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Full Length Research Paper

Screening of antioxidant potential of *Lonicera tatarica, Viburnum opulus* and *Sambucus ebulus* L. by multiple *in vitro* assays

Maria-Viorica Bubulica[1]*, Liviu Chirigiu[1], Alexandru Mihai Grumezescu[1], Alexandru Popescu[2] and Andreea Simionescu[3]

[1]Faculty of Pharmacy, University of Medicine and Pharmacy, Craiova, Romania.
[2]Faculty of Applied Chemistry and Material Science, Politechnica University of Bucharest, Bucharest, Romania.
[3]Faculty of Chemistry, University of Craiova, Craiova, Romania.

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In this study, three medicinal plants known as (*Sambucus ebulus*, *Lonicera tatarica* and *Viburnum opulus*) were investigated for their antioxidant properties. Total phenolic and total flavonoid content was also determined. All three phytopharmaceutical products have high amounts of phenols but the highest values were observed at *V. opulus* bark extract (42.38±0.12 gallic acid equivalents GAE/g). The 1,1 -diphenyl-2-picrylhydrazyl (DPPH) test showed also high antioxidant potential of them in comparation with other medicinal plant species (IC_{50} was 1.089+1.09 mg/ml for *V. opulus* bark, 0.918+0.46 mg/ml for *S. ebulus* leaves, 1.947±0.98 mg/ml for *L. tatarica* flowers and 2.145±1.34 mg/ml for *L. tatarica* fruits). Therefore, the medicinal products studied have the potential to substitute artificial antioxidants in food and nutraceutical industry.

Key words: Antioxidant activity, *in vitro,* total phenols, medicinal plants.

INTRODUCTION

The technological importance of natural products has driven efforts to fabricate hybrid materials, which are of fundamental interest to modern science due to their vast applications in controlled drug release (Mihaiescu et al., 2011), drug targeting (Grumezescu et al., 2011), inhibition of microbial biofilm growth (Saviuc et al., 2011a), biosensors (Chirea et al., 2011), antimicrobial therapy (Saviuc et al., 2011b) or medical diagnostics.

Free radicals (such as superoxide radicals, peroxyl radicals and hidroxyl radicals) are involved in the development of chronic diseases (cancers, cardiovascular diseases, artherosclerosis and cerebrovascular diseases) (Miron and Stanescu, 2003). Reducing the risk of occurrence of these diseases is related with the intake of natural antioxidants (Lopaczynski and Zeisel, 2001; Alonso et al., 2004). Scientists are continuously searching natural sources of antioxidants. Groups of compounds which possess antioxidant properties are: polyphenols, vitamins A, C and E, carotenoids. The number of publications regarding health benefits of polyphenols has increased in the last period of time and the antioxidant activity of some medicinal plants was determined by scientists (Moein et al., 2008; Dogan et al., 2010). *Viburnum opulus* bark (Cortex Viburni) is harvested from April to early June when sap circulates and it is easy to detach it. It is used in the form of decoction, 2% infusion or alcoholic extracts in the treatment of gastric or uterine bleeding, hemorrhoids.

It has calming action, sedative, uterine and according to some author's cardiotonic and vasodilator actions (Cam et al., 2007; Ovodova et al., 2000). *Lonicera* species are well established antitumoral, tonic and anti-inflammatory agents in traditional Chinese medicine (Chun-Whan et al., 2007). *L. tatarica* is a member of the Caprifoliaceae family known by the common name Tatarian honeysuckle and widespread in China, Europe, India and North America. *Sambucus ebulus* (common danewort, dwarf elderberry) is a less demanding plant

*Corresponding author. E-mail mariaviorica.bubulica@gmail.com. Tel:/Fax: 0040251533713

found on uncultivated lots all over Europe along roadsides or water flowing (Ciocârlan, 2010). Dwarf elderberry is a herb known in romanian phytotherapy from which roots are harvested and used on diets being a good inhibitor of food appetite (Gross et al., 2004), leaves are used to treat rheumatism being an excellent antiinflammatory and antirheumatic agent (Süntar et al., 2010) and flowers and fruits are used as a natural detoxifier and antioxidant (Ebrahimzadeh et al., 2009). These parts of the plant are prepared: decoction, tinctures or poultices. The objective of this study was to determinate the total polyphenol content, total flavonoid content and antioxidant capacity of *L. tatarica, V. opulus* and *S. ebulus via* a set of *in vitro* assays. As far as we know there are no previous studies (*L. tatarica*, *V. opulus* bark) or little information (*S. ebulus*) (Ebrahimzadeh et al., 2008) regarding antioxidant activity and chemical composition of these plants.

MATERIALS AND METHODS

Plant material

Samples were collected from Botanical Garden of Craiova City, Dolj County, Romania between May and June 2011. The plants were identified by Prof. Botanist Dr. Tita Ion of the Pharmaceutical Botany Department, Faculty of Pharmacy, University of Medicine and Pharmacy, Craiova, Romania and voucher specimens were deposited in his herbarium. Samples were washed with doubly distilled water to remove dust and damaged portions. The leaves (*S. ebulus*), flowers (*L. tatarica*), fruits (*L. tatarica*) and bark (*V. opulus*) were air-dried (25 to 27°C) in a dark room. After drying, the collected samples were ground to fine powder and passed through a sieve (24 mesh) then the ground samples were dried again in a convection oven at 45°C until there was no change in weight (Mohd et al., 2002).

Extract preparation

1 g of each sample was refluxed with 100 ml methanol 80% (v:v) at 35°C for 24 h. Extracts were filtered through Wathman no. 42 filter paper and were centrifuged for 8 min at 8000 rpm. Supernatants were stored at 4°C prior to use within 48 h.

Chemicals and reagents

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, USA) or Merck (Darmstadt, Germany).

Determination of total polyphenol content

Total phenols were determined by the Folin-Ciocalteu colorimetric method described previously (Borneo et al., 2009). First, a standard curve was plotted using gallic acid as standard with concentrations ranging from 0 to 1000 mg/L. Two hundred microliters of each extract were separately mixed with 2, 5 ml Folin-Ciocalteu reagent (1:10 diluted with bidistilled water). After 3 to 4 min, 2 ml of sodium carbonate 75 g/L were added in each sample. The reaction mixture was allowed to react for 2 h at room temperature in the dark. The absorbance of all samples was measured at 765 nm with a Lambda 950 (Perkin-Elmer) spectrophotometer and the results were expressed as mg GAE/g of dry sample. Results are represented as mean ± standard deviation.

Determination of total flavonoid content

Total flavonoid content of each extract was determined by Aluminum Chloride ($AlCl_3$) 10% colorimetric method (Nayan et al., 2011). The calibration curve ($y=0.0173x+0.0216$, $R^2=0.9949$) was prepared with concentrations ranging from 12.5 to 100 mg/L of quercetin. 0.5 ml from each extract was mixed with 1.5 ml methanol, 0.1 ml aluminium chloride 10%, 0.1 ml potassium acetate and 2.8 ml bidistilled water. The mixture was left at room temperature for 40 min and then the absorbance was measured at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents/g of dry sample.

Antioxidant activity

DPPH free radical scavenging activity

The DPPH radical was used for the determination of the free radical scavenging activity of the extracts. 0.05 ml from methanolic extracts of 0.1 to 0.9 mg/L was mixed with 2.9 ml of a 0.004% (w/v) methanol solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm against a blank (Koksal et al., 2011). Ascorbic acid was used as reference positive control.

Ferrous ion chelating activity assay

This method was performed according to the method of Dinis et al. (1994) with some modifications. 0.2 ml of extract (with concentrations ranging from 0.1 to 2.5 mg/ml) was added to a solution of 1mM of $FeCl_2$ (200 µl). After 2 min, 0.2 ml of ferrozine were added and the mixture was left for 10 min at room temperature to react. Te absorbance was measured at 562 nm spectrophotometrically. The metal chelating activity of extracts was determined by comparison with the chelating activity of Na_2 ethylene diamine tetraacetic acid (EDTA) (positive control). The percentage of inhibition was calculated as:

$$\% \text{ of inhibition} = [(A_0-A_s)/A_s] \times 100$$

Where, A_0, is the absorbance of the control (before adding the sample); A_s, is the absorbance of the mixture containing the sample.

Cyclic voltammetry studies

Cyclic voltammetry studies of plant extracts were carried out in a potentiostat PGZ-402 Universal Pulse Dinamic EIS Voltammetry model (from Radiometer Copenhagen, 2007) equipped with Volta Master 4 soft. A three electrode system was employed with vitreous carbon (active surface 1 cm^2) as the working electrode, Ag/AgCl as the reference electrode and a platinum wire (active surface 0.25 cm^2) as the counter electrode. Supporting electrolyte preparation. For pH=2.8 and 0.138 g NaH_2PO_4 were dissolved in 100 ml distilled water; for pH=7.1, a solution of 0.05 g NaH_2PO_4 and 100 ml water was used as the supporting electrolyte. The scan was done in the potential range of -200 to 1000 mV at a scan rate of 100 mV/s. Plant extracts were prepared in a 1:1 ratio (solid sample:

Table 1. Total polyphenols, flavonoid content and inhibitory concentration of *V. opulus*, *S. ebulus* and *L. tatarica* extracts.

No	Sample	Total polyphenols[a]	Total flavonoids[b]	IC_{50} (DPPH assay) (mg/ml)	IC_{50} (mg/ml) ($Fe^{2\pm}$ chelating activity)
1	*Viburnum opulus* bark	42.38±0.12	19.21±1.02	1.089±1.09	1.865±0.05
2	*Sambucus ebulus* leaves	35.73±1.26	13.55±1.36	0.918±0.46	1.753±0.14
3	*Lonicera tatarica* fruits	27.23±0.62	15.23±0.14	1.947±0.98	28%***
4	*Lonicera tatarica* flowers	25.17±0.84	22.00±1.21	2.145±1.34	25%***
	Ascorbic acid	-	-	0.05±0.00	-
	EDTA	-	-	-	0.02±0.00

[a] mg gallic acid equivalents/g of dry sample; [b]mg quercetin equivalents/g of dry sample; ***at 2.5 mg/ml. Values are given as mean ± SD (n=3).

ethanol 80%). At 10 ml extract, 5 ml supporting electrolyte were added.

Statistical analysis

Statistical analysis was obtained by Student's t-test (the values of $p < 0.05$ were considered significant) or using the correlation and regression in Microsoft Excel v. 2007. All data were expressed as mean ± standard deviation of three measurements (n=3).

RESULTS AND DISCUSSION

Total polyphenol and flavonoid content

The Folin-Ciocalteu method was used to estimate the amount of phenolic compounds in extracts based on chemical reduction of this reactive, a mixture of tungsten and molybdenum oxides - with the appearance of blue color of which intensity can be measured at 765 nm. Total polyphenol compounds, as determined by Folin-Ciocalteu method were reported as shown in Table 1 by reference to standard curve (y=0.0008x+0.0291, R^2=0.9957). *V. opulus* bark showed highest polyphenol content: 42.38 mg GAE/g followed by *S. ebulus* leaves 35.73 mg GAE/g while *L. tatarica* flower extract have the highest flavonoid content: 22.00 mg QE/g. The results are comparable with those obtained for medicinal plants known for their antioxidant capacity such as: *Sanguisorba officinalis*, *Rosa chinensis*, *Teminalia chebula* (Cay et al., 2004) and *V. opulus* (Rop et al., 2010).

Antioxidant activity

DPPH radical scavenging activity

For antioxidant activity evaluation of extracts method was used based on the reduction of DPPH radical in methanol extracts causing an absorbance drop at 517 nm. This method has been widely used not only in plant analysis but in food industry also (Zielinska et al., 2007). The antioxidant activity was calculated as a decrease in the absorbance value using the formula:

$$\text{Antioxidant activity (\%)} = 100 \times (A_i - A_f)/A_i$$

Where Ai, is the absorbance of the control (before adding the sample); A_f, is the absorbance of the mixture containing the sample.

The IC_{50} values (inhibitory concentration required to scavenge 50% of free radicals) were calculated from the regression equation and results are given in Table 1. A lower IC_{50} value indicates greater antioxidant activity. All extracts have high DPPH scavenging activity, but leaves extract of *S. ebulus* has the higher activity, even at low concentrations (Figure 1). IC_{50} values obtained for *L. tatarica* are comparable with IC_{50} values obtained for *L. japonica* extracts, an herb known for its antineoplastic activity (Dung et al., 2011). In terms of reducing properties, studied plant products can be ordered as follows: *S. ebulus* leaves > *V. opulus* bark > *L. tatarica* fruits > *L. tatarica* flowers. A possible explanation of increased antioxidant activity for the two samples is steric accessibility (Kumari and Kakkar, 2008). Thus, small molecules that have better accessibility to the site of radical give a better response in this test (Prior et al., 2005). Not a very good correlation was found between antioxidant capacity and total phenolic content (R^2=0.8399) fact also met in the specialty literature in similar studies (Tachakittirungrod et al., 2007).

Ferrous ion chelating activity assay

A high level of ferrous ions can promote Fenton reaction, which generates hydroxyl radicals (the most reactive oxygen species is known as):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\cdot$$

Since Fe^{2+} ions causes production of oxygen radicals, minimize their concentration protects the body against

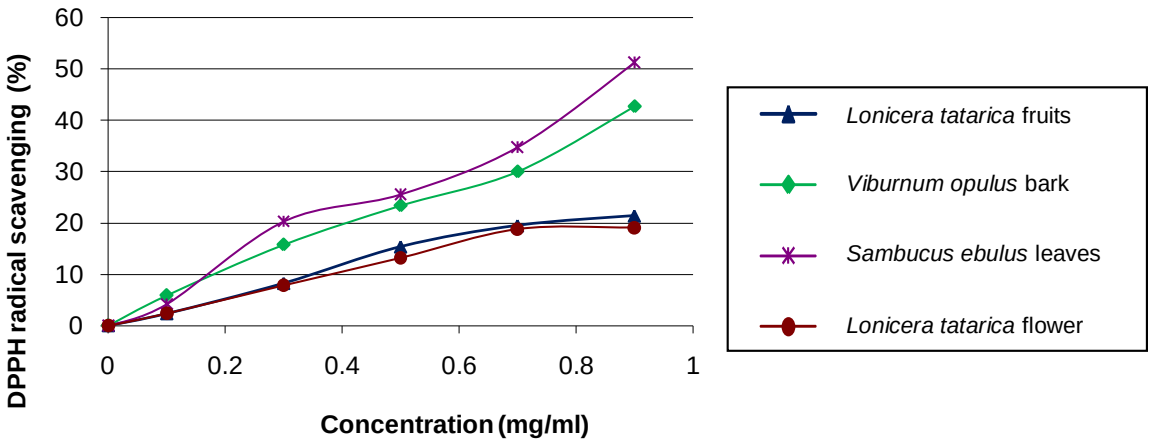

Figure 1. Antioxidative capacity (DPPH radical scavenging activity) of methanolic extracts of *L. tatarica*, *V. opulus* and *S. ebulus.*

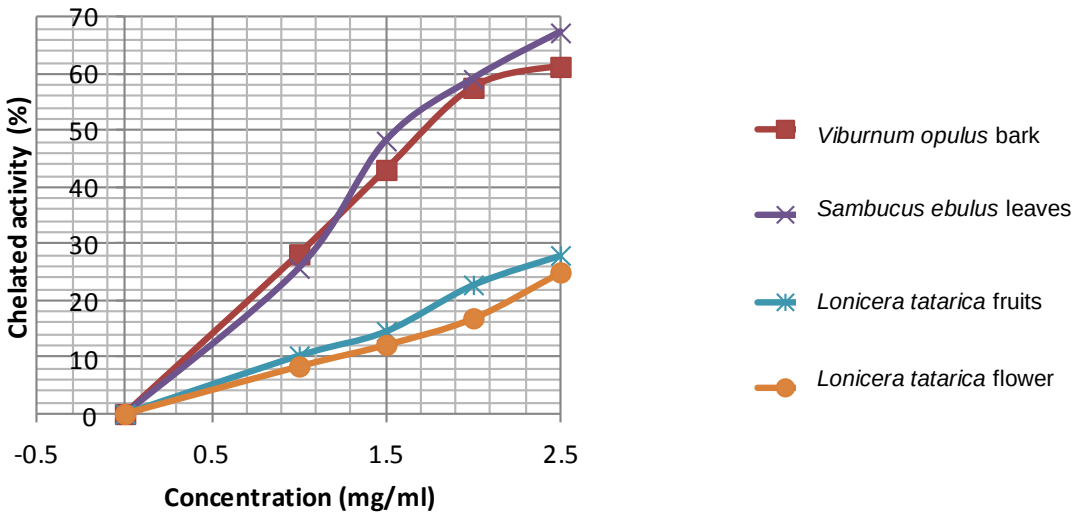

Figure 2. Inhibition of ferrozine-Fe^{2+} complex at different extract concentrations (0 to 2.5 mg/ml).

oxidative damage. Ferrous ions also participates in the process of lipid peroxidation (Halliwell and Gutteridge, 1990). Ferrozine forms with ferrous ions red complexes Fe (Ferrozine)$_3^{2+}$. In the presence of chelating agents (plant extracts), formation of this complex is disrupted; a decrease in coloration intensity occurs that can be measured at 562 nm and allows estimation of coexisting chelating activity. Extracts from the leaves of *S. ebulus* and bark of *V. opulus* showed the largest chelating activity suggesting that they form complexes with ferrous ions before ferrozine (Figure 2).

Cyclic voltammetry studies

Cyclic voltammetry studies are intensive used in the last years to determinate the redox potential of plants (Khopde et al., 2001; Pisoschi et al., 2011; Sousa et al., 2004). Two anodic peaks were observed around 0.45V (corresponding routine) and around 0.7V (corresponding vitexin) for fruit and flower extract of *L. tatarica* (Figures 3 to 5) at both basic and acid pH values. Electrochemical behavior is similar but a stronger antioxidant capacity can be attributed to fruit tincture. For *V. opulus* bark extract was observed a single anodic peak around 0.45V corresponding to routine for both alkaline and acid pH's. It also appears a slight shift of anodic peak to positive values with decreasing pH and an increased peak intensity at pH 2.8 (Figures 6 to 7). *S. ebulus* extract observed the movement to negative values of oxidation peak with pH increasing (Figures 8 to 9). The observed anodic peaks are due to the polyphenols, flavonoids and soluble vitamins which may be present in these extracts (Chevion et al., 2000; Zielinska et al., 2007). The study

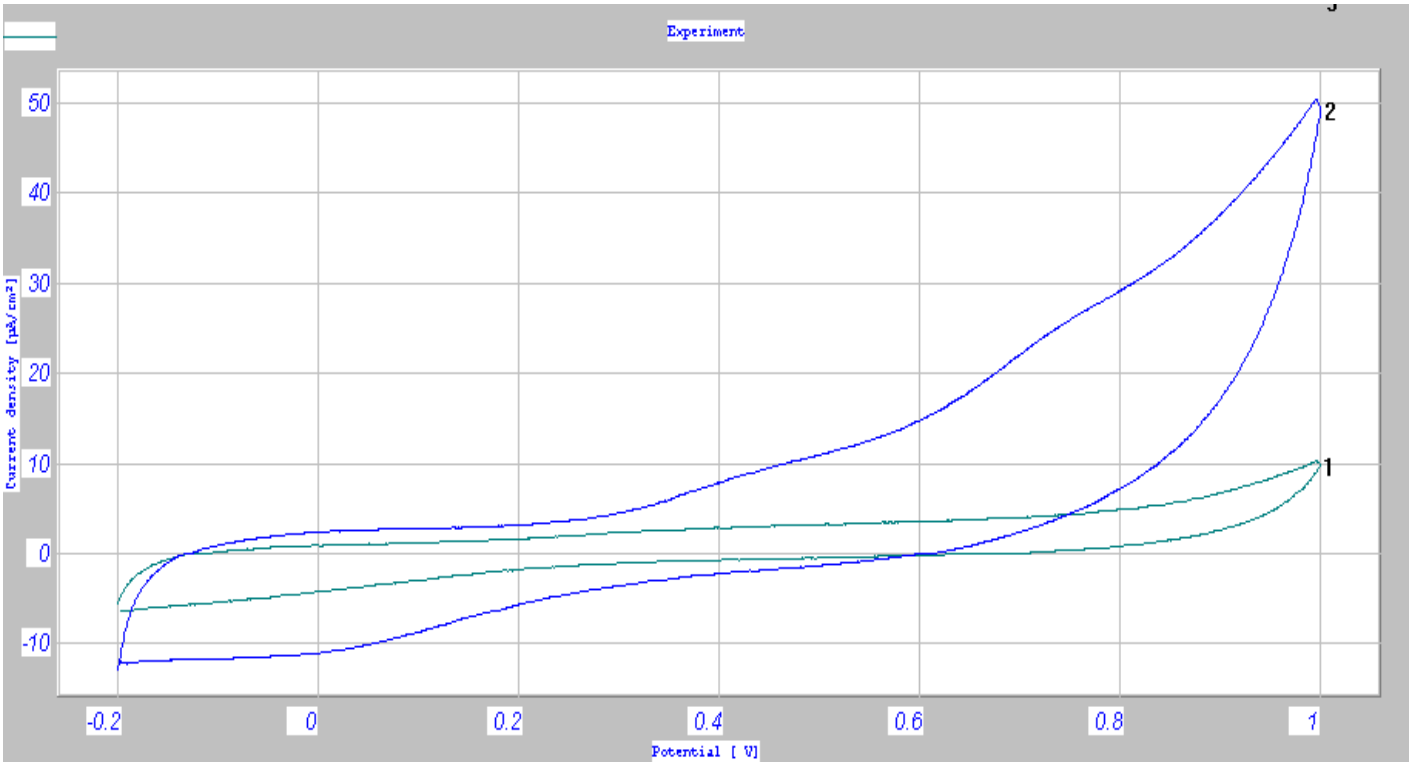

Figure 3. Cyclic voltammograms of *L. tatarica* flowers at pH=7,1: 1- electrolyte solution; 2 – *Lonicera* extract.

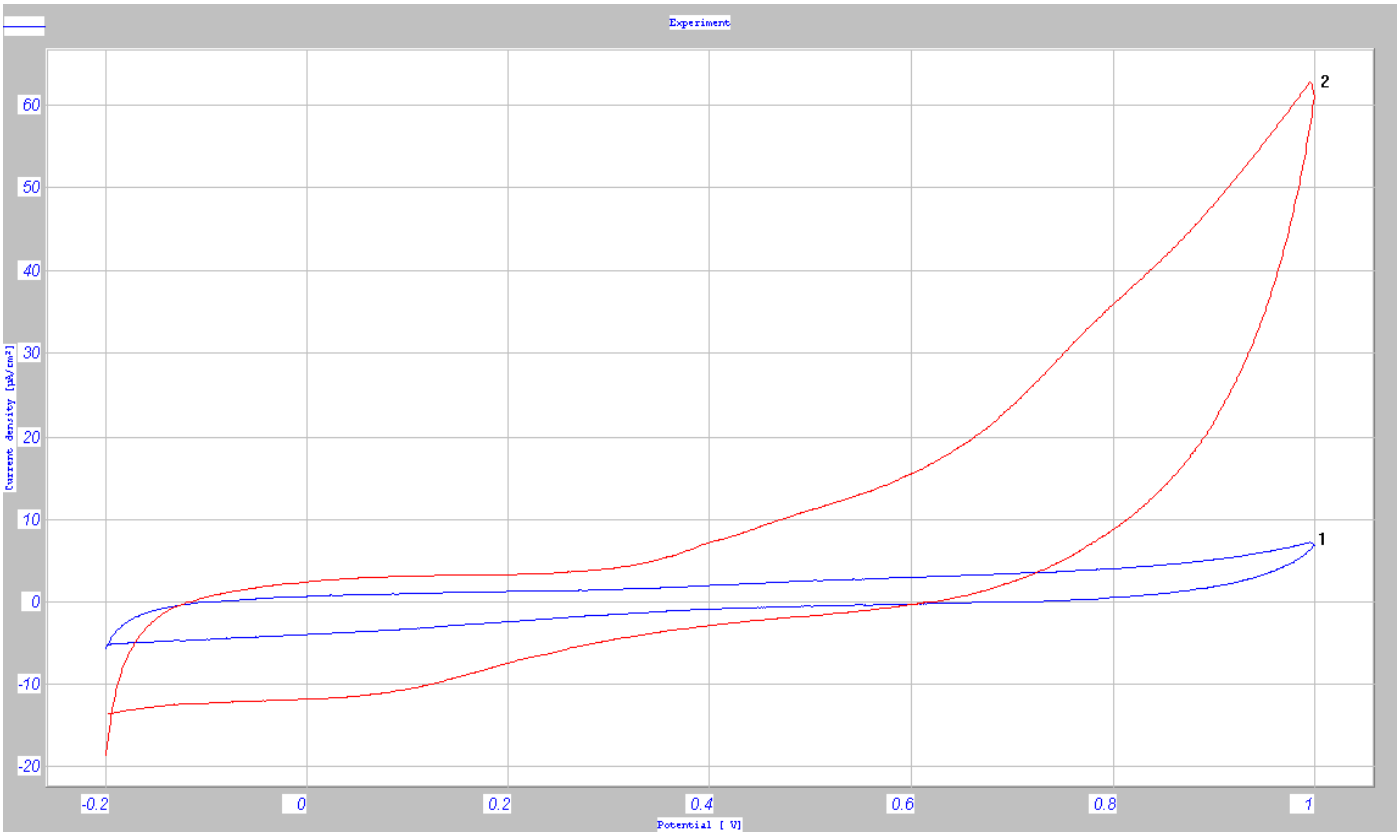

Figure 4. Cyclic voltammograms of *L. tatarica* flowers at pH=2,8: 1- electrolyte solution; 2 – *Lonicera* extract.

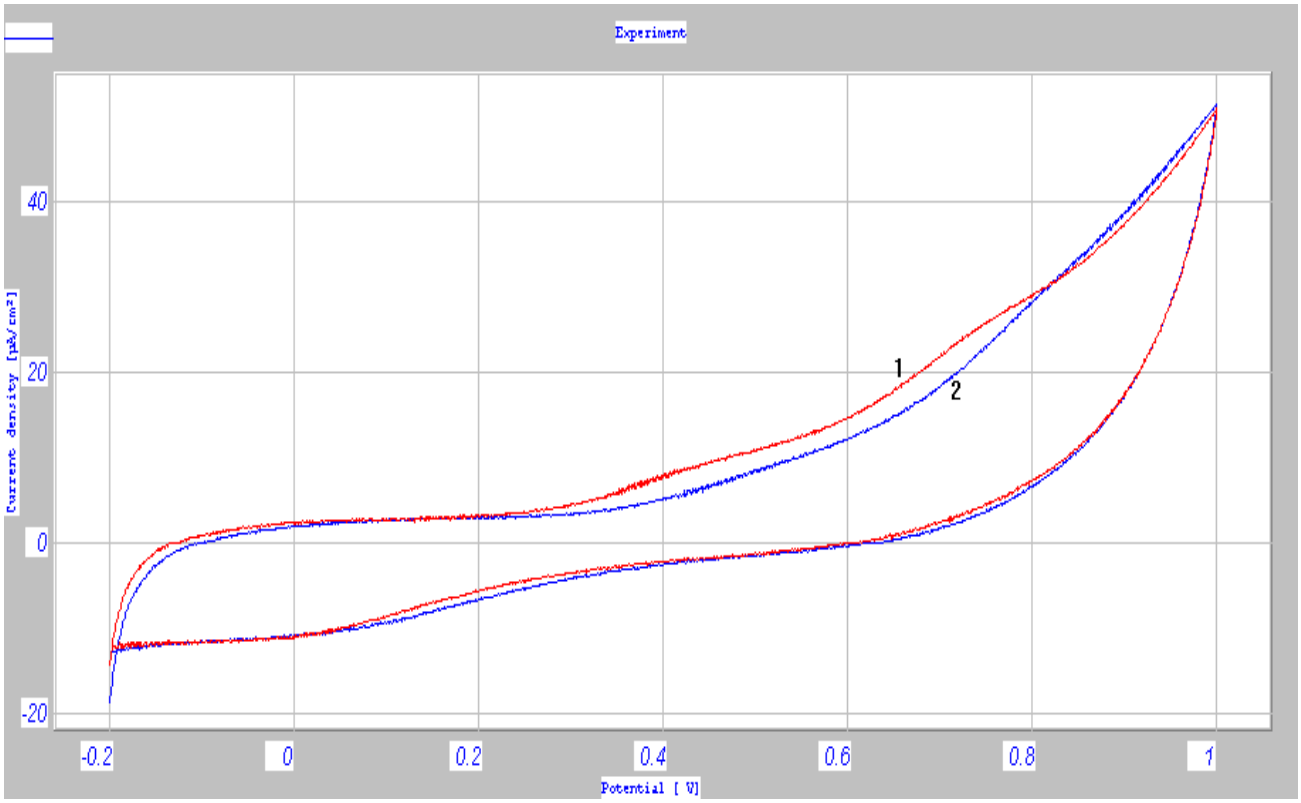

Figure 5. Cyclic voltammograms of *L. tatarica* fruits at: 1- pH=7, 1; 2- pH=2, 8.

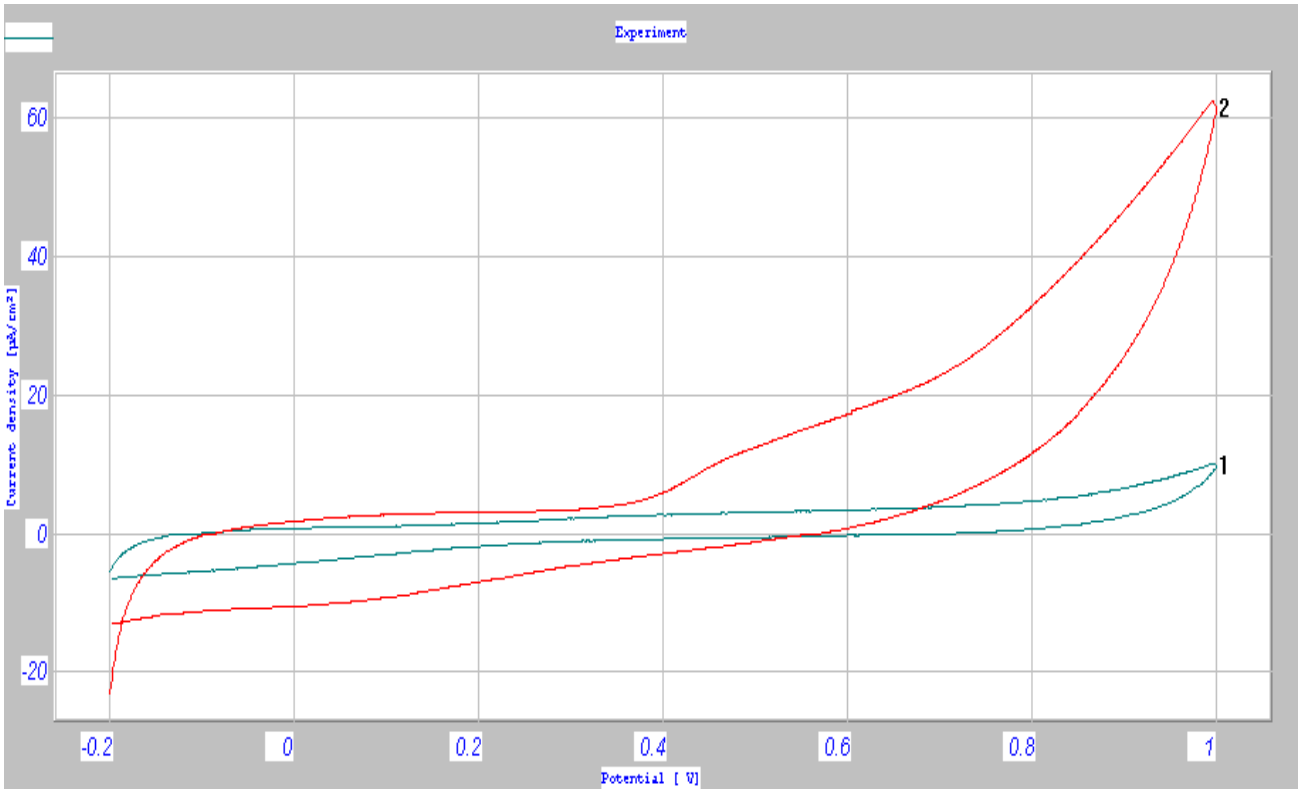

Figure 6. Cyclic voltammograms of *V. opulus* bark at pH=7,1: 1- electrolyte solution; 2 –*Viburnum* extract.

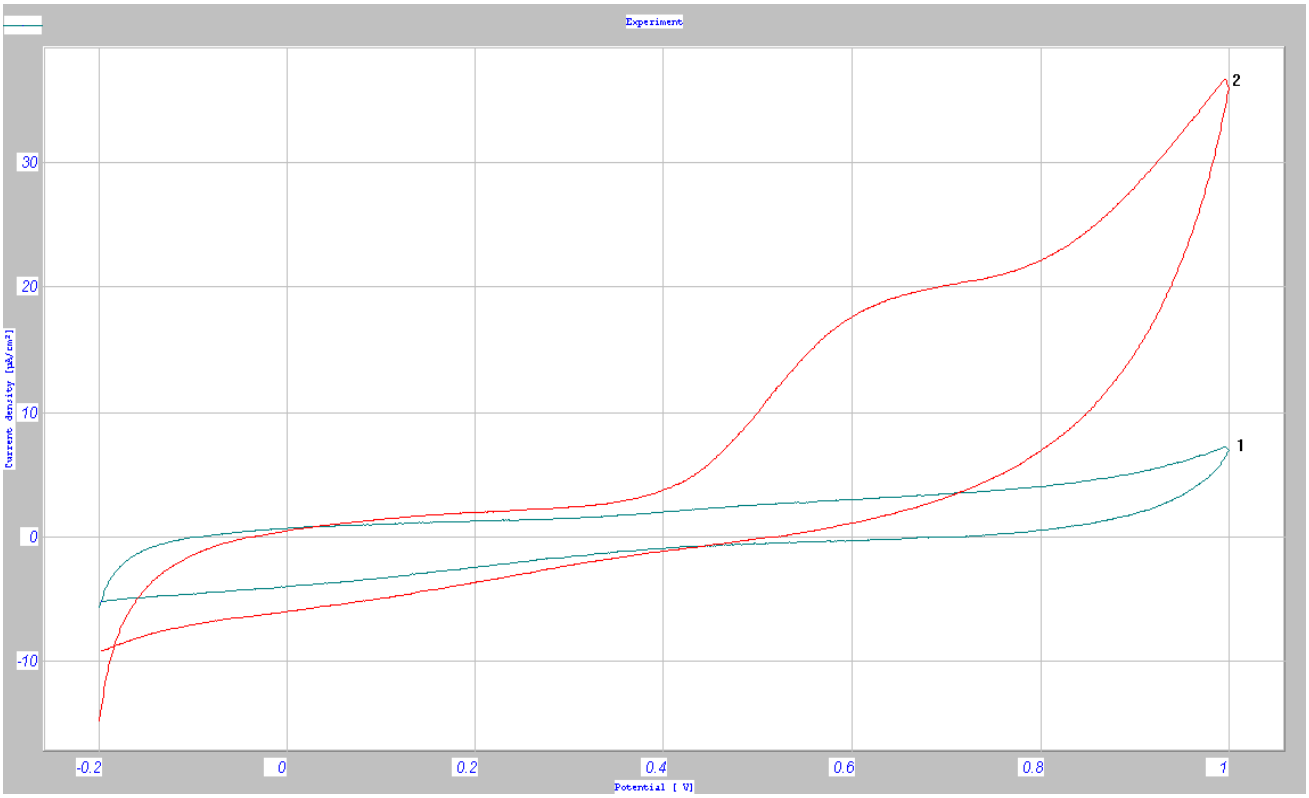

Figure 7. Cyclic voltammograms of *V. opulus* bark at pH=2,8: 1- electrolyte solution; 2 –*Viburnum* extract.

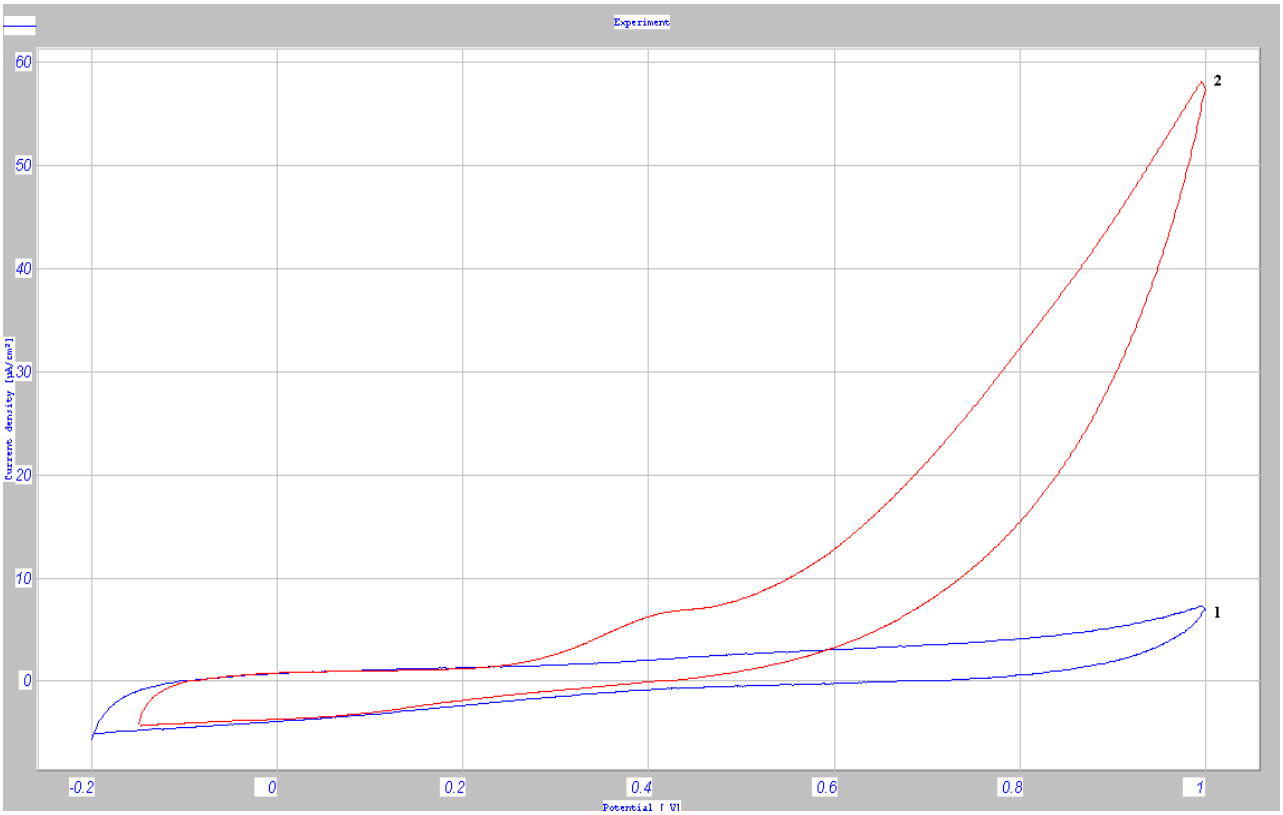

Figure 8. Cyclic voltammograms of *S. ebulus* leaves at pH=7, 1: 1- electrolyte solution; 2 –*Sambucus extract*.

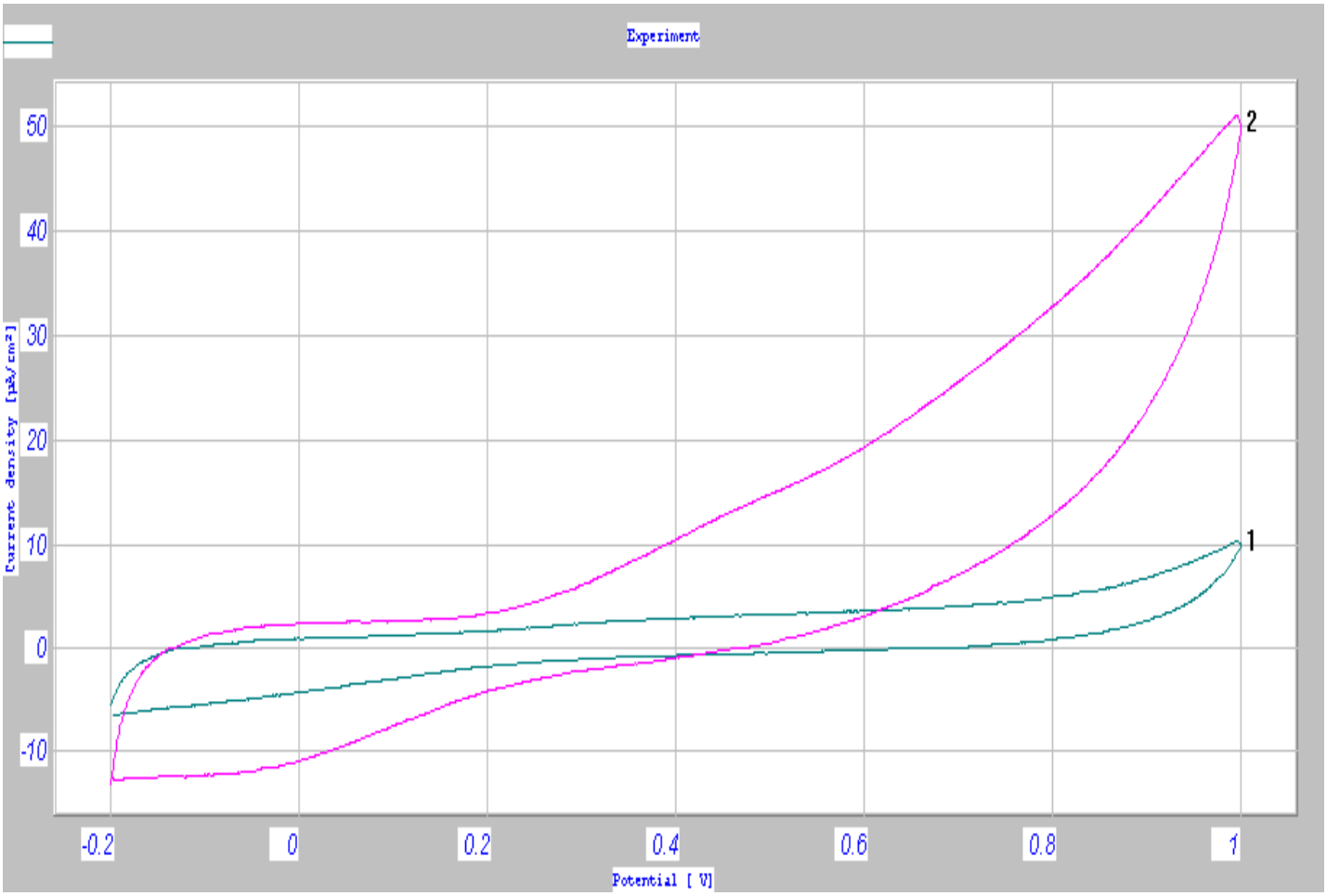

Figure 9. Cyclic voltammograms of *S. ebulus* leaves at pH=2,8: 1- electrolyte solution; 2 –*Sambucus* extract.

showed that the cyclic voltammetry method is an efficient tool for evaluating the total antioxidant capacity. Cyclic voltammetry measurements are easy to perform and can be used for the convenient monitoring of the antioxidant capacities of plant extracts (Llesuy et al., 2001).

Conclusion

Due to lack of information in the literature, the aim of the present study was to evaluate antioxidant capacity, total polyphenols and total flavonoid content of aerial parts of *L. tatarica*, *V. opulus* and *S. ebulus*. All *in vitro* tests performed in this study confirms that the plant extracts studied have a role in protection against oxidative stress, acting primarily as a scavenger - prevent oxidative stress by trapping free radicals, behave as preventive antioxidants, act by seizing ferrous ions, preventing Fenton reaction. The medicinal plants used in this study may become a valuable source of antioxidants for human nutrition.

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