Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Antioxidant and antifungal activity of Romanian propolis

Received for publication, April, 23, 2015
Accepted, September, 22, 2015

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Abstract

Propolis, a known bee product for its beneficial effects on the human health, was the focus of many investigations recently carried out. In the present study we evaluated antioxidant and antifungal activity of propolis fractions and ethanolic extracts of propolis (EEP) from different regions of Romania. The evaluation of the antimicrobial activity of propolis samples was performed by an adapted disc diffusion and serial microdilutions methods. The EEP influence on biofilm development on the inert substratum was investigated by the microtiter method. The antioxidant activity was evaluated using the free radicals1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) scavenging methods. The propolis fractions and EEPs used in this study exhibited antifungal and antibiofilm activity against all tested Candida albicans strains and revealed a strong free radical scavenging capacity. These results recommend Romanian propolis as a natural antioxidant and a potential therapeutic agent useful in the prevention and therapy of oxidation-related diseases and Candida albicans infections. An updated data analysis in the changing foodborne pathogens evolution in Romania and improvements in food chain traceability.

Keywords: Romanian propolis, antioxidant activity, antifungal activity, microbial biofilm, *Candida albicans*

1. Introduction

Propolis, a product collected by bees, is a resinous, dark color material with various vegetal origins. The complex and varied composition of propolis, as well as the synergism of its bioactive constituents confer it multiple pharmacological properties: antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, antitumoral etc. The accumulation of free radicals generated by biochemical oxidative reactions induces the occurrence of several human pathologies (cardiovascular disease, cancer, diabetes) (RUSSO & al. [1], MELO & al. [2], NAGAI & al. [3]). Antioxidants are substances which are capable to neutralize the free radicals. Among these substances, the phenolic compounds have free radical-scavenging properties, blocking the oxidative processes (SOARES [4]). In last years, many scientific studies were conducted in order to find new antioxidants of natural origin without adverse effects. The studies performed in different countries have revealed the capacity of propolis to scavenge free radicals, being considered one of the most effective natural antioxidants.

Beside to its known antibacterial activity, propolis exhibits also antifungal properties. The evaluation of propolis extracts antifungal activity was carried out mainly by conventional methods (OZCAN [5]; KUJUMGIEV & al. [6]; OTA & al. [7]; HEGAZI & al. [8]; TRUSHEVA & al. [9]; DOBROWOLSKI & al. [10]), on various strains belonging to the genera *Candida, Aspergillus, Saccharomyces* and dermatophytous filamentous fungi (like *Trichophyton, Fusarium, Mycrosporum*). An increased sensitivity of dermatophytes was highlighted even at low concentrations of propolis (LONGHINI & al. [11]; DE CASTRO [12]). The *C. tropicalis, C. albicans, C. guilliermondii* and *C.parapsilosis* strains proved to be susceptible to Brazilian EEP (FERNANDES & al. [13]). The antifungal activity of the propolis extracts varies depending on chemical composition and concentration of propolis bioactive constituents. The main antifungal compounds identified in propolis are considered to be in the mixture pinocembrin, triterpenes and caffeates (BANKOVA [14]; BANKOVA & al. [15]). *Candida albicans* can be a commensal or opportunistic pathogen, being one of the main causes of nosocomial infections. *C. albicans* can cause local or systemic infections, the predisposing factors for their appearance including antibiotics, immunosuppressive therapy, HIV infection, the use of implants, stents, endotracheal tubes, various catheters [POPA & al., [16]; BERTESTEANU &al [17]). This ability to adhere to various surfaces represented by cellular substrates or biomaterials is required for microbial colonization and biofilm formation, and for further tissue invasion and infection dissemination. *C. albicans* biofilm formation is associated with a decreased susceptibility to antimicrobial agents and resistance to the host defense mechanisms. MELLO & al. (2006) suggested that Brazilian propolis antifungal activity is based on inducing changes in the cell wall (alteration of cellular permeability) that have as consequence an increasing volume of the cell and cellular membrane rupture. The inhibition of fungal growth and germination tube formation of *C. albicans* could be attributed to the interaction of propolis with proteins sulfhydryl groups (MELLO & al. [18]). The aim of this study was to evaluate the antifungal and antioxidant activity of propolis fractions and ethanolic extracts of propolis from different regions of Romania and to establish potential correlations between the antioxidant capacity and antifungal and antibiofilm activities of propolis samples.

2. Materials and Methods

EEP preparation

Propolis samples used in this study were collected from eight Romanian regions (Arad, Bihor, Cluj, Mures, Calarasi, Dambovita, Dolj and Mehedinti). Ethanolic extracts of propolis (EEP) 30 % were obtained by extraction of raw propolis (30 g) with 96% v/v ethanol (70 ml), filtration and addition of extraction solvent to a final volume of 100 ml.

Propolis fractions

The propolis samples (10 g) collected from South Romania were finely powdered in a chilled mortar and subjected to extraction with 80 % ethanol (1:10 w/v), three times. After the waxes were removed by centrifugation the sample was concentrated in a water bath to obtain a crude extract which was then added to an 8% *L*-lysine solution at 51-60 ºC in a 1:15 (w/v) ratio (NIKOLOV & al. [19]). The *L*-lysine-based propolis extract was filtered (Whatman paper) and then was subjected to fractionating into major compounds, flavonoids and phenolic acids, using solid phase extraction technique (SPE). The pH of propolis solution was adjusted to pH=7 with 1 N NaOH solution. In this procedure, the polyphenols from propolis are fractionated into neutral and acid fractions. Phenolic acids are completely ionized at pH=7 and unionized at pH=2. Based on this fact, it can be achieved a separation of

the neutral polyphenols at pH=7 and acid polyphenols at pH=2. Two separately preconditioned C18 cartridges (Bond Elut C18 Varian) were used for separation, one for neutral polyphenols (flavonoids) and the other for acid polyphenols. The C18 cartridges was preconditioned with water and methanol and then the propolis extract with pH=7 was passed onto the cartridges. The effluent was collected and adjusted to pH=2 with 1N HCL. The acid fraction was passed through the second (acid) cartridges to absorb the polyphenolic acids. Both neutral and acid polyphenols were eluted from their cartridges with absolute methanol. The collected methanolic fractions were evaporated with a concentrator plus Eppendorf. The two resulted fractions were solubilized in absolute ethanol, and 60% ethanol, respectively.

Free radical scavenging activity on DPPH

The free radical-scavenging capacity of samples was tested by their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (GOVINDARAJAN & al. [20]). One ml of $DPPH^{\bullet}$ solution (25 mM in MeOH) was added to 0.1 mL of ethanolic solutions of propolis and 1.9 mL MeOH (LITESCU & al. [21]). The absorbance decrease was recorded for 30 min at 517 nm and compared to a control with 0.1 mL of MeOH. The antioxidant activity was expressed as mmol Trolox g^{-1} propolis using a calibration curve: $y = 7.477x + 0.001$, $R^2 = 0.998$.

Scavenging activity of ABTS radical cation

The ABTS radical cation ($ABTS^{\bullet+}$) scavenging activity was measured according to the method described by EREL with some modifications (EREL [22]). ABTS was dissolved in water to a 7 mM concentration. The ABTS radical cation was produced by reaction of the ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the stock solution to stand in the dark at room temperature for at least 12 h to allow the completion of radical generation. Next, 2.5 mL of this $ABTS^{\bullet+}$ solution was added to 0.1 mL of ethanolic solution of propolis and 0.4 mL distilled water and the absorbance decrease was recorded for 15 min at 734 nm. Control sample contained 0.1 mL of MeOH instead of sample. The antioxidant activity was expressed as mM Trolox g^{-1} propolis using a calibration curve: $y = 12.45x + 0.008$, $R^2 = 0.999$.

Fungal strains

In the present work we used one *Candida albicans* reference strain ATCC 26790 for the evaluation of propolis fractions antifungal activity and seven *Candida albicans* strains which were isolated from tracheal secretions in hospitalized patients with respiratory tract associated infections (for testing the EEP antifungal activity).

Qualitative screening of propolis antifungal activity

An adapted disc diffusion method was used in order to assess the sensitivity of *Candida albicans* strains to propolis fractions and EEPs action. A volume of 5µl both EEP and propolis fraction was spotted on the medium surface inoculated with the fungal suspension. The inoculum was obtained from *Candida* strains seeded on Sabouraud medium for 24 hours. The solvent was used as control for testing its antifungal activity. After the plates incubation (24 hours, 37°C), the growth inhibition zone around the spot was measured for determining the antifungal activity of propolis fractions and EEPs.

Determination of MIC

The antifungal activity of EEP was quantitatively evaluated by serial two-fold microdilution method in liquid medium and the MIC (minimum inhibitory concentration) was established. Serial dilutions of EEP (0.058 - 30 mg/ml) were performed in a volume of 200 µl, in 96-well microtiter plates. The solvent was diluted in the same conditions as EEP and its antifungal activity was comparatively evaluated. The used volume of fungal suspension was 20 µl. The microplates incubation was carried out at 37°C for 24 hours. The

macroscopic evaluation and the spectrophotometric reading at 620 nm indicated the MIC values defined as the lowest concentration of EEP able to inhibit the visible fungal growth.

Influence of EEP and propolis fractions on biofilm development on inert substratum

For evaluating the antibiofilm activity of EEP, the fungal cells were cultivated in 96-well plates with nutrient broth in the presence of various concentrations of EEP. After incubation at 37°C for 24 h, the microplates were washed twice and then 100 µl cold methanol 80% was added in order to fit the adhered cells. After 5 minutes methanol was removed and a 1% crystal violet solution was used for staining the adhered cells (15 minutes). The biofilm developed on inert substratum was resuspended in 33% acetic acid and the MBEC (minimum biofilm eradication concentration) values were established by measuring the absorbance at 490 nm.

3. Results and discussion

Antioxidant activity of propolis samples

DPPH scavenging is a rapid, simple and practical method, based on the reduction of DPPH radical to a stable molecule by hydrogen or electron donation in the presence of an antioxidant substance (LAGOURI & al. [23]). In table 1 there are presented the results obtained by DPPH and ABTS methods concerning the EEPs and propolis fractions antioxidant activity.

Table 1. Antioxidant activities of propolis samples.

Propolis sample	Collecting site	DPPH[a] (mM Trolox g^{-1} propolis)	ABTS[a] (mM Trolox g^{-1} propolis)
1	Mehedinti	4.78±0.19	7.00 ± 0.10
2	Dambovita	4.92±0.10	8.41 ± 0.17
3	Calarasi	6.34±0.04	8.49 ± 0.07
4	Dolj	5.41±0.13	7.83 ± 0.09
5	Bihor	4.38±0.10	7.69 ± 0.16
6	Mures	4.33±0.12	7.82 ± 0.09
7	Arad	5.57±0.22	8.32 ± 0.07
8	Cluj	5.55±0.04	7.78±0.13
9	Fraction I (flavonoids)	9.69±0.75	17.89±0.70
10	Fraction II (40%) (polyphenolic acids)	12.10±1.28	21.22±0.42

[a]Values are expressed as means ± standard deviation of three independent experiments.

The free radical scavenging activity on DPPH varied between 4.33±0.12 (Mureş) and 6.34±0.04 mM Trolox/g propolis (Călăraşi) for EEP samples; these results are in accordance with some previous studies (GATEA & al. [24, 25]) which stipulate that propolis from plain zone (Călăraşi, Arad, Dambovita, Dolj) have, generally, higher polyphenolic content and antioxidant activities than propolis from plateau and upper hills zone (Mureş, Cluj). The same observations can be made regarding the results obtained with ABTS method for EEPs. The ABTS method was used for evaluation of total antioxidant activity of studied samples. This method indicates the ability of an antioxidant to reduce the $ABTS^{+}$ radical formed by oxidation of ABTS (BONVEHÍ & GUTIÉRREZ [26]). The values obtained by ABTS method ranged from 7.00 ± 0.10 (Mehedinti) to 8.49 ± 0.17 (Calarasi) mM Trolox/g propolis

for propolis extracts. Concerning the propolis fractions, the antioxidant activities obtained by both methods are much higher than those obtained for EEPs. Fraction I, which contains mostly flavonoids (neutral polyphenols) presented a lower antioxidant activity (9.69±0.75 mM Trolox/g propolis with DPPH and 17.89±0.70 mM Trolox/g propolis with ABTS) in comparison with Fraction II which contains mainly polyphenolic acids and presented 12.10±1.28 (DPPH) and respectively 21.22±0.42 mM Trolox/g propolis with ABTS method. The antioxidant activity of propolis samples (EEP and propolis fractions) evaluated by DPPH and ABTS methods was different, recording a higher scavenging activity towards $ABTS^{\bullet+}$. This result might be attributed to the affinity of the DPPH radical to the lipophilic antioxidants, in contrast to the radical ABTS which reacts with both lipophilic and hydrophilic antioxidants (Prior & al. [27]). Also, the various chemical compositions of propolis samples could be the reason of the differences in antiradical activity determined by DPPH and ABTS assays (SOCHA & al. [28]). The propolis samples from various regions of Poland were less active in terms of antioxidant activity compared to our samples (GATEA & al. [24]). The values of antiradical activity of propolis from Poland both on DPPH and ABTS radicals were much lower, ranging between 1.92 and 2.69mM TE/g, respectively 3.96–4.98mM TE/g (SOCHA & al. [28]). Our results indicated also a higher antioxidant activity of Romanian propolis comparatively with propolis from Northeastern Spain which had lower values for free radical scavenging activity on ABTS radical (560 - 1,430 µmol Trolox/g) (BONVEHÍ & GUTIÉRREZ [26]).

Qualitative screening of the antifungal activity of propolis fractions and EE

The results of the qualitative analysis showed that all tested *C. albicans* strains were susceptible to both EEPs and propolis fractions for antifungal action.

EEPs and the propolis fractions led to the appearance of growth inhibition zones with diameters between 6 and 20 mm (table 2). EEP Bihor, EEP Cluj and EEP Mures exhibited a week antifungal action, the inhibition zone diameter being 6 mm.

The qualitative assessment of the *C. albicans* susceptibility to different propolis extracts, carried out by several workgroups, indicated different values for inhibition zones diameters: 0 - 10 mm (MARGHITAS & al. [29]; GONSALES & al. [30]); 20.5 - 25.5 mm (SALMAÕ & al. [31]), 10 - 13.5 mm (KOSALEC & al. [32]), 16 -18 mm (AFROUZAN & al. [33]), 16 -22 mm (NAJMADEEN & KAKAMAND [34]). Contradictory results were obtained by NAHER & al. (2011) reporting no antimicrobial effects of propolis against *C. albicans* (NAHER & al. [35]). Our results showed that the maximum inhibition zone diameter (20 mm for EEP Calarasi) against *C. albicans* was situated in the range reported by NAJMADEEN & KAKAMAND (16-22 mm) for a propolis extract from Sulaimani province-Kurdistan region. An interesting observation can be done about the antifungal effect of propolis fractions. Fraction I (flavonoids) presented a higher antifungal activity (16 mm inhibition diameter) than Fraction II (polyphenolic acids) with 11 mm, therefore the antifungal effect is determined by individual compounds (especially flavonoids). Generally, the EEP samples with higher antioxidant activities (from plain zone) presented higher antifungal activities, but this statement is not applicable in the case of propolis fractions.

The results obtained by other groups are in accordance with our obtained data. Therefore, a study conducted by YARFANI (2010) showed that MIC values of the propolis extract on *C. albicans* strains were in the range of: 2 - 20 mg/ml, and 15 mg/ml concentration was fungicidal for all fluconazole-resistant clinical isolates *C. albicans* strains used in the experiment (YARFANI & al. [36]).

Table 2. Antifungal activity of propolis samples on *Candida albicans*

Propolis samples	Diameter zone inhibition (mm)							
	C.albicans 47	*C.albicans 58*	*C.albicans 600*	*C.albicans 601*	*C.albicans 1668*	*C.albicans 1726*	*C.albicans 1760*	*C.albicans 26790*
EEP Arad	10	16	13	13	18	10	11	-
EEP Bihor	6	16	15	16	17	7	11	-
EEP Cluj	6	11	14	10	15	7	5	-
EEP Mureş	6	15	15	15	18	7	7	-
EEP Călăraşi	13	20	15	19	19	12	17	-
EEP Dâmboviţa	7	14	14	19	16	10	11	-
EEP Dolj	12	16	13	16	19	10	13	-
EEP Mehedinţi	8	15	15	16	19	13	14	-
Propolis fraction I	-	-	-	-	-	-	-	16
Propolis fraction II	-	-	-	-	-	-	-	11

Quantitative testing of the antifungal activity of propolis samples for MIC determination on C. albicans

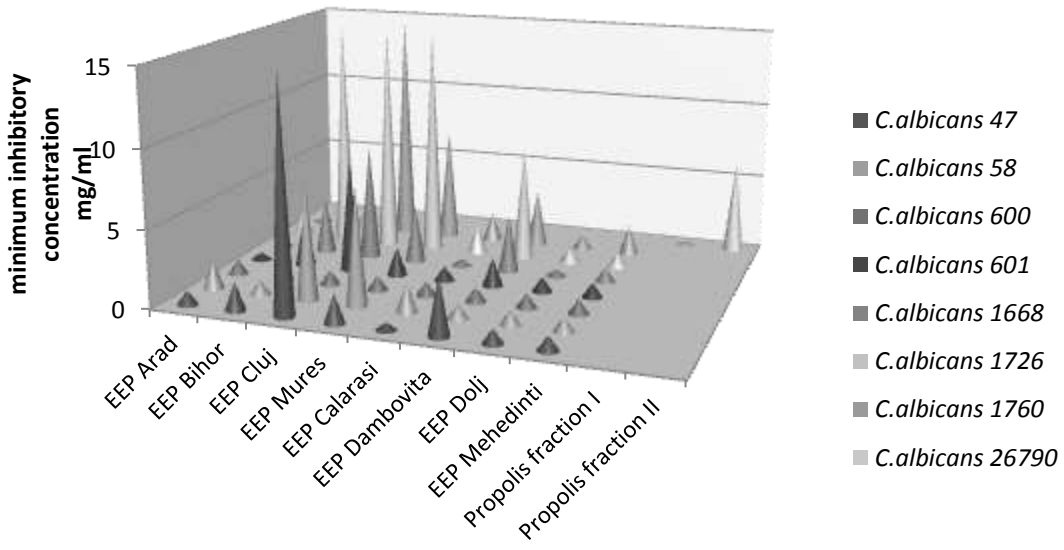

Fig. 1 Minimum inhibitory concentration (MIC) of Romanian propolis on *C. albicans*

Other MIC values obtained were: 0.25 mg/ml for *Candida albicans* ATCC 10231 (MORA & al. [37]) between 1400 and 3200 µg /ml for *Candida albicans* (HEGAZI & HADY [38]), 2.59 mg/ml for *Candida albicans ATCC 10231* (KOSALEC & PEPELJNJAK [39]), between 6 and 12 mg/ml for *Candida sp.* (SAWAYA & al. [40]).

Antibiofilm activity of propolis fractions and EEP

The evaluation of the antibiofilm capacity of EEP showed the inhibition of adhesion on the inert substratum of all tested *Candida* strains at MBEC values between 0.2343 and 15 mg/ml (fig. 2) for all tested samples. MBEC lowest value was registered for EEP Arad

against *C. albicans* 1760, and the highest value of MBEC was obtained for EEP Cluj in the case of *C. albicans* 47. For propolis fraction I we obtained the inhibition of *C. albicans* 26790 biofilm development at 0.095 mg/ml and for propolis fraction II at 6.08 mg/ml.

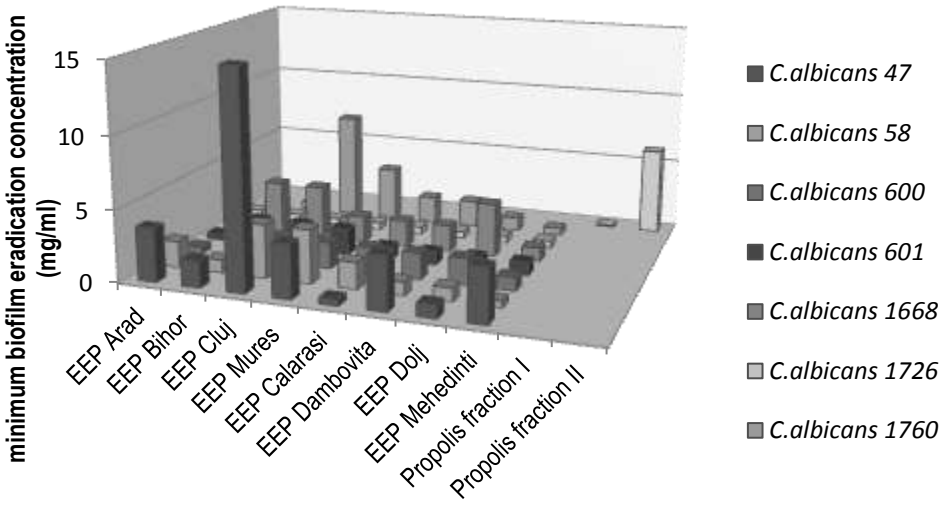

Fig. 2 Minimum biofilm eradication concentration (MBCE) of Romanian propolis on *C. albicans*

4. Conclusion

The evaluation of free radical scavenging capacity of Romanian propolis by DPPH and ABTS assays revealed a high antioxidant activity of EEPs, and very high one for the propolis fractions. The highest values of the antiradical activity determined by both methods was obtained for EEP Calarasi (South, plain climate) from all the analyzed EEP samples. The same sample presents also the highest antifungal activity. The results of qualitative screening and quantitative assay of Romanian propolis antifungal activity showed that both propolis fractions and ethanolic extracts of propolis exhibited a more or less intensive inhibitory effect on the tested *C. albicans* strains. Also, it was noticed that *C. albicans* biofilm development was inhibited in the presence of all propolis samples, proving the antibiofilm activity of Romanian propolis. The variability of propolis antifungal activity could be attributed to different chemical composition of propolis samples. In our opinion, flavonoids content influence more the antifungal activity than polyphenolic acids. Because of antioxidant and antifungal properties, Romanian propolis could be considered a natural antioxidant and a potential therapeutic agent useful in the prevention and therapy of oxidation-related diseases and *C. albicans* infections.

5. Acknowledgements

The financial support of the UEFISCDI project PN-III-P2-2.1-BG-2016-036 is gratefully acknowledged.

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