
# International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 7 (1): 113-118, 2011
ISSN 1811-7775 / DOI: 10.3923/ijp.2011.113.118


# *In vitro* Evaluation of Antioxidant Capacity of Algerian Propolis by Spectrophotometrical and Electrochemical Assays

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**Abstract:** The aim of the present study was to evaluate the antioxidant capacity and the total phenolic content of propolis extract, obtained from colonies of honeybees located in El-Oued (south of Algeria). The total phenolic content was evaluated using the Folin-Ciocalteu method and the antioxidant capacity was measured using the following methods: 2, 2-diphenyl-1-picryhydrazyl free radical scavenging capacity and reducing power capacity for spectrophotometrical techniques, ascorbic and gallic acids equivalent antioxidant capacity assays for electrochemical techniques. Both techniques allowed an evaluation of the antioxidant capacity of methanolic propolis extract. The electrochemical techniques were performed by cyclic voltammetry, the results suggest that propolis extract do not reveal similar electrochemical responses to that of ascorbic and gallic acids, suggesting a different electroactive chemical composition and oxidation potential more positive than that of the standard (ascorbic acid), however this does not suggest that propolis has a lower antioxidant capacity. The results suggest also that propolis extract possess antioxidant capacity *in vitro* conditions.

**Key words:** Propolis, antioxidant capacity, honeybee, cyclic voltammetry, spectrophotometry

## INTRODUCTION

Bee propolis or bee glue is a very sticky valuable resinous mixture produced by honeybees from trees bubs and various plants sources around the hive, it is masticated by the bees, salivary enzymes and beeswax added, then used as a construction material in bee hives for filling cracks and repairing combs thereby insulating and reinforcing the hives, also protecting the hive and its nutritious contents from attack by micro-organisms (Burdock, 1998; Johnson *et al*., 1994). Due to biological and pharmacological activities, propolis has been extensively used in folk medicine since ancient times (Galvao *et al*., 2007) and is now known to be a natural medicine with antibacterial, antifungal, antitumoral, antioxidative, imunomodulatory and other beneficial activities (Bankova *et al*., 2002; El-Kott and Owayss, 2008).

Now is presently used in health food and various pharmaceutical and cosmetic products such as mouthwash preparations, face creams, lotions and tablets (Burdock, 1998). Propolis contains a diversity of compounds of plant origin basically is composed of 55% vegetable resins and balsam, 30% bee wax, 10% essential oil and 5% pollen (Cizmarik and Matel, 1970; Pepeljnjak *et al*., 1985; Serkedjieva *et al*., 1992).

The chemical composition and antioxidant capacities of propolis of many countries have been widely studied by a lot of scientific research groups (Zadeh *et al*., 2007; Moreira *et al*., 2008; Gulcin *et al*., 2010) but only a few reports can be found in literature on Algerian propolis. This motivated us to explore the antioxidant capacity of Algerian propolis and its total polyphenol and flavonoid contents.

The aim of this study is to measure the *in vitro* antioxidant activity of the methanolic extract of south Algerian propolis. We used the following three assay systems: (1) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay, (2) reducing antioxidant power assay (RP) and (3) Cyclic Voltammetry assay (CV). Total polyphenol and Flavonoid contents of the methanolic extract of propolis were determined by standard colourimetric methods. (Chang *et al*., 2002; McDonald *et al*., 2001; Mello *et al*., 2010).

## MATERIALS AND METHODS

**Chemical:** Methanol (99%), Folin Ciocalteu reagent, trichloroacetic acid (99%), potassium chloride (99.8%) were all purchased from Biochem Chemopharma Co. (Canada). 1,1-Diphenyl-2-picryl hydrazyl (DPPH) (99%),

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potassium ferricyanide (99%), ascorbic acid (99.7%), gallic acid (99%) ferric chloride (99%), sodium carbonate (99%), $AlCl_3$ (99%), rutin (99%) were all purchased from Merck Co.

Orthophosphoric acid (85%) was purchased from Riedel-de Haen Co., all other reagents used were of analytical grade.

**Propolis:** Crude propolis sample was brought from hives of honeybees located in El-Oued (south of Algeria) in May-April, 2010. Samples, once received were stored at 4°C in airtight/dark plastic containers until analysis.

**Instrument:** UV-Visible spectrophotometer (PRIM Advanced SCHOTT Instruments Gmbh), centrifuge Machine (SLW centryge, Ultra-8TL), PGP301 potentiostat with voltamaster 4 version 7.08 software (radiometer analytical SAS), rotary evaporator (IKA Evaporator RV 06-ML).

**Extraction of propolis compounds:** Extraction of propolis contents was achieved using methanol as a solvent. The propolis, is cut into small portions; ground into a coarse powder; dived in methanol (1 g/30 mL) for 24 h, the mixture was then centrifuged for 30 min at 3500 rpm. The insoluble residue (mostly beeswax) was removed by filtering through Whatman No. 4 paper and evaporated to 40°C.

**Determination of total polyphenolics:** Total polyphenolic content was determined using Folin-Ciocalteu reagents according to the method of Scalbert *et al.* (1989), briefly described as 0.5 mL of Folin and Ciocalteu's phenol reagent was mixed with 100 μL extract solution. After 3 min, 2 mL of 20% aqueous sodium carbonate solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 30 min, after which the absorbance was read at $\lambda = 760$ nm.

Gallic acid was used to calculate the standard curve (0.03-0.3 mg $mL^{-1}$; $y = 3.3974x$; $R^2 = 0.994$) and the results were expressed as mg of Gallic Acid Equivalents (GAEs) $g^{-1}$ of extract. The amount of total phenols found for the methanolic propolis extracts was equal to 10.99 mg $g^{-1}$ of GAEs. This value is very low compared to that of Portuguese propolis samples from Bornes and Fundão regions which were of 329 and 151 mg $g^{-1}$ of GAE, respectively (Moreira *et al.*, 2008). Present values still also lower than the Korean propolis from Yeosu, Yangpyeong, Boryung and Cheorwon regions which was, respectively equal to 212.7, 160.6, 172.3 and 180.3 mg $g^{-1}$ of GAEs (Choi *et al.*, 2006).

**Determination of total flavonoids:** For flavonoid contents determination, the methanol extracts of propolis was retaken in 1 mL of methanol and treated with $AlCl_3$ methanol solution (2%, 1 mL). After 30 min the solution was mixed well and the intensity of pink color was measured at $\lambda = 430$ nm. Rutin was used to calculate the standard curve, the calibration graph obtained for rutin presents linearity between 0.1 and 0.02 g $L^{-1}$ ($y = 13.686$ x where y represents the value of absorbance and x, the value of rutin concentration expressed as g $L^{-1}$; $R^2 = 0.999$) and the results were expressed as mg of Rutin Equivalents (REs) $g^{-1}$ of extract. All the samples and the standards were analyzed in triplicate. The amount of total flavonoids found for the methanolic propolis extracts was equal to 2.12 mg $g^{-1}$ of REs. Present results still lower compared with Brazilian propolis samples which contained 6.95 mg $g^{-1}$ of flavonoids using quercetin as standard (Mello *et al.*, 2010). Also lower than propolis from Greece and Cyprus which contained flavonoids at levels of and 8.8 mg $g^{-1}$ using caffeic acid as standard (Kalogeropoulos *et al.*, 2009).

**Evaluation of antioxidant capacity by spectrophotometrical techniques**

**Using the free radical scavenging determination:** The free radical scavenging capacity of propolis was measured in terms of hydrogen donating or free radical scavenging ability by using the stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) (Molyneux, 2004), propolis extract and standard ascorbic acid solution (0.1 mL) of different concentrations viz., 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg $L^{-1}$ was added to 1 mL of a 0.004% methanol solution of DPPH. An equal amount of methanol and DPPH served as control. After 30 min incubation in the dark, absorbance was recorded at 517 nm and the percentage inhibition capacity was calculated from the following relation:

$$\% \text{ inhibition} = \frac{A_0 \quad A_1}{A_0} \times 100\%$$

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 $IC_{50}$. The $IC_{50}$ value was defined as the concentration (in μg $mL^{-1}$) 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 equation obtained from the linear calibration graph in the studied concentration range for ascorbic acid is $y = 271.04$ x (where y represents the value of absorbance and x, the value of ascorbic acid

Table 1: Comparison of DPPH free radical inhibitory of the propolis extract and ascorbic acid

| Samples | Equation | $R^2$values | $IC_{50}$values (g $L^{-1}$) |
|---|---|---|---|
| Ascorbicacid | y = 271.04x | 0.9942 | 0.18447 |
| Propolis | y = 19540x | 0.9982 | 0.02558 |

concentration, expressed as g $L^{-1}$) with a correlation coefficient of $R^2$ = 0.9942. Under the same conditions the Eq of the calibration graph for propolis is y = 1954 x with a correlation coefficient of $R^2$ = 0.9982. According to $IC_{50}$ values, the propolis extract has a higher antioxidant capacity; the results are summarized in Table 1.

**Using Reducing Power (RP) determination:** Different concentrations of propolis extract and standard ascorbic acid solution viz., 10, 20, 40, 60, 80 and 100 mg $L^{-1}$ in 1 mL of methanol were mixed with phosphate buffer (2.5 mL, 0.2 M pH 6.6) and potassium ferricyanide $K_3Fe(CN)_6$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A volume of 2.5 mL of aqueous tricholoroacetic acid solution (10%) was added to the mixture. A volume of 2.5 mL of the resulting mixture was mixed with 2.5 mL distilled water and (0.5 mL, 0.1%) of ferric chloride. The absorbance was then recorded at 700 nm. All the tests were performed in triplicate and the graph was plotted with the average of three observations (Chevion *et al*., 2000).

Both calibration graphs for ascorbic acid and the propolis are linear in the range of the studied concentrations, with an standard curve equation for the ascorbic acid of y = 673 x with a correlation coefficient of $R^2$ = 0.9937 (where y represents the value of absorbance and x, the value of acid ascorbic or propolis concentration, expressed as mM). The standard curve equation for the propolis is, y = 0.9314 x with a correlation coefficient of $R^2$ = 0.9994, (where y represents the value of absorbance and x, the value of the inverse of the dilution coefficient of propolis concentration).

The result of Reducing Power (RP) of the propolis extract, in terms of ascorbic Acid Equivalent Antioxidant Capacity (AEAC) calculated from the calibration graph using linear regression analysis is found to be equal to 1.384 mM.

**Evaluation of antioxidant capacity by electrochemical techniques:** The measurement of the antioxidant capacity of the studied samples of propolis was performed using an electrochemical method based on cyclicvoltammetry techniques (Campanella *et al*., 2001; Cosio *et al*., 2006). Cyclicvoltammetry measurements were performed in an electrochemical cell with a volumetric capacity of 50 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 +800 mV with a scanning rate of 100 mV $sec^{-1}$. To avoid reducing the sensitivity of the working electrode, the latter was polished after each cycle by rubbing its surface using alumina oxide (particle size 0.3 μm) before every electrochemical assay. After polishing it was rinsed thoroughly with bidistilled water for 30 sec.

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. 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 (Laskar *et al*., 2010; Oyaizu, 1986).

## RESULTS AND DISCUSSION

The cyclic voltammetry voltammograms obtained for 1 mM of ascorbic and gallic acids in pH 7, 0.1 M phosphate buffer solution (pH = 2, Britton-Robinson buffer solution for gallic acid) and 0.1 M 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 0.26 V for ascorbic acid (Fig. 1A) and two oxidation peaks at 0.58 and 0.85 V for gallic acid (Fig. 1B).

The same irreversible electrochemical behavior was observed for propolis sample extract (Fig. 2) although, with oxidation potential value of propolis extract is more positive than ascorbic acid, around 0.44 V and less positive than gallic acid, however these results do not indicate that, under the electrochemical conditions used, the propolis extract has an antioxidant capacity less than gallic acid and more than ascorbic acid but it indicates that the propolis extract do not contain any of the constituents of the standards ascorbic nor gallic acids.

Although the oxidation potential value of propolis extract is less positive than gallic acid, the antioxidant capacity of propolis is higher than gallic acid, this stands in sharp contrast with the results of Kilmartin and Hsu (2003) (extracts with lower oxidation potential values have higher antioxidant capacity). This may be due to the fact that the obtained voltammograms do not have the same allure.

The antioxidant activity of propolis methanolic, ethanolic and aqueous extracts has been investigated

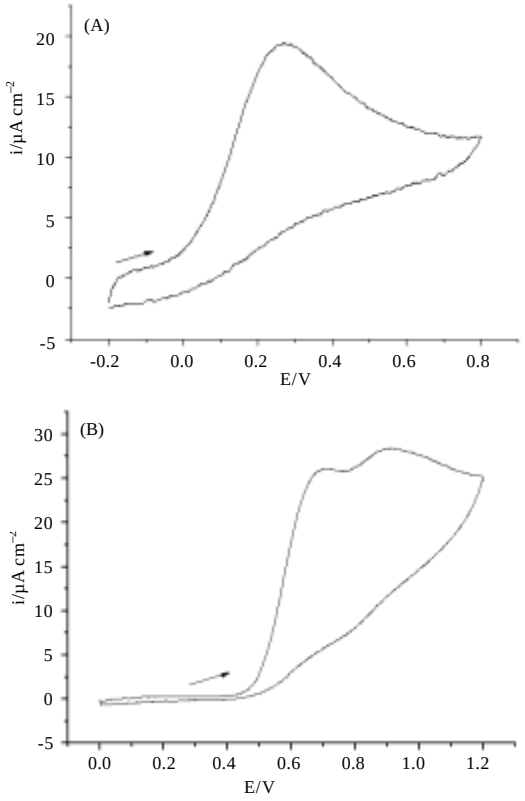


Fig. 1: Cyclic voltammograms obtained in 1 mM of ascorbic acid (A) and gallic acid (B) in pH 7 and pH 2 buffer solutions containing 0.1 mol $L^{-1}$KCl at scan rate 100 mV $sec^{-1}$

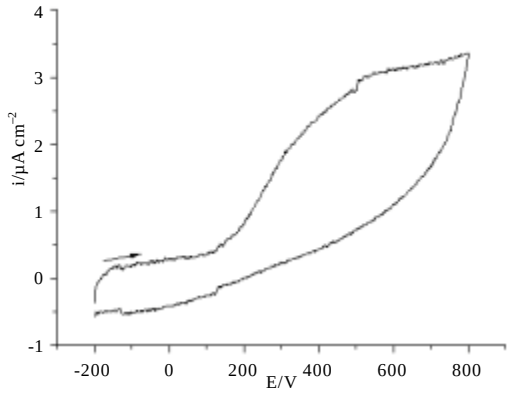


Fig. 2: Cyclic voltammogram of propolis extract in pH 7, 0.1 M phosphate buffer solution containing 0.1 mol $L^{-1}$ KCl at scan rate 100 mV $sec^{-1}$

by many scientific research groups in different countries and has been reported in different spectrophotometrical methods including Ferric Reducing Antioxidant Potential (FRAP) (Kalogeropoulos *et al*., 2009), b-carotene-linoleic acid system (Ahn *et al*., 2007)

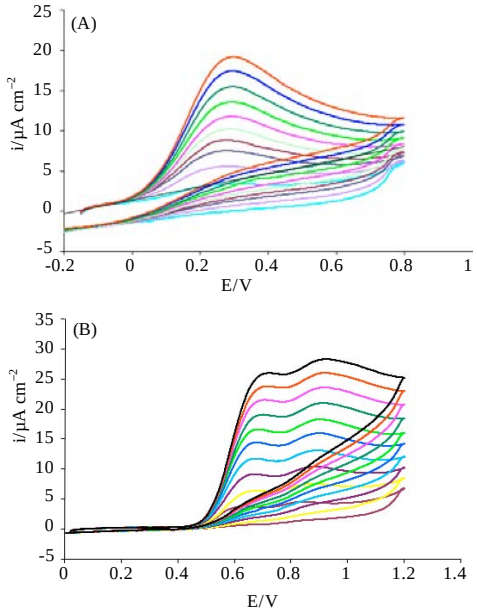


Fig. 3: Cyclic voltammograms referring to different ascorbic (A) and gallic acids (B) concentrations

and the free radical-scavenging capacity with reduction of radical diphenylpicrylhydrazyl (DPPH) (Russo *et al*., 2004), but only a few reports can be found in literature using electrochemical assays as means of evaluation of the antioxidant capacity of propolis or any other natural materials.

The cyclic voltammograms, at different concentrations of ascorbic and gallic acids, are shown in Fig. 3a, b. As can be seen there is an increase in peak current with the increase in ascorbic or gallic acids concentrations which leads to a linear relation between these two parameters.

In order to express the antioxidant capacity of the propolis extract in equivalent terms of Ascorbic Acid Equivalent Antioxidant Capacity (AEAC) and Gallic Acid Equivalent Antioxidant Capacity (GEAC), different concentrations of the standards ascorbic and gallic acids were plotted verses the Area of the Anodic Wave (AAW). The anodic area displays excellent linearity toward both ascorbic and Gallic acids concentrations Fig. 4A, B. The values are presented in Table 2.

The equation obtained from the linear calibration graph in the studied concentration range for ascorbic and gallic acids is, respectively, $y = 5.0628 x + 1.7674$ and $y = 8.418 x + 1.169$ (where y represents the value of the area of the anodic wave and x, the value of standards concentration, expressed as g $L^{-1}$), with a correlation coefficient of $R^2 = 0.999$ for both equation.

Table 2: Area of the anodic wave obtained from cyclic voltammetry of ascorbic and gallic acids

| | AAW ($\mu W\ cm^{-2}$) | |
|---|---|---|
| C (mmol $L^{-1}$) | Ascorbic acid | Gallic acid |
| 0.1 | 2.3216 | 1.9307 |
| 0.2 | 2.9153 | 2.8508 |
| 0.3 | 3.3234 | 3.6988 |
| 0.4 | 3.8196 | 4.5749 |
| 0.5 | 4.2026 | 5.4457 |
| 0.6 | 4.6211 | 6.236 |
| 0.7 | 5.1756 | 7.093 |
| 0.8 | 5.6535 | 7.9 |
| 0.9 | 6.1691 | 8.729 |
| 1 | 6.687 | 9.531 |

Table 3: The antioxidant capacity of propolis calculated using (RP), (DPPH) values and (AEAC), (GEAC) equivalent

| Techniques | Equation | $R^2$ values | Antioxidant capacity |
|---|---|---|---|
| Spectrophotometrical | y = 0.9314 x | 0.9994 | 1.384 (RP) mM |
| Electrochemical | y = 1954.7x | 0.9982 | 0.0258 (DPPH) g $L^{-1}$ |
| | y = 5.0628 x + 1.7674 | 0.999 | 5.125 mg $g^{-1}$ (AEAC) |
| | y = 8.418 x + 1.169 | 0.999 | 8.8205 mg $g^{-1}$ (GEAC) |
| | y = 19.439 x + 2.0023 | 0.999 | 2.773 mg $g^{-1}$ (AEAC) * |

*calculated using the current density of the anodic peak

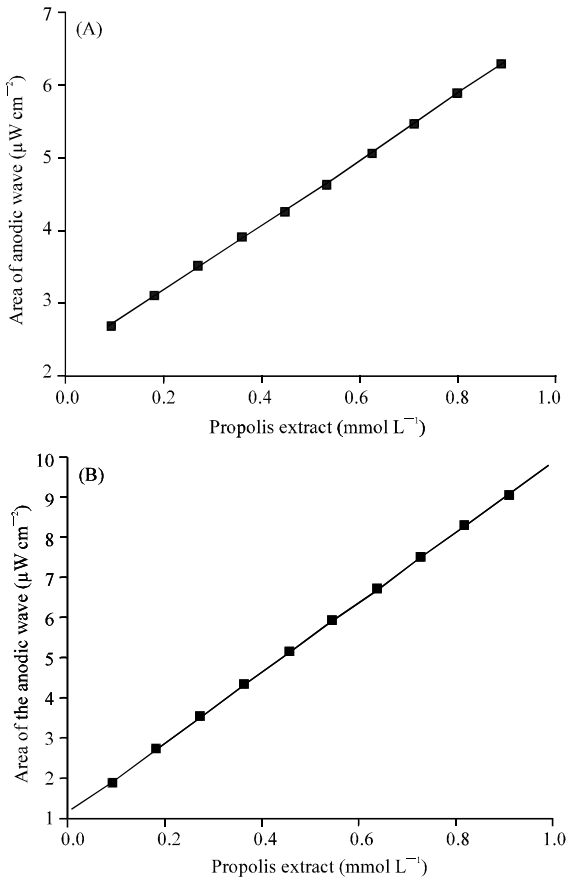


Fig. 4: Calibration curve obtained by cyclic voltammetry method expressed as ascorbic (A) and gallic (B) acids equivalents/L

The Ascorbic Acid Equivalent Antioxidant Capacity (AEAC) and Gallic Acid Equivalent Antioxidant Capacity (GEAC) of the propolis extract calculated from the calibration graphs is equal to 5.125 and 8.8205 mg $g^{-1}$. Table 3 summarizes the values of antioxidant capacity of propolis extract estimated by spectrophotometrical and electrochemical assays.

Laskar *et al.* (2010) have studied the antioxidant capacity of Indian propolis by cyclic voltammetry assay and found that the propolis ethanolic extracts reducing power values of Indian samples, expressed as mg ascorbic acid equivalents, ranged between 15.32 and 23.95 mg $g^{-1}$ ascorbic acid equivalents, these values are relatively higher values than Algerian propolis samples. This may be due to its higher polyphenol content. Hence aqueous extract may well be a substitute of organic solvent extracts of propolis.

The antioxidant capacity of Italian propolis was also quantified by Buratti *et al.* (2007) using galangin as reference. Quantification was based on peak height using amperometric flow injection analysis. The antioxidant capacity of propolis samples varies from 2 to 169 mg $g^{-1}$ galangin equivalents, these values still relatively higher than those observed in Algerian propolis samples. The differences in values of the antioxidant capacity may be attributed to the standards used.

## CONCLUSIONS

Both the spectrophotometrical (DPPH and RP) and electrochemical (AEAC and GEAC) assays suggest that the methalonic extract of propolis shows *in vitro* antioxidant activities by inhibiting DPPH and reducing power ability which may be due to presence of flavonoids and phenolic compounds found in the preliminary phytochemical screening. The results show that the antioxidant capacity, expressed in terms of ascorbic (AEAC) and gallic acids (GEAC) equivalent antioxidant capacity obtained from electrochemical experiments is higher than that obtained from spectrophotometrical experiment using (RP) and (DPPH). This outcome can be attributed to the overestimation of the total polyphenolic content due to the interferences of other non-phenolic species like reduction sugars.

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