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Evaluation of bioactive compounds and biological activities of Tunisian propolis

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1 **Evaluation of bioactive compounds and biological activities of Tunisian**
2 **propolis**

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Abstract

The aim of this study was to investigate the phytochemical composition and biological properties 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

33 1. Introduction

34 Propolis, or bee glue, is a resinous substance collected by honeybees (*Apis mellifera* L.) from
35 buds and exudates of several plants and then used to smooth out the internal walls of the hive
36 in order to protect it against intruders (Toreti, Sato, Pastore, & Park, 2013). Propolis chemical
37 composition greatly varies with the site of collection and thus, with the geographical and
38 climatic conditions (Bankova, Popova, & Trusheva, 2014). Propolis mainly consists of 50%
39 resin, 30% wax, 10% essential oils, 5% of other organic compounds, as well as 5% pollen
40 (Gómez-Caravaca, Gómez-Romero, Arráez-Román, Segura-Carretero, & Fernández-
41 Gutiérrez, 2006). This natural substance has been widely used in folk medicine in many
42 regions of the world, being one of the few natural remedies that has preserved its popularity
43 for a long time (Castaldo & Capasso, 2002). There are several studies, that describe some
44 potentially interesting properties of propolis, among which we can cite its antibacterial
45 (Graikou, Popova, Gortzi, Bankova, & Chinou, 2016), antioxidant (Campos et al., 2014),
46 antiviral (Ahmad, Kaleem, Ahmed, & Shafiq, 2015), anti-inflammatory (Shi, Yang, Zhang, &
47 Yu, 2012) and antitumoral (Silva-Carvalho et al., 2014). Nevertheless, propolis is not directly
48 employed as such, being necessary the removal of unwelcome compounds. Therefore, a
49 propolis extraction is indispensable before using. The most common method to obtain a
50 propolis extract is by solvent extraction, but this procedure is being increasingly replaced by
51 ultrasound extraction, whose efficiency for such vegetal compounds as phenolics has been
52 reported. On the basis of the above, it would be very interesting to research which extraction
53 procedure yields the best values for potential functional properties of propolis. Different
54 solvents, such as ethanol, water, methanol and ethyl acetate, among others were used for
55 propolis extraction (Ma, Ma, Pan, Luo & Weng, 2016; Usman, Abu Bakar, & Mohamed,
56 2016), showing ethanol extract the highest activity for most of them. Furthermore, ethanol is

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

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

69 **2. Materials and methods**

70 **2.1. Analytical standards and reagents**

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

75 **2.2. Propolis samples and extracts preparation**

76 Propolis samples were collected by beekeepers in four areas of Tunisia. Fig. 1 shows the
77 collection sites of each sample. The samples were harvested using a plastic propolis trap and
78 stored in the dark at -20 °C until use. Propolis samples were grounded in a marble mortar at -
79 30 °C. Extraction was carried out according to two methods.

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

83 *Procedure 2:* Two grams of grinding propolis were extracted in dark conditions with 30 ml of
84 80% ethanol in an ultrasonic bath (Selecta, Abrera, Barcelone, Spain) with heating frequency
85 of 40 KHz for 20 min. Then, the mixture was filtered (Whatman filter paper No. 4), and the
86 solid was re-extracted two times more using the same conditions, in order to extract the
87 maximum possible quantity of bioactive compounds from the crude propolis. After the third
88 extraction, all the extracts were combined in a 100 ml volumetric flask and the volume was
89 adjusted with 80% ethanol. The extraction procedure was performed in triplicate for each
90 sample, obtaining a final volume of 300 ml.

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

92 **2.3. Total phenolic content**

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

99 **2.4. Total flavonoids content**

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

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

109 **2.5. Identification and quantification of phenols compounds using HPLC-UV and** 110 **HPLC-ESI-MS system**

111 Qualitative and quantitative analysis were performed using a liquid chromatograph Varian
112 Pro Star 310. The chromatographic separation was carried out on a reversed-phase Microsorb-
113 MV 100-5 C18 column (150 × 4.6 mm, 5 µm particle size) provided by Agilent Technologies.
114 The chromatographic conditions were described by Falcão et al. (2013) and modified for our
115 purposes. The mobile phase comprised (A) 0.1% formic acid in miliQ water and (B) 0.1%
116 formic acid in acetonitrile. The solvent gradient was: 0-7 min, 0% B, 7-12 min, 2% B, 12-20
117 min, 8% B, 20-23 min, 10% B, 23-33 min, 20% B, 33-45 min, 23% B, 45-50 min, 30% B, 50-
118 55 min, 32% B, and 55-60 min, 50% B. The injection volume for all samples was 20 µL and
119 the flow rate was 1 mL/min. Detection was carried out at 280 nm.

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

127 In order to identify the unknown compounds, a HPLC-ESI-MS system consisting of a HPLC
128 1260 Infinity chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), connected

129 to a quadrupole-time of flight (6545-Q-TOF) system was used. Electrospray ionization (ESI)
130 in the negative and positive ion mode was done by the source Dual AJS-ESI under the
131 following conditions: Gas temperature 325 °C, drying gas 10 L/min, nebulizer 45 psi, Vcap
132 3500 V, nozzle voltage 200V and sheath gas at 350 °C. MS-TOF with fragmentor at 100 V,
133 skimmer 45 V and OCT 1 RF VPP 750 V was used, acquiring data between 100 and 1000
134 m/z. Nitrogen was used as collision and as nebulizing gas. The compounds were identified by
135 comparison of their ESI-MS fragmentation spectra with the literature data (Andrade et al.
136 2018; Kasiotis et al. 2017; Nina et al. 2016; Sobral et al. 2017) and with data from on-line
137 chemical database Phenol-Explorer (<http://phenol-explorer.eu>). The column, mobile phase
138 and flow conditions were those described for the previous HPLC-UV analysis.

139 **2.6. Assessment of antioxidant activities**

140 **2.6.1. ABTS scavenging activity test (TEAC Assay)**

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

147 **2.6.2. Radical-scavenging effect on hydroxyl radicals (AOA assay)**

148 Hydroxyl radicals scavenging activity of extracts was determined using the method reported
149 by Koracevic, Koracevic, Djordjevic, Andrejevic, & Cosic (2001). Each sample (A1) had its
150 own control (A0) and for each series of analysis a negative control (K1 and K0) was prepared
151 where the sample was replaced with phosphate buffer. Standards containing 1 mmol/l uric
152 acid (UA₁ and UA₀) were used for calibration. The antioxidant activity was calculated as
153 $AOA (mmol/l) = CU \times (K-A)/(K-U)$ 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 = $(A - B/A) \times 100$, 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 C₁₈ column (4.6 \times 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

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

180 **2.9. Antimicrobial activity**

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

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

197 **2.10. Statistical analysis**

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

202 **3. Results and discussion**

203 **3.1. Total phenolics and flavonoids contents**

204 The amounts of total polyphenols' and flavonoids' contents of Tunisian propolis significantly
205 varied depending on both, the samples harvesting region and the extraction method ($P < 0.05$)
206 (Fig. 2). Polyphenols extraction was more efficient by sonication ($P < 0.05$) as has been
207 previously reported (Ristivojević et al., 2018). Propolis polyphenols ranged from a minimum
208 value of 1734 mg GA/100 g for a conventionally extracted propolis from Monastir, to a
209 maximum value of 3344 mg GA/100 g for an ultrasonic extracted propolis from Béja. Such
210 values were in the same range as those obtained for the Algerian propolis (Mouhoubi-
211 Tafinine, Ouchemoukh, & Tamendjari, 2016), and Moroccan propolis (Miguel et al., 2014).
212 In contrast, total polyphenols contents of the Turkish, Brazilian and Chinese propolis were
213 considerably higher than those found in our research (Alencar et al., 2007; Ristivojević et al.,
214 2018; Wang et al., 2014). These variations are very likely due to the propolis different
215 botanical origins, being also influenced by the harvesting year, geographic origins, as well as
216 environmental conditions and seasonal variation.

217 In this research, three groups of flavonoids were analyzed. The first one involved flavones
218 and flavonols, whose values ranged from 378 mg Q/100 g to 1661 mg Q/100 g. The second
219 one was made up of flavanones and dihydroflavonols, whose results varied from 1098 mg
220 N/100 g to 2391 mg N/100 g. The third group was made up flavanols, whose contents
221 fluctuated from 117 mg C/100 g to 559 mg C/100 g. Our flavones and flavonols amounts
222 were comparable to those described by Miguel et al. (2014) for the Moroccan propolis (from
223 20 to 3427 mg Q/100 g). Nevertheless, in general our values for the different flavonoids
224 groups were lower than the data described in the literature for the propolis from other
225 continents (Alencar et al., 2007). These differences in flavonoids quantities could be

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

228 **3.2. Identification of phenolic compounds in propolis by HPLC**

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

251 Caffeic acid phenethyl ester (CAPE) and galangin were among the major compounds in the
252 studied Tunisian propolis samples. CAPE was also described as the major compound of
253 Indian (Kasote et al., 2017) and Chinese propolis (Kumazawa, Hamasaka, & Nakayama,
254 2004). CAPE and, to lesser extent, galangin were cited as responsible for the anti-
255 inflammatory potential of propolis (Rossi et al., 2002). Furthermore, CAPE was also related
256 to a large number of biological activities such as antimicrobial and anticancer activities
257 (Murtaza et al., 2014). Genistein and chrysin are frequently found in propolis from different
258 geographical locations (Andrade et al. 2018), and are among the predominant bioactive
259 constituents presents in the studied Tunisian propolis. Genistein, showed a good potential in
260 treating some irregularities related to metabolic syndrome an cancer (Mukund, Mukund,
261 Sharma, Mannarapu, & Alam, 2017). Chrysin was reported in the literature as advantageous
262 for human health. In fact, several studies described its therapeutic effects against various
263 human diseases (Mani & Natesan 2018).

264 **3.3.Antioxidant properties**

265 Two assays (ABTS and AOA) were chosen to estimate the antioxidant capacity of different
266 propolis extracts. For the ABTS assay (Fig. 3a), TEAC of propolis extracts ranged from
267 109.76 and 252.9 $\mu\text{mol Trolox/g}$ ($P < 0.05$). Furthermore, the propolis extracts showed a
268 radical-scavenging effect on hydroxyl radicals ranging between 5.26 and 6.83 mmol UA/100
269 g , which corresponded to 0.1 to 0.13 mmol UA/100 ml (Fig. 3b). Such values were similar to
270 those obtained by Osés et al. (2016). ABTS and AOA assays showed that the propolis from
271 Béja was the richest source of antioxidants, while that from Monastir had the lowest
272 antioxidant capacity ($P < 0.05$). The highest antioxidant activities values of propolis from
273 Béja could be due to its higher content of phenolics and flavonoids, as well as to the
274 contribution of other reducing compounds from bee and pollen origin (Bogdanov, 2017),
275 among them some minerals, carbohydrates, organic acids, nitrogen compounds and vitamins.

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

281 **3.4. Anti-inflammatory activity**

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

295 **3.5. Antihypertensive activity: ACE inhibitory activity**

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

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

313 **3.6. Antimicrobial activity**

314 Table 3 shows the antimicrobial activity of different propolis samples. All propolis showed
315 antimicrobial activity against all the assessed microorganisms. Ethanol was used as a control
316 sample. In most cases, ethanol showed no antimicrobial activity. However, when an ethanol
317 halo was observed, this halo was subtracted from the total inhibition halo. As expected, the
318 propolis from Béja showed significantly higher antifungal and antibacterial activity ($P <$
319 0.05). In fact, this propolis was the richest in phenolics and flavonoids, and the importance of
320 these compounds for propolis antimicrobial activity has been proved in several studies
321 (Popova, Silici, Kaftanoglu, & Bankova, 2005; Stepanović, Antić, Dakić, & Švabić-Vlahović,
322 2003). *Penicillium commune* and *Fusarium sp.* appeared to be the most susceptible
323 microorganisms while *Aspergillus flavus* was the most resistant microorganism to propolis
324 extracts. As in previous studies (Cardoso et al., 2016; Kouidhi et al., 2010) Tunisia propolis
325 confirmed antimicrobial activity against *S. mutans*, a cariogenic bacterium.

326 **4. Conclusions**

327 Tunisian propolis has demonstrated to be an interesting natural source of polyphenols and
328 flavonoids. Furthermore, it has shown high antioxidant, anti-inflammatory, antihypertensive
329 and antimicrobial activities. With regard to antioxidants and the vast majority of biological
330 activities, the best results have been obtained by ultrasonication extraction. In contrast, the
331 conventional extraction procedure has shown to be the most adequate for analysing anti-
332 inflammatory activity. HPLC-UV and HPLC-ESI-MS procedures have successfully identified
333 24 phenolic compounds, being genistein, galangin and CAPE the predominant phenols in
334 Tunisian propolis. Propolis from Béja have exhibited the highest amount of phenolic
335 compounds, also showing a stronger potential of almost all biological activities.

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

339 **Conflicts of interest**

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

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492

ACCEPTED MANUSCRIPT

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 $\mu\text{mol Trolox}/100\text{g}$ and by AOA assay (b) expressed as $\text{mmol UA}/100\text{ g}$.
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 | <i>p</i> -Coumaric acid | Standard |
| 7 | 27.5 | 195 | - | Ferulic acid | Standard |
| 8 | 29.6 | - | 623 | Isorhamnetin-3- <i>O</i> -rutinoside | Sobral et al., 2017 |
| 9 | 33.4 | 281 | - | <i>p</i> -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 caffeic 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 |
| <i>p</i> -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- <i>O</i> -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 |
| <i>p</i> -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 ^f | 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 caffeic 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.

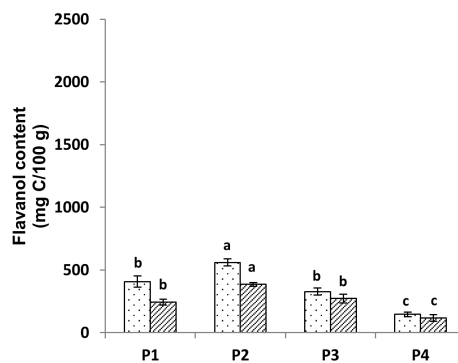
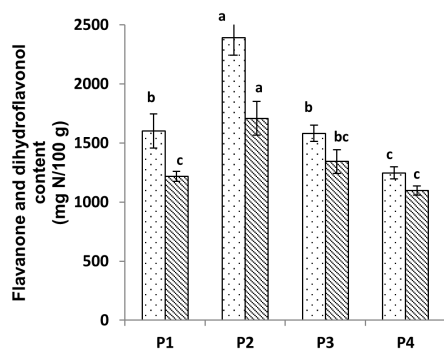
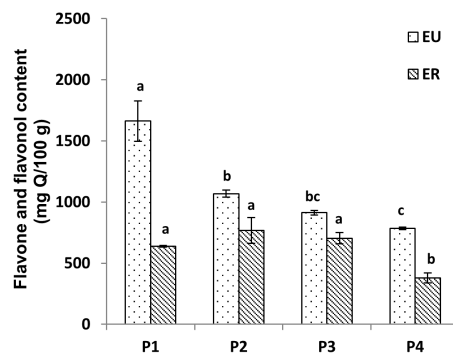
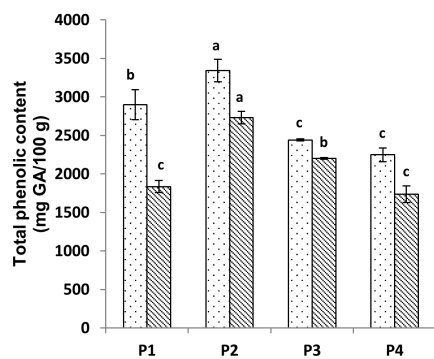
Antimicrobial activity of different extracts of propolis (P1: Kasserine, P2: Béja, P3: El Kef, P4: Monastir) extracted with ultrasonic (U) and reactor extraction (R), expressed as inhibition diameter (mm) including disc (6.0 mm) by agar well diffusion method.

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

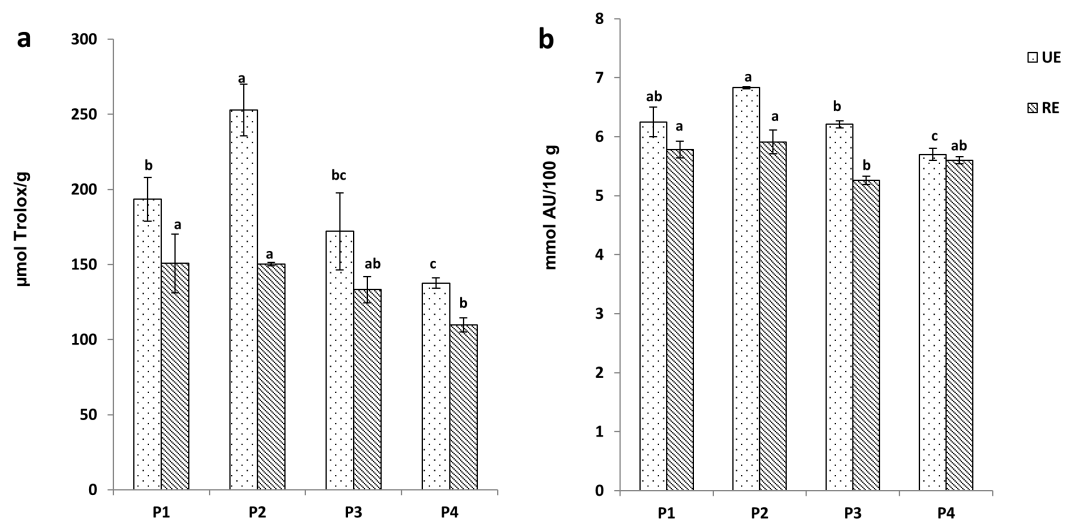
Different superscript letters (a-f) in the same column for each microorganism indicate significant differences and different capital letters (A-F) in the same row for each sample indicate significant differences between microorganisms according to Tukey's test at significance level $P < 0.05$.



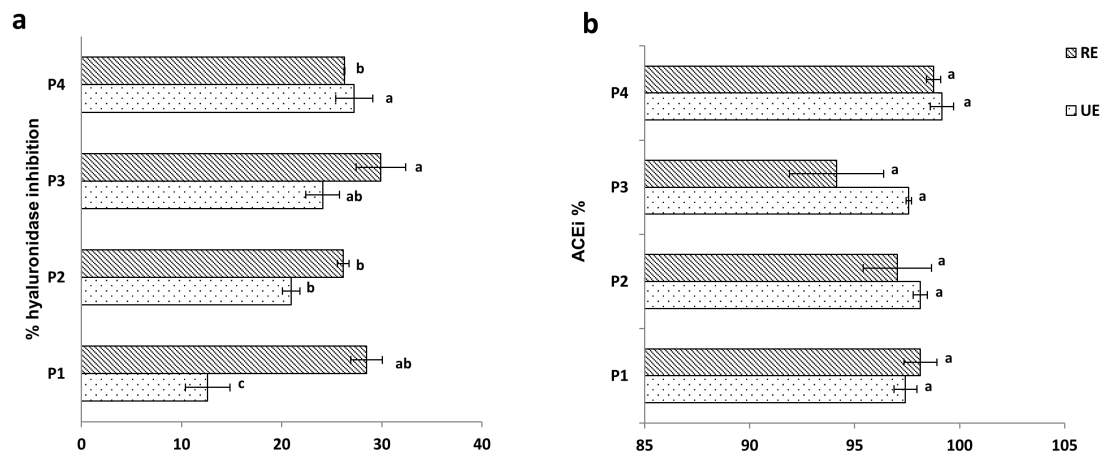
ACCEPTED MANUSCRIPT



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1 Highlights

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