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Journal of Ethnopharmacology 76 (2001) 165–170

Journal of ETHNO-PHARMACOLOGY

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Antioxidant activity of Argentine propolis extracts

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Received 22 February 2000; received in revised form 22 February 2001; accepted 14 March 2001

Abstract

Propolis is used in Argentine folk medicine. We have examined its possible protective action against oxidative modification of lipid in unfractionated serum. The kinetics of copper-induced oxidation was continuously monitored by measuring the formation of conjugated dienes, as the increase in the absorbance at 234 nm. According to the kinetics of oxidation, the propolis were classified in three different groups. Group I (CE, CO, BO, MO, BE) inhibited lipid oxidation during the initiation and propagation phases even at low concentrations. Group II (SP, CA, AM) increased the lag-phase for conjugated diene formation. All propolis in groups I and II diminished the maximal rate of diene production and the maximal amount of dienes produced. Group III (PA, RA, FE, VR, TV) had no effect on the lipid oxidation. The extent of lipoprotein oxidation was measured by the thiobarbituric acid reactive substance assay. Generation of malondialdehyde–like substances was inhibited and delayed by the presence of propolis extracts from group I and II. Our results justify the use of propolis (groups I and II) as a source of natural antioxidants.

Keywords: Antioxidants; Propolis; Low density lipoproteins (LDL); High density lipoproteins (HDL); Very low density lipoproteins (VLDL)

1. Introduction

Propolis have been used in folk medicine to maintain health. Pharmacological activities such as anticancer (Matsuno, 1995), antiinflammatory (Wang et al., 1993), antibiotic (Grange and Davey, 1990; Park et al., 1998; Nieva Moreno et al., 1999; Koo et al., 2000), antioxidative (Scheller et al., 1990; Yamauchi et al., 1992; Pascual et al., 1994; Basnet et al., 1997; Nieva Moreno et al., 2000), antiviral (Manolova et al., 1985; Ghisalberti, 1979; Amoros et al., 1992; Marcucci, 1995; Kujumgiev et al., 1999), antifungal (Kujumgiev et al., 1999), anaesthetic and cytostatic (Ghisalberti, 1979) have been ascribed to ethanolic extracts of propolis. The biological activities of propolis have been studied extensively in Europe but only a few reports can be found on Argentine propolis. Analysis of Argentine propolis alcoholic extracts showed the presence of antibacterial and free radical-scavenging activities (Nieva Moreno et al., 1999, 2000). Epidemiological studies showed that the consumption of fruits and vegetables is related to reduced risks of cancer and cardiovascular diseases (Steinmetz and Potter, 1991; Criqui and Ringel, 1994). The flavonoid content of foods may be a major dietary factor responsible for this effect (Hertog et al., 1993, 1994; Knekt et al., 1996, 1997).

Flavonoids may reduce free radical formation and consequently might have a protective effect on serum lipids against oxidation. Argentine propolis contain high total flavonoid levels and the correlation between this and free radical-scavenging activity is significant (Nieva Moreno et al., 2000). The healthy function of the brain, cardiovascular system, heart, and most organs depends on the unobstructed flow of blood for the delivery of oxygen and nutrients and the removal of harmful metabolites and waste. Cholesterol and other fats cannot dissolve in the blood; they need to be transported in the blood stream by special carriers called lipoproteins. Among them, two of great importance are low-density lipoprotein (LDL), which is the

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0378-8741/01/$ - see front matter
PII: S0378-8741(01)00231-8

major carrier of cholesterol in the blood, and high-density lipoprotein (HDL), which carries near one-third to one-quarter of blood cholesterol. Atherosclerosis, is characterized at an early stage by macrophage cholesterol accumulation and foam cell formation (Aviram et al., 1998). The cholesterol accumulated in these cells is mainly derived from LDLs which had undergone oxidative modification (Steinberg et al., 1989; Aviram, 1995a,b; Berliner et al., 1995; Aviram, 1996). Thus, inhibition of LDL oxidation is considered to be antiatherogenic. Very low density lipoprotein (VLDL) and HDL oxidation also occurs during oxidative stress (Parthasarathy et al., 1989; Keidar et al., 1992; Kita et al., 1992; Nagano et al., 1991) and may also contribute to atherogenesis. LDL particles contain endogenous antioxidants including α and γ tocopherols, β carotene, lycopene, and retinyl stearate (De Whalley et al., 1990; Jessup et al., 1990; Frei and Gaziano, 1993). LDL oxidation by copper ions in vitro exhibits a lag phase corresponding to the time required for the endogenous antioxidants in LDL to be consumed (De Whalley et al., 1990; Jessup et al., 1990). Exogenous antioxidants can protect the lag phase or even prevent LDL oxidation in vitro (Negre-Salvayre and Salvayre, 1992).

In this work we describe the protective action of Argentine propolis against copper-mediated oxidative modification of lipids in unfractionated serum.

2. Materials and methods

2.1. Propolis origins

Propolis were gathered from different regions of Argentine: Arroyo Mixta (AM), Raco (RA), Tafí del Valle (TV), El Paraiso (PA), La Banda Este (BE), La Banda Oeste (BO), El Molino (MO) and El Corte (CO), province of Tucumán; Cerrillos (CE), Fernández (FE), province of Santiago del Estero; Roque Saenz Peña (SP), Juan José 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)

Ethanolic extracts of 13 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 centrifugation at 27 000 × *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 at room temperature in the dark until use.

2.3. Reagents

Silica TLC plates containing a fluorescent indicator and all solvents were purchased from Merck (Damstadt, Germany). $CuCl_2$ was purchased from Sigma, USA. All reagents used were of analytical grade.

2.4. Measurement of absorption spectra and total flavonoid concentration

The absorption spectra of the propolis extracts were measured in a Beckman spectrophotometer DU 650.

Flavonoid concentration was determined according with Nieva Moreno et al. (2000).

2.5. Collection of blood samples

Blood was obtained by venepuncture of a forearm vein from 12 h fasted individuals. Samples were received into tubes without anticoagulant and centrifuged at 1000 × *g* for 20 min at 4°C. Serum was aspirated and used immediately for the assays. Excess serum was stored at −20°C until required. Cholesterol content, HDL-cholesterol, LDL-cholesterol and triglycerides were determined using the commercially available kit of Wiener Lab., Rosario, Argentina.

2.6. Protein determination

Protein concentrations in serum preparations were determined according to Lowry et al. (1951). Protein content of LDL was determined using the commercially available kit of Wiener Lab., Rosario, Argentina.

2.7. Lipid oxidation in unfractionated serum

Serum preparations containing 1.23 mg of protein/ml; 0.035 mg of LDL-cholesterol/ml; 0.04 mg of protein of LDL/ml in 10 ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl) were incubated with or without $CuCl_2$ (final concentration 11.7 mM) with or without propolis extract (25 and 110 μg/ml). Oxidation was terminated by the addition of 100 μmol of EDTA or 10 μM butylated hydroxytoluene (BHT) and refrigeration at 4°C (Aviram et al., 1998).

2.8. Measurement of the kinetics of copper-induced oxidation

The kinetics of copper-induced oxidation was continuously monitored by measuring the formation of conjugated dienes. The increase in the absorbance at 234 nm

(Esterbauer et al., 1989) was assayed at 37°C during 130 min in a Beckman UV-visible recording spectrophotometer. Lag times (min) were determined from the intercept of lines drawn through the linear portions of the lag phase and propagation phase. The maximal propagation rate (expressed as Molar concentration of dienes produced per minute) was obtained from the slope of the absorbance curve during the propagation phase. The molar absorptivity $\varepsilon_{234\ nm}$ of 29 500 M^{-1} cm^{-1} for conjugated dienes was used (Abuja et al., 1998). Maximum diene concentration (expressed as M 10^5) was calculated from the difference in absorbance at zero time and at the end of the propagation phase.

2.9. Thiobarbituric acid reactive substance (TBARS) assay

The extent of lipoprotein oxidation mediated by $CuCl_2$ addition was measured by the thiobarbituric acid reactive substance (TBARS) assay. Malondialdehyde (MDA) was used as standard (Buege and Aust, 1978). After oxidation with $CuCl_2$, for different times, serum samples (containing 1.23 mg of protein/ml) were mixed with 1 ml of reagent (15% trichloroacetic acid (w/v); 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid), and then heated for 15 min in a boiling water bath. After cooling and centrifugation at $1000 \times g$ for 10 min, the absorbance at 535 nm was determined in the supernatant. The MDA concentration of the sample was calculated using an extinction coefficient of 1.56×10^5 mol^{-1} cm^{-1} (Laplaud et al., 1997).

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

Regions of propolis recollection	Dominant plant species in each region
Tafí del Valle (TV) Tucumán, Argentina	*Budleja tucumanses*, *Baccharis salicifolia*, *Baccharis conidifolia*, *Chuquiraga* sp., *Prosopis* sp.
Arroyo Mixta (AM) Tucumán, Argentina	*Schinopsis* sp., *Apidosperma quebracho blanco*, *Cercidium* sp.
Raco (RA) Tucumán, Argentina	*Tipuana tipu*, *Solanum riparicum*, *Cupania vernalis*, *Rapanea laetevirens*
Paraiso (PA) Amaicha del Valle Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata*, *Prosopis alba*
Banda Este (BE) Amaicha del Valle Tucumán, Argentina	*Larrea cuneifolia*, *Larrea divaricata 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*, *Prosopis alba*
El Corte (CO) Tucumán, Argentina	*Eucalyptus* sp., *Liquidambar stiracifolia*
Verónica (VR), La Plata Buenos Aires, Argentina	*Eucalyptus* sp.
Fernández (FE) Santiago del Estero, Argentina	*Schinus molle*, *Prosopis* sp., *Eucalyptus* sp
Cerrillos (CE) Santiago del Estero, Argentina	*Schinus molle*, *Prosopis* sp., *Casuarina* sp.
Juan José Castelli (CA) Chaco, Argentina	*Prosopis* sp., *Cercidium australis*, *Schinopsis* sp., *Opuntia* sp.
Saenz Peña (SP) Chaco, Argentina	*Schinopsis* sp., *Cercidium australis*

3. Results and discussion

A series of experiments were performed in an attempt to assess a possible protective action against oxidative lipid modification for Argentine propolis. Propolis samples from different phytogeographical formations were collected. Table 1 shows the collecting regions, and prevalent plant species visited by bees. Propolis contain a wide variety of phenolic compounds, mainly flavonoids (García-Viguera, 1992; Bonvehi et al., 1994; Park et al., 1997; Koo and Park, 1997; Martos et al., 1997; Koo et al., 1999). Propolis flavonoid patterns were attributed to the preferred foraging plants used by the bee colonies. Propolis extracts containing the same content of dry matter were scanned in an extended spectrum of wavelengths between 200–500 nm. The appearance of absorption at 270–330 nm was demonstrated to be attributable to flavonoids and phenols, in general (Markham, 1982). The obtained flavonoid absorption spectra showed similar positions for their absorbance peaks, with maximum absorption at 290 nm (not shown). AM, RA and TV propolis extracts showed higher flavonoid content (Table 2) than those from other phytogeographical regions.

Serum dilution in copper-containing media at 37°C resulted in lipid oxidation and in a consequent increase of absorbance of UV light. The absorbance at this wavelength (234 nm), usually attributed to conjugated dienes, is in fact a sum of absorbances of dienic and 7-keto cholesterol hydroperoxides. Such oxidation did not occur when the diluting media contained no copper ions (Martos et al., 1997). The propolis effect was examined during initiation and propagation phases. The kinetics of oxidation has been monitored by continuous recording of absorbance at 234 nm and analyzed in terms of the preceding oxidation lag, the maximal rate of accumulation of absorbing products (V_{max}) expressed in mmol per min and the maximal concentration of absorbing products ($M \times 10^5$) (Table 3). According to the kinetics of oxidation, propolis were classified in three different groups. The first group was those from CE, CO, BO, MO, and BE which inhibited lipid oxidation during the initiation and propagation phases even at low concentration (Fig. 1). Group II is composed by propolis from AM, CA, and SP which increased the lag-phase for conjugated diene formation. Indeed starting from 10 min (control experiments), the

Table 2
Inhibition of malondialdehyde production and total flavonoid concentration of propolis extracts

Group	Extract	Total flavonoid concentrations (mg/g of propolis)[a]	Inhibition of malondialdehyde production (%)[b]
I	BO	42.7 ± 0.80	40
I	BE	39.3 ± 0.66	45
I	MO	37.6 ± 0.75	43
I	CE	20.0 ± 0.72	20
I	CO	20.0 ± 0.60	20
II	AM	62.0 ± 0.99	80
II	CA	15.3 ± 0.99	50
II	SP	13.3 ± 0.90	20
III	RA	50.0 ± 1.01	20
III	TV	45.0 ± 0.99	20
III	PA	30.3 ± 0.67	45
III	FE	20.3 ± 1.02	20
III	VR	17.5 ± 1.11	45

[a] mg of total flavonoid content/g of propolis. Values represent mean of six determinations ± the standard deviations.
[b] Values represent mean of five determinations.

Table 3
Effect of propolis from Group II on Cu-induced lipid oxidation[a]. Conjugated diene formation was measured as change in absorbance at 234 nm during Cu-induced lipid oxidation

Parameter	Control	AM	CA	SP
Lag time (min)	10	15	20	30
Maximal oxidation rate (mmol/min 10^5)	13.6	2.9	7.5	1.9
Maximal concentration of conjugated dienes ($M \times 10^5$)	1.59	1.08	1.18	0.71

[a] Group I propolis inhibited serum lipid oxidation during initiation and propagation phases. Otherwise, Group III propolis had not effect on serum oxidation.

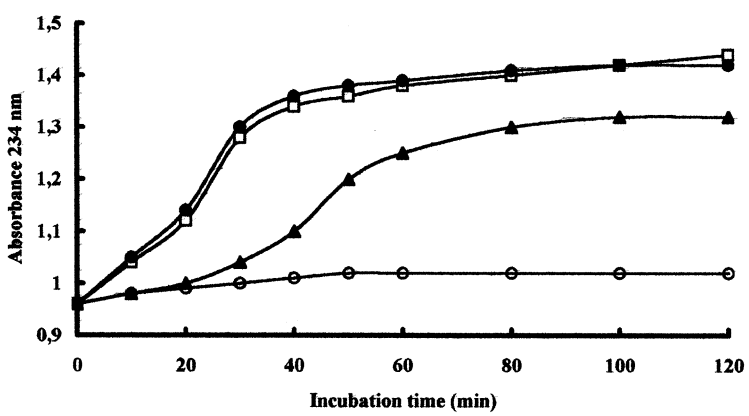

Fig. 1. Inhibition of Cu-induced diene-formation by ethanolic propolis extracts. Propolis can affect the lag and/or propagation phase duration. Oxidative modification of lipids was carried out by incubation with $CuCl_2$ at 37°C without (●) and with propolis extracts: (□) FE, group III; (▲) SP, group II; (○) CE, group III. Diene-formation was determined by recording absorption at 234 nm.

lag-phase increased to 15, 20 or 30 min in the presence of 72 μg/ml of AM, CA and SP propolis extracts, respectively. All these types of propolis analyzed diminished the maximal rate and maximal amount of diene production. Group III, being the propolis from FE, PA, TV, RA and VR had no effect on serum lipid oxidation (Fig. 1).

Generation of malondialdehyde–like substances was inhibited and delayed by the presence of the propolis extracts (Table 2). Malondialdehyde concentration measured after 1 h of serum incubation in the presence of $CuCl_2$ and of 50 μg/ml of CO, RA, TV, SP, FE, CE propolis extracts was only 20% of those observed in the control experiment. Otherwise, propolis from Amaicha del Valle, Castelli and Verónica inhibited 40–50% of MDA production. The AM propolis (Fig. 2) was the most active (80% inhibition). It inhibited lipid oxidation during the initiation (0 min) and propagation (20 or 30

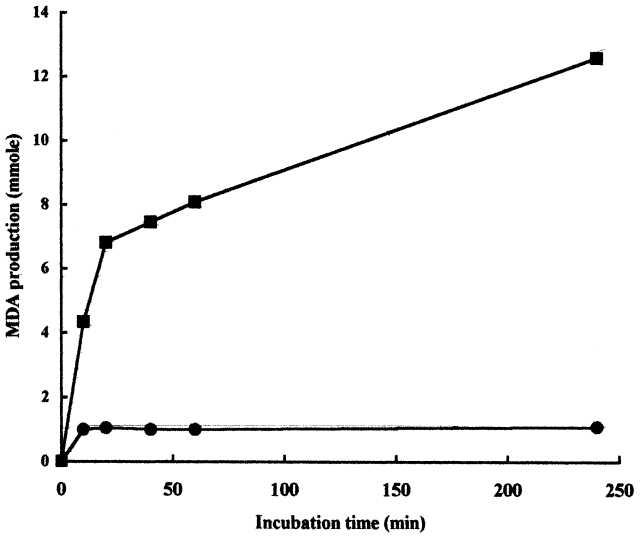

Fig. 2. Effect of the incubation time on serum lipid oxidation. Cu-induced malondialdehyde production was measured in the presence (●) and absence (■) of propolis extract from Arroyo Mixta (AM).

min) phases even at low concentrations of soluble principles.

Our results demonstrate that propolis extracts (Groups I and II) exhibit potent antioxidant activity protecting serum lipids from copper-induced modification in vitro, and reveal a marked dose-response effect. Otherwise, a positive correlation between flavonoid content and % of inhibition of MDA production was observed for group I ($R^2 = 0.96$) and group II ($R^2 = 0.88$). However, though RA and TV extracts have a high flavonoid content, they belong to group III which has no effect on peroxidation. This result suggests that other factors, in addition to flavonoid content, would be involved in the propolis antioxidant activity.

In the absence of pharmacokinetic data on the intestinal absorption and/or metabolic fate of these extracts (multi-component preparations of natural origen), we can only speculate with respect to its actual antioxidant effect under physiological or hyperlipidemic conditions in vivo in humans.

Acknowledgements

This research was partially supported by the Consejo de Investigaciones de la Universidad Nacional de Tucumán, Argentina, and by the Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina.

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