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USAGE OF ROSMARINUS OFFICINALIS L. EXTRACT AS NATURAL ANTIOXIDANT IN EXTRA VIRGIN OLIVE OIL

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ABSTRACT

Olive oil is one of the oldest plant oils. It has pleasant taste and high nutritional value, where it is considered the favorite component of Mediterranean countries diet. Extra virgin olive oil (EVOO) is the highest grade of olive oil. During storage and heat treatment (EVOO) is subjected to oxidation resulting in deterioration of its sensory quality and nutritional value. There is increasing interest to enhance oil stability and delay the oxidation during storage and heat treatment therefore many synthetic and natural antioxidants are used. Extracts from herbs -such as *Rosmarinus officinalis* L- are rich sources of natural antioxidants including phenolic compounds. Therefore, the aim of this study is to investigate the effect of *Rosmarinus officinalis* L. ethanolic extract (RE) on reducing EVOO oxidation compared to Butylated hydroxyanisole (BHA) during storage and heat treatment. The phenolic content of the dried ethanolic *Rosmarinus officinalis* L. extract was 95.18 ± 5.38 mg GAE/g. Peroxide Value (PV), p-Anisidine value (p-An-V) and totox value increased in EVOO and reached after 30 min in oven 43.02 ± 3.3 mEqO2/kg, 25.75 ± 1.84 and 111.79 ± 6.7, respectively. Similarly, PV, p-An-V and totox value increased in EVOO and reached after 30 min in microwave 52.57 ± 3.3 mEqO2/kg, 50.56 ± 1.84 and 155.7 ± 6.7, respectively. The addition of RE and BHT led to decrease PV, p-An-V and totox value compared to EVOO (p-value <0.05) both during microwave and conventional heat treatment. The oxidation process in microwave was clearly higher than traditional heating in oven (p-value <0.05). PV, p-An-V and totox value increased gradually in EVOO during storage and reached after 9 months 38.59± 2.99 mEqO2/kg, 39.56 ± 0.42 and 116.74± 4.8, respectively. The addition of RE and BHT decreased PV, p-An-V and totox value during storage compared to EVOO (p-value <0.05). The results indicate that the ethanolic extract of *Rosmarinus officinalis* L. decreased EVOO oxidation, which enable it to be used as natural antioxidant in olive oil instead of the synthetic antioxidant BHT.

KEYWORDS: Extra virgin olive oil, oxidation, *Rosmarinus officinalis* L, phenolic compounds, Butylated hydroxyanisole.

INTRODUCTION

Olive oil is one of the oldest plant oils. It has an outstanding position because of its pleasant taste and smell as well as its high nutritional value, where it is considered the favorite component of Mediterranean countries diet [Obeid *et al*,. 2007] and now throughout the world (Oueslati *et al*,. 2018). Olive oil contains high amount of monounsaturated fatty acids (oleic acid) and minor amounts of phenolic compounds with strong antioxidant properties (Obeid *et al*,. 2007), additionally it contains vitamins and sterols (Rahmanian *et al*,. 2014). Olive oil is among rare vegetable oils which can be used in its raw state directly (Oueslati *et al*,. *2018)* additionally is used for cooking meat, poultry, seafood, and different vegetables (Kishimoto, 2019).

Extra virgin olive oil (EVOO), which is produced mechanically by pressing olive fruit without preliminary refining or heating and without using chemicals or solvents, is considered a valuable and nutritional vegetable oil (Ammar *et al*,. 2014). EVOO is the highest grade of olive oil and has free acidity (FA), expressed as oleic acid content, of not more than 0.8 g/100 g oil (IOC, 2018). EVOO is claimed to have the highest quality among olive oils for its pleasing and especial flavor which is superior than other edible vegetable oils (Oueslati *et al*,. 2018).

Another important minor component of EVOO is tocopherol, which protects the oil from oxidation at elevated temperatures (Quiles *et al*,. 2002,. Allalout *et al*,. 2009), however, the oxidative stability of EVOO

correlates mainly with the concentration of hydrophilic phenolic compounds. Phenolic compounds give olive oils their unique taste and they represent an important contribution to its oxidative stability. On the other side, phenolic compounds have many beneficial health effects as natural antioxidant (Al-Asaad and Aldiab, 2017, Aldiab, 2018), anti-inflammatory (Aldiab *et al,*. 2021, Nezam *et al,*. 2021,), anti-bacterial (Kaddar *et al,*. 2023, Alahmad *et al,*. 2023) anti-diabetic effect (Asalti *et al,*. 2022) and anticoagulant (Saeed *et al,*. 2023,. Alaa *et al,*. 2023). Therefore, Olive oil is related to protection against cardiovascular diseases and cancer due to the presence of phenolic constituents as well as to its fatty acid profile (Gharby *et al,*. 2011).

During storage and heat treatment such as frying and microwave heating, fat is subjected to hydrolysis, oxidation and polymerization. The previous reactions lead to deterioration of quality with respect to both its sensory quality and its nutritive value. The mechanism of the oxidation processes is the same for different fats and oils, however, the reaction rates vary between them. The antioxidants in oil react with free radicals, and the peroxide value (PV) is expected to increase only when insufficient antioxidants are left to protect against the formation of free radicals (Gharby *et al,*. 2019).

There is increasing interest to strength oil stability in order to delay the oxidative degradation during heating; therefore, many synthetic antioxidants are used such as Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT). The previous antioxidants protect oils effectively, however they undergo during frying and cooking thermal decomposition leading to decrease there protection, additionally they have potential harmful effects on health that limit their use (Aladedunye *et al,*. 2017).

Many studies showed that the exposure to high doses of BHT leads to a severe internal bleeding and death in mice (Shahidi and Ambigaipalan 2015). BHT turns in mice into toxic compounds that enhance hepatic and pulmonary injury (Guyton *et al,*. 1991), additionally BHT caused DNA damage in animals (Oikawa et al., 1998). In recent years the attention on phytochemical such as phenolic compounds increased due to its antioxidant activity, since they can effectively reduce oil rancidity caused by heat treatment (Cheng *et al,*. 2007).

Extracts from herbs and spices are a rich source of natural antioxidants. Their properties are related mainly to the presence of phenolic compounds, which may act as antioxidants by scavenging of free radicals (Antolovich *et al,*. 2002).

Rosmarinus officinalis L. is a medical plant of *Rosmarinus* that belongs to Lamiaceae. *Rosmarinus officinalis* L. originates from Mediterranean region and grows there. It is used since ancient time for its medical characteristics, where it was traditionally used to relieve muscle pain, improve memory, enhance immunity and promote hair growth. Besides its beneficial effects on health it is used for cooking and perfume production (Kompelly *et al,*. 2019). *Rosmarinus officinalis* L. contains many active compounds most important are terpenes and phenolic compounds. It is considered one of the most important natural sources of phenolic compounds, which provide an antioxidant efficiency such as Carnosol and Carnosic Acid (Andrade *et al,*. 2018). These antioxidant properties made *Rosmarinus officinalis* L. *extracts* ideal as preservative instead of synthetic antioxidants (Kompelly *et al,*. 2019).

Thus, due to the richness of *Rosmarinus officinalis* L. in phenolic compounds and considering them as safe and active natural antioxidant, there is a possibility to use them to prevent EVOO oxidation and thus to protect the nutritional value of EVOO as well as to increase its shelf life. Therefore, the aim of this study is to investigate the effect of *Rosmarinus officinalis* L. ethanolic extract to reduce and delay the oxidation of EVOO during storage and heat treatment both conventional oven and microwave.

MATERIAL AND METHODS

EVOO was obtained from local farm in Latakia, Syria.

Instruments

Rotavapor (BÜCHI Rotavapor R-200), Spectrophotometer (Jasco V-530 UV), Ultrasonic bath (K & H Industries), Analytical balance (RADWAG, AS 220/C/2).

Chemicals

Gallic acid was purchased from Biotech LTD, India. Folin-Denis reagent was purchased from Sigma- Aldrich, Switzerland. Sodium carbonate was obtained from BDH, England. Sodium thiosulfate from Tekkim. Butylated Hydroxy Anisole (BHA) and Potassium iodide were obtained from Qalikema Fine Chemicals Pvt.Ltd. p-Anisidine reagent was purchased from Sigma- Aldrich. Ethyl acetate was from Carbolite. n-hexane, ethanol (75%) were from Tekkim. Glacial acetic acid was from Qalikema Fine Chemicals Pvt.Ltd.

Preparation of phenolic extract

Fresh *Rosmarinus officinalis* L. was harvested from latakia, Syria. The plant washed by water and dried at room temperature without exposure to sunlight. The dry plant was grounded by a home blender and stored in the refrigerator until analysis.

To prepare *Rosmarinus officinalis* L. ethanolic extract (RE), 12 g of grounded plant were soaked in 100 ml of ethanol 70% and the mixture was sonicated for 25 min at room temperature. The mixture was then centrifuged at 2000 rpm for 5 min and filtered (Aldiab, 2018). The filtrate was concentrated by a rotary evaporator until dryness. The dry extract was stored at -20 °C until analysis.

Determination of phenolic content in *Rosmarinus officinalis* L. ethanolic extract

The phenolic content of RE was determined using Folin-Ciocalteu reagent. 10 mg of the dried extract was dissolved in 10 ml distilled water. 0.1 ml of the previous solution was mixed with 2 ml of sodium carbonate 2% and left to stand for 5 min followed by the addition of 0.1 ml of Folin-Denis' reagent (prepared by dilution with distilled water 1:1) and then left for 30 min at room temperature. A blank was prepared like samples; however, 0.1 ml of distilled water was added instead of RE solution. A serial dilution of Gallic acid was prepared in concentrations 0.1, 0.2, 0.4, 0.6, 0.8 g/L. The absorbance was measured by spectrophometer at 750 nm for both Gallic acid solutions and RE solution. The equation was y=1.517x+0.0243 and R^2 was 0.995. The total phenolic content was expressed as mg of Gallic acid equivalents (GAE) per 1 g of the dried extract (Aldiab and Sahunie, 2023). The experiment was carried out in triplicate.

Preparation of EVOO samples

EVOO was devided into three portions. The ethanolic extract of *Rosmarinus officinalis* L. was added to the first portion of EVOO to achieve the concentration of 200 ppm (EVOO+RE), where 2.1g of the dried ethanolic extract was first dissolved in 1 g of ethyl acetate and then added to 996.9 g of EVOO. The mixture of dissolved extract and oil was homogenized with vortex for 10 min. BHT was added to the second portion of EVOO to achieve 200ppm and considered as positive control (EVOO+BHT). The third portion without any addition was used as negative control (EVOO).

The heating process of EVOO samples in oven and microwave

The oven was heated at 180 °C for ten minutes before placing samples. 100 g of each portion of oil (EVOO, EVOO + RE, EVOO+BHT) were entered the oven. An aliquot was taken before heat treatment and after heating for 3, 5, 10, 15, 20 and 30 min. Similarly, 100 g of each portion of oil (EVOO, EVOO + RE, EVOO+BHT) were entered the microwave. An aliquot was taken before microwave heat treatment and after heating for 3, 5, 10, 15, 20 and 30. All aliquots were put in ice in order to stop oxidation reaction. Experiments were carried out in triplicate for each oil sample and for each oxidation indicator. Finally, the steps needed for peroxide value (PV), p-Anisidine (p-AV) value and totox were applied.

Storage of EVOO samples

1000 g of each of EVOO samples (EVOO, EVOO+RE, EVOO+BHT) were placed in dark glass bottle, closed strictly and kept in a dry and dark place. PV, p-AV and totox value were determined at the beginning of storage and after 1, 2, 3, 6 and 9 months. Experiments were carried out in triplicate for each oil sample and for each oxidation indicator. Finally, the steps needed for PV, p-AV and totox were applied.

Determination of PV

PV was determined according to AOAC (1997) as the following: 1g of olive oil sample was mixed with 6 ml of glacial acetic acid/chloroform mixture (v/v, 3:2). 0.1 ml of saturated potassium solution was added with stirring for 1 min followed by addition of 6 ml water. The titration was carried out by 0.01M Sodium thiosulfate until the appearance of yellow color. Finally, 0.5 ml of starch suspension 1% was added where the color changed into blue, and then the titration was completed until the blue color disappeared. The blank was prepared by replacing the olive oil sample with distilled water and repeating the same previous steps. PV was determined according to the following equation (Zrekah *et al*,. 2015):

$$PV = \frac{(S - B) * M * 1000}{g}$$

PV= [(S - B) * M * 1000] / G

PV: peroxide value (milli-equivalent (mEq) Oxygen per 1 KG oil)

S: volume of 0.01M Sodium thiosulfate for the sample

B: volume of 0.01M Sodium thiosulfate for the blank

M: Molarity of Sodium thiosulfate (0.01M)

1000 = conversions of unit (g/kg)

g: olive oil sample weight

Determination of p-AV

1g of olive oil sample was dissolved in n-hexan and the volume completed until 25 ml, the absorption of the mixture was measured versus n-hexane alone at 350 nm (A1). 1ml of p-Anisidine solution (0.25% w/v in glacial acetic acid) was added once to 5ml of olive oil/n-hexan mixture and twice to 5 ml of n-hexan with stirring for 10 minutes in darkness. After that, the absorption of olive oil / n-hexan / p-Anisidine was measured versus n-hexan /p-Anisidine at 350 nm (A2). p-AV was calculated following the equation : (Aldiab and Sahunie, 2023)

$$\text{p} - \text{AV} = \frac{25 * (1.2\,A2 - A1)}{m}$$

p-AV: p-Anisidine value

A2: The absorption of olive oil/n-hexane/p-Anisidine mixture

A1: The absorption of olive oil/n-hexane mixture

m: oil weight

Determination of totox value

The totox value was determined according to the following equation:

Totox Value = p-AV + 2 * PV (Shahidi and Zhong 2005).

Statistic analys

All results were presented as mean ± standard deviation. The differences of oxidation indicator values between negative control (EVOO), positive control (EVOO+BHT) and samples of olive oil with phenolic extracts (EVOO+RE) were tested by Students' t-test. Differences were considered to be significant at p value

< 0.05. All statistical analyses were performed using the Microsoft Excel 2016 Software.

RESULTS AND DISCUSSION

The phenolic content of the ethanolic RE was 95.18 ± 5.38 mg GAE/g, which is similar to our previous study (Aldiab and Sahunie, 2023). Other study mentioned that the ethanolic RE contained 201 mg GAE/g (Aljabri, 2020). The differences in phenolic content of RE compared to other studies may return to many factors such as the origin of the plant, the harvest time as well as the method of extraction. For example, temperature, humidity, quantity of rainfall and the different altitude from sea surface all can affect the different phenolic content of Rosmarinus *officinalis* L. (Yeddes *et al.*, 2019). In addition to phenolic compounds, plant extracts may contain other compounds such as carbohydrate, fat, ascorbic acid, tocopherols and others (Gulcin, 2020), which make the ethanolic extract has better antioxidant activity than aqueous extract since the later will contain many impurities such as organic acids, proteins, and dissolved sugars, which do not have antioxidant activity (Okhli *et al,*. 2020).

Oxidation stability after heat treatment

Figures 1, 2 and 3 show that PV, p-An-V and totox value increased gradually in EVOO by increasing heating time in oven where they reached after 30 min 43.02 ± 3.3 mEqO2/kg, 25.75 ± 1.84 and 111.79 ± 6.7, respectively. The addition of RE and BHT led to decrease PV, p-An-V and totox value compared to EVOO (p-value <0.05). PV exceeded the allowed limit (10 mEqO2/kg) in EVOO after 3 min versus after 5 min in EVOO+BHT (positive control) and after 10 min in EVOO+RE. PV is a widely used indicator for fat oxidation, measuring lipid peroxides and hydroperoxides that formed during the initial stages of oxidation. However, PV is not a good indicator to evaluate and measure secondary and total oxidation because hydroperoxides are unstable (Norihito Kishimoto., 2019) and are rapidly decomposed to form a variety of secondary products such as aldehydes, ketones, alcohols, hydrocarbons and polymers. The aldehydes and other secondary products of lipid oxidation are well absorbed and may show toxic effects on the liver, kidneys and spleen. Aldehydes can cause fast oxidation of the cell lipids resulting in cell destruction (Gharby *et al,*. 2016).

Therefore, other indicators of secondary oxidation are used such as p-AV that measures the aldehydes generated during the secondary oxidation by the reaction of aldehydes with p-Anisidine reagent under acidic conditions (Shahidi and Zhong 2005). Our results showed that the addition of ethanolic RE prevented the primary oxidation (PV), secondary oxidation (p-AV) as well as the total oxidation (totox value) of EVOO better than BHT until 20 min heating in oven, while BHT prevented oxidation better than RE extract up 20 min, however both with no statistic difference between BHT and RE (p-value >0.05). This can be interpreted by losing some of the phenolic compounds where many of them are unstable at high temperatures and transform into small compounds or ineffective compounds (Azizah *et al,*. 2009).

The microwave oven is a common kitchen appliance that used for a broad range of food processing such as heating, drying, baking. The microwave oven become gradually simpler and more convenient. Microwave heating has substantial advantages over conventional heating, especially in terms of energy efficiency. Domestic microwaves are often used at powers of 150, 500, and 700 W according to the required food processing technique (Kishimoto, 2019).

Figures 4, 5 and 6 show an increase in PV, p-An-V and totox value by increasing heat treatment of EVOO in microwave, where they reached after 30 min 52.57 ± 3.3 mEqO2/kg, 50.56 ± 1.84 and 155.7 ± 6.7, respectively. The addition of RE and BHT led to decrease PV, p-An-V and totox value compared to EVOO during heating in microwave. On the other hand, the oxidation process in microwave was clearly higher than traditional heating in oven (p-value <0.05). Some studies described the effect of microwave heating on the thermo-oxidative stability of olive oil during cooking where it resulted in the formation of free radicals, hydroperoxides and secondary oxidation products (Kishimoto, 2019). One study revealed that intense microwave heating of EVOO significantly increased the free acidity and specific extinction coefficients (K270) in addition to severe loss of phenolic compounds compared to conventional heating. Microwave treatment also resulted in higher amounts of acrolein in EVOO after a short processing period compared to conventional heating (Kishimoto, 2019).

Many studies investigated the effects of *Rosmarinus officinalis* L. on fat and oil stability during heat treatment, for example, methanolic extract of *Rosmarinus officinalis* L. decreased significantly PV in flaxseed oil compared to the negative control and flaxseed oil with BHA (Wang *et al,*. 2018). Likewise, the addition of RE to sunflower oil has contributed to prolonging the first stage of the oxidation reaction and this related to its high content of phenolic compounds, especially Carnosic acid, Rosmarinic acid, and Chlorogenic acid (Soldo *et al,*. 2019). Another study was carried out in Tunisia found that adding RE to sunflower oil reduced p-AV to 38.51 compared to the negative control, in which p-AV reached 90.32 after 6 hours of frying at 180 °C (Saoudi *et al,*. 2016). It was found that ethanolic RE reduced p-AV to 68.0 versus 75.4 in the negative control; additionally RE enhanced oil stability better than BHA when they were used at the same concentration (Guo *et al,*. 2016).

Similarly, The ethanolic extract of *Rosmarinus officinalis* L. reduced totox value of soybean oil to 17.2±0.3 compared to 141.6±1.4 in the negative control both after

heat treatment in the oven for 20 days at 60°C (Dias *et al*,. 2015). Studies expended and included olive oil where the addition of RE to olive oil decreased free acidity, peroxide value, K232 and led to increase the phenolic content and antioxidant activity (Habibi *et al*,. 2024)

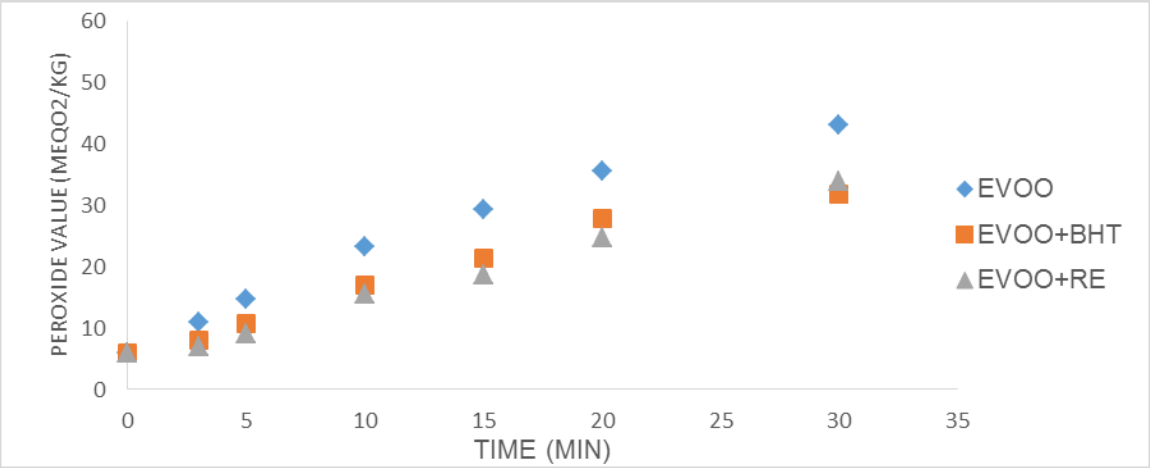

Figure 1: PV during heating in oven.

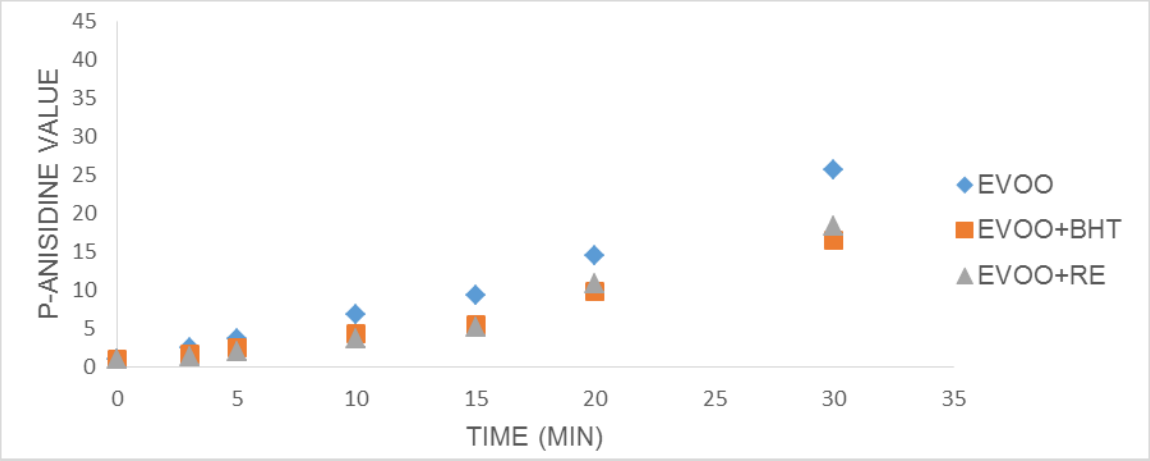

Figure 2: p-AV during heating in oven.

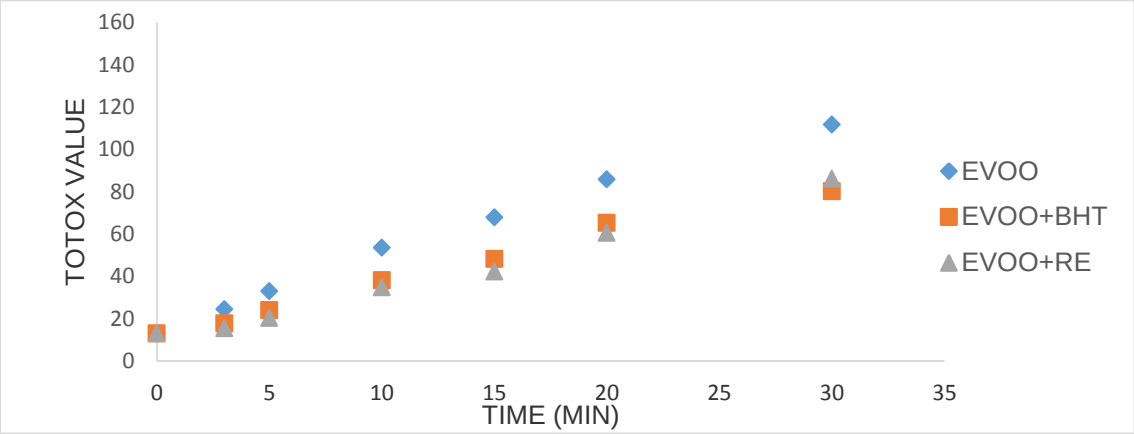

Figure 3: Totox value during heating in oven.

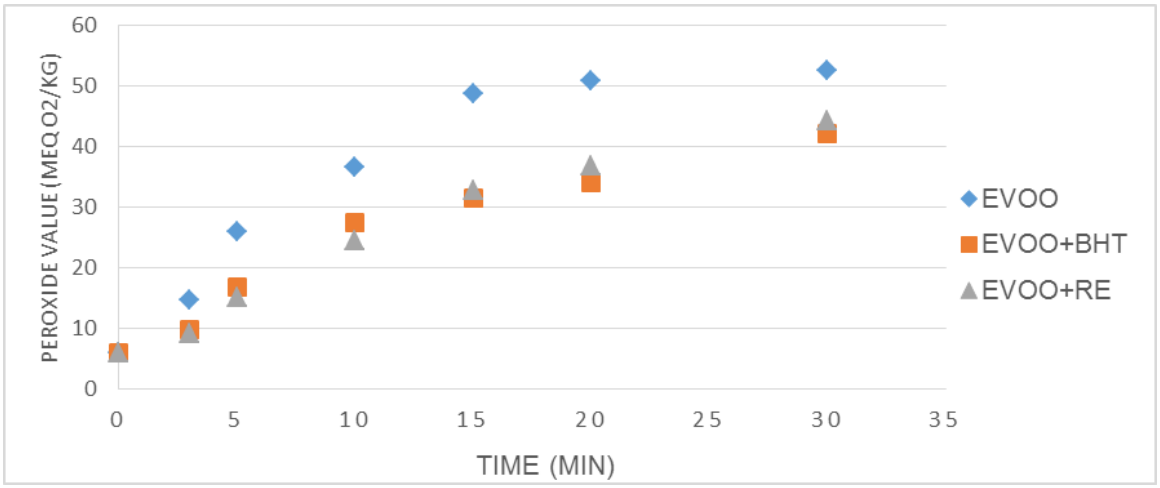

Figure 4: PV during heating in microwave.

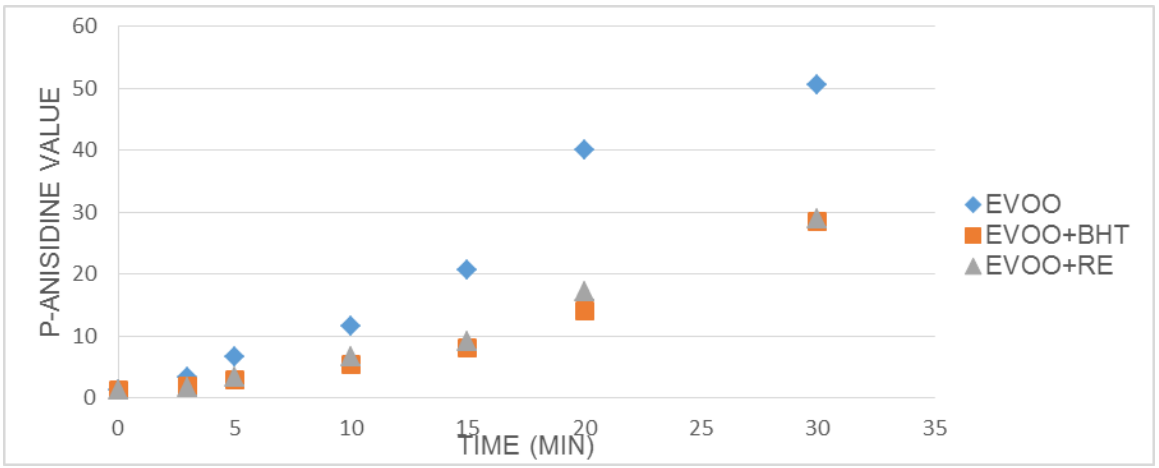

Figure 5: p-AV value during heating in microwave.

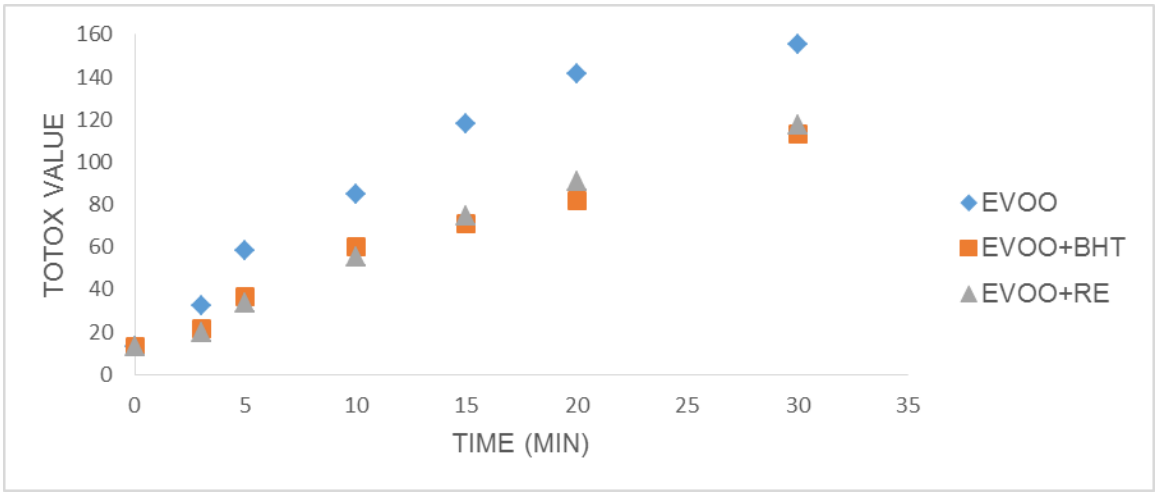

Figure 6: Totox value during heating in microwave.

Oxidation stability during storage

Long-term storage of oils at room temperature is one of the main causes of the spontaneous oxidation reaction, which occurs because of the interaction between oxygen and fatty acids and leading to formation of free radicals (Choe and Min 2006). As seen in Figures 7, 8 and 9, PV, p-An-V and totox value increased gradually in EVOO during storage and reached after 9 months 38.59± 2.99 mEqO2/kg, 39.56 ± 0.42 and 116.74± 4.8, respectively. Oxidation indicators reached in the positive control (EVOO+BHT) 29.34 ± 1.99, 24.52 ± 0.3 and 83.2 ± 4.9 for PV, p-AV and totox value, respectively, while they reached in EVOO+RE 24.97±2.5, 26.92 ±2 and 76.89 ± 3.7, respectively.

PV in EVOO reached the permissible limit (10 meq/kg) after one-month storage. The addition of BHA and RE extract contributed to maintain PV below the permissible limit until the second and third month, respectively, even though, no important difference was found between them (p-value > 0.05). RE showed better protection against oxidation presented in lower PV and totox value and this was almost along storage period, however, EVOO+BHT and EVOO+RE exceeded the permissible limit after three-month storage at room temperature. The changes in lipids influence their nutritive value, where polyunsaturated fatty acids are degraded and toxic compounds may appear, as a result, the absorption of some other food constituents may be limited (Gharby et al, 2016).

Many studies investigated the possibility of using phenolic extracts as an alternative to synthetic antioxidants in order to prevent and delay the oxidation of oils during storage. One study found that the ethanolic extract of Thymus Vulgaris decreased totox value in sunflower oil to 19.56 compared to 30.42 in the negative control when both stored for 29 days at 18 °C (Zaborowska *et al.*, 2012). Another study was carried out in 2015 found that the addition of ethanolic *RE* to soybean oil reduced PV significantly but it was less effective than synthetic antioxidant tertiary-butylhydroquinone (TBHQ) during storage for 20 days at 60 °C (Dias *et al.*, 2015).

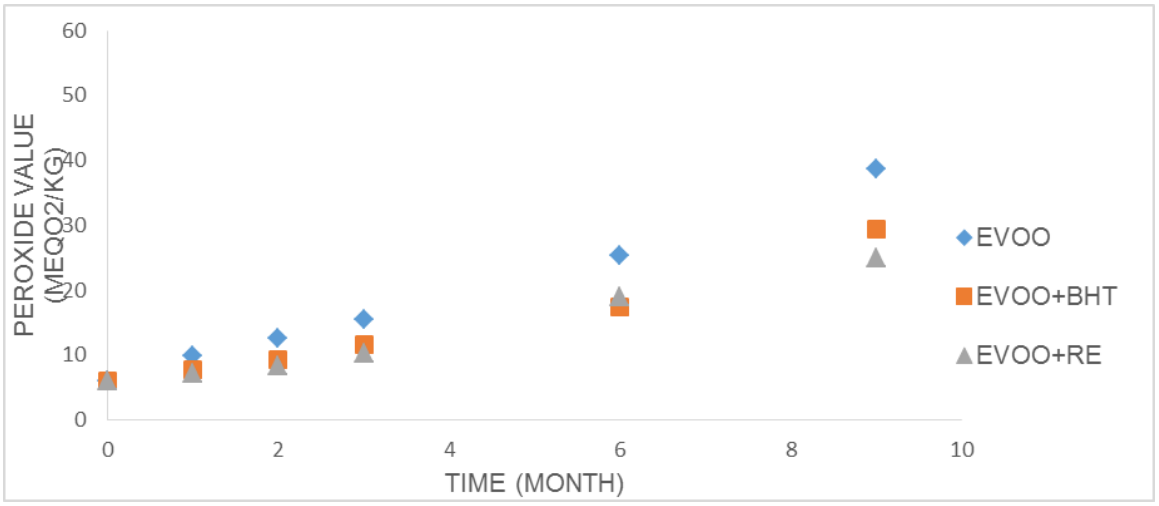

Figure 7: PV during storage.

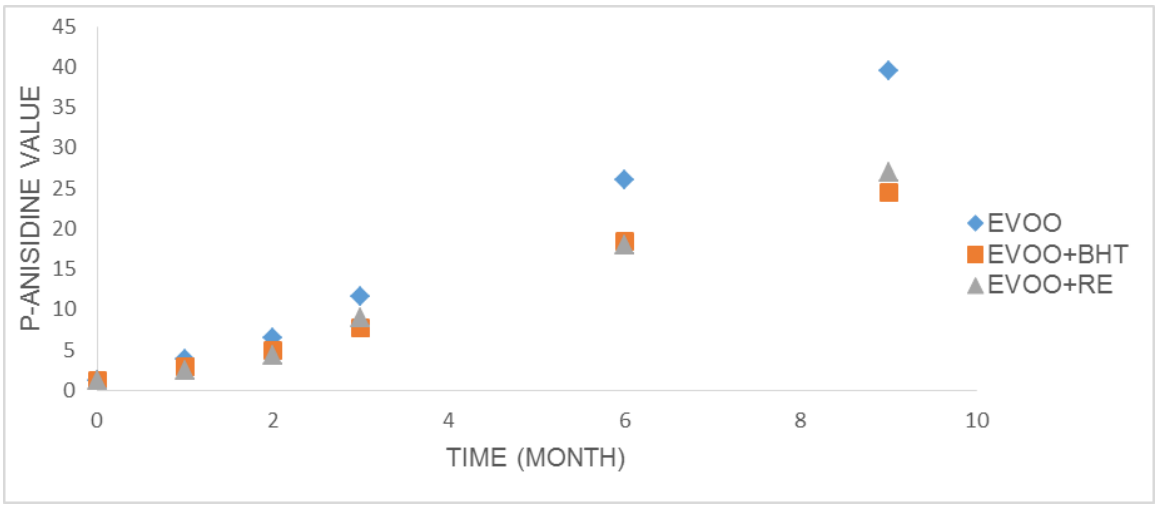

Figure 8: p-AV during storage.

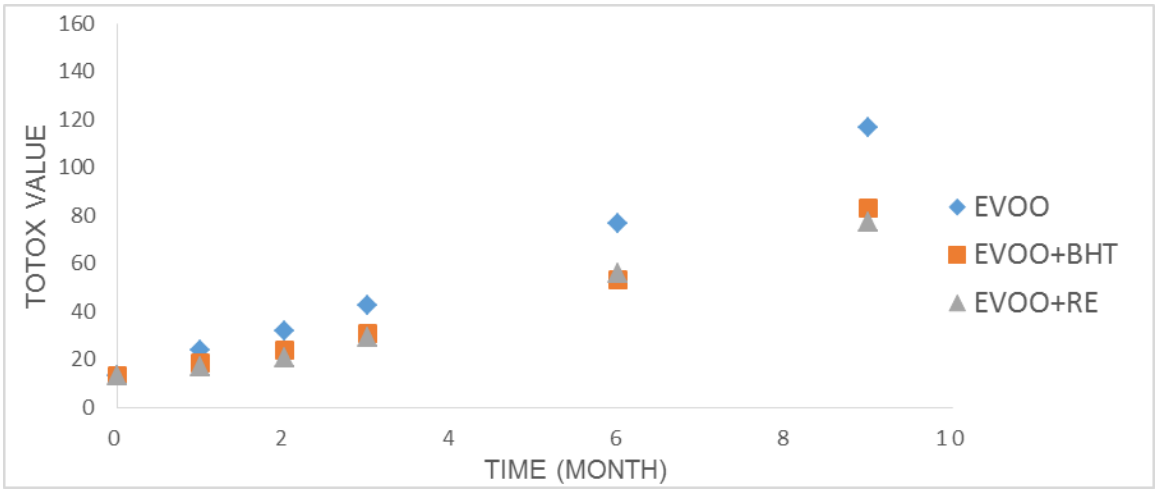

Figure 9: Totox value during storage.

CONCLUSION

The ethanolic extract of *Rosmarinus officinalis* L. decreased oxidation index (PV, p-AV, Totox value) in EVOO during storage and heat treatment compared to the negative control. This enable RE to be used as natural antioxidant in olive oil instead of the synthetic antioxidant BHT. More studies should be done to investigate the effect of RE on olive oil during longer storage periods and under other heat treatment conditions.

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