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# Effect of Punicalagin as Natural Antioxidant on The Oxidative Stability of Canola Oil during Storage

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THE present study intends to determine the effect of punicalagin as natural antioxidant on stabilising canola oil during 90 days storage and also to compare its strength with the synthetic antioxidant Butylated hydroxytoluene (BHT). Canola oil sample was categorized into three groups; pure oil (control), oil with Punicalagin (600 ppm) and oil with BHT (600 ppm). Peroxide value (PV) and free fatty acids (FFA) were used to estimate the primary products of oil oxidation while Thiobarbituric Acid Reactive Substances (TBARS) and P-Anisidine values (PAV) were used to estimate the secondary products. Finally, total oxidation (TOTOX) was calculated to evaluate the overall oxidation of oil samples. The results showed that PV, FFA, PAV and TOTOX were significantly increased in all canola oil samples with increased in storage time. In contrast, The TBARS values continued to increase from the starting storage period until 60 days and then decreased significantly until the end of the storage period. Punicalagin effectively reduces the production of the first and second oxidation products of canola oil during storage as indicated by the reduction in the PV, FFA, PAV and TOTOX of canola oil. When compared to BHT, punicalagin showed similar effect in inhibiting primary oxidation products whereas BHT showed stronger effect in reducing the secondary oxidation products. In conclusion, punicalagin can be used as a suitable replacement for chemically synthetic antioxidants on stabilising canola oil. Future studies should focus on evaluating the effect of higher concentrations of punicalagin to help in reducing the secondary oxidation products.

**Keywords:** Oxidative stability, Natural antioxdant, Punicalagin, Canola oil, Storage.

## Introduction

The use of edible oils has become an indispensable part of the world today. This is evident from its production of 203.3 million metric tons in 2019 only [1] . One of the best edible oils is canola oil which considered as the third largest vegetable oil by volume after palm and soybean oil [2]. Canola oil is characterized by low level (7%) of saturated fatty acids (SFAs); substantial amounts of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), including 61% oleic acid, 21% linoleic acid, and 11% alpha-linolenic acid (ALA); plant sterols (0.53–0.97%); vitamin K (71 µg/100g) and tocopherols (17 mg/100g)– all of which have data indicating they

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are cardioprotective substances [3; 4]. In addition to its nutritional value, canola oil has light flavour, high smoke point, and smooth texture making it one of the most used cooking oils globally [5] .

The quality of oils is substantially deteriorating as an outcome of oxidation. Asnaashari and Sharif (2014) [6] demonstrated that light and heat, trace metal, oxygen partial pressure, and unsaturated content of fatty acids impact the oil oxidation rate. This oxidation consists of two main processes; namely, primary oxidation and secondary oxidation. In the former, the oxygen is added to fatty acids at the double bond site to create peroxides. However, the physical changes of oils occur in the secondary oxidation when the peroxides break down into different volatile compounds [7]. This deterioration can lead to decrease both nutritional contents and shelf life, while simultaneously adversely affecting the human health [8]. Interestingly, the rate of oxidation can be reduced with the use of antioxidants, which reduce the oil deterioration in food products. Therefore, the enhancement of oil's quality as a consequence of increased oxidative stability has become a popular subject of the present food industries. It is resonated that this would not only improve oil products' shelf life but would also decrease the toxic compounds generated by oxidation [9]. Accordingly, edible oils that are rich with monounsaturated and polyunsaturated fatty acids and are subjected to heat or storage are often exposed to oxidation, stressing the instigation of an antioxidant regardless of its type i.e., synthetic or natural [8, 10, 11].

Natural antioxidants are observed to be more beneficial in contrast to the synthetic antioxidants such as BHT (butylated hydroxytoluene) or BHA (butylated hydroxyanisole), which have accounted to various safety concerns for consumers. Moreover, naturally occurring antioxidants were found to have similar activity as the chemically synthesized antioxidants against oxidation [12]. One of the predominant sources of natural antioxidants is pomegranate, which peel and rind are considered to be rich sources of flavonoids, tannins, and anthocyanins [13]. Bazargani-Gilani, Aliakbarlu, and Tajik (2015) [14] have highlighted that its antioxidant activity is significantly related to the increased level of punicalagin (An Ellagitannin Polyphenol extracted from pomegranate peel) [15, 16, 17] . Considering this, the study intends to determine first the effect of punicalagin on stabilising canola oil during 90 days storage and also to compare its strength with the synthetic antioxidant BHT.

## Methodology

*Preparation of Oil Samples*

A fresh canola oil was purchased from a local press oil shop in Jeddah City, Saudi Arabia. The obtained oil sample was categorized into three groups; pure oil (control), oil with Punicalagin (natural antioxidant) and oil with BHT (synthetic antioxidant). Both lyophilized antioxidants were obtained from Sigma Aldrich (St. Louis, MO). canola oil samples were prepared according to the method provided by Rashid et al. (2010) [18] with minor modifications. Initially, a 600 ppm stock antioxidant solution was prepared by adding 10 mg of the antioxidant to 16.6 ml of pyrogallol. Then, the antioxidant was added to 1L of canola oil. Once the oil samples were prepared, they were stored in a dark place for 90 days. To monitor the lipid peroxidation process and the antioxidant activity of both antioxidants, all the experiments were performed at 0, 30, 60 and 90 days. The preparation of samples was done in triplicate for obtaining independent measurements each time.

*Peroxide Value (PV)*

Peroxide value is used to monitor the amount of primary lipid oxidation present in oils. It detects the free radicals formed as peroxides and which indicate the presence of rancidity. PV states the milliequivalents of peroxide oxygen combined in a kilogram of oil and able, under testing, to liberate iodine from potassium iodide; the iodine is then estimated using a standard sodium thiosulfate [19]. In brief, five grams of oil sample were placed into 250ml flask. Then, acetic acid-chloroform solution (30ml) was added until the oil was dissolved. Saturated potassium iodide solution (o.5 ml) was added and swirled for exactly 1 minute followed by an immediate addition of deionized water (30ml). Starch (1ml) was also added as an indicator. The oil samples were then titrated against (0.1N) sodium thiosulfate with constant and vigorous agitation until the blue-gray color disappeared. The volume of titrant was used to calculate the peroxide value according to the following equation:

$$\text{Peroxide value} = \frac{(S - B) \times N\ thiosulphate\ \times 1000}{weight\ of\ oil}$$

Where,
S is titrant volume for the sample

B is titrant volume for the blank

*Free Fatty Acids (FFA)*

Free Fatty Acids was used as an indicator of fat (triglycerides) hydrolysis by using the method of AOAC Te 1a-64 [20] with some modifications. One gram of oil was added to 250ml flask. After this, 10ml of ethanol and 2 drops of phenolphthalein were added to the oil samples as solvent and indicator, respectively. Finally, oil samples were tittered against potassium hydroxide (KOH) until the faint pink colour appeared. The volume of KOH was then used to calculate the free fatty acid values according to the following equation:

$$Free\ Fatty\ Acids\ \% = \frac{(V - B) \times N \times 0.503}{W}$$

Where,
V = titrant volume for the sample
B = titrant volume for the blank
N = normality of KOH
W = weight of oil (g)

*Thiobarbituric Acid Reactive Substances (TBARS)*

TBARS measures the formation of secondary oxidation products i.e. carbonyl or aldehydes such as Malondialdehyde (MDA), which may contribute to off-flavour of oxidized oils. It is a test that involves the reaction between TBA and MDA (a product of lipid hydroperoxide decomposition) forming a red chromophore. This coloured complex was measured using Ohkawa, Ohishi, and Yagi (1979) method [21]. A 50 microliter of oil samples was drawn with a microsyringe and added to test tubes containing a mixture of 0.8 mL distilled water, 0.2 mL sodium dodecyl sulphate (8.1%,w/v), 1.5 mL of 20% acetic acid (w/v) (pH 3.5) and 1.5 mL of 0.8% 2-thiobarbituric acid solution in water (w/v). The samples were heated at 100 C for 1 hour. After cooling, the samples were centrifuged at 4300 g for 10 min. A spectrophotometer was used to measure the upper layer absorbance at 532 nm. The concentration of MDA in the sample was then determined by comparing the average optical density (absorbance) of the samples to a standard curve. A Five-point standard curve was used for the determination of unknown. First, a stock of Tetraethoxypropane (TEP) solution at a concentration of 1 µg/ml was prepared by adding 0.1 µg TEP to 9.9ml distilled water. Next, from this stock solution, four aliquots of 0.2, 0.4, 0.6, 0.8 and 1µg were taken and added to each volumetric flask, and the volume was completed by adding distilled water to prepare the four standard solutions with 0.2, 0.4, 0.6, 0.8 and 1 µg/ml concentrations, respectively.

*P-Ansidine Value (PAV)*

PAV is applied to determine the secondary products of oil oxidation. This method is based on the reaction of P methoxy aniline (Ansidine) and aldehydic compounds especially 2,4 dienals and 2-alkenals, as principal metabolites of decomposition of hydroperoxide compounds (International Union of Pure and Applied Chemistry (IUPAC)). The PAV was determined using the United States Pharmacopeia method USP 401 [22] "Fats and Fixed Oils". Two solutions were prepared, one with the reagent (P-Anisidine), and the other was without it. First, the solution A was prepared by dissolving 0.5g of oil samples with 25 mL of isooctane. The absorbance was measured at 350nm using isooctane as a blank. After that, the solution B was prepared by adding 1 ml of P-anisidine in glacial acetic acid (2.5g/ L) to 5 ml of solution A. the mixture was shacked and stored in the dark. The absorbance was measured at 350nm exactly 10 min after preparation. The blank of solution B was prepared by adding 5 ml of isooctane to 1ml of p-anisidine solution. The p- anisidine value was then calculated according to the following equation:

$$P - ansidine\ value = \frac{[25 \times (1.2AS - AB)]}{m}$$

Where,

$AS$ = absorbance of Test solution B at 350 nm
$AB$ = absorbance of Test solution A at 350 nm
m = weight of the substance to be examined in Test solution A (g)

*Total Oxidation (TOTOX)*

The overall oxidation state of canola oils was calculated according to De Abreu et al. (2010) [23] method using the following equation:

$$TOTOX = 2 * PV + PAV$$

PV= peroxide Value
PAV= P-anisidine Value

*Statistical Analysis*

To evaluate the oxidative stability of canola oil

by punicalagin during 90 days storage, two -way ANOVA followed by tukey's multiple comparison test was used. The same approach was used to assess the differences in antioxidant activity between punicalagin and BHT. All statistical analysis was performed using the statistical software GraphPad prism 7. Data were presented as means ± SEM and the statistical significance was taken as $P < 0.05$.

## Results

*Peroxide value (PV)*

Figure 1 shows the PV for pure canola oil, canola oil with BHT and punicalagin during storage for 90 days. It can be observed that storage time promoted oxidation in all canola oil samples and the mean PV increased significantly during the entire period. Pure canola oil showed the highest PV value at the end of the storage period (33 meq/kg). Oxidation was significantly reduced by the addition of both antioxidants and the PV measured in these samples were lower than in pure canola oil during all time points. Interestingly, PV of canola oil with punicalagin and BHT were both decreased by 8 meq/kg compared to pure canola oil at the end of storage time.

*Free Fatty Acids (FFA)*

FFA are formed due to hydrolysis of triglycerides and may get promoted by reaction of oil with moisture [24]. FFA content went on increasing with the increase in storage period for pure canola oil, but no regular pattern of increase could be observed (Figure 2). In contrast, FFA

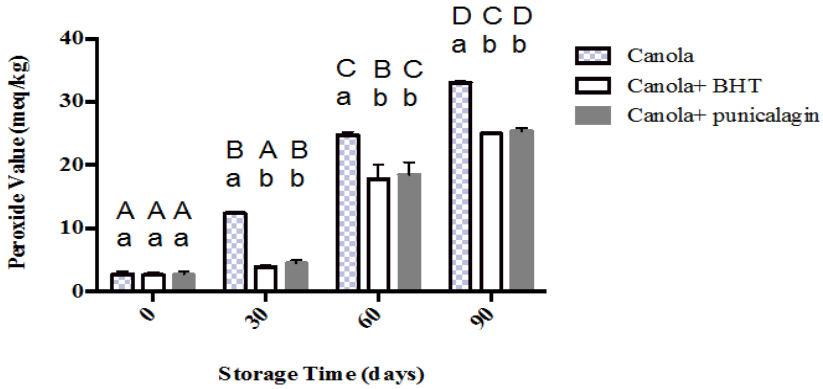


**Fig. 1. Mean peroxide value (PV) for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period(90 days). Error bars show SEM. Different upper -case letters mean significant difference ($p < 0.05$) between PV of the same type of oil during different time points. Different lower-case letters mean significant difference ($p < 0.05$) between pure canola oil and canola oil with antioxidants at the same time point.**

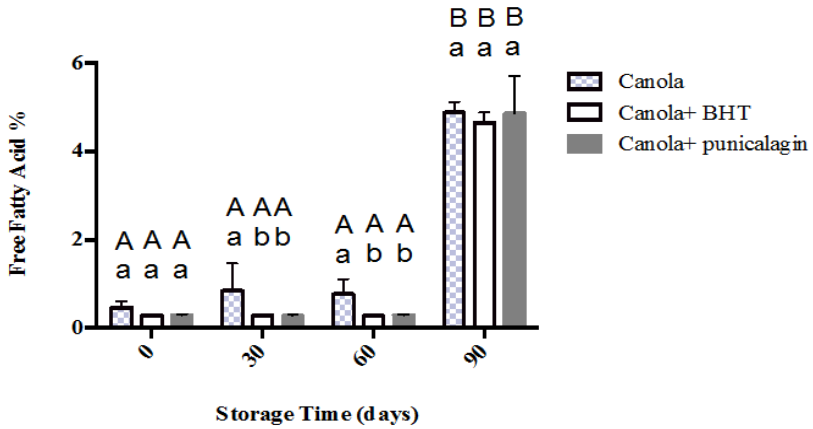


**Fig. 2. Mean Free Fatty Acids (FFA) for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period (90 days). Error bars show SEM. Different upper -case letters mean significant difference ($p < 0.05$) between free fatty acid % of the same type of oil during different time points. Different lower-case letters mean significant difference ($p < 0.05$) between pure canola oil and canola oil with antioxidants at the same time point.**

content of canola oil with both antioxidants were stable until 60 days of storage before it sharply increased at the end of the storage period. pure canola oil as well as canola oil with both antioxidants exhibited the highest FFA content after 90 days of storage.

Canola oil with punicalagin had FFA equal to canola oil with BHT and both are significantly slightly lower than pure canola oil only at 30 and 60 days of storage.

*Thiobarbituric Acid Reactive Substances (TBARS)*

Figure 3 shows mean TBARS value for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period (90 days). The TBARS values of all canola oil samples continued to increase from the starting storage period until 60 days and then decreased significantly until the end of the storage period. BHT antioxidant significantly decreased the formation of MDA in canola oil during all storage period compared to pure canola oil. On the other hand, punicalagin significantly decrease the formation of MDA in canola oil at day 30 only.

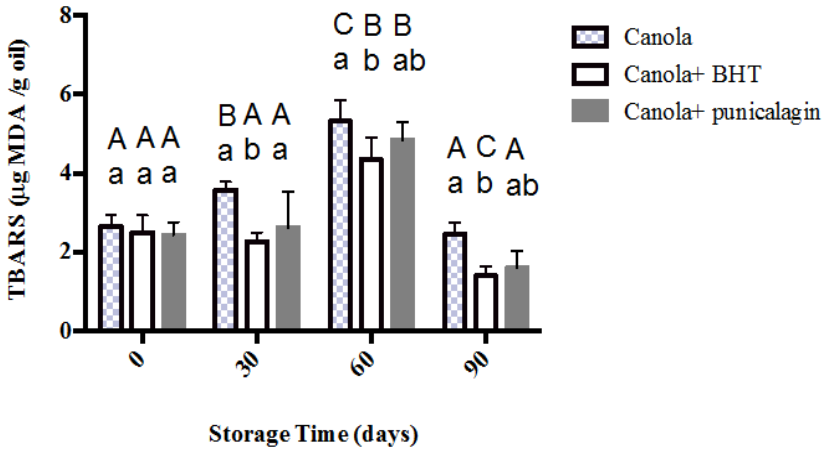


**Fig. 3. Mean Thiobarbituric Acid Reactive Substances(TBARS) for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period (90 days). Error bars show SEM. Different upper-case letters mean significant difference ($p < 0.05$) between TBARS value of the same type of oil during different time points. Different lower-case letters mean significant difference ($p < 0.05$) between pure canola oil and canola oil with antioxidants at the same time point.**

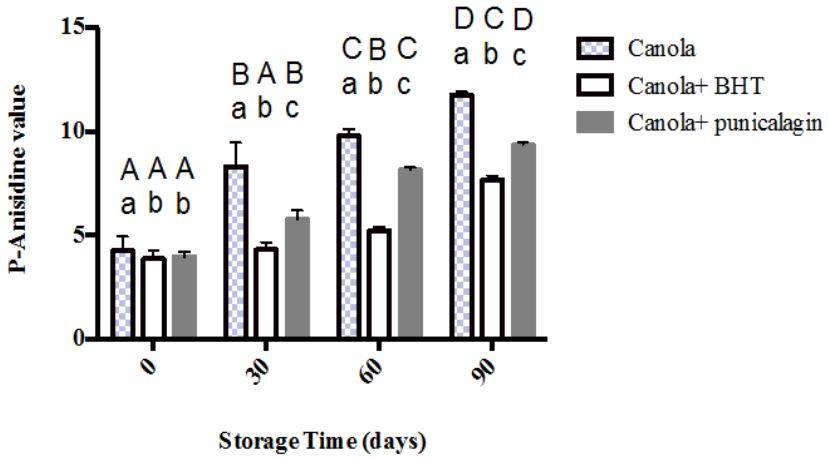


**Fig. 4. Mean P-Ansidine value (PAV) for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period (90 days). Error bars show SEM. Different upper -case letters mean significant difference ($p < 0.05$) between P-Ansidine value of the same type of oil during different time points. Different lower-case letters mean significant difference ($p < 0.05$) between pure canola oil and canola oil with antioxidants at the same time point.**

*P-Anisidine Value (PAV)*

The p-anisidine value relates to secondary oxidation products (carbonyls), reflecting the magnitude of aldehydes formation in oils [25]. Pure canola oil showed a significant upsurge of PAV as compared to canola oil with BHT and Punicalagin, which experienced a gradual increase (Figure 4). The addition of either BHT or punicalagin to canola oil resulted in a significant decrease in PAV compared to pure canola oil. When comparing the effect of punicalagin and BHT in preventing the secondary oxidation, BHT found to be more effective than punicalagin.

*Total Oxidation (TOTOX)*

Total oxidation values represent a deterioration oxidative index, because it accounts for both primary and secondary products (i.e. peroxides and aldehydes) [26]. Figure 5 shows the changes in the total oxidation of pure canola oil, canola oil with BHT and canola oil with punicalagin. There was a marked increase in TOTOX index during 90 days storage in all canola oil samples. TOTOX index for canola oil with BHT and punicalagin were significantly lower than pure canola oil. Interestingly, TOTOX of canola oils treated with both antioxidants gave a comparable result..

## Discussion

The present study assessed the punicalagin effect as a natural antioxidant on the oxidative stability of canola oil during storage. It compared its effect with pure canola oil and canola oil with BHT. Different assays including PV, FFA, TBARS, PAV and TOTOX were used to determine the primary and secondary oxidative compounds produced during storage.

*Effect of punicalagin and BHT on PV*

In this study, A regular increase in PV as a function of storage time was observed for all canola oil samples at all time intervals indicating a primary oxidation of the samples. Popa et al (2017) [27] and Maszewska et al (2018) [28] found a similar increase in PV during storage of several edible oils. This increase in PV is because the high degree of unsaturation of canola oil. As the degree of unsaturation increases, both the rate of formation and the amount of primary oxidation compounds will increase and accumulates at the end of the induction period [29]. The addition of either punicalagin or BHT to canola oil reduced the PV of these samples compared to pure canola oil. This reduction was in the same pattern for both antioxidants indicating that punicalagin is an effective antioxidant and stable as BHT until 90 days of storage. These results are consistent with data reported recently by Sarojini et al (2019) [30] who found that Pomegranate peel extract (PPE) contain a considerable amount of phenolic compounds which has the ability to scavenge free radicals during the oxidation process explaining the slow down formation of peroxide in sardine fish oil compared to control.

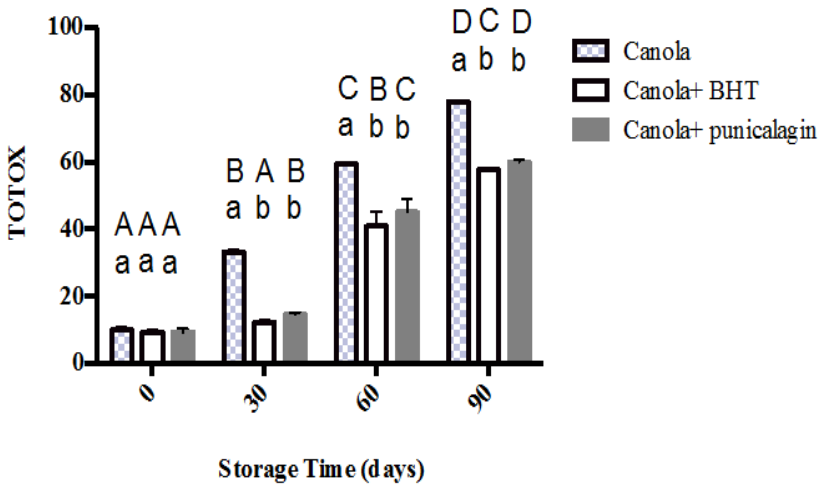


**Fig. 5. Mean Total Oxidation (TOTOX) for pure canola oil, canola oil with BHT and canola oil with punicalagin during storage period (90 days). Error bars show SEM. Different upper-case letters mean significant difference ($p < 0.05$) between TOTOX of the same type of oil during different time points. Different lower-case letters mean significant difference ($p < 0.05$) between pure canola oil and canola oil with antioxidants at the same time point.**

*Effect of punicalagin and BHT on FFA*

Free fatty acids are more susceptible to autoxidation than esterified fatty acids. Thus, free fatty acids act as pro-oxidants (compounds that initiate, facilitate, or accelerate lipid oxidation) in edible oil. These compounds have a hydrophobic and a hydrophilic group in their structure. The hydrocarbon chain is the hydrophobic group and the carbonyl group is the hydrophilic group. The carbonyl group of these compounds are preferably concentrated on the surface of edible oil, decreasing the surface tension and increasing the diffusion rate of oxygen from the headspace into the oil, so accelerating oil oxidation. Normally, 0.5–0.8% FFA is encountered [31]. In the present study, all types of canola oil showed acceptable percentage of FFA (0.2 to 0.7%) until 60 days of storage. After that, a sharp increase in FFA% (reached above 4%) for all oil samples were observed. The increase in FFA content during storage were previously reported in different oils such as sunflower, palm and fish oil [32, 33, 34] The results of this study also showed that the antioxidant efficiency of punicalagin and BHT in reducing the FFA content in canola oil were similar up to 60 days before they show a weaker effect at the end of the storage period. Based on this, it is recommended to add a higher concentration of both antioxidants (>600 ppm) to have longer effect

*Effect of punicalagin and BHT on TBARS*

The TBARS is the most widely used method for the measurement of secondary oxidation products such as MDA which generated from lipid hydroperoxide decomposition. In general, A continuous increase in TBARS values were observed for several oil types with the increase of storage time [25, 30, 33, 35]. These findings are in line with the results of the present study, where TBARS values of all canola oil samples continued to increase from the starting storage time until 60 days. This gradual increase in TBARS values proves the conversion of primary oxidation products to secondary oxidation products [30]. After 60 days of storage, TBARS values for all oil samples showed a sudden drop. The findings of Agregan et al. (2017) [36] corroborate the present study outcomes demonstrating that this may due to the degradation of MDA as a result of its unstability and the formation of carboxylic acids. The addition of BHT showed better antioxidant property than punicalagin in improving the oxidative stability of canola oil, which might be due to the difference in their chemical structure. The BHT has only one OH group attached to the aromatic ring while punicalagin has around 15 OH groups attached to several aromatic rings. Thus, BHT provide more sites for forming complexes between free radicals and antioxidant groups for lipid stabilization purposes [37]. Another possible reason is the low concentration of punicalagin used in this study (600 ppm) compared with other studies. For example, Sarojini et al (2019) [30] found that the addition of 2000 ppm pomegranate peel extract to sardine fish oil showed a similar effect in reducing TBARS values as BHA antioxidant. They concluded that pomegranate peel extracts at higher concentration have the potential to act as an effective natural antioxidant instead of synthetic antioxidants in arresting oxidation in oil samples.

*Effect of punicalagin and BHT on PAV*

In order to confirm results for primary oxidation, the simultaneous detection of primary and secondary lipid products is necessary. For this reason, PAV was also determined in our canola oil samples. This index relates to secondary oxidation products (carbonyls), reflecting the magnitude of aldehyde formation in oils [34, 38]. The results showed a rising trend in the PAV of pure canola oil, canola oil with BHT and canola oil with punicalagin during storage. These changes in PAV of canola oil with time were also obtained by Zhang et al (2018) and Li et al (2012) [37, 39] in pecan, peanut and rapeseed oils. Addition of BHT and punicalagin to canola oil were significantly delayed the formation of secondary oxidation products compared to pure canola oil. Our results are in general agreement with previous studies investigating the effect of both natural and synthetic antioxidants on PAV of canola oil [40, 41]. However, punicalagin was less effective in inhibiting secondary products formation than BHT. The previous studies obtained by Zhang et al. (2018) [37] and Li et al (2012) [39] have a similar findings, as they added different synthetic and natural antioxidants to pecan oil and rapeseed oils, respectively. They found that samples with synthetic antioxidant show lower PAV compared with natural antioxidants.

*Effect of punicalagin and BHT on TOTOX*

At the end of the study period, all canola oil samples showed an increase in TOTOX. These findings are in line with the results of a previous study, where TOTOX values of peanut, corn and rapeseed oils significantly increased with storage time [28]. The addition of BHT and punicalagin

resulted in lower TOTOX index compared to pure canola oil indicating a slower oxidation rate in the former. these results revealed that punicalagin is effective as BHT in improving the oxidative stability of canola oil.

## Conclusion

In summary, different assays were used to examine antioxidant efficacies of punicalagin in canola oil. It effectively inhibits the production of the first and second oxidation products of canola oil during storage as indicated by the reduction in the PV, FFA, PAV and TOTOX of canola oil. When compared to BHT, punicalagin showed similar effect in inhibiting primary oxidation products such as peroxides and free fatty acids. In contrast, BHT showed stronger effect in reducing the secondary oxidation products such as aldehydes. Therefore, punicalagin could be used as a good natural antioxidant to supress canola oil oxidation. Future studies should focus on evaluating the effect of higher concentrations of punicalagin to help in reducing the secondary oxidation products in canola oil.

## Conflicts of interest

There are no conflicts to declare..

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