Extraction and Identification of Natural Antioxidants from Liquorices (*Glycyrrhiza glabra*) and Carob (*Ceratonia siliqua*) and its Application in El-Mewled El-Nabawy Sweets (Sesames and Folia)

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Abstract: The objective of this study was to determine, identify and to investigate the effects of natural antioxidants of licorice and carob. Besides, their effects as powder and antioxidant extracts addition on refined sunflower oil stability as natural antioxidants were evaluated. Total polyphenol contents as total phenols, total carotenoids and total tannins were 353.93mg/100g (gallic acid), 10.62mg/100g (carotenoids) and 83.33mg/100g (tannic acid), respectively in licorice, while in carob, it was 186.07, 18.66 and 106.67, respectively. Polyphenol compounds of the studied licorice and carob extracts were determined and identified by HPLC. The stability of refined sunflower oil (which determined by peroxide value and Rancimat) was increased with increasing the level of polyphenols extracts addition. Also, our study shows the effect of addition of these polyphenols extracts to El-mewled El-nabawy sweets fortified by full cream milk powder (sesames and folia). We found that, licorice and carob as powder and polyphenols extracts were delayed the rancidity of sesame and peanut significantly. That encourages using licorice and carob as powder and polyphenols extracts as a good natural antioxidants source instead of synthetic antioxidants.
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1. Introduction

Plants produce a variety of phenolic antioxidants. Among the various phenolic compounds, the flavonoids are perhaps the most important group (Kuo *et al*., 1992and Belinky *et al*., 1998). Flavonoids are components of a wide variety of edible plants, fruits, vegetables and grains, and are an integral part of the human diet (Berge and Daniel, 1988 and Belinky *et al*., 1998). Spices and herbs are added to food, not only for flavor, but also for preservation. Research has shown that constituents of these aromatic plants can function as natural antioxidants and there by prevent/ retard rancidity of food lipids, improve sensory scores and offer greater consumer acceptance of food products (Nakatani, 1997).

Although, certain culinary spices and herbs, or their fractions, possess marked antioxidant capacities, their practical application to foods may be restricted, due to a pungent and/ or characteristic flavor imparted by the plant species. A number of synthetic antioxidants, such as 2- and 3- tert-butyl-4-methoxy phenol (i.e. butylated hydroxyl anisole, BHA), 2, 6-di-tert- butyl-4-methyl phenol (i.e. butylated hydroxyl toluene, BHT) and tert- butyl hydroquinone (TBHQ) have been added to food stuffs but, because of toxicity issues, their use is being questioned (Valentao *et al.,* 2002). Attention has therefore been directed toward the development/ isolation of natural antioxidants from botanical sources, especially edible plants. The use of natural antioxidants in food is limited, however, on account of the lack of knowledge concerning their molecular composition, the content of active compounds in the raw materials and the availability of relevant toxicological data. Unlike synthetic antioxidants, which are phenolic compounds with varying degrees of alkyl substitution, natural antioxidants can be phenolic compounds (Flavonoids, phenolic acids and tannis), nitrogen- containing compounds (alkaloids, chlorophyll derivatives, amino acids, peptides and amines), carotenoids, tocopherols or ascorbic acid and its derivatives (Velioglu *et al.,* 1998). Crude extracts of species, herbs and other plant materials rich in polyphenolics are increasingly of interest to the food industry because they have the capacity to retard oxidative degradation of lipids and there by improve the quality and nutritional value of food. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary disease and cancer is also raising interest among scientists, food manufactures and consumers since the future trend is toward functional foods with specific health effects (Amarowicz *et al*., 2004).

Carob is the beanlike fruit of *ceratonia siliqua*, which grows widely in the Mediterranean region and belongs to the genus *leguminosae*. Carob is used in many Arab countries to make a popular drink which is consumed mainly in the month of Ramadan. Carob

is also used in preparation of special traditional types of Arabic confectionery (Yousf and Alghzawi, 2000).

Often the physiological effects of diets or foods rich in dietary fiber cannot be liked to a single component because of the presence of additional health-promoting constituents such as phytochemicals, among which the polyphenols can exhibit antioxidative (Rice-Evans *et al.,* 1995), antimutagenic (Yamagishi *et al.,* 2000), anticarcinogenic (Mukthar *et al.,* 1992), antiproliferative (Manthey and Guthrie, 2002), or antioestrogenic activity (Messina and Barnes, 1991). Polyphenols are a very heterogeneous group and include simple phenolic acids, cinnamic acid and its derivatives, flavonoids, isoflavoves, lignans, anthocyans and tannins. Therefore, when included in the daily diet, these compounds may play an important role in the prevention and reduction of cancer and heart disease.

Licorice is one of the oldest and most popular herbal medicines in the world are reduced in the pharmacopoeias of many countries. Licorice is derived from the dried roots and rhizomes of *Glycyrrhiza* species (*Leguminosae* Family). The *Glycyrrhiza* genus contains about 30 species, and is widely distributed all over the world. It shows a variety of pharmacological activities, including antiulceric, antioxidative, antiallergic and antiviral... etc (Baltina, 2003).

More than 300 flavonoids have been isolated from Glycyrrhizin species. These flavonoids belong to various types, including flavanones or flavanols, chalcones, isoflavans, isoflavenes, flavones or flavonols, isoflavones and isoflavanones (Qingying Zhang and Min Ya, 2009).

The purpose of this study is to: (1) evaluate two plant species indigenous for their radical-scavenging properties and antioxidant activity, by use of a number of classical assays and compare by some synthetic antioxidants and another natural antioxidant; (2) identify and quantify major phenolic and flavonoids compounds present in the tested species by using HPLC technique (3) assess whether these herbs could be sources of natural antioxidants for food applications.

2. Material and Methods

Materials:

1-Carob (*Ceratonia siliqua*), licorice (*Glycyrrhiza glabra*), sesame (*Sesamus indicum*), peanut (*Arachis hypogeae, L.*), full cream milk powder (NIDO) and sucrose were purchased from a local market at Cairo, Egypt.

2- All chemicals reagents were purchased from El-Gomhoria Co. at Cairo, Egypt.

Methods:

1- Preparation of extracts and its yield of tested spices:

Twenty five grams sample was put into flask a fine powder in a mill and was mixed with 500 ml ethanol. The obtained extracts were filtered over whatman No.1 paper. The residue was re-extracted until extraction solvents became colorless. The filtrate was collected, and then ethanol was removed by a rotary evaporator at 50 °C to obtain dry extract. The dry extracts were placed in a plastic bottle, and then stored at refrigerator until used (Gulein *et al.,* 2003).

2- Determination **of total phenolic compounds**

Total soluble phenolics in carob and licorice extracts were determined with Folin-Ciocalteu reagent according to the method of (Slinkard and Singleton, 1977) using gallic acid as standard phenolic compound. Briefly, 1.0 ml of extract solution containing 1.0g extracts in a volumetric flask was diluted with distilled water (46 ml). One mil Liter of Folin-Ciocalteau reagent was added and the content of the flask mixed thoroughly. Three minutes later, 3 ml of Na_2CO_3 (2%) was added, and the mixture was allowed to stand for 2 hrs with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the samples extracts were determined as microgram of gallic acid equivalent using an equation obtained from the standard gallic acid graph: Absorbance=0.0028 x gallic acid (mg).

3-Determination of Total Carotenoids

The total carotenoids were determined according to Heinonen and Marina (1989).

4- Determination of tannins (as tannic acid)

Total tannins were determined calorimetrically as described by A.O.A.C (2000). Sample (5 gm) was mixed with 50 ml methanol in a closed conical flask and left for 20 hrs at room temperature (25 ± 2°C). The mixture was centrifuged for 30 min. at 3000 rpm. The tannins in the supernatant were measured at 760 nm using Jen way 6505 UV/VIS spectrophometer. Tannic acid was used to prepare the standard curve.

5- Separation of polyphenols and flavonoids by HPLC

High performance liquid chromatography (HPLC) technique using HPLC Agilent 1100 Series equipped with Quaternery pump, set at flow 1 ml/ min. Autosampler, degaser, column compartment set at 35 ^{0}C and variable wavelength detector set at 330 for flavonoid compounds and 280 for phenolic

compounds, column: Hypersil ODS 5 um, 250 x 4 mm was used. Pure phenolic compounds: P. coumaric, pyrogallol, catechein, salycilic, coumarin, protocatchoi, chlorogenic, vanillic, cinnamic, syrinic, caffeic, chrisin, ferullic, caffeine, p. OH. Penzoic and naringnin and pure flavonoid compounds: kampferol, Apegnin, luteolin, Quecettin and Hypersoid were used as standard obtained from El-Gomhoria-chemical Company, Egypt.

6- Antioxidant efficiency determination

a- Determination of antioxygenic activity using the peroxide value:-

Samples (100g) of refined sunflower oil, both with and without 0.25g, each of carob and licorice as well as 200 ppm of Rutin and 200 ppm of butylated hydroxyl anisole (BHA). All treatments were placed in an oven at 60 °C for 3 hours daily. The experiment was repeated for 7 days. Peroxide value was determined for each sample according to the A.O.A.C. (2000) method. Antioxygenic activity was calculated as the ratio between the peroxide value of control and the peroxide value of sample (Gerard and Roberts, 2004).

$$\% \text{ anti oxygenic activity} = \frac{\text{peroxide value of control}}{\text{peroxide value of sample}}$$

b- Oxidation systems by Rancimate

Different concentrations of licorice and carob ethanolic extract (1.5, 3 and 4.5 ml/ 25 ml refined sunflower oil), Rutin and BHA (200 ppm) were individually added to refined sunflower oil (25g) to study their antioxidant efficiency. The designation of induction period by rancimate instrument was taken as a tool to compare the effectiveness of herbs extract fractions on refined sunflower oil stability according to the method of Mendez *et al.* (1997). 679 Rancimate (Metrobm Ltd. CH.9100 Herisau, Switzerland) was used for the determination of the oxidative stability of refined sunflower oil mixed with herbs extract. The 679 Rancimate comprises of control unit and wet section containing 6 reactions vessels.

The induction period was used as a mean for measuring the antioxidant activity of the various spices extracts added individually to refined sunflower oil and was compared it with the antioxidants used. The control sample was refined sunflower oil without any antioxidants.

Preparation of El-mewled El-nabawy sweets (sesames and folia):-

1- **Roasting of sesames and peanut**:

Sesames and peanut were roasted at 160 °C for 20 – 30 min. (Refaat, 1988) and the peanut hulls were removed.

2- **Processing procedure of oriental sweets:**

Sugar and water were heated with stirring until light caramel is obtained and the consistency of the syrup became thick, then, other ingredients were added and thoroughly mixed with the hot syrup. The mass was left to cool partially, then re-stirred and poured on a marble surface (recoated lightly with oil) and left for cooling to ambient temperature. The mass was extended with a rolling pin to 1.5 cm thickness and cut with a knife into bars which were packed in tightly closed cellophane pouches and were stored. The ingredients were given in Table (1).

Table (1) Formula of El-mewled El-nabawy sweets (sesames and folia) samples

Ingredients	Weight (gm)
Sesame or peanut	800
Dry milk powder	200
Sucrose	500
Water	100 ml
Antioxidant addition:- *As extracts *As powder of carob and licorice	5 and 7.5 ml 3 and 6 gm/100 gm

3. Results

Antioxidant contents:

Total phenols, carotenoids and tannins were determined and results are presented in Table (2). The table shows that total phenolic compounds content of licorice and carob were 353.93 and 186.07 mg/100g, respectively. While, carotenoids contents were 10.62 and 18.66 (mg/100g), respectively. Results in the same Table revealed that tannins content in the same samples represented 83.33 and 106.67 (mg/100g), respectively. These results are in agreement with Ibanoglu and Ibanoglu (2000). On the other side, Yousif and Alghzawi (2000) measured tannins as ratio percentage and they found that, the carob powder contained 3.15% tannins. While, Hanan Al-sayed (2008) found that, carob was contained 11.19 mg tannins as gallic acid/ mg dry extract.

Table (2): Total phenols, carotenoids and tannins (mg/100g on dry weight basis) content of licorice and carob

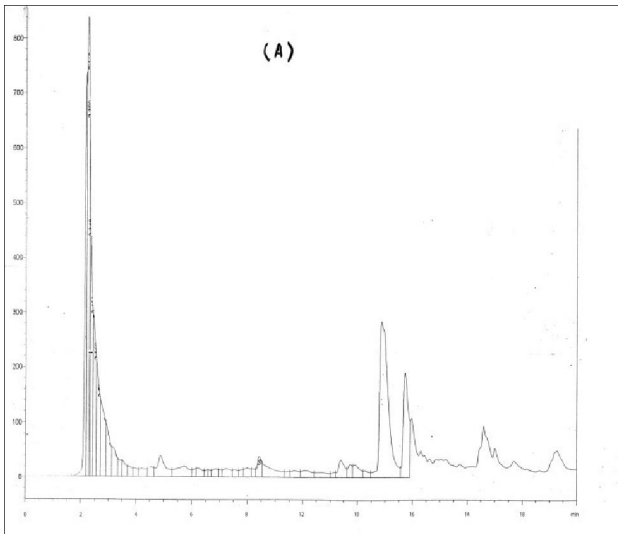
Constituents (mg/100g)	Licorice	Carob
Total phenols (as mg gallic acid)	353.93	186.07
Carotenoids	10.62	18.66
Tannins (as mg tannic acid)	83.33	106.67

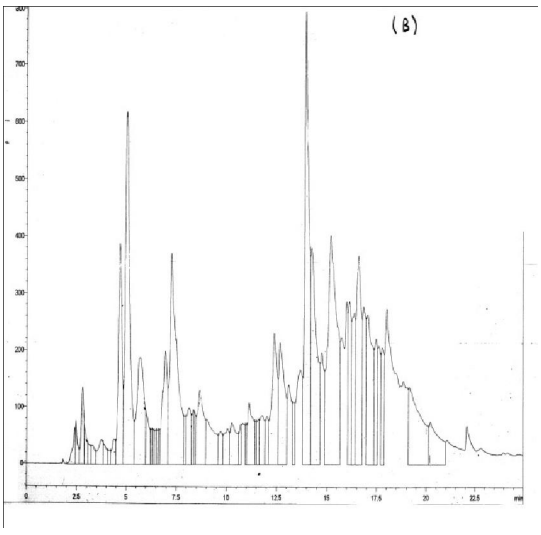
Fractionation of natural phenolic and flavonoid compounds from extracted licorice and carob

Data illustrated in Fig. (1) and presented in Table (3) show the HPLC- chromatograms of the two ethanolic extracts of licorice and carob and representing the identified components monitored at 280 nm for phenolic compounds and at 330 nm for flavonoid compounds. Table (3), shows the area ratios and the concentrations by ppm/g of the identified phenolic and flavonoid compounds. As seen the response intensity as well as the total area under chromatogram for the licorice extract was higher than those of carob extract. Some components such as pyrogallol, chlorogenic, caffeine and ferullic were absent in carob extract but there were present in licorice extract. On the other hand, some components such as catechein, caffeic, syrinic, cinnamic, hypersoid, luteolin and kampferol were absent in licorice extract but there were found in carob extract. These results are in agreement with those of Duke *et al.* (2002), Owen *et al.* (2003) and Heba (2009).

Table (3): Polyphenols and flavonoids contents of ethanolic extracts of licorice and carob

compound	licorice			carob		
	R_t	Area (%)	ppm/g	R_t	Area (%)	ppm/g
Protocatchoi	2.451	5.635	39.46	2.467	0.403	0.53
Pyrogallol	nd	nd	nd	2.437	0.439	1.38
Catechein	2.563	4.317	10.82	nd	nd	nd
Chlorogenic	nd	nd	nd	2.782	0.962	0.22
Caffeic	3.334	0.831	0.37	nd	nd	nd
Syrinic	3.950	0.547	0.28	nd	nd	nd
Caffeine	nd	nd	nd	4.391	0.414	58.94
Ferullic	nd	nd	nd	6.316	0.285	0.015
Salycilic	6.552	0.273	0.067	6.547	0.245	0.124
Cinnamic	8.419	1.146	0.26	nd	nd	nd
Chrisin	11.718	0.723	0.35	11.773	1.069	0.096
Hypersoid	4.900	0.238	0.107	nd	nd	nd
Quecethin	7.412	0.745	0.402	7.378	2.816	24.58
Luteolin	7.727	1.1791	3.81	nd	nd	nd
Kampferol	8.727	0.378	0.195	nd	nd	nd
Apegnin	8.880	1.496	1.19	8.858	0.833	10.67
Total area of phenolic compounds	3.48664×10^4			1.30471×10^5		
Total area of flavonoid compounds	7217.45094			1.16391×10^5		





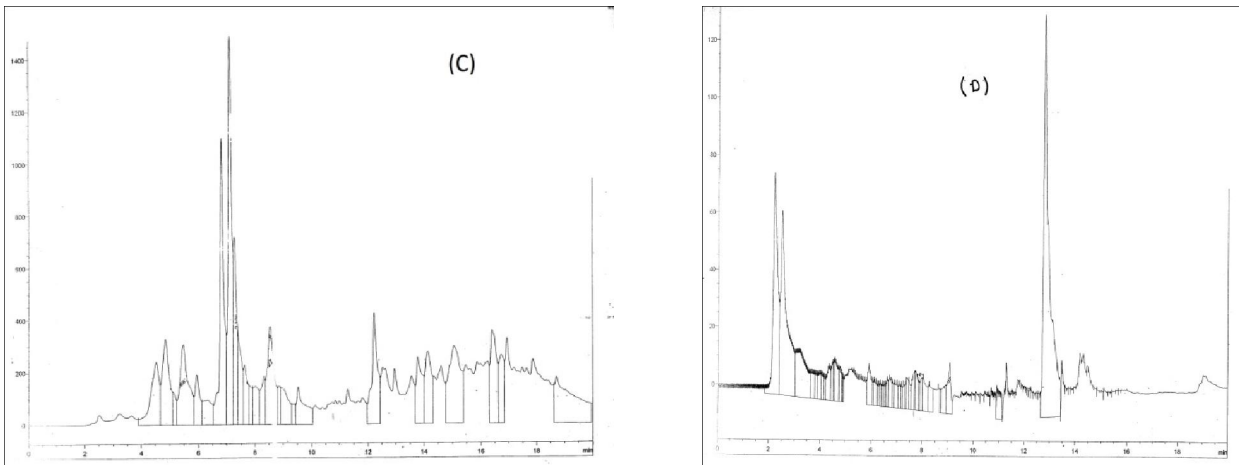


Fig. (1): Polyphenols and flavonoids contents of ethanolic extracts of licorice and carob
(a) **Phenol compounds of carob, (b) Phenol compounds of licorice, (c) Flavonoids of licorice and (d) Flavonoids of carob.**

Antioxygenic activity of licorice and carob powders, Rutin and BHA in refined sunflower oil:

Proper selection of antioxidants and through dispersion into the fat or oil portion of the product can ensure adequate protection against oxidation (Byrd, 2001).

Measuring changes in peroxide value of treated or untreated oils is considered a suitable potent method to characterize oxidative changes in refined sunflower oil exposed to 60° C in an oven for 3 hrs daily for consecutively 7 days. Table (4) shows the effects of licorice and carob powders (2.5 g/ kg oil), as well as Rutin (200 ppm), on peroxide formation in refined sunflower oil in comparison to BHA (200 ppm). The tabulated data revealed that, the development of rancidity was more pronounced in control sample reaching 29.43 meq/ kg oil after 7 days. The antioxygenic activity of herbs powder was slightly higher. It was interesting to observe that licorice powder showed strong antioxidant activity in refined sunflower oil than carob powder.

Table (4): Peroxide value and antioxygenic activities of licorice and carob powders, Rutin and BHA in refined sunflower oil

Stored period (days)	Peroxide value of control	Licorice powder		Carob powder		Rutin		BHA	
		Peroxide value	Antioxygenic activity	Peroxide value	Antioxygenic activity	Peroxide value	Antioxygenic activity	Peroxide value	Antioxygenic activity
0	0.66	0.66	-----	0.66	-----	0.66	-----	0.66	-----
1	5.25	1.19	4.41	1.16	4.53	1.03	5.10	1.02	5.15
2	5.95	2.82	2.11	2.61	2.28	2.89	2.06	2.34	2.54
3	8.08	4.47	1.81	5.18	1.56	4.73	1.71	5.78	1.40
4	10.08	5.21	1.93	6.81	1.48	7.91	1.27	6.52	1.55
5	15.39	8.13	1.89	9.50	1.62	8.61	1.79	7.85	1.96
6	27.42	10.72	2.56	12.56	2.18	17.75	1.54	9.46	2.90
7	29.43	24.81	1.18	25.52	1.15	25.81	1.14	19.99	1.47

*Antioxygenic activity values > 1 indicate Antioxygenic activity and <1 indicate pro-oxygenic activity.
*Antioxygenic activity = peroxide value of control / peroxide value of sample

Effect of total polyphenols addition on refined sunflower oil

The total polyphenols was extracted from Licorice and carob powders and Rutin as well as BHA were added to refined sunflower oil. Stability of refined sun flower oil was measured by Rancimate method. Polyphenols extracts were added at levels of 1.5, 3 and 4.5 ml from concentrate ethanolic extracts. Data of oxidative stability were tabulated in Table (5), it is clear that the addition of licorice and carob extracts increased the stability of refined sunflower oil at all used concentrations up to induction 9.43 hrs in refined sunflower oil without any additions compared with that induction for 10.2 hrs in refined sunflower oil with 200 ppm butylated hydroxyl anisole (BHA) to 9.63 hrs in the same oil with 200

ppm Rutin. The results revealed that the addition of carob antioxidants extracts increase the stability of refined sunflower oil compared to licorice antioxidants extracts.

Moreover, increasing the concentration of polyphenol compounds extracts from both spices remarkable exhibited the antioxidant activity of refined sunflower oil. Therefore, it could be used licorice and carob extracts instead of using synthetic antioxidants.

Table (5): Effect of adding different concentrations of licorice and carob extracts, Rutin (200 ppm) and BHA (200 ppm) on the oxidative stability of refined sunflower oil

Items	Oxidative stability (Induction periods = hrs)
Refined sunflower oil (control)	9.43
Refined sunflower oil +200 ppm BHA	10.2
Refined sunflower oil +200 ppm Rutin	9.63
Refined sunflower oil (25 ml) + 1.5 ml licorice extract	8.20
Refined sunflower oil (25 ml) + 3 ml licorice extract	9.43
Refined sunflower oil (25 ml) +4.5 ml licorice extract	12.7
Refined sunflower oil (25 ml) +1.5 ml carob extract	8.83
Refined sunflower oil (25 ml) +3 ml carob extract	10.10
Refined sunflower oil (25 ml) +4.5 ml carob extract	10.20

Effect of licorice and carob (as powder and antioxidant extracts) addition on El-mewled El-nabawy sweets (sesames and folia):-

Data presented in Tables (6 &7) show the effect of addition of licorice and carob as powders and ethanolic extracts to sesames (Table 6) and folia (Table 7). Licorice as powders and ethanolic extract gave higher effect on preserving the sweets from rancidity during storage than carob as powders and ethanolic extract.

Table (6): Effect of Effect of licorice and carob (as powder and antioxidant extracts) addition on sesames sweets

Samples	Peroxide values (days)						
	0	1	2	3	4	5	6
Sweet without any antioxidants (control)	2.01	2.21	3.68	4.54	6.83	6.92	9.74
Sweet +3g. carob powder	1.91	2.14	3.28	3.55	4.65	5.48	7.07
Sweet + 6g. carob powder	0.97	1.68	3.19	3.46	5.48	5.58	8.22
Sweet + 5ml.con.carob extract	1.61	1.78	2.06	2.70	3.87	4.01	9.38
Sweet + 7.5ml. con. Carob extract	1.38	1.42	1.62	2.00	4.21	4.56	7.23
Sweet +3g. licorice powder	1.22	1.66	1.66	2.88	4.27	5.57	8.31
Sweet + 6g. licorice powder	0.76	1.17	1.35	2.69	5.20	5.16	7.29
Sweet + 5ml.con. licorice extract	1.51	1.70	1.73	2.75	4.13	4.32	8.73
Sweet + 7.5ml. con. licorice extract	1.01	1.42	1.55	2.03	3.48	3.56	6.36

Table (7): Effect of Effect of licorice and carob (as powder and antioxidant extracts) addition on Folia sweets

Samples	Peroxide values (days)						
	0	1	2	3	4	5	6
Sweet without any antioxidants (control)	2.85	4.68	5.76	5.92	7.01	7.14	9.90
Sweet +3g. carob powder	2.58	2.83	3.08	4.03	4.13	5.29	7.53
Sweet + 6g. carob powder	2.27	2.29	3.04	3.27	3.96	4.70	7.19
Sweet + 5ml.con.carob extract	1.63	2.42	2.46	2.54	2.57	3.56	6.91
Sweet + 7.5ml. con. Carob extract	1.61	1.68	2.22	2.26	2.46	3.40	6.60
Sweet +3g. licorice powder	2.03	2.17	2.80	3.02	3.18	3.98	6.98
Sweet + 6g. licorice powder	1.68	2.09	2.12	2.91	3.11	3.67	6.94
Sweet + 5ml.con. licorice extract	1.33	2.20	2.41	2.54	2.68	3.16	6.40
Sweet + 7.5ml. con. licorice extract	1.28	2.17	2.23	2.26	2.37	3.01	5.12

Discussion

Licorice and carob were found to contain a rich

variety of phenolic compounds. A comparison with other studies on the content of phenolic compounds in carob is not because none exist (Owen *et al*., 2003). It is obvious that the total phenolic content measured by the Folin-Ciocalteu procedure does not give full picture of the quality or quantity of the phenolic constituents in the extracts (Katsube *et al*., 2004; Wu *et al*., 2004 and Wajdylo *et al*., 2007).

Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Selected phenolics in two herbs separated and identified by the HPLC method. Considerable variation was found in phenolic compounds of two herbs, because of the diversity and complexity of the natural mixtures of phenolic compounds. It is rather difficult to characterize very compound and elucidate its structure, but it is not difficult to identify major groups and important aglycones of phenolic compounds (Luo *et al*., 2004 and Wojdylo *et al*., 2007).

It is clear that the addition of antioxidants extracted from licorice and carob were gave slightly increased the stability of refined sunflower oil. Also, we can say, when we add 5 ml or more from these extracts may be used as natural antioxidative additive to improve the quality and stability of oils and food products. These compounds are considered to be beneficial to health science they act as antioxidants in the body by inhibiting lipid peroxidation scavenging, free radicals and displaying antimutagenic properties. These results suggest El-mawled El-nabawey sweets that the phenolic compounds extracted from licorice and carob posses antioxidant properties and could be as alternatives natural antioxidants in food applications.

Finally, it could be concluded that it is possible to produce untraditional sorts of El-mawled El-nabawey sweets of good storage property. This may be achieved by using some new materials not used before in processing of El-mawled El-nabawey sweets, such as natural antioxidants, which were extracted from licorice and carob. The addition of these extracts gave strong ant oxidative efficiency in El-mawled El-nabawey sweets. Sesames sweet gave higher storage period than folia sweets, because sesame hulls contain phenolic compounds (Awatif *et al.*, 2004).

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