

Free Radical Scavenging Properties and LC/MS Analysis of Bulgarian Crataegus Oxycantha Fruits Ethanol Extract

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Citation

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Abstract

Investigations on procyanidin profile on Bulgarian Crataegus oxycantha fruits extract has been carried out by different analytical approaches based on chromatographic methods. We retained of interest to test the validity of direct infusion of Crataegus extract in ESI conditions to achieve an immediate fingerprinting of the procyanidins therein contained. The results are compared with those obtained by classical HPLC/MS approach.

The antioxidant properties of Crataegus extract on iron-dependant lipid peroxidation and on UV irradiation induced deoxyribose oxidation were investigated. On the basis of the experimental data C-50 value was calculated: 0.70 ± 0.02 mg/ml and 0.26 ± 0.01 mg/ml for LP and UV irradiation systems respectively. The LP measured for the same model system but in the presence of six of the main biologically active Crataegus fruits extract components proved by GC/MS analysis.

We identified polyphenols in this extract and we proved the antioxidant effect in two biologically relevant systems.

INTRODUCTION

Crataegus oxycantha (Cr. oxycantha) is used for treatment of cardiovascular diseases such as hypertension, hyperlipidemia, and in particular congestive heart failure, cancer, diabetes and sexual weakness in Arabic traditional medicine. Hawthorn may induce anti-ischemia/reperfusion-injury and anti-arrhythmic effects ^{1,2,3,4}.

The observed effects may be due to inhibition of free radicals generation as well as suppression growth of free radicals damage of biologically important molecules. There are investigations on antioxidant properties of hawthorn on in vitro systems. Investigations on medical effects of leaves, fruits, green fruits and flowers of many Crataegus species extracted by different methods showed antioxidant effects. It was established that Cr. monogynya, Cr. pinnatifida and Cr. aronia inhibits metal-induced lipid peroxidation ^{5,6,7,8,9}. These beneficial effects may in part be due to the presence of antioxidant flavonoid components ².

In the last decade new and interesting information on the content of polar components of natural extracts have been gained by the coupling of LC and MS technique mainly based on APC and electro spray ionization (ESI) systems. The recent availability of these ionization methods coupled with simple and effective MS/MS systems has suggested the possibility of direct infusion of the natural extract and the

characterization of the different components by MS/MS. Of course this approach fails in the presence of isometric compounds and in general exhibits a specificity lower than that of LC-MS and LC-MS/MS systems. However, considering the time saving aspect of this approach, it can be thought of interest for a fast and preliminary description of natural extract mixtures.

The present study is devoted to the identification of flavonoid and procyanidin profile from Bulgarian Crataegus oxycantha fruit ethanol extract and the antioxidant properties of this extract on iron-dependant LP and on UV irradiation induced oxidation. Both LC- and direct injection – MS/MS approaches were employed in order to evaluate the power of the former in this specific frame.

MATERIAL AND METHODS

PREPARATION OF THE ETHANOL EXTRACT

Fresh fruits from Bulgarian Crataegus oxycantha (Rosaceae) were extracted with 95 % ethanol and were distilled at vacuum rotating evaporator. The yielded viscous residues were analyzed.

COMPOUNDS ANALYSIS

Instrumentation: LCQ DECA Thermoquest

ESI Parameters: Negative ion mode; Mass range: 50 – 1500;

Spray voltage: 4 kV; Capillary temperature: 270 °C;
Capillary voltage: -8 V

HPLC

Column: Luna C18 150 x 4.5 mm; guard column: C18;
injection volume: 20 µl; solvents

A = H₂O + 0.05 % acetic acid (HAC) and B = MeOH.

Gradient profile of solvent B: 10 % for 5 min, 10 – 100% in 55 min; flow rate: 0.5 ml/min.

DIRECT INFUSION

5 µl/min; solvent H₂O : MeOH 50%

Registration of tiobarbituric acid reactive species (TBA-RS) induced by Fe²⁺.

The TBA-RS of LP was measured in liposomal suspension obtained from phospholipids of egg yolk extracted according to Folch et al.¹⁰. Each sample was prepared in PBS: 1 mg lipid/ml and diluted substances or a buffer for the controls. After addition of 0.1 mmol/l FeCl₂ samples was incubated at 37 °C for 30 min. The 0.5 ml of 2.8% trichloroacetic acid and 0.5 ml of 0.5 % TBA were added. The solution was heated at 100 °C for 20 min. The absorption was measured at 532 nm.

DEOXYRIBOSE ASSAY

The deoxyribose assay was carried out as Halliwell et al.¹¹ with small modifications. One ml samples of PBS, pH 7.4 containing: 0.3 mmol/l 2-deoxy-D-ribose and tested drug at concentrations between 1 and 100 µmol/l. or a buffer for the controls. After 25 min of UV irradiation (UV 220-400 nm) 0.5 ml of 2.8% trichloroacetic acid and 0.5 ml of 1 % TBA were added. The solution was heated at 100 C for 20 min. The chromophore absorption was measured at 532 nm.

The antioxidant activity (AOA) was calculated by:

Figure 1

$$AOA = \frac{A_0 - A}{A_0} \cdot 100\%$$

A - absorption at 532 nm for control and A - absorption at 532 nm for substances.

Calculation of C-50

The value concentration that provide AOA = 50 % was termed C-50. C-50 was calculated by:

Figure 2

$$AOA = 100 / [1 + 10^{B(\lg C - \lg(C-50))}]$$

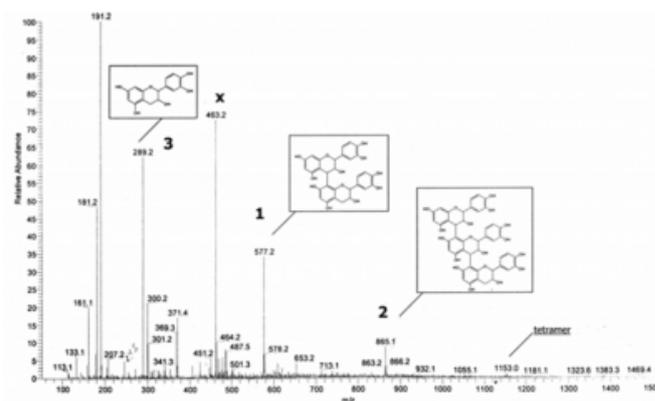
The calculations used fitting of the data to the “sigmoid” model, where B is the coefficient (hill slope) and C is the substance concentration¹².

RESULTS AND DISCUSSION

The TIC LC-MS chromatograms of the natural extract is reported in Fig. 1. The system has been set to direct negative ions and in these conditions some of the most abundant peaks are due to procyanidins and to anthocyanidin with [M-H]⁻ at m/z 463. This has been verifying by reconstructed ion chromatograms of components related to [M-H]⁻ species (peaks 1, 2, 3 and x of Figs 1 and 2). Their ESI spectra do not show the deprotonated molecules only but also a series of fragment peaks which can be well related to the original structure (Fig. 2).

Figure 3

Figure 1: TIC LC-MS chromatogram of the *Crataegus oxycantha* fruits ethanol extract.

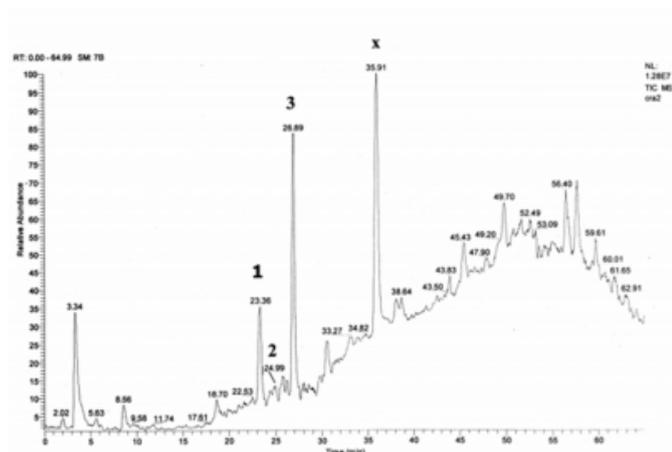


Interestingly the same fragments are observed by MS/MS of the [M-H]⁻ species. The most abundant peak detected in the LC run (peak x) can be attributed to the glucoside of peonidine. His spectrum showed an abundant [M-H]⁻ at m/z 463 and a fragment a at m/z 301. The loss of a species with mass 162, due to the glucose, is a typical behavior of anthocyanidins. MS² and MS³ spectra of the species at m/z 463 and m/z 301 confirm the structure of x (Scheme 1). The fragmentation of the ion a at m/z 301 lead to the formation of the daughter ions d at m/z 179 (100%), originating from

the loss of ring B (3'-methoxy-4'-hydroxyphenyl) and e at m/z 151 (90%), formed from the loss of ring B and CO. Other relevant daughter ions originate by the loss of formaldehyde (b m/z 271) and by the loss of water (c m/z 283).

Figure 4

Figure 2: Direct infusion ESI TIC MS of the *Crataegus oxycantha* fruits ethanol extract.



With the same molecular and fragment ions, m/z 463 and 301, could be a glucoside flavonoid of quercetin type, but it is easy to distinguish from isobaric anthocyanidins by their IR spectrum ¹³.

The natural extract has been injected by direct infusion leading the TIC MS in Fig. 2. Worth nothing is the fact that, aside the ion at m/z 191, which do not belong to the anthocyanidin class, the most abundant anions are due to the glucoside of peonidine and to procyanidins (monomer, dimer, trimer and, tetramer). Their nature has been confirmed by comparing their MS/MS spectra with those of the $[M-H]^-$ species of the peaks detected in LC-MS conditions. Interestingly the same trend of relative abundance is observed by measuring the relative abundance of the three mass spectrometric peaks (Fig. 2) and the relative areas of the three chromatographic peaks (see Table 1). This result is not surprising considering that both the data originate from the same ionization mechanism, i.e. procyanidin deprotonation, and put in evidence the validity of direct infusion spectrum for a semiquantitative evaluation of the main procyanidin components present in the natural extract. In the same table the times requested for the two analytical approaches are reported, making evident the advantage the direct infusion.

Figure 5

Schema 1: Scheme of fragmentation.

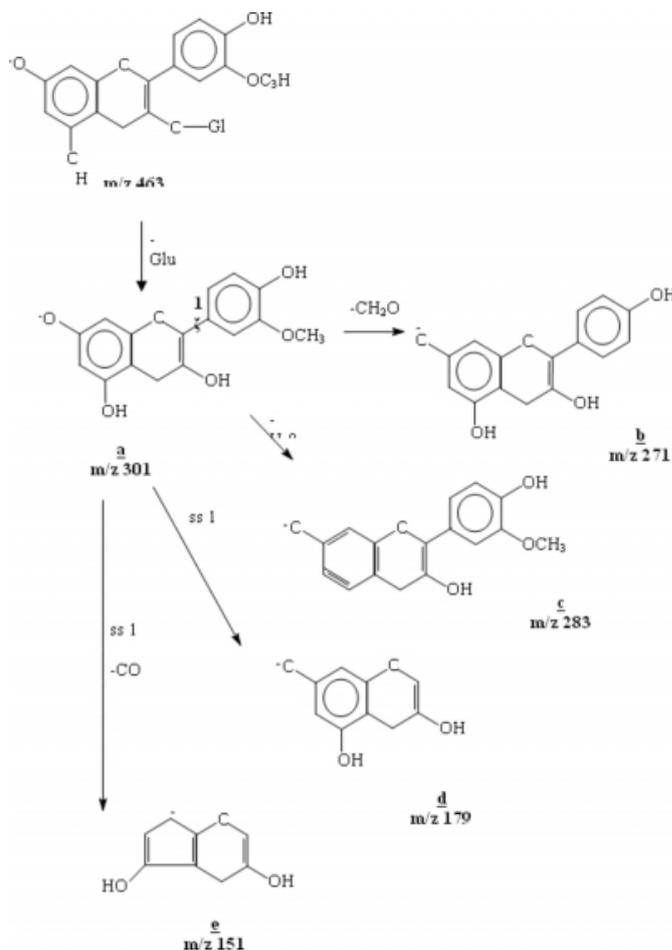


Figure 6

Table 1: Relative areas of the three procyanidins chromatographic peaks.

m/z	Relative percentage	
	Direct infusion	HPLC
	Analysis time: 5 min	Analysis time: 60 min
289	59 %	66 %
577	32 %	29 %
865	8 %	5 %

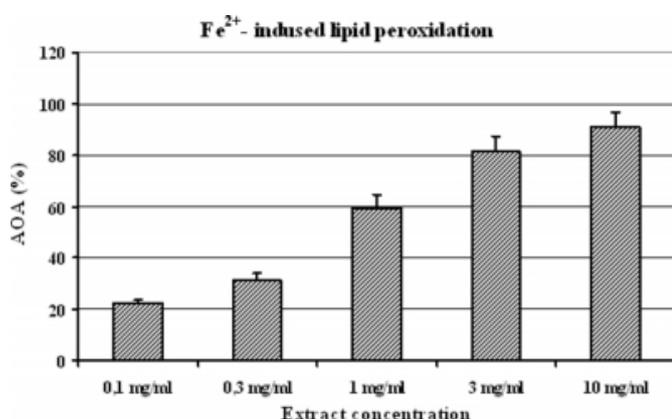
It is consider the presence of these anthocyanidins and procyanidins leads to the elucidation of cardiotoxic action of *Cr. oxycantha* ethanol extract used in the medicine. In the present study we investigated the antioxidant properties of the analyzing extract in habitual systems.

Our research on antioxidant properties of this extract includes two methods. They investigate the influence of the extract on Fe^{2+} -induced LP and $\cdot OH$ scavenging and UV

protective effects. The choice of the methods is based on the healing Cr. oxycantha effect, which successfully influences cardiac performance, high blood pressure and protection against ischemia/ reperfusion brain damage; against chronic diseases such as atherosclerosis and coronary heart disease ¹⁴. All these medical conditions are accompanied with heightened radical production and an increased LP level.

Figure 7

Figure 3: Antioxidant activity of Crataegus oxycantha fruits extract measured in lipid consisted system. AOA is calculated as percentage from control. The control absorption was about 0.250.



The ability of the extract to affect LP processes induced by catalytically active iron and hence to protect cell membranes from oxidative damages can be evaluated by a system of Fe²⁺ induced LP (Fig 3).

Antioxidant activity values between 20% and 90% were found within the tested concentration range of 0.1 to 10 mg/ml. The last case means that only 10% of membrane lipids have been oxidized (and 90% are not) which permits us to classify the extract as a good antioxidant for the system tested. Those values are obtained as a result of three independent experiments for each of shown in Fig. 3 concentrations. On the basis of data shown in Fig. 3 C-50 value was calculated. In the tested model system we found a value of C-50 = 0.70 ± 0.02 mg/ml.

The absorbances measured for the same model system but in the presence of one of the main biologically active Crataegus fruits extract component proved by GC/MS analysis are shown in Table 2 ¹⁵. The tested concentration of 0.3 mg/ml is the same for all substances. Six among eight investigated components showed antioxidant properties. Surprisingly the nicotinic acid showed prooxidant activity. Asiatic acid did not show significant difference from the controls.

Figure 8

Table 2: Absorption at 532 nm. obtained by assay 1 for same of components consisting in the Crataegus extract. AO – substance with antioxidant activity. PO - substance with prooxidant activity.

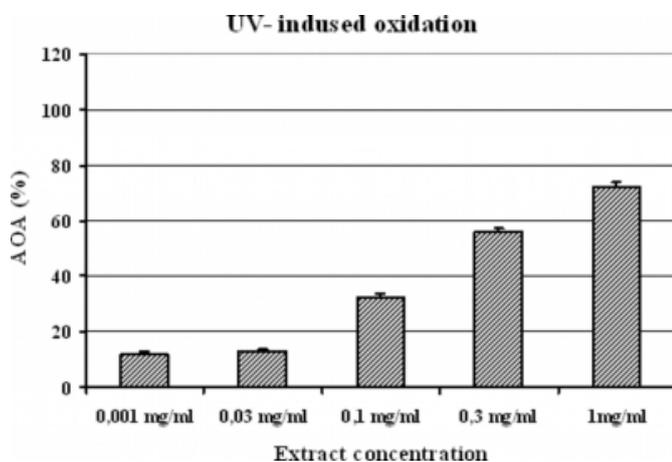
	Abs.	± Stdev	
Control	0.249	0.003	
Quercetin	0.012	0.001	AO
Nicotinic acid	0.328	0.020	PO
α - tocopheryl acetat	0.195	0.011	AO
Phytol	0.129	0.014	AO
Esculin	0.192	0.012	AO
Crategolic acid	0.176	0.005	AO
Ursolic acid	0.196	0.006	AO
Asiatic acid	0.236	0.029	n.s.

In the UV irradiation oxidation of deoxyribose system mainly hydroxyl radicals are generated and they are mostly responsible for the DNA damages ¹¹

In our experiments the Crataegus fruits extract was tested within the concentration range of 0.01 to 1 mg/ml. Absorbance values found were calculated as an average of absorbance of 3 independent experiments for each of shown in Fig. 4 concentrations. On the basis of these data C-50 value (which produces twofold decrease of deoxyribose oxidation) was calculated. In the tested here system we found a value of C-50 = 0.26 ± 0.01 mg/ml.

Figure 9

Figure 4: Antioxidant activity of *Crataegus oxycantha* fruits extract measured in UV irradiation hydroxyl radicals generating system. AOA is calculated as percentage from control. The control absorption was about 0.250.



The comparison of C-50 of the *Crataegus* extract for the both tested systems showed that its antioxidant activity is about 2.5 fold stronger in the systems of UV irradiation generated hydroxyl radicals.

Hawthorn polyphenol prepared by ethyl acetate treatment of the ethanol extract of Chinese hawthorn fruit were detected 15 polyphenols comprise flavonoids and procyanidin (monomer, dimer, trimer, tetramer and pentamer) (2) ¹⁶. Similar components were found in ethanol fruit extract of Bulgarian *Crataegus oxycantha* (Fig. 1 and 2). Those polyphenols manifested a strong superoxide and hydroxyl radical scavenging effect with C-50 values of 6.3 µg/ml and 1.1 µg/ml respectively

We obtained ·OH scavenging effect lower than Chinese hawthorn but we used whole *Crataegus oxycantha* extract not polyphenols only.

It has been shown a positive correlation between antioxidant activity and polyphenol contents in different Bulgarian herbs water extract ^{17,18}. In this paper we identified polyphenols in *Cr. oxycantha* fruits ethanol extract and we proved the antioxidant effect in two biologically relevant and complementary systems.

The application of direct infusion – ESI – MS for the procyanidin content analysis, coupled with an evaluation of free radicals scavenging properties of the plant extract are important approaches for the phytotherapy.

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