be more effective during oxidation of refined sardine oil containing trace water, whereas rutin showed relatively mild activity. This was attributed to the ability of water to form association colloids with other minor components in sardine oil. As a result, oxidation mechanism in bulk oil could resemble the mechanism in oil-water emulsions thus requiring a lipophilic antioxidant for better activity in sardine oil. This further implies the ramifications involved in the oxidation and antioxidation process involved in complex media such as sardine oil. Studies on varying concentration of these antioxidants and the combination of these two antioxidants could throw more insights into the effective strategy for reducing oxidation in sardine oil.

Conflict of interest

The authors have no conflict of interest to declare.

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