Int. J. Electrochem. Sci., 10 **(2015)** 9641 - 9651

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Evaluation of Antioxidant Capacity of Propolis Collected in Various Areas of Algeria Using Electrochemical Techniques

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Received: 12 April 2015 / *Accepted:* 9 September 2015 / *Published:* 30 September 2015

The present study examined the antioxidant activity of methanolic extracts of propolis samples from different regions of Algeria. Total phenol content (TPC) was determined by using Folin-Ciocalteau Reagent. Total flavonoid content (TFC) was determined by using aluminum chloride method. Resulting ranged between (210.884±0.754-19.626±0.301 mg/100g) and (262,338±0.810-81.141±0.538 mg/100g) crude extract of propolis, respectively. Thereafter, evaluate propolis' antioxidant activity, measured by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Total antioxidant activity was measured, based on the phosphomolybdenum assay (PM). A novel system to evaluate the Algerian propolis' antioxidant activity is reported. The methods use a cyclic and square wave voltammetry. These techniques are realized to compare the results from spectroscopic method and to electrochemically characterize the propolis polyphenols. Our results justify the use of as a natural source of antioxidant compounds could be used in the prevention of free radical-related diseases.

Keywords: Algerian propolis; Antioxidant; DPPH; Total polyphenol; Cyclic voltammetry ; Electrochemical techniques.

1. INTRODUCTION

Propolis is an important natural substance contains more than 160 components and used by bees to fill gaps in their hive [1]. Historically, it has been used for various purposes, especially as a medicine [2]. Propolis has gained popularity also as a health drink and is extensively used in food to improve health and prevent diseases. In fact, numerous studies have reported a broad spectrum of biological activities such as anticancer [3]. Antimicrobial [4], antiinflammatory [5-7], antiviral [8,9], as antibiotic, antifungal [9], antineoplasic [10], antioxidative [11-13]. These activities are associated with

the phenolic constituents, especially flavonoids and phenolic acids [14,15]. The flavonoid content of foods may be a major dietary factor responsible for this effect [16,17].

Numerous analytical techniques are available to evaluate oxidative stress namely, electron spin resonance, chromatography, spectroscopy or mass spectrometry [18-23], ESR spectrometry, fluorescence, chemiluminescence, and electrochemistry. Electrochemical approaches are of special advantage in studies of the antioxidant properties of polyphenols [23]. In particular, voltammetric techniques have been successfully employed to detect phenolic compounds in water solutions [24-26] and in complex aqueous media such as teas, wines, beers, etc. [27-29]. Antioxidant compounds can act as reduction agents and, in solutions, they tend to be easily oxidised at inert electrodes. Based on this fact, some of the previous cited authors established an interesting relationship between electrochemical behaviour of the antioxidant compounds and their resultant ‘‘antioxidant power” [30].

For that, we developed a new technique for the determination of antioxidant capacity using less complicated methods, compared to chromatographic and spectroscopic techniques, which used far more complicated apparatus [31]. I am not sure if you need the above sentence at all? It does not make senesce to me.

Our method uses electrochemical techniques, for the determination of the propolis antioxidant capacities.

The objective of the present work thus was to examine the *in vitro* antioxidant activities of Algerian propolis (R_B, R_M, R_{BJ} and R_G, respectively). by different antioxidant assay that are 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, Total antioxidant activity was estimated by phosphomolybdenum, cyclic and square wave voltammetry. Furthermore, the estimation of total phenolic contents (TPC) and Total flavonoid.

2. MATERIALS AND METHODS

2.1. Propolis sample

Propolis samples were obtained from colonies of honeybees located in four different geographical regions of Algeria in 2012 (Table.1). The locations of hives were: Boumerdes (**R_B)**) and was green propolis; Mostaganem (**R_M**), Bejaia (**R_{BJ}**) and Ghardaia (**R_G**). After collection, propolis sample was frozen at -4 °C until use.,

Table 1. List of samples and their geographical origins

Sample	Latitude	Longitude
Boumerdes (R_B)	36°46'3.35"N	3°42'10.44"E
Mostaganem (R_M)	35°56'23.42"N	0°5'23.16"E
Bejaia (R_{BJ})	36°44'60.00"N	5°4'0.00"E
Ghardaia (R_G)	32°29'20.61"N	3°40'42.74"E

2.2. Chemical

Methanol (99%), Folin- Ciocalteu reagent, Trichloroacetic Acid (99%), and Potassium Chloride (99.8%) were all purchased from Biochem Chemopharma Co (Canada). 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) (99%), Potassium Ferricyanide (99%), Ascorbic Acid (99.7%), Gallic Acid (99%), Ferric Chloride (99%), Sodium Carbonate (99%), $AlCl_3$ (99%), Rutin (99%), Sulfuric Acid (98%). Sodium Phosphate(High- purity**)** and Ammonium Molybdate (99%). were all purchased from Merck Co. Orthophosphoric Acid (85%) was purchased from Riedel-de Haen Co, all the other reagents used were of analytical grade.

2.3. Instrument

The following equipment's have been used in this study: UV-Visible spectrophotometer (UV-1800 shimadzu), Bath ultrasound Machine (3.2L 120W 110/220V CE RoHS), PGP301 Potentiostat with Voltamaster 4 version 7.08 software (radiometer analytical SAS) and rotary evaporator (IKA Evaporator RV 06-ML).

2.4. Preparation of methanolic extracts of propolis

Extraction of propolis contents was achieved using methanol as a solvent. The propolis, were cut into small portions; grounded into a coarse powder; dived in methanol (1g/30ml) for 30 min, the mixture was then Bath ultrasound Machine. The insoluble residue (mostly beeswax) was removed by filtering through Whatman N° 4 paper and evaporated to 40°C.

2.5. Determination of total phenolics contents (TPC)

Total polyphenol contents of the extracts of Algerian propolis were determined by Folin-Ciocalteu reagent [32]. Extract solutions 0.1 ml were mixed with 0.5 ml of the Folin-Ciocalteau reagent (1:10) and aqueous Na_2CO_3 (2 ml, 20%). The absorbance of the reaction mixture was measured at 760 nm after 30 min incubation at room temperature, in the dark. Gallic acid standard solutions were used for constructing the calibration curve (0.03-0.3 mg/ml). The mean of three readings was used and the total polyphenol contents were expressed as mg of Gallic acid equivalent (GAE) per 100g of extract (mg/100g).

2.6. Determination of total flavonoid content (TFC)

Total flavonoid contents (TFC) in the extracts were determined using to the aluminum chloride colorimetric method described by Chang et al. [33]. 1ml of methanol extracts of propolis was mixed with 1 ml of 20 % aluminum trichloride in methanol. The absorption at 430 nm was read after 30 minutes. Rutin was used to calculate the standard curve (0.02 and 0.1 g/L) and the results were expressed as mg of Rutin Equivalent (RE) per 100g of extract. All determinations were carried out in triplicates.

2.7. Evaluation of the antioxidant effect (in vitro) by spectrophotometrical techniques

2.7.1. DPPH radical-scavenging activity

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. [34]. Propolis extract (0.1 ml) was added to 1ml of a 250 µmol.l^{-1} Methanol solution of DPPH. Absorbance at 517nm was determined after 30 min, and the percentage inhibition activity $I\%$ was calculated using the formula: $I\% = ((A_0 - A_1)/A_0) * 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/ standard.

The antioxidant capacity of the extract was expressed as an IC_{50} value. The IC_{50} value was defined as the concentration (in mg/l) of extracts that inhibits the formation of DPPH radicals by 50%. All the tests were performed in triplicate and the graph was plotted with the average of three observations. The antioxidant capacity is also obtained using anti-radical power ARP values which increase with the increase of the antioxidant capacity.

2.7.2. Phosphomolybdenum Assay

The preparation of different concentrations of Gallic acid (standard composite) be sandwiched between (0.03 g/l) and (0.250 g/l) .The total antioxidant capacities of the propolis extracts were evaluated by the phosphomolybdenum method(PM) as described by Prieto, Pineda, and Aguilar (1999). [35]. The assay is based on the reduction of Mo(VI) to Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.2 ml of each sample solution and Gallic acid were combined with 2 ml of reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated for 90min at 95ºC. The mixture was cooled to room temperature and the absorbance was read at 695nm against blank using a UV–Vis spectrophotometer. The experiment was performed in duplicates.

2.8. Evaluation of the antioxidant effect (in vitro) by electrochemical techniques

The measurement of the antioxidant capacity of the studied samples of propolis was performed using an electrochemical methods based on cyclic voltammetry and square wave techniques [36-38] were performed in an electrochemical cell with a volumetric capacity of 25 ml containing a glassy carbon electrode (GCE) working electrode (radiometer analytical SAS), a Pt wire counter electrode, and an Hg/Hg_2Cl_2 reference electrode (saturated with KCl). The potential was swept in inverse scanning mode starting from -200 to +1000 mV with a scanning rate of 100 mV/s to avoid reducing the sensitivity of the working electrode. The electrode was polished daily with silicon carbide 4000 paper in, then rinsed with distilled water, and dried with a dry tissue paper. This cleaning procedure was applied always before any electrochemical measurements.

After polishing, the electrode was rinsed thoroughly with bidistilled water for 30 s. All experiments were conducted at ambient laboratory temperature (25°C). Potentials were measured with

respect to a saturated calomel electrode. The samples in the electrochemical cell were de-aerated by purging with high purity nitrogen during the electrochemical measurements [36, 38,39].

The antioxidant capacity of the studied samples of propolis was obtained using the area below the anodic curve of the Voltammogram. The calibration graph is obtained by plotting the area below the anodic curve of the Voltammogram of each sample of the standard versus its concentration [36,38,40]. Ascorbic and Gallic acids were used as standards in the calculation of antioxidant capacity of the studied sample of propolis because of their wide spreading in nature and also because their anodic area displays excellent linearity toward ascorbic or Gallic acids concentrations [36,41].

3. STATISTICAL ANALYSIS

All analyses were carried-out in triplicate and the results were expressed as means ± standard deviation (SD). Statistical analysis of the results was calculated using the Microsoft Excel 2010 and the Origin Pr8 programs. P-values less than 0.05 were considered significant.

4. RESULTS AND DISCUSSION

4.1. Determination of Total polyphenols and Flavonoids

Table 2 shows the total polyphenol and flavonoid contents of propolis samples. Total polyphenol content in methanolic extract of R_B, R_M, and R_{BJ}, R_G propolis as estimated by Folin-ciocalteu Reagent method shows 262.338±0.810 The largest value (R_B) and less value 81.141±0.538mg (R_{BJ}) Gallic acid equivalent per 100 mg of propolis powder respectively. The order of TPC in propolis extracts is: **R_B>R_G>R_M>R_{BJ}**. These results showed that the Algerian propolis has a higher phenolic content compared with those reported by Da Silva et al. [42] and Potkonjak et al. [43]. It has a lower phenolic content compared with those reported by Kumazawa et al. [32] and Choi et al. [44]. Significant differences in TFC were observed among the propolis samples ranging from 210.884 ±0.754 to 19.626±0.301mg RE/100 g, with the following ranking order: **R_B>R_G>R_M>R_{BJ}**. It has a higher flavonoid content compared with those reported by Da Silva et al [42] and Shiva Mohammadzadeh et al [45].The composition of propolis depends upon the vegetation of the area and the season from which it is collected [32].

Table 2. The total polyphenol and flavonoid contents of propolis samples

Compound (concentration)	R_B	R_M	R_{BJ}	R_G
Extraction yield (%)	41,10	30,01	23,18	15,57
(TPC)(mg/100g)	262.338±0.810	86.213±0.416	81.141±0.538	185.074±1.336
(TFC) (mg/100g)	210.884±0.754	28.304±0.232	19.626±0.301	74.827±0.995

4.2. Antioxidant activity

DPPH is a free radical compound that has been widely used to test the free radical scavenging ability of various samples [46]. It is accepted that the DPPH free radical scavenging by antioxidants is

due to their hydrogen- donating ability [47]. To evaluate the scavenging effect of DPPH on methanol extract of propolis, DPPH inhibition was investigated and these results are shown as relative activities against control. As shown in Table 3 the activities of propolis samples and ascorbic acid as free radical scavenging increased as a function of concentration increment. All of the extracts exhibited concentration dependent radical scavenging activity.

Table 3. DPPH radical scavenging activities (%) of ascorbic acid (**A**) and methanol propolis extracts collected from (R_B), (R_M), (R_{BJ}) and (R_G).

Samples	**A**	**R_B**	**R_M**	**R_{BJ}**	**R_G**
IC_{50} (g/l)	0.184	0.010	0.048	0.066	0.007
ARP	5.42	91.70	20.46	15.10	133.40

Generally, The comparative data of DPPH radical scavenging activity, as determined by the IC_{50} values (the concentration required to inhibit radical formation by 50% and was obtained from interpolation from linear regression analysis) of the different propolis. Highest activity was found in **R_G**, followed by **R_B**, **R_M** and the lowest activity was found in **R_{BJ}**. This may be due to the higher polyphenol content of this extracts (**R_G,R_B**). The analysis (ARP) of (**Table 3.**) that the radical scavenging activity of the extracts of the different propolis increases with increasing in concentration and follows the given orders **R_G > R_B > R_M > R_{BJ} > A.**

From the present results it may be postulated that the extracts of Algerian propolis reduces the DPPH radical to corresponding hydrazine when its reacts with hydrogen donors in antioxidant principles.

4.3. PM assay

PM assay is based on the reduction of Phosphate-Mo (VI) and its transformation to Phosphate Mo (V) in the sample which is a bluish green colored phosphate that is complex at acid pH. The phosphomolybdenum method is regularly used in the laboratory to estimate the total capacity of antioxidant of propolis extracts and to calculate the amount of antioxidants in each extract. Where this estimate showed that there is a direct proportion between the increase in the optical density and the concentration of acid Gallic (0.03 µg/ml - 0.250 µg/ml).

Table 4. PM Assay

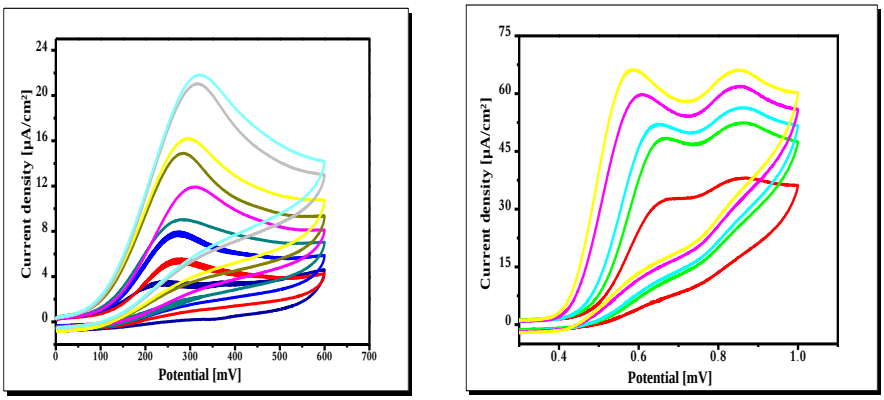
The sample	(TAC) (mg/g)	Order
R_B	149.015*±0.454	1
R_M	112.543±4.181	4
R_{BJ}	118.525±1.909	3
R_G	136.301±1.050	2

Values are Mean ±SD (n=3); propolis Extract – Gallic acid

As shown in Table 4, the study reveals that the antioxidant activity of the extract is in growing trend between the different studied areas where the largest recorded value is R_B=149.015*±0.454. The values take the following order: $\mathbf{R_B} > \mathbf{R_G} > \mathbf{R_{BJ}} > \mathbf{R_M}$. The results do not differ much from the DPPH.

4.4.Evaluation of antioxidant capacity by electrochemical techniques

The cyclic voltammetry (CV) and square wave (SWV) voltammograms obtained for 40 mmol/l of ascorbic and Gallic acids in pH= 2 (12.5ml) and methanol (12.5ml), 0.1 mol.l^{-1} KCl as a supporting electrolyte using a 3 mm-diameter glassy carbon electrode present typical irreversible oxidation processes with the existence of an irreversible one oxidation peak at 0.3 V for ascorbic acid (Fig. 1.a). It corresponds to what was found in previous studies. Two oxidations peaks at 0.60 and 0.83 V for Gallic acid (Fig. 1.b).



(b) (b)

Figure 1. Cyclic voltammograms obtained in 40 m.mol.l^{-1} of ascorbic acid (a) and gallic acid (b) in pH 2 (12.5ml) and methanol (12.5ml), 0.1 mol.l^{-1} KCl at scan rate 100 mV/s.

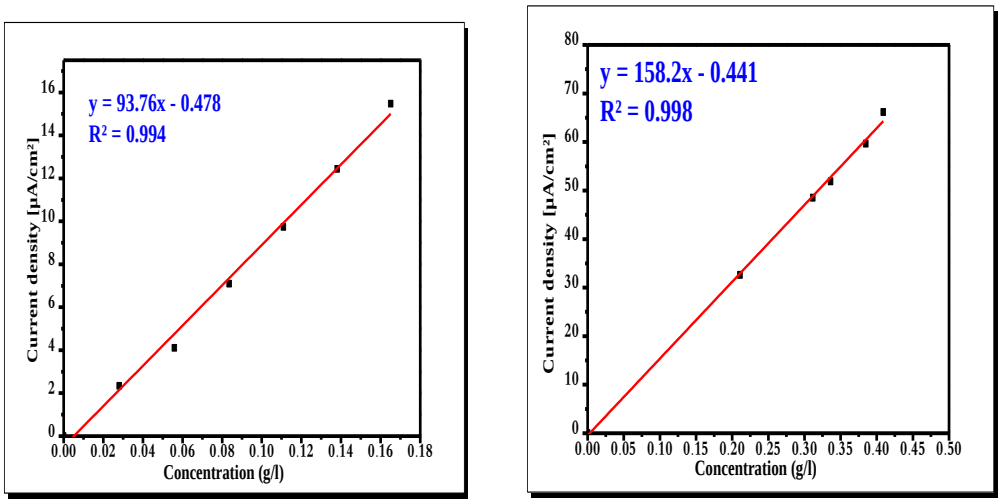
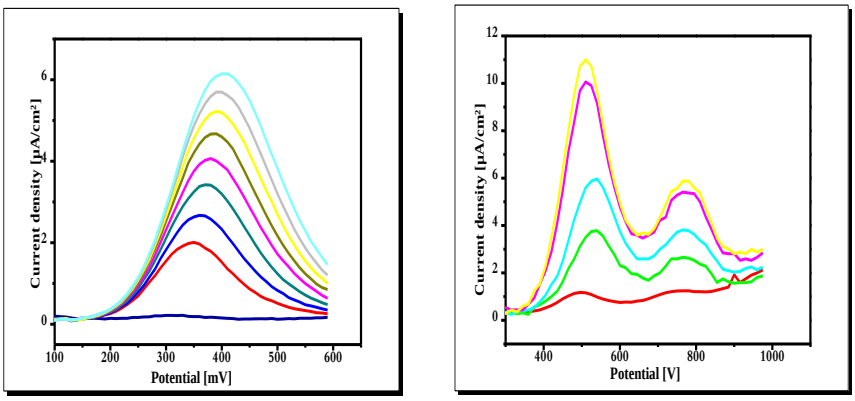


Figure 2. Calibration curve obtained by cyclic voltammetry method expressed as ascorbic (a) and Gallic (b) acids equivalents/l.



(a1) (b1)

Figure 3. SWV voltammograms obtained (0.1-1ml) in 40 mmol.l^{-1} of ascorbic acid (a1) and Gallic acid (b1) in pH 2 (12.5ml) and methanol (12.5ml), 0.1 mol.l^{-1} KCl at scan rate 100 mV/s.

The equation obtained from the linear calibration graphs in the studied concentration range for ascorbic and Gallic acids is respectively, y = 93.76 x -0.478 and y = 158.2 x -0.441 by cyclic voltammetry method and , y = 26.67 x +0.143 and y = 29.77 x +0.032 by SWV method (where y represents the value of the area of the anodic wave and x, the value of standards concentration, expressed as g/l), with a correlation coefficient of R^2 = 0.99 for both equations.

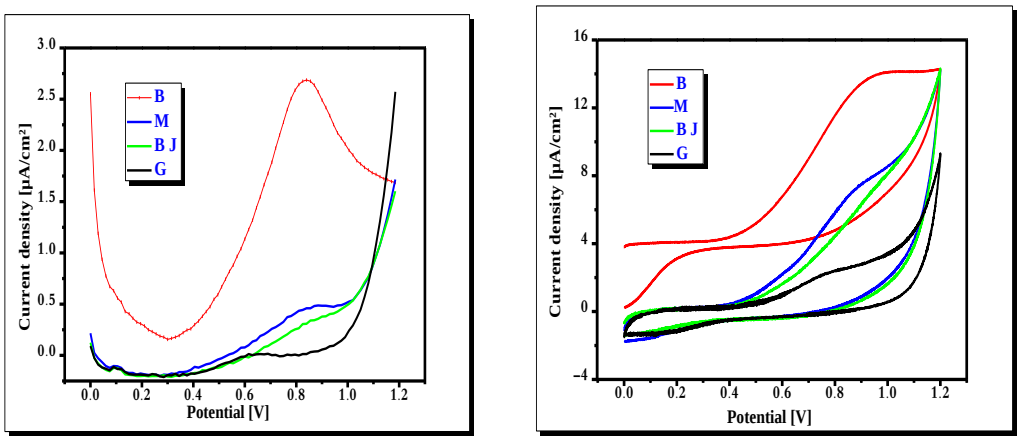


Figure 4. SWV, CV voltammograms respectively obtained for propolis extracts in pH 2 (12.5ml) and methanol (12.5ml), 0.1 mol.l^{-1} KCl at scan rate 100 mV/s.

In Table 5 the ascorbic acid equivalent antioxidant capacity (AEAC) and gallic acid equivalent antioxidant capacity (GEAC) of the **R_B** propolis extract calculated from the calibration graphs is equal to 177.251* and 104.897* mg/g.

Table 5. The antioxidant capacity of propolis calculated using cyclic voltammetry (CV).

propolis extracts	AEAC (mg/g)	GEAC (mg/g)
R_B	177.251*	104.897*
R_M	74.568	43.984
R_{BJ}	81.794	48.351
R_G	82.617	48.559

Table 6. The antioxidant capacity of propolis calculated using square wave voltammetry.

propolis extracts	AEAC (mg/g)	GEAC (mg/g)
R_B	62.688*	58.609*
R_M	22.197	23.240
R_{BJ}	22.754	24.727
R_G	23.062	27.127

In Table 6 The same thing ascorbic acid equivalent antioxidant capacity (AEAC) and Gallic acid equivalent antioxidant capacity (GEAC) of the $\mathbf{R_B}$ propolis extract calculated from the calibration graphs is equal to 62.688* and 58.609* mg/g.

In all the antioxidant assay systems, R_B showed higher activity compared to the three extacts ($\mathbf{R_M}$, $\mathbf{R_{Bj}}$ and $\mathbf{R_G}$). This may be due to its higher polyphenol content and flavonoid contents. The similarity in the oxidation potential between the Three extracts ($\mathbf{R_M}$, $\mathbf{R_{BJ}}$ and $\mathbf{R_G}$) indicates that these three extracts should have an analogous chemical composition in respect to the electro-active species.

5. CONCLUSIONS

All the assays confirmed the good antioxidant potential of the four samples of Algerian propolis (R_B, R_M, R_{BJ}, and R_G). The work described in this study showed that cyclic voltammetry and square wave voltammetry can be considered as important techniques for the evaluation of propolis antioxidant properties. The results suggested that the propolis has important benefits to human health, and could serve as a source of antioxidants with potential applications.

ACKNOWLEDGEMENT
This material is based upon work supported by a grant from both University El-Oued, and University Kasdi Merbah Ouargla.

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