Effect of Egyptian Propolis on the Susceptibility of LDL to Oxidative Modification and its Antiviral Activity with Special Emphasis on Chemical Composition

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The antioxidant activity of eight Egyptian propolis samples from different localities was evaluated by the antioxidative potential and capacity of the DPPH-ESR signal, superoxide anion generated in the xanthine-xanthine oxidase (XOD) system and low density lipoprotein (LDL) peroxidation assay. As, F, Is and D samples showed the highest antioxidative capacity and potential, respectively. The El, IsR, Is, D and So samples exhibited highly significant antioxidant activity in the XOD system and in LDL peroxidation assays.

The antiviral activity of propolis samples was investigated. They showed variations in their activity; sample D induced the highest antiviral activity against Newcastle disease virus and infectious bursal disease virus.

42 Polyphenolic compounds were identified by HPLC; 13 aromatic acids, esters and alcohols were present, 29 flavonoids were identified, 6 of them being new to propolis.

Key words: LDL Peroxidation, Antiviral, Polyphenolics

Introduction

Oxidation of lipids is assumed to be implicated in the pathophysiology of atherosclerosis. It has been suggested that scavenging of lipid peroxyl radicals contributes to the antiatherosclerotic effects of naturally occurring compounds such as polyphenol compounds. These compounds are capable of inhibiting the lipoprotein oxidation *in vitro* and suppressing the formation of plasma lipid oxidation products *in vivo* (Stocker and Keany, 2004). Therefore, inhibition of LDL oxidation might be an important step in preventing atherosclerosis. Humans protect themselves from reactive oxygen species, in part, by absorbing dietary antioxidants (Kamiya, *et al*., 2004). This group of polyphenolics includes flavonoids, phenolic acids and their esters and is present in relatively high concentrations in propolis (Hegazi and Abd El Hady, 2001, 2002; Abd El Hady and Hegazi, 2002).

Propolis is a resinous hive product collected by bees. It is rich in polyphenolic compounds (Greenaway *et al*., 1990) and has antioxidant (Basnet *et al*., 1997; Hegazi and Abd El Hady, 2002), antiinflammatory (Marcucci, 1995), antibacterial (Hegazi *et al*., 2000), antifungal (Hegazi and Abd El Hady, 2002), antiviral (Hegazi *et al*., 2003, 2006; Abd El Hady and Hegazi, 2002) and antitumour activities (Hegazi *et al*., 1998).

The presence of polyhydroxy flavonoids can not be well detected by GC/MS studies due to the higher molecular weight of tetrahydroxy and pentahydroxyflavones and their methyl ethers. In most cases the mass spectra with such small peaks are not sufficiently detailed to accurately identify the compounds present. Also their percentage occurrence may be seriously underestimated by GC/MS analysis (Garcia-Viguera *et al*., 1993). So, HPLC seems to be a more convenient technique for the analysis (identification and quantification) of flavonoids from propolis (Garcia-Viguera *et al*., 1993)

The aim of this study was to evaluate the antioxidant activity of propolis from eight different localities to find out the highly effective antioxidant one which could protect the human low density lipoprotein (LDL) against copper-induced oxidation *in vitro* – a study provides primary evidence – for further *in vivo* studies, as well as to

Abbreviations: F, Fayoum; As, Assiut; So, Souhag; D, Dakahlia; Sh, Sharkia; Is, Ismailia; El, El-Saff; IsR, Ismailia-R.

estimate their antiviral activity, in correlation with investigating their chemical composition by HPLC.

Materials and Methods

Propolis

Eight Egyptian propolis samples were collected from different localities. Three samples came from Fayoum (F), Assiut (As) and Souhag (So) provinces (Upper Egypt). Three further samples were collected from Dakahlia (D), Sharkia (Sh) and Ismailia (Is) provinces (East Nile Delta) and two samples from new reclaimed lands, El-Saff (El) and Ismailia-R (IsR).

Extraction and sample preparation

1 g of each sample was cut into small pieces and extracted at room temperature with 50 ml of 70% ethanol (twice within 24 h). The alcoholic extract was evaporated to dryness under vacuum at 50 °C. The percentage of extracted matter was as follows (g/g dry weight): Fayoum, 0.13; Assiut, 0.24; Souhag, 0.10; Dakahlia, 0.80; Sharkia, 0.40; Ismailia, 0.33; El-Saff, 0.45; and Ismailia-R, 0.21. The dry extract was dissolved in methanol and filtered through a 0.45-μm filter before HPLC analysis.

Estimation of antioxidant activity by kinetic ESR measurements

The scavenging activities of the propolis samples from different sources were determined by ESR measurements of the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical using the method adopted by Calliste *et al.* (2001). Reaction mixtures contained 100 μl test samples (10 μg/ml) and 100 μl 60 μM DPPH; the samples and DPPH were dissolved in ethanol. Due to its paramagnetic properties, DPPH exhibits an ESR signal. Concerning the kinetics measurements, the degradation of the DPPH radical was observed by monitoring the ESR signal: The more the ESR signal decreases during the first 2 min of the measurement, the higher is the antioxidative potential (Gardner *et al.*, 1998); the more the decrease in the signal intensity is after 25 min, the higher is the antioxidative capacity (Gardner *et al.*, 1999). ESR spectra were obtained with a Bruker Elexsys 500 spectrometer using micro-pipettes at room temperature under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.77 GHz; time constant, 5.12 ms.

Determination of superoxide anion radical scavenging activity

The superoxide anion radical ($O_2^{-\bullet}$) scavenging activity by generating free superoxide anion radicals in the xanthine-xanthine oxidase (XOD) system was measured following the method of Matsushige *et al.* (1996). The colour obtained was measured at 560 nm. The mean of three measurements of each sample was calculated.

Measurement of copper-induced LDL oxidation in vitro

Isolation of LDL

LDL was isolated according to the method of Gugliucci and Menini (2002). LDL (1.019–1.055 g/ml) was separated from plasma by sequential ultra-centrifugation using a TL-100 ultracentrifuge (Beckman, USA). LDL was then extensively dialyzed against phosphate-buffered saline (PBS), pH 7.2, containing 0.01% EDTA at 4 °C. Samples were stored at 4 °C in the dark and used within 24 h. Protein content was determined according to Lowry *et al.* (1951) (determination of protein by a kit).

Thiobarbituric acid reactive substances (TBARSs) assay

LDL was oxidized using 5 μM $CuSO_4$ (Masaki *et al.*, 1989). Oxidation of LDL was monitored in the presence or absence of the propolis sample by measuring the absorbance of thiobarbituric acid reactive substances (TBARSs) at 534 nm using a UV spectrophotometer (UNICAM UV300). Malondialdehyde-bis-(dimethylacetal), which yields malondialdehyde (MDA) by acid treatment, was used as a standard. The mean of three measurements per sample was calculated.

Antiviral activity

Viral strains

Two viruses were used in this investigation. These viruses were: Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV). The viruses were kindly supplied by Animal Health Research Institute, Dokki, Giza, Egypt.

Viral titration

The viral activity of NDV and IBDV were determined to evaluate the infectivity titer in chicken fibroblast cell cultures. Embryonated chicken eggs were obtained from the Faculty of Agriculture, Cairo University, Egypt. Primary monolayer cell cultures were prepared from embryonated chicken eggs (9- to 11-day-old chick embryos). Chicken embryo fibroblasts (CEFs) were prepared in plastic plates (Falcon 3002, Becton Oxnard, CA, USA). CEFs which were grown in microtiter plates were used for virus titration. The titration was done by inoculation of each virus dilution into 5 wells of CEF fibroblast cultures. The tissue culture infectivity dose that causes a cytopathic effect in 50% of the cell culture ($TCID_{50}$) was calculated.

Antiviral activity of propolis

The ethanolic extract from 1 g (dry weight) of each propolis raw sample was dissolved in PBS (pH 7.2) to obtain a 1% stock solution. Titration of antiviral activity was done by mixing an equal volume of serial ten-fold dilutions of each virus with 1% stock solution of propolis. The mixture was incubated at 37 °C for 30 min at room temperature. 50 μl of each mixture were inoculated into CEF cell cultures using 5 wells/mixture. Back titration of NDV (Reavc and Poste, 1971) and IBDV (Kibeng *et al*., 1988) was done using 5 wells/virus dilution where 5 wells were used for control (to test for cell cytotoxcity) and 5 wells were left as cell control. After 120 h, cells were observed microscopically for cytopathic effects. The monolayer cells were stained with crystal violet. The effect of propolis on different viruses was calculated according to Reed and Muench (1938) as mean $TCID_{50}$.

HPLC analysis of propolis

The HPLC analysis was achieved with an Agilent 1100 series liquid chromatograph with a UV detector and an auto-sampler. The column used was a Lichrochart RP-18 (Merck, Darmstadt, Germany; 12.5 × 0.4 cm, 5 μm particle size).

Elution was with water/formic acid (19:1 v/v; solvent A) and acetonitrile (solvent B), and the flow rate was 1 ml/min. Gradient elution started with 20% B, reaches 25% B at 25 min and 30% B at 35 min, and then the system became isocratic until 50 min, reaches 50% B at 60 min and 70% B at 67 min. The compounds were detected with a UV detector and the chromatograms were recorded at 340 and 290 nm for flavones and flavanones, respectively, and at 290 nm for phenolic acids (Tomàs-Barberàn *et al*., 1993; Gil *et al*., 1995).

Polyphenolic compounds (flavonoids and phenolic acids) were quantified by HPLC on a C-18 reverse phase column, using a UV detector. Their identification was carried out by direct HPLC comparison with authentic markers and was based on co-chromatography at 290 nm for phenolic acids and flavanones and 340 nm for flavones. Response factors for the authentic markers and the concentration of compounds in each propolis sample were calculated according to Ogan and Katz (1981) and Annual Book of ASTM Standards (1983). Some of the authentic markers were commercially available; others were available in E. W.′s laboratory.

Results and Discussion

Increasing the body's antioxidant content may help to protect against cellular damage and the development of chronic diseases. Research indicates that propolis contains numerous phenolic and non-phenolic antioxidants (Scheller *et al*., 1990; Hegazi and Abd El Hady, 2002). LDL peroxidation is considered to be essential in the pathogenesis of atherosclerosis (Stocker and Keaney, 2004). Compounds with antioxidant activity could have some beneficial effects in the prevention of atherosclerosis (Fuhrman and Aviram, 2001).

Estimation of antioxidant activity by ESR measurements

The antioxidative capacity and potential of eight Egyptian propolis samples from different provinces were assessed on the basis of their scavenging activity for the stable free DPPH radical. To distinguish the activities of the eight samples, they were tested at a lower concentration (10 μg/ml), as all DPPH radicals have been reduced already and can not be detected by means of ESR spectroscopy within 2 min at a concentration of (100 μg/ml). The DPPH measurements showed interesting results. They showed different anti-oxidative parameters. The kinetics of the decrease of the DPPH-ESR signal of the samples are displayed in the Fig. 1; from these kinetics the anti-oxidative potentials and capacities were calculated for each sample.

The results of the antioxidative capacity measurements of the different Egyptian propolis samples are shown in Fig. 2. Samples As, F, Is and D

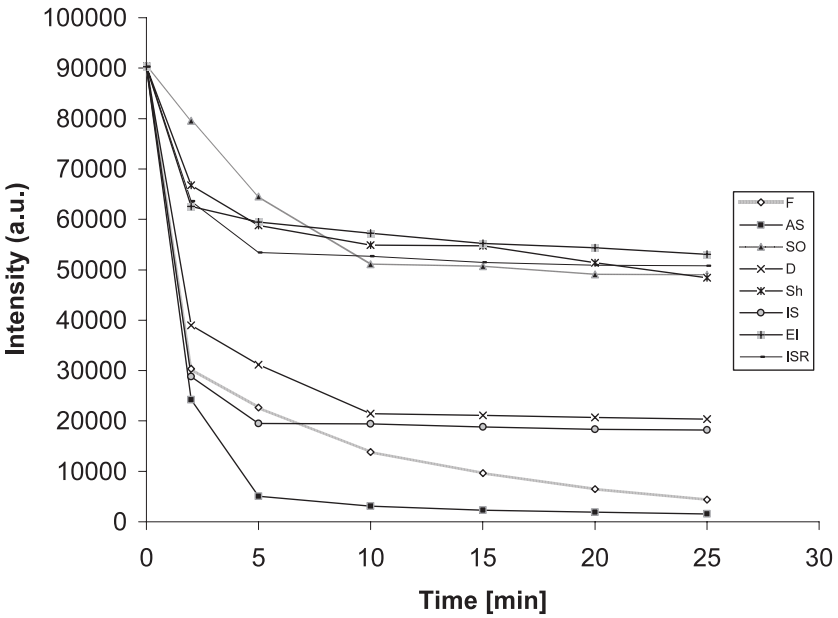

Fig. 1. Kinetics of the decrease of the DPPH-ESR signal by Egyptian propolis samples in ethanol.

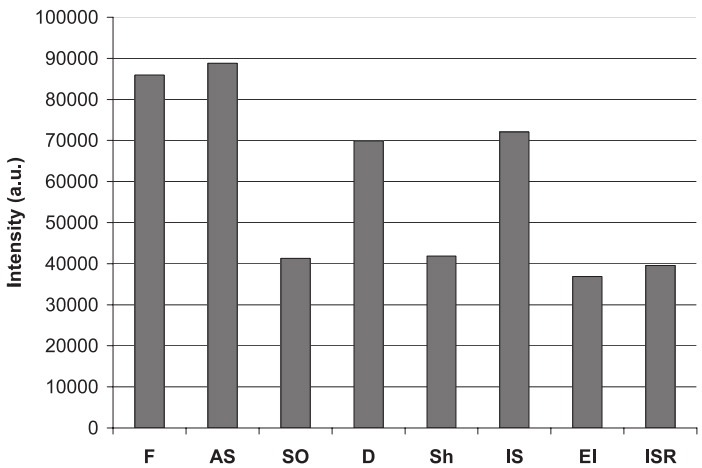

Fig. 2. Antioxidant capacity of propolis samples in ethanol tested with DPPH.

showed the highest antioxidative capacity, respectively, while the samples So, Sh, IsR and El had moderate antioxidative capacity. The decrease in the signal intensity is a measure for the antioxidative capacity (quantitative parameter related to the total amount of radicals which are reduced by a defined amount of antioxidant) (Gardner *et al*., 1999). This decrease occurred when a single electron of the radical is paired (Calliste *et al*., 2001). Electron spin resonance (ESR) spectroscopy can provide direct evidence of the formation of free radicals in chemical and biological reactions. Because ESR generates signals only in molecules with unpaired electrons, it is very selective and can be used even in situations where thousands of other compounds are present (Fukuhara *et al*., 2003). Some authors demonstrated such results of free radical scavenging activities of propolis and some plants were examined using ESR spectroscopy (Scheller *et al*., 1990; Rapta *et al*., 1995; Cal-

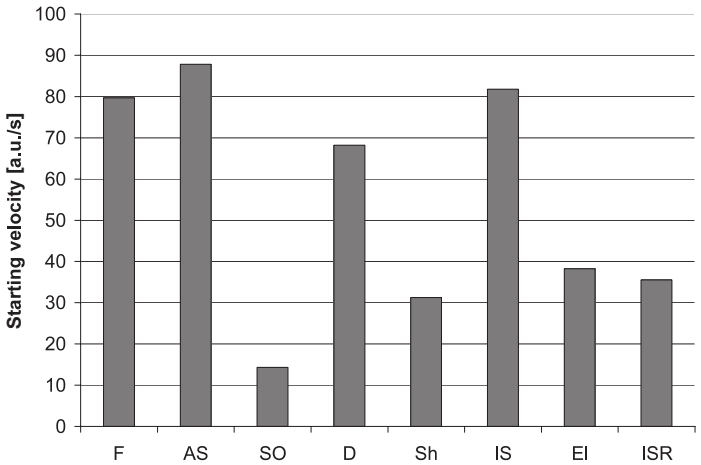

Fig. 3. Antioxidative potential of propolis samples in ethanol tested with DPPH.

liste *et al*., 2001). Also Scheller *et al*. (1990) stated that the DPPH signal intensity was inversely related to the concentration of the ethanolic extract of propolis (EEP) and due to the reaction time. They also assumed that the ability of components in an ethanolic extract of propolis to donate a hydrogen atom is responsible for the lowering of the DPPH-EEP signal, and reflect the antioxidative nature of the EEP.

The antioxidative potentials of the propolis samples are shown in Fig. 3. It was observed that all the propolis samples except for sample So (14.37 a. u./s) possess good antioxidative potential. Sample As has the highest antioxidative potential (87.85 a. u./s) followed by Is, F and D; the other remaining propolis samples ranged from 31.27 up to 38.00 a. u./s. The velocity by which the signal decreases determined the antioxidant potential (kinetic parameter) (Rapta *et al*., 1995; Gardner *et al*., 1998).

Scavenging ability for superoxide anion radical

The free radical scavenging activity on superoxide anion radicals generated by an enzymatic method was evaluated. The results are shown in Fig. 4. The propolis samples El, IsR, Is and So exhibited highly significant antioxidant activity within the concentration of 100 μg/ml (79.25–67.00%). Sh and D samples had moderate activity

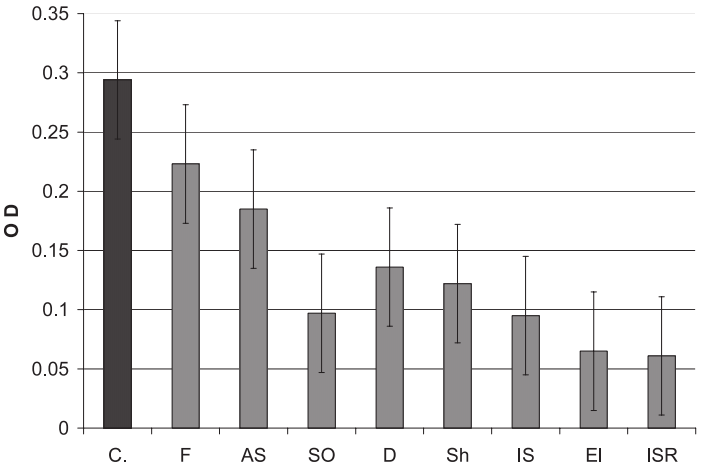

Fig. 4. Free radical scavenging activity of propolis samples in the xanthine-XOD system.

(58.5–53.74%), but F and As propolis samples showed very low activity (24.14–37.07%). Basnet *et al*. (1997) evaluated the free radical scavenging activity of water, methanol and chloroform extracts of Brazilian propolis in xanthine-xanthine oxidase (XOD)-generated superoxide anion assay systems. They found that the water extract was more potent than the most common antioxidants such as vitamin C and vitamin E. Tiwari (2001) suggested that flavonoids which possess pyrogallol (adjacent trihydroxy) and/or catechol (adjacent dihydroxy) moieties in their structure show strong H_2O_2-generating activity via an $O_2^{\bullet}$ anion radical and also possess inhibitory activities in rat liver microsomal lipid peroxidation. Flavonoids which generate H_2O_2 can scavenge freeradicals. Flavonoids generate H_2O_2 by donating a hydrogen atom from their pyrogallol or chatechol structure to oxygen through a superoxide anion radical. The pyrogallol-type flavonoids generate more H_2O_2 than catechol. The higher the H_2O_2 generation, the more potent is the radical trapping.

Superoxide anion has been of intense interest due to its increased dominance *in vivo* in different disease conditions (inflammation, atherosclerosis and cancer; Tiwari, 2001). XOD enzyme is considered as an important source of superoxide radicals. The compound bearing both superoxide scavenging as well as XOD inhibitory activity may offer better therapeutic potential. Flavonoids with both these properties possess in common hydroxy groups either at C-5, C-3 or C-3′ and C-4′ (Tiwari, 2001).

Susceptibility of LDL to Cu^{2+}-induced oxidation

Pre-incubation of LDL with propolis samples resulted in significant inhibition of TBARS accumulation. From the data shown in Fig. 5, it is clear that in the LDL peroxidation assay of propolis samples, IsR, Is, So and D exhibited very highly significant antioxidant activity within the concentration of 100 μg/ml (117.39–102.17%). While El and As samples had moderate activity (93.48%, 84.78%), but F and Sh propolis samples showed very low activity (17.39%, 19.56%).

TBARSs, as an index of lipid peroxidation, were undetectable in control LDL, slightly rising only after 3 h of incubation. Incubation with the oxidant resulted in a marked elevation of TBARSs. After 24 h of incubation in the presence of the oxidant, TBARS level did not further increase significantly (data not shown).

The results showed that not all the propolis samples had the same activity to suppress LDL peroxidation *in vitro*. Although the propolis sample Sh contained about 187.4 mg/g propolis polyphenolic compounds and Is and IsR propolis samples contained about 73.9 and 9.1 mg/g propolis, respectively (Fig. 5 and Table I), the propolis samples Is and IsR exhibited highly significant antioxidant activity, *i. e.* prevent the detection of TBARSs. Our data are in agreement with Isla *et al*. (2001) who found that groups I and II of Argentine propolis extracts diminished the maximal rate of diene production and the maximal amount of dienes produced. Group III had no effect on the lipid oxidation. The extent of lipoprotein oxidation was

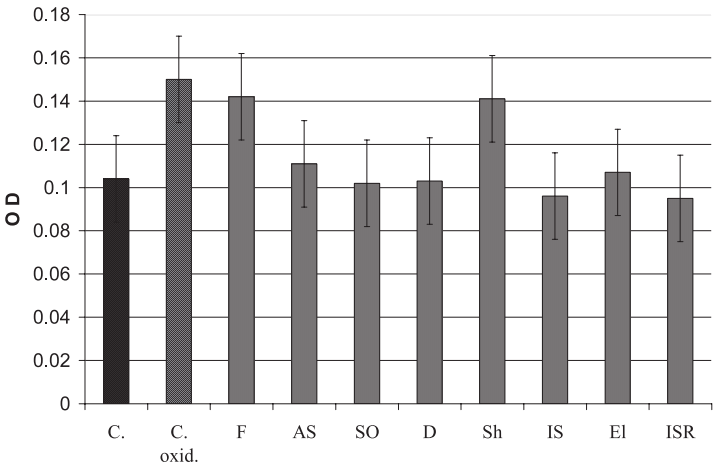

Fig. 5. Antioxidant activity of propolis samples on copper-induced human LDL peroxidation *in vitro*.

Table I. Polyphenolic compounds of Egyptian propolis collected from different provinces assessed by HPLC (in mg/g propolis).

No	Name	Chemical name	F	As	So	D	Sh	Is	El	IsR
1	Hydrocinnamic acid	3-Phenylpropanoic acid	4.86	2.38	1.27	1.72	18.05	42.55	8.88	1.00
2	Hydrocaffeic acid	3,4-Dihydroxyhydrocinnamic acid	0.35	6.14	0.27	0.87	15.70	22.86		0.46
3	Cinnamic acid	3-Phenylpropenoic acid	3.47	6.14		0.51			0.24	0.06
4	Vanillic acid	4-Hydroxy-3-methoxybenzoic acid			0.12	0.13		2.49	0.32	0.04
5	Ellagic acid	4,4′,5,5′,6,6′-Hexahydroxydiphenic acid-2,6,2′,6′-dilactone		0.32		0.45		0.22		
6	Hydrocoumaric acid	3-(4-Hydroxyphenyl) propionic acid				0.12				
7	------	2-Hydroxy-3-methoxybenzyl alcohol	0.09	0.54	0.10			2.44		0.2
8	Coniferyl alcohol	4-Hydroxy-3-methoxycinnamyl alcohol	0.50		2.06	0.58	2.79			0.15
9	Coumaric acid	*trans*-4-Hydroxycinnamic acid				1.41				
10	Ferulic acid	4-Hydroxy-3-methoxy cinnamic acid		0.56		0.30				
11	Caffeic acid	3,4-Dihydroxy cinnamic acid	0.13	10.16	0.96	0.33	1.58		0.31	
12	Eriodictyol	5,7,3′,4′-Tetrahydroxyflavanone	1.70	2.00	0.13		4.70			0.12
13	Liquiriteginin	7,4′-Dihydroxyflavanone	0.13		0.10		1.96			
14	Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone	2.5	34.29	0.34	0.06	5.56		0.18	0.24
15	Luteolin	5,7,3′,4′-Tetrahydroxyflavone		38.74	0.67	0.96	3.66			
16	Quercetin	3,5,7,3′,4′-Pentahydroxyflavone	0.28				1.36	0.07	0.73	0.15
17	Naringenin	5,7,4′-Trihydroxyflavanone	0.17		0.60		14.46			
18	Pinobankasin	3,5,7-Trihydroxyflavanone	1.82	1.19	0.80	1.48	0.83			
19	Quercetin-3-methylether	5,7,3′,4′-Tetrahydroxy-3-methoxy-flavone	0.16	9.35	0.15	1.25	1.33		0.53	0.08
20	Genistein	5,7,4′-Trihydroxyisoflavone					1.88			
21	Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone	0.22	2.78			3.85	0.18		
22	8-Methoxykaempferol	3,5,7,4′-Tetrahydroxy-8-methoxyflavone		0.17			0.75	0.23		0.44
23	Apigenin	5,7,4′-Trihydroxyflavone	0.14	7.50	0.44	0.37	1.02	0.47	0.48	0.05
24	Kaempferol	3,5,7,4′-Tetrahydroxyflavone			2.00			0.86		0.47
25	Luteolin-3′-methylether	5,7,4′-Trihydroxy-3′-methoxyflavone	0.80	12.22	0.44	2.30	5.39		3.37	0.13
26	Kaempferol-3-methylether	5,7,4′-Trihydroxy-3-methoxyflavone 5.43	0.77	12.68				1.86		
27	Quercetin-3,3′-dimethylether	5,7,4′-Trihydroxy-3,3′-dimethoxyflavone	1.57	10.31	0.73	1.00	22.82	0.03	0.44	0.42
28	Formonontin	7-Hydroxy-4′-methoxyisoflavone	5.45		1.73	3.44	10.14			
29	Quercetin-7-methylether	3,5,3′,4′-Tetrahydroxy-7-methoxy-flavone	3.23	6.30	0.61	1.18	4.56		3.11	0.18
30	Dimethylallylcaffeate	3-Methylbut-2-enyl caffeate	1.52	13.04	5.07	5.11	2.42	0.09	31.09	1.43
31	Pinocembrin	5,7-Dihydroxyflavanone	4.22	47.05	0.79	0.89	39.36	0.03		0.49
32	Phenylethylcaffeate	Phenylethyl-*trans*-caffeate				1.89				
33	Chrysin	5,7-Dihydroxyflavone	0.51	7.56		1.26				
34	Pinobankasin-3-acetate	5,7-Dihydroxy-3-acetyloxyflavanone	0.78		1.06	2.92				
35	Prunetin	5,4′-Dihydroxy-7-methoxyisoflavone	0.95				2.98			
36	Formononetin	7-Hydroxy-4′-methoxyflavone				1.15	1.82			
37	Galangin	3,5,7-Trihydroxyflavone	0.63			5.00				
38	Acacetin	5,7-Dihydroxy-4′-methoxyflavone		24.52			7.00		4.84	0.41
39	Quercetin-7,3′-dimethylether	3,5,4′-Trihydroxy-7,3′-dimethoxyflavone			0.17					
40	Biochanin A	5,7-Dihydroxy-4′-methoxyflavanone	3.18	0.64	1.19	3.15	10.16	0.07		0.70
41	Pinostrobin	5-Hydroxy-7-methoxyflavanone	0.58	0.55	0.48	2.06	1.24	1.19	2.48	
42	Galangin-7-methylether	3,5-Dihydroxy-7-methoxyflavone						0.12	10.64	
Total			39.98	249.82	23.05	111.04	187.40	73.91	72.50	9.10

measured by the TBARS assay. The results also demonstrated that the reactivity of the polyphenolics in protecting low-density lipoprotein (LDL) against Cu^{2+}-induced oxidation is dependent on their structural properties in terms of the response of the particular polyphenolics to Cu^{2+} ions, either chelation or oxidation, and their partitioning abilities between the aqueous compartment and the lipophilic environment within the LDL particle; their hydrogen-donating antioxidant properties are important aspects, too.

The fewer the number of OH groups, the lower is the probability of hydrogen loss and the lower is the probability of oxidation of the flavonoid and the reduction of the metal. In addition, the presence of the 2,3-double bond in conjugation with the 4-oxo group in the C ring is particularly important. It is interesting to consider possible structures of the oxidized species of each flavonoid. In the cases of myricetin, quercetin and kaempferol, the oxidized structure is probably the ketone structure as all three flavonoids have a hydroxy group in position 3. Luteolin does not possess the 3-hydroxy group (Table I) and is less oxidized than the above flavonoids. Naringenin has no double bond between positions 2 and 3 in the C ring, which prevents the formation of structures equivalent to the ketone structure discussed above (Brown *et al.,* 1998; Pietta, 2000). Also it was observed that analysis of eight Egyptian propolis samples by HPLC (Table I) and GC/MS (Hegazi and Abd El Hady, 2001, 2002; Abd El Hady and Hegazi, 2002) confirmed the results obtained by Silva *et al.* (2000) who observed a structure-activity relationship of caffeic acid and derivatives. They also noted that esterification of the carboxy group of dihydrocaffeic acid dramatically enhanced the radical scavenging potency of the compound. However, similar effects were not observed with caffeic acid. The authors suggested that the *n*-alkyl esters of both phenolic series had similar potencies, and their antiradical activities are independent of the alkyl chain length. Our data are in agreement whit their data, as the IsR propolis sample which had very significant high radical scavenging activity contained a low amount of polyphenolic compounds (9.09 mg/g propolis) identified by HPLC, but contained a very high amount of dihydrocinnamate and dihydroferulate esters, 13.27 and 33.83%, respectively (Table I and Hegazi and Abd El Hady (2002).

Kitani *et al*. (1999) found that ursolic acid in herbal medicines increased the endogenous antioxidant enzyme activity in mice liver. Also our GC/MS data are in agreement with that, where samples Is and El contained low polyphenolic compounds and gave high antioxidant activity; their activity may be attributed to the ursolic acid derivatives which were identified before by GC/MS analysis (Hegazi and Abd El Hady, 2002; Abd El Hady and Hegazi, 2002).

Antiviral activity

The antiviral activity of 8 propolis samples was detected by their minimum lethal dose on embryonic chicken fibroblasts. The mean tissue culture infectivity dose that caused a cytopathic effect in

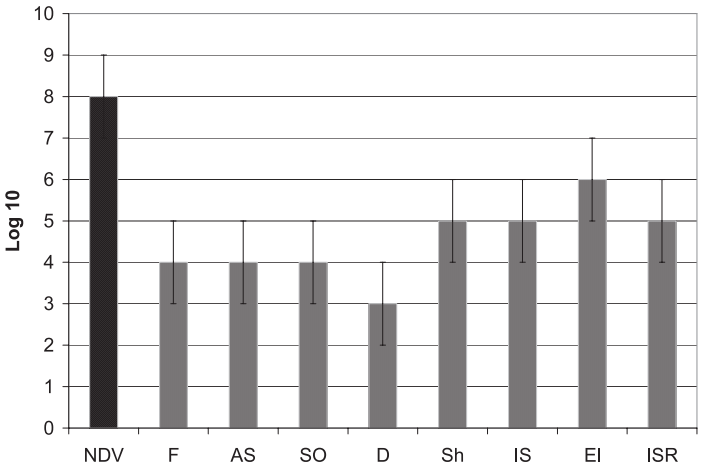

Fig. 6. Effect of propolis samples on Newcastle disease virus (NDV) infectivity mean titer.

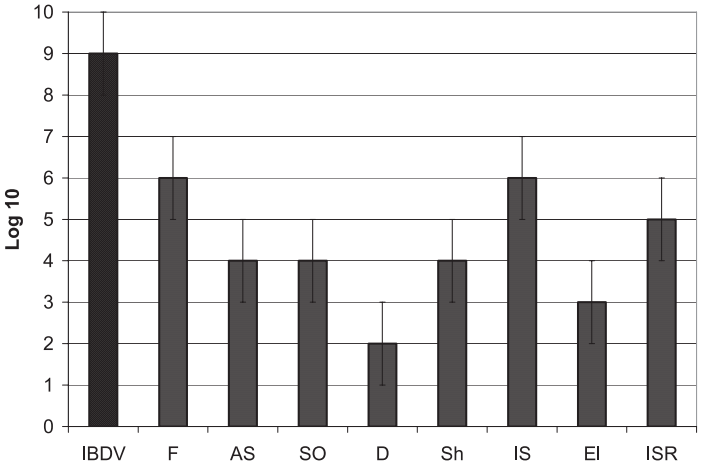

Fig. 7. Effect of propolis samples on infectious bursal disease virus (IBDV) infectivity mean titer.

50% of the cell culture ($TCID_{50}$) of Newcastle disease virus (NDV) was 3.52×10^8/ml and infectious bursal disease virus (IBDV) was 1.37×10^9/ml (Figs. 6 and 7). All propolis samples from different provinces showed reduction in the infectivity mean titers of NDV (Fig. 6) and IBDV (Fig. 7). It was obvious that the reduction varied according to the propolis origin. The highest reduction in the mean $TCID_{50}$ value against NDV was detected in the case of D propolis followed by F, As and So propolis. Also the highest reduction was observed in the case of IBDV by D propolis followed by El, As, So and Sh propolis. It was clear that propolis induced different variations in the inhibitory effect of all viral strains. Similar results were observed previously by Amoros *et al*. (1994) on herpes simplex virus, Kujumgiev *et al*. (1999) on avian influenza virus, and Hegazi *et al*. (2000) on infectious bursal disease virus and reo virus.

The infectivity of those viruses was reduced, but this reduction varied according to the propolis origin that reflected on the chemical composition of different propolis samples. These findings of the difference in the chemical composition were previously reported as considerable different in the biological activities (Kujumgiev *et al*., 1999; Hegazi *et al.,* 2000; Abd El Hady and Hegazi, 2002; Hegazi *et al*., 2003).

HPLC analysis

Quantitative comparison of the polyphenolic profile of propolis collected from 8 Egyptian governorates showed by comparison with authentic markers 42 polyphenolic compounds which are characteristics of propolis. Table I shows the results of the HPLC analysis.

13 Aromatic acids, esters and alcohols were present. Hydrocinnamic acid and hydrocaffeic acid were present with highly significant contents in the samples Is and Sh (42.55, 18.05 and 22.86, 15.70 mg/g propolis, respectively). Caffeic and cinnamic acids were highly present in the sample As (10.16, 6.14 mg/g propolis).

29 Flavonoids were identified, six of them were identified for the first time in propolis: liquiritegenin, genistein, formonontin, prunetin, formononetin and biochanin A (Table I). Myricetin and luteolin were present in significant contents in sample As (34.29, 38.74 mg/g propolis). Naringenin was present with large amount in sample Sh (14.46 mg/g propolis). Quercetin-3,3′-dimethylether and pinocembrin were highly present in samples As and Sh (10.31, 22.82 and 47.05, 39.36 mg/g propolis, respectively). Luteolin-3′-methylether and acacetin were present with and highly significant contents in sample As (12.22 and 24.52 mg/g propolis, respectively). Also it has the highest contents of quercetin-3-methylether, apigenin quercetin-7-methylether and chrysin (9.35, 7.50, 6.30 and 7.56 mg/g propolis, respectively). While sample Sh has the highest contents of formonontin and biochanin A (10.14 and 10.16 mg/g propolis, respectively), sample El showed the highest contents of dimethylallylcaffeate and galangin-7-methylether (31.09 and 10.64 mg/g propolis, respectively). These findings also were observed by Wollenwe-

ber and Buchmann (1997) who identified in propolis flavonoids typical for leaf exudates of *Ambrosia deltoidea,* among them 5,7,4′-trihydroxy-6,8-dimethoxyflavone and sideritiflavone which are also being new for propolis.

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