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Assessment of the antioxidant activities of Brazilian extracts of propolis alone and in topical pharmaceutical formulations

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Abstract

The antioxidant activity of extracts of propolis and of formulations added with these extracts were measured by scavenging different radicals in different systems. For the ethanolic extract of propolis (EEP) and the glycolic extract of propolis (GEP) the IC_{50} observed were respectively of 0.024 and 0.035 μL/mL in scavenging hydroxyl radical, 0.016 and 0.012 μL/mL in inhibiting lipid peroxidation, 0.22 and 0.24 μL/mL in inhibiting chemiluminescence produced in the H_2O_2/luminol/horseradish peroxide (HRP) system and about 0.005 μL/mL for both extracts in inhibiting chemiluminescence produced in the xanthine/luminol/xanthine oxidase (XOD) system.

The antioxidant activity of extracts of propolis in the formulations was not able to be assessed neither using the deoxyribose assay, since the formulation components interfered in the assay measurements, nor using chemiluminescence in the H_2O_2/luminol/HRP system, since this method did not show to be sensitive for the extract of propolis evaluation. However, the antioxidant activity of extracts of propolis could be successfully evaluated in the formulations using both lipid peroxidation and chemiluminescence generated in the xanthine/luminol/XOD system inhibitions.

Keywords: Propolis; Antioxidant; Topical formulation; Chemiluminescence; Lipid peroxidation; Deoxyribose assay

1. Introduction

Skin is a biological interface with the environment and functions as the first line of defense against noxious external stimuli such as ultraviolet, visible irradiation, prooxidant chemicals, infection and ionizing radiation [1].

However, by acute or chronic exposure to UV light the skin prooxidant/antioxidant equilibrium can be overwhelmed due to severe decrease of its antioxidant content and to striking formation of reactive oxygen species (ROS). This way the ROS generated in excess can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA. So the deleterious effects of sunlight and particularly UV radiation on the skin can lead to a variety of ravages as inflammation, skin aging, tumour promotion, cutaneous auto-immune disease, and phototoxicity/photosensitivity [1].

Topical administration of antioxidants provides an efficient way to enrich the endogenous cutaneous protection system, and thus may be a successful strategy for diminishing ultraviolet radiation-mediated oxidative damage in skin [2].

Extracts of propolis are receiving renewed attention worldwide because of their beneficial effects, among them, the effective antioxidant activity and a general “back to nature trend”. Propolis typically conserts of waxes, resins, water, inorganics, phenolics and essential oils [3], the exact composition of which is dependent upon the source plant(s).

Despite the chemical differences, it is well known that samples of different geographical origin and chemical com-

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position usually demonstrate similar pharmacological activity. Propolis has shown pharmacological activities such as antioxidant [4–9], antiviral, antibacterial, antifungal, antiamoebic [3,10,11], antiinflamatory [9], etc. This fact has led some researchers to the assumption that biological and biochemical test are the best way to standardization and evaluation of propolis. Among these tests, the assessment of the antioxidant activity by different methodologies, using enzymatic or non-enzymatic methods, it would be indicated [12].

Knowing that propolis has high antioxidant activity, it would be very important the development of topical formulations added with extracts of propolis. Also the antioxidant activity of these formulations should be accordingly assessed, using several methodologies, in order to choose the most adequate one. So raises the necessity to be concerned about formulations, since one of most challenging tasks in evaluating topical formulations is to deal with the presence of the formulations compounds that may cause interference if using a non specific method.

So, one of the objectives of this work was standardization of ethanolic and glycolic extracts of propolis using enzymatic or non-enzymatic antioxidant methodologies performed against different radicals and in different systems. It was also intended the development of different topical formulations added with extract of propolis and evaluation of these formulations using the same methods for the extracts, in order to verify, if the antioxidant activity is lost in the presence of the formulations compounds or if these compounds interfere with the method of evaluation. This way it will be possible to choose the most suitable method to perform the quality control and stability studies of the antioxidant activity of formulations added with propolis extract.

2. Materials and methods

2.1. Chemicals

Brazilian extracts of propolis were purchased from APIS FLORA (Ribeirão Preto, SP, Brazil. The extracts were standardized using propolis from several sites of Brazil. Patent number PI 0405483-0, published in Revista de Propriedade Industrial no. 1778 from 01/02/2005). One of these extracts was ethanolic (EEP) and the another one was glycolic (GEP). The GEP was obtained from the EEP after evaporating the ethanolic portion and adding propylene glycol in the same amount. Luminol, horseradish peroxidase (HRP), thiobarbituric acid (TBA), xanthine, xanthine-oxidase (XOD) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), hydrogen peroxide 36% was purchased from Calbiochem (California, USA), deoxyribose and quercetin were purchased from Acros (New Jersey, USA), Gallic Acid and Folin–Ciocalteu were purchased from Merck (Darmstadt, Germany). All other chemicals were of reagent grade and were used without further purification.

2.2. Total polyphenol and flavonoid contents in the Brazilian extracts of propolis

Total polyphenol contents in Brazilian extracts of propolis were determined by the Folin–Ciocalteau colorimetric method. 0.5 mL of EEP or GEP solution was mixed with 0.5 mL of the Folin–Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenol contents were expressed as mg/g (gallic acid equivalents) [13].

Total flavonoid contents were determined using the aluminium chroride colorimetric method. To 0.5 mL of EEP or GEP solution, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin (mg/g) from an analytical curve. The amount of 2% aluminium chloride was substituted by the same amount of distilled water in blank [13].

2.3. Test formulations

All the raw materials for the formulations were purchased from Galena (Campinas, SP, Brazil) or were a gift from Clariant (São Paulo, SP, Brazil). Emulsions were developed varying the emulsifying agent and in formulation 3 the anionic hydrophilic colloid (carboxypolymethylene, Carbopol® 940) was also added as a stabilizing agent. Macadamia nut oil, isodecyl oleate and isopropyl palmitate were added as emollient, and glycerol as a moisturizer. The preservative was a mixture of phenoxyethanol and parabens. Deionized water was used for the preparation of all formulations (Table 1). Extracts of propolis (2.5%) and preservatives were firstly solubilized in propylene glycol and next incorporated to the formulations at room temperature.

Table 1
Percent composition (w/w), of the emulsion media of the formulations

Component	*F*1 (%)	*F*2 (%)	*F*3 (%)
Hostacerin® SAF[a]	–	–	–
Croda®[b]	–	8	2
Isodecyl oleate	0.5	1	0.5
Isopropyl palmitate	0.5	1	1
Macadamia nut oil	1.5	2	–
Propylene glycol	5	5	5
Glycerol	–	3	2
Carbopol® 940 (dispersion 2%)	–	–	40
Phenoxyethanol and parabene	0.5	0.5	0.5
Water	86.6	79.5	49

[a] Self-emulsifying agent prepared without heating (ammonium acryloyldimethyl-taurate/VP copolymer + rapeseed oil sorbitol esters + trilaureth-4 phosphate + mineral oil + isopropyl palmitate).

[b] Self-emulsifying wax (mineral oil + petrolatum + lanolin alcohol + fatty alcohol + ethoxilated fatty alcohol).

2.4. Preparation of samples

Five hundred microlitres of each extract of propolis was solubilized with propylene glycol and diluted using the medium of each reaction to final concentrations of 0.4, 0.3, 0.2, 0.1, 0.5, 0.025, 0.0125, 0.00625, and 0.00325 μL/mL, for the following methodologies: inhibition of lipid peroxidation assay; H_2O_2/luminol/HRP chemiluminescence assay; and the deoxyribose assay. For the xanthine/luminol/XOD chemiluminescence assay the final concentrations were 0.15, 0.075, 0.045, 0.037, 0.03, 0.019, 0.0093, and 0.0047 μL/mL. Formulations containing 2.5% of ethanol extract of propolis or glycolic extract of propolis and propolis-free formulations, were diluted 1:5 with the medium of each reaction. Next, the antioxidant activities found in the formulations were compared to the extracts of propolis in the same final concentration.

2.5. Inhibition of lipid peroxidation assay

Ten microlitres of each sample was added to 1.0 mL of a reaction mixture sucrose (125 mM), KCl (65 mM) and Tris–HCl (10 mM) (pH 7.4; medium I), and mitochondria was added to yield a final concentration of 1 mg of protein. Then, plus 50 μM ferrous ammonium sulfate and 2 mM sodium citrate was added and the samples were incubated at 37 °C for 30 min. Mitochondria was isolated by differential centrifugation from livers of male Wistar rats as described for Pedersen et al. [14] and mitochondrial protein content was determined by the biuret reaction [15]. For TBA-reactive compounds determination, 1 mL of 1% thiobarbituric acid (TBA) (prepared in 50 mM NaOH), 0.1 mL of NaOH (10 M) and 0.5 mL of H_3PO_4 (20%) were added, followed by incubation for 20 min at 85 °C. The TBA-reactive compounds were extracted with 2mL of *n*-butanol. The samples were then centrifuged at $9800 \times g$ for 10 min. The measurement was performed on the supernatant at 535 nm [16]. The amount of TBA-reactive compounds were evaluated as malondialdehyde (MDA), and was calculated from $\varepsilon = 1.56 \times 10^5$/M.

2.6. Chemiluminescence assay

2.6.1. H_2O_2/luminol/HRP system

Changes of chemiluminescence intensity of the H_2O_2/luminoL/HRP system were measured as follows: 10 μL of each sample was mixed with phosphate buffer (0.1 M; pH 7.4) (medium II), and a 2 mg/mL luminol solution in DMSO was added to yield a final concentration of 1.13×10^{-4} M. H_2O_2 was then added to a final concentration of 5×10^{-5} M. The reaction was started by adding HRP at a final concentration of 0.2 IU/mL, yielding final volume of 1 mL of solution [17]. Chemiluminescence was measured for 10 min at 25 °C with an Autolumat LB 953 apparatus.

2.6.2. Xanthine/luminol/XOD system

Chemiluminescent mixture was prepared immediately before analysis by mixing: 400 μL glycine buffer (0.1 M pH 9.4, 1 mM EDTA) (medium III), 150 μL xanthine (6 mM in glycine buffer), 10 μL sample, 10 μL of luminol (0.6 mM). Adding 100 μL xanthine-oxidase (XOD) solution (20 IU/mL) started the reaction. The buffer and the xanthine solution were stable for 4 and 2 weeks, respectively, when kept at 4 °C, while the XOD and luminol solutions were freshly prepared each time. Chemiluminescence was measured for 5 min at 25 °C with an Autolumat LB 953 apparatus [18].

2.7. Deoxyribose assay

The degradation of the deoxyribose by the hydroxyl radical was evaluated by mixing: 1 mL of KH_2PO_4-KOH buffer (20 mM, pH 7.4) (medium IV), 10 μL ascorbate (100 μM), 10 μL sample, 10 μL deoxyribose (2.8 mM), 10 μL H_2O_2 (1 mM), 10 μL of Fe-EDTA solution ($FeCl_3$ 100 μM, EDTA 104 μM). Reaction mixtures were incubated at 37 °C for 30 min, next it was added 1 mL tiobarbituric acid (1%), 100 μL of NaOH (10 M) and 500 μL H_3PO_4 (20%), and the reaction mixtures were incubated at 85 °C for 20 min. Solutions of $FeCl_3$ and ascorbate were made up immediately before use in deaerated water. The measurements were made at 535 nm at room temperature [19].

2.8. Statistical analysis

Data were expressed as mean ± S.E. determined of triplicate or duplicate analysis. The percentage which caused 50% of inhibition of the system assessed (IC_{50}) by the extracts of propolis were determined using GraphPad Prism® software. Extracts of propolis data and formulations containing these extracts data were evaluated using Student's *t*-test, at *P*-values < 0.05 determined significant differences among means.

3. Results and discussion

A series of experiments were performed in an attempt to evaluate the antioxidant activity of two extracts of propolis in order to show the ability of these extracts in scavenging different radicals in different systems. It was also intended to assess if the antioxidant activity expected for both extracts of propolis alone could be measured in the presence of the formulation components.

The antioxidant activity may be related to polyphenol and flavonoid content since it has been reported that these phenolic compounds can act breaking the chain reaction of lipid [20], inhibiting chemiluminescence reactions [21,22], scavenging several ROS [23], etc.

The Folin–Ciocalteau method and the $AlCl_3$ coloration, to determine the total polyphenol and flavonoid contents,

Table 2
Polyphenol and flavonoid content in Brazilian extracts of propolis

Extract	Polyphenol content (mg/g)	Flavonoid content (mg/g)
EEP	13.3	4.7
GEP	13.33	4.79

respectively, are currently used to analyze plants and food materials. In the present study, these methods were applied to determine total polyphenol and flavonoid contents of two extracts of propolis.

The total flavonoid and polyphenol contents were similar for both extracts and are demonstrated in Table 2. The polyphenol content in both extracts was about 2.8 times of the flavonoid content. The most common constituents of propolis from Brazil and other tropical zones are prenylated derivatives of *p*-coumaric acid, various diterpenes and flavonoids. This composition is different if compared to extracts from other origins, since the composition of propolis depends upon the vegetation of the area and the season from which it is collected [13,24].

Regarding to lipid peroxidation induced by Fe^{2+}/ascorbate, the initiation of peroxidation sequence of a membrane in a free lipid peroxide system refers to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid.

Considering that membrane fractions (mitochondria) isolated from disrupted cells, as used in this experiment, may contain some lipid peroxides, which are formed enzymatically in tissues by cyclooxygenase and lypoxygenase enzymes when iron salts are added, these lipid peroxides can be decomposed to generate peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals that can abstract hydrogen from polyunsaturated acyl chains and propagate lipid peroxidation [25].

The peroxides breaking down will produce carbonyl compounds known as TBA-reactive compounds. The 3-carbon compound malondialdehyde (MDA) is one such carbonyl, which forms a characteristic chromogenic adduct with two molecules of thiobarbituric acid (TBA), that is evaluated in 535 nm.

Table 3 shows the TBA-reactive compounds formed in lipid peroxidation when extracts of propolis were added in this system and in Fig. 1 it is shown the

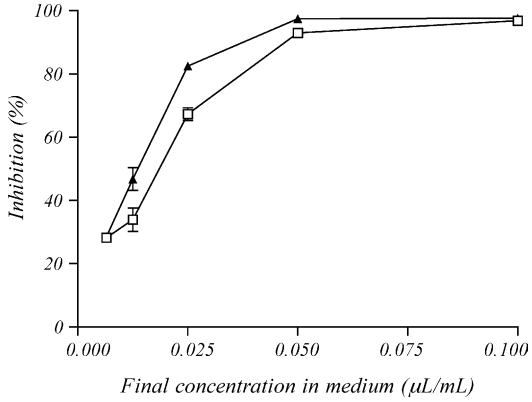

Fig. 1. Inhibition of lipid peroxidation induced by Fe^{2+} found for different concentrations of: (▲) glicolic extract of propolis and (□) ethanolic extract of propolis. Results are mean ± S.E. of three experiments run in parallel.

concentration–response curves obtained for this method. The percent of peroxidation inhibition was plotted against different concentrations of the extract of propolis examined and the concentration which caused 50% inhibition was taken as the IC_{50} value. The same procedure for expressing results was used for all the methodologies employed.

The percentage of inhibition was calculated using the following equation

$$\text{Inhibition}\,(\%) = 100 - \frac{100A_s}{A_0} \quad (1)$$

where A_s is the absorvance observed when experimental sample was added, and A_0 the absorvance of the positive control (sample absence or the extract of propolis-free control formulation).

The IC_{50} for EEP and GEP were 0.016 and 0.012 μL/mL, respectively. α-Tocopherol, a well known lipophilic antioxidant of endogenous origin found in tissues showed in this methodology IC_{50} of 0.48 μg/mL [26]. These results suggest that both extracts of propolis act inhibiting the lipid peroxidation in a more effective way than α-tocopherol. The inhibition of lipid peroxidation may be probably due to the scavenging activity against lipid peroxides, peroxyl and alkoxyl radicals and, in same extend, to the Fe^{2+} chelating activity. The precision of this method was also evaluated, showing 2.58% within-assay precision and 4.33% between-day repeatability.

Table 3
TBA-reactive compounds formed in lipid peroxidation induced by Fe^{2+}

Final concentration in medium (μL/mL)	EEP		GEP	
	TBA-reactive compounds (nM/mg of protein)	TBA-reactive compounds (%)	TBA-reactive compounds (nM/mg of protein)	TBA-reactive compounds (%)
0.1	0.17	3.22	0.13	2.47
0.05	0.36	7.05	0.13	2.61
0.025	1.70	32.82	0.90	17.38
0.0125	3.42	66.04	2.76	53.29
0.0065	3.72	71.83	3.70	71.44
Control[a]	5.18	100	5.18	100

[a] Antioxidant absence.

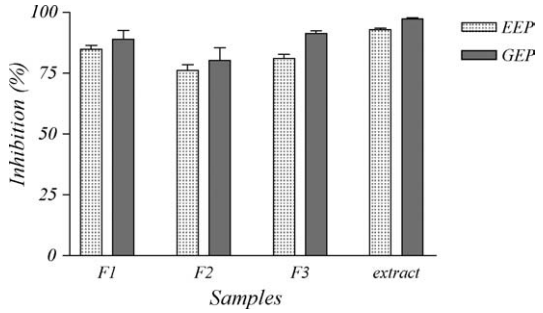

Fig. 2. Inhibition of lipid peroxidation induced by Fe^{2+}, when formulations containing extracts of propolis were evaluated: F, formulations; EEP, ethanolic extract of propolis; GEP, glycolic extract of propolis. The samples of extract of propolis were proportional to the content of the extract in the formulations and were evaluated in a final concentration of 0.05 μL/mL. Results are mean ± S.E. of three experiments run in parallel.

The antioxidant activity observed in our studies was also kept when both extracts were added to different formulations, as observed in Fig. 2. The inhibition of lipid peroxidation by extracts of propolis when analyzed in a final concentration of 0.05 μL/mL were similar to that found for formulations added with the extracts (no significant difference), suggesting that the antioxidant activity may be properly measured since the formulations components showed no interference.

The chemiluminescence assays were evaluated based on the measurements of the areas under the time courses of the luminescent emission in the presence of the extracts of propolis and the formulations containing these extracts. We estimated the relative inhibitory activity of each sample at different concentrations and in both systems used.

The percent inhibition caused by each sample was calculated as:

$$\text{Inhibition}\,(\%) = 100 - \frac{100\,\text{AUC}_1}{\text{AUC}_0} \quad (2)$$

where AUC_0 represents the area under the curve observed for the control (extract absence or propolis-free control formulations) and AUC_1 (experimental samples).

Among the assays for antioxidant activity, chemiluminescence is advantageous because of its high sensitivity and rapidity. Light emission can be markedly amplified using the H_2O_2/luminol/HRP system [27], where in the presence of H_2O_2, HRP catalyses the one-electron oxidation of various divalent redox molecules (luminol radical) through the catalytic cycle of the enzyme. The luminol radicals generated reacts with O_2 to yield oxidized luminol and $O_2^{\bullet -}$. This superoxide radical reacts with luminol radical to yield an endoperoxide, which then decomposes to yield an eletronically excited 3-aminophthalate dianion, which in returning to its ground state emits light [28].

When compounds like propolis are added to the chemiluminescent solution, the light emission should be reduced, indicating antioxidant activity by scavenging any radical generated in this system.

The xanthine/luminol/XOD assay was evaluated by the ability of extract of propolis and the formulations containing these extracts in scavenging superoxide radical ($O_2^{\bullet -}$) generated in the following reaction:

$$\text{Xanthine} + O_2 \xrightarrow{\text{XOD}} \text{Uric acid} + O_2^{\bullet -}$$

Luminol is used as a detector, which is oxidized by the superoxide anions. The inhibition of luminescent emission caused by the decrease of superoxide anions was measured.

Concerning the possibility of enzyme inhibition in both chemiluminescent assays, the enzymes (HRP and XOD) were incubated with extracts of propolis at different temperatures varying the contact time. No enzyme inhibition was found (data not shown), showing that extracts of propolis act exclusively scavenging free radicals generated in these systems.

The IC_{50} calculated in the H_2O_2/luminol/HRP system for EEP and the GEP were 0.22 and 0.24 μL/mL, respectively. In the xanthine/luminol/XOD system the IC_{50} found for EEP and GEP were both about 0.005 μL/mL, showing that the strongest antioxidant activity for propolis was found in scavenging superoxide radical (see Figs. 3 and 4). The precision of these methods were also evaluated, showing 0.78% within-assay precision and 1.10% between-day repeatability for the H_2O_2/luminol/HRP system, and 1.57% within-assay precision and 1.17% between-day repeatability for the xanthine/luminol/XOD system.

Extracts of propolis showed about 40 times more effective in the xanthine/luminol/XOD (pH 9.4) system compared to the H_2O_2/luminol/HRP (pH 7.4) system, this may probably be due to the pH of the medium which permitted different redox potential of the propolis antioxidant compounds, and also due to the different kind of radicals formed. May be in this more basic reaction medium (pH 9.4) the propolis antioxidant compounds can scavenge the radicals in an easier way.

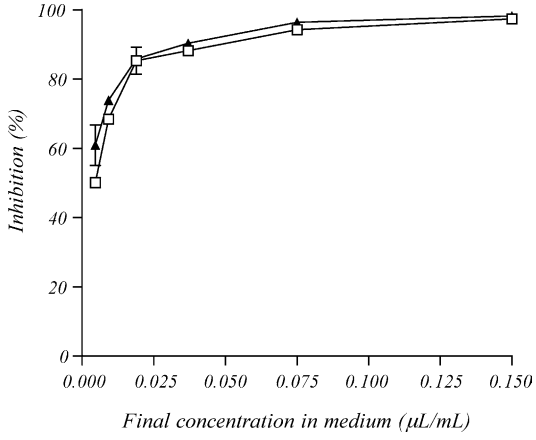

Fig. 3. Inhibition of light emission from xanthine/luminol/XOD luminescent reactions with luminol found for different concentrations of: (▲) glycolic extract of propolis and (□) ethanolic extract of propolis. Results are mean ± S.E. of three experiments run in parallel.

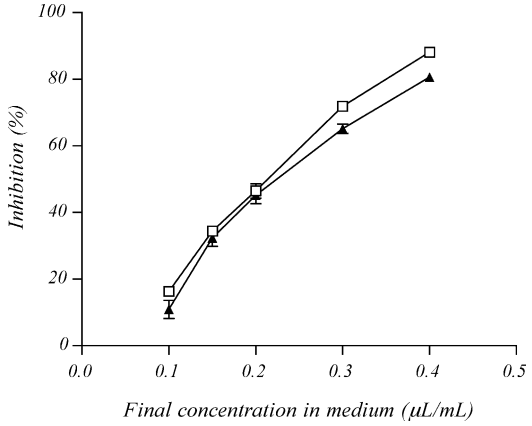

Fig. 4. Inhibition of light emission from H_2O_2/luminol/HRP luminescent reactions with luminol found for different concentrations of: (▲) glycolic extract of propolis and (□) ethanolic extract of propolis. Results are mean ± S.E. of three experiments run in parallel.

These methods were also evaluated for α-tocopherol and showed IC_{50} of 3,3 μg/mL in the H_2O_2/luminol/HRP assay [26], and this potent antioxidant didn't show any activity in xanthine/luminol/XOD assay (data not shown).

The chemiluminescence assay using xanthine/luminol/XOD system was also performed to assess the formulations (Fig. 5). The inhibitions of chemiluminescence in this system by extracts of propolis when analyzed in a final concentration of 0.075 μL/mL were similar to those observed for formulations (no significative variance). These results suggest that the varying formulation components caused no interference with the antioxidant measurement.

However, the H_2O_2/luminol/HRP system was not able to evaluate the formulations, since the final concentration of extract of propolis in the medium when formulations are diluted (1:5) was 0.05 μL/mL, and the activity found for the extracts of propolis in this concentration was less than 5%. So it would be necessary a 20% extract of propolis formulation to reach the same activity (about 90% inhibition) found for the other methodologies used. Nevertheless, this antioxidant methodology could be successfully applied in evaluating other antioxidant compounds which had a lower IC_{50} than the extracts in study (0.22 and 0.24 μL/mL for EEP and GEP, respectively).

The very low IC_{50} (about 0.005 μL/mL) found for both extracts of propolis in the xanthine/luminol/XOD system confirms that besides the high antioxidant activity of the extracts of propolis, this method presents high sensitivity. This implies that this method could have high applicability when intending to evaluate the antioxidant activity in very diluted formulations or in formulations which the antioxidant compound is used in low concentration.

The inhibitory capacity of chemiluminescence was also reported by Pascual [10] evaluating extracts of propolis from Cuba. These extracts had strong antioxidant activity against superoxide radicals, alkoxyl radicals and in the oxidation reaction of luminol on hydrogen peroxide.

In order to find out if propolis is active against hydroxyl radicals, the deoxyribose assay was used. The sugar deoxyribose (2-deoxy-D-ribose) is degraded on exposition to hydroxyl radical generated by a mixture of Fe^{3+}, ascorbate, and H_2O_2 in a presence of a slight molar excess of EDTA over the Fe^{3+} salt. The $^{\bullet}OH$ radicals attack the deoxyribose and set off a series of reactions that eventually result in formation of MDA. MDA may be detected by its ability to react with thiobarbituric acid (TBA) in acid conditions [19,29].

Any other molecule added to the reaction mixture that is capable of reacting with $^{\bullet}OH$ should compete with deoxyribose for $^{\bullet}OH$, hence, it will decrease the rate of deoxyribose degradation. The precision of this method was also evaluated, showing 2.03% within-assay precision and 3.5% between-day repeatability.

The activity of extracts of propolis in scavenging $^{\bullet}OH$ radicals can be seen in Fig. 6, the percentage inhibition was calculated as in Eq. (1), and it was observed that these extracts scavenge $^{\bullet}OH$ in a dose-response way, showing IC_{50} for EEP and GEP of 0.024 and 0.035 μL/mL, respectively. These results are also better when compared to α-tocopherol,

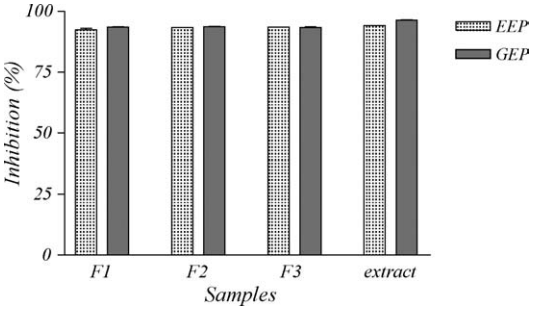

Fig. 5. Inhibition of light emission from xanthine/luminol/XOD luminescent reactions with luminol when formulations containing extracts of propolis were evaluated: F, formulations; EEP, ethanolic extract of propolis; GEP, glycolic extract of propolis. The samples of extract of propolis were proportional to the content of the extract in the formulations and were evaluated in a final concentration of 0.075 μL/mL. Results are mean ± S.E. of three experiments run in parallel.

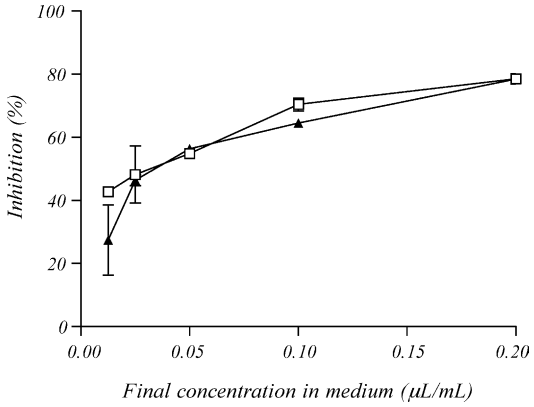

Fig. 6. Inhibition of deoxyribose degradation found for different concentrations of: (▲) glycolic extract of propolis and (□) ethanolic extract of propolis. Results are mean ± S.E of two experiments run in parallel.

Table 4
IC_{50} values for the antioxidant activities of extracts of propolis

Methodologies	IC_{50}[a]	
	EEP[b]	GEP[c]
Lipid peroxidation assay	0.016	0.012
H_2O_2/luminol/HRP assay[d]	0.22	0.24
Xanthine/luminol/XOD assay[d]	0.005	0.005
Deoxyribose assay	0.024	0.035

[a] Concentration which caused 50% inhibition.
[b] Ethanolic extract of propolis.
[c] Glycolic extract of propolis.
[d] Chemiluminescence.

which showed IC_{50} of 0.3 μg/mL [26]. However, this method was not able to evaluate this activity when the extracts were added in the formulations. All the formulations with propolis extract tested produced the same content of MDA as the formulation without propolis extract, implying no antioxidant activity. This result is probably due to the high reactivity of •OH and its unspecificity, thus •OH may react fast to the components of the formulations. Hence, the propolis antioxidant compounds do not have enough •OH to react, since the formulation compounds are present in a higher concentration.

This unspecific activity of hydroxyl radicals was reported for Halliwell and Gutteridge [25] showing the ability of •OH to hydroxylate easily compounds such as salicylate, benzoate, phenylalanine or phenol, attack tryptophan, convert dimethyl sulfoxide to methanal, methanesulfinic acid, or methane decarboxylate benzoic acid to CO_2, etc.

4. Conclusion

The IC_{50} values observed for the extracts of propolis tested in the present study showed how different activities these extracts may have, and the extension that each radical may be scavenged (Table 4). In all the methodologies studied, it was possible to build a dose-response curve, showing that these methodologies may be adequate in evaluating the antioxidant activity of different extracts of propolis. And this kind of evaluation could successfully lead to standardization of propolis extracts from different geographic areas.

After evaluating the IC_{50} found for both propolis, it was concluded that the best antioxidant activity of the extracts of propolis studied was found for the superoxide radical generated in the xanthine/luminol/XOD system, followed by lipid peroxidation inhibition and scavenging •OH radicals in the deoxyribose assay.

But only lipid peroxidation and xanthine/luminol/XOD assays were able to evaluate the antioxidant activity of formulations. In these methodologies the diluted extract of propolis-free formulation caused no effect on the observed controls, showing no interference of the different formulations components.

From the results observed in this work, it can be deduced that both propolis extract can be added to topical formulations and their antioxidant activity are maintained in order to protect skin against damage caused by free radical. Nevertheless, it is essential to choose the correct method to evaluate the antioxidant activity of these formulations, with the purpose of performing a quality control work. Besides this it would be of very importance the antioxidante activity evaluation in stability studies, knowing that an antioxidant formulation could become pro-oxidant, without altering the marker compound stability that is usually used in these studies.

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