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Comparison of the free radical-scavenging activity of propolis from several regions of Argentina

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Abstract

Propolis is extensively used in Argentine folk medicine. Alcoholic extracts of propolis from different regions of Argentina were prepared. The extracts were analysed for the determination of total flavonoid content (from 13.3 to 42.6 mg/g of propolis) by using the aluminum nitrate method, UV spectrophotometry and thin layer chromatography. All of them contained high total flavonoid content. It was also observed that all samples of ethanolic extracts of propolis showed free radical-scavenging activity in terms of scavenging of the radical DPPH but the highest activities were found for samples from Tucumán and Santiago del Estero. In all cases with 20 μg/ml of soluble principles, the percentage of DPPH degradation was different (Banda Oeste: 67.5%; Verónica: 45%; Forres: 35%; Saenz Peña: 20% and Juan José Castelli: 55%). These results may justify their use as a source of natural antioxidants.

Keywords: Argentine propolis; Biological activities; Antioxidant activity; Free radical-scavenging activity

1. Introduction

Propolis is a natural product derived from plant resins collected by honey bees. It is used as a glue, a general-purpose sealer and a draught excluder in the construction of beehives. Propolis contains a variety of chemical compounds such as polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes, alcohols and ketones), sesquiterpene quinones, coumarins, steroids, amino acids and inorganic compounds. Propolis samples contain more than 160 constituents, and differs greatly due to variation in its geographical and botanical origin (Ghisalberti, 1979; Greenaway et al., 1991; Bonvehi et al., 1994). It is used in folk medicine all over the world. Many

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medical properties, including antiseptic, bacteriostatic, antimycotic, antiprotozoan, antiviral, spasmolytic, astringent, anti-inflammatory and immunostimulatory activities, have been ascribed to propolis. It has been suggested that the biological activities of propolis mainly depend on the presence of a large number of flavonoids (Bankova et al., 1987; Grange and Davey, 1990; Vennat et al., 1995). These are reported to have antioxidant (Scheller et al., 1990; Basnet et al., 1997), anti-inflamamtory, anticancer and antiviral activities, as well as antimicrobial effects (Rice-Evans and Packer, 1998). In addition, it was suggested that some propolis produce allergic contact dermatitis (Hausen et al., 1987, 1992). This effect was attributed to the presence of cinnamic acid and caffeic acid esters (Miyataka et al., 1997) in propolis.

The biological activities of propolis have been studied extensively in Europe, mainly in eastern countries. Some of them, such as antioxidant properties, have been found in the ethanolic extracts of propolis (Silesian propolis). Scavenging of free radicals generated by neutrophils in inflammatory processes may be an important mechanism of the anti-inflammatory effect. Acceleration of the regenerative process have been observed in clinical trials (Krol et al., 1996) after treatment with ethanolic propolis extract. Recently, Cuban red propolis has been reported to possess scavenging action against oxygen radicals as well as hepatoprotective effects (Pascual et al., 1994; González et al., 1994; Rodríguez et al., 1997).

Available literature indicates that no previous antioxidant property studies have been done on Argentine propolis and there are no reports on their chemical constituents. This is the first time that the antioxidant properties of propolis from different regions of Argentina (Tucumán, Santiago del Estero, Chaco and Buenos Aires) are described. In this study, we have used scavenging of diphenyl picryl-hydrazyl, a simple and rapid colorimetric method for free radical-scavenging activity determination and the aluminum nitrate method for total flavonoid content determinations.

2. Materials and methods

2.1. Propolis origins

Propolis were gathered from different regions of Argentina: El Paraiso (PA), La Banda Este (BE), La Banda Oeste (BO), El Molino (MO) and El Corte (CO), province of Tucumán; Cerrillos (CE), Forres (FO), Fernández (FE), province of Santiago del Estero; Roque Saenz Peña (SP), Juan Jose Castelli (CA), province of Chaco and Verónica (VR) province of Buenos Aires.

Hand collected propolis were kept desiccated and in the dark up to their processing.

2.2. Preparation of ethanolic extracts of propolis (EEP)

Ethanol extracts of 11 propolis samples were prepared and used throughout this work. Briefly, propolis was frozen at -20°C, and ground in a chilled mortar. Then, the ground powder was extracted with ethanol (15 ml of 80% ethanol/g of propolis) with continuous stirring at room temperature for 24 h. The suspension was separated by centrifuged at $27\,000 \times g$ for 20 min. The supernatant was then concentrated in a rotary evaporator under reduced pressure at 40°C and the residue redissolved in a minimal volume of 96% ethanol and kept in the dark at room temperature until use.

2.3. Reagents

Silica TLC plates containing a fluorescent indicator and all solvents were purchased from Merck (Damstadt, Germany).

All reagents used were of analytical grade. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), rutin, quercetin and aluminum nitrate were purchased from Sigma, USA.

2.4. Free radical-scavenging activity measurement

Dilutions of propolis extracts (6.58–300 μg/ml) were added to 0.5 ml of 300 μM DPPH in 96% ethanol. The mixtures were vigorously shaken and

(Mensor et al., 1997; Yamaguchi et al., 1998) left to stand at room temperature for 20 min in the dark. Absorbance at 514 nm was measured versus ethanol as a blank. Standard samples (rutin and quercetin, 30 μg/ml) were used. The degradation of DPPH was evaluated by comparison with a control (0.5 ml of DPPH solution and 1.5 ml of ethanol, $n = 5$). Results were expressed by the proportion of DPPH degradation compared with the control.

2.5. Determination of total flavonoid concentration

Flavonoid concentration was determined as follows: EEP (0.1 ml) was diluted with 80% aqueous ethanol (0.9 ml). An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M aqueous potassium acetate and 4.3 ml of 80% ethanol. After 40 min at room temperature, the absorbance was determined spec-

Table 1
Regions of propolis recollection and dominant plant species visited by bees

Propolis source	Regions of propolis recollection	Dominant plant species in each region
Paraiso (PA)	Amaicha del Valle, Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata* and *Prosopis alba*
Banda Este (BE)	Amaicha del Valle, Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata* and *Prosopis alba*
Banda Oeste (BO)	Amaicha del Valle, Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata* and *Prosopis alba*
El Molino (MO)	Amaicha del Valle, Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata* and *Prosopis alba*
El Corte (CO)	Tucumán, Argentina	*Eucalyptus* sp. and *Liquidam-bar stiracifolia*
Verónica (VR)	La Plata, Buenos Aires, Argentina	*Eucalyptus* sp.
Forres (FO)	Santiago del Estero, Argentina	*Schinus molle*, *Prosopis* sp., *Eucalyptus* sp., *Geoffrora decorticons*
Fernández (FE)	Santiago del Estero, Argentina	*Schinus molle*, *Prosopis* sp., *Eucalyptus* sp.
Cerrillos (CE)	Santiago del Estero, Argentina	*Schinus molle*, *Prosopis* sp., *Casuarina ainminghemia*, *Vallesia gloilra*
Juan José Castelli (CA)	Chaco, Argentina	*Prosopis* sp., *Cercidium australis*, *Schinopsis* sp., *Opuntia* sp.
Saenz Peña (SP)	Chaco, Argentina	*Schinopsis* sp., *Cercidium australis*

Table 2
Propolis extraction with 80% ethanol

Propolis source	Propolis weight (g)	Soluble principles (g) in 80% (v/v) ethanol	Yield (%, w/w)	Extract concentration (g/100 ml)
PA	10.16	6.6	65	38.82
BE	9.17	5.45	59	32.06
BO	7.18	3.71	52	30.92
MO	7.31	3.77	52	31.42
CO	24.86	10.35	42	15.44
VR	20.00	10.80	54	36.00
FO	33.10	16.81	51	30.56
FE	24.56	12.68	52	27.56
CE	35.22	16.88	48	29.10
CA	41.38	12.94	31	17.97
SP	51.80	21.71	42	24.12

Table 3
Total flavonoid amount in propolis samples

Source of propolis	Group	Total flavonoid concentrations* (mg/g of propolis**)
PA	I	30.3 ± 0.67
BE	I	39.3 ± 0.66
BO	I	42.6 ± 0.80
MO	I	37.6 ± 0.75
CO	II	20.0 ± 0.62
VR	II	17.5 ± 1.11
FO	III	19.0 ± 0.70
FE	III	20.3 ± 1.02
CE	III	20.0 ± 0.72
CA	IV	15.3 ± 0.99
SP	V	13.3 ± 0.90

* Values represent mean of six determinations ± the standard deviations.

** Milligrams of total flavonoid content/g of propolis based on quercetin as standard.

trophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard (Park et al., 1997).

2.6. Thin-layer chromatography (TLC) of EEP

Samples of 5 μl (1/10 dilution of EEP) of each solution were spotted on the silica plates. Two development systems were used: toluene:chloroform:acetone (40:25:35, v/v) and hexane:ethyl acetate:acetic acid (60:40:3, v/v). The detection of flavonoid was carried out using 1% $FeCl_3$ (w/v).

3. Results and discussion

Propolis samples from different phytogeographical formations were collected. Table 1 shows the collecting regions and prevalent plant species visited by bees. Propolis samples were extracted with 80% ethanol. Table 2 shows the yield of ethanol soluble principle extractions. All samples of EEPs were analyzed for total flavonoid content by the method of aluminum nitrate. As shown in Table 3, the flavonoid content of the different samples varied between 13 and 42.6 mg/g of propolis. All samples of EEP were examined by TLC. According with TLC patterns the EEPs (Fig. 1) were classified in five different groups. The first being those from Amaicha del Valle, Tucumán (PA, BO, BE and MO) which showed similar TLC patterns. Group II is composed by propolis from El Corte and Verónica (CO, VR). They also showed similar TLC patterns and these patterns are different from those of group I. Probably; this can be attributed to a different plant ecology. In this way *Eucalyptus* sp. is present in both regions. Group III being the propolis from Santiago del Estero (CE, FO and FE), group IV constitutes those from Juan José Castelli (CA) and Group V from Roque Saenz Peña (SP), these two last localities of Chaco province. Our results demonstrate that the qualities and quantities (Table 3) of

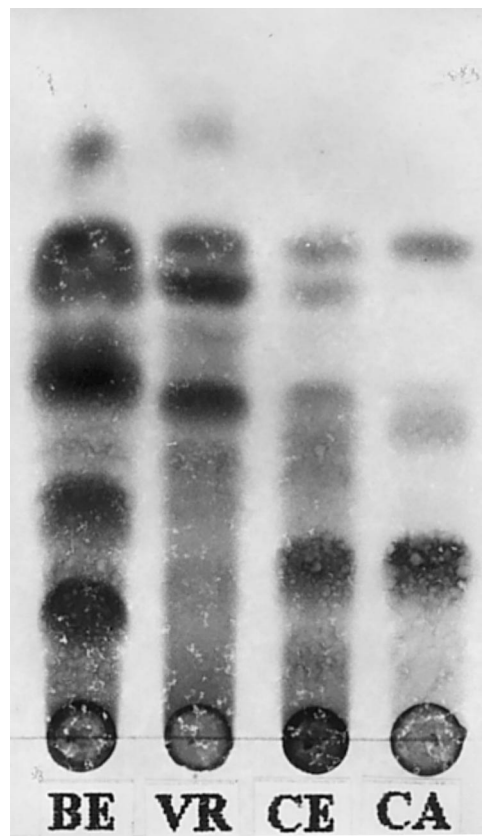

Fig. 1. Comparison of the thin layer chromatography (TLC) profiles of propolis extracts from: Banda Este (BE); Verónica (VR); Cerrillos (CE) and Juan José Castelli (CA). Samples of 5 μl of 1/10 dilution of each propolis extract were seeded in each position.

Table 4
Degradation of 1,1-diphenyl-2-picrylhydrazyl (DPPH) by propolis extracts from different regions

Propolis source	Group	DPPH degradation* (%) ± S.D.
PA	I	50.0 ± 2.8
BE	I	62.5 ± 2.3
BO	I	67.5 ± 1.5
MO	I	62.5 ± 1.2
CO	II	52.5 ± 2.3
VR	II	45.0 ± 2.0
FO	III	35.0 ± 1.8
FE	III	45.0 ± 1.9
CE	III	57.5 ± 2.1
CA	IV	55.0 ± 2.4
SP	V	20.0 ± 1.1

* The concentrations of propolis extracts in all cases were 20 μg/ml. 2.5 μg/ml of rutin and 1.5 μg/ml of quercetin were necessary to obtain 25% of DPPH degradation.

flavonoids are different among the provinces of the northwest of Argentina. Consequently, a comparative study of free radical-scavenging activity of this propolis was undertaken.

In order to evaluate the free radical-scavenging activity of EEP we used a method based on the reduction of DPPH, a stable free radical. Table 4 summarizes the percentage of DPPH degradation with 20 μg/ml of material extracted from different propolis. All propolis studied showed free radical-scavenging activity. The free radical-scavenging activities of rutin and quercetin were higher than the activities of the assayed propolis extracts (2.5 μg/ml for rutin and 1.5 μg/ml of quercetin were necessary to obtain 25% of DPPH degradation). The highest activity was shown by samples from Amaicha del Valle (MO, BO, BE, PA), Tucumán and Santiago del Estero (CE, FO, FE) (7.5 μg/ml of propolis extracts were necessary to obtain 25% of DPPH degradation). Propolis from Roque Saenz Peña (SP), a region of Chaco appears less active than the other samples (31.25 μg/ml of propolis extract were necessary to obtain 25% of DPPH degradation). Fig. 2 shows the dose-response curve for the free radical-scavenging activity of BE (Group I), VR (Group II), CE (Group III), CA (Group IV) and SP (Group V).

These findings demonstrate that the antioxidant activity is correlated with the propolis chemical composition and this with the plant ecology. Linear regression for the data in Tables 3 and 4 reveals that the flavonoid content explains about

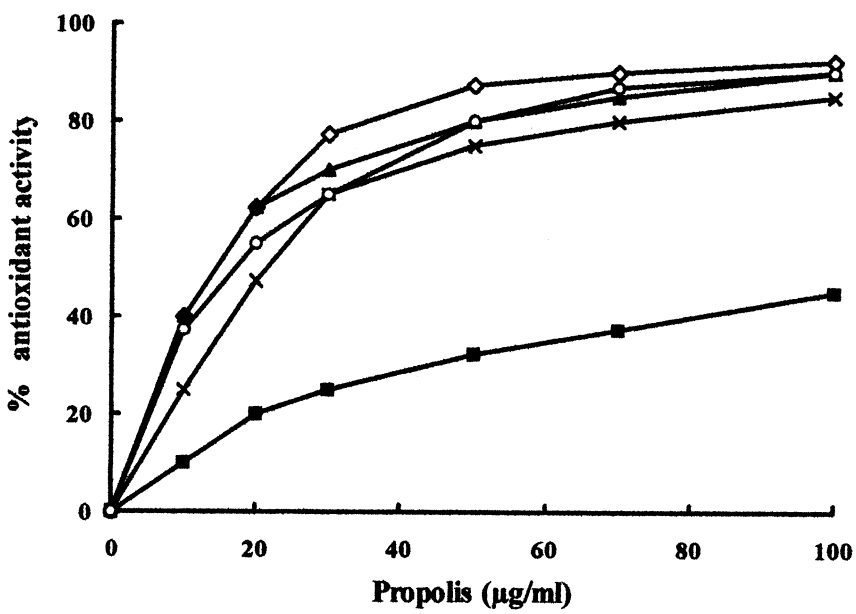

Fig. 2. Free radical-scavenging activity of propolis from: Banda Este (BE) (◇); Cerrillos (CE) (▲); Verónica (VR) (○); Roque Saenz Peña (SP) (■) and Juan José Castelli (CA) (×). The propolis amount was contained in1.5 ml of volume sample.

half of the difference in activity ($R^2 = 0.53$). The correlation between flavonoid content and scavenging activity is significant (P for trend < 0.025), but obviously, other factors are involved. These might be different flavonoid composition or the presence of non-flavonoid scavengers. Our results also show that all propolis analyzed possess free radical-scavenging activity which justify their use as a source of natural antioxidants.

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