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Evaluation of bioactive compounds and biological activities of Tunisian propolis

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Abstract

The aim of this study was to investigate the phytochemical composition and biological proporties of Tunisian propolis from four different regions: Kasserine, Béja, Kèf and Monastir. Ethanolic extracts of propolis were prepared using two extraction methods; solvent and ultrasonic extraction. Total phenolics, flavonoids, ABTS free radical and hydroxyl radicals scavenging abilities, anti-inflammatory, anti-hypertensive, as well as antimicrobial activities of propolis extracts were determined. Identification and quantification of phenolic and flavonoid compounds were performed by using both HPLC-UV and HPLC-ESI-MS. The results revealed high contents of total phenolics and flavonoids and polyphenols extraction was more efficient by sonication. Caffeic acid phenethyl ester (CAPE), galangin, and genistein were the major identified compounds. Antihypertensive activity, evaluated in propolis extracts for first time by HPLC-UV, was higher than 90% for all extracts. Tunisian propolis is an important natural source of polyphenols and flavonoids. The best extraction method was ultrasonic for antioxidants and most of biological activities; conventional method seems to be more suitable for anti-inflammatory activity. Propolis from Béja contains the highest amount of antioxidants and have a stronger potential biological activities. Tunisian propolis could be, therefore, a promising raw material for food and pharmaceutical industry.

Keywords: propolis; phenolics; HPLC-UV; antioxidant activity; ACE

1. Introduction

Propolis, or bee glue, is a resinous substance collected by honeybees (*Apis mellifera L.*) from buds and exudates of several plants and then used to smooth out the internal walls of the hive in order to protect it against intruders (Toreti, Sato, Pastore, & Park, 2013). Propolis chemical composition greatly varies with the site of collection and thus, with the geographical and climatic conditions (Bankova, Popova, & Trusheva, 2014). Propolis mainly consists of 50% resin, 30% wax, 10% essential oils, 5% of other organic compounds, as well as 5% pollen (Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2006). This natural substance has been widely used in folk medicine in many regions of the world, being one of the few natural remedies that has preserved its popularity for a long time (Castaldo & Capasso, 2002). There are several studies, that describe some potentially interesting properties of propolis, among which we can cite its antibacterial (Graikou, Popova, Gortzi, Bankova, & Chinou, 2016), antioxidant (Campos et al., 2014), antiviral (Ahmad, Kaleem, Ahmed, & Shafiq, 2015), anti-inflammatory (Shi, Yang, Zhang, & Yu, 2012) and antitumoral (Silva-Carvalho et al., 2014). Nevertheless, propolis is not directly employed as such, being necessary the removal of unwelcome compounds. Therefore, a propolis extraction is indispensable before using. The most common method to obtain a propolis extract is by solvent extraction, but this procedure is being increasingly replaced by ultrasound extraction, whose efficiency for such vegetal compounds as phenolics has been reported. On the basis of the above, it would be very interesting to research which extraction procedure yields the best values for potential functional properties of propolis. Different solvents, such as ethanol, water, methanol and ethyl acetate, among others were used for propolis extraction (Ma, Ma, Pan, Luo & Weng, 2016; Usman, Abu Bakar, & Mohamed, 2016), showing ethanol extract the highest activity for most of them. Furthermore, ethanol is

nontoxic and can also be easily removed after extraction if propolis extracts are going to be used as food ingredients.

Despite there being studies on propolis from several world areas, research of Tunisian propolis is still very limited. In fact, the only identification of phenolic compounds of Tunisian propolis was achieved by Martos, Cossentini, Ferreres, & Francisco (1997). In addition, biological activities of Tunisian propolis were limited in literature to anti-cariogenic, anti-biofilm and antifungal activities (Kouidhi, Zmantar, & Bakhrouf, 2010). The main objective of this study was to identify and quantify for the first time phenolic compounds in Tunisian propolis, comparing the conventional solvent and the ultrasound-assisted procedures. Biological activities comprising antioxidants, anti-inflammatory, antihypertensive and antimicrobial are equally studied in order to explore their beneficial properties for applications in food and pharmaceutical industry.

2. Materials and methods

2.1. Analytical standards and reagents

Gallic acid and catechin from Panreac (Barcelona, Spain). Caffeic acid phenethyl ester (CAPE) and galangin from TargetMol (Boston, EEUU). Apigenin, chlorogenic acid, kaempferol and pinocembrin from Cymit Quimica, S.L. (Barcelona, Spain). The other standards are from Sigma–Aldrich (Stein-heim, Germany).

2.2. Propolis samples and extracts preparation

Propolis samples were collected by beekeepers in four areas of Tunisia. Fig. 1 shows the collection sites of each sample. The samples were harvested using a plastic propolis trap and stored in the dark at -20 °C until use. Propolis samples were grounded in a marble mortar at -30 °C. Extraction was carried out according to two methods.

Procedure 1: Ten grams of ground propolis were extracted in darkness with 500 ml of 80% ethanol in a 2-l jacketed glass reactor with temperature control and mechanical agitation for 3 h at 40 °C with an agitation speed of 300 rpm.

Procedure 2: Two grams of grinding propolis were extracted in dark conditions with 30 ml of 80% ethanol in an ultrasonic bath (Selecta, Abrera, Barcelone, Spain) with heating frequency of 40 KHz for 20 min. Then, the mixture was filtered (Whatman filter paper No. 4), and the solid was re-extracted two times more using the same conditions, in order to extract the maximum possible quantity of bioactive compounds from the crude propolis. After the third extraction, all the extracts were combined in a 100 ml volumetric flask and the volume was adjusted with 80% ethanol. The extraction procedure was performed in triplicate for each sample, obtaining a final volume of 300 ml.

Then, propolis extracts were stored in the dark at -20 °C until analyzed.

2.3. Total phenolic content

Total phenolic compounds content was essayed using the Folin-Ciocalteau reagent (Singleton, Orthofer, & Lamuela-Raventós, 1999). An aliquot (0.2 ml) of extract was added to 1.5 ml of distilled water and 0.4 ml of the Folin-Ciocalteau reagent 2N. After 5 min, 0.6 ml of sodium carbonate solution 20% (w/v) was added to the mixture. The absorbance was read at 760 nm after 2h of incubation in dark at room temperature. The standard for the calibration curve was gallic acid (25-300 µg/ml), expressing the results as mg gallic acid (GA)/100g sample.

2.4. Total flavonoids content

Total flavonoids content was determined by three colorimetric methods in order to determine different types of flavonoids. The total flavone and flavonol were determined according to the method proposed by Meda, Lamien, Romito, Millogo, & Nacoulma (2005), using quercetin as standard (5-250µg/ml) and expressing the results as mg of quercetin (Q)/100 g of propolis sample. Total flavanone and dihydroflavonol were assessed using the method described by

Popova et al. (2004). Calibration curve of naringenin was prepared (0.1-2.5 mg/ml), expressing the results as mg of naringenin (N)/100 g of sample. Total flavanol content was determined following the procedure described by Pękal & Pyrzynska (2014). Catechin (5-250 µg/ml) was the standard and the results were expressed as mg catechin (C)/100 g of sample.

2.5. Identification and quantification of phenols compounds using HPLC-UV and HPLC-ESI-MS system

Qualitative and quantitative analysis were performed using a liquid chromatograph Varian Pro Star 310. The chromatographic separation was carried out on a reversed-phase Microsorb-MV 100-5 C18 column (150 × 4.6 mm, 5 µm particle size) provided by Agilent Technologies. The chromatographic conditions were described by Falcão et al. (2013) and modified for our purposes. The mobile phase comprised (A) 0.1% formic acid in miliQ water and (B) 0.1% formic acid in acetonitrile. The solvent gradient was: 0-7 min, 0% B, 7-12 min, 2% B, 12-20 min, 8% B, 20-23 min, 10% B, 23-33 min, 20% B, 33-45 min, 23% B, 45-50 min, 30% B, 50-55 min, 32% B, and 55-60 min, 50% B. The injection volume for all samples was 20 µL and the flow rate was 1 mL/min. Detection was carried out at 280 nm.

Quantification was carried out using calibration curves for gallic acid, caffeic acid, catechin, clorogenic acid, *p*-coumaric acid, ferulic acid, naringenin, quercetin, apigenin, kaempferol, pinocembrin, galangin and CAPE, at eight concentration levels (0.0005-0.5 mg/ml). When the standard was not available, the compound quantification was expressed in equivalent of caffeic acid. The linearity of all compounds was satisfactory with R^2 values > 0.9925. Furthermore, the linear ranges included the usual concentration of these compounds in propolis.

In order to identify the unknown compounds, a HPLC-ESI-MS system consisting of a HPLC 1260 Infinity chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), connected

to a quadrupolo-time of fight (6545-Q-TOF) system was used. Electrospray ionization (ESI) in the negative and positive ion mode was done by the source Dual AJS-ESI under the following conditions: Gas temperature 325 ºC, drying gas 10 L/min, nebulizer 45 psi, Vcap 3500 V, nozzle voltage 200V and sheath gas at 350 ºC. MS-TOF with fragmentor at 100 V, skimmer 45 V and OCT 1 RF VPP 750 V was used, acquiring data between 100 and 1000 m/z. Nitrogen was used as collision and as nebulizing gas. The compounds were identified by comparison of their ESI-MS fragmentation spectra with the literature data (Andrade et al. 2018; Kasiotis et al. 2017; Nina et al. 2016; Sobral et al. 2017) and with data from on-line chemical database Phenol-Explorer (http://phenol-explorer.eu). The column, mobile phase and flow conditions were those described for the previous HPLC-UV analysis.

2.6. Assessment of antioxidant activities

2.6.1. ABTS scavenging activity test (TEAC Assay)

Trolox equivalent antioxidant capacity (TEAC) of propolis samples was carried out by the ABTS (3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolorization assay reported by Miguel, Doughmi, Aazza, Antunes, & Lyoussi (2014) with some modifications. A volume of 1490 µl of $ABTS^{\bullet+}$ was mixed with 10 µl of extract. After 6 min of the mixture, the absorbance was read at 734 nm against a blank of ethanol. Trolox was used as standard for the calibration curve (0.625-5 mM) and results were expressed as µmol Trolox (T)/g of sample.

2.6.2. Radical-scavenging effect on hydroxyl radicals (AOA assay)

Hydroxyl radicals scavenging activity of extracts was determined using the method reported by Koracevic, Koracevic, Djordjevic, Andrejevic, & Cosic (2001). Each sample (A1) had its own control (A0) and for each series of analysis a negative control (K1 and K0) was prepared where the sample was replaced with phosphate buffer. Standards containing 1 mmol/l uric acid (UA_1 and UA_0) were used for calibration. The antioxidant activity was calculated as AOA (mmol/l)= CU x (K-A)/(K-U) where CU is the concentration of the uric acid (1 mM), K

154 is the control absorbance (K1-K0), A is the sample absorbance (A1-A0) and U is the uric acid
155 solution absorbance (U1-U0).

156 **2.7. Anti-inflammatory activity**

157 Anti-inflammatory activity was determined by measuring the inhibitory effect of propolis on
158 the reaction catalyzed by hyaluronidase, using the method reported by Ferreres et al. (2012).
159 When the color developed, absorbance was read at 586 nm against a blank (where enzyme
160 and samples were substituted by buffer). N-acetyl-D-glucosamine (NAG) solutions (in the
161 range between 0 and 2 µmol per test) were used as standard for calibration curves. Based on
162 the NAG formed in each enzymatic reaction, inhibition enzyme percentage was calculated as
163 % Inhibition = (A - B/A) × 100, where A was µmol of NAG in the positive control (where the
164 sample was substituted by a buffer) and B was µmol of NAG of each sample reaction.

165 **2.8. Antihypertensive activity: ACE inhibitory activity in vitro**

166 Angiotensin converting enzyme inhibitory activity percentage (ACE %) was determined as
167 reported by Gonzalez-Gonzalez, Tuohy, & Jauregi (2011). This method is based on the
168 hydrolysis of N-hippuryl-histidyl-leucine (HHL) into hippuric acid (HA) and His-Leu (HL)
169 by the ACE enzyme. The ACE activity was measured in terms of HA at the end of the
170 hydrolysis reaction. The evaluation of the HA concentration liberated at the end of the
171 reaction was carried out on a HPLC system (Agilent Technologies Inc, CA, USA),
172 comprising a C_{18} column (4.6 × 250 mm) at 25 °C, a mobile phase consisting of 0.1%
173 trifluoroacetic acid and 12.5% acetonitrile in milliQ water, at a flow rate of 1 mL/min, and a
174 Pro star 325 UV-Vis detector measuring the optical density at 228 nm during 15 min. Data
175 were quantified using star chromatography workstation version 6.41 Software. The injection
176 volume was 25 µl and peaks corresponding to the HA concentration were identified by
177 comparison of their retention times with peaks of the HA standard solutions of HA. A control

was also prepared in the same conditions where the sample was replaced by a buffer. ACE% assays were made by duplicate and each sample was injected twice into the HPLC system.

2.9. Antimicrobial activity

The antifungal and antibacterial evaluations of the propolis samples were performed by the agar disc diffusion method according to Osés et al. (2016) against six fungi species: *Aspergillus flavus* (CECT 2687), *Penicillium nordicum* (CECT 20766), *Penicillium expansum* MP75, *Penicillium commune* M35 (fungi collection of the Department of Food Hygiene and Food Technology, at León University), *Fusarium sp.* NB1 and *Aspergillus niger* NB1 (fungi collection of the Department of Biotechnology and Food Science, at Burgos University), two Gram-positive bacteria: *Streptococcus mutans* (CECT 479), *Lactobacillus plantarum* (CECT 220) and Gram-negative bacteria: *Escherichia coli* (CECT 434).

Organisms were maintained on MEB (Malt Extract Broth) for fungi, Nutrient broth (NB) for *E. coli*, MRS (De Man, Rogosa and Sharpe) for *Lb. plantarum* and BHI (Brain Heart Infusion) for *S. mutans*. Agar plates (NA, MRS, BHA and MEA) were inoculated with 100 µl of suspensions of the tested microorganisms, containing 8 log CFU/ml for bacteria and 5 log conidia/ml for the fungal strains. After two hours, the filter paper discs (6 mm in diameter) were placed onto the surface of the agar plates, and then impregnated with 10µl of the extracts. Plates were incubated at 37 °C for 24 h for bacteria and 25 °C for 3 days for fungal strains. Ethanol, where the propolis extracts were diluted was also used.

2.10. Statistical analysis

Analyses were performed in triplicate and the statistical analysis was carried out with SPSS version 20 (SPSS Inc., Chicago, IL, USA). The values of the analytical determinations were subjected to ANOVA procedure and significant differences ($P < 0.05$) between the means were determined by Tukey's test.

3. Results and discussion

3.1. Total phenolics and flavonoids contents

The amounts of total polyphenols' and flavonoids' contents of Tunisian propolis significantly varied depending on both, the samples harvesting region and the extraction method ($P < 0.05$) (Fig. 2). Polyphenols extraction was more efficient by sonication ($P < 0.05$) as has been previously reported (Ristivojević et al., 2018). Propolis polyphenols ranged from a minimum value of 1734 mg GA/100 g for a conventionally extracted propolis from Monastir, to a maximum value of 3344 mg GA/100 g for an ultrasonic extracted propolis from Béja. Such values were in the same range as those obtained for the Algerian propolis (Mouhoubi-Tafinine, Ouchemoukh, & Tamendjari, 2016), and Moroccan propolis (Miguel et al., 2014). In contrast, total polyphenols contents of the Turkish, Brazilian and Chinese propolis were considerably higher than those found in our research (Alencar et al., 2007; Ristivojević et al., 2018; Wang et al., 2014). These variations are very likely due to the propolis different botanical origins, being also influenced by the harvesting year, geographic origins, as well as environmental conditions and seasonal variation.

In this research, three groups of flavonoids were analyzed. The first one involved flavones and flavonols, whose values ranged from 378 mg Q/100 g to 1661 mg Q/100 g. The second one was made up of flavanones and dihydroflavonols, whose results varied from 1098 mg N/100 g to 2391 mg N/100 g. The third group was made up flavanols, whose contents fluctuated from 117 mg C/100 g to 559 mg C/100 g. Our flavones and flavonols amounts were comparable to those described by Miguel et al. (2014) for the Moroccan propolis (from 20 to 3427 mg Q/100 g). Nevertheless, in general our values for the different flavonoids groups were lower than the data described in the literature for the propolis from other continents (Alencar et al., 2007). These differences in flavonoids quantities could be

attributed to the fact that flavonoids are characteristic of zones and harvesting years, being highly dependent on natural environments, plants and climates (Falcão et al., 2013).

3.2. Identification of phenolic compounds in propolis by HPLC

Phenolics' composition of Tunisian propolis extracted by reactor and sonication were identified by HPLC-ESI-MS (Table 1) and quantified by HPLC-UV (Table 2). In general, all propolis extracts showed the same qualitative phenolic profiles, but with quantitative differences. Table 2 shows that adipic acid, caffeic acid, *p*-coumaric acid, isorharmnetin-3-*O*-rutinoside, *p*-coumaroyl malic acid, luteolin, rosmarininc acid, naringenin, quecetin, kaempferol, pinocembrin, genistein, chrysin, CAPE, galangin and 4- cinnamoyloxy caffeic acid were found in all the samples, while catechin was not detected in any sample and chlorogenic acid was only detected in propolis from Béja and El Kef extracted by sonication. Genistein, galangin and CAPE were the most abundant compounds found in all the Tunisian propolis samples independently of their geographical origin, followed by chrysin and apigenin. Martos et al. (1997) also found pinobanksin, pinocembrin, CAPE, chrysin and galangin in Tunisian propolis. It is interesting to highlight the fact that extracts obtained by sonication contained more compounds and in higher concentrations than extracts obtained with reactor. Propolis from Béja contained all the studied compounds, being also the samples with the highest amount of phenolics and flavonoids, both as a group and also as individual components. Gallic, caffeic, *p*-coumaric and ferulic acid, rutin, luteolin, apigenin, kaempferol, chrysin, galangin and CAPE were obtained in similar amounts than those previously described for Greek propolis (Kasiotis et al., 2017). In comparison with other propolis (Andrade et al. 2018; Kasiotis et al. 2017), our quantities of pinocembrin, quercetin, naringenin were lower, while our amounts of rosmarinic acid and genistein were higher. Isorhamnetin-3-*O*-rutinoside and *p*-coumaroyl malic acid were identified in propolis for the first time, although the first was already detected in bee bread (Sobral et al. 2017).

Caffeic acid phenethyl ester (CAPE) and galangin were among the major compounds in the studied Tunisian propolis samples. CAPE was also described as the major compound of Indian (Kasote et al., 2017) and Chinese propolis (Kumazawa, Hamasaka, & Nakayama, 2004). CAPE and, to lesser extent, galangin were cited as responsible for the anti-inflammatory potential of propolis (Rossi et al., 2002). Furthermore, CAPE was also related to a large number of biological activities such as antimicrobial and anticancer activities (Murtaza et al., 2014). Genistein and chrysin are frequently found in propolis from different geographical locations (Andrade et al. 2018), and are among the predominant bioactive constituents presents in the studied Tunisian propolis. Genistein, showed a good potential in treating some irregularities related to metabolic syndrome an cancer (Mukund, Mukund, Sharma, Mannarapu, & Alam, 2017). Chrysin was reported in the literature as advantageous for human health. In fact, several studies described its therapeutic effects against various human diseases (Mani & Natesan 2018).

3.3.Antioxidant properties

Two assays (ABTS and AOA) were chosen to estimate the antioxidant capacity of different propolis extracts. For the ABTS assay (Fig. 3a), TEAC of propolis extracts ranged from 109.76 and 252.9 µmol Trolox/g ($P < 0.05$). Furthermore, the propolis extracts showed a radical-scavenging effect on hydroxyl radicals ranging between 5.26 and 6.83 mmol UA/100 g, which corresponded to 0.1 to 0.13 mmol UA/100 ml (Fig. 3b). Such values were similar to those obtained by Osés et al. (2016). ABTS and AOA assays showed that the propolis from Béja was the richest source of antioxidants, while that from Monastir had the lowest antioxidant capacity ($P < 0.05$). The highest antioxidant activities values of propolis from Béja could be due to its higher content of phenolics and flavonoids, as well as to the contribution of other reducing compounds from bee and pollen origin (Bogdanov, 2017), among them some minerals, carbohydrates, organic acids, nitrogen compounds and vitamins.

Indeed, several studies also reported a high correlation between the total phenolic compounds and the extracts antioxidant activity (Mouhoubi-Tafinine et al., 2016). Béja is located in north-western Tunisia and it is characterized by its fertile soil and wide mountainous areas densely covered with trees. This could be a strong reason justifying the best quality of propolis from Béja.

3.4. Anti-inflammatory activity

Fig. 4a shows the anti-inflammatory activities of the Tunisian propolis extracts. The inhibition percentage varied with the samples geographical origin from 12.61% (Kesserine) to 28.46% (El Kef). These results were in the same range as those of some Moroccan propolis (El-Guendouz et al., 2016), for which different anti-inflammatory activities were described depending on the harvesting region. However, anti-inflammatory activities were not related to phenols and/or flavonoids, which was concordant with the results obtained by Silva, Rodrigues, Feás, & Estevinho (2012) and El-Guendouz et al. (2016), suggesting that polyphenols are not the sole substances involved in this activity. Other compounds, namely, vitamins and proteins could play a role in the anti-inflammatory activity. Contrary to the results of total phenols and antioxidant activities, the anti-inflammatory capacity of propolis was higher if the extraction was carried out by the conventional method rather than sonication. These results could suggest that other compounds extracted by the latter procedure (but not by the former), could interfere with anti-inflammatory activity.

3.5. Antihypertensive activity: ACE inhibitory activity

Hypertension and related diseases are controlled by angiotensin converting enzyme which indirectly increases blood pressure and hypertension. The ACE inhibition is considered as an important therapeutic way in the treatment of hypertension. Tunisian propolis (Fig. 4b) showed an ACE inhibition percentage higher than 90%. There were neither significant differences among the values depending on the geographical origin of the samples nor

between the two extraction methods ($P > 0.05$). Antihypertensive activity of Brazilian propolis was briefly described by Mishima, Yoshida, Akino, & Sakamoto (2005). The review of Bogdanov (2017) included this activity among other propolis biological effects. To the best of the authors' knowledge, this is the first report, in which the HPLC-UV procedure has been used to assess the ability of Tunisian propolis extracts to inhibit (*in vitro*), the activity of angiotensin converting enzyme. When compared the antihypertensive activity of propolis and honeys, it was found that higher concentrations of honeys (50% v/v) showed lower ACE inhibitory activities (max. 71%) (León-Ruiz et al., 2013). Propolis' antihypertensive activity could be attributed to their richness in flavonoids (García-Lafuente, Guillamón, Villares, Rostagno, & Martínez, 2009), suggesting that flavonoids might be protective against cardiovascular diseases by several mechanisms such as antioxidant, anti-platelet and anti-inflammatory effects.

3.6. Antimicrobial activity

Table 3 shows the antimicrobial activity of different propolis samples. All propolis showed antimicrobial activity against all the assessed microorganisms. Ethanol was used as a control sample. In most cases, ethanol showed no antimicrobial activity. However, when an ethanol halo was observed, this halo was subtracted from the total inhibition halo. As expected, the propolis from Béja showed significantly higher antifungal and antibacterial activity ($P < 0.05$). In fact, this propolis was the richest in phenolics and flavonoids, and the importance of these compounds for propolis antimicrobial activity has been proved in several studies (Popova, Silici, Kaftanoglu, & Bankova, 2005; Stepanović, Antić, Dakić, & Švabić-Vlahović, 2003). *Penicillium commune* and *Fusarium sp.* appeared to be the most susceptible microorganisms while *Aspergillus flavus* was the most resistant microorganism to propolis extracts. As in previous studies (Cardoso et al., 2016; Kouidhi et al., 2010) Tunisia propolis confirmed antimicrobial activity against *S. mutans*, a cariogenic bacterium.

4. Conclusions

Tunisian propolis has demonstrated to be an interesting natural source of polyphenols and flavonoids. Furthermore, it has shown high antioxidant, anti-inflammatory, antihypertensive and antimicrobial activities. With regard to antioxidants and the vast majority of biological activities, the best results have been obtained by ultrasonication extraction. In contrast, the conventional extraction procedure has shown to be the most adequate for analysing anti-inflammatory activity. HPLC-UV and HPLC-ESI-MS procedures have successfully identified 24 phenolic compounds, being genistein, galangin and CAPE the predominant phenols in Tunisian propolis. Propolis from Béja have exhibited the highest amount of phenolic compounds, also showing a stronger potential of almost all biological activities.

The results of this study have shown that Tunisian propolis, especially those from Béja, could be efficiently used as promising raw materials in food and pharmaceutical industries, due to their rich phenolic composition and their potential health benefits.

Conflicts of interest

The authors declare that there are no conflicts of interest concerning this article.

5. ACKNOWLEDGMENTS

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493 **Figure captions**

494 **Figure 1.** Geographical areas from Tunisia where propolis samples were collected. P1:
495 Kasserine, P2: Béja, P3: El Kef, P4: Monastir.

496 **Figure 2.** Total phenol content of propolis samples (P1: Kasserine, P2: Béja, P3: El Kef, P4:
497 Monastir) extracted with ultrasonic (UE) and reactor extraction (RE), flavone flavonol content
498 expressed as mg Q/100 g, flavanone and dihydroflavonol content expressed as mg N/100g
499 and flavanol content as mg C/100 g of propolis samples. Different superscript letters (a-c) by
500 each extraction method indicate significant differences according to Tukey's test at
501 significance level $P < 0.05$.

502 **Figure 3.** Antioxidant activity of propolis samples (P1: Kasserine, P2: Béja, P3: El Kef, P4:
503 Monastir) extracted with ultrasonic (UE) and reactor extraction (RE) by TEAC assay (a)
504 expressed as µmol Trolox/100g and by AOA assay (b) expressed as mmol UA/100 g.
505 Different superscript letters (a-c) for the same extraction method indicate significant
506 differences according to Tukey's test at significance level $P < 0.05$.

507 **Figure 4.** Anti-inflammatory activity (a) of propolis samples (P1: Kasserine, P2: Béja, P3: El
508 Kef, P4: Monastir) extracted with ultrasonic (UE) and reactor extraction (RE) expressed as %
509 of hyaluronidase inhibition and ACE-inhibitory activity (b) of samples expressed as ACEi%.
510 Different superscript letters (a-c) for the same extraction method indicate significant
511 differences according to Tukey's test at significance level $P < 0.05$.

Table 1. Phenolic compounds identified in Béja propolis (Tunisia) extracted by sonication, characterized by HPLC-ESI-MS analysis.

Peak	RT (min)	MS^+ $[M-H]^+$ (m/z)	MS^- $[M-H]^-$ (m/z)	Proposed compounds	Reference/standard used
1	1.8	-	145	Adipic acid	Kasiotis et al., 2017
2	3.2	171	169	Gallic acid	Standard
3	17.6	181	179	Caffeic acid	Standard
4	18.3*	291	289	(+)- Catechin	Standard
5	20.9	355	353	Chrologenic acid	Standard
6	23.1	-	163	*p*-Coumaric acid	Standard
7	27.5	195	-	Ferulic acid	Standard
8	29.6	-	623	Isorhamnetin-3-*O*-rutinoside	Sobral et al., 2017
9	33.4	281	-	*p*-Coumaroyl malic acid	http://phenol-explorer.eu
10	34.3	611	609	Rutin	Andrade et al., 2017
11	35.0	287	285	Luteolin	Kasiotis et al., 2017
12	37.2	-	271	Pinobanksin	Kasiotis et al., 2017
13	38.4	361	359	Rosmarinic acid	Kasiotis et al., 2017
14	40.3	-	271	Naringenin	Standard
15	40.7	303	301	Quercetin	Standard
16	41.5	-	315	Isorhamnetin	Andrade et al., 2017
17	45.8	271	269	Apigenin	Standard
18	46.3	-	285	Kaempferol	Standard
19	51.3	257	255	Pinocembrin	Standard
20	52.2	-	269	Genistein	(Kasiotis et al., 2017)
21	54.1	255	253	Chrysin	(Kasiotis et al., 2017)
22	55.3	-	283	CAPE	Standard
23	55.7	271	269	Galangin	Standard
24	58.8	-	295	4-Cinnamoyloxy cafeic acid	Nina et al., 2016

*Only found in standard.

Table 2. Phenolic compounds of Tunisian propolis (P1: Kasserine, P2: Béja, P3: El Kef, P4: Monastir) extracted with ultrasonic (U) and reactor (R) extraction by HPLC-UV (mg/g of propolis) (n=3)

Compound	RT (min)	P1U	P1R	P2U	P2R	P3U	P3R	P4U	P4R
Adipic acid†	1.8	0.178±0.004^{bc}	0.023±0.003^{d}	0.380±0.078^{a}	0.157±0.000^{c}	0.257±0.033^{b}	0.165±0.002^{bc}	0.230±0.044^{bc}	0.183±0.007^{bc}
Gallic acid	3.2	0.013±0.007^{b}	0.016±0.000^{b}	0.082±0.035^{a}	0.011±0.005^{b}	0.032±0.022^{ab}	ND	0.015±0.000^{b}	0.016±0.018^{b}
Caffeic acid	17.6	0.285±0.036^{b}	0.094±0.011^{c}	0.353±0.044^{ab}	0.398±0.024^{a}	0.078±0.002^{c}	<LQ	<LQ	<LQ
(+)- Catechin	18.3	ND	ND	ND	ND	ND	ND	ND	ND
Chrologenic acid	20.9	ND	ND	0.046±0.009	ND	<LQ	ND	ND	ND
p-Coumaric acid	23.1	0.105±0.013^{b}	0.071±0.011^{bc}	0.196±0.050^{a}	0.043±0.003^{c}	0.073±0.006^{bc}	0.029±0.002^{c}	0.040±0.010^{c}	0.026±0.000^{c}
Ferulic acid	27.5	0.103±0.001^{b}	0.086±0.003^{b}	0.155±0.038^{a}	0.086±0.003^{b}	0.082±0.002^{b}	0.064±0.007^{b}	0.072±0.001^{b}	ND
Isorhamnetin-3-*O*-rutinoside†	29.6	0.034±0.002^{b}	0.004±0.000^{c}	0.191±0.027^{a}	0.004±0.001^{c}	0.025±0.009^{bc}	0.032±0.003^{bc}	0.009±0.005^{bc}	0.004±0.001^{c}
p-Coumaroyl malic acid†	33.4	0.150±0.006^{e}	0.030±0.004^{g}	0.872±0.031^{a}	0.407±0.005^{b}	0.099±0.004^{f}	0.266±0.011^{d}	0.325±0.004^{c}	0.246±0.015^{d}
Rutin†	34.3	0.043±0.003^{b}	0.033±0.002^{b}	0.134±0.042^{a}	0.098±0.007^{a}	0.027±0.004^{b}	<LQ	<LQ	ND
Luteolin†	35.0	0.169±0.005^{d}	<LQ	0.444±0.021^{a}	0.170±0.009^{d}	0.111±0.002^{e}	0.270±0.002^{bc}	0.259±0.007^{c}	0.311±0.035^{b}
Pinobanksin†	37.15	ND	ND	0.255±0.026^{a}	ND	0.130±0.010^{b}	<LQ	ND	ND
Rosmarinic acid†	38.4	0.760±0.004^{a}	0.465±0.007^{b}	0.745±0.028^{a}	0.439±0.051^{bc}	0.385±0.006^{c}	0.060±0.006^{d}	0.089±0.005^{d}	0.030±0.004^{d}
Naringenin + Quercetin*	40.5	0.020±0.004^{b}	0.014±0.004^{b}	0.150±0.099^{a}	0.057±0.031^{b}	0.037±0.006^{b}	0.044±0.009^{b}	0.028±0.000^{b}	0.033±0.006^{b}
Isorhamnetin†	41.5	<LQ	<LQ	0.041±0.006	<LQ	ND	ND	<LQ	ND
Apigenin	45.8	0.268±0.067^{bc}	ND	0.465±0.056^{a}	0.249±0.057^{bc}	0.315±0.036^{bc}	0.275±0.000^{bc}	0.338±0.000^{b}	0.221±0.007^{c}
Kaempferol	46.3	0.100±0.063^{b}	0.031±0.008^{b}	0.229±0.027^{a}	0.050±0.042^{b}	0.036±0.011^{b}	<LQ	0.114±0.007^{b}	<LQ
Pinocembrin	51.3	0.023±0.000^{b}	<LQ	0.436±0.039^{a}	0.108±0.059^{b}	<LQ	<LQ	<LQ	<LQ
Genistein†	52.2	1.026±0.083^{bc}	0.737±0.057^{d}	1.652±0.030^{a}	1.106±0.090^{b}	0.854±0.067^{cd}	0.416±0.035^{e}	0.411±0.107^{e}	0.197±0.022^{f}
Chrysin†	54.1	0.934±0.045^{b}	0.572±0.034^{cd}	1.165±0.008^{a}	0.490±0.111^{d}	0.683±0.022^{c}	0.287±0.048^{e}	0.260±0.118^{e}	0.212±0.051^{e}
CAPE + Galangin*	55.5	0.746±0.125^{cd}	0.572±0.000^{cd}	2.455±0.412^{a}	0.916±0.031^{bc}	1.452±0.182^{b}	1.127±0.061^{bc}	0.655±0.42^{cd}	0.178±0.023^{d}
4-Cinnamoyloxy cafeic acid†	58.8	0.437±0.055^{b}	0.187±0.025^{de}	0.608±0.061^{a}	0.335±0.013^{bc}	0.275±0.042^{cd}	0.104±0.022^{ef}	0.078±0.025^{f}	0.067±0.017^{f}

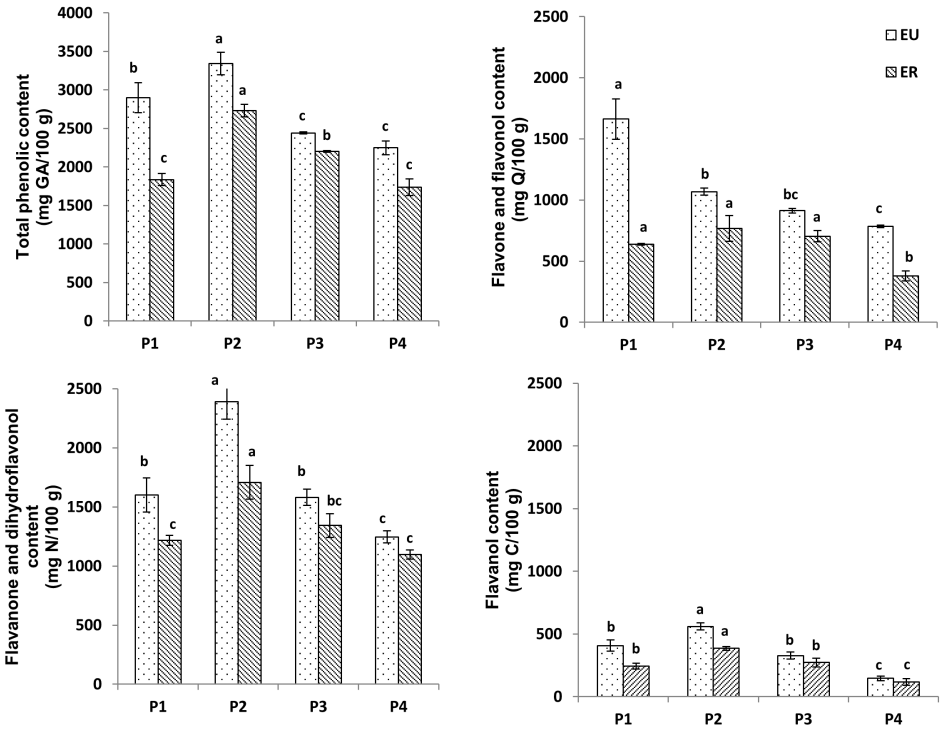
a-g: different letters means significant difference ($P < 0.05$) for the same phenol compound. RT: Retention time; LQ: Limit quantification; ND: Not detected; † These compounds were quantified as mg of caffeic acid/g of propolis. *These compounds elute at the same RT, so they were quantified together.

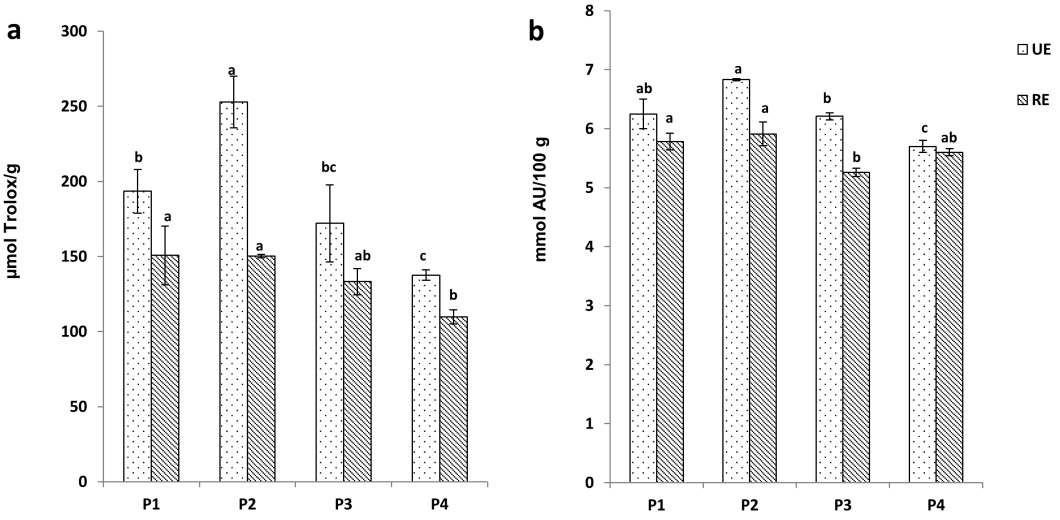
Table 3.

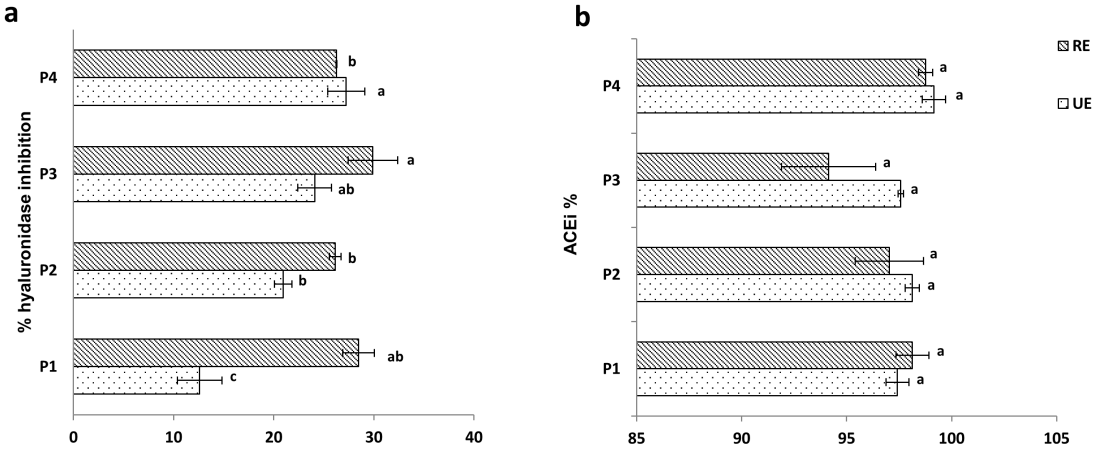
Antimicrobial activity of different extracts of propolis (P1: Kasserine, P2: Béja, P3: El Kef, P4: Monastir) extracted with ultrasonic (U) and reactor extraction (R), expressed as inhibition diameter (mm) including disc (6.0 mm) by agar well diffusion method.

Sample	*P. expansum*	*P. nordicum*	*P. commune*	*A. flavus*	*A. niger*	*Fusarium sp.*	*S. mutans*	*Lb. plantarum*	*E. coli*
P1U	$_{E}7.39^{d}$	$_{D}10.44^{bc}$	$_{B}13.22^{ab}$	$_{E}7.52^{a}$	$_{CD}11.32^{abc}$	$_{A}15.45^{a}$	$_{CD}11.07^{bcd}$	$_{D}10.41^{ab}$	$_{BC}12.27^{b}$
P1R	$_{D}7.21^{d}$	$_{BC}9.89^{cd}$	$_{BC}11.08^{bcd}$	$_{CD}9.37^{a}$	$_{B}12.30^{ab}$	$_{A}16.65^{a}$	$_{BC}10.23^{cde}$	$_{BC}10.87^{ab}$	$_{BC}10.13^{d}$
P2U	$_{C}11.89^{a}$	$_{C}12.07^{a}$	$_{AB}14.17^{a}$	$_{D}9.45^{a}$	$_{BC}12.90^{a}$	$_{A}14.90^{a}$	$_{BC}12.68^{a}$	$_{BC}12.63^{a}$	$_{C}11.83^{bc}$
P2R	$_{BCD}9.97^{bc}$	$_{CD}9.89^{cd}$	$_{ABC}11.86^{abc}$	$_{D}8.07^{a}$	$_{AB}12.30^{ab}$	$_{ABC}11.93^{b}$	$_{D}9.00^{e}$	$_{ABC}11.42^{ab}$	$_{A}12.92^{a}$
P3U	$_{ABC}11.36^{ab}$	$_{ABC}11.51^{ab}$	$_{A}13.20^{ab}$	$_{C}9.18^{a}$	$_{BC}9.95^{bc}$	$_{BC}10.72^{b}$	$_{BC}9.80^{de}$	$_{BC}9.57^{b}$	$_{AB}11.78^{c}$
P3R	$_{CDE}9.10^{c}$	$_{DEF}8.82^{de}$	$_{ABC}10.32^{cd}$	$_{EF}7.80^{a}$	$_{BCD}9.53^{c}$	$_{A}11.13^{b}$	$_{AB}10.48^{bcd}$	$_{ABCD}9.75^{b}$	$_{F}7.68^{e}$
P4U	$_{B}8.69^{cd}$	$_{B}8.24^{e}$	$_{A}12.75^{abc}$	$_{B}7.70^{a}$	$_{A}11.85^{abc}$	$_{A}11.30^{b}$	$_{A}11.55^{ab}$	$_{A}11.07^{ab}$	$_{A}11.28^{cd}$
P4R	$_{A}11.37^{ab}$	$_{C}6.74^{f}$	$_{BC}8.28^{d}$	$_{C}7.82^{a}$	$_{AB}9.75^{c}$	$_{A}10.97^{b}$	$_{A}11.15^{bc}$	$_{A}11.43^{ab}$	$_{A}11.17^{cd}$

Different superscript letters (a-f) in the same column for each microorganism indicate significant differences and different capital letters (A-F) in the same row for each sample indicate significant differences between microorganisms according to Tukey's test at significance level $P < 0.05$.







Highlights

- Phenolic compounds were determined in propolis from Tunisia.
- Propolis ultrasonic extraction yielded higher bioactive properties.
- Antihypertensive activity was evaluated for the first time in propolis extracts.
- Tunisian propolis has properties that may be useful in industrial applications.