Correlation between antioxidant activity and phenolic acids profile and content of Algerian propolis: Influence of solvent

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Abstract: We aimed in the reported study to investigate the impact of using various solvents in the extraction of potentially active compounds from Algerian propolis. Phenolic and flavonoids contents in association with antioxidant activity of the tested extracts were evaluated. Moreover phenolic composition was determined using UFLC-MS/MS. The tested parameters varied according to the used solvent. Total phenolic and flavononid contents ranged from 0.81±0.16 to 8.97±0.25 EGA mg/g and from 0.57±0.01 to 3.53±0.84 EQ mg/g respectively. All the investigated extracts demonstrated notable antiradical and reducing activities. Ethyl acetate and n-butanol were found to contain the highest amounts of phenolic and flavonoid compounds and the strongest antioxidant properties. The antioxidant activity of propolis extracts appears to be largely influenced by total phenolic and flavonoid contents. Rutin, chlorogenic, ferulic, caffeic and gallic acids were found to be the main phenolic compounds in Algerian propolis. Our results suggest that Algerian propolis may be a poplar-type propolis

Keywords:Algerian propolis, phenolic profile, UFLC-MS/MS, antioxidant activity.

INTRODUCION

In recent years, natural products have become the subject of a widespread food producers and consumers interest. Among them bee products are worth of particular attention (Socha *et al.,* 2015). Propolis is one of bee products described as a viscous material collected from different parts of plant and mixed with salivary secretions and wax (Burdock, 1998). This material found numerous uses in the beehive and is considered as a very interesting product for human with many biological effects such as antiinflammatory (Sforcin, 2007), antioxidant (Yang *et al.,* 2011; Jug *et al.,* 2014), antitumor (Orsolic *et al.,* 2006), hepatoprotective (Banskota *et al.,* 2001) and immunostimulatory activities (Orsolic *et al.,* 2003).

Algerian northeast regions propolis tested *in vivo* have been reported to reduce toxic effects of doxorubicin (Lahouel *et al.,* 2004). In a previous study, we reported that another propolis collected from the same location of the northeast of Algeria (Jijel) suppress overexpression of MMP-3 in UVA irradiated cells making it an attractive candidate as a control agent of proteolytic cascade involved in several pathologic disorders (rheumatoid arthritis, periodontitis and atherosclerosis (Segueni *et al.,* 2011). Investigation of ethanolic extract of some Algerian propolis showed a remarkable antibacterial activity (Nedji *et al.,* 2014; Segueni *et al.,* 2014) and a potent antioxidant activity (Rebai *et al.,* 2011; Benhanifia *et al.,* 2013; Belfar *et al.,* 2015).

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Phenolic compounds are major bioactive constituents of propolis generally prepared with solvent extraction method (Hatano *et al.,* 2012). Among extraction solvents, polar ones such as ethanol, methanol and water, as well as non-polar ones were used (Sun *et al.,* 2015). In previous research results dealing with the choice of the best solvent for maceration of propolis and leading to the highest antioxidant activity and the highest phenolic and flavonoid contents were inconsists and sometimes with great contradictions. Yang *et al* (2011) reported that in comparaison to ethanol, chloroform and butanol extracts, ethyl acetate extract exhibited the highest content of total phenolic and the strongest antioxidant activity. Many studies revealed that ethanol / water solvents were more effective in extracting phenolic compounds and exhibited a higher antioxidant activity than aqueous extract (Mello *et al.,* 2010). Moreover, phenolic compounds and antioxidant properties of propolis extracts were significantly dependent on the concentration of ethanol / water solvents (Sun *et al.,* 2015). On the contrary, other studies revealed that water extract of propolis showed significantly greater activity than ethanolic extract of propolis (Laskar *et al.,* 2010).

The purpose of the present study is to investigate the influence of different solvents used for maceration on the antioxidant activity and phenolic profile of Algerian propolis. Phenolic profile was characterized by the determination of polyphenol and flavonoid contents. Phenolic composition was also analyzed using UFLC-MS/MS. Antioxidant activity of tested propolis extracts

was investigated using four assays : 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, 2,2 azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, ferric Reducing Antioxidant Power (FRAP) and Cupric ion Reducing Antioxidant Capacity (CUPRAC). Obtained results were correlated in order to select the most convenient solvent for the preparation of propolis extracts.

MATERIALS AND METHODS

Extraction

Propolis was collected from Beni Belaid, Jijel (northeast of Algeria) and extracted by the a combinaison of two previously extraction method used in our laboratory (Segueni *et al.,* 2011 and 2013) with minor modification. Propolis (500g) was first extracted with a mixture of CH_2Cl_2-MeOH in 1:1 (v/v) ratio, tree times. After filtration the extract was concentrated to dryness. The residue was then extracted with MeOH-H_2O (70:30 v/v), concentrated and dissolved in boiling water. After storage in cold for 24 hrs, the aqueous solution was filtered. The filtrate was then extracted successively with $CHCl_3$, ethyl acetate (EtOAc) and butanol (*n*-BuOH) to yield 0.9 g, 2,7g and 8,3g. The $CHCl_3$ extract seems to contain an oily part with a distinguished green colour. The oily part of chloformic extract was separated in a funnel filtered, dried under vacuum to yield 0,42g. CH_2Cl_2-MeOH (1:1) and MeOH-H_2O (70:30 v/v) were expressed as DMEP and MEP. $CHCl_3$, EtOAc and *n*-BuOH extracts were expressed as CEP, ETEP and BEP. The oily part of $CHCl_3$ extract was expressed as OCEP.

Total phenolics and flavonoids contents

Total phenolic content (TPC) of propolis extracts was determined by the Folin-Ciocalteu colorimetric method described by (Spanos and Wrolsted, 1999). A volume of 100µl of propolis extracts at a final concentration of 20 µg/ml, and 900µl of distilled water were mixed with 5ml of Folin-Ciocalteu reagent (0.2 N). After 4min, 4ml of saturated Na_2CO_3 (75g/L) were added. The mixture was incubated 2 h then the absorbance was measured at 765 nm. Total phenolic content was expressed as mg of Gallic acid equivalent /g of the extract.

Total flavonoids contents (TFC) was determined by a colorimetric method described by Dewanto *et al,* (2002) using aluminium chloride. A volume of 250µl of propolis extracts at a final concentration of 100 and 1000µg/ml was mixed with 1.25ml of distilled water and 75µl of 5% $NaNO_2$ solution. The mixture is allowed to stand 6 min then 150µl of a 10% $AlCl_3.6H_2O$ solution were added. After 5min 0.5ml of 1M NaOH was added. Before measuring the absorbance at 510 nm, 275µl of distilled water were added. Total flavonoids contents were expressed as mg quercetin equivalent (QE) /g of the extract.

DPPH radical scavenger activity

The radical scavenger activity of propolis extracts was evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical. About 100µl of propolis extracts at a concentration of 10-100µg/ml and a blank were added to 2ml of DPPH solution. The absorbance was then determined at 517nm after 30 min incubation at 37°C (Rai *et al.,* 2006). Results are expressed as µM trolox equivalent by 1g of propolis extract.

ABTS radical-scavenger activity

The radical scavenger activity of propolis extracts was measured using ABTS (2,2 azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical decolourization assay by the method described by (Toor *et al.,* 2006). A volume of 100µl of propolis extracts at concentrations of 10-100µg/ml and 1ml of the ABTS solution were vortexed for 10s. After exactly 1min, the absorbance at 734 nm was measured. Results are reported as µM trolox equivalent antioxidant capacity by 1g propolis extract.

Ferric ion Reducing Antioxidant Power

The Ferric ion Reducing Antioxidant Power (FRAP) was evaluated according to the procedure described by Deinghon *et al*. (2000). FRAP reagent was freshly prepared with 1mM 2,4,6-tripyridyl-2-tiazine (TPTZ) and 2mM ferric chloride in 0,25M sodium acetate. A volume of 100µl of propolis extracts at a concentration range 10-100µg/ml was added to 900µl of FRAP reagent and mixed. After 4 min of incubation, absorbance at 593 nm was measured. Results are expressed as µM trolox equivalent by 1g of propolis extract.

Cupric ion reducing antioxidant capacity

The cupric ion (Cu^{2+}) reducing antioxidant capacity (CUPRAC) of propolis extracts was evaluated according to the method described by Apak *et al*, (2004). In tested tubes containing 100µl of propolis extracts at a concentration range 10-100µg/ml, 1ml $CuCl_2$ solution (0,01M), 1ml ethanolic neocuproine solution (7,5. 10^{-3} M), 1ml ammonium acetate buffer solution (1M) and 1 ml distilled water were added. 30 min later, absorbance was measured at 450nm. Results are expressed as µM trolox equivalent by 1g of propolis extract.

Phenolic acids analysis by UFLC-MS/MS

Phenolic acids analysis of propolis extracts was performed using a Shimadzu 20A series ultra fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan), a micro vacuum degasser (Prominence Degasser DGU-20A3, Schimadzu), an autosampler (Prominence AutoSampler SIL-20AHT, Schimadzu), a column oven (Prominence Column Oven CTO-10ASVP, Schimadzu), a controller (Prominence Controller CBM-20A Lite, Schimadzu) and an MS detector with electrospray ion source (ESI) and a triple quadrupole analyzer (API-3200 QTRAP, ABSciex, USA).

Separation was performed on an Inertsustain C18 column (150 mm x 4,6 mm, 3µm) with guard column (4.0 x 10 mm x 2) using a gradient of mobile phase A and B (7.5 mM formic acid and acetonitrile). The injection volume was 20µL for each standard mixture and the flow rate was 0.5mL min^{-1}. The column temperature was set to 40°C. An increasing gradient of B starting from 5% up to 95% in 20 min was used. Standard phenolic acids (chlorogenic acid, caffeic acid, 4 (p-)Hydroxybenzoic acid, trans-cinnamic acid, p-coumaric acid, ferulic acid, sinapic acid, syringic acid, vanillic acid and gallic acid) and a glycoside flavonol (rutin) were used for identification and quantification of phenolic acid composition of Algerian propolis extracts by comparaison of their retention time and peak areas with that of detected compounds in propolis tested extracts (Gültekin-Özgüven *et al.*,2015).

STATISTICAL ANALYSIS

Total polyphenols and flavonoids contents as well as antioxidant assays of propolis extracts were quantified using methods adapted to 96-well plate assay. Each sample assay was performed in triplicate. Data were reported as mean ± SD and subjected to statistical analysis using SPSS software (version 20.0) and origin 8. One-way and two-way analysis of variance (ANOVA) followed by Tukey post-hoc and Bonferroni's tests were used to analyze significant differences between treatments ($P<0.05$). The correlation coefficients (R^2) for spectrophotometric assays were calculated using the Microsoft Office Excel 2011 software (Microsoft Corporation, Redmond, WA).

RESULTS

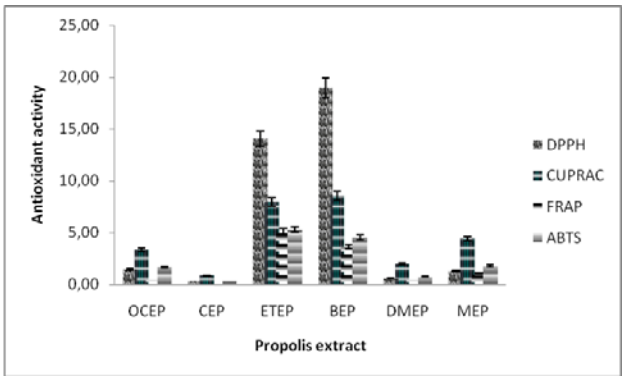
Total polyphenol and flavonoid contents

Total polyphenol (TPC) and flavonoids (TFC) contents of Algerian propolis extracts were determined by Folin-Ciacalteu and aluminium trichloride methods. TPC of various propolis extracts distinctly varied according to the used solvent and ranged from 0.81±0,16 to 8.97±0.25 EGA mg/g. Compared with TPC, TFC ranged from 0.57 ±0.01 to 3.53±0.84 EQ mg/g. In addition, the highest TPC and TFC were both observed in ETEP and BEP followed by MEP, DMEP and OCEP (table 1). The lowest values were observed in CEP. Difference between the tested extracts was found to be significant.

Antioxidant activity

Oxydation is a very complex process involving different mechanisms (Sun *et al.,* 2015). Therefore, antioxidant properties of Algerian propolis extracts was investigated using four assays.

DPPH radical-scavenging activity has been widely used in propolis studies (Mohdaly *et al.,* 2015). In this assay, results are expressed as µM trolox equivalent by 1g of propolis extract (µM trolox/g) (fig. 1). In agreement with finding of TPC and TFC, ETEP and BEP demonstrated higher scavenging activity. However, BEP activity was the highest with 18.97±0,36µM trolox/g. CEP had the lower scavenger activity. DPPH assay showed a strong correlation with TPC *(R^2=0.91)* and a positive correlation with TFC *(R^2 = 0.75)*.



Note: Propolis extracts were expressed as OCEP, CEP, ETEP, BEP, DMEP and MEP. DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2-azobis(2-amidinopropane) dihydrochloride), feric reduciong power (FRAP) and cupric ion reducing antioxidant capacity (CUPRAC) were expressed as micromole of trolox per 1g of propolis (µmole trolox/g). Data are expressed as mean ± SD (n = 3). Values in the same column followed by the same lowercased letter are not significantly different.

Fig. 1: Antioxidant properties of Algerian propolis.

The ABTS radical reactivity is more sensitive than the DPPH radical (Mohdaly *et al.*, 2015). In the present study, ABTS radical scavenging activity assay presented similar results of the DPPH assay (fig. 1). ETEP and BEP were the most actif extracts. In contrast, ETEP demonstrated the highest scavenging activity with 5.28± 0.16µmole trolox/g. CEP was the lowest extract regarding this assay. Difference between the tested extracts was found to be significant. ABTS assay showed a strong correlation with TPC *(R^2 = 0.98)* and TFC *(R^2 = 0.88).*The ability of propolis extracts to scavenge DPPH and $ABTS^+$ free radicals suggests that they might be electrons donors. This propriety leads to the conversion of free radicals to more stable products. The radical chain reaction is then terminated (Apak *et al.,* 2007).

The reductive capabilities of propolis extracts was also measured using FRAP and CUPRAC assays. The reducing properties are generally associated with the presence of reductones (Kumaran *et al.,* 2006). The reducing power of propolis extracts is shown in fig. 1. Similar to the DPPH radical assay, the highest reducing power was shown by ETEP and BEP. The CEP showed the weakest reducing power. A high correlation was observed between reducing power and TPC and TFC in this test system too, with correlation coefficient R^2 = *0.96* and *0,94* respectively.

Table 1: Total phenolic and flavonoid contents of propolis extracts.

Tested extracts	Total polyphenol content EGA mg/g	Total flavonoid content EQ mg/g
OCEP	3.29 ± 0.41^{a}	0.72 ± 0.02^{a}
CEP	0.81 ± 0.16^{b}	0.57 ± 0.01^{a}
ETEP	9.97 ± 0.25^{c}	3.53 ± 0.84^{b}
BEP	$8.23 \pm 0{,}51^{d}$	2 ± 0.41^{c}
DMEP	2.17 ± 0.61^{e}	0.70 ± 0.02^{a}
MEP	$2.95 \pm 0.22^{a,e}$	$1.43 \pm 0.06^{a,c}$

Note: Propolis extracts were expressed as OCEP, CEP, ETEP, BEP, DMEP and MEP. TPC was expressed as milligram of gallic acid equivalent per gram of propolis extract (EGA mg/g). TFC was expressed as milligram of quercetin equivalent per gram of propolis extract (EQ mg/g). Data are expressed as mean ± SD (n = 3). Values in the same column followed by the same lowercased letter are not significantly different.

CUPRAC assay was also used to evaluate the reductive capabilities of propolis extracts (fig. 1). This method based on reduction of Cu^{2+} to Cu^{+} by antioxidants might be more efficiently extended to the possible *in vivo* reactions of antioxidants (Gulçin *et al.,* 2010). Similar to previous assays, ETEP and BEP demonstrated higher scavenger activity. However, BEP activity was the highest with 8.56±0.28 µM trolox/g. CEP had the lower scavenger activity. CUPRAC assay showed a strong correlation with TPC *(R^2 = 0.95)* and TFC *(R^2 = 0.82)*. Confirming the results obtained for TPC and TFC, antioxidants assays (DPPH, ABTS, FRAP and CUPRAC) also showed a significant difference between the tested extracts.

Phenolic acids analysis

Phenolic acids composition of Algerian propolis extracts was investigated using HFLMS/MS. Chlorogenic (48.79 ±5.01ng/ml), ferulic (15.7±2.29ng/ml), caffeic (29.18± 4.95ng/ml) and gallic acids (44.25±6.40ng/ml) were found to be the main phenolic compounds in MEP. In addition, rutin a glycoside flavonol was also investigated (21.89±3.57ng/ml) (fig. 2). The mentioned phenolic acids in association with rutin were representing 32% of MEP. While, ETEP considered as the richest extract in caffeic acid, contained 44.1±5.64ng/ml representing 9%. Gallic and ferulic acids were also detected in this extract but in traces. Among the detected compounds, trans-cinnamic acid was detected in traces in all propolis extracts. Vanillic acid was detected in traces in OCEP, CEP, DMEP and MEP. In addition, sinapic acid was detected in traces only in MEP.

DISCUSSION

The polyphenol and flavonoid contents in MEP are in the same order than those reported in other Algerian regions by Belfar *et al,* (2015), (0.81 to 2.62 EGA mg/g for propolis of Boumerdes, Mostaganem, Bejaîa and Ghardaia) and lower than those reported in north (100.90 to 257.40 EGA mg/g in four regions of Annaba namely Seraidi, Chetaibi, Berrahal and El-bouni by Nedji *et al.,* 2014), south (10.99 EGA mg/g for propolis of El-oued by Rebai *et al.,* 2011) and western Algeria (9. 99 to 46. 63 EAG mg/g for propolis f Tiaret, Sidi bel abbés and Mascara by Benhanfia *et al.,* 2013). Compared to other studies, our results are lower than those reported in China (Yang *et al.,* 2011;Sun *et al.,* 2015), Croatia (Jug *et al.,* 2014), Brazil (Schmidt *et al.,* 2014), India(Laskar *et al.,* 2010),Turkey (Gulçin *et al.,* 2010), Portugal (Moreira *et al.,* 2008) and Iran (Mohammadzadeh *et al.,* 2007).

Generally, phenolics of propolis are prepared with solvent extraction method. Among organic solvents, polar one are widely used such as water, methanol and ethanol. Many researches revealed that ethanol/water solvents were more effective in extracting phenolic compounds than water (Jug *et al.,* 2014; Sun *et al.,* 2015). According to Yang *et al.* (2011), the ethyl acetate extract contained the most content of polyphenols and flavonoids. The quantities were found to decrease in the following order: ethyl acetate extract > chloroformic extract > ethanolic extract > butanolic extract. On the contrary, our study revealed that ethyl acetate and butanol extracts exhibited higher phenolic and flavonoid contents. Chloroform seemed to be less effective in extracting phenolics than methanol and a mixture of methanol and dichloromethane (1/1).

The antioxidant activity of Algerian propolis was found to be largely influenced by the TPC and TFC. This observation is in agreement with the previous research concerning Indian (Laskar *et al.,* 2010), Chinese (Yang *et al.,* 2011; Hatano *et al.,* 2012) and Beijing propolis (Sun *et al.,* 2015).The most active extracts in this study were those prepared using ethyl acetate and n-butanol. Among the tested extracts, the cited ones were also the richest in TPC and TFC. In conclusion, the concentration of such compounds in propolis extracts might be responsible of the antioxidant activity.

Caffeic acid, ferulic and cinnamic acids have been previously detected by GC-MS after silylation in Algerian propolis collected from M'Sila (Velikova *et al.,* 2000). The three cited phenolic acids were also isolated, separated and identified by Boufadi *et al.,* (2014) from Algerian propolis. In the present study, gallic acid, vanillic acid, sinapic acid, chlorogenic acid and rutin are reported for the first time from Algerian propolis.

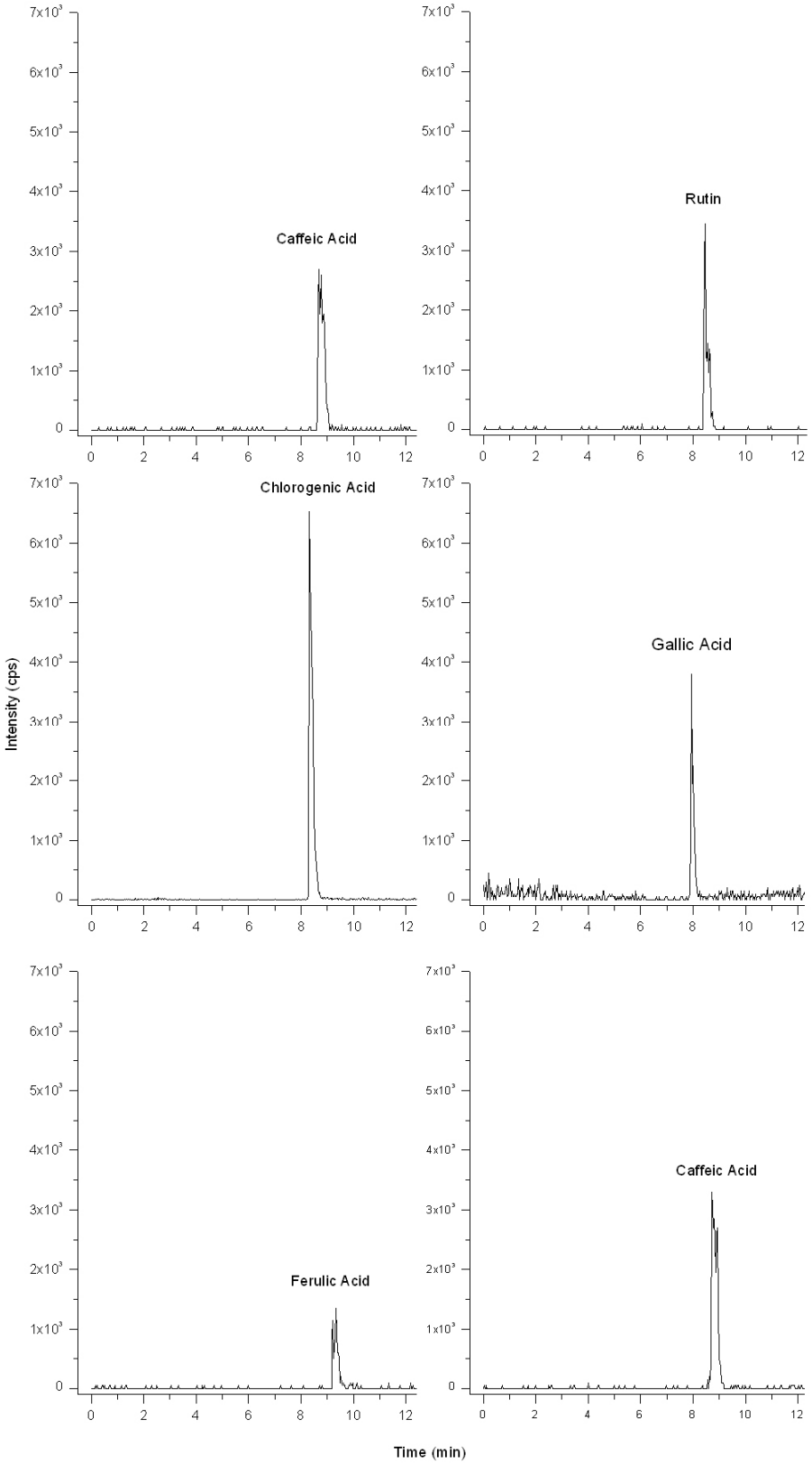


Fig. 2: UFLC Chromatogram of detected phenolic acids in Algerian propolis. (a) caffeic acid, (b) rutin, (c) chlorogenic acid, (d) gallic acid, (e) ferulic acid and (f) caffeic acid.

Previous research on Algerian propolis detected the presence of p-hyroxycinnamic acid and its ethyl ester (Boutabet *et al.,* 2011), isopentyl caffeate, 3-hydroxy-4-methoxycinnamic acid, ferulic acid methylester and prenyl caffeate (Boufadi *et al.*, 2014).

Regarding antioxidant activity of the tested extracts and their composition on phenolic acids, it appeared that there is no correlation between the detected phenolic acids and antioxidant capacities of the tested extract suggesting that other compounds were responsible for the observed variation in antioxidant activity of Algerian propolis such as flavonoids. More investigations are needed to determine the chemical composition of Algerian propolis and to identify compounds responsible of the observed antioxidant activity.

Results of the chemical investigation carried out on Algerian propolis extracts indicated the presence of phenolic acids. Our previous study on Algerian propolis leads to the isolation and identification of some flavonoids and caffeic acid derivatives considered as markers of propolis from Populus resin (Segueni *et al.*, 2011; 2013; 2014). The present study confirm the presence of others markers of the same type of propolis such as caffeic, ferulic and trans cinnamic coumaric acids.

CONCLUSION

We aimed in the present study to investigate the influence of used solvent in the extraction of potentially active compounds from Algerian propolis. Potentially active constituents were tested for their antioxidant activity. Chemical composition was evaluated using total phenolic and flavonoid contents. Moreover, phenolic composition was determined using UFLC-MS/MS. TPC and TFC of various propolis extracts distinctly varied according to the used solvent. Among tested solvent ethyl acetate and n-butanol were found to have the strongest antioxidant activity and the highest amount of TPC and TFC. Rutin, chlorogenic, ferulic, caffeic and gallic acids were found to be the main phenolic compounds in Algerian propolis.

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