Antioxidant Activity of Water Extract of Propolis from Different Regions In Kafr El-Sheikh Governorate

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ABSTRACT

Water extracts of propolis collected from three geographic regions (Motobes, Kafr El-Sheikh and Desouk) in Kafr El-Sheikh Governorate, Egypt were prepared. The extracts were analyzed for the determination of total polyphenols which ranged from 5.70 to 8.79 g/100 g of the sample and from 22.80 to 34.30 g/100 g of the freeze-dried extract. the total flavonoid content ranged from 3.05 to 4.85 g/100 g of the sample. Water extracts of propolis were evaluated for antioxidant activities using the β- carotene bleaching and 1,1-diphenyl- 2- picrylhydrazyl (DPPH) free radical-scavenging assay systems. It was observed that all propolis had strong antioxidant activities due to their contents of total polyphenol and flavonoid. The highest activities were found for samples from Desouk followed by these from Kafr El-Sheikh, then those from Motobes. Freeze-dried extracts of propolis can be used as natural antioxidants in sunflower oil as compared to BHT and TBHQ. Propolis from Desouk and Kafr El-Sheikh at 200 and 300 ppm were similar in reducing peroxide values and both of them at 300 ppm were better than BHT but lower than TBHQ, added at 200 ppm concentration, in reducing peroxides and hydroperoxides production in sunflower oil at 63°C for 4 days.

Keywords: propolis; antioxidants; water extracts; DPPH; free radical scavenging.

INTRODUCTION

Propolis (bee glue) is a resinous substance of complex mixture of several compounds collected by honeybees from trees and leaf buds. The sources of propolis were poplar (*Populus* spp.), birch (*Betula alba*), beech (*Fagus stylvatica*), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus glutinosa*) and various conifers (Ghisalberti, 1979, Amoros *et al*., 1992, Bankova *et al*., 2000). Park *et al*. (2002) reported that the botanical origin of propolis was resinous coatings from young leaves of *Hyptis divaricata* (Lamiaceae) and *Baccharis dracunculifolia* (Asteracea). Also, *Baccharis dracunculifolia*, is an established source of propolis (Santos *et al*., 2003). Propolis used mainly to cover the hive interior and the breeding cells and also to repair cracks and fissures. These uses are interest, because propolis avoids hive colonization with diseases (Walker & Crane, 1987).

Propolis contains a variety of chemical compounds such as polyphenols (flavonoid aglycons, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones), sesquiterpene quinines, coumarins, steroids, amino acids, and inorganic compounds (Bankova *et al*., 2000). Propolis samples contain more than 160 constituents and differ greatly due to the variation in their geographical and botanical origins (Kujumgiev *et al*., 1999, Moreno *et al*., 2000, Kumazawa *et al*., 2004). It has been used in folk medicine to maintain health. Pharmacological activities such as anticancer (Marcucci, 1995) anti-inflammatory (Wang *et al*., 1993), antibiotic (Koo *et al*., 2000), antioxidative (Moreno *et al*., 2000), antiviral, antifungal (Kujumgiev *et al*., 1999), anaesthetic and cytostatic effects (Ghisalberti, 1979) have been ascribed to ethanolic extract of propolis. Although ethanol extract of propolis is the most common, it is known that this extract poses immunological properties in animals and patients (Scheller *et al*., 1988).

Most propolis components are of phenolic nature, mainly flavonoids. It is known that simple phenols, phenolic acids and polyphenols are active antimicrobial agents (Cowan, 1999). Flavonoids are synthesized by plants as a response to microbial infections and are recognized to have effective antimicrobial effects against a wide range of microorganisms (Recio *et al*., 1989).

Studies concerning water extract of propolis are increasing (Basnet *et al*., 1996). Nagai *et al*. (2003) reported that the quantity of phenolic compounds in fresh propolis from Brazil was about 168 µg/mg powder of lyophilized water extract. They suggested that water extract of propolis contains a mixture of natural substances, such as amino acids, phenolic acids, phenolic acid esters, flavonoids, cinnamic acid, and caffeic acid.

The purpose of the present study was to examine the antioxidative effects of water extract of propolis collected from three different regions in Kafr El-Sheikh Governorate.

MATERIALS AND METHODS

Materials

Fresh propolis was obtained from three regions in Kafr El-Sheikh Governorate; namely, Kafr El-Sheikh, Desouk and Motobes during 2007 season. Survey of propolis sources in the previous regions was done. Sunflower oil (free of antioxidants) were purchased from Tanta Company for oils and soaps, Tanta, Egypt.

Chemicals

Linoleic acid, ascorbic acid and butylated hydroxy toluene (BHT) were purchased from Al Gomhoria Company for Chemical and Drugs in Cairo. 1,1-diphenyl- 2- picrylhydrazyl (DPPH), β- carotene and quercetin were purchased from Sigma. Tert-butyl hydroquinone (TBHQ) was obtained from Tanta Company for oils and soaps in Tanta City.

Preparation of water extract of propolis

Water extract of propolis was obtained as described by Suzuki, (1990) with slight modifications by Nagai *et al*. (2003) as follows: 20.0 g of propolis were suspended and extracted with 5 volumes of distilled water with shaking using shaker at laboratory temperature (25°C) for 24 hrs. The extracts were centrifuged at 3000 g for 20 min., and the supernatants were taken. The residue was re-extracted under the same conditions. The extracts were centrifuged under the same conditions and the supernatants were taken. The obtained supernatants were combined and dialyzed against distilled water, and then the dialysate was lyophilized. Each solution (10, 50, 100 mg/ml water) was used as the sample solution for the following tests.

Determination of total polyphenol and flavonoid contents

Total polyphenol contents in extracts were determined colourimetrically by the Folin- Ciocalteau method (Singleton *et al*., 1999). Extract solution (0.5 ml) was mixed with 0.5 ml of the Folin- Ciocalteau reagent and 0.5 ml of 100 mg/ml Na_2CO_3, and the absorbance was measured at a wavelength of 760 nm after 1 hr of incubation at room temperature. Extract samples were evaluated at a final concentration of 20 µg/ml. Total polyphenol contents were expressed as mg/g (tannic acid equivalents).

Total flavonoid contents in extract were determined by the method of Woisky & Salatino (1998) with minor modifications by Nagai *et al*., (2003). To 0.5 ml of the extract solution, 0.05 ml of 20 mg/ml $AlCl_3$ ethanol solution was added. After 1 hr at room temperature, the absorbance was measured at a wavelength of 420 nm. Extract samples were evaluated at a final concentration of 20 µg/ml. Total flavonoid contents were calculated as quercetin (mg/g) from a calibration curve.

Antioxidant assay to determine DPPH scavenging activity

The scavenging effect of propolis samples as well of ascorbic and chlorogenic acid (as positive control samples) corresponded to the quenching intensity of 1,1-diphenyl -2–picrylhydrazyl (DPPH) was carried out by the method described by Yamaguchi *et al*. (1998) as follows: Dilutions of propolis extracts (10, 50 and 100 µg/ml) were added to 0.5 ml of 300 m mol/l DPPH in ethanol. The mixtures were shaken vigorously and left to stand at room temperature for 20 min in the dark. Absorbance at a wavelength of 514 nm was measured using ethanol as a blank. The degradation of DPPH was evaluated by comparison with a control (0.5 ml of DPPH solution and 1.5 ml of ethanol). Results were expressed by the proportion of DPPH degradation compared with the control.

Antioxidant activity on linoleic acid oxidation

This experiment was carried out according to the method of Emmons *et al*. (1999) with some modification. ß- Carotene (3 mg) was dissolved in 30 ml of chloroform, then 3 ml were added to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was removed under a stream of nitrogen gas. Then distilled water (100 ml) was added, and the solution was well mixed. Aliquots (3 ml) of the ß- carotene/linoleic acid emulsion were mixed with 50 µl of sample solution and incubated in a water bath at 50°C. Oxidation of the emulsion was monitored spectrometrically by measuring absorbance at a wavelength of 470 nm after 60 min. The control sample contained 50 µl of solvent instead of the extract. The antioxidant activity was expressed as percent inhibition relative to the control after a 60 min incubation using the equation:

AA = (DRc – DRs)/ DRc × 100

Where: AA is the antioxidant activity, DRc is the degradation rate of the control [=1n (a/b)/60], DRs is the degradation in the presence of the sample [=1n (a/b)/60], a is the initial absorbance at time 0, and b is the absorbance after 60 min. Propolis extract samples were evaluated at a final concentration of 10 µg/ ml, and ascorbic acid and BHT at 1 µg/ml were used as a reference samples.

Assay of propolis freeze -dried extracts as antioxidants in sunflower oil

Propolis phenolic extracts (as powder) were assayed as natural antioxidants for sunflower oil (free of antioxidants). Five ml of Tween 40 was used as emulsifier. These freeze-dried extracts were added at concentrations of 200 and 300 ppm (as phenolic compounds in powder) compared to 200 ppm for BHT and TBHQ as synthetic antioxidants. Triplicate portions of each solution (50g) were put in an open 100- ml beaker. The solutions were incubated in an oven, thermostatically controlled at 63°C, for 4 days. Peroxide values in the stored oil samples were determined every 12 hrs (Rodriguez de Sotillo *et al*., 1994).

Peroxide value (PV)

Peroxide value (PV) was determined as described by Leonard *et al*. (1987) by dissolving 1 g of oil samples in a 30 ml glacial acetic acid- chloroform solution (60:40, v/v), adding of 1 ml potassium iodide (15%) and titrating the iodine librated with 0.1 N sodium thiosulphate solution. The peroxide value was expressed as milliequivalents of peroxide per 1000 g of sample.

Statistical analysis

The obtained data were statistically analyzed using General Linear Models Procedure Adapted by Statistical Package for the Social Sciences (SPSS, 1997).

RESULTS AND DISCUSSIONS

Total polyphenol and flavonoid contents

Table (1) shows the total polyphenol and flavonoid contents of propolis samples. The results indicated that propolis samples collected from Desouk had significantly the highest amounts of total polyphenols in crude samples and freeze-dried extract, being 8.57 and 34.38 g/100g; respectively. The samples collected from Kafr El-Sheikh were significantly, the second, and those collected from Motobes were the third.

No significant differences in the total flavonoid contents were found between the samples collected from Desouk and Kafr El-Sheikh and both of them were better than those collected from Motobes.

Phenolic compounds are commonly found in both edible and non-edible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Propolis contains a wide variety of phenolic compounds, mainly flavonoids. Variation in the flavonoid content of propolis is mainly attributable to the difference in the preferred regional plants collected by honeybees (Kahkonen *et al*., 1999).

DPPH radical scavenging activity

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Hatano *et al*., 1997). It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen- donating ability (Tang *et al*., 2002). To evaluate the scavenging effect of DPPH on water extract of propolis, DPPH inhibition was investigated and these results are shown as relative activities against control.

As shown in Table (2), the activities of propolis samples and synthetic antioxidants as free radical scavenging increased as a function of concentration increment.

Table 1: Total polyphenol and total flavonoid contents of propolis from three regions in Kafr El-Sheikh Governorate

Sample region	Total polyphenols (g/100g)		Total flavonoids (g/ 100g)
	Propolis sample	Lyophilized extract	
Motobes	5.70 ± 0.123 [c]	22.80 ± 0.564 [c]	3.05 ± 0.233 [b]
Kafr El-Sheikh	7.32 ± 0.233 [b]	29.30 ± 0.322 [b]	4.11 ± 0.122 [a]
Desouk	8.57 ± 0.242 [a]	34.38 ± 0.227 [a]	4.85 ± 0.253 [a]

Values are Means±S.D. Means of treatments having the same letter(s) within a column are not significantly different (P > 0.05)

All propolis samples showed free radical scavenging activity less than synthetic antioxidants. The samples collected from Desouk had the highest free radical scavenging at all the used concentrations compared to others propolis samples. It may be related to its contents of total polyphenol and flavonoid contents.

Generally, the abilities of synthetic and natural extracts as free radical scavenging at all the used concentrations are in the descending order: BHT > ascorbic acid > Desouk propolis extracts > Kafr El-Sheikh propolis extracts > Motobes propolis extracts.

Nagai *et al*. (2003) reported that the activities of water extract of fresh propolis from Brazil (at concentrations 1, 5, 10, 50 and 100 mg/ml of extract) as DPPH radical scavenger were between those of 0.1 and 1.0 mM ascorbic acid. Hegazi & Abd El Hady, (2002) found that caffeic and vitamin C at concentrations of 1, 10 and 100 µg showed the highest activity as free radical scavenger compared to the same concentrations of propolis samples collected from a reclaimed land in Egypt. Ahn *et al*., (2007) observed that propolis samples collected in various area of China showed free radical scavenging activity and there were positive correlation between the activities and total polyphenol contents.

Effect of various propolis samples on linoleic acid oxidation

The antioxidant assay, using the discoloration of β- carotene is extremely susceptible to free radical- mediated oxidation. β- Carotene is discolorized easily by the oxidation of linoleic acid, due to its double bonds being sensitive to oxidation (Unten *et al*., 1997 & Singh *et al*., 2002).

Water extracts of propolis samples were evaluated at the final concentration of 10 µg powder/ml for the assay, and ascorbic acid and BHT were compared at 1 µg/ml under the same conditions. As shown in Table (3), antioxidant activities of synthetic antioxidants were higher than those of natural freeze dried extracts, and BHT was the highest. Freeze dried extract of propolis from Desouk had a strong antioxidant activity than those from other regions followed by those collected from Kafr El-Sheikh, then from Motobes. That may be related to its high contents of both total polyphenol and flavonoid compared with the other samples. Flavonoids

Table 2: The DPPH radical scavenging activities (%) of water propolis extracts collected from three regions in Kafr El-Sheikh Governorate as compared with ascorbic acid and BHT

Samples	Concentration µg powder/ ml water					
	10 µg		50 µg		100 µg	
	Absorbance	Activity	Absorbance	Activity	Absorbance	Activity
Control	0.173±0.0002	0.00	0.173±0.0002	0.00	0.173±0.0002	0.00
Ascorbic	0.117±0.0004	32.37^{d}	0.054±0.0003	68.79^{b}	0.033±0.0004	80.92^{b}
BHT	0.102±0.0004	41.04^{a}	0.045±0.0004	73.99^{a}	0.020±0.0002	88.44^{a}
Motobes	0.125±0.0003	27.75^{e}	0.085±0.0003	50.87^{e}	0.057±0.0003	67.05^{e}
Kafr El-Sheikh	0.115±0.0003	33.53^{c}	0.073±0.0002	57.80^{d}	0.041±0.0002	76.30^{d}
Desouk	0.107±0.0002	38.15^{b}	0.067±0.0002	61.27^{c}	0.038±0.0004	78.03^{c}

Values are means ± S.D.

Table 3: Antioxidant activities (%) of water propolis extracts collected from different regions in Kafr El-Sheikh Governorate as compared with ascorbic acid and BHT

Samples	Antioxidant activities (%)
Ascorbic acid[1]	79.58 ± 0.132 ^{b}
BHT[1]	85.13 ± 0.437 ^{a}
Metobes[2]	65.49 ± 0.257 ^{e}
Kafr El-Sheikh[2]	71.68 ± 0.193 ^{d}
Desouk[2]	77.13 ± 0.325 ^{c}

1= Synthetic antioxidants at 1 µg/ml solvent. 2= Propolis extracts at 10 µg powder/ ml water.
Values are Means ± S. D. Means of treatments having the same letter(s) within a column are not significantly different (P > 0.05)

have been reported to be the most abundant and most effective antioxidant in propolis from Argentine (Bonvehi & Coll, 1994, Isla *et al*., 2001). Kumazawa *et al*., (2004) reported that the antioxidant activity is correlated with total flavonoid contents of ethanolic extracts of propolis.

The DPPH free radical-scavenging activity seemed to relate with the antioxidant activity. However, more detailed qualitative and quantitative analyses of the compounds with antioxidant activity are necessary to elucidate the antioxidant activity of propolis (Choi *et al*., 2006).

Assay of Freeze-dried extracts of propolis as antioxidant in sunflower oil

Freeze-dried extracts of propolis samples were applied on sunflower oil (free of antioxidants) as natural antioxidants. The used concentrations were 200 and 300 ppm (total polyphenols in freeze dried extract) as compared to the recommended concentration of synthetic antioxidants (200 ppm).

The results in Table (4) show peroxide values (PV) of the untreated and treated sunflower oil with natural and synthetic antioxidants with incubation at 63°C for 4 days. The results indicated that there were significant differences (at $P > 0.05$) between PV of the untreated and treated samples. PV of samples treated with natural and synthetic antioxidants were lower than those of untreated samples with storage time for 4 days. Peroxide values of oil treated with TBHQ were significantly the lowest among all treated samples. So that TBHQ had strong antioxidant activity higher than those of others additives. No significant difference was found in PVs for oil treated with Kafr El-Sheikh propolis extracts and Desouk propolis extracts at 300 ppm concentration, and both of them were the second. BHT was significantly the third in reducing peroxides and hydroperoxides production. Also, Kafr El-Sheikh and Desouk propolis extracts at 200 ppm concentration were similar in reducing peroxide values and both of them were the fourth. Propolis extracts collected from Motobes at 300 ppm concentration was the fifth, followed by 200 ppm concentration. Generally, these findings demonstrate that the antioxidant activity was correlated with total polyphenol contents in propolis samples. Also, these activities increased depending on the concentration of the added total polyphenol content.

CONCLUSION

In this study, the *in vitro* antioxidant activity of propolis collected from three regions in Kafr El-Sheikh Governorate was investigated. Differences were observed in total polyphenol and flavonoid contents. Propolis samples had strong antioxidant activities, and the highest activities were found in Desouk propolis. Also, freeze-dried extracts of propolis can be used as natural antioxidants instead of synthetic antioxidants in edible oils and fatty foods against oxidative deterioration.

Table 4: Peroxide values* of sunflower oil treated with different levels of propolis phenolic extracts and incubated at 63 oC for 4 days as compared with ascorbic acid and BHT

Treats. / Storage time (day)	Control	Synthetic		Natural extracts					
		BHT	TBHQ	Motobes		Kafr El-Sheikh		Desouk	
	0 ppm	200 ppm	200 ppm	200 ppm	300 ppm	200 ppm	300 ppm	200 ppm	300 ppm
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	3.78^{a}	1.49^{c}	1.35^{c}	2.83^{b}	2.75^{b}	1.50^{c}	1.41^{c}	1.39^{c}	1.35^{c}
1.0	5.09^{a}	3.95^{b}	3.10^{c}	3.75^{b}	3.00^{c}	3.63^{bc}	4.00^{b}	4.00^{b}	3.75^{b}
1.5	7.72^{a}	5.73^{b}	4.25^{d}	6.11^{b}	5.09^{c}	5.64^{bc}	5.53^{bc}	6.08^{b}	5.70^{b}
2.0	15.82^{a}	9.56^{d}	7.00^{f}	13.87^{b}	13.51^{b}	11.22^{c}	8.41^{e}	10.79^{c}	8.33^{e}
2.5	23.71^{a}	14.72^{d}	10.21^{f}	19.44^{b}	18.86^{b}	16.45^{c}	12.36^{e}	17.00^{c}	12.00^{e}
3.0	29.45^{a}	17.31^{e}	12.16^{g}	25.13^{b}	21.92^{c}	19.73^{d}	14.29^{f}	19.32^{d}	13.95^{f}
3.5	37.18^{a}	19.86^{e}	16.35^{g}	29.52^{b}	27.14^{c}	22.86^{d}	18.00^{f}	23.11^{d}	18.23^{f}
4.0	48.32^{a}	22.14^{e}	17.89^{g}	37.05^{b}	35.07^{c}	25.19^{d}	19.43^{f}	24.78^{d}	19.00^{f}

* Peroxide values are expressed as meq. O_2/kg oil.
Values are means of three replicates.
Means of treatments having the same letters(s) within a row are not significantly different ($P > 0.05$).

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النشاط المضاد للأكسدة في المستخلص المائي لصمغ النحل المجمع من أماكن مختلفة في محافظة كفرالشيخ

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تم إعداد المستخلص المائي لصمغ النحل المتحصل عليه من أماكن مختلفة في محافظة كفرالشيخ وهى مطوبس – كفرالشيخ – دسوق٠ تم تقدير الفينولات العديدة الكلية في المستخلص المائي منسوبة للعينة الأصلية وكذلك في المستخلص المائي بعد تجفيده حيث تراوحت النسبة من ٥,٧ إلى ٨,٨٧ جم/١٠٠ جم عينة أصلية، ومن ٢٢,٨٠ إلى ٣٤,٣٠ جم/ ١٠٠ جم مستخلص مجفد٠ أما محتوى الفلافونويدات الكلية فقد تراوح من ٣,٠٥ – ٥,٨٥ جم/ ١٠٠ جم عينة أصلية٠ أيضا" تم اختبار النشاط المضاد للأكسدة للمستخلص المائي لهذه العينات باستخدام اختبار تبييض البيتا كاروتين وكذلك اختبار إيقاف نشاط الأصل الحر DPPH ٠

أظهرت النتائج أن كل المستخلصات المستخدمة ذات نشاط مضاد للأكسدة قوى وكان ذلك مرتبطاً بمحتوى المستخلصات المائية من المركبات الفينولية والفلافونويدات٠ وكان النشاط المضاد للأكسدة للعينات المأخوذة من دسوق الأعلى يليها المأخوذة من كفرالشيخ ثم المأخوذة من مطوبس٠ كما تم استخدام المستخلص المائي المجفد للعينات المختلفة بتركيزات التي تحتوي على الفينولات بتركيزي ٢٠٠ ، ٣٠٠ جزء فى المليون كمضاد أكسدة طبيعي لزيت عباد الشمس مقارنة بالـ TBHQ ، BHT بتركيزات ٢٠٠ جزء فى المليون كمضادات أكسدة صناعية حيث أظهرت النتائج أن العينات المأخوذة من مدينتى دسوق وكفرالشيخ والتي تحتوي على الفينولات بتركيز ٣٠٠ جزء في المليون كانت ذات تأثير مشابه (لا يوجد فروق معنوية بينهما) في خفض رقم البيروكسيد لزيت عباد الشمس وكانت أفضل من الـ BHT ولكنها كانت أقل كفاءة من الـ TBHQ٠