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A RAPID DETERMINATION OF RADICAL SCAVENGER PROPERTIES OF PLANT EXTRACTS USING ELECTROCHEMICAL APPROACH

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The present paper addresses the radical scavenger properties evaluation of different extracts from plants and berries, in order to prove the feasibility of electrochemical approach to such an application. The radical scavenging properties of extracts of acclimatised *Lonicerae caerulea fructus*, *Myrtilli fructus, Equiseti herba, Millefolii herba* and *Medicagini herba* were evaluated using as model a long lifetime free radical 2,2'-diphenyl-1-pycrilhydrazil (DPPH). An electro-analytical method (square-wave voltammetry) devoted to the evaluation of extract properties was developed and results were confirmed using spectrochemical methods. The results were expressed in Trolox equivalent radical scavenging activity on mg dry base. In the case of *Lonicera caerulea* fruits the results (12385.63 μmol TE/mg dry fruit for aqueous extract; 12455.27 μmol TE/mg dry fruit for alcoholic extract and 3918.05 μmol TE/mg dry fruit for total extract) proved that berries from acclimatised plant provide a high content of antioxidants.

INTRODUCTION

The main defensive system against ageing processes free radical initiated – either for living organisms or synthetic polymeric products – is ensured by the antioxidants. Oxidative stress has been one of the most studied processes for several years as it is involved in the apparition of many diseases like: atherosclerosis, Parkinson's disease, Alzheimer's disease and it plays an important role in ageing process. The oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the produced damage. The prevention or repairing effects against injury produced by ROS is exerted, generally by antioxidants, which are at the living organisms level both endogenous and exogenous ones. [1] Generally the suppression of the oxidative damaging of a substrate by a certain compound presumed to act as antioxidant is denominated as "antioxidant efficacy" when compound is present at low concentration with respect to substrate concentration. [2]

In the general category of exogenous compounds are included phenols and phenolic derivatives, most effective ones being those present in plants, fruits and vegetables. Polyphenols occurring in plants are either genuine – wild species and crop fields – or "in vitro" synthesised via elicitation or exerting an oxidative stress against *in vitro* cultivated plants. As consequence these compounds have received considerable attention and there are intensively studied as potential antioxidants, their activity being assessed. In order to obtain reliable analytical information on the efficacy of polyphenols as antioxidants, at least two main determinations are mandatory: (1) assessment of polyphenols amount and (2) evaluation of the polyphenols efficiency against oxidative damage of a biological significant substrate (effectiveness against free radicals injury leading to oxidative stress).

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Evaluation of the antioxidant features of phenol-like compounds has to be done with respect to few well-defined factors: the specificity of antioxidant action against the critical target, free radical nature and free radical flow (or the free concentration level in the analysed system). [3,4]

There are several known methods usually used in evaluation of antioxidant capacity for plants extracts, depending on the level of action of presumed antioxidants. When polyphenols has to be characterised as potential nutritional supplements or antioxidants food additives three approaches are employed, namely: determination of each poly-phenolic component by high-performance chromatography (HPLC) or capillary electrophoresis, [5,6] determination of total phenolics content using Folin-Ciocalteu reagent[7] and, in order to determine the total antioxidant capacity, the assessment of polyphenols reducing activity. The antioxidant capacity could be performed by different approaches, the frequently used being: the oxygen radical absorbance capacity assay (ORAC)[8, 9] and the Trolox equivalent antioxidant capacity (TEAC) assay. [10]

Amongst the most reported compounds having an important antioxidant capacity are polyphenols for which preferred sources are plants extracts. Generally, the extraction is performed using various plant sources such as leaves, roots, fruits, grapes, berries-seeds etc. either as fresh or dried material. The main polyphenols classes are flavonoids and anthocyans. Flavonoids themselves involve several subclasses, the main important being: the flavonols (which include quercetin, kaempferol and myricetin), the flavones (which include apigenin, luteolin and tangeritin), the flavanones (which include catechins, catechin gallates, naringenin and hesperetin) and the isoflavones (which include genistein, daidzen and glycitein).

In our study several plants are studied as potential sources of antioxidants, the antioxidant capacity determination being performed either on aerial parts (*herba)* of the plant, or on fruits/berries extracts.

As reported, several fruits belonging to *Caprifoliaceae* family[11] are used as food supplements due to their protective properties against blood vessels injury. They actually act as dietary source of antioxidant active principles, extremely important in ensuring the human organism defence against free radicals damage. As consequence it was presumed that even *L. caerulea*'s berries would support the same action taken into account the fact that important amounts of anthocyanins were reported as present in crude fruits extracts. [6, 12] *Lonicera caerulea* is a fruit bearing bush species originated from the half isle of Kamchatka forests that was started to be cultivated in Roumania in 1987, after the plant adjustment to the specific climatic conditions. The *L. caerulea* fruits are similar to bilberries, used in food industry. [13]

The aim of our work was to evaluate the radical scavenging effect of various extracts using based on electro-analytical approach and to prove the electrochemical approach feasibility to antioxidant capacity evaluation. To be more precise the radical scavenging properties of extracts were evaluated using as model a long lifetime free radical, [14, 15] DPPH (2,2'-diphenyl-1-pycrilhydrazil).

RESULTS AND DISCUSSION

When developing an antioxidant evaluation protocol, there are many steps that have to be carried out from analytical point of view. It started to the total phenols quantification followed by the active principles identification and quantification and ending with the evaluation of the extract behaviour against significant oxidative markers. [16] This paper addressed only few of those stages, because the idea was first to verify if the developed electrochemical method is applicable to a wide range of samples and second to optimise the extraction procedure in the case of berries extract.

As consequence the sample preparation had a main significance, and for the *L. caerulea* berries several types of extraction were tested.

The *Myrtilli fructus, Equiseti herba, Millefolii herba* and *Medicagini herba* extracts were obtained according to the following procedure: a sample (5 g dry plant) was cut, grinded and then heated (60° C) with 20 mL mixture of alcohol ethylic 70 % and distilled -demineralised water (2:1, v:v), for 10 minutes, after filtered on a funnel using a cotton filter. The filter cake was twice re-extracted with 10 mL alcohol, in the same conditions as previous. The extract was made up to 50 mL with acidified (2% HCl) water and stored at 4°C until analysed. [17]

Three extraction procedures were performed for *L. caerulea* berries.

A sample (1.2g fruits) was blended and then heated (100° C) with 20 mL distilled and demineralised water, for 10 minutes, and then filtered on a funnel using cotton filter. The filter cake was twice re-extracted with 10 mL water, in the same conditions as previous. The extract was made up to 50 mL with water and stored at 4°C until further analysed. [18] In the further

measurements the sample will be denominated as *L. caerulea* total aqueous extract.

The second sample (1.2g fruits) was blended and then heated (60-70°C) with 20 mL alcohol ethylic 50 %, for 10 minutes, and then filtered on a funnel using cotton filter. The filter cake was twice re-extracted with 10 mL ethanol 50%, in the same conditions as previous. The extract was made up to 50 mL with ethanol 50% and stored at 0-4°C until further analysed. In the further measurements the sample will be denominated as *L. caerulea* total alcoholic extract.

The third sample (10g fruits) was blended and then heated (60-70°C) with 20 mL of acetone 99 % for 10 minutes, and then filtered on a funnel using cotton filter. The filter cake was re-extracted two times with 10 mL of 70% aqueous acetone, in the same conditions as previous. Filtrates were combined and washed two times with 20 mL of chloroform, then separated using a separation funnel. The polar fraction was collected and distilled at 70 -80°C in order to remove the traces of organic solvents (chloroform and acetone). The extract was made up to 50 mL with acidified (2% HCl) water and stored at –4°C until further analysed. [18] In the further measurements the sample will be denominated as *L. caerulea* total phenolic extract.

In the first step the quantification of the total phenolics (TP) was performed**,** according to Folin - Ciocalteu procedure. The method calibration was completed versus gallic acid. The determined total phenolic content, as equivalent of gallic acid, are presented in Table 1. As could be observed from the obtained data, excepting *L. caerulea* total aqueous extract, all other extracts had an acceptable content of total phenols as expected, in fact, considering the composition profiles of the tested extracts, which proved as main components flavonoid derivatives.

Second step of experiment performing consisted in rationalising on the electrochemical approach suitability to the evaluation of plants extracts radical scavenging properties. As consequence, the electrochemical characterisation of all involved compounds was performed on a concentration range significant to the study of radical scavenging properties (10^{-6} - 10^{-4} molL^{-1}). The electroanalytical parameters of Trolox and, respectively DPPH process on working electrode were determined (Table 2). As could be observed from Table 2 the potential range of interest is 0.5 - 1.3 V. The SWV was performed using a measuring cell and a system of solvent: supporting electrolyte as mentioned in the experimental section, the frequency used being 15 Hz and the amplitude of 25 mV.

Table 1

Total phenolics content determined using Folin-Ciocalteu method

No.	Extract	TP assay – Folin-Ciocalteu (Equivalent gallic acid)
1.	*Myrtilli fructus*	15.21± 0.04 μg/g dry basis
2.	*Equiseti herba*	8.43 ± 0.04 μg/g dry basis
3.	*Millefolii herba*	12.1 ± 0.03 μg/g dry basis
4.	*Medicagini herba*	6.55 ± 0.03 μg/g dry basis
5.	*L. carulea fructus* total aq. extract	1.81 ± 0.02 μg/g dry basis
6.	*L. carulea fructus* total alc. extract	7.48 ± 0.05 μg/g dry basis
7.	*L. carulea fructus* total phen. extract.	18.42 ± 0.01 μg/g dry basis

Table 2

Electroanalytical parameters for SWV characterisation of Trolox and DPPH

Analyte	Electrode potential V	Equation of calibration curve*	R	Linear domain of concentration mol L^{-1}	Detection Limit (3S/N) mol L^{-1}
Trolox	0.785 ± 0.015	i (μA) = 8891.5·C(molL^{-1}) + 0.0061	0.9848	$2.74\cdot10^{-6}$ – $1.05\cdot10^{-4}$	$1.02\cdot10^{-6}$
DPPH	0.840 ± 0.015	i (μA) = 5401.5·C(molL^{-1}) + 0.0116	0.9932	$4.68\cdot10^{-6}$ –$1.2\cdot10^{-4}$	$1.54\cdot10^{-6}$

*Mean of three determinations

Since Trolox is a chain-breaking donor type of antioxidant, it is know that the reaction has to have a lag time, when the system seems to register no changes. From this reason several experiments were carried out at the different times after antioxidant addition. The next step was to draw up the calibration for scavenging properties against DPPH, and to set up the most appropriate time of voltammogram registering/the proper end-point. The experiment consisted into the determination of antioxidant properties of Trolox against DPPH by measuring the decreasing of the maximum peak intensity of DPPH (constant concentration) with Trolox addition in the measuring cell (Fig. 1). Than the response linearity was checked in terms of Trolox concentration variation. The linear correlation between DPPH peak intensity decreasing and Trolox concentration could be considered as calibration equation for the free radical scavenging activity.

From Table 3 it could be noticed that the appropriate time for registering the maximum of antioxidant effect is 3 minutes, due to the better compromise settled between the method sensitivity (a better slope value) and response linearity (R value).

In Fig. 2 is given the calibration graph, corresponding to 2 minutes measuring time.

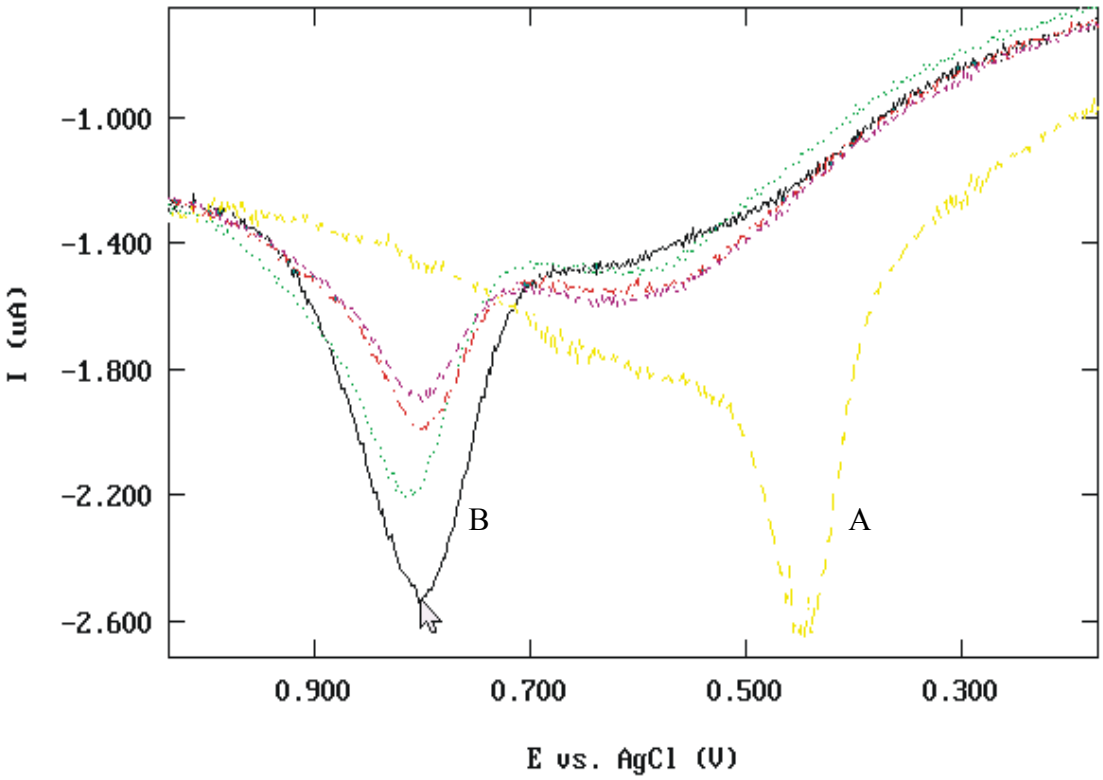

Fig. 1 – SW voltammograms (15 Hz) of Trolox 10^{-4} molL^{-1} (A) and DPPH itself (3 10^{-4} molL^{-1}) and in Trolox presence (B), multiple additions (of 2 10^{-6} mol L^{-1}).

Table 3

The evolution in time of Trolox antioxidant capacity against DPPH, on gold-bare electrode

No.	Reaction time (min.)	Equation	Correlation coefficient	Trolox concentration range, molL^{-1}
1	0	$\Delta i\ (\mu A) = -0.0028 \cdot C\ (molL^{-1}) + 0.8136$	$R^2 = 0.9253$	$2 \cdot 10^{-6} – 1.2 \cdot 10^{-4}$
2	2	$\Delta i\ (\mu A) = -0.0067 \cdot C\ (molL^{-1}) + 1.333$	$R^2 = 0.99$	
3	4	$\Delta i(\mu A) = -0.0052 \cdot C(mol\ L^{-1}) + 2.2607$	$R^2 = 0.993$	

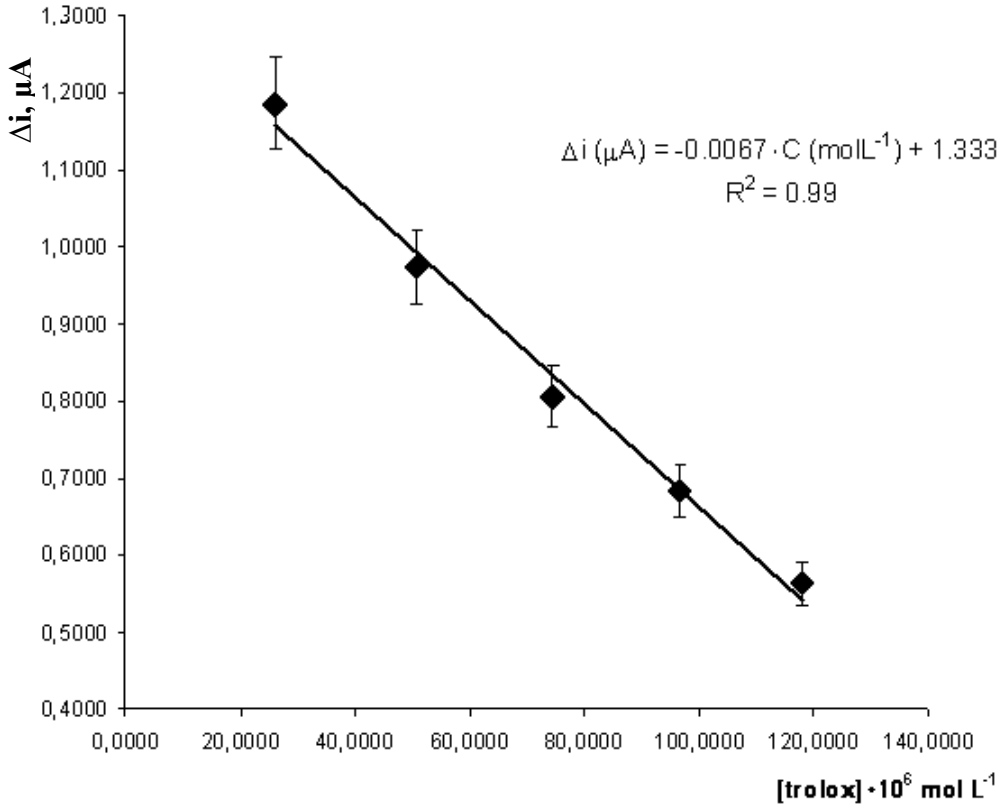

Fig. 2 – Variation of current intensity with Trolox concentration, reaction time 2 minutes.

Then, in the same electrochemical conditions (same supporting electrolyte, same electrode, same SWV parameters) experiment was carried out for different dilutions (dilution factor f =100; 10 and 1) and volumes (20 μL up to 100 μL) of plant extracts. The volumes were added into the measuring cell in order to find out the probable electrochemical characteristics of each extract, for applied potential ranging between 0.1 and 1.3 V vs. RE. No electrochemical peak to interfere with DPPH 0.940 V signal rose for any of the investigated extracts.

When those experiments were carried out the antioxidant 'definition' was taken into account so the molar ratio between antioxidant and free radical in the measuring cell was 1: 100.[1,4]

The procedure was as follows: the appropriate volume of supporting electrolyte was de-oxygenated by 4 minutes Argon purging, after that 1 mL DPPH stock solution was added and purged more 15 seconds, then 10 μL to 70 μL of Trolox stock solution were added and each time the corresponding voltammograms were registered at different times: immediately after Trolox addition (0 minute), after 1 minute respectively 2 and 4 minutes, and the maximum peak intensity of 0.840 V DPPH peak was measured. The same measurements were performed using instead of Trolox the addition of the corresponding volume of blank solution. It was noticed that the optimum reaction time was of 4 minutes, the decrease of the measured current intensity corresponding to DPPH reached a constant value for the next 30 minutes. Since the reaction between DPPH and Trolox, respectively polyphenolic antioxidants, is a combined mechanism of hydrogen transfer and electron transfer between the free radical and the antioxidant[16], determinations were performed separately for each Trolox (or antioxidant test solution) concentration, in order to avoid artefacts created by the secondary radicals generated in the precedent reaction.

The calibration graphs of scavenging properties of Trolox against DPPH were obtained by plotting the value of the difference of measured intensities versus Trolox concentrations. The value of the difference resulted from the subtraction of the intensity obtained for blank solution from that intensity corresponding to Trolox addition. It should be mentioned that due to particularities of

the measured properties, meaning the radical scavenging effect, even if we are discussing about a linear dependence between measured signal and the radical concentration it is difficult to define a detection limit of the developed method, since the paper goal was to characterise plant extracts with respect to antioxidant definition as mentioned above.

The results were calculated with the following formulas:

$$TEAC_{sample} = C_{Trolox} \cdot f \cdot \frac{i_{sample} - i_{blank}}{i_{Trolox} - i_{blank}},$$

where C – the molar concentration of the Trolox used as reference; f the sample dilution (which is 1 in all our cases), i_{sample} – maximum intensity of DPPH anodic peak after sample addition, i_{blank} – maximum intensity of DPPH anodic peak when the corresponding blank volume was added, i_{Trolox} – maximum intensity of DPPH anodic peak after Trolox addition. In order to have a rational observation of exhibited free radical scavenger properties the obtained results for sample solution were reported to the berries dry-bases (dry substance determined elsewhere[19] is 16.46 %), taking into account the weight amount for each type of extract. The obtained results are presented in Table 4.

Spectrochemical approach provided measurable results only for two samples, due to the interferences from the components of the extract, on the wavelength range 500-520 nm (the DPPH maximum raised at 519 nm). The calculus was made according to:

$$TEAC_{sample} = C_{Trolox} \cdot f \cdot \frac{A_{sample} - A_{blank}}{A_{Trolox} - A_{blank}},$$

where A is the maximum absorbancy at 519 nm, the other parameters having the same significance as previous. Data are presented in Table 4.

Table 4

Trolox equivalent FR scavenger capacity of plant extracts

No.	Extract	$TEAC_{spectrochemical}$ µmol / mg dry basis	$TEAC_{electrochemical}$ µmol / mg dry basis
1	*Myrtilli fructus*	190	260
2	*Equiseti herba*	7 300	7 800
3	*Millefolii herba*	5 900	6 300
4	*Medicagini herba*	1 400	1 310
5	*L. carulea* total aq. extract	15 801.6	12 385.63
6	*L. carulea* total alc. extract	ND	12 455.27
7	*L. carulea* total phen. extract	6 794.66	3 918.05

Few discussions seem necessary when reported results are analysed.

As could be observed from the data presented in Table 4, the direct values reported without taking into account the dry-bases, lead to an expected conclusion: the alcoholic extracts are slightly more efficient than aqueous one. The assertion is valid both when comparing results of all considered extracts and when comparing the different types of *L.caerulea* berries extracts. This feature could be explained on behalf of the used extraction solvent: polyphenols had a better extraction in medium polar solvents, such alcohols.

Another conclusion when the values reported in Table 4 are analysed is a sort of false-positive one, which the total phenolics extract seems to be more active than the total alcoholic extract. However, when results were reported to dry-bases, the first conclusion rests.

The *L caerulea* alcoholic extract is more efficient than *L caerulea* aqueous extract (12455.27 µmol TE for the first one versus 12385.63 µmol TE, of the second one). But, in the case if the total phenolics extract, when dry-bases is used in calculus, its activity per mg dry substance has a smaller value than that of *L caerulea* alcoholic extract, namely, 3918.05 µmol equivalent Trolox/1mg dry substance versus 12455.27 µmol equivalent Trolox/1mg dry substance. This could support various explanations. One consists in better extraction of ascorbic acid (known efficient antioxidant), sugars

and glycosilated polyphenols[9,10,19] by water instead of acetone or alcohol.

Another explanation is provided by the used electrochemical conditions (0.12 mol L^{-1} sulphuric acid in methanol) the slightly acidic media help to the partial hydrolysis of glycosides to aglycons and favoured the exhibit of electro-analytical features of phenolic OH, the same that is reported as antioxidant responsible group.

A fast and easy to use electrochemical method was developed and successfully applied to quantify the antiradical activity of different plant extracts. The obtained results proved that the electrochemical method is compatible to evaluate the radical scavenging properties of various types of vegetal samples.

The developed method, based on the scavenging of stable radical 2,2'-diphenyl-1-pycrilhydrazil - proves to be more reliable than the spectrophotometric one, avoiding the 520 nm interferences. In addition it has to be stressed that this approach could provide a more direct related data on phenolic OH behaviour, a further deep investigation on the electrode mechanism consisting in a potential source of information regarding the pathway of antiradical effect, as H donor or electron transfer depending on specific media.

The results, expressed in Trolox equivalent radical scavenging activity on mg dry fruit proved that the *L. caerulea* berries could be effectively used as supplements for improving food/soft drinks quality. It has to be taken into account even the fact that no other secondary effects were registered when administrated to Wistar.

EXPERIMENTAL

1. Materials and Methods

1.1. Chemical Reagents

All used reagents – methanol, sulphuric acid, Trolox, 2,2'-diphenyl-1-pycrilhydrazil (DPPH) were analytical grade, Sigma provided. All voltammetric measurements were performed at 25 ^{o}C under argon atmosphere after oxygen removal from solution (3 minutes de-aerated solution). Trolox stock solutions were alcoholic (methanol) ones, 10^{-3} mol L^{-1}, freshly prepared at the beginning of the day and stored under argon atmosphere, at 4 ^{o}C, avoiding light, during all day, to pass up the oxidative degradation. DPPH stock solutions were in methanol 3×10^{-3} mol L^{-1} maintained under the same conditions as Trolox.

1.2. Apparatus

The electrochemical experiments were performed using an EG&G Princeton Applied Research potentiostat 273A and a three electrodes measuring cell (working electrode: bare gold millielectrode; counter electrode: platinum wire; reference electrode: Ag/AgCl, KCl 3M). The cell volume was 3 up to 15 mL.

The extracts investigation was performed in a solvent-electrolyte system consisting of methanol: sulphuric acid (0.12 mol L^{-1}), positive results being expected due to enhancing of the radical scavenging properties in alcohol-acidic media[17]. The used techniques were cyclic voltammetry and square-wave voltammetry.

The spectrometric determinations were performed on V-530 JASCO spectrometer.

1.3. Methods

The quantification of total phenolic content was made using Folin-Ciocalteu method, measuring the maximum of the developed blue colour at 752 nm, the results being reported in gallic acid equivalent.

In order to rationalise on the reductive potential of some of the extracts, the effect of pH against antioxidant potential was investigated, using buffers of appropriate pH.

The electrochemical evaluation of free radical scavenging capacity of investigated extracts was based on an indirect method of determination. The method consists in evaluation of potential antioxidant properties of the extracts against evolution of a long life-time free radical, DPPH. That involved the direct correlation between the decreasing of free radical electrochemical signal with the increase of the scavenging properties of the extracts. Finally the results were reported with respect to the equivalent activity gave by a well-known antioxidant, Trolox.

The electrochemical assay was performed using the following solutions:

Blank reagent: 20 μL water, 1 mL DPPH solution 10^{-4} mol L^{-1} (obtained by appropriate dilution of stock in supporting electrolyte system) and a volume of supporting electrolyte up to a total volume of 5 mL in cell.

Trolox/standard: 20 μL Trolox, 1 mL DPPH solution 10^{-4} mol L^{-1} (obtained by appropriate dilution of stock in supporting electrolyte system) and a volume of supporting electrolyte up to a total volume of 5 mL in cell

Sample: 20 μL of extract, 1 mL DPPH solution 10^{-4} mol L^{-1} (obtained by appropriate dilution of stock in supporting electrolyte system) and a volume of supporting electrolyte up to a total volume of 5 mL in cell.

The UV-VIS procedure were carried out by measuring the changes in the value of the DPPH maximum absorbancy to 519 nm and followed the same steps, using the appropriate dilutions for measuring cells with a path length of 1.00 cm.

Determinations were performed according to the optimum time of system stability after antioxidant additions (details above).

All samples and all performed measurement were at least three times replicated.

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