



Relationship between antioxidant properties and GC-MS component composition of extracts from flowers, leaves and fruits of *Crataegus Oxycanta*

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ABSTRACT

The aim of this study was to perform a comparative investigation of the antioxidant effect of *Crataegus oxycanthas*' flowers, leaves, green and ripe fruits probes and to relate the obtained results with the ones of the accomplished qualitative GC-MS analysis of the antioxidant components participating in the studied standardized ethanol extracts. We established that the three extracts have well manifested antioxidant properties in three biological relevant model systems with different mechanism of generation of ROS: Fe-induced lipid peroxidation, UV induced deoxyribose damage and horse-radish peroxidase (HRP)-H₂O₂ chemiluminescent system. In the UV system the strongest effect is observed for the extract from flowers and leaves (C-50 = 0.109±0.009 mg/ml), followed by the ones from ripe fruits (C-50 = 0.259±0.015 mg/ml) and finally – by green fruits (C-50 = 0.557±0.014 mg/ml). In the Fe-induced lipid peroxidation system the antioxidant effect of the studied samples decreases in the following order: effect of flowers and leaves (C-50 = 0.287±0.018 mg/ml) > effect of green fruits (C-50 = 0.495±0.021 mg/ml) > effect of ripe fruits (C-50 = 0.704±0.035 mg/ml). Similar results were obtained in the HRP-H₂O₂ system. The generalized results show that the strongest antioxidant effect is observed for the plant extract from flowers and leaves of *Crataegus*. The GC-MS analyses we carried out indicate that the observed differences are due to the presence of a bigger number of constitutes possessing antioxidant properties in flowers and leaves extracts, compared with the fruits extracts.

Indexing terms/Keywords

Crataegus oxycantha, GC-MS, free radicals

Academic Discipline And Sub-Disciplines

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INTRODUCTION

Contemporary pharmaceuticals and cosmetic industry turn nowadays more frequently towards natural products when preparing drugs. This is connected to the fact that many patients prefer to be treated with herbal drugs, as it is believed that such preparations have less side effects than their synthetic analogues. The usage of drugs for medication purposes however requires numerous investigations of their composition and biological effect.

Crataegus species have been known with their beneficial therapeutics effects since ancient times. [1,2,3]. The evaluation of the evidences in the literature reviews clearly demonstrates the immense effect of several hawthorn species used mainly for cardiovascular disease which are among the leading causes of mortality, disability and reduced quality of life in developed countries[1,4].

Crataegus oxycantha possesses valuable medical application in releasing the symptoms of cardiovascular diseases such as hypertension, hyperlipidemia, and in particular - congestive heart failure [5]. There are scientific proofs that the Hawthorn may induce anti-ischemia/reperfusion-injury and possess anti-arrhythmic effects [6, 7, 2, 8]. These beneficial effects may be due, in part, to the presence of antioxidant components in its flowers, leaves and fruits extract [1, 9, 10]. During the years several pharmaceutical products containing standardized extracts from *Crataegus oxycanthas'* flowers, leaves and deep red fruits have been developed [11].

Several authors have suggested possible connection between the observed therapeutic effects of extracts from *Crataegus* species and their antioxidant properties they have indicated the need of comparative aviation of their antioxidant potential [1, 4]. The performed *in vitro* and *in vivo* investigations of the medical effects of the obtained by different methods *Crataegus* species leaves, fruits, green fruits and flowers extracts have proved antioxidant capability. For example, leaves extract of *Crataegus monogyna*, *Crataegus pinnatifida* fruits, *Crataegus aronia*, and *Crataegus sinaica* decrease the process of lipid peroxidation and have scavenging effects against hydroxyl and nitroxyl radicals [9, 12, 13, 14, 15].

The purpose of the present study was to perform a comparative investigation of the antioxidant potential of *Crataegus oxycanthas'* flowers, leaves, and green and ripe fruits probes and to relate the obtained results with ones of the accomplished qualitative GC-MS analysis of the antioxidant components participating in the studied standardized ethanol extracts.

Free radicals are generated upon natural function of plants and other organisms. Due to the fact that plants use peroxides and other reactive species in redox signaling and defense processes they expose themselves to high levels of these reagents. The observed rates of nitroxyl radicals' generation, basal levels of H_2O_2 and lipid hydroperoxides in plant tissues are much higher compared to animals' samples [16]. ROS are produced as a normal product of plant cell metabolism. They are always formed by the unavoidable leakage of electron onto O_2 from the electron transport activities taking place in chloroplasts, mitochondria and plasma membrane or as a product of the metabolic pathways. Investigating the main trigger and processes associated with ROS generation in plants we have come to the conclusion that the most suitable biological relevant model system for comparative evaluation of the antioxidant properties of the plant extracts would be the one based on the main damaging mechanisms of *in vivo* radical generation. To perform the experimental work we have chosen two spectrophotometric assays – Fe – generated lipid peroxidation and UV induced ROS generation, and one chemiluminescent assay - HRP- H_2O_2 system.

The Fe-induced lipid peroxidation and UV ROS generation assays are among the most commonly used model systems for evaluation of the antioxidant properties of plant extracts. The reason is that during the photosynthesis processes plants are exposed to risk of solar UV radiation induced photo oxidative destruction of the main cellular components. The oxidative breakdown of biological phospholipids occurring in most cellular membranes including chloroplast, mitochondria, peroxisomes and plasma membrane is the main harmful mechanism implicated in oxidative stress cellular damage. To these both methods we have decided to add a sensitive chemiluminescent assay which measures the oxidation of luminol in the presence of H_2O_2 by horse-radish peroxidase. H_2O_2 is generated in the plant cell under normal and stressful conditions such as drought, UV irradiation and exposure to intense sunlight. It has been proved that cell walls peroxidase is implicated in the catalyzed the formation of H_2O_2 in the presence of NADH. The obtained results from the chosen model system will give us the possibility to investigate the efficiency of the studied extracts upon different mechanism of free radical induction.

Crataegus oxycantha is used successfully for relief of cardiovascular diseases. It is known that these pathological conditions are associated with increased levels of oxidative stress markers [17]. Due to this fact numerical experiments performed by other authors have shown increased plasma levels of oxidized LDL. Our investigation includes oxidation in model lipid system of a classic type.

MATERIALS AND METHODS

Preparation of ethanol extract:

Fresh flowers, leaves and green and deep red (ripe) fruits from Bulgarian *Crataegus oxycantha* (Rosaceae) were extracted with ethanol according to Long et al. (2006) [18]. The obtained extracts were standardized based on the content of flavonoid [11]. Three different plant extracts were obtained: from flowers and leaves; from green fruits and from deep red (ripe) fruits. All extracts were prepared strictly following the same procedure which is essential for correct comparison of the obtained by the used analytical methods results.

Compounds analysis:

GC-MS analysis was carried out with the following conditions. Instrumentation: LCQ DECA Thermoquest ESI Parameters: Negative ion mode; Mass range: 50 – 1500; Spray voltage: 4 kV; Capillary temperature: 270 °C; Capillary voltage:-8V.

Registration of TBA-RS induced by Fe²⁺:

The TBA-RS of lipid peroxidation were measured in liposomal suspension of phospholipids from egg yolk extracted according to Folch et al. (1957) [19]. Each sample comprises 1ml PBS, pH 7.4, containing 1 mg lipid/ml, the studied extracts or buffer for the controls. After addition of 0.1 mmol/l FeCl₂ samples were incubated at 37 °C for 30 min. Subsequently 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.

UV induced deoxyribose damage:

The deoxyribose assay was performed as given by Halliwell et al. (1987) [20] with small modifications. The final volume for all probes was one ml. The samples mixture comprised PBS. pH 7.4 containing: 0.3 mmol/l 2-deoxy-D-ribose and the tested extracts at concentrations between 0.01 and 1 mg/ml. In control samples plant extracts were omitted. 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 mixture was vortexed, heated in a 1000C water bath for 20 min and cooled in a cold water bath. The chromophore absorption was measured at 532 nm and the antioxidant activity (AOA) was calculated by the following equation:

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

where A₀ is the absorption of the control sample and A the absorption the studied probes.

HRP-H₂O₂ assay:

The horse-radish peroxidase (HRP) - luminol- hydrogen peroxide system is a sensitive assay for monitoring antioxidant activity. The assay was carried out using 1 ml samples of 50 mmol/l PBS containing: 100 μmol/l luminol, 0.5 IU/l HRP enzyme and the tested extracts at concentrations from 0.01 to 1 mg/ml. In the control samples the extract solutions were omitted. The reaction was started by adding of 50 μl H₂O₂ (1 mmol/l). Chemiluminescence (CL) was measured for 5 min at temperature of 25 °C. We calculated chemiluminiscent scavenging index - CL-SI (%) as a ratio of CL in the presence and in the absence of the extract.

Calculation of C-50:

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

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

The calculations used fitting of the data to the “sigmoid” model, where B is the coefficient (hill slope) and C is the substance concentration [21].

RESULTS AND DISCUSSION

The results of the experiments carried out in a system with Fe-induced LP are shown on Fig. 1. In this system we have established that the extract of flowers and leaves had the strongest antioxidant properties, followed by the extracts of green and ripe fruits. In order to compare quantitatively AOC of the three extracts we calculated C-50. The smaller is the C-50 value – the stronger is the antioxidant effect of the investigated extract. The obtained C-50 data decrease in the following order: of flowers and leaves(C-50 = 0.287 ± 0.018 mg/ml) > green fruits (C-50 = 0.495 ± 0.021 mg/ml) > ripe fruits (C-50 = 0.704 ± 0.035 mg/ml). The values for green and ripe fruits are respectively 2 and 2.5 times bigger, compared to the C-50 values for flower extracts.

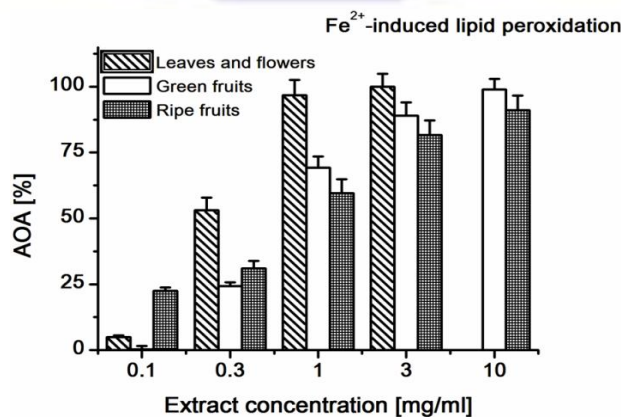


Fig 1: Effect of Crataegus oxycanthas’ extracts on TBARS production in a model system of Fe²⁺ - induced lipid peroxidation.

In later experiments we used UV radiation as a free radicals source. This method causes formation of OH^* , leading to degradation of deoxyribose molecules. The performed experiments prove the protective efficiency of the studied extracts in the investigated model system. The obtained results let us establish strong AOA and diminished DNA oxidative damage for the three investigated extracts (Fig. 2). They demonstrated different efficiency in this process, though the effect as a whole was stronger in comparison to the previous model system. The investigated concentration range includes concentration above 0.1 mg/ml which indicates stronger efficiency of the action upon elimination of OH^* .

Like in the previous studied model system the flower extracts have demonstrated the strongest AOA ($C-50 = 0.109 \pm 0.009$ mg/ml). They were followed by ripe fruits ($C-50 = 0.259 \pm 0.015$ mg/ml) and finally – by green fruits ($C-50 = 0.557 \pm 0.014$ mg/ml). Compared to the previous model system of Fe-induced lipid peroxidation there is an increase in the effectiveness of ripe fruits and slight decrease in green fruits extracts' antioxidant properties.

Recent publications prove radio protective effect of *Crataegus* extract in in vivo mouse model [22] and in vitro human lymphocytes test system [23]. Given the fact that the hydroxyl radical is considered to be the main damaging factor of ultraviolet irradiation the presented in vitro results from the UV deoxyribose model system and the chemiluminescent assay, which are in agreement with the date of the mentioned reviews, could be considered as an independent experimental proof of the observed by the authors radio protective effect. The extracts of flowers and leaves that we have studied could prove most effective in this process.

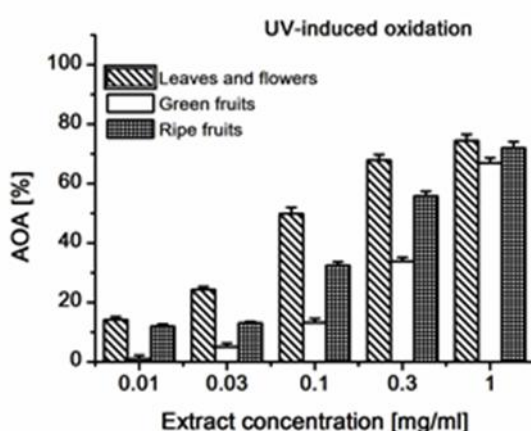


Fig 2: DNA protection activity of *Crataegus oxycanthas*' flowers, leaves, green and ripe fruits extracts in a model system of UV induced deoxyribose damaging.

The peroxidase activity of the extracts is studied by HRP-H₂O₂ assay. Luminol dependent CL is used for registration of the process. In this method HRP is used as an electron donor of luminol upon destruction of H₂O₂. This method gives us the possibility to determine the total antioxidant effect of extracts in an enzyme system, using wide variety of substrates to initiate the decomposition of H₂O₂ to water. According to the obtained results the addition of extracts has led to dramatic reduction of CL (Fig. 3). The values measured at concentrations above 0.03 mg/ml show a 100 times decrease of CL with regard to control. Again the effect is the strongest for extracts from flowers and leaves ($C-50$ is out of tested concentration range) and weaker, but still well expressed, for green ($C-50 = 0.014 \pm 0.005$ mg/ml) and for ripe ($C-50 = 0.020 \pm 0.003$ mg/ml) fruits samples.

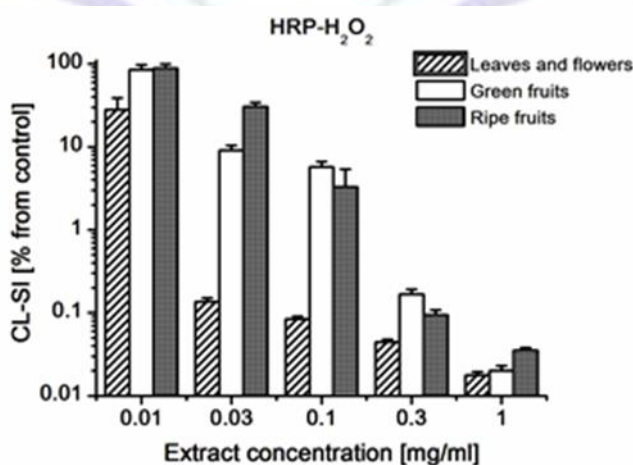


Fig 3: Effect of *Crataegus oxycanthas*' flowers, leaves, green and ripe fruits extracts on luminol-dependent CL induced by HRP/H₂O₂.



From the generalized experimental data it is evident that extracts from flowers and leaves possess the strongest antioxidant potential. The obtained values for green and ripe fruits suggest considerable antioxidant effect.

Many of the authors who have investigated the antioxidant properties of plant extracts have tried to establish relationship between the detected antioxidant effect and the amount of total phenols and procyanidins in the investigated samples. In previously performed experiments we have determined the procyanidin composition of the tested samples using HPLC and LC/MS analysis, but the obtained results could not explain the observed antioxidant-induced changes [24, 25]. Additional GC-MS analysis was performed in order to determine other constituents (beyond the procyanidins) determining samples' effect.

The TIC-mass chromatographs obtained for the three extracts shows that each extract contains over 50 different components.

Table 1. Components with antioxidant properties proved GC-MS analysis of flowers, leaves, green and ripe fruits ethanol extracts obtained from *Crataegus oxycantha*.

Components with antioxidant properties proved by GC-MS analysis	Flowers and leaves	Green fruits	Ripe fruits
Isoprenoids			
<i>Monoterpenoids</i>			
Fenchol	+	—	—
Myrcene	+	—	+
Carene	+	—	—
Myrtenal	+	—	—
Linalooloxide	+	—	+
Lynalyl	+	—	+
propionate			
Terpineol	+	—	—
<i>Sesquiterpenoids</i>			
Farnesene	+	—	—
Bisabolol oxide	—	+	—
<i>Triterpenoids</i>			
Erythrodiol	+	+	—
α, β - Amyrines	+	+	+
Ursenal	+	+	+
Squalene	—	+	—
Ursolic acid	+	+	—
<i>Steroids</i>			
Campesterol	+	+	+
Sigmasterol	+	—	—
Sitosterol	+	—	+
Ergosterol	+	—	+
Phytol	+	+	+
Tocopherols	—	+	+
$\alpha, \beta, \gamma, \delta$			
Phenol	+	—	—
components (anisic acid, anisyl alcohol, etc)			

From the obtained results we can conclude there is difference in the component composition between the three studied extracts. Constituents with proven in literature AOA are shown on Table 1. According to the performed analysis the 4 types of tocopherols, phytol, $\alpha, \beta, \gamma, \delta$ – amyrynes, ursenal, campesterol are constitutes to all of the studied extracts. As seen from Table 1, the flowers and leaves extract contains the broad spectrum of antioxidant components in comparison with the two fruit extracts. They contain more monoterpenoids, steroids and phenol components compared to the fruits sample. Triterpenoids are detected mostly in green fruits extracts.

On the grounds of these data we can suggest that the bigger antioxidant effect of flowers and leaves samples is due not only to the bigger amount of procyanidins, but also to the presence of additional components with antioxidant properties. The observed differences in the composition, defining the differences in the investigated properties we ascribe to the fact that in the different parts of the plant, the sources and magnitude of oxidative damages are different. In order to sustain the balance between oxidants and antioxidants, more antioxidant components are necessary on the sites with increase possibility of formation of reactive oxygen and nitrogen species.

Our survey shows that the three investigated extracts have well manifested antioxidant properties in the used systems. The strongest effect is observed for the extract from flowers and leaves. Ripe and green fruits show similar but statistically different antioxidant activity. Ripe fruits demonstrate stronger effect in the model system with UV irradiation and chemiluminescent HRP-H₂O₂ systems, but green fruits antioxidant potential in the spectrophotometric system of Fe induced LP is stronger. The GC-MS analysis we carried out indicates that the observed differences are due to the presence of a bigger number of components possessing antioxidant properties in flowers and leaves extract, compared with samples.



REFERENCES

- [1] Chang, Q., Zuo, Z., Harrison, F., Chow, MS. 2002. Hawthorn. *J. Clin. Pharmacol.* 42, 605-12.
- [2] Ljubuncic, P., Portnaya, I., Cogan, U., Azaizeh, H., Bomzon, A. 2005. Antioxidant activity of *Crataegus aronia* aqueous extract used in traditional Arab medicine in Israel. *J. Ethnopharmacol.* 101, 153-61.
- [3] Cui, T., Nakamura, K., Tian, S., Kayahara, H., Tian, YL. 2006. Polyphenolic content and physiological activities of Chinese hawthorn extracts. *Biosci. Biotechnol. Biochem.* 70, 2948-2956.
- [4] Hwang, HS., Bleske, BE., Ghannam, MM., Converso, K., Russell, MW., Hunter, JC., Boluyt, MO. 2008. Effects of hawthorn on cardiac remodeling and left ventricular dysfunction after month of pressure overload-induced cardiac hypertrophy in rats. *Cardiovasc. Drugs Ther.* 22, 19-28.
- [5] Jayalakshmi, R., Thirupurasundari, C.J., Devaraj, SN. 2006. Pretreatment with alcoholic extract of *Crataegus oxycantha* (AEC) activates mitochondrial protection during isoproterenol - induced myocardial infarction in rats. *Mol. Cell Biochem.* 292, 59-67.
- [6] Miller, AL. 1998. Botanical influences on cardiovascular disease. *Altern. Med. Rev.* 3, 422-31.
- [7] Chang, WT., Dao, J., and Shao, ZH. 2005. Hawthorn: potential roles in cardiovascular disease. *Am. J. Chin. Med.* 33, 1-10.
- [8] Zhao, B. 2005. Natural antioxidants for neurodegenerative diseases. *Mol. Neurobiol.* 31, 283-93.
- [9] Bahorun, T., Trotin, F., Pommery, J., Vasseur, J., Pinkas, M. 1994. Antioxidant activities of *Crataegus monogyna* extracts. *Planta Med.* 60, 323-8.
- [10] Kiselova, Y., Ivanova, D., Chervenkov, T., Gerova, D., Galunska, B., Yankova, T. 2006. Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. *Phytother. Res.* 20, 961-5.
- [11] Barnes, J., Anderson, LA., Phillipson, JD. 2007. Hawthorn. In: *Herbal Medicines*, Third edition, London-Chicago, Pharmaceutical Press (RPS Publishing), 346-351.
- [12] Bahorun, T., Aumjaud, E., Ramphul, H., Rycha, M., Luximon-Ramma, A., Trotin, F., Aruoma, OI. 2003. Phenolic constituents and antioxidant capacities of *Crataegus monogyna* (Hawthorn) callus extracts. *Nahrung. Jun;47(3):191-8.*
- [13] Chu, CY., Lee, MJ., Liao, CL., Lin, WL., Yin, YF., Tseng, TH. 2003. Inhibitory effect of hot-water extract from dried fruit of *Crataegus pinnatifida* on low-density lipoprotein (LDL) oxidation in cell and cell-free systems. *J. Agric. Food Chem.* 17, 7583-7588.
- [14] Quettier-Deleu, C., Voiselle, G., Fruchart, JC., Duriez, P., Teissier, E., Bailleul, F., Vasseur, J., Trotin, F. 2003. Hawthorn extracts inhibit LDL oxidation. *Pharmazie* 58, 577-81.
- [15] Wang, W., Chen, WW. 1991. Antioxidative activity studies on the meaning of same original of herbal drug and food. *Zhong Xi Yi Jie He Za Zhi* 11, 159-161.
- [16] Halliwell, B., Gutteridge, J. 2007. *Free Radicals in Biology and Medicine* (4-th ed.) Bioscience Oxford.
- [17] Willcox, BJ., Curb, JD., Rodriguez, BL. 2008. Antioxidants in cardiovascular health and disease: key lessons from epidemiologic studies. *Am. J. Cardiol.* 101, 75-86.
- [18] Long, SR., Carey, RA., Crofoot, KM., Proteau, PJ., Filtz, TM. 2006. Effect of hawthorn (*Crataegus oxycantha*) crude extract and chromatographic fractions on multiple activities in a cultured cardiomyocyte assay. *Phytomedicine* 13, 643-50.
- [19] Folch, J., Lees, M., Shoane-Stoaley, CH. 1957. A simple method for the isolation and purification of total lipid from animal tissues. *J. Biol. Chem.* 226, 497-507.
- [20] Halliwell, B., Gutteridge, JM., Aruoma, OI. 1987. The deoxyribose method: a simple test-tube assay for determination of rate constants for reactions of hydroxyl radical. *Anal. Biochem.* 165, 215-219.
- [21] Traykov, T., Hadjimitova, V., Goliysky, P., Ribarov, S. 1997. Effect of phenothiazines on activated macrophage - induced luminol - dependent chemiluminescence. *Gen. Physiol. Biophys.* 16, 3-14
- [22] Hosseinimehr, SJ., Azadbakht, M., Mousavi, SM., Mahmoudzadeh, A., Akhlaghpour, S. 2007. Radioprotective effects of hawthorn fruit extract against gamma irradiation in mouse bone marrow cells. *J. Radiat. Res.* 48, 63-8.
- [23] Leskovic, A., Joksic, G., Jankovic, T., Savikin, K., Menkovic, N. 2007. Radioprotective properties of the phytochemically characterized extracts of *Crataegus monogyna*, *Cornus mas* and *Gentiana austriaca* on human lymphocytes in vitro. *Planta Med.* 73, 1169-75.
- [24] Hatalcheva, N., Hadjieva, P., Ganeva, Y., Sheitanova, T., Kaisheva, PG., Seraglia, R., Traldi, P. 2007. GC/MS analysis of Bulgarian *Crataegus oxycantha* flowers, leaves and fruits ethanol extracts. *Asian J. Spectrosc.* 11, 105-114.
- [25] Hadjimitova, V., Traykov, T., Hadjieva, P., Traldi, P. 2009. Free radical scavenging properties and LC/MS analysis of Bulgarian *Crataegus oxycantha* fruits ethanol extract. *The Internet Journal of Alternative Medicine.* 6.