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Antioxidant activity of some celandine (*Chelidonium majus L.*) carotenoidic extracts

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Abstract

The paper presents the antioxidant activity determination of some celandine (*Chelidonium majus L.*) carotenoidic extracts, using a method with 2,2-diphenyl-1-picrylhidrazyl (DPPH) free radical and the results correlation with the carotenoids content. The carotenoidic extracts obtained from celandine leaves, flowers and stem was analysed by reverse phase – high performance liquid chromatography (RP-HPLC), in view of β-carotene and total carotenoids content determination, using an Agilent 1100 RP-HPLC system with a Zorbax SB-C18 column. The highest carotenoids content was found in the celandine flowers 1377.70 μg/g, followed by the stem (1041.11 μg/g) and leaves (1007.06 μg/g). Celandine flowers also contain the greatest β-carotene amounts (369.50 μg/g), the smallest content being in stem (53.30 μg/g). The obtained data concerning the carotenoidic extracts antioxidant activity was compared with those of the ascorbic acid at different concentration (0.1 mM, 0.01 mM and 0.001 mM) , as antioxidant standard. The best antioxidant activity presents the celandine flowers extract and the smallest, the stem extract, the results being propotionally with the extracts carotenoids concentration.

Keywords: carrotenoids, antioxidant activity, celandine, 2,2-diphenil-1-picrylhidrazil, HPLC chromatography

1. Introduction

Antioxidants are defined as (Halliwell, 1990): any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. An oxidizable substrate might be a lipid, DNA, protein or any molecule found *in vivo* (Nälsén, 2006).

Since 1981, when Peto et al. (1981) raised the idea dietary □-carotene from fruits and vegetables as a protective agent against cancer, carotenoids have received wideresearch interest as potential antioxidants. According to Omaye et al. (1997), much of the evidence has supported the hypothesis that lipid oxidation or oxidative stress may be the underlying mechanism in chronic diseases and that □-carotene would act as an antioxidant *in vivo*. Furthermore, Burton and Ingold suggested in their pioneering work that □-carotene would be an unsual type of lipid antioxidant working at low oxygen concentrations (Burton & Ingold, 1984). In all, carotenoids have been considered as antioxidants, rather than pro-oxidants based on experimental evidence *in vitro* (Astorg, 1997; Burton, 1989; Edge et al. 1997; Krinsky, 1989; Krinsky, 1993). It is known that carotenoids may act as antioxidants by quenching singlet oxygen or by reacting with free radicals. Moreover, the

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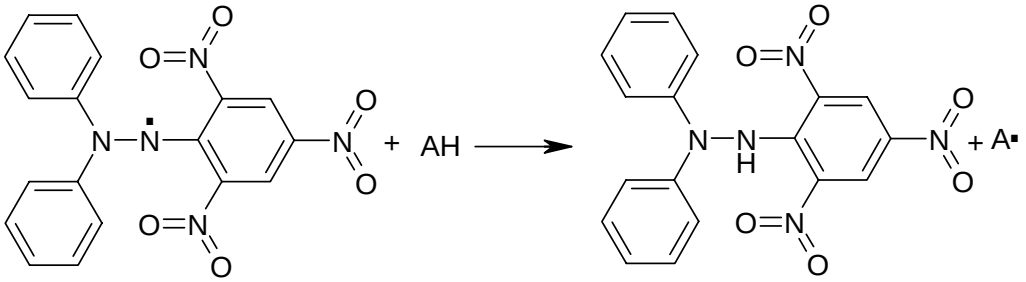
antioxidant/pro-oxidant properties of carotenoids are affected by the concentration of carotenoid, oxygen partial pressure and the nature of the environment. The mechanisms of reactions between carotenoids and radical species may involve radical addition, hydrogen abstraction and electron transfer, but the precise antioxidant/pro-oxidant mechanisms remain unclear (Edge et al. 1997; Britton 1995; Tsuchihashi et al. 1995; Leibler & McClure, 1996). Further chemically based understanding on antioxidant/pro-oxidant action of carotenoids is necessary.

Several methods have been developed during last decade to measure the antioxidant capacity of plasma, serum, and of other biological samples and in foods (Wayner et al., 1985; Whitehead et al., 1992; Cao et al., 1993; Miller et al., 1993; Benzie & Strain, 1996; Prior et al., 2005). Most of these methods measure the extent to which free radical generation is inhibited by antioxidants present in the sample. The methods are based on different technologies using different free radical generators, target molecules and endpoints. Therefore it is possible that the response of various antioxidants will depend on the particular assay used. The relative advantages and disadvantages of the various antioxidant capacity assays vary according to the situation and the type of study. However, opinions regarding various antioxidant capacity assays vary considerably, probably depending on the complexity of antioxidants. Recently, it was proposed that the procedures and applications of the oxygen radical absorbance capacity (ORAC) assay, and possibly those of the Trolox equivalent antioxidant capacity (TEAC) assay, should be standardized (Prior et al., 2005).

DPPH• is one of a few stable and commercially available organic nitrogen radicals bearing no similarity to the highly reactive and transient peroxyl radicals involved in various oxidative reactions in vivo (Huang et al., 2005; Wu, 2004). This assay is based on the measurement of the reducing ability of antioxidants toward DPPH•. The ability can be evaluated by electron spin resonance or by measuring the decrease of its absorbance. The measurement of the loss of DPPH color at 517 nm following the reaction with test compounds is what the antioxidant assays are based on (Prior et al., 2005). In the reduction moment, DPPH• solution are discoloured from purple to yellow, the DPPH• free electron pairing with the hydrogen from an antioxidant, with reduced DPPH-H formation:



DPPH• Antioxidant DPPH-H

Using the DPPH assay is simple, rapid and needs only a UV/VIS spectrophotometer to carry out (Prior et al., 2005).

Greater celandine (*Chelidonium majus L.*), *Papaveraceae* family, contains alkaloids, carotenoids, chelidonic acid and other plant acids, such as malic and citric acids, flavonoids, a saponin, and other ubiquitous substances. For centuries, celandine has been used as a pain reliever, a cough suppressant, antitoxin, and anti-inflammatory drug in Chinese medicine. The fresh, bright yellow-orange stem latex was once a popular folk medicine treatment for warts, eczema, ringworm and corns, due, especially, to their carotenoids content. Traditionally, celandine was also used as a remedy for jaundice, scurvy, scrofula, gout, toothache, peptic ulcers, piles, and most

notably as a topical to treat abnormal growths (Taborska et al., 1995; Foster & Duke, 1990).

The scope of this study is to obtain some carotenoidic extracts from celandine flowers, leafs and stem, determination of β-carotene and total carotenoids content from this by RP-HPLC, evaluation of the antioxidant capacity using the DPPH method and comparing the obtained results with those for different ascorbic acid solutions, as standard antioxidant.

2. Materials and method

Reagents and materials. As raw material for carotenoids extraction was used celandine fresh plants from Didactic Station of the Agricultural Science and Veterinary Medicine Banat's University Timisoara. The extraction solvents: ethanol (96%), petroleum ether and acetone (analitical purity) was purchased from Merck company.

Butylhydroxytoluene (BHT) used for carotenoids oxidative degradation prevention was from Merck company like the potassium hydroxide used for saponification process. The solvents for RP-HPLC analysis: acetonitrile and methanol were from Merck & Co., Inc., New Jersey, and the β-carotene standard (>97% purity), proceeding from Sigma Chemical Company. The free radical DPPH• used for antioxidant capacity determination was from MP Biomedicals, LLC-Germany and the standard ascorbic acid from Merck company.

*Apparatus.*The laboratory apparatus used was:

- rotative evaporator model RV-05 basic 1-B (Shimadzu, Japan);
- digital analytical balance model AW 320 (Shimadzu, Japan);
- HPLC system Agilent 1100 (Agilent, USA).
- spectrophotometer Perkin Elmer, Lambda EZ Series, the obtained data being processed with PESSW program, 1.2. version, Revision E.

Obtaining of the carotenoidic extracts. For the carotenoidic extracts obtaining, the fresh vegetal material (celandine flowers, leaves and respectivelly, stem) was fine cutted, weighed and then triturated with quartz sand and acetone. The carotenoids extraction was achieved with a solvents mixture of petroleum ether: ethanol (96%): acetone (8:1:1, v:v:v), in which was aded 0,1% BHT (reported to the raw material) for carotenoids oxidative degradation prevention. The extraction was repeated for many times with fresh solvents mixture, to colourless. After concentration under vacuum at 35 ºC, the extracts were submitted to saponification process with 25 ml of 20% ethanolic KOH, 12 hours, at room temperature, under nitrogen atmosphere, in dark (Rodriquez- Amaya & Kimura, 2004; Rodriguez Amaya, 2001). For removal of soaps, alkalies and chlorophylls, the solution was washed many times with a sodium chloride saturated solution and then with distilled water. The organic layer was dried over anhydrous sodium sulphate, evaporated to dryness and then weighed and dissolved in petroleum ether. The samples were kept under nitrogen at -20 ºC until further utilization and were filtered through 0.45 mm Whatman filters prior HPLC analysis (Britton et al., 1995). From every raw material were achieved three samples in the same conditions.

Raw material and final extracts quantities (average values) are presented in table 1.

Table 1. Raw material and final extracts quantities

Raw material	$m_{\substack{vegetal \\ material}}$ (g)	$m_{extract}$ (g)
Celandine flowers	132.00	0.2547±0.0101
Celandine leaves	125.00	0.1704±0.0097
Celandine stem	180.00	0.2948±0.0103

RP-HPLC analysis of carotenoidic extracts. The carotenoidic extracts obtained were submitted to reverse phase high performance liquid chromatography (RP-HPLC) analysis in view of □-carotene concentration determination. The HPLC system Agilent 1100 was equiped with a Zorbax SB-C18 column, 250 x 4,6 mm and particles size of 5 □m. As mobile phase was used a mixture of acetonitrile: methanol (20: 80 v/v), with 1 ml/min eluent flow, a temperature of 30ºC and the wavelength of 450 nm. Were injected samples of 20 □l and for □-carotene concentration determination was used a standard curve obtained with pure □-carotene.

Antioxidant capacity determination. From each carotenoidic extract the samples were diluted with petroleum ether (1:100). In a test tube were introduced 0.3 ml diluted sample, 2.6 ml petroleum ether and then 0.3 ml DPPH 1mM solution (in petroleum ether). For this blend was recorded the absorbance at 517 nm and the time variation of this. The ascorbic acid was evaluated at final concentrations of 0.001 mM, 0.01 mM, and 0.1 mM in ethanol 96%. For determination of the DPPH concentration variation in the standard antioxidant (ascorbic acid) or extracts presence, in the aim to average reaction speeds determination, was obtained the DPPH calibration curve. Also, for sample antioxidant activity comparison, was calculated and recorded the relative absorbance (*A*%) as report between the t time absorbance at 517 nm and the initial absorbance (t=0). As the relative absorbance is lower, the antioxidant activity of the sample is better.

3. Rusults and discussion

β-Carotene and total carotenoids RP-HPLC data. Standard curve obtained with pure □-carotene conduct to the following calibration equation:

$$Area = 7.8 + 862.7 \cdot c\,; \quad r = 0.969$$

where: -*Area* represents the peak area expressed as mAU·min,

-*c* is the β-carotene concentration in g/100 ml,

-*r* is the correlation coefficient.

The RP-HPLC superposed chromatograms obtained for standard □-carotene are presented in figure 1.

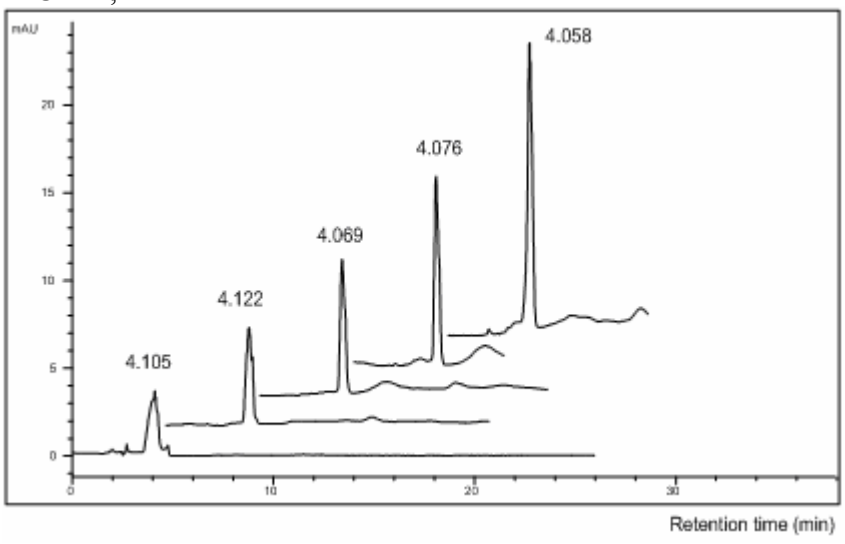


Fig.1. RP-HPLC superposed chromatograms for standard β-carotene

β-Carotene was identified in all celandine carotenoidic extracts (figures 2). The chromatographic peaks area and the β-carotene concentration in the raw material are presented in tables 2.

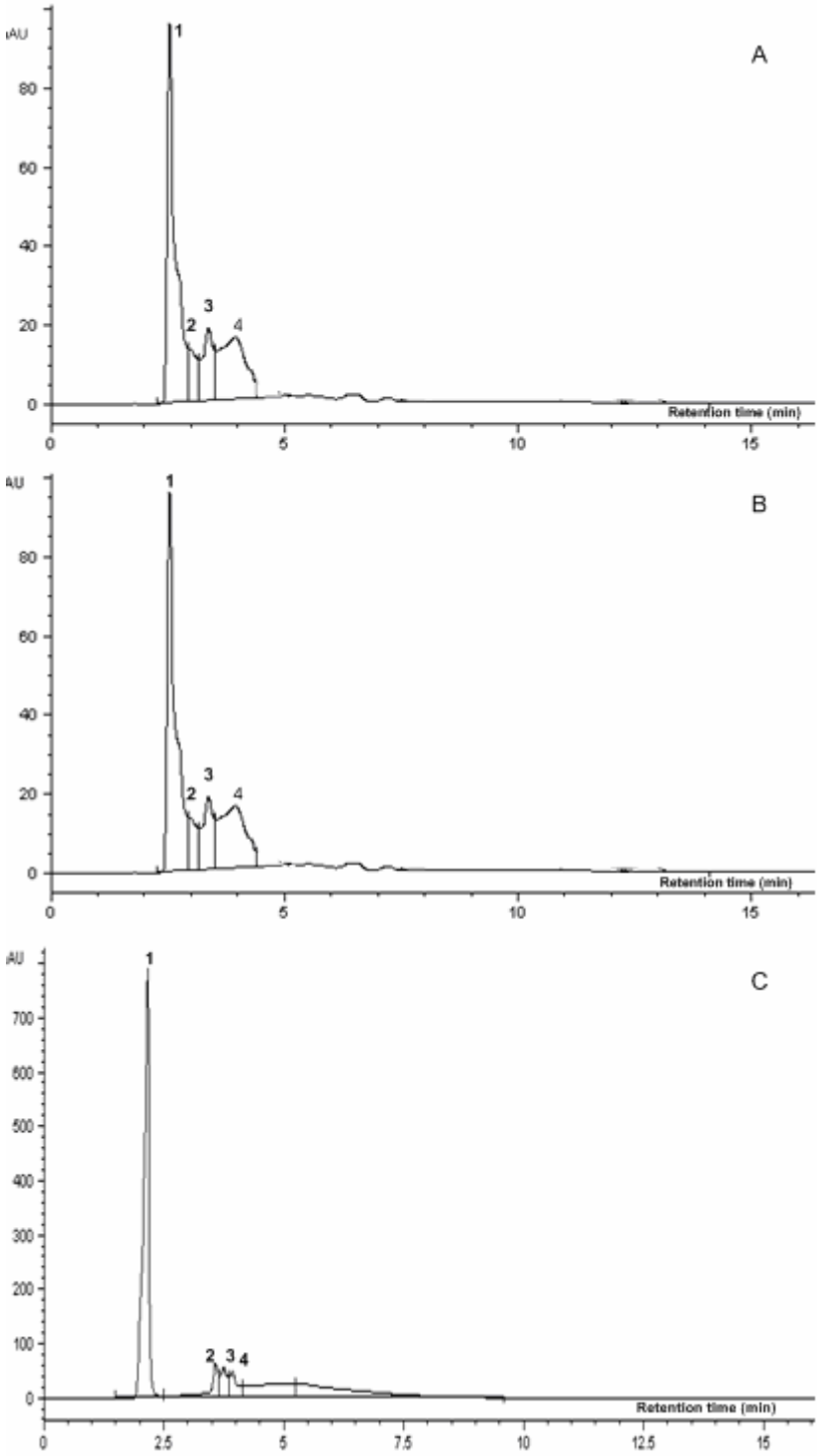


Fig. 2. RP-HPLC chromatograme for celandine flowers (A), leaves (B) and stem (C) carotenoidic extracts

Table 2.–Chromatographic peaks area and β -carotene concentration in the raw material for celandine flowers, leaves and stem

Peak no.	Retention time (min)	Area (mAU)	A%	Compound identified with standard	Content in the raw material (µg/g)
Celandine flowers carotenoidic extract					
1	2.514	1205.10	52..84	-	727.98
2	2.91	159.30	6.98	-	96.16
3	3.365	304.60	13.36	-	184.06
4	3.925	611.70	26.82	β-carotene	369.50
Total carotenoids					1377.70
Carotenoids concentration (%) in extract flowers					*71.72%±0.72%*
Celandine leaves carotenoidic extract					
1	2.146	80.10	9.64	-	97.07
2	2.481	585.50	70.45	-	709.42
3	3.363	46.60	5.61	-	56.49
4	3.884	118.90	14.31	β-carotene	144.10
Total carotenoids					1007.06
Carotenoids concentration (%) in extract leaves					*73.87%±0.72%*
Celandine stem carotenoidic extract					
1	2.15	792.25	82.27	-	856.44
2	3.561	63.18	6.56	-	68.29
3	3.737	58.31	6.06	-	63.08
4	3.918	49.28	5.12	β-carotene	53.30
Total carotenoids					1041.11
Carotenoids concentration (%) in extract stem					63.57%±0.65%

It could be observed that the highest total carotenoids content are present in celandine flowers (1377.70 µg/g), then in stem (1041.11 µg/g) and in leaves (1007.06 µg/g). Celandine flowers also contain the greatest β-caroten concentration (369.50 µg/g), followed by the leaves (144.10 µg/g) and stem (53.30 µg/g).

Antioxidant activity results. On basis of the DPPH absorbtion VIS spectra was obtained the DPPH calibration curve *Absorbance* (517 nm) = *f* (*c*, mM) with the following calibration equation:

$$Abs = 0.025+10.96 \cdot c$$

Analysing the time variation of DPPH solution relative absorbance (A%) in the presence of ascorbic acid solutions it could be observed that very good antioxidant activity showed the samples with ascorbic acid solution of 0.1 mM (A%= 3%) and 0.01 mM (A%= 5.5%); the sample with ascorbic acid solution of 0.001 mM presents a much lower antioxidant activity (A%= 76%).

Evaluation of the ascorbic acid samples antioxidant activity conducted to average reaction speeds (calculated on the curve cvasilinear part, as the differential of concentration linerar dependence function of reaction time: *c* (mM) = *f* (*t*, s)) between 1.1 and 3.6 µM/s. Thus, in the case of 0.1 mM ascorbic acid solution, the average reaction speed was of 3.6 µM/s, for the 0.01 mM solution this was of 2.5 µM/s, and for the smallest concentration ascorbic acid solution (0.001 mM) the average reaction speed was of 1.1 µM/s (figure 3).

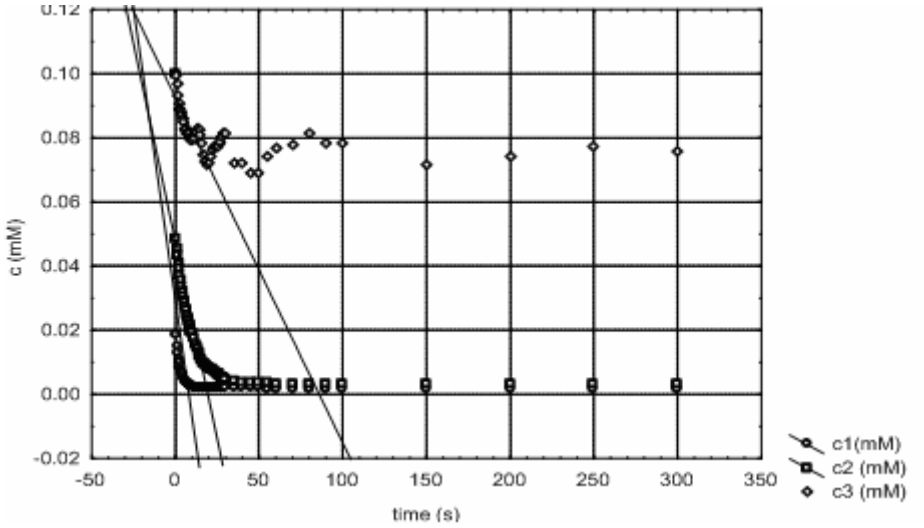


Fig. 3.- DPPH concentration time variation in the presence of the ascorbic acid solutions

Spectrophotometric analysis of celandine extracts at 517 nm (figure 4) showed the best antioxidant activity for leaves, respectively, flowers carotenoidic extracts (relative absorbance A%=36% in both cases). For celandine stem carotenoidic extract was obtained a relative absorbance value A%=50%, that means a smaller antioxidant activity.

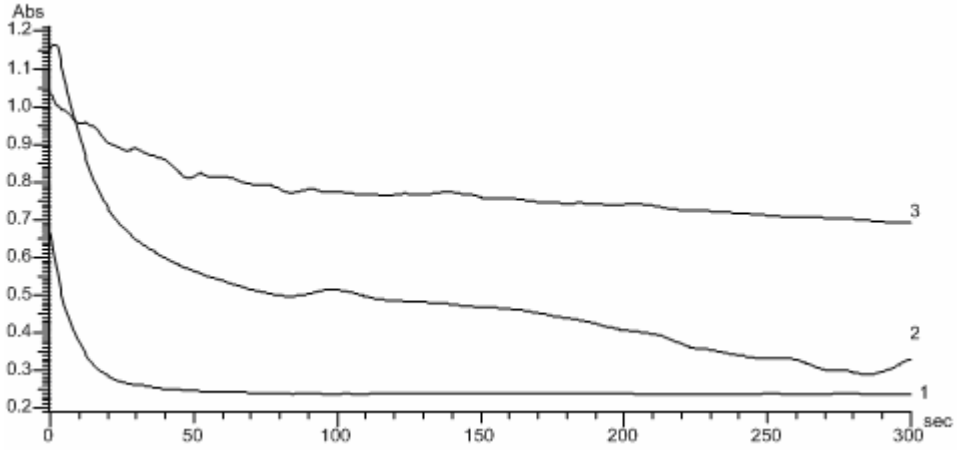


Fig. 4. – Time variation of the DPPH solution absorbance at 517 nm, in the presence of the carotenoidic extracts (1- leaf, 2- flower, 3- stem)

The average reaction speeds determination for the quasi-linear c (μM) = f (t, s) curve region (figure 5.), showed a maximum value for the celandine leaves extract (2.6 μM/s), greater than that for the 0.01 mM ascorbic acid solution, followed by the mean reaction speed of the flowers extract (2.2 μM/s), higher than that of the 0.001 mM ascorbic acid solution. The smallest reaction speed was registered in the case of the celandine stem extract (0.7 μM). A little less high value of the average reaction speed for the leaves extract than that for the flowers one, are due to the total carotenoids concentration that are greater in the celandine leaves sample (7.387±0.072 mg/ml) than that in the flowers sample (7.172±0.072 mg/ml), even if the β-carotene is present in a higher amount in the flowers sample (1.923±0.019 mg/ml)

than in the leaves one (1.057±0.010 mg/ml) ; in the case of celandine stem sample the total carotenoids and β-carotene concentrations are smallest (6.357±0.065 mg/ml, respectively 0.325±0.003 mg/ml). Thus, it could be concluded that the antioxidant activity of carotenoidic extracts from different celandine anatomical parts are direct proportionally with the sample total carotenoids concentration value.

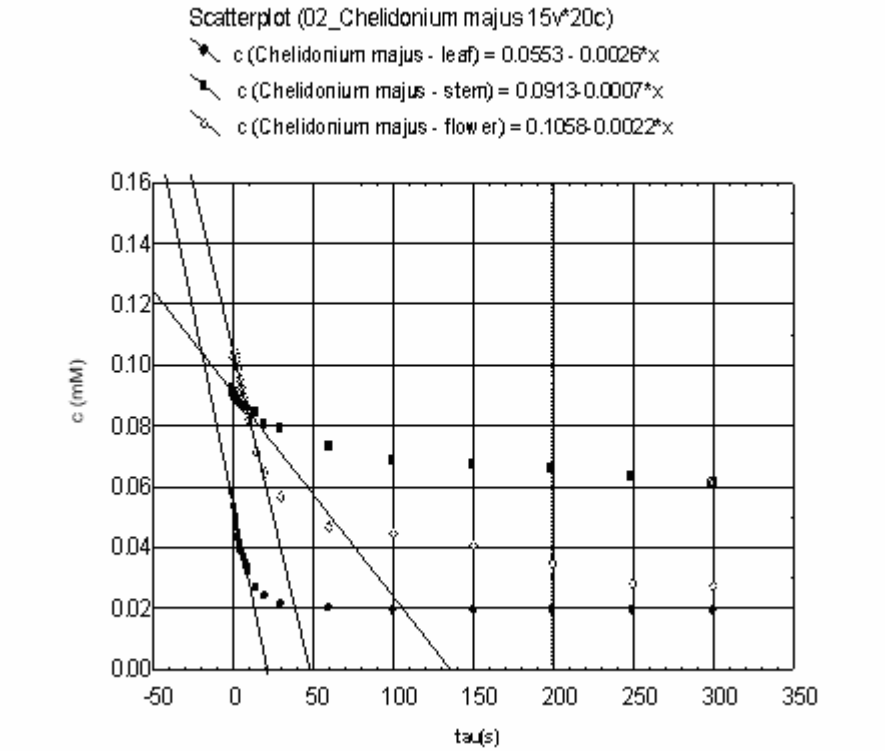


Fig.5. Time reaction (s) -DPPH concentration (mM) dependence in the reaction with celandine carotenoidic extracts

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