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The Effect of *Moringa oleifera* Leaf Extract as Antioxidant on Stabilization of Butter Oil with Modified Fatty Acid Profile

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ABSTRACT

Effect of methanolic leaf extract of *Moringa oleifera* for the stabilization of butter oil with modified fatty acid profile at ambient temperature was investigated. Twelve Sahiwal cows of first and early lactation were randomly stratified into two groups in a completely randomized design and fatty acid profile of milk fat was modified by feeding 300 grams calcium salts of fatty acids (per cow per day) to one group (G-1) and the second group was not fed on calcium salts of fatty acids (G-2). Concentration of long chain fatty acids (C18:1 to C18:3) in milk of G-1 was increased from 30.33±0.174b to 35.36±0.14a% as compared to G-2. Milk fat of G-1 was turned into butter oil. *Moringa oleifera* leaf extract (MOLE) was incorporated into butter oil (from milk of G-1) at three different concentrations: T_1= 400, T_2= 600, and T_3= 800 ppm. All these treatments were compared with a control, without any addition of MOLE. Peroxide value of T_2 in Schaal oven test (after 90 days of storage) was 5.35±0.29b as compared to control 16.64±0.42a (meq /kg). *p*-anisidine value and induction time (after 90 days of storage at ambient temperature) of T_2 and control were 12.45±0.63b, 28.67±1.36a (meq kg^{-1}) and 10.84±0.28a and 3.95±0.14b hours, respectively. It was concluded that *Moringa oleifera* leaf extract at 600 ppm concentration may be used for the enhancement of oxidative stability of butter oil with modified fatty acid profile at ambient temperature.

Keywords: *Moringa oleifera*, Natural antioxidants, Oxidative stability, Peroxide value.

INTRODUCTION

The ambient temperature of the subcontinent is around 40°C for almost eight to nine months of the year and poise a major threat for the storage of fat rich foods, especially those which have higher contents of unsaturated fatty acids (Garcia *et al*., 2003). Butter oil is widely used for cooking and coating of "chapatti" (unleavened flat bread) and manufacturing of precious traditional sweets etc. Milk fat is characterized by higher contents of saturated fatty acids about 70% of which possess many health risks and are considered to be a potential risk of coronary heart disease (Mc Sweeney and Fox, 2003). Fatty acids composition of milk fat may be significantly modified and the concentration of several bio active compounds may be increased many times by manipulating the rumen feeding regime (Ashes *et al*., 1992; AbuGhazaleh and Holmes, 2009). Calcium salt of fatty acids is a rumen bypass fat; the objective of bypassing rumen is to save the beneficial unsaturated fatty acids from microbial bio-hydrogenation and formation of *trans* isomers. Role of calcium salts of fatty acids in modifying the composition of milk fat has been studied by many authors (Gonzalez *et al*., 2001; Fahey *et al*., 2001). Fats containing higher content of unsaturated fatty acids are susceptible to

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auto oxidation (Gonzalez *et al*., 2010). Higher ambient temperatures further speed up the auto oxidation process and reduce the shelf stability of modified fats. Chemical antioxidants like BHA (butylated hydroxyl anisole), BHT (butylated hydroxyl toluene), and TBHQ (tertiary butylhydroquinone) possess excellent antioxidant properties, but the use of synthetic antioxidants from the safety point of view is controversial and questioned by many people. Many researchers have studied the presence of poly phenolic antioxidants in higher plants, which possess strong antioxidant activity (Jeong *et al*., 2004). These antioxidants have anti carcinogenic and cardio-protective activity. Studies have demonstrated that diets rich in phenolic antioxidants have many health benefits and confer longer life expectancy (Kris-Etherton *et al*., 2002). Phenolic antioxidants are mainly present in leaves and seeds of some plants and have been implicated in preventing the food from free radical mechanism. *Moringa oleifera*, locally known as, "Sohanjna", is a common backyard tree and widely grown in many parts of southern Punjab (Anwar *et al*., 2004). Fresh tender pods are used for cooking and animal feeding and roots of young trees are turned into one of the tastiest pickles. *Moringa oleifera* leaves and seeds are a good source of antioxidants and oil is highly resistant to oxidation (Anwar *et al*., 2006). *Sahiwal* cattle is one of the most important breeds of dairy cows in Pakistan and is naturally resistant against ticks and many diseases and has the capability of adjusting in hot and humid climate (Garcia *et al*., 2003). No study has been done so far in Pakistan on exploration of antioxidant potential of *Moringa oleifera* leaves for the stabilization of modified butter oil containing higher concentration of unsaturated fatty acids. Keeping in view the above mentioned facts, this investigation was planned to improve the unsaturated fatty acids content of milk of "Sahiwal" cows and enhance oxidative stability of butter oil containing higher content of unsaturated fatty acids by using various concentrations of *Moringa oleifera* leaf extract on the basis of certain chemical parameters.

MATERIALS AND METHODS

Experimental Station

The study was performed at Livestock Production Research Institute (LPRI), Bahadur Nagar, Okara. Twelve Sahiwal cows of first and early lactation were randomly divided into two groups (each containing six cows) in a completely randomized design. Fatty acid profile of milk fat was modified by feeding 300 grams calcium salts of fatty acids (per cow per day) to one group (G-1) and the second group (G-2) was not fed on calcium salts of fatty acids. To minimize variation in the experiment iso-caloric and iso-nitrogenous feed was given to both groups.

Extraction of Phenolic Extract from *Moringa oleifera* Leaves

Leaves of *Moringa oleifera* were dried in the sun, ground to pass through 1-mm sieve. 500 mL of 80% methanol (and 50-g of leaf powder) was taken in 1,000 mL glass beaker, covered with aluminum foil to prevent solvent evaporation, agitated at 100 rpm on magnetic stirrer at room temperature for 48 hours as prescribed by Siddhuraju and Becker (2003).

Manufacturing of Butter Oil

Milk of G-1 (containing higher concentration of unsaturated fatty acids) was collected in the morning and evening, pooled, passed through the cream separator, cream was pasteurized at 85 ^{0}C in a thermostatically controlled water bath

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(Memmert Germany) for two minutes, cooled immediately to 21°C and inoculated with 2% bulk starter of CSK-; *Lactococcus lactis* sub. Spp. *lactis* (CSK Food Specialties Friesland, The Netherlands) and was ripened at this temperature for 16 hours (acidity 0.85% lactic acid) and churned in a laboratory scale butter churn (Speer, 2005). Butter was washed with cold water at 10-12°C and, after proper working, transferred to 2,000 mL Buckner flask equipped with laboratory scale vacuum pump and heated to 80°C on an electrical hot plate, the curd was removed by using fine muslin cloth. Each treatment was performed in triplicate.

Experimental Plan

Butter oil made from milk of G-1 was augmented with MOLE at three different concentrations i.e. 400 (T_1), 600 (T_2), and 800 ppm (T_3). All these treatments were compared with a control (T_0), which did not contain any addition of MOLE.

Analysis

Unmodified Butter Oil and Butter Oil Containing Higher Concentration of Unsaturated Fatty Acids

Fatty Acid Profile: Fatty acid profile of butter oil was determined as fatty acid methyl esters on the gas chromatograph. Fatty acid methyl ester was prepared by dissolving 0.4 gram samples in 3-mL *iso*-octane in the screw capped test tubes, then 1 mL 2.5N sodium methoxide was added and vortexed for two minutes, the layers were allowed to separate for five minutes and supernatant was injected into Gas Chromatograph model Shmidzu, Japan 17-A, fitted with a methyl lignoserate-coated (film thickness 0.25 l m), SP-2330 (SUP ELCO Inc. Supelco Park Bellefonte, PA 16823- 0048, USA) polar capillary column (30 m x 0.32 mm) and a flame ionization detector as in method described by Sukhija and Palmquist (1988).

Characterization of *Moringa oleifera* Leaf Extract

Total Phenolic Content: The total phenolic contents of the *Moringa oleifera* leaf extract was determined by following the method of Saeedeh and Asna (2007). 20 µL of *Moringa oleifera* leaf extract was mixed with 1.6 mL distilled water and 100 µL Folin–Ciocalteu reagent, then, 300 µL sodium carbonate (20%) solution was added and incubation was carried out in shaking water bath at 40°C for half an hour and absorbance (intensity of blue color) was measured at 760 nm in visible region of spectrum on double beam spectrophotometer (Schimadzu, Japan). Gallic acid was used as standard for the determination of total phenolic contents from the standard curve and measured as Gallic acid equivalent.

Analysis of Butter Oil with Higher Content of Unsaturated Fatty Acids Supplemented with Leaf Extract of *Morinaga oleifera*

Peroxide value and *p*-anisidine value were determined for three months, at the interval of one month, using methods of AOCS (1990). Schaal oven test was performed by keeping 20±0.1 g samples, in triplicate 50 mL beakers in an oven at 63±1°C, for 5 days. Peroxide values were analyzed in the experimental samples as prescribed in IUPAC (1987). Induction time was measured on Metrohm Rancimat 679 by taking 10±0.1 g samples in the reaction vessels and oxidizing by passing steady stream of oxygen till the straight lines were curved, which was automatically recorded by the electrodes by following the method as given in instruction manual of Metrohm Corporation, Switzerland.

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Conjugated Dienes and Trienes

Butter oil samples were dissolved in *iso*-octane and oxidation products were determined by measuring the absorbance in the ultraviolet region of the spectrum at 232 and 268 nm, respectively, for conjugated dienes and trienes according to the method of IUPAC (Paquot, 1979).

Statistical Analysis

Triplicate samples were analyzed for every treatment and data was collected as mean±SD of three samples of each treatment. Data was analyzed by using one- and two-way analysis of variance techniques to find out the effect of treatments and storage. Significance difference among the treatments ($P< 0.05$) was determined by using Duncan's Multiple Range Test (DMR-Test) as prescribed by Steel *et al*. (1997).

RESULTS AND DISCUSSION

Total Phenolic Content

Phenolic compounds are present in many higher plants, they donate hydrogen atom or electron to make stable radical intermediates and serve as good antioxidants (Cuvelier *et al*., 1992). The total phenolic content in the present study was 7.6 g 100 g^{-1} dry mass (dry leaves). Canola hull contains appreciable amount of phenolic compounds but the concentration is significantly less than total phenolic content of *Moringa oleifera* (Naczk and Shahidi, 1998). The probable reason for higher total phenolic contents was due to the higher polarity of methanol, and further methanol water system has high polarity/ dielectric constant (Saddiq *et al*., 2005). Anwar *et al*. (2006) extracted the phenolic extracts from dry leaves of *Moringa oleifera* and reported that yield of methanolic extract was 7.6%.

Effect of CSFA on Fatty Acid Composition

The fatty acids profile of butter oil is given in Table-2, which shows that the concentration of short chain fatty acids significantly decreased in milk of cows fed on calcium salts of fatty acids, which was 32% lower than the control ($P< 0.05$). Concentration of long chain fatty acids (C18:1 to C18:3) increased from 30.33 to 35.36%. The decrease in the content of short chain fatty acids and significantly higher values of long chain fatty acids was probably due to the occurrence of higher proportions of long chain fatty acids in CSFA

Table 1. Detail of feed ingredients.

Ingredients	G-2	G-1
Cotton seed cake (%)	25	50
Calcium salts of fatty acids (%)	----	10
Maize gluten (60%)	15	3
Maize grains Ground (%)	10	23
Wheat bran (%)	8	12
Rice polishing (%)	15	----
Molasses (%)	10	----
Sunflower meal (%)	15	----
Mineral mixture (%)	2	2
Total	100	100
Crude protein (%)	17.6	17.49
Metabolizable energy (Mega Calories)	2.76	2.81

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Table 2. Effect of feeding calcium salts of fatty acids on fatty acid profile of milk of Sahiwal cows.

Fatty Acid	G-2[a]	G-1[b]
n	6	6
C4:0	3.74±0.21a[c]	2.63±0.11c
C6:0	2.45±0.09a	1.76±0.18c
C8:0	1.34±0.11a	0.81±0.08c
C10:0	2.64±0.08a	1.75±0.10c
C12:0	2.68±0.06	1.36±0.22c
C14:0	9.75±0.08a	8.09±0.14c
C16:0	24.99±0.46c	31.14±0.15a
C18:0	14.22±1.34a	14.05±0.19c
C18:1	27.34±0.47c	31.99±0.33a
C18:2	2.19±0.29c	3.14±0.19a
C18:3	0.80±0.05a	0.23±0.04c

[a] Cows who were not fed on 300-g Calcium salts of fatty acids; [b] Cows fed on 300-g Calcium salts of fatty acids, [c] Means of triplicate experiment; means with same letters in same columns are statistically non-significant 0.05 level of significance.

(oleic acid 45% and linoleic acid 9.7%). Fahey *et al*. (2002) reported that addition of CSFA in feed causes substantial increase in the concentration of long chain fatty acids in the milk of Holstein-Friesian cows. In another study, determined the effect of added dietary lipids on lactating cows and reported higher contents of long chain unsaturated fatty acids in the milk. Similar results were reported by Abu-Ghazaleh *et al*. (2001) and Whitlock *et al*. (2002). Elliott *et al*. (1996) studied the impact of feeding with calcium salts of palm fatty acids on fatty acid composition in milk of Holstein Friesian cows and reported significantly higher concentration of long chain fatty acids.

Effect of MOLE on Peroxide Value at Elevated Temperature

Peroxide value of butter oil samples and the control sample in the Schaal oven test linearly increased throughout the storage period. At the end of 90 days storage period, control sample exhibited the highest peroxide value of 16.65 as compared to 5.5 (meq kg^{-1}) in T_2 (600 ppm MOLE). The stabilization of butter oil containing higher content of unsaturated fatty acids at ambient temperature up to 600-ppm level was due to the presence of higher concentration of phenolic antioxidants in MOLE which inhibited the formation of oxidation products. Schaal oven test is a good indicator of oxidation in fats and oils at elevated temperature (Ramadan and Morsel, 2004; Mahuya *et al*., 2008). Mohdaly *et al*. (2011) studied the impact of S*esaum indicum* cake extract on stabilization of sunflower and soybean oils and observed that addition of S*esamum indiucm* cake extract was quite useful in the inhibition of hydroperoxides and aldehydes. Zia *et al*. (2003) studied the effect of ginger extract on oxidative stability of sunflower oil and recorded significantly lower values of peroxides as compared to the control. Anwar *et al*. (2003) stabilized corn oil with some natural extracts and reported that addition of natural extracts significantly decreased the

Table 3. Peroxide value (meq kg^{-1}) of modified butter in Schaal oven test at 63ºC for 5-days in oven.

Treatments[a]	0-Day	30-Days	60-Days	90-Days
T_0	4.79±0.41a[b]	7.53±0.55a	11.85±1.03a	16.64±0.42a
T_1	3.52±0.55b	4.97±0.34b	6.25±0.64b	8.41±0.93b
T_2	1.85±0.32c	3.74±0.16c	4.12±0.48c	5.35±0.29c
T_3	1.76±0.19c	4.58±0.26d	4.02±0.31b	5.18±0.51c

[a] T_0: Control (without any augmentation of *Moringa oleifera* leaf extract); T_1: Augmented with 400 ppm *Moringa oleifera* leaf extract; T_2: Augmented with 600 ppm *Moringa oleifera* leaf extract , T_3: Augmented with 800 ppm *Moringa oleifera* leaf extract

[b]Means of triplicate experiment; means with same letters in same columns are not statistically different at 0.05 level of significance

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Table 4. Effect of *Moringa oleifera* leaf extract on conjugated dienes of butter oil[a].

Treatments	0-Day	30-Days	60-Days	90-Days
T_0	0.45±0.13a[b]	5.19±0.23a	13.72±1.56a	21.34±2.67a
T_1	0.45±0.13a	4.38±0.16b	10.48±0.83b	14.62±1.27b
T_2	0.45±0.13a	1.95±0.09c	7.42±0.65c	10.84±0.39c
T_3	0.45±0.13a	2.12±0.34c	7.05±0.49c	10.49±0.87c

Table 5. Effect of *Moringa oleifera* leaf extract on conjugated trienes of butter oil[a].

Treatments	0-Day	30-Days	60-Days	90-Days
T_0	0.24±0.09a[b]	0.45±0.13a	0.75±0.18a	1.10±0.22a
T_1	0.24±0.09a	0.43±0.05a	0.55±0.12b	0.78±0.14b
T_2	0.24±0.09a	0.35±0.11b	0.45±0.6c	0.57±0.12c
T_3	0.24±0.09a	0.39±010b	0.41±0.08c	0.61±0.11c

[a] Refer to Table-3 for the detail of treatments.
[b] Means of triplicate experiment; means with same letters in same columns are not statistically different at 0.05 level of significance. n= 3

rise in peroxide value of the experimental samples.

Conjugated Dienes and Trienes

The value of Conjugated dienes is a good parameter to assess the oxidative break down in fats and oils (Pritchard, 1991). Conjugated dienes in modified butter oil samples increased during the 90-day storage. The maximum value of conjugated dienes was recorded in the control, the lowest values of conjugated dienes were determined in 800 ppm MOLE added butter oil samples. It is evident that phenolic antioxidants of *Moringa oleifera* leaf extract significantly inhibited the formation of conjugated dienes. The results of conjugated trienes evaluation of fatty acids modified butter oil revealed that oxidation products in the form of conjugable trienes were significantly higher in the control sample as compared to all the treatments (Table 5). The concentration of conjugated trienes linearly increased in all the treatments and in the control throughout the storage period. Anwar *et al*. (2006) studied the stabilization of sunflower oil by methanolic and acetonic extracts of *Moringa oleifera* leaves and found that addition of 80% methanolic extract of *Moringa oleifera* leaves decreased the production of conjugated dienes and trienes. Measurement of conjugated trienes in fats and oils can serve as a good indicator of the oxidative degradation (Yoon *et al*., 1985). While studying the antioxidant activity of few plants from the *Labiatae* family for the stabilization of soybean oil, Economu *et al*. (1991) concluded that augmentation of soybean oil with these extracts significantly inhibited the formation of oxidation products. Thus, the results of the present study are in line with the findings of other researchers.

Effect of MOLE on *p*-anisidine Value*:*

p-anisidine value is a useful parameter to detect the potential of antioxidants (Liu and White, 1992). As with all other parameters discussed above, *p*-anisidine value of the control sample was higher than the respective values for the MOLE added butter oil samples. Addition of MOLE at 600 ppm was more efficient in the retardation of oxidation products i.e. aldehydes, ketones, alcohols, etc, and significantly lower *p*-anisidine values of experimental samples could be attributed to the phenolic antioxidants naturally present in leaves of *Moringa oleifera*. Lalas and Tsakins (2002) characterized the antioxidants in seeds of *Moringa oleifera* grown in Malawi and reported that seeds

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contained appreciable amount of phenolic antioxidants that could be used to prolong the shelf life of edible oils without affecting sensoric characteristics. Anwar *et al*. (2005) investigated the antioxidant activity of different extract of *Moringa oleifera* leaves and concluded that leaves contain abundant amount of antioxidants, especially the phenolic antioxidants. Anwar *et al*. (2006) studied the stabilization of sunflower oil by leaf extract of *Moringa oleifera* and reported that augmentation of sunflower oil with 600 ppm *Moringa oleifera* leaf extract significantly dropped the formation of oxidation products at primary, secondary, and tertiary stages of auto-oxidation. Similar results were reported by Mohdaly *et al*. (2011).

Induction Period

From the analysis of the results shown in Table-7, it is obvious that addition of *Moringa oleifera* leaf extract at T_2 level significantly ($P< 0.05$) increased the induction period as measured in terms of hours on Metrohm Rancimat. The shortest period of induction time was found for the control sample. The induction period of 600 and 800 ppm *Moringa oleifera* leaf extract were equal ($P> 0.05$) with each other. The reason for having appreciably higher induction time in T_2 and T_3 was due to the presence of phenolic antioxidants which resisted against the development of auto oxidations products for longer time. The evaluation of induction time is used as an assay to assess the thermal and oxidative stability of fats and oils.

CONCLUSIONS

The main objective of this research was to find out the suitability of M*oring oleifera* leaf extract for the stabilization of butter oil containing higher content of unsaturated fatty acids. The addition of *Moringa oleifera* leaf extract at 600 and 800 ppm significantly inhibited the formation of peroxides, conjugated dienes, trienes, and lowered *p*-anisidine value. The induction time of 600 ppm *Moringa oleifera* leaf extract added-butter-oil sample was 10.84 hours, even after 90 days of storage at ambient temperature. Hence, butter oil with modified fatty acids may be efficiently

Table 6. Effect of *Moringa oleifera* leaf extract on *p*-ansidine value of butter oil.

Treatments	0-Day	30-Days	60-Days	90-Days
T_0	6.75±0.19a	10.48±0.35a	16.35±0.65a	28.67±1.36a
T_1	6.75±0.19a	9.62±0.27b	13.44±0.39b	19.89±0.88b
T_2	6.75±0.19a	7.53±0.21d	9.23±0.58d	12.45±0.63c
T_3	6.75±0.19a	8.65±0.36c	11.74±0.25c	15.35±1.12d

Means of triplicate experiment; means with same letters in same columns are not statistically different at 0.05 level of significance.
n=3 (n is the no. of replicates)
Refer to Table-3 for the detail of treatments

Table 7. Effect of *Moringa oleifera* leaf extract on induction period of butter oil**.**

Treatments	0-Day	30-Days	60-Days	90-Days
T_0	7.68±0.09c	6.55±0.13c	5.10±0.17c	3.95±0.54c
T_1	8.57±0.05b	8.14±0.19b	7.05±0.25b	5.50±0.16b
T_2	12.28±0.32a	12.10±0.64a	11.66±0.05a	10.84±0.12a
T_3	12.45±0.45a	11.90±0.73a	11.72±0.31a	10.50±0.46a

Means of triplicate experiment; means with same letters in same columns are not statistically different at 0.05 level of significance.
n=3 (n is the no. of replicates)
Refer to Table-3 for the detail of treatments

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stored at ambient temperature for 90 days by augmenting with 600 ppm *Moringa oleifera*leaf extract.

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اثر عصاره برگ *Moringa oleifera* به عنوان آنتی اکسیدان روی پایدارسازی روغن کره با اسید های چرب اصلاح شده

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چکیده

در این بررسی، اثر متانولیک عصاره برگ *Moringa oleifera* روی پایدار سازی روغن کره با اسید های چرب اصلاح شده در حرارت محیط مطالعه شد. ۱۲ راس گاو ساهیوال (Sahiwal) در اولین شیردهی یا در مراحل اولیه شیردهی در یک طرح کاملا تصادفی در دو گروه مطالعه شدند. برای اصلاح اسیدهای چرب موجود در چربی شیر آنها، به گاوهای گروه اول (G-1) روزانه مقدار ۳۰۰ گرم نمک کلسیم اسیدهای چرب (به هر گاو) داده شد ولی این نمک ها به گروه دوم (G-2) خورانده نشد. غلظت اسیدهای چرب با زنجیره طولانی (C18:1 to C18:3) در شیر گروه G-1 در مقایسه با گروهG-2 از مقدار30.33±0.174b به35.36±0.14a% افزایش یافت. چربی شیر گروه G-1 تبدیل به روغن کره شد.سپس، عصاره برگ *Moringa oleifera* (MOLE) در سه غلظت مختلف شاملppm T_1=400، T_2= ۶۰۰ppm و T_3 =800ppmبا کره به دست آمده مخلوط شد. تمام این تیمارها با یک تیمار شاهد که (MOLE) به آن اضافه نشده بود مقایسه شدند. عدد پراکسید مربوط به T_2 در آزمون تنوریSchaal (بعد از۹۰ روز انبارداری) برابر 5.35±0.29b بود در حالی که این عدد برای تیمار شاهد به16.64±0.42a (meq /kg). میرسید. ۹۰ روز بعد از انبارداری، عدد مربوط به *p*-anisidine و طول عمر روغن (induction time) برای تیمارT2 و شاهد به ترتیب برابر12.45±0.63b meq /kg و 28.67±1.36a meq /kgو 10.84±0.28a ساعت و3.95±0.14b ساعت بود. از این مطالعه چنین نتیجه گرفته شد که عصاره برگ *Moringa oleifera* در غلظت ۶۰۰ppm می تواند در درجه حرارت محیط برای افزایش پایداری اکسیدی روغن کره با اسیدهای چرب اصلاح شده، به کار رود.

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