Romanian Reports in Physics **71**, 706 (2019)

ANTIOXIDANT CAPACITY OF SOME *CALENDULA* EXTRACTS BY EPR SPECTROSCOPY

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Received February 12, 2018

Abstract. Antioxidant activity of some ethanol extracts of *Calendula* (marigold) flowers and chloroform fraction of extracts were evaluated. By an optimized electron paramagnetic resonance (EPR) spectroscopy method using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined antioxidant activity of chloroform fraction of each extract. The decrease in time of DPPH• concentration in the reaction medium was studied and it was noted that the reaction mechanism is characterized by two different reaction rates. Antioxidant activity of ethanol extracts was determined by spectrophotometric DPPH free radical scavenging activity method and the content of polyphenols in extracts was performed by LC-MS and spectrophotometric analysis methods. Results showed that fat soluble compounds are mainly responsible of the antioxidant activity of extracts. Analyzed extracts showed higher antioxidant activity than BHT (butylated hydroxytoluen), synthetic antioxidant compound.

Key words: Calendula extract, antioxidant activity, EPR, LC-MS/MS, polyphenols.

1. INTRODUCTION

Calendula sp. (marigold, *Asteraceae*) is often used in traditional and modern medicine as tea or as different pharmaceutical products like ointments, tinctures, in homeopathic treatments [1–4], because of its properties and greater availability. Products from marigold flowers are used in skin disorders [2, 4–7], the treatment of abdominal cramps [8], as potent antidiabetic [9], hepato-protective [10, 11], antimicrobial [12–16], anti-inflammatory [2, 17–18], antigenotoxic [19], antitumoral and cytotoxic [20, 21], etc.

Flowers of marigold are well known as being rich in bioactive principles; the main compounds include: carotenoids and lycopene [22–25], phenolic acids (protocatechuic, vanillic, syringic acids), hydroxycinnamic acids (*p*-coumaric, caffeic, chlorogenic acids), flavonoids and their glycosides (quercetin, isorhamnetin, isoquercitrin, rutin) [24, 26–29], coumarins (scopoletin, umbelliferone, aesculetin) [4, 10], volatile oil with terpenoids and terpenoid esters (oleanolic acid glycosides, taraxasterol, faradiol, lupeol, arnidiol, erythrodiol, calenduladiol, manilladiol [9, 12, 17], lipids [10, 13], etc.

Due to the presence of compounds with good antioxidant activity (polyphenol acids, flavonoids, lycopene) the extracts from marigold flowers exhibit antioxidant activity [30–33].

In this work is presented a characterization regarding chemical content and antioxidant activity of *Calendula* sp. (marigold) extracts from flowers provided by: Plafar (Bucharest), Larix (Sovata, Mures), Fares (Orastie, Hunedoara) and Belin (Poznan, Poland). The extracts were obtained in ethanol 96% using reflux and ultrasounds methods. Antioxidant activity of extracts was determined by two different techniques: *i*) the electron paramagnetic resonance spectroscopy (EPR) and *ii*) the DPPH free radical scavenging assay; the results were compared with antioxidant activity of synthetic butylated hydroxytoluene (BHT). The main polyphenols of extracts were estimated by liquid chromatography coupled with mass spectrometry, LC-MS/MS. Total phenols (TP) content was determined by Folin-Ciocalteu method (FCM). The amount of total flavonoids was quantified by colorimetric aluminum chloride assay.

2. MATERIALS AND METHODS

2.1. MATERIALS

Chemicals. Ethanol (S.C. Chemical Company S.R.L., Romania), Folin-Ciocalteu reagent, rutin monohydrate, gallic acid monohydrate, caffeic acid, chlorogenic acid, acid ferulic, catechin hydrate chloroform, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) (Sigma-Aldrich, Germany), sodium bicarbonate (Reactivul, Bucharest, Romania), aluminum chloride hexahydrate, methanol p.a., potassium acetate (Scharlau, Spain), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), quercetin dihydrate (Fluka, Buchs, Switzerland), salicin (Wako, Japan), 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\cdot}$), butylated-hydroxytoluene (BHT – Alfa Aesar, Germany).

Apparatus. Varian 310 – MS LC-MS/MS triple quadrupole mass spectrometer fitted with an electrospray ionization interface (ESI), Adani CMS 8400-EPR spectrometer provided with a H_{102} cylindrical resonant cavity, Shimadzu UV mini 1240 UV-Vis spectrophotometer; ultrasonic cleaning bath ELSA-MATNANTECH (60 Hz frequency, 750 W power).

2.2. EXTRACT PREPARATION

For each extract, 10 grams of grounded vegetable material and 100 ml 96% ethanol were extracted by reflux (3 h) and ultrasounds assisted extraction (1 hour), which can be considered as an efficient tool for improving the analytical performance of procedures employed in analytical chemistry and natural products chemistry [26, 31] and exhibited excellent repeatability and recoveries [31–32]. Extracts were filtered through filter paper and kept at 4°C till further analyses.

Extracts obtained from four vegetable samples using two extraction methods were labelled as follows: 1-R – Plafar reflux, 2-R – Fares reflux, 3-R – Larix reflux, 4-R – Belin reflux, 1-US – Plafar ultrasounds, 2-US – Fares ultrasounds, 3-US – Larix ultrasounds, 4-US – Belin ultrasounds extracts.

2.3. LC-MS ANALYSIS OF EXTRACTS

The characterization of polyphenols in alcoholic extracts was performed using liquid chromatography – mass spectrometry (LC-MS) method [33–35].

Standard mixture for LC-MS multicomponent analysis. Stock solutions of 0.1 mg/ml concentration in methanol for each standard (gallic acid, caffeic acid, chlorogenic acid, ferulic acid, salicin, (+)-catechin, quercetin, rutin) were prepared. 1 ml of each stock solution of standard were added in a volumetric flask and completed with methanol to 25 ml, thus obtaining a final concentration of 4 µg/ml for each component of the standard mixture.

LC analyses were acquired using an Alltech ALLTIMA C18 5U column (100 × 3.2 mm, 5 µm particle size). The mobile phase was methanol: bidistilled water = 70:30 (v/v), flow rate 0.6 ml/min in an isocratic elution, and injection volume was 20 µl.

The triple quadrupole mass spectrometer fitted with an electrospray ionization interface was operated in negative-ion mode (M-H)- which is more sensitive for identifying water soluble phenolics compounds. MS parameters were as follow: ion spray voltage –4500 V; nebulizer gas (nitrogen), 40 psi; dry gas (air), 19 psi at 250°C temperature, collision gas (argon) 1.5 mTorr pressure. Thus, deprotonated molecular ion obtained was selected by the first quadrupole. Into the second quadrupole, the deprotonated molecular ion was fragmented by collision with the inert gas (argon). Fragments were analyzed by the third quadrupole.

Triplicate injections were made for standard mixture and for each sample. Results were expressed as mg standard/100 g dry weight plant (dw).

2.4. ANTIOXIDANT ACTIVITY BY EPR SPECTROSCOPY ANALYSIS

Electron paramagnetic resonance spectroscopy (EPR) is the only technique capable of providing direct free radicals detection. The experimental variables of the method were studied and optimized.

It was used an internal reference standard of CaO: Mn^{2+} with a well known signal given by the hyperfine interaction of the nuclear spin $I = 5/2$ [36]. The internal standard reference was kept for all measurements in the same position with respect to the samples and to the resonant cavity. The EPR signal of the DPPH in solution is due to the hyperfine interaction of an unpaired electron with the two ^{14}N atoms, each with nuclear spin $I = 1$. The overall spectra are given by the overlapped of the two spectra and consist in five lines with the relative intensity 1:2:3:2:1 [37, 38].

The free radicals TEMPOL and DPPH were used in this study. Because in the experimental conditions TEMPOL was less reactive (data not shown), DPPH• was selected. To find the optimal concentration of the DPPH• in chloroform work solution we used two criteria: *i)* to have a constant EPR line-widths *i.e.,* at high concentration due to dipole-dipole interaction a broadening of the EPR line-widths is observed; *ii)* the best signal/noise ratio. From the experimental analysis (data not shown) of the EPR line-widths as a function of DPPH concentration, we have obtained 0.625 mM as the optimal bulk concentration of DPPH• in chloroform. The main reason for using chloroform as solvent for EPR studies is due to his week polar character. It is well know that solution with high degree of polarity absorbs the microwave waves and in consequence is difficult to be used in EPR spectroscopy.

The phenomena which appear in disorder system are very complex [39–41]. However, in this case, since the signal line-widths are constant, the experimental parameter used in the analysis was the amplitude of the EPR spectra. Furthermore, the same criteria was used to choose the EPR operation parameters and for all spectra the following set-up was used: 100 kHz, modulation amplitude 2 G, receiver gain 2×10^4 ms, centre field 3350 G, sweep width 100 G, sweep time 70 s, microwave power 10 mW.

2.4.1. Sample preparation

6 ml of each marigold homogenous ethanol extract (see extracts preparation) was left to evaporate at room temperature. The residue was extracted in 3 ml chloroform, filtrated and also left to evaporate at room temperature. The obtained residue was weighed (approximately 26 mg semi-solid extract) and re-dissolved in 1 ml chloroform right before the analysis.

2.4.2. Working procedure

A volume of 30 µl of DPPH• chloroform work solution was placed in a capillary tube and inserted in the resonant cavity to obtain the EPR spectra. Then, 30 µl of each marigold re-extract in chloroform were added in the capillary tube

over the DPPH• work solution. The EPR spectra was registered during 1 hour at different times (10 spectra/hour). The same procedure was applied to obtain the EPR spectra of 0.05 mg/ml BHT in chloroform solution as comparison sample.

2.4.3. Calculation

The EPR signal area (Fig. 1) is proportional to the number of the unpaired electron and from this, one can obtain the absolute concentration of the free radicals [42]. In this work, the absolute concentration of the DPPH• was obtained by normalizing the amplitude of the third DPPH• signal *i.e.*, the most intense one, to the average amplitude of the third and fourth lines of CaO: Mn^{2+} reference signal, as can see from Fig. 1. Figure 1 shows the EPR spectra of DPPH• blank and spectra of a mixture of DPPH• and marigold Larix ultrasounds extract (noted 3-US in the Fig. 1) after 1 hour.

The amplitude of the reference signal (noted A_{ref}) is given by equation (1):

$$A_{ref} = \frac{A_{III} + A_{IV}}{2}, \quad (1)$$

where A_{III} and A_{IV} are the amplitude of the third and respectively fourth line of the paramagnetic ion Mn^{2+} (Fig. 1).

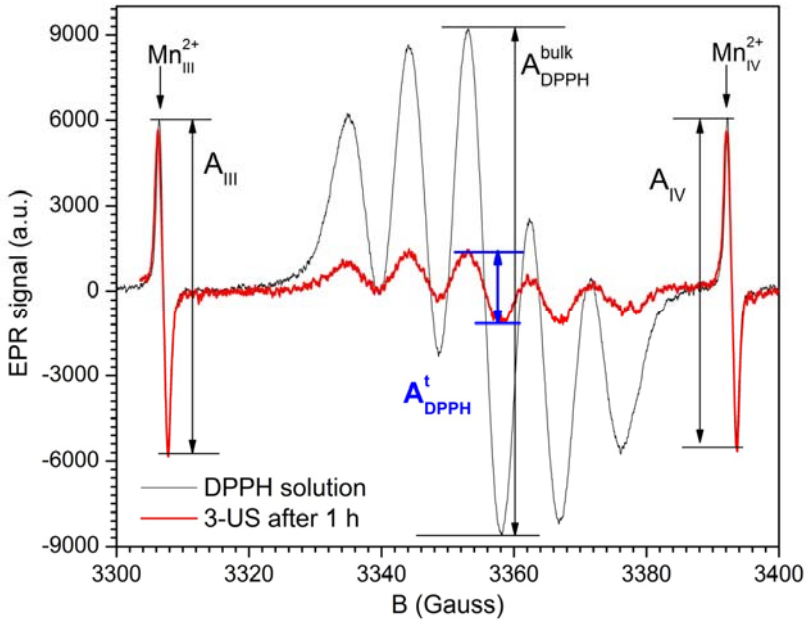

Fig. 1 – The EPR spectra of the bulk DPPH solution and a marigold extract after 1 hour (a.u. = arbitrary units).

For the amplitude of the DPPH• signal the most intense line of the EPR spectra was considered A_{DPPH} (see Fig. 1). It was calculated the percent of the DPPH• left in the sample according to equation (2):

$$\text{DPPH} = \left(\frac{A_{DPPH}^{bulk}}{A_{ref}^{bulk}} - \frac{A_{DPPH}^{t}}{A_{ref}^{t}} \right) \times 100, \quad (2)$$

where A_{DPPH}^{bulk} and A_{ref}^{bulk} are the amplitude of the signal of DPPH• solution and respectively reference EPR signal of bulk solution; A_{DPPH}^{t} and A_{ref}^{t} are the amplitude of the remained DPPH• signal and references EPR signal at each moment of time.

From this, it was obtained the percent of the DPPH• scavenging, which is proportional to antioxidant activity of marigold extracts.

2.4.4. DPPH free radical scavenging activity

DPPH• scavenging activity of plant extracts was made according to [43–46]. Thus, 500 µl standard/sample were mixed with 500 µl DPPH• solution (0.135 mM in ethanol 96%) and kept in dark (30 minutes). Absorbance measurements were done at the wavelength of 514 nm and as standards were used caffeic acid, chlorogenic acid and rutin. Calibration curves inhibition percent (% I) *vs*. standard concentration (mg/l) were drawn. Antioxidant activity of samples was expressed as mg standard equivalents/100g dw.

2.5. TOTAL PHENOLS AND FLAVONOID CONTENT DETERMINATIONS

Total phenols (TP) content was determined according to Folin-Ciocalteu method (FCM) [43, 47–49]. Absorbance was measured at λ = 761 nm and caffeic acid and chlorogenic acid were used as standards.

Total flavonoids content of extracts was measured calorimetrically using aluminium chloride method [43, 48, 50]. Absorbance was measured at λ = 425 nm and rutin was used as standard.

The calibration curves absorbance *vs*. concentration of standards (mg/l) were drawn. Results were expressed as mg standard equivalents/100 g dw.

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS ANALYSIS OF EXTRACTS

The results of the LC-MC/MS analysis are shown in Table 1. Of the used standards, caffeic acid, chlorogenic acid and rutin were found in all samples.

As one can see from Table 1, the concentration (mg/100 g dw) of these polyphenols varies between: 0.22 ± 0.02 – 1.1 ± 0.3 caffeic acid, 0.85 ± 0.03 – 9.0 ± 0.8 chlorogenic acid and, respectively, 0.27 ± 0.05 – 2.2 ± 0.6 rutin. Chlorogenic acid is found in highest concentration in all extracts, followed by rutin and caffeic acid. The same variation of compunds content was also reported in the literature for marigold extracts [4, 33, 51]. The other authors [52] reported following results by HPLC-UV method: 0.20–1.14 mg/g dw (20–114 mg/100 g) caffeic acid and 0.15–0.92 mg/g dw (15–92 mg/100 g) rutin for marigold extracts obtain in 60% ethanol, similar to our results. The reflux and ultrasounds extracts of marigold from Larix (3-R, 3-US, Table 1) have the highest amounts of determined hydroxycinnamic acids and rutin.

Of the other used standards, small amounts have been quantified only in some extracts, mentioned in Table 1.

Table 1

Quantification of the main phenolic compounds of ethanolic *Calendula* extracts

Extract	Quantity (mg/100g dw)				
	Caffeic acid	**Chlorogenic acid**	**Rutin**	**Gallic acid**	**Quercetin**
1-R	0.28 ± 0.003[a]	**0.85 ± 0.03**	0.69 ± 0.03	0.015 ± 0.001	nd[b]
1-US	0.62 ± 0.05	3.6 ± 0.4	0.81 ± 0.09	nd	0.046 ± 0.002
2-R	0.64 ± 0.02	1.6 ± 0.2	1.5 ± 0.07	nd	0.051 ± 0.002
2-US	**0.22 ± 0.02**	0.86 ± 0. 1	**0.27 ± 0.05**	nd	nd
3-R	**1.10 ± 0.3**	7.0 ± 0.7	**2.2 ± 0.6**	nd	nd
3-US	0.66 ± 0.02	**9.0 ± 0.8**	1.2 ± 0.3	nd	nd
4-R	0.29 ± 0.06	1.6 ± 0.2	0.47 ± 0.05	nd	nd
4-US	0.56 ± 0.04	4.9 ± 1.1	0.69 ± 0.10	0.049 ± 0.002	nd

[a] Results are expressed as mean ± standard deviation of three determinations.
[b] Nedetectable.

3.2. ANTIOXIDANT ACTIVITY OF EXTRACTS BY EPR SPECTROSCOPY

The decrease of DPPH• inhibition percentage in time as a result of the reaction with the marigold extracts is illustrated in Fig. 2. In the analysis of the antioxidant activity by EPR spectroscopy one must first consider the time dependence of the EPR spectra. The reaction is not an instant one and depends on the sample. Therefore, to analyse the antioxidant activity one must compare the results after a sufficiently long time in order to consider that the reaction is finished. After one hour the DPPH• concentration remain constant for all samples and in consequence the reaction was considered finished, moreover, any attempt to analyse the antioxidant activity for shorter time will be affected by the speed of the reaction, because each sample has a different time dependence of the reaction mechanisms.

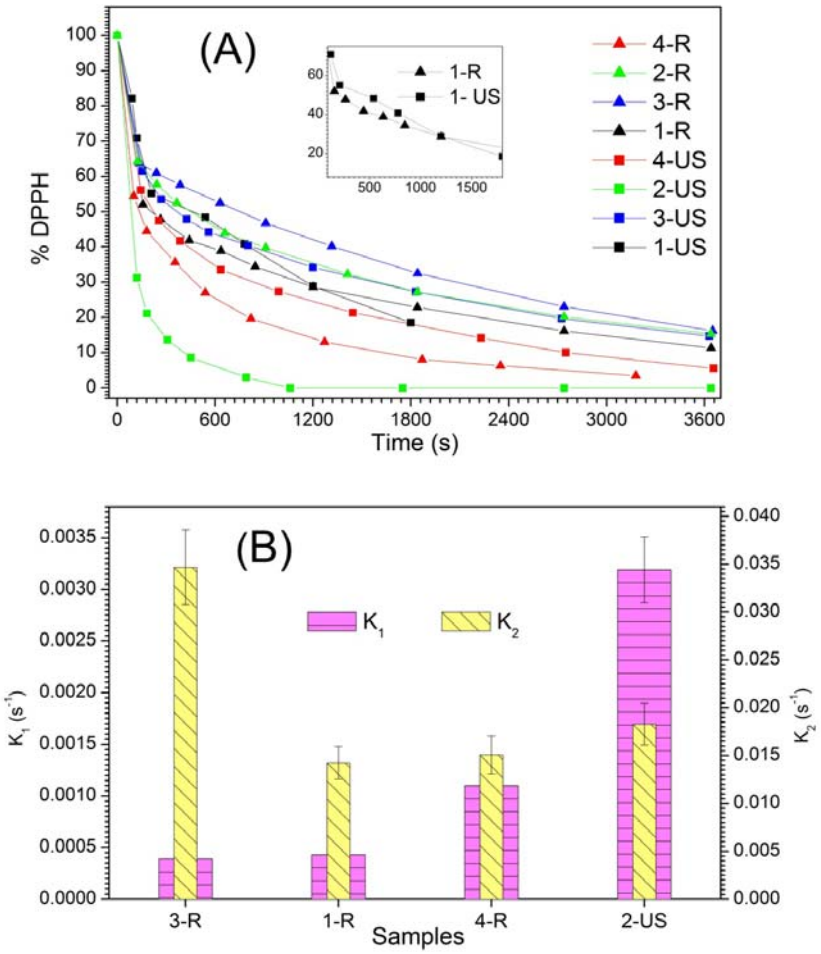

Fig. 2 – The time dependence of the percents of DPPH• scavenged for different samples. The EPR signal decay for different sample (A) and the characteristics reaction rates from some samples (B).

The percent of DPPH• scavenged by extracts determined using EPR spectroscopy after 1 hour varied between 81.5% and 97% (Fig. 2A). The highest antioxidant activity was obtained for the marigold ultrasounds Fares (2-US) and reflux Belin (4-R) extracts, with only 3% of the DPPH free radicals remained in the samples. In addition, it can be observed that, in the case of first mentioned extract, the reaction was ended after only 20 minutes, so 2-US extract had better antioxidant activity. The rate of reaction of other extracts is slower and comparable one to each other. The *Calendula* extract concentrations of 1.6 to 0.20 μg/ml induced a significant decrease in the lines intensity of the DPPH• spectrum with a reduction from 56.35 to 12.17% in their experimental conditions [38].

A slight increase in EPR spectra were observed for 1-R and 1-US marigold extracts in the time range between 540 and 780 s for ultrasounds extract and at 630 s for reflux extract (see insertion on Fig. 2A). This is a clear signature of an increase in the free radicals concentration. The same results are obtained with spectrophotometric methods by Romano *et al*. [53], for rosemary extract. This increase of the EPR signal could be explained by the production of some intermediary free radicals during the reaction between antioxidants from complexes samples (extracts) and DPPH•. However, all this issues are beyond the purpose of this work and need further investigation.

To analyze the time dependence of the concentration of the DPPH• scavenged it was considered a reaction mechanism described by the equation (3):

$$C = C_0 e^{-kt}, \tag{3}$$

where C is the concentration at moment t and C_0 is the initial concentration; k – is the reaction rate.

The best fit results were obtained by considering not one mechanism but two, with two different reaction rates. The analysis of the time dependent of the DPPH• concentration was described by equation (4):

$$C = C_1 e^{-k_1 t} + C_2 e^{-k_2 t}, \tag{4}$$

where C is the total concentration of the free radicals, C_1 and C_2 are the initial concentration and k_1 and k_2 are the reaction rate for the two mechanisms.

Figure 2B shows the results obtained for the representative samples: 3-R, 1-R, 4-R and 2-US. As one can see, the reaction rate are very different, K_1 describe the mechanism at long time, with a reaction rate between 3.90×10^{-4} s^{-1} and $3.19 \times \times 10^{-3}$ s^{-1} and K_2 gives the reaction rate al short time with a value of the order of 10^{-2} s^{-1}. In the same time, the ration between the concentration C_1 and C_2 is changed, from a ration of C_1/C_2=2.12 found for the 3-R sample the ration is changed to 1.02 for 1-R, at 0.93 for 4-R and 0.53 for 2-US.

According to Figs. 2A and 2B, the ultrasounds Fares (from 26 mg residue/ml chloroform) extracts was found to have the best antioxidant activity when DPPH• was neutralized after only 20 minutes.

In case of synthetic BHT (0.05 mg/ml chloroform) used for comparison, free radical was neutralized after only 2 minutes (Fig. 3), while Fares ultrasounds extract (noted 2-US) reacted much slowly, neutralizing 97% of the radical after one hour.

All extracts had a slower reaction rate than BHT, but after 60 minutes percent of DPPH• scavenging is over 80% (Fig. 2A), even if these contain only the chloroform soluble fraction.

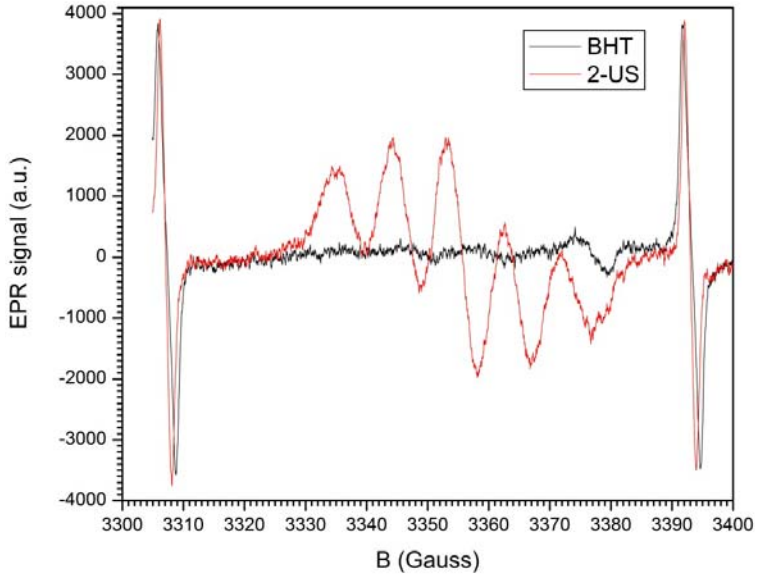

Fig. 3 – The EPR spectra of BHT and 2-US samples.

From the analysis of the EPR spectra was found the highest antioxidant activity for the marigold Fares ultrasounds (2-US) and Belin reflux (4-R) extracts.

3.3. DPPH FREE RADICAL SCAVENGING ACTIVITY OF EXTRACTS

The DPPH free radical scavenging activity of marigold flowers ethanolic extracts is illustrated in Fig. 4.

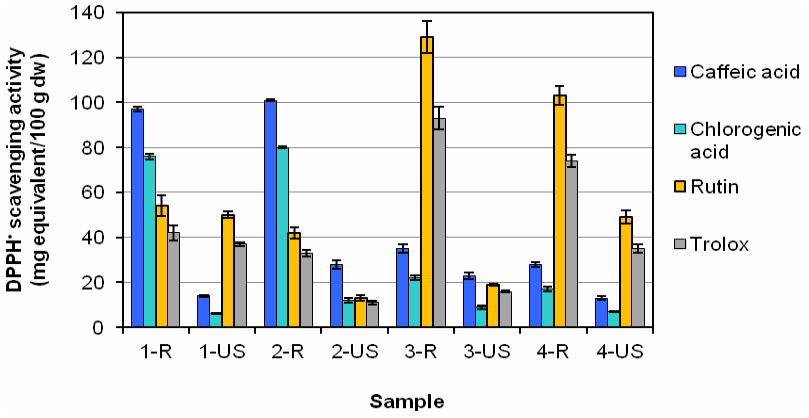

Fig. 4 – DPPH free radical scavenging activity of extracts.
Error bars represent ± SD (n = 3).

Antioxidant activity of analyzed alcoholic extracts (Fig. 4) expressed as mg equivalents/100 g dw varies between 13.0 ± 0.73 and 101 ± 0.27 caffeic acid; 6.00 ± 0.25 and 80.0 ± 0.32 chlorogenic acid; 13.0 ± 1.2 and 129 ± 7.1 rutin. Antioxidant activity expressed as Trolox equivalents reflect the contribution of both fat and hydro-soluble compounds at total antioxidant activity of extracts and was found between 11.0 ± 0.8 and 93.0 ± 5 mg equivalents/100 g dw.

As can be observed from Fig. 4 all reflux extracts have the higher antioxidant activity than ultrasounds extracts (excepting of Plafar marigold extract expressed as rutin equivalents).

Antioxidant activity of marigold studied extracts was compared with the one of the synthetic BHT. A solution of 5 mg/100 ml BHT leads to DPPH• inhibition percent of 52. 1 ± 1.5 while for the marigold reflux ethanolic extracts (1g dry plant/ 100 ml) the average of percent of DPPH• scavenged was 71%. In case of ultrasounds extracts approximately the same percentage was obtained for 2 g dry plant/100 ml solvent, showing that ultrasounds is less efficient as extraction method (Fig. 4).

3.4. POLYPHENOLS CONTENT DETERMINATION OF EXTRACTS

Experimental data of TP and flavonoids content are shown in Fig. 5. In ethanol marigold extracts total polyphenols (TP) content (mg standard equivalents/100 g dw) varied between 180 ± 0.80 ÷ 609 ± 8.6 caffeic acid and 327 ± 1.7 ÷ 1273 ± 18 chlorogenic acid, respectively. Of the analyzed extracts, Plafar marigold reflux extract has the highest TP amount.

TP values expressed in terms of equivalents of chlorogenic acid in all extracts are approximately twice higher than the ones expressed as caffeic acid equivalents. These results are in agreement with the ones obtained by LC/MS analysis, where concentrations of chlorogenic acid determined in extracts are higher than the ones of caffeic acid.

Values of flavonoids content were between 455 ± 29 ÷ 1350 ± 86 mg rutin equivalents/100 g dw (Fig. 5).

The polyphenols of the analyzed extracts were found in moderate amounts, aspect mentioned by some others [26, 27]. Similar results were reported by Cetkovic [31]: 14.49–55.07 mg chlorogenic acid/g extract (376–399 mg/100 g dw), respectively 5.26–18.48 mg rutin/g extract (136–509 mg/100 g dw). By stepwise injection analysis based on the chromogenic reaction between flavonoid and aluminium ions Falkova *et al*. [54] has determined 1.09 ± 0.03 % for a *Calendula* flowers extract.

As can be seen in Fig. 5, reflux proved to a better extraction method for polyphenols for marigold than ultrasounds assisted extraction, confirming the results obtain by spectrophotometric DPPH method.

4. CONCLUSIONS

Ethanol extracts of marigold flowers from the Romanian market were obtained by reflux and ultrasounds methods and have been characterized for their antioxidant activity and polyphenols content. The experimental results obtained by LC-MS/MS and spectrophotometric methods showed that marigold extracts contain especially hydroxycinnamic acids and rutin.

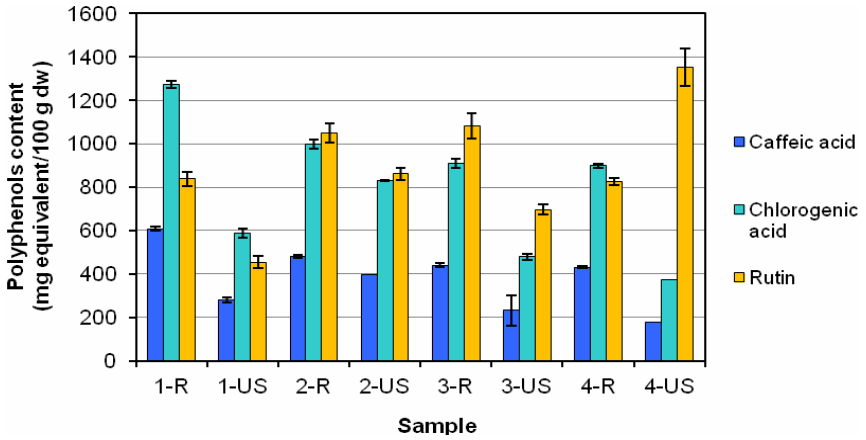

Fig. 5 – Total phenols and flavonoids content of extracts. Error bars represent ± SD (n = 3).

Antioxidant activity was determined by electron paramagnetic resonance spectroscopy optimized method and spectrophotometric method using DPPH free radical. In case of EPR method, the percent of the DPPH$^{\bullet}$ scavenged by the chloroform re-extracts after one hour (when EPR spectra had constant amplitude) was calculated. From the variation in time of the EPR signal was observed a mechanism by which the free radicals are neutralized in two steps, with two different reaction rate and the reaction constant for each of them was calculated. Furthermore, it was observed at some samples a slight increase in the EPR signal, a clear signature of an increase in the free radicals concentration, probably due to the presence of some intermediate products. Further studies must be done to identify and establish the pathway by which DPPH$^{\bullet}$ and extracts reacts.

Percentage of DPPH• scavenged determined by spectrophotometric method were higher than the ones obtained by EPR, probably because in the last case analyzed re-extracts in chloroform contain only the fat-soluble fraction of the extracts. Antioxidant activity of ethanol extracts is tenfold higher than that of a 0.05 mg/ml BHT solution.

The results of the study suggest that marigold plant is a source of strong antioxidants and a potential replacement for the synthetic antioxidants, used today especially in food, drugs and cosmetics industry.

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