

# Evaluation of the antioxidant potential of oregano leaves (*Origanum vulgare* L.) and their effect on the oxidative stability of ghee

Akshay Kumar Singh<sup>a</sup>, Vivek Kumar<sup>a</sup>, Karunakar Singh<sup>a</sup>

Correspondence to: Akshay Kumar Singh, akshayhbt@rediffmail.com

ABSTRACT

Antioxidants were extracted from oregano leaves (*Origanum vulgare* L.) using the solvents water, ethanol, methanol, acetone and diethyl ether. Although a higher extract yield was obtained with water, in general higher total phenolic and flavonoid content and better antioxidant activity were found in the extracts prepared using ethanol. The antioxidant potential of the ethanolic extract of oregano leaves in ghee during storage and frying was evaluated in comparison with the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Three quality parameters, namely peroxide value (PV), free fatty acid (FFA) content and p-anisidine value (PAV), of ghee were analyzed. The PV, FFA content and PAV of control ghee samples increased significantly during storage and frying as compared to the ghee samples containing oregano extract (OGE), BHA or BHT. The frying period had a greater effect on changes in PV, FFA content and PAV of ghee than the storage period. Ghee samples with added 1.0% (w/w) OGE showed the least increase in PV, FFA content and PAV, which was comparable with samples with added 0.02% (w/w) BHA and BHT. The study revealed that oregano leaves could be a good natural source of antioxidants and can be used in ghee to retard oxidative deterioration during storage and frying.

## Keywords

Ghee (butter oil)  
Frying time  
Antioxidant activity  
Oregano extracts  
Proximate analysis

## Introduction

Oils and fats have a wide range of applications and are very important in the human diet. Ghee is a traditional dairy product exclusively obtained from milk, cream or butter from various animal species with a maximum moisture content of 0.3%. Ghee is prepared by melting butter at 110–140°C, which produces its characteristic flavour. India ranks first in milk production globally, producing 146.3 million tonnes annually and accounting for 18.5% of world production. About 25–30% of the milk produced in India is converted into ghee [1]. Ghee is the most widely used milk product in the Indian sub-continent and is considered the best cooking and frying medium [2]. Despite its low water content, it starts to deteriorate during storage because of oxidation which changes its colour, flavour, aroma and nutritional value. Oxidation has significant economic and health consequences due to the loss of product quality and the generation of toxic substances [3]. There is a lot of evidence indi-

cating oxidized lipids have a negative impact on health. For instance, oxidized oils and fats cause cancer, heart disease and early aging in consumers. The use of antioxidants is the most effective way to stabilize oils and fats, delay or prevent lipid oxidation, and protect oils from the damage caused by oxidized products such as free radicals.

Antioxidants are effective compounds that can delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of an oxidizing chain reaction [4]. Butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) are some of the most widely used synthetic antioxidants employed to retard oxidation and prolong the shelf-life of foods. Synthetic antioxidants have been used for many years in the food industry but are suspected of harming human health and the environment because of their possible toxic properties [5]. Consequently, consumers prefer natural antioxidants, so the food industry is looking for natural products to replace, or reduce the use of, synthetic antioxidants as food additives. The benefits resulting from the consumption of natural products rich in antioxidant compounds have prompted a renewed interest worldwide. Therefore, naturally occurring antioxidants such as ascorbic acid, vitamin

<sup>a</sup>Harcourt Butler Technological Institute, Kanpur (U.P.), India

E and certain crude plant extracts, are being considered as replacements for synthetic antioxidants.

Numerous studies have demonstrated that spices have potent antioxidant properties, mostly due to the quantity and quality of the polyphenolic compounds they contain [6]. Many studies have also reported the extraction of natural antioxidants from orange peel [7], arjuna [8], vidarikand [9], tulsi (*Ocimum sanctum* L.) leaves [10], food processing waste [11], herbs [12], and cloves [13], and their effect on the oxidative stability of ghee.

Oregano (*Origanum vulgare* L.) is a perennial plant widely used in Europe. Its leaves have been employed as a herbal remedy due to its purported analgesic, anti-inflammatory, anti-fungicidal, anti-bacterial and antioxidant properties [14]. Dried oregano contains vitamin A, potassium, iron, magnesium, calcium and phosphorus. It also showed high antioxidant activity due to the presence of large amounts of phenolic compounds, flavonoids, rosmarinic acid and carotenoids. As ghee is susceptible to oxidation and natural antioxidants are increasingly being used to reduce nutritional and organoleptic losses in oxidised ghee, the antioxidant potential of oregano added to ghee was investigated for its effect during storage and frying.

## Materials and methods

### Materials

Oregano leaves collected from the campus of Harcourt Butler Technological Institute, Kanpur were washed thoroughly under running water and dried in a tray drier (Armfield, UK) at  $60 \pm 0.5^\circ\text{C}$  with an air velocity of 1.2 m/s. The dried leaves were ground into fine powder using an MG600 grinder (Polar Bear) and passed through a 100-mesh sieve. The sieved powder was then packed in air-tight zipper bags and stored at room temperature. Fresh cream procured from the local dairy was used to make ghee by the direct cream method.

### Chemicals and reagents

Sodium sulfate, copper sulfate, sulfuric acid, sodium hydroxide, ascorbic acid, glacial acetic acid, charcoal, methanol, ethanol, diethyl ether, acetone, gallic acid, cyanidin chloride, tannic acid, sodium carbonate, aluminium chloride, potassium hydroxide, potassium iodide, sodium thio-sulphate, iso-octane, BHA, BHT and p-anisidine were of AR grade and procured from Thomas Baker Ltd (New Delhi, India).  $\beta$ -Carotene, Folin-Ciocalteu's reagent, rutin and 2,2-diphenyl-1-picrylhydrazine (DPPH) were obtained from Sigma Chemical Co. (St Louis, MO, USA).

### Proximate analysis

The prepared oregano powder was analyzed for moisture, protein, fat, ash, fibre and carbohydrate content. Moisture content was determined by drying the sample at  $105^\circ\text{C}$  in a hot air oven until a constant weight was obtained [15]. Protein content ( $N \times 6.25$ ) was determined by the Kjeldahl method [15]. Fat content was estimated using an automatic Soxhlet system (SOCS PLUS, Pelican Equipments, Chennai, India) with petroleum ether as solvent. Ash content was determined by taking 10 g of moisture-free powder and incinerating it in a muffle furnace at  $600^\circ\text{C}$  for 6 h. The residue was cooled in a desiccator and weighed. The fibre content was determined using the standard AOAC method [15]. The carbohydrate content was determined by subtracting the sum of the mean values of the other parameters (moisture, protein, fat, ash and fibre) from 100. The calorific value was determined using the formula:

$$\text{Calorific value} = (\% \text{ protein} \times 2.44) + (\% \text{ carbohydrate} \times 3.57) + (\% \text{ fat} \times 8.37)$$

### Ascorbic acid and $\beta$ -carotene

The ascorbic acid content of the sample was estimated using the 2,6-dichlorophenol indophenol titration method [16]. Briefly, 5 g of sample was macerated in 15 ml of metaphosphoric and glacial acetic acid mixture and then heated at  $40^\circ\text{C}$  for 10 min with 1 g of added activated charcoal. The contents were then filtered through Whatman No. 1 filter paper into a conical flask. Distilled water was added to the flask to make the solution up to 100 ml. A blank solution was titrated against 2,6-dichlorophenol indophenol dye in a beaker until a pink colour was obtained, and the quantity of dye used was recorded. The quantity of ascorbic acid was calculated as:

$$\text{Ascorbic acid} \left( \frac{\text{mg}}{\text{g}} \right) = V \times S \times D$$

where V is the volume of the dye used to titrate, S is the standardization value in mg ascorbic acid, and D is the dilution factor.

The  $\beta$ -carotene content was determined by mixing 100 mg of oregano powder with 10 ml of 80% acetone. The mixture was shaken for 30 min to extract the carotene and was then centrifuged at 3000 rpm for 10 min. The supernatant was recovered and its volume made up to 10 ml. Absorbance was then read at 480 nm in a UV-spectrophotometer (LAB UV 3000plus, Lab Systems & Biotech, India). The amount of  $\beta$ -carotene was calculated using the formula:

$$\beta\text{-Carotene} \left( \frac{\text{mg}}{\text{g}} \right) = \frac{(4 \times \text{OD} \times \text{Total volume of sample})}{\text{Weight of sample}}$$

### Extraction of antioxidants

Antioxidants were extracted from oregano powder using different solvents: water, ethanol, methanol, acetone and diethyl ether. Extraction was carried out at normal temperature to avoid thermal degradation of the antioxidants [17]. Oregano powder was mixed with solvent (1:10) and placed in a conical flask in an orbital shaker (Orbitek, India) and shaken at 120 rpm and 30°C overnight. The mixture was then filtered and concentrated using a rotary vacuum evaporator (DLAB, Los Angeles, CA, USA) at reduced pressure. The concentrated oregano extract (OGE) was then lyophilized to obtain dried extract.

### Extraction yield

Extraction yield was calculated using the formula:

$$\text{Yield (\%)} = \frac{\text{Amount of extract}}{\text{Weight of sample}} \times 100$$

### Total phenol component

The total phenol component (TPC) was determined using Folin-Ciocalteu's method [18]. Oregano powder extract (0.2 ml, 1 mg/ml) and Folin-Ciocalteu's reagent (1.5 ml of 10%) were dissolved in 3.75 ml of distilled water. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml of 6% Na<sub>2</sub>CO<sub>3</sub> solution. After incubation for 90 min at room temperature in the dark, the absorbance of the mixture was read in a spectrophotometer at 725 nm against a blank prepared with solvent instead of extract solution. The samples were prepared in triplicate and the mean absorbance value obtained. The same procedure was repeated for gallic acid, which was used to calibrate a standard curve. Total phenol content is reported as gallic acid equivalents expressed as milligram of gallic acid equivalent per gram of extract (mg GAE/g of extract).

### Total flavonoid content

The concentrations of flavonoids were determined using the aluminium chloride method [19]. A 2 ml sample of extract (1 mg/ml) was mixed with 2 ml of 2% methanolic aluminium chloride, vigorously shaken and incubated for 10 min at room temperature. The absorbance was then read at 430 nm using a spectrophotometer. The samples were prepared in triplicate and the mean absorbance value obtained. Rutin was used to calibrate a standard curve. The concentrations of flavonoids were expressed as milligram of rutin equivalent per gram of extract (mg of RU/g of extract).

### Condensed tannin content

Condensed tannin content (CTC) was determined using the

butanol-HCl method [20]. The reaction mixture was prepared in a tube by mixing 0.5 ml of extract (1 mg/ml) with 3 ml of the butanol-HCl reagent (butanol:HCl 95:5, v/v) and 0.1 ml of the ferric reagent (2% ferric ammonium sulfate in 2N HCl). The covered tubes were heated in a water bath at 100°C for 60 min. After cooling, absorbance was read at 550 nm using a spectrophotometer. The samples were prepared in triplicate and the mean absorbance value was recorded. Cyanidin chloride was used to calibrate a standard curve. The concentrations of condensed tannins were expressed as milligram of cyanidin chloride equivalent per gram of extract (mg of CCh/g of extract).

### DPPH radical scavenging activity

The ability of oregano extracts to donate hydrogen atoms or electrons was determined by measuring their ability to bleach purple-coloured DPPH solution. The DPPH radical scavenging activity of the oregano extracts was determined using the method reported by Yu *et al* [21] with some modifications. The reaction mixture of 3 ml of 0.3 mM DPPH and 1 ml of extract (2.5 mg/ml) in a solvent was kept in the dark for 30 min. Absorbance was then measured on a UV visible spectrophotometer at 517 nm. The decreasing amplitude of the signal at the selected wavelength confirmed high radical scavenging activity. The same solvent was used as a blank and solution measured without a sample was used as the control. Inhibition of DPPH radicals by the samples was calculated as follows:

$$\text{DPPH inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A is absorbance.

### Preparation of extract for the determination of ghee stability

A stock mixture of ethanolic extract of oregano powder with ghee was prepared by adding 1.0% (w/w) extract to 300 g of ghee. Five 100 g samples of ghee each having different ethanolic concentrations of 0.2%, 0.4%, 0.6%, 0.8% and 1.0% were formulated by diluting the stock mixture with the required quantity of ghee. Two separate samples of ghee were prepared by adding the synthetic antioxidants BHA and BHT at a concentration of 0.02% (w/w). Ghee without any added synthetic antioxidant or oregano powder extract served as control. Each sample was put in a 250 ml reagent glass bottle, placed in a hot air oven (Yorko, New Delhi, India) and maintained at 80°C for 12 days. The samples were analyzed at regular intervals of 0, 3, 6, 9 and 12 days for peroxide value (PV), free fatty acid (FFA) content and p-anisidine value (PAV).

To study the effect of the oregano extract on the frying stability of ghee, three samples of ghee with oregano extract concentrations of 0.2%, 0.6% and 1.0% (w/w) were prepared. Two samples of ghee were prepared with added synthetic antioxidants BHA and BHT at a concentration of 0.02% (w/w). Pure ghee without added antioxidant served as a control sample. Each sample of ghee (250 g) was placed in a shallow pan and heated to 180°C. Then 15 g of potato fries in a stainless-steel fine-meshed basket were immersed in the ghee. After 9 min of frying, the sample was removed and replaced with a fresh sample. This process was repeated for up to 90 min of frying. The quality of ghee was analyzed during frying at a regular interval of 0, 18, 36, 54, 72 and 90 min by measuring PV, FFA content and PAV.

### Quality analysis of ghee

#### Peroxide value

The PV indicates the extent of primary oxidation in oil or fat. The PV was determined by the standard method (Cd 8-53) of the American Oil Chemists' Society [22]. A solution of acetic acid and chloroform was prepared in a 3:2 ratio (v/v). A saturated KI solution was freshly prepared by dissolving an excess of KI in boiled distilled water. Then 0.1N sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ) was prepared for titration. A 5 g sample of ghee was weighed in a 250 ml flask, 30 ml of acetic acid chloroform solution was added and the flask was shaken. A 0.5 ml sample of the freshly prepared KI solution was then added using a pipette. The flask was shaken again for 1 min and 30 ml of distilled water was added. The solution was slowly titrated with 0.1N sodium thiosulfate solution with vigorous shaking until the yellow colour had almost disappeared. Then 0.5 ml of 1% starch solution was added and titration was continued, with vigorous shaking to release all iodine from the chloroform layer until the blue colour had just disappeared. The PV was calculated as follows:

$$\text{Peroxide value (meq } \frac{\text{peroxide}}{\text{kg}} \text{ sample)} = \frac{S \times N}{\text{Weight of sample (g)}}$$

where S is the volume in millilitres of sodium thiosulfate used in titration and N is the normality of the sodium thiosulfate solution.

#### Free fatty acids

FFA content is a relative measure of rancidity as free fatty acids are formed due to the decomposition of ghee during storage and frying. The American Oil Chemists' Society standard method (Ca 5a-40) was used to estimate FFA content [22]. A 5 g sample of ghee was placed in a 250 ml flask and 50 ml of

hot absolute alcohol and 2.0 ml of phenolphthalein solution (1% in 95% alcohol) were added. Titration was carried out with 0.1N potassium hydroxide solution until a permanent faint pink colour was seen and persisted for  $\geq 30$  s. FFA was calculated as follows:

$$\text{FFA (\%, oleic acid)} = \frac{\text{Volume of alkali (ml)} \times \text{Normality of alkali (N)} \times 28.2}{\text{Mass of fat (g)}}$$

#### p-Anisidine value

The PAV was determined according to the American Oil Chemists' Society standard method (Cd 18-90) [22]. The technique is based on the spectrophotometric determination of products formed during the reaction between aldehydic compounds in the ghee and p-anisidine. Ghee samples (0.5 g) were dissolved in 25 ml of iso-octane and absorbance was measured at 350 nm using a spectrophotometer. Then 5 ml of the mixture was mixed with 1 ml of 0.25% p-anisidine in glacial acetic acid (w/v), and after 10 min absorbance was read at 350 nm using a spectrophotometer. A 5 ml sample of iso-octane was then added to 1 ml of 0.25% p-anisidine solution and used as a blank for determining the absorbance of the fat solution after reaction with the p-anisidine reagent, while iso-octane was used as a blank for determining the absorbance of the fat solution. The PAV was calculated according to the equation:

$$\text{p-anisidine value} = \frac{1.2A_s - A_b}{\text{Weight of sample}}$$

where  $A_s$  is the absorbance of the fat solution after reaction with the p-anisidine reagent and  $A_b$  is the absorbance of the fat solution.

### Accelerated oxidation test using a Rancimat

The oxidative stability of ghee samples was measured using a Rancimat (Metrohm, Switzerland). Ghee samples (3 g) were placed in the reaction vessels and the instrument was operated at 120°C and an air flow rate of 20 l/h for the oxidation of ghee [2]. Oxidative stability was expressed as induction period (IP), which is the time required to reach an end point of oxidation.

## Result and discussion

### Proximate composition

The proximate composition of the oregano powder is shown in Table 1. The proximate composition of any plant is important for determining its nutritional significance. Oregano powder was rich in fibre (40.98%) as shown in Table 1, and so would help to regulate intestinal transit by increasing the

Parameter	Value
Moisture (%)	8.50±0.31
Fat (%)	11.23±0.19
Fibre (%)	40.98±0.71
Protein (%)	10.25±0.22
Ash (%)	19.10±0.53
Carbohydrate (%)	9.94±1.96
Calorific value (kJ)	154.47±0.83
β-Carotene (µg/100 g)	4712±18
Vitamin C (mg/100 g)	42.08±0.55

**Table 1** - Proximate composition of oregano leaf powder

bulk of faeces due to its ability to absorb water [23]. The ash content was relatively high (19.10%), which indicates that oregano powder is a better source of minerals than cereals and tubers (2–10%) [24]. The high fat content (11.23%) indicates oregano contains a good amount of essential oils which give the herb its flavour. The estimated calorific value was 154.77 kcal/100 g which is in agreement with the fact that plants have low energy values. The β-carotene content was 4.712 mg/100 g which was much less than that reported for carrot powder (23.9 mg/100 g) [25]. The vitamin C content of the oregano powder was 42.08 mg/100 g which was very close to values reported earlier [26].

### Extraction yield

Extraction is an important method for isolating phenolic compounds from plant material. Extraction efficiency is affected by several factors such as the chemical nature of compounds, the extraction method, sample particle size and the solvent used, as well as the presence of interfering substances. The extraction yield depends on the solvent polarity, solvent:sample ratio, pH, temperature, extraction time, and composition of the sample. In this study, the phenolic compounds in oregano leaf powder were extracted using various polar and non-polar solvents: water, ethanol,

methanol, acetone and diethyl ether. Extraction yield ranged from 1.72% to 7.06% (w/w), depending on the solvent used (Table 2). Extraction yield decreased in the order water>methanol>ethanol>acetone>diethyl ether, indicating that the extraction yield of methanol (6.82%) was the highest among the organic solvents used. This shows that extraction yield increases with increasing polarity of the solvent. It was also found that the yield of the water extract (7.06%) was slightly higher than that of the methanol extract (6.82%) because compounds other than phenolics might be extracted in water extract. This may be due to the higher solubility of proteins and carbohydrates in water and methanol than in ethanol, acetone and diethyl ether [27]. Therefore, methanol is considered a more appropriate extraction solvent for phenolic compounds than ethanol, acetone, diethyl ether or water [28]. Our results were in good agreement with the findings of other researchers [29, 30].

### Total phenolic content

The TPC values of the extracts ranged from 133.2 mg GAE/g for water extract to 457.5 mg GAE/g for ethanolic extract (Table 2). The TPC of ethanolic extract was significantly ( $p<0.05$ ) higher than that of the other solvent extracts, while the TPC of water extract was significantly lower than that of the other solvent extracts. Of the extracts, the ethanolic and methanolic extracts showed the highest TPC contents at 457.5 mg GAE/g and 321.5 mg GAE/g, respectively, possibly because of their higher polarity indices. These results were similar to previous oregano leaf powder findings [29, 31]. Ethanolic extract of oregano powder is rich in rosmarinic acid, quercetin, coumaric acid, caffeic acid and protocatechuic acid [32]. Water extract showed the lowest TPC content at 133.2 mg GAE/g despite its high polarity index, probably because of prolonged exposure to higher temperatures during concentration of the extract, which may have caused the degradation of phenolic compounds. The TPC contents

of diethyl ether and water oregano extracts, respectively, as reported by Albano and Miguel, were 20-fold and 25-fold lower than in our study [33], probably because their values were reported in mg GAE/ml. Weerakkody *et al* [34] reported TPC values of 49.69 mg GAE/g of dry sample in water extract and 26.16 mg GAE/g of dry sample in ethanol extract, which values are lower than found in our study.

Solvent	Yield (%)	TPC (mg GAE/g)	TFC (mg RU/g)	CTC (mg CCh/g)	DPPH radical scavenging activity (%)
Water	7.06±0.35 <sup>a</sup>	133.2±1.6 <sup>e</sup>	57.40±1.27 <sup>d</sup>	2.98±0.10 <sup>c</sup>	3.48±1.11 <sup>d</sup>
Ethanol	5.48±0.56 <sup>b</sup>	457.5±3.4 <sup>a</sup>	72.04±1.20 <sup>a</sup>	3.68±0.08 <sup>b</sup>	10.30±0.93 <sup>a</sup>
Methanol	6.82±0.48 <sup>a</sup>	321.5±2.8 <sup>b</sup>	64.08±1.11 <sup>c</sup>	3.05±0.07 <sup>c</sup>	8.10±0.92 <sup>c</sup>
Acetone	2.48±0.36 <sup>c</sup>	262.6±2.6 <sup>c</sup>	60.35±1.32 <sup>d</sup>	2.32±0.11 <sup>d</sup>	9.66±0.82 <sup>b</sup>
Diethyl ether	1.72±0.23 <sup>c</sup>	219.2±2.1 <sup>d</sup>	68.18±1.05 <sup>b</sup>	4.25±0.12 <sup>a</sup>	9.88±0.70 <sup>b</sup>

Values are means±SD. Values with different superscripts in a column differ significantly ( $p<0.05$ ) (n=3) CTC condensed tannin content, DPPH 2,2-diphenyl-1-picrylhydrazine, TFC total flavonoid content, TPC total phenol component

**Table 2** - Extraction yield, TPC, TFC, CTC and DPPH radical scavenging activity of oregano powder extract

### Total flavonoid content

Flavonoids are the most common phenolic compounds and are divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones and anthocyanins according to the degree of oxidation of the heterocyclic central third ring [35]. Quantitative analysis of total flavonoid content (TFC) revealed that the highest flavonoid concentration was in ethanolic extract (72.04 mg RU/g), followed by diethyl ether (68.18 mg RU/g), methanol (64.08 mg RU/g), acetone, (60.35 mg RU/g) and, finally, water (57.40 mg RU/g) (Table 2). The TFC of acetone and diethyl ether extracts of oregano powder in the study by Bendini *et al* were higher than reported in our study [29]. On the other hand, Barros *et al* [36] measured very high concentration of flavonoids, reporting 224.1 mg CE/g extract in methanolic extract of oregano powder. Jain and Bhagia [37] also reported a high concentration (19.53 mg QE/g) of flavonoids in aqueous extract of fresh oregano leaves.

### Condensed tannin content

Condensed tannins, also known as oligomeric proanthocyanidins, are polymeric flavonoids. These compounds give a specific flavour to natural extracts or foods prepared from tannin-rich plants [38]. Tannins inhibit lipid peroxidation and scavenge radicals such as hydroxyl, superoxide and peroxyl. The CTC of the extracts in this study was much lower than the content of other bioactive compounds (Table 2). The oregano extracts prepared with diethyl ether showed higher CTC (4.25 mg CCH/g) than with the other solvent extracts. This result agrees well with the findings of Licina *et al* [30] who reported tannin contents ranging from 1.25 to 4.02 mg CCh/g of extract, with the highest content found in diethyl ether extract of oregano leaf powder. Also, Skerget *et al* [39] reported 2.53 g/kg of proanthocyanidins in methanol extract. Babili *et al* [40] found a variable amount of CTC in oregano leaf essential oil (12.4–510.3 g CEq/kg), while the amount of extractable CTC varied between 2.32 and 4.25 mg CCH/g of extract in our study. These varying results may be due to differences in plant variety, extraction procedure, extraction time and the extraction phase used for the recovery of extract.

### DPPH free radical scavenging activity

The relatively stable DPPH radical has been widely used in the determination of the antioxidant activity of single compounds and plant extracts through the ability of compounds to act as free radical scavengers or hydrogen donors. DPPH is a stable nitrogen-centred free radical that changes colour from purple to yellow on reduction by a hydrogen-donating antioxidant. In our study, ethanolic extract of oregano leaf

powder showed the highest DPPH radical scavenging activity at 10.30%, while water extract showed the lowest at 3.48% (Table 2). Solvent type had a significant ( $p \leq 0.05$ ) influence on the radical scavenging activity of samples. Oregano samples extracted with organic solvents showed the greatest scavenging activity, while no significant difference was observed in acetone and diethyl ether extracts ( $p \leq 0.05$ ). The scavenging activity of methanolic extract of oregano powder was less than that of acetone and diethyl ether extract, even though the TPC was higher in the methanolic extract. This suggests that the physico-chemical nature of the individual phenolics in the extracts may be more important in contributing to the antioxidant activity than the TPC present in the extract. Licina *et al* [30] reported the DPPH radical scavenging activity of different extracts of *O. vulgare* L. in terms of  $IC_{50}$  values and found that ethanol extract was the most active with an  $IC_{50}$  value of 34.5  $\mu\text{g/ml}$ , while acetone extract was the least active with an  $IC_{50}$  value of 86  $\mu\text{g/ml}$ . Other researchers reported similar scavenging activity in oregano samples extracted with different solvents [31, 32]. On the other hand, Albano and Miguel [33] reported that ethyl acetate extract ( $IC_{50}$  of 1.8 mg/ml) was the most effective scavenger of DPPH free radicals followed by water extract (2.6 mg/ml) and diethyl ether extract (3.7 mg/ml). As shown by TPC, TFC, CTC and DPPH radical scavenging activity was highest in the ethanolic extract of oregano powder, as indicated in Table 2. Consequently, the ethanolic extract of oregano powder was further examined to analyze its effect on the oxidative stability of ghee during storage and frying.

### Accelerated oxidation of ghee during storage at 80°C Peroxide value

The PV is a measure of the concentration of peroxides and hydroperoxides in the initial stages of lipid oxidation. It indicates the quantity of active oxygen (mg) contained in 1 g of lipid. The changes in PV during storage are expressed in milli-equivalents of oxygen per kg of fat (meq. $O_2$ /kg). A continuous rise in PV was observed when samples were stored under accelerated oxidation condition at 80°C, probably due to the formation of hydroperoxides. However, it was observed that OGE significantly ( $p \leq 0.05$ ) inhibited peroxide development throughout the 12 days of storage at 80°C as compared to control. The PVs of ghee containing 0.8% and 1.0% ethanolic OGE were similar to those of ghee containing BHA (0.02%) and BHT (0.02%) until a peroxide value of 1.0 meq. $O_2$ /kg was reached. Thereafter, the PV of OGE was higher than that of BHT throughout storage but comparable with that of BHA until a PV of 12 meq. $O_2$ /kg was reached

(Fig. 1a). The PV of ghee samples significantly ( $p \leq 0.05$ ) decreased as the concentration of ethanolic OGE increased as compared to the control ghee sample. These results indicated that OGE acts as a natural antioxidant to retard the oxidation of ghee.

Ghee with added orange peel extract was more effective than BHA-treated ghee in retarding primary oxidation [7]. The effect of agri-industrial waste on ghee stability was evaluated by El-Shourbagy and El-Zahar [11] who found that peanut skin was most effective, followed by pomegranate peel and olive pomace, in inhibiting the formation of peroxides in ghee. Shende *et al* [13] reported that ghee containing steam-distilled clove extract was more effective in retarding peroxide development than its oleoresin counterpart. Arjuna bark ethanolic extract was found to be very effective in retarding peroxide development in ghee as compared to control [8]. When extracts of rosemary, green tea and Shatavari were incorporated into ghee, the extract of Shatavari was less effective than the green tea and rosemary extracts in retarding oxidation [2].

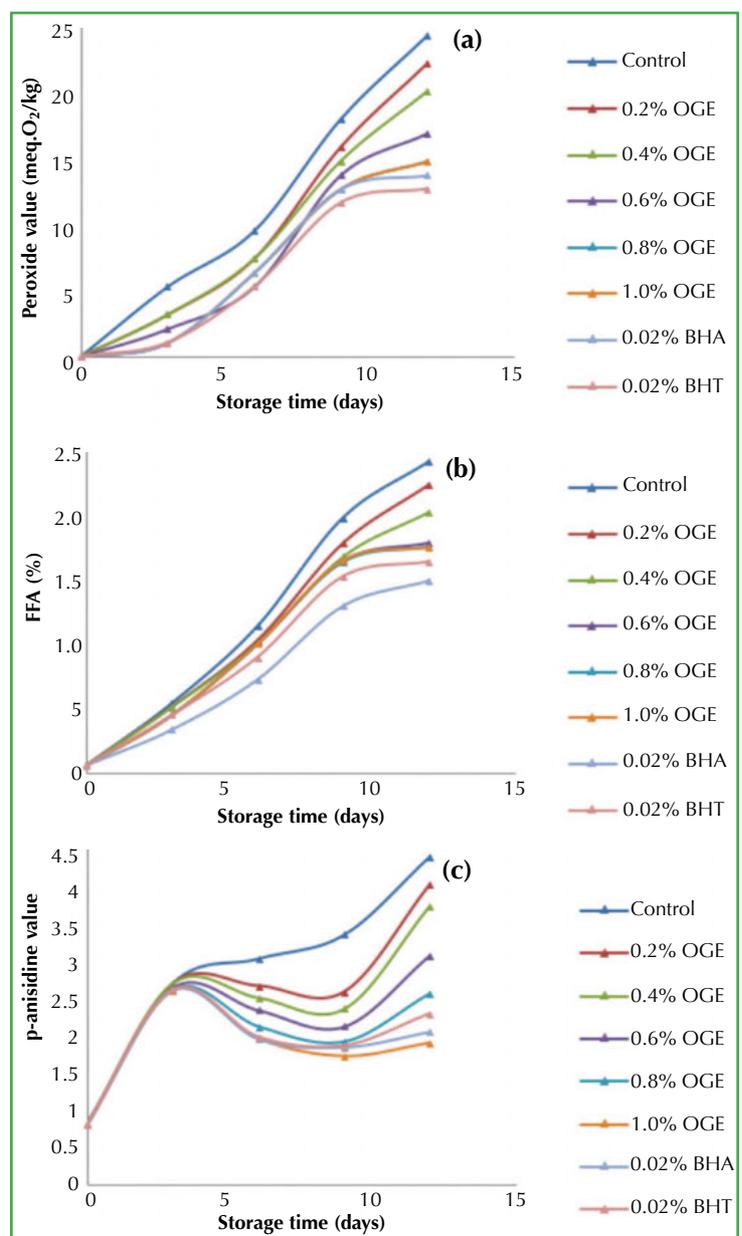
#### FFA content

Although FFA content is a measure of the extent of hydrolytic and lipolytic rancidity in ghee, it was nevertheless measured as free fatty acids contribute to the development of off-flavours and off-odours and are an important indication of the rancidity of foods. The changes in FFA content in ghee samples during storage are presented in Fig. 1b. It was observed that the FFA content increased with increased storage time for all ghee samples. The least increase in FFA was observed in ghee samples with added OGE, BHA and BHT throughout accelerated storage at 80°C, with a significantly higher value seen in the control sample. The FFA content of control ghee had increased from 0.0564% to 2.3421% oleic acid after 12 days of storage, compared to an increase from 0.0564% to 1.699% in ghee containing 1.0% OGE, from 0.0564% to 1.5892% in ghee containing 0.02% BHT, and from 0.0564% to 1.447% in ghee containing 0.02% BHA. All ghee samples containing OGE showed a higher FFA content than ghee containing BHA and BHT. A non-significant ( $p \leq 0.05$ ) difference in FFA content was observed in the samples containing 0.8% and 1.0% OGE. The inhibitory effect of BHA was better than that of BHT and OGE. Shende *et al* [13] reported a lower FFA value in ghee containing steam-distilled

clove extract than that containing oleoresin. It was also reported that the FFA content of ghee containing alcoholic extract of Arjuna was lower than that of the control sample [8].

#### *p*-Anisidine value

The PAV is used extensively to measure secondary oxidation products, mainly non-volatile carbonyls formed during lipid oxidative degradation. Hence, an increase in the PAV indicates a greater concentration of aldehydes and lower oxidative stability of the oil. The primary products of lipid (i.e., hydroperoxides) decompose to produce undesirable secondary flavours and odours. The effect of different concentrations of OGE on the PAV of ghee during storage at accelerated storage conditions of 80°C is given in Fig. 1c,



**Figure 1** - Effect of changes in (a) peroxide value, (b) free fatty acid (FFA) content, and (c) *p*-anisidine value during storage of ghee. BHA butylated hydroxyl anisole, BHT butylated hydroxyl toluene, OGE oregano extract

which shows that the PAV continuously increased with storage time. The control ghee sample had the highest PAV, which indicated a higher rate of oxidation. The PAV of the control sample reached a maximum of  $4.270 \pm 0.14$  from an initial value of  $0.783 \pm 0.13$  after 12 days of storage at an accelerated temperature of  $80^\circ\text{C}$ . As the concentration of OGE increased, there was a gradual decrease in the PAV, with 1.0% OGE having the lowest PAV. All ghee samples treated with OGE showed a higher PAV than ghee treated with BHA and BHT. The PAV of ghee treated with BHA (0.02%) and BHT (0.02%) ranged from 0.783 to 1.988 and from 0.783 to 2.223, respectively. The efficiency of natural antioxidants in retarding the oxidation of various oils and fats was highlighted by Mohdaly *et al* [41] who studied the antioxidant efficacy of potato peel and sugar beet pulp extract in protecting against the oxidation of vegetable oil.

**Oxidation of ghee during frying at  $180^\circ\text{C}$**

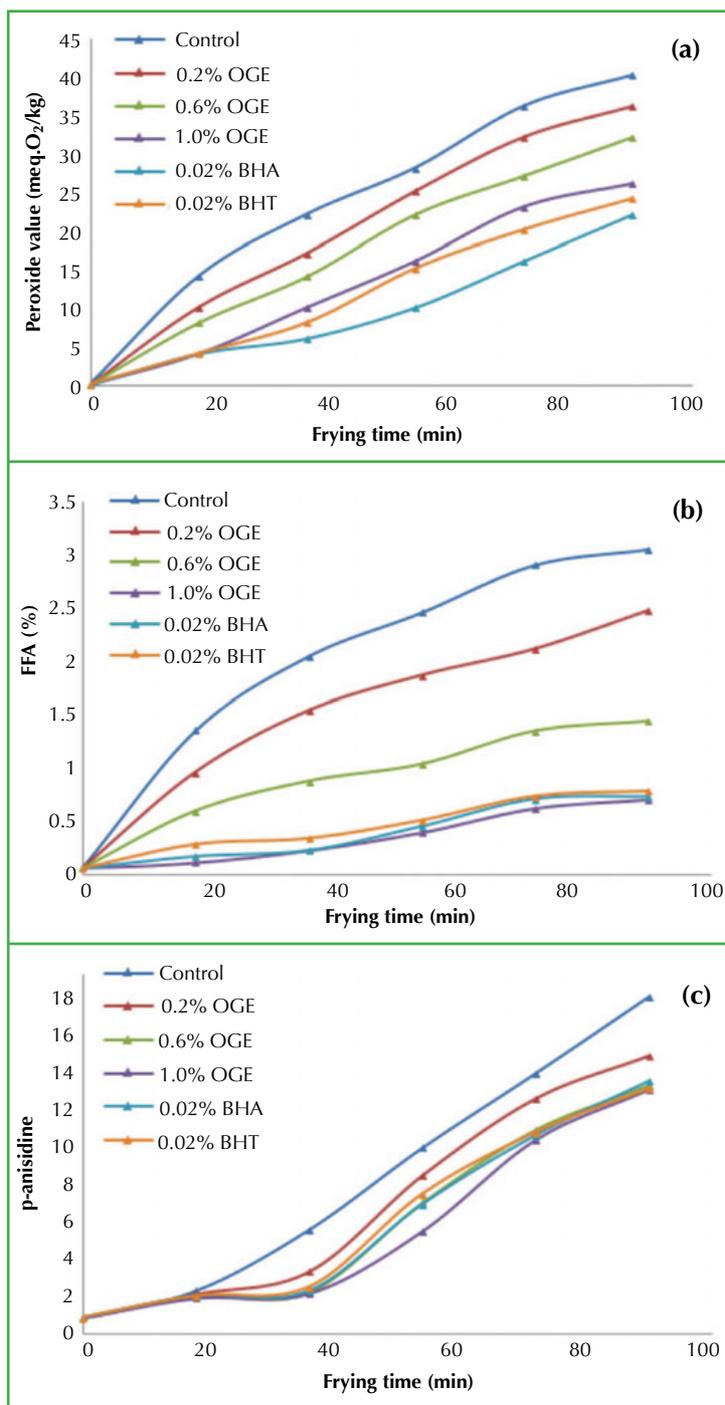
*Peroxide value*

Results indicated that the ghee samples containing OGE, BHA and BHT had a significantly lower PV than the control sample (Fig. 2a). At the beginning of frying, the PV of the ghee samples containing OGE, BHA and BHT were almost the same, whereas the control ghee had a significantly higher PV. However, after 36 min of frying, a significantly higher rate of peroxide formation was observed in samples containing 0.2% and 0.4% OGE as compared to 1.0% OGE, BHA and BHT. Almost similar antioxidant activity was observed in the samples containing 1.0% OGE and 0.02% BHT throughout the frying period of 90 min. The PV of the control sample was significantly higher ( $40 \text{ meq.O}_2/\text{kg}$  of extract) than that of the samples with 1.0% OGE ( $26 \text{ meq.O}_2/\text{kg}$ ), BHT ( $24 \text{ meq.O}_2/\text{kg}$ ) and BHA ( $22 \text{ meq.O}_2/\text{kg}$ ). BHA offered more inhibition against the formation of peroxides than BHT and OGE throughout the frying process. The effectiveness of natural antioxidants in retarding oil deterioration has been described by various researchers. It was reported that the use of rosemary and sage extract in palm olein oil was more effective in retarding palm olein deterioration than BHT or BHA [42].

*FFA content*

The fact that FFA content increased more during

frying might be due to greater hydrolysis of triglycerides in the presence of heat and moisture. FFA content increased with increasing frying time in all ghee samples (Fig. 2b). However, the ghee samples containing OGE, BHA and BHT had significantly lower amounts of FFA than the control at all sampling times, indicating control ghee was the least stable against oxidative deterioration during the entire frying process. In addition, 1.0% OGE demonstrated significantly higher protection against the development of FFA in ghee



**Figure 2** - Effect of changes in (a) peroxide value, (b) free fatty acid (FFA) content and (c) p-anisidine value during frying of ghee. *BHA* butylated hydroxyl anisole, *BHT* butylated hydroxyl toluene, *OGE* oregano extract

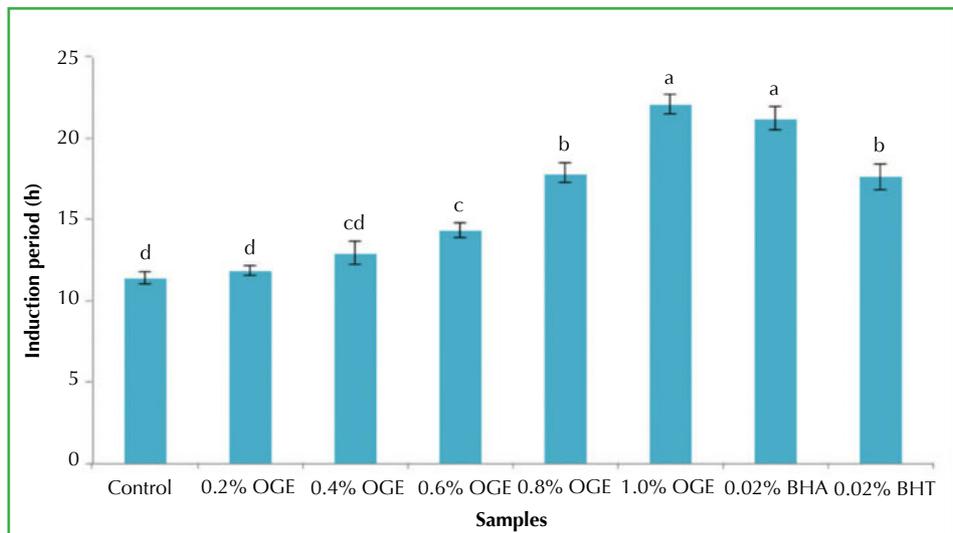
than both synthetic antioxidants. The antioxidant potential of rosemary oleoresin and sage extract was comparable with the synthetic antioxidants, BHA and BHT in lowering the % FFA of palm olein during the deep fat frying of potato chips [43].

*p*-Anisidine value

The PAV of ghee containing OGE, BHA and BHT was significantly lower than that of the control sample as shown in Fig. 2c. The PAV of the control sample rapidly increased during 20 min of frying, probably due to the formation of secondary components such as aldehydes and ketones. The ghee sample containing 1.0% OGE maintained a lower PAV than the ghee with added BHA or BHT. The sample with 0.6% OGE had a PAV value similar to that of the samples with BHA or BHT. The lower antioxidant activity of BHA might be due to the destruction of antioxidant compounds during frying. The decrease in the antioxidant potential of BHA at high temperatures has been reported in many studies [13, 44]. It was also shown that BHA volatilized during frying and was ineffective in enhancing the oxidative stability of frying oil [45].

**Evaluation of ghee stability using a Rancimat**

The effect of OGE and the synthetic antioxidants BHA and BHT on the oxidative stability of ghee was evaluated using a Rancimat. As shown in Fig. 3, a significant ( $p \leq 0.05$ ) increase in IP was observed with increased OGE concentration in ghee. The ghee with 1.0% OGE showed the highest antioxidant potential (IP  $21.1 \pm 0.57$  h) among the ghee samples with different concentrations of OGE, BHA and BHT. The antioxidant effect of 1.0% OGE and BHA was comparable with no significant difference observed. Ghee with added BHT showed a significantly lower antioxidant effect than ghee with 1.0% OGE and BHA but a higher antioxidant effect than other OGE concentrations and control. Pawar *et al* re-



**Figure 3** - Induction period of different ghee samples. The mean changes between the samples were analyzed using Duncan's test. Samples with different letters are significantly different ( $p < 0.05$ ) from each other. BHA butylated hydroxyl anisole, BHT butylated hydroxyl toluene, OGE oregano extract

ported that ghee with added BHA (IP  $20.14 \pm 0.28$  h) showed significantly higher ( $p < 0.05$ ) antioxidant effectiveness than ghee with added extracts of other herbs [2].

Table 3 shows the IP of control ghee, and ghee with added OGE, BHA and BHT during different periods of frying. The results suggest that both 1.0% OGE and BHA can protect ghee from oxidative deterioration. Before frying, all samples displayed a higher IP which decreased significantly during frying at  $180^\circ\text{C}$ . Ghee containing 1.0% OGE showed the highest IP of all samples throughout frying, indicating 1.0% OGE was the most effective antioxidant for stabilizing ghee. The antioxidant effectiveness of 1.0% OGE was very similar to that of 0.02% BHA, a synthetic antioxidant. Ghee with added 0.02% BHA had a lower IP during frying than ghee with added 0.5% steam-distilled clove extract [13]. A study of the effect of steam-distilled extract of coriander leaves on the oxidative stability of ghee during frying showed it had a higher IP compared to samples containing BHA and oleoresin [44].

Ghee sample	Frying time (min)					
	0	18	36	54	72	90
Control	$11.4 \pm 0.36^d$	$5.68 \pm 0.41^e$	$2.72 \pm 0.53^e$	$1.05 \pm 0.31^e$	$0.61 \pm 0.27^e$	$0.16 \pm 0.07^e$
0.2% OGE	$11.8 \pm 0.29^d$	$7.35 \pm 0.38^d$	$5.56 \pm 0.32^d$	$4.22 \pm 0.12^d$	$1.69 \pm 0.32^d$	$0.94 \pm 0.10^d$
0.6% OGE	$14.3 \pm 0.44^c$	$11.61 \pm 0.52^c$	$8.82 \pm 0.15^c$	$5.49 \pm 0.19^c$	$3.14 \pm 0.16^c$	$1.88 \pm 0.13^c$
1.0% OGE	$22.1 \pm 0.57^a$	$18.70 \pm 0.45^a$	$16.23 \pm 0.18^a$	$12.96 \pm 0.32^a$	$7.81 \pm 0.22^a$	$4.91 \pm 0.29^a$
0.02% BHA	$21.2 \pm 0.69^a$	$19.07 \pm 0.62^a$	$16.46 \pm 0.20^a$	$12.83 \pm 0.22^a$	$6.54 \pm 0.21^b$	$4.05 \pm 0.37^b$
0.02% BHT	$17.6 \pm 0.78^b$	$14.33 \pm 0.42^b$	$10.29 \pm 0.11^b$	$6.19 \pm 0.14^b$	$3.27 \pm 0.10^c$	$2.08 \pm 0.41^c$

Values are means  $\pm$  SD. Values with different superscripts in a column differ significantly ( $p < 0.05$ ) ( $n = 3$ )  
*BHA* butylated hydroxyl anisole, *BHT* butylated hydroxyl toluene, *OGE* oregano extract

**Table 3** - Oxidative stability of ghee samples during frying expressed as induction period in hours

## Conclusions

The results indicate that OGE can protect ghee against oxidative degradation during accelerated storage and frying. Among the different solvent extracts, ethanol extract was demonstrated to be rich in TPC, TFC and DPPH free radical scavenging activity. The addition of 1.0% OGE significantly inhibited the formation of peroxides and FFA and lowered the PAV during accelerated storage and frying. The 1.0% OGE showed higher antioxidant activity than the synthetic antioxidants BHA and BHT under accelerated storage conditions. Rancimat analysis revealed that there was no significance difference in the IP values of ghee samples with added 1.0% OGE or 0.02% BHA until 18 min of frying, but thereafter the IP value of BHA decreased throughout the remaining frying time. Therefore, it is suggested that oregano leaves (*O. vulgare* L.) could be used as a natural food antioxidant.

## Conflict of Interest

The authors declare that they have no conflict of interest.

## Human and Animal Rights

The article does not report any studies with human or animal subjects performed by any of the authors.

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