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Research Article

TOTAL POLYPHENOL CONTENTS, RADICAL SCAVENGING AND CYCLIC VOLTAMMETRY OF ALGERIAN PROPOLIS

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ABSTRACT

Objective: In this study, we will determine the antioxidant properties of methanolic extract of propolis from Ghardaia and Khanchla provinces of Algeria and will correlate the values with total levels of polyphenolic compounds.

Methods: The total polyphenol contents of methanolic extract of propolis were measured by using Folin–Ciocalteu spectrophotometric method. Thereafter, the antioxidant properties of these polyphenols were determined by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging. All polyphenols extracted were tested using cyclic voltammetry (CV) in aqueous media. The CV was realised to compare the results from spectroscopic method and to electrochemically characterise the propolis polyphenols.

Results: The total polyphenolic content in methanolic extract of propolis from Ghardaia and Khanchla was 493.49 and 1423.32 mg gallic acid equivalent/100g of extract, respectively. The IC50 values for scavenging DPPH radical for Ghardaia and Khanchla propolis were 0.03917 mg/mL, 0.01211mg/mL respectively. Antioxidant activity measured by cyclic voltammetry method indicated that methanolic extract of Khanchla propolis had AEAC of 15,61 mg/g.

Conclusion: Propolis samples had strong antioxidant activities, and the highest activities were found in Khanchla propolis. Also, among three assays employed in this study (DPPH, RP, CV), the cyclic voltammetry method was recommended as it represented a relatively clean chemical system.

Keywords: Propolis, Polyphenol, Flavonoid, Cyclic voltammetry, Antioxidant.

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 [1], 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 [2], also protecting the hive and its nutritious contents from attack by micro-organisms [2,3]. Due to biological and pharmacological activities, propolis has been extensively used in folk medicine since ancient times and is now known to be a natural medicine with antibacterial, antifungal, antitumoral, antioxidative, imunomodulatory and other beneficial activities [4]. Now, propolis is presently used in health food and various pharmaceutical [4,5] and cosmetic products such as mouthwash preparations, face creams, lotions and tablets [5]. Propolis contains a diversity of compounds of plant origin. It is, basically, composed of 55% vegetable resins and balsam, 30% bee wax, 10% essential oils and 5% of pollen [2].

Propolis, in more of their high content in vitamins and minerals [6], contain important content of polyphenols [7]. These compounds are known today for their antioxidant capacities.

Although the considerable importance of antioxidants, there is not a unique method or protocol for the determination of antioxidant capacity (ORAC, FRAP, TEAC). Therefore, the results obtained from all these methods are not constantly compatible and the materials used for these analyses are costly (AAPH, ABTS, DPPH). For those reasons, we applied a new technique to determine antioxidant capacities using less complicated methods, compared to chromatographic and spectroscopic techniques which use complicated apparatus.

A highly attractive, convenient and especially sensitive voltammetry approach for the study of antioxidant properties [8], antioxidants are substances, which interrupt radical-chain oxidation in organic and inorganic molecules. Our method consists in using electrochemical techniques, cyclic voltammetry (CV), for the determination of propolis antioxidant capacities.

Voltammetry methods appear as simple methods giving good estimations of the global amount of polyphenols in vegetables and plants [7,8,9]. Furthermore, these techniques have been capable to give the global amount of different types of polyphenols in the same time and to characterise new compounds containing polyphenols which could play important role in food [8].

Thus, in the present paper, the first part is focused on spectroscopic techniques used to study propolis extracts, in purpose to determine their total polyphenol contents (TPC). It should be noted that in the spectroscopic section we used Folin–Ciocalteu method to determine their polyphenols consistence. In the second part of this study we were interested to study their antioxidant capacity using the 2,2-diphenyl-1-picrylhydrazyl DPPH as a scavenging radical reagent. Also, and for the main reason in the third part, these extract's were tested electrochemically, using cyclic voltammetry, to determine their electrochemically response. This technique can provide us the composition of each extracts, qualitatively, and can give us the global amount of polyphenols in each extract.

MATERIALS AND METHODS

Chemical

Methanol (99%), Folin Ciocalteu reagent, trichloroacetic acid (99%), potassium chloride (99.8%) was 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%) 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

Two Crude propolis samples were brought from hives of honeybees located in Ghardaïa (south of Algeria) and Khenchela (east of Algeria) in May-April, 2007. The samples, once received, were stored at 4°C in airtight /dark plastic containers until analysis.

Methods

Instrument

UV-Visible spectrophotometer (PRIM Advanced SCHOTT Instruments Gmbh), centrifuge Machine (SLW centryge, Ultra-8TL), PGP301

potentiostat with voltamaster 4 version 7.08 soft ware (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 (1g/30ml) for 24 hours, the mixture was then centrifuged for 30 minutes 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 polyphenolic

Total polyphenolic content was determined using Folin- Ciocalteu reagents according to the method of Kumazawa et al [10], 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 λ = 760 nm.

Gallic acid was used as the standard to produce the calibration curve (0.03-0.3 mg/ml). The mean of three readings was used and the total polyphenolic content expressed in mg of gallic acid equivalents (GAEs) (mg/100g).

Determination of total flavonoid

For flavonoid contents determination [11], 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 λ = 430 nm. Rutin was used to calculate the standard curve (0.1 and 0.02 g/L) and the results were expressed as mg of rutin equivalents (REs) per g of extract. All the samples and the standards were analyzed in triplicate.

Evaluation of antioxidant capacity by spectrophotometrical techniques

Using the (DPPH) 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) [10], 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 was added to 1 ml of a 250 $mmol.l^{-1}$ methanol solution of DPPH [10]. An equal amount of methanol and DPPH served as control. After 30 minutes incubation in the dark, absorbance was recorded at 517 nm, and the percentage inhibition capacity was calculated from the following relation:

$$\text{Inhibition percentage} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

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) 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 was also obtained by using anti-radical power ARP values which increase with the increase of the antioxidant capacity.

Using the Reducing Power Determination (RP)

Different concentrations of propolis extract and standard ascorbic acid solution, viz. 10, 20, 40, 60, 80 and 100 mg/L 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. Then, 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. After that, the absorbance was recorded at 700 nm. All the tests were performed in triplicate and the graph was plotted with the average of three observations [12].

The result of reducing power (RP) of the propolis extract, in terms of ascorbic acid equivalent antioxidant capacity (AEAC), was calculated from the calibration graph using linear regression analysis.

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 cyclic voltammetry techniques [7,8,9,13]. Cyclic voltammetry 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/s. 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 0.3µm) before every electrochemical assay. After polishing it was rinsed thoroughly with bidistilled water for 30 s.

The samples in the electrochemical cell were de-aerated by purging with high purity nitrogen during the electrochemical measurements.

The antioxidant capacity of the studied samples of propolis was obtained using the area below the anodic curve of the voltammogram. The calibration graph was obtained by plotting the area below the anodic curve of the voltammogram of each sample of the standard versus its concentration [7,13]. 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 [7].

RESULTS AND DISCUSSION

Determination of the total polyphenolics and flavonoids

Table 1. shows the total polyphenol and flavonoid contents of propolis samples. Total polyphenol content in methanolic extract of Khanchla and Ghardaia propolis. As estimated by Folin-Ciocalteu Reagent method shows 1423.32 and 493.49 mg gallic acid equivalent per 100 gm of propolis powder respectively. The result indicates that both methanolic extract of Khanchla and Ghardaia propolis contain satisfactory amount of phenolic compounds but the phenolic compounds present in methanolic extract of Khanchla are more.

Table 1: The total polyphenol and total flavonoid (mg/100 g) contents of methanolic extracts of propolis from Algeria

Compound (concentration)	Ghardaia	Khanchla
Extraction yield (%)	7.26	24.13
Total phenol (mg/100g)	493.49	1423.32
Total flavonoid (mg/100g)	194.93	345.99

Kumazawa, Hamasaka, and Nakayama previously reported that the polyphenol content of EEP of Europe and China was in the range of 200–300 mg GAE g1. The polyphenol content of MEP of Algeria propolis was lower than the reported value [10]. This confirms the influence of the origin of the material in the result.

This study shows that total flavonoid contents in the selected propolis as: Khanchla 345.99 mg/100g> Ghardaia 194.93 mg/100g. Variation in the flavonoid content of propolis is mainly attributable to the difference in the preferred regional plants collected by honeybees [14].

Using the free radical scavenging determination (DPPH)

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples [15]. It is accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen- donating ability [16]. 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 2. and Fig. 1, the activities of propolis samples and ascorbic acid as free radical scavenging increased as a function of concentration increment.

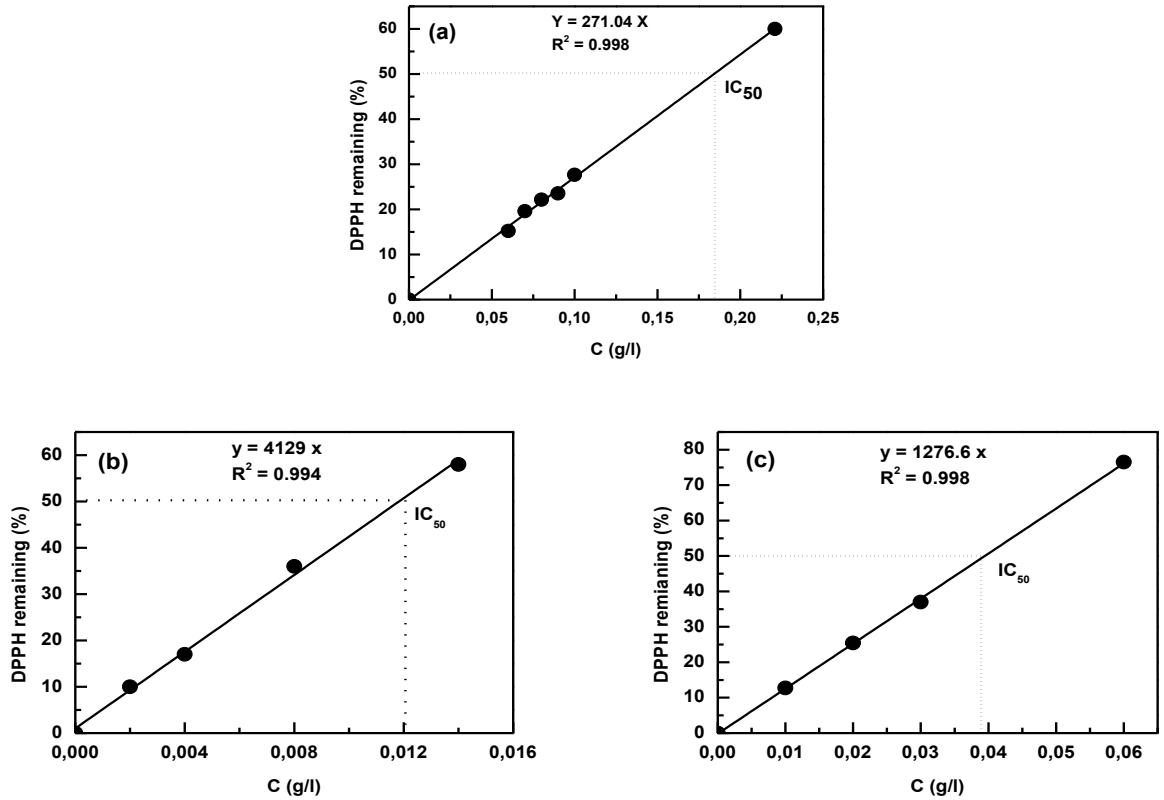


Fig. 1: The DPPH radical scavenging activities (%) of ascorbic acid (a) and methanol propolis extracts collected from khanchla (b) and ghardaia (c).

Table 2: The DPPH radical scavenging activities (g/l) of ascorbic acid and methanol propolis extracts collected from Khanchla and Ghardaia

Samples	Ghardaia	Khanchla
IC50 (g/l)	0.03917	0.01211
ARP	25.529	82.576

Both propolis samples show free radical scavenging activity but less than ascorbic acid. The sample collected from Khanchla has the highest free radical scavenging. It may be related to its contents from total polyphenol and flavonoid contents.

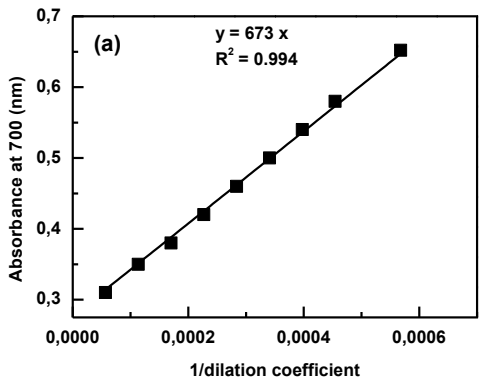
Generally, the abilities of ascorbic and natural extracts as free radical scavenging at all used concentrations in order: ascorbic acid > Khanchla propolis > Ghardaia propolis.

Ahn et al observed that propolis samples collected in various area of China showed free radical scavenging activity and there were positive correlation between the activities and total polyphenol contents [17].

Using the Reducing Power Determination (RP)

The reducing power of a propolis is also a supporting feature for its antioxidant activity [18]. Reducing power characteristics of methanol extract of propolis and ascorbic acid (standard compound) are given in Fig. 2. The concentration dependent reducing power followed the order of: ascorbic acid > Khanchla propolis> Ghardaia propolis. Reducing power of ascorbic acid is significantly higher than that of Khanchla propolis and Ghardaia propolis.

Table 3. shows reducing power (RP) of Khanchla propolis extract 3.20 m.mol.l^{-1} is relatively more pronounced than that of Ghardaia propolis extract 2.37 m.mol.l^{-1}. The figure also shows the reductive capabilities significant elevation of reducing power. This may be due to the higher polyphenol content of this extract. Because being a good electron donor, phenolic compounds have the ability to convert Fe^{3+} to Fe^{2+} and hence show higher reducing activity.



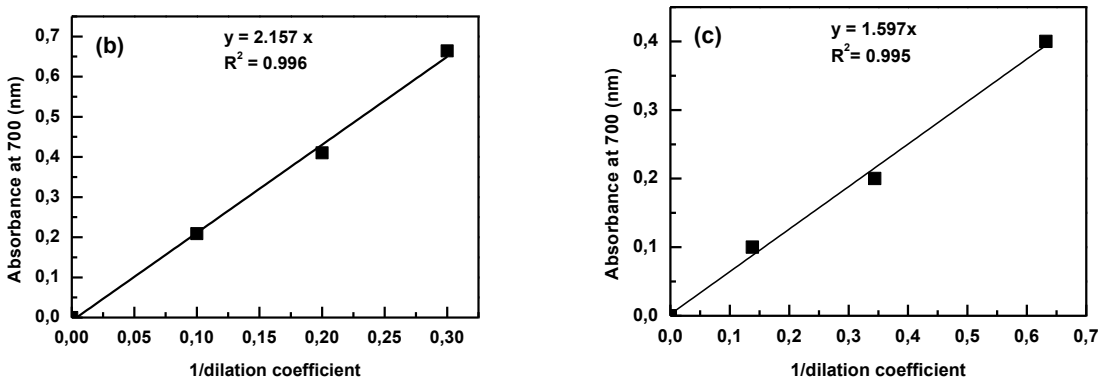


Fig. 2: The Reducing Power (RP) of ascorbic acid (a) and methanol propolis extracts collected from Khanchla (b) and Ghardaia (c).

Table 3: The Reducing power (RP) of Khanchla and Ghardaia propolis extracts

	AEAC (m.mol.l^{-1})
Khanchla propolis	3.20
Ghardaia propolis	2.37

Evaluation of antioxidant capacity by electrochemical techniques

The cyclic voltammetry voltammogram, obtained for 1 m.mol.l^{-1} of ascorbic and gallic acids in pH 7, 0.2 mol.l^{-1} phosphate buffer solution and 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 0.26 V for ascorbic acid (Fig. 3.a) and two oxidation peaks at 0.58 and 0.85 V for gallic acid (Fig. 3.b).

The same irreversible electrochemical behavior is observed for propolis sample extract Fig. 4, 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 but it indicates that the propolis extract do not contain any of the standards ascorbic nor gallic acids.

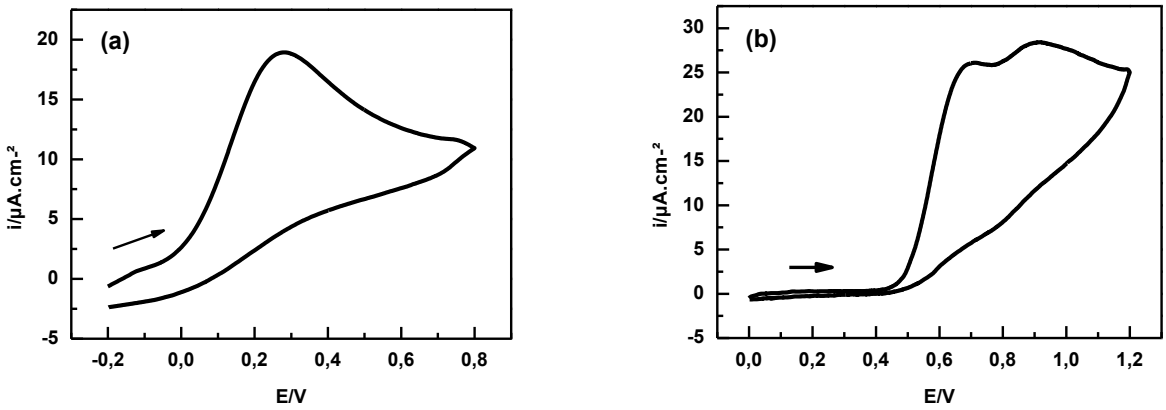


Fig. 3: Cyclic voltammograms obtained in 1 m.mol.l^{-1} of ascorbic acid (a) and gallic acid (b) in pH 7, 0.1 mol.l^{-1} phosphate buffer solution containing 0.1 mol.l^{-1} KCl at scan rate 100 mV/s.

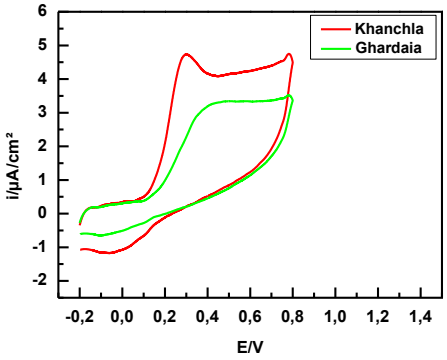


Fig. 4: Cyclic voltamograms of 20 mg/ml methanolic extracts of propolis in pH 7, 0.2 mol.l^{-1} phosphate buffer solution containing 0.1 mol.l^{-1} KCl at scan rate 100 mV/s.

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 P.A. Kilmartin [19] (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 Cyclic voltammograms, at different concentrations of ascorbic and gallic acids, are shown in Fig. 5. 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 versus the area of the anodic wave (AAW) [20].

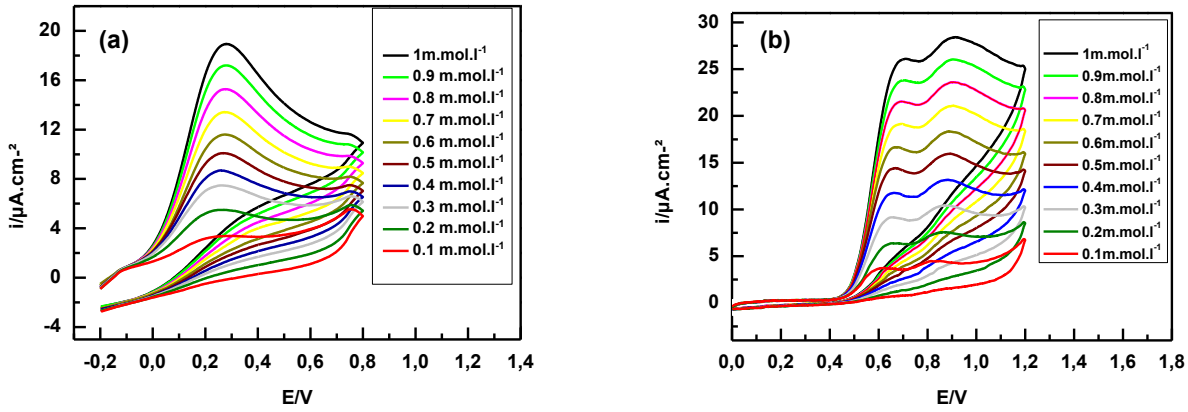


Fig. 5: Cyclic voltammograms referring to different ascorbic (a) and gallic acids (b) concentrations.

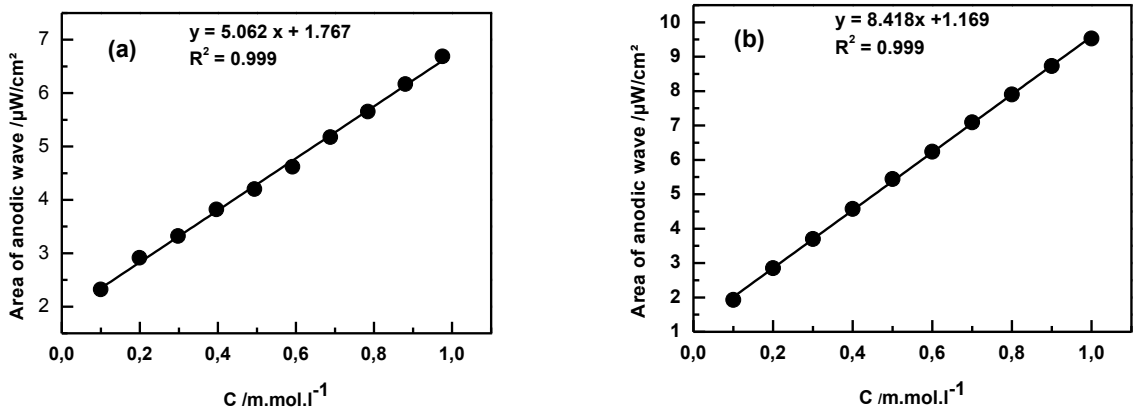


Fig. 6: The calibration curve obtained by cyclic voltammetry method expressed as ascorbic (a) and gallic (b) acids equivalents/l

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), with a correlation coefficient of R^2 = 0.999 for both equations.

In Table 4. the ascorbic acid equivalent antioxidant capacity (AEAC) and gallic acid equivalent antioxidant capacity (GEAC) of the Khanchla propolis extract, calculated from the calibration graphs, is equal to 15,61 and 14,40 mg/g.

Table 4: The antioxidant capacity of propolis calculated using cyclic voltammetry (CV).

	AEAC (mg/g)	GEAC (mg/g)
Khanchla propolis	15,61	14,40
Ghardaia propolis	8,20	10,95

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 ferric reducing antioxidant power (RP). This outcome can be attributed to the over estimation of the total polyphenolic content due to the interferences of other non-phenolic species like reduction sugars [21,22].

CONCLUSION

In this study, it was concluded that the antioxidant activity of propolis collected from Khanchla and Ghardaia was investigated. Differences were observed in total polyphenol and flavonoid contents. Propolis samples had strong antioxidant activities, and the highest activities were found in Khanchla propolis. Also, among three assays employed in this study (DPPH, RP, CV), the cyclic voltammetry method is recommended as it represents a relatively clean chemical system, easy to control, is not affected by turbid solutions of the extracts and is fairly rapid and cost-effective.

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