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Antioxidant activity of *Humulus lupulus* and *Vaccinium myrtillus* individual and combined extracts

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Abstract

Oxidative stress, that generates reactive oxygen and nitrogen species, is caused by an imbalance occurred at the cellular level and causes the appearance of several disease, including atherosclerosis, diabetes, neurodegenerative diseases and cancer. We investigated by two methods: DPPH and total antioxidant capacity, antioxidant capacity and free radical scavenger potential of three hydroalcoholic extracts from: Humulus lupulus (cones) and Vaccinium myrtillus (leaves) and a combined extract obtained as a mixture of the two species, compared with the reference substances chlorogenic acid and rutin, highlighting the chemical composition of the extracts in total polyphenolic compounds. Reference substances have a similar behavior in both test methods, meanwhile the studied samples have opposite behaviors. In DPPH assay Vaccinium myrtillus extract has a higher scavenger capacity than Humulus lupus extract at the most concentrations and test times leading to a direct dependence between the inhibition potential of DPPH and composition in total polyphenolic compounds. In phosphomolybdenum assay Humulus lupulus extract has total antioxidant capacity higher than the Vaccinium myrtillus extract, at all tested concentrations, which may be due to the different reactivity of the extracts compounds with phosphomolybdic reagent, taking into account both time and reaction temperature, as well as the chemical complexity of the extracts. Antioxidant activity of combined extract tends to achieve a balance compared with the results for individual extracts, in the determinations made by the two methods, including in the case of quantitative chemical composition.

Keywords: *Vaccinium myrtillus, Humulus lupulus, antioxidant, Dpph, total antioxidant capacity*

1. Introduction

Oxidative stress, that generates reactive oxygen and nitrogen species, is caused by an imbalance occurred at the cellular level and causes the appearance of several disease, including atherosclerosis, diabetes, neurodegenerative diseases and cancer (Fatmah A. Matough & al [1]; Erika Szaleczky & al [2]). Deficiency of antioxidants in the body leads to enzymatic imbalances, modifications of genetic material or transformation of normal cells into atypical cells (Lobo V. & al [3]). Currently there is an increased interest in natural antioxidants, especially towards a group of compounds present in plants, namely polyphenolic compounds. Polyphenolic compounds have been the subject of many research studies that have shown their

anti-inflammatory, anti-corrosion, antioxidant properties, from which the antioxidant properties are a major concern (Kanti Bhooshan, Syed Ibrahim [4]; Lucia Camelia Pirvu [5], [6]).

Antioxidant activity of polyphenolic compounds consists in that they have redox properties, acting as a reducing agent and hydrogen donor (Rice-Evans C al [7]).

Vaccinium myrtillus (fam. *Ericaceae)* blueberry, is a plant species that currently from which is being used both for food and curator as well as scientific purposes, in particular the fruit. Studies on other organs of the species are found in very small number. Thus, high exploitation of as many parts of this species is of great interest. Active compounds that have been isolated from the fruit and leaves of the species are anthocyanins, vitamins, carbohydrates and pectines isolated in fruits and phenolic compounds (quercetin, catechins, tannins) in the leaves (Benigni R & al [8]; Baj A. & al [9]). Blueberry leaves contain high amounts of phenolic compounds, in particular hydroxycinnamic acid, flavonols, catechins and proanthocyanidins (Martz F & al [10]). The compound with the highest concentration found in leaves was chlorogenic acid (5-O-cafeoilquinic acid). Flavonic compounds most abundant in leaves were quercetin derivatives (Witzell Johana & al [11]. The content of phenolic compounds and tannins in leaves was distinctly higher than in fruits from 11 blueberry populations analyzed. Flavonoid content was between 400 and 600 mg/100g (Rolson W & al [12]). Blueberry (fruit) is now promoted especially for its potential to improve vision (Benzie IFF, Wachtel-Galor S [13]).

Humulus lupulus (fam. *Canabaceae)* the hops - is being used especially in brewing industry. The female cones of the species are also used for pharmaceutical purposes. Chemical composition includes: 0.3-1% volatile oil, resins, bitter principles, 2-4% tannins, flavones (rutin, quercetin, astragaloside), chalcone (xanthohumol), flavanones (izoxanthohumol) (Istudor Viorica [14]). In traditional medicine the species is used to treat abdominal cramps, anemia, bacterial infections, dermatitis, diarrhea, dysmenorrhea, leucorrhea, migraine and edemas. It is also used as a analgesic, antihelmintics, antipyretic, aphrodisiac, carminative, depurative, digestive, diuretic, diaphoretic and tonic (Farnsworth NR [15]; WHO monographs on selected medicinal plants [16]).

In this article are analyzed by two methods: DPPH and total antioxidant capacity assays, antioxidant and free radical scavenger potential of three hydroalcoholic extracts from: *Humulus lupulus* (cones) and *Vaccinium myrtillus* (leaves) and a combined extract obtained from a mixture of the two species, compared with the reference substances chlorogenic acid and rutin, highlighting the chemical composition of the extracts in total polyphenolic compounds.

2. Materials and methods

Raw materials: Humulus lupulus (cones), *Vaccinium myrtilus (*leaves) - was purchased from Plafar (Romania).

Plant extracts preparation:

Extracts were obtained by: extraction with ethylic alcohol 50%: vegetal material (*Vaccinium myrtilus* leaves; *Humulus lupulus* cones/ solvent rate 1:10; 1:15 (w/v) at boiling temperature for 3 hours, under continuous stirring. After filtration the solution was concentrated under reduced pressure at 50°C to a vegetal material/water extract rate - 1:1 (w/v) and spray dried. For the combined extract was used an equal quantity of each plant of raw material.

Humulus lupulus and *Vaccinium myrtillus* plant analysis was obtained by extraction of raw material in the same conditions, for 5 minutes.

HPTLC Analysis for Polyphenols

For flavones and total phenols have been performed qualitative analyses according to TLC Atlas - *Plant Drug Analyses* (Wagner H., Bladt S.[17]). The development of charac-

teristic finger printing profile was performed by a densitometric HPTLC analysis. The extracts were dissolved with HPLC grade 50% (v/v) methanol to 1% (w/v) solution concentration. Then, 0.6-3,2µl of the samples and 0.8-2.8µl of references substances (10^{-3} M rutin, hyperoside, chlorogenic acid, caffeic acid, - *Sigma-Aldrich*) were loaded as 10mm band length in the 20 x 10 Silica gel $60F_{254}$ TLC plate. The mobile phase was constituted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v). After development, plates were dried and derivatised in NP-PEG reagent. The fingerprints were evaluated at 366nm in fluorescence mode with a WinCats and VideoScan software.

Quantitative analytical determination

Quantitative measurements were realized by standard colorimetric method; total phenols content was measured by Folin – Ciocalteau's method. Results were expressed as mg/100g extract (Romanian Pharmacopoeia, X,[18]).

Free radical scavenging assay.

The scavenging activity of the extracts against 2,2-diphenyl-1-picrylhydrazyl free radical DPPH (*Sigma-Aldrich*) was determined by measuring UV absorbance at 517nm according to Sanchez-Moreno et al. (1998) (Sanchez-Moreno & al.[19]) assay. The extracts concentration were 10mg/ml, 1mg/ml, 0.1mg/ml and 0.01mg/ml in methanol. 50µl aliquots of the extract were mixed with 2950µl of the methanolic DPPH solution (0.025g/l). A blank solution was prepared containing the same amount of methanol and DPPH, and measured after standing at room temperature 5, 15, 30 and 60 minutes. Chlorogenic acid and rutin methanolic solution (10mg/ml) were used as positive controls.

The radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = \{(AB - AA)/AB\} \times 100.$$

Where AB is the absorption of blank sample and AA is the absorption of tested extract solution.

Total antioxidant capacity assay

Was assesed by phosphomolybdenum method, according to Prieto et al. (Prieto & al [20]). To 0.3 ml ethanolic solution of the sample (concentration 1 mg / ml, 0.1 mg / ml, 0.01 mg / ml and 0.001 mg / ml) was added 2.7 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium molybdate, and 4 mM ammonium phosphate). The mixtures were incubated at 95^0C for 90 minutes. After cooling the samples to room temperature, their extinction was measured at 695 nm at UV-VIS spectrophotometer. Ethanol was used as negative control. The antioxidant capacity was expressed as ascorbic acid equivalent to 1 mg of active substance. The calibration curve is linear for ascorbic acid in the range of 0.001 to 1 mg / ml, $n = 6$, $r^2 = 0.999$.

Apparatus

Extraction system (Jena, Germany), Concentrator (Bűchi, Switzerland), Spectrophotometer UV-VIS - Hélios γ (Thermo Electron Corporation); HPTLC system - CAMAG LINOMAT 5, Hamilton- Bonaduz, Schweiz.

Statistical Analysis.

The experiments were repeated three times. The results were expressed as means ± S.D and the difference was tested by Student's t-test.

3. Results and discussions

HPTLC profile the samples: *Humulus lupulus* (cones) extract and plant analysis, *Vaccinium myrtilus* (leaves) extract and plant analysis, combined extract (*Humulus lupulus* (cones), *Vaccinium myrtilus* (leaves) in equal parts) was recorded in Figure 1. The presence of polyphenols was confirmated by blue and orange fluorescent spots detected in the chromatogram, after derivatization.

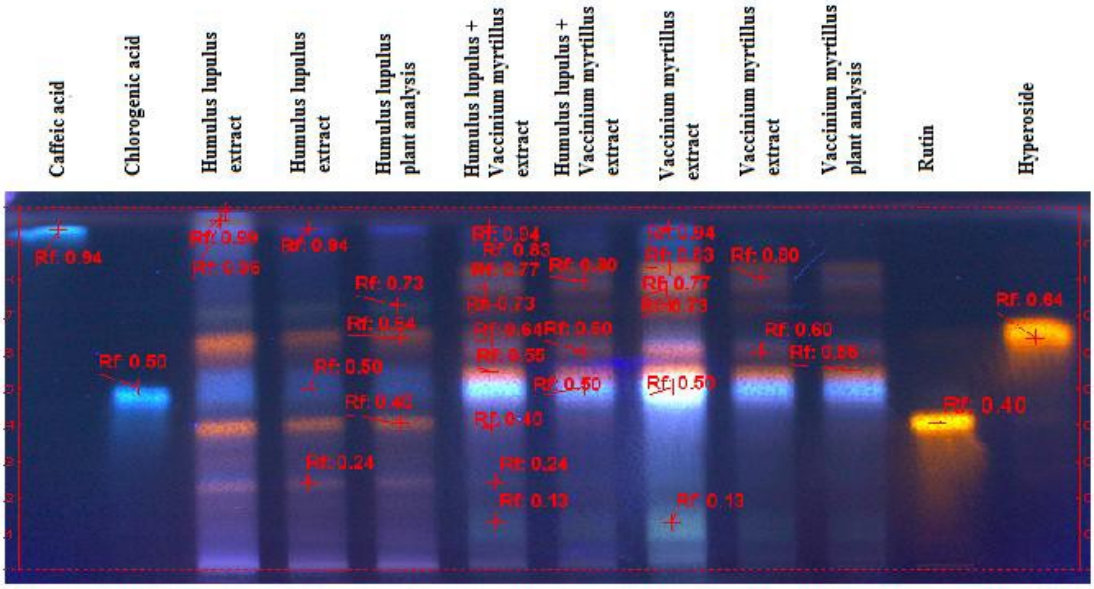


Figure 1 . Extracts chromatogram in HPTLC analysis

Caffeic acid (Rf~ 0.94), chlorogenic acid (Rf~ 0.50), rutin (Rf~ 0.40), hyperoside (Rf~ 0.64) were identified in *Humulus lupulus* extract and combined extract (*Humulus lupulus* + *Vaccinium myrtillus* extract). In *Vaccinium myrtilus* extract and plant analysis the compound identified was chlorogenic acid (Rf~ 0.50) and according to with Wagner H., Bladt S., (1996) (17), probably isochologenic acid (Rf~ 0.80) and based on the relationship spot colour - Rf, quercetin derivates - orange sports, caffeic acid derivetes - blue spots. The qualitative differences observed in cromatograms obtained for the extracts and plant analysis of plant species, consists in a better extraction of polyphenolic compound on tree hour extraction technique. Comparative analyses of qualitative chromatographic profiles of *Humulus lupulus* extract and plant analysis compared to reference substance caffeic acid are represented in Figure 2.

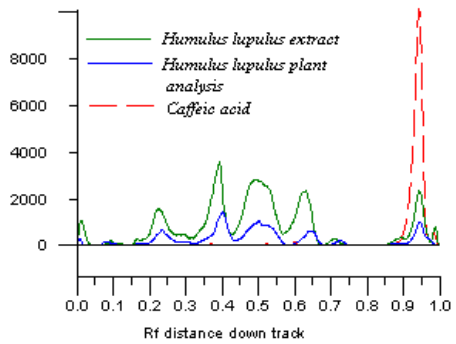


Figure 2. Comparison between *Humulus lupulus* extract, plant analysis and caffeic acid references substance fingerprints

The results of the Folin–Ciocalteu total phenols photometric assay content are reported in Table 1.

Table 1. Quantitative composition of extracts

Extracts	Total phenols content (expressed as caffeic acid equivalents) (mg/100g)	Total phenols content (expressed as chlorogenic acid equivalents) (mg/100g)
Humulus lupulus	3.5	4.8
Vaccinium myrtillus	14.7	20.8
Combined extract	9.7	13.8

DPPH assay

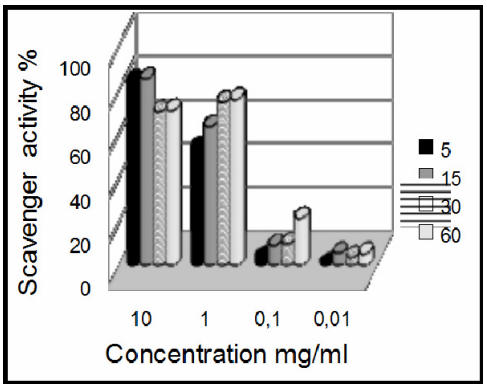
A rapid method for measuring antioxidant capacity involves the use of free radical 2,2-diphenyl-1-picrilhidrazil (DPPH). DPPH is used for testing free radical scavenger ability and the assessment of antioxidant capacity. It is also used, in recent years for the quantification of anti-oxidants in biological systems. The DPPH method can be used for liquid or solid samples and is not specific to a certain antioxidant compound, being indicative for the total antioxidant capacity of the sample. Method of determining the antiradical activity using the DPPH free radical establishes the antioxidant ability to participate in redox reactions through hydrogen transfer mechanism.

The potential of the free radical scavenger DPPH was evaluated for *Humulus lupulus*, *Vaccinium myrtillus* extracts and the combined one at different concentrations (10mg/ml, 1mg/ml, 0.1mg/ml and 0.01mg/ml) and also for reference substances chlorogenic acid and rutin at concentrations of 10mg/ml to 5, 15, 30 and 60 minutes (Figure 1-3). All evaluations were performed in triplicate, obtaining the average values of the inhibitory capacity for extracts of the stable radical with the nitrogen.

All tested samples showed an important capacity of inhibition of the free radical. It can be seen that the ability of inhibition of the stable radical decreases with the samples concentration. *Vaccinium myrtillus* extract (84.83%) has a potential to inhibit DPPH free radical compared to the reference substances chlorogenic acid (84.81%) and rutin (83.52%) at a concentration of 10 mg/ml at 15 minutes from the start of the reaction. Also, at this time and this concentration, *Vaccinium myrtillus* extract has the strongest scavenger action. For the same extract at a concentration of 1 mg/ml, the highest activity was recorded at 60 minutes after initiation of the reaction as being of 75.54%. The combined extract (85.07%) has the potential to inhibit DPPH free radical compared to the reference substances chlorogenic acid (84.81%) and rutin (83.52%) at a concentration of 10mg/ml at 15 minutes after the initiation of the reaction. Also, at this time and this concentration combined extract has the strongest scavenger action. For the same extract at a concentration of 1mg/ml the strongest activity was recorded at 60 minutes after initiation of the reaction as being of 74.54%. For *Humulus lupulus* extract at a concentration of 10mg/ml inhibiting effect of nitrogen radical is more pronounced in the recording at 60 minutes being of 71.83%, lower than the reference substances chlorogenic acid and rutin with 86.20% and respectively 84.46%. It can be observed the pronounced decrease of scavenger activity for *Humulus lupulus* extract with decreased concentrations in all measurements read regardless of time (Fig. 3-5).

The calculated EC50 (half maximal effective concentration) resultes are: *Vaccinium myrtillus* extract - concentration 10mg/ml at 15 minutes – 5,89mg, *Humulus lupulus* extract – concentration 10mg/ml at 60 minutes – 6,96mg, combined extract – concentration 10mg/ml at 15 minutes – 5,87mg, chlorogenic acid concentration of 10mg/ml at 60 minutes – 5,80mg, and for rutin of concentration 10mg/ml at 30 minutes – 5,25mg.

The results are expressed as the average±standard deviation, 95% confidence interval (t-test).



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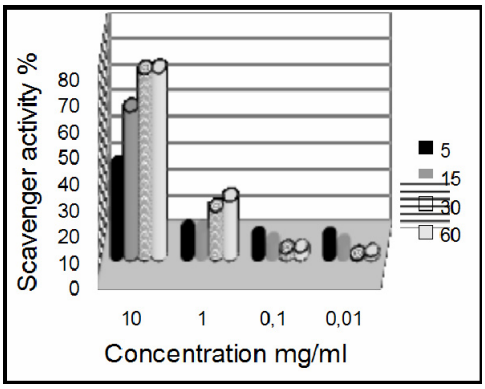

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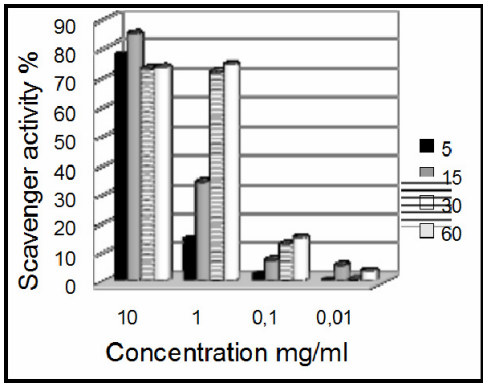

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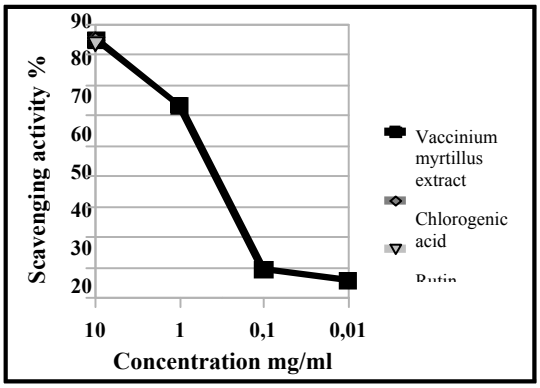
Figure 3. Scavenger activity of 1 - *Vaccinium myrtillus* extract , 2- *Humulus lupulus* extract
3- Combined extract at 5, 15, 30 60 minutes for concentrations between 10-0,01 mg/ml



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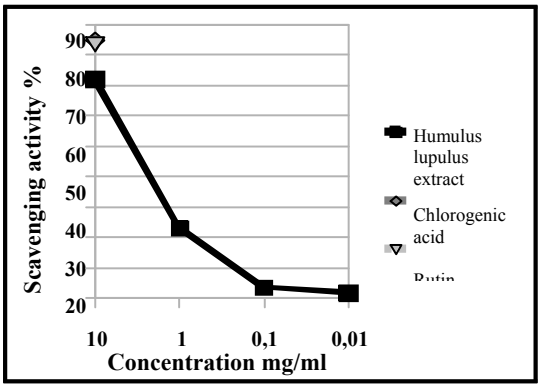

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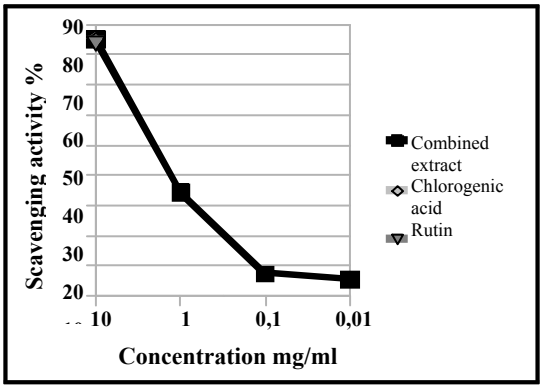

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Figure 4. Scavenger activity of 1 - *Vaccinium myrtillus* extract at 15 minutes, 2- *Humulus lupulus* extract at 60 minutes, 3- Combined extract at 60 minutes for concentrations between 10-0,01 mg/ml comparative with references substances

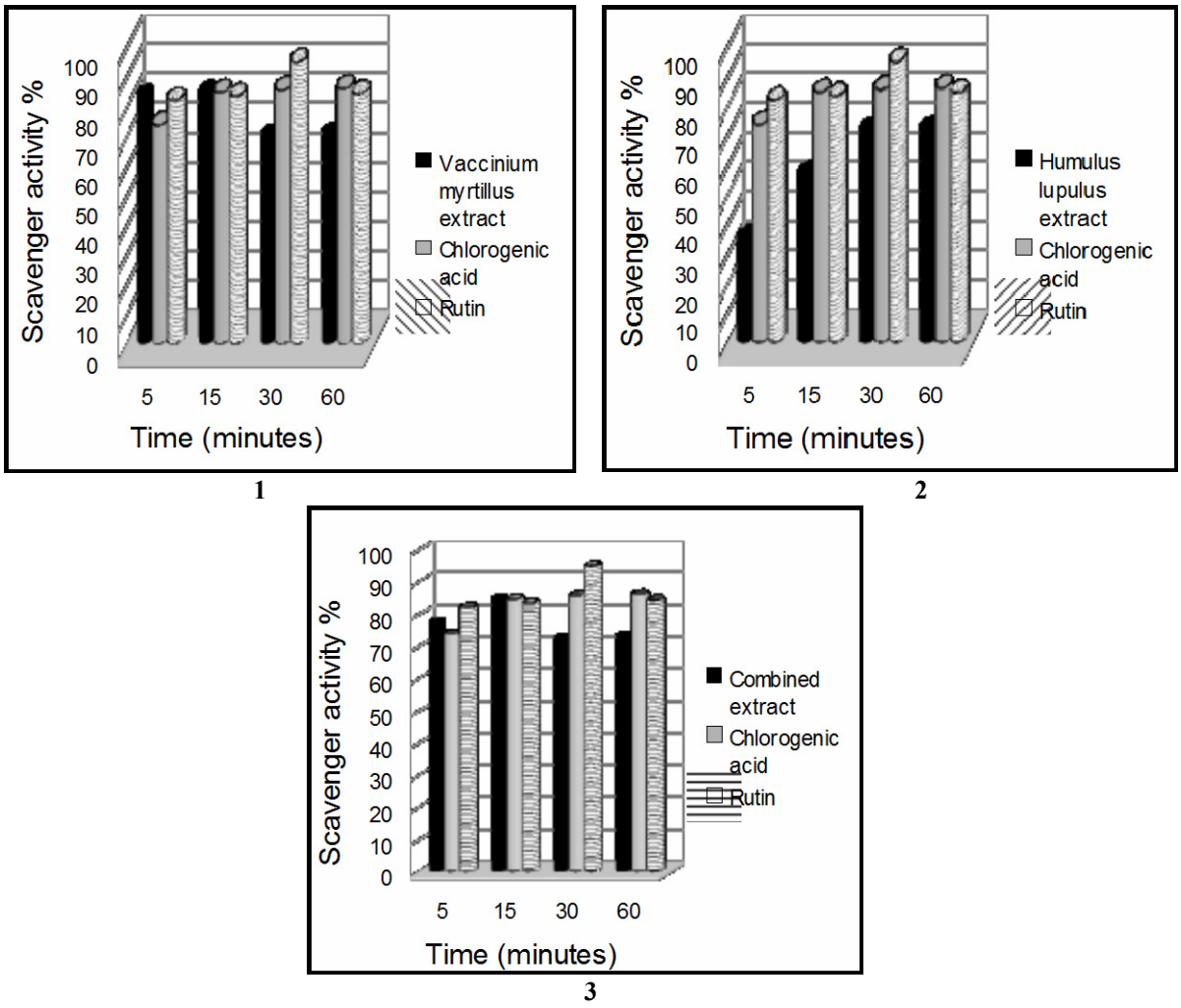

Figure 5. Scavenger activity of 1 - *Vaccinium myrtillus* extract 2- *Humulus lupulus* extract, 3- Combined extract at 10 mg/ml concentration and 5, 15, 30 and 60 minutes comparative with references substances

Total antioxidant capacity assay

One of the usual methods that establishes the capacity of the tested antioxidant to participate in the oxidation-reduction reaction as a reducing is the method of determining the antioxidant activity by the reduction reaction of the reagent solution (0.6 M sulfuric acid, 28 mM sodium molybdate, and 4 mM ammonium phosphate). The method shows that, in an indirect way, the antioxidant tested has the ability to reduce the radicals by electron donor mechanism.

The phosphomolybdenum method results are shown in figure 4 and indicates a strong antioxidant activity for samples at concentration of 1mg/ml for *Vaccinum myrtilus* extract equivalent to 1.77 mg of ascorbic acid, for *Humulus lupulus* equivalent to 0,628 mg ascorbic acid and respectively equivalent to 0,996 mg ascorbic acid for combined extract. Total antioxidant capacity of the samples decreases with the concentration. Reference substances chlorogenic acid and rutin have total antioxidant capacity compared with the samples analyzed.

The results are expressed as medium values ± standard deviation, 95% confidence interval (t -test).

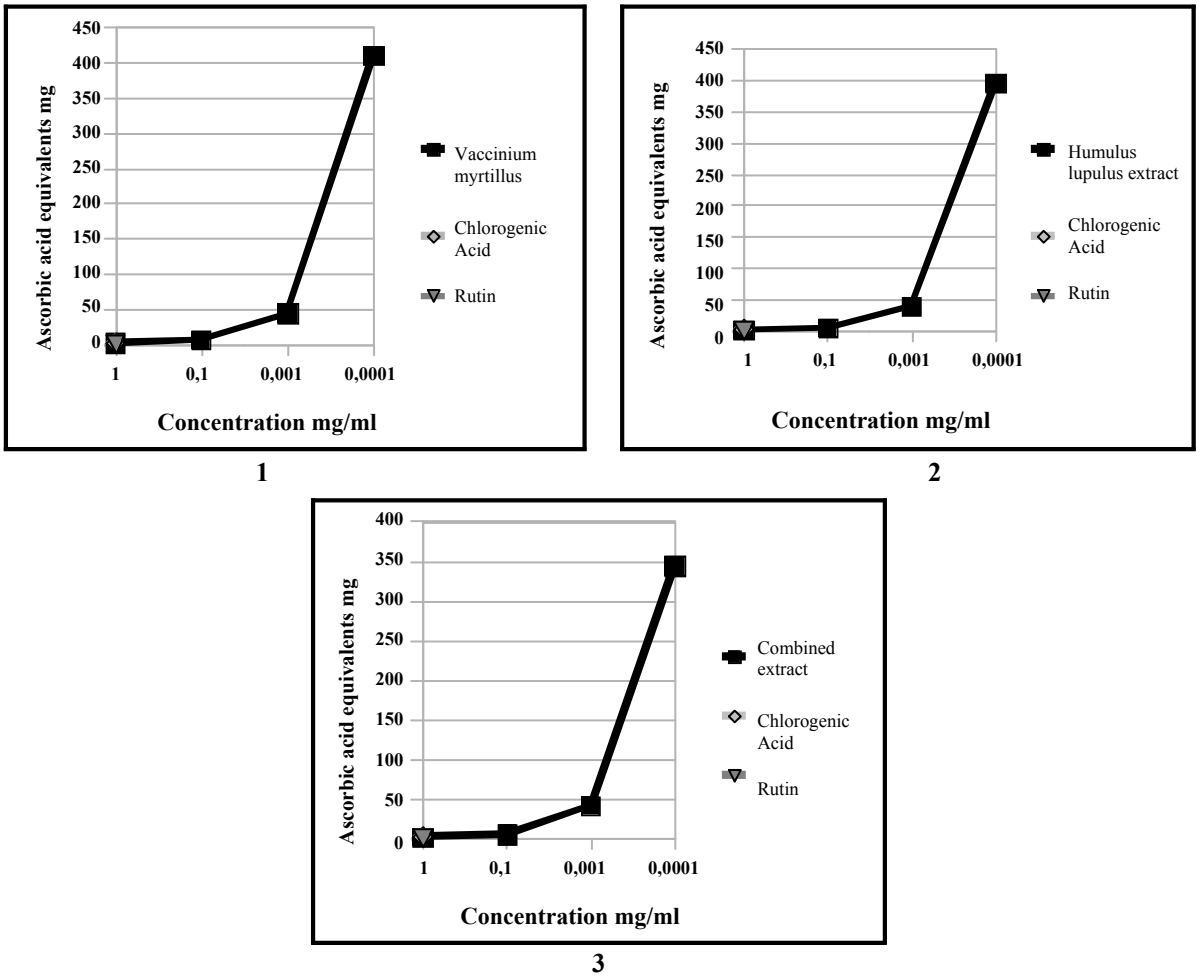

Figure 6. Total antioxidant capacity of 1- *Vaccinium myrtillus* extract, 2- *Humulus lupulus* extract, 3- Combined extract

4. Discussions

In most studies about antioxidant capacity of the species *Humulus lupulus* are used aqueous extracts, due to the especially use of this species in the brewing process. Thus aqueous extract of hops (plants collected from European Union countries between 2004-2006), showed the DPPH radical scavenger potential ranging between 40-80%. Also, there is a potentially increased by approximately 5% for the fresh plant extracts compared with the obtained one from the dried plant extracts (Karel Krofta1 & al [21]).

Water, ethyl alcohol and ethyl acetate extracts at various concentrations of the fruit and leaves of *Vaccinium myrtillus,* have demonstrated neutralizing activity of DPPH. The most pronounced activity was measured for alcoholic extract obtained from the fruit (~ 94%), followed by the aqueous one obtained from the leaves (~ 91%), at the same time having a direct relationship between the content of polyphenolic compounds and antioxidant action (Dragana M. Vučić & al [22]).

Individual hydroalcoholic extract and the combined extract obtained from the two species used in this study have demonstrated antioxidant activity revealed by the results obtained within the two methods. Meanwhile, reference substances chlorogenic acid and rutin have similar behavior in both test methods, the studied samples have opposite ones. In DPPH assay *Vaccinium myrtillus* (with a content of 14.7 mg/100 polyphenols expressed in caffeic acid and

20.8 mg/100g total polyphenols expressed in chlorogenic acid) has a more pronounced scavenger capacity than *Humulus lupus* extract (with a content of 3.5mg/100 polyphenols expressed in caffeic acid and 4.8 mg/100g total polyphenols expressed in chlorogenic acid) at the most concentrations and test times leading to a direct dependence between the inhibition potential of DPPH and composition in total polyphenol compounds. In total antioxidant capacity assay *Humulus lupulus* extract has a higher antioxidant capacity than the *Vaccinium myrtillus* extract, at all tested concentrations. The different resultes obtained in the two methods may be due to the different reactivity of the extracts compounds with phosphomolybdenum reagent, taking into account both time and reaction temperature as well as the chemical complexity of extracts. Antioxidant activity of combined extract tends to achieve a balance compared with the results for individual extracts, in the determinations made by the two methods, including in the case of quantitative composition (9.7 mg/100g total polyphenols expressed in caffeic acid and 13.8 mg/100g total polyphenols expressed in chlorogenic acid).

5. Conclusions

The three extracts showed significant antioxidant activity in this study, demonstrated by the two methods tested. The association of the two species to obtain an extract combined where the two species may potentiate their antioxidant action may be of great interest for applications in phytopharmacy industry, and also can be a novel approach in brewing industry.

References

(1) FATMAH A MATOUGH, SITI B BUDIN, ZARIYANTEY A HAMID, NASAR ALWAHAIBI, JAMALUDIN MOHAMED, The Role of Oxidative Stress and Antioxidants in Diabetic Complications- Sultan Qaboos Univ Med J. Feb 2012; 12(1): 5–18

(2) ERIKA SZALECZKY, JÓZSEF PRECHL, JÁNOS FEHÉR, ANIKÓ SOMOGYI- Alterations in enzymatic antioxidant defence in diabetes mellitus − a rational approach, Postgrad Med J 1999; 75:13–17

(3) V. LOBO, A. PATIL, A. PHATAK, AND N. CHANDRA, Free radicals, antioxidants and functional foods: Impact on human health, Pharmacogn Rev. 2010 Jul-Dec; 4(8): 118–126.

(4) KANTI BHOOSHAN PANDEY AND SYED IBRAHIM RIZVI, Plant polyphenols as dietary antioxidants in human health and disease Oxidative Medicine and Cellular Longevity 2:5, 270-278; November/December; © 2009 Landes Bioscience

(5) LUCIA CAMELIA PIRVU, IBD Pathomechanism at the Basis of Herbal-Based Anticolitis Products, LAP Lambert Academic Publishing (2013-03-28)

(6) LUCIA CAMELIA PIRVU "Development in Corosion Protection", Chapter 10 Polyphenols and Herbal-Based Extracts at the Basis of New Antioxidant, Material Protecting Productsbook edited by M. Aliofkhazraei, ISBN 978-953-51-1223-5, Published: February 20, 2014 under CC BY 3.0 license

(7) RICE-EVANS C, MILLER NJ, PAGANGA G (1996). Structure-antioxidant activity relationship of flavonoids and phenolic acids. Free Rad. Bio. Med., 20: 933-956.

(8) BENIGNI R, CAPRA C, CATTORINI PE. Plante Medicinali – Chimica Farmacologia E Terapia. Vol. II. Milano, Italia: Inverni della Beffa; 1962: 951-958.

(9) BAJ A, BOMBARDELLI E, GABETTA B, MARTINELLI EM. Qualitative and quantitative evaluation of Vaccinium myrtillus anthocyanins by high-resolution gas chromatography and high-performance liquid chromatography. J Chromatography 1983; 279: 365-372.

(10) MARTZ F, JAAKOLA L, JULKUNEN-TIITTO R, STARK S. Phenolic composition and antioxidant capacity of bilberry (Vaccinium myrtillus) leaves in Northern Europe following foliar development and along environmental gradients. J Chem Ecol. 2010 Sep; 36(9): 1017-28. Epub 2010 Aug 19.

(11) WITZELL JOHANNA, ROLF GREF, TORGNY NASHOLM -Plant-part specific and temporal variation in phenolic compounds of boreal bilberry (*Vaccinium myrtillus*) plants Biochemical Systematics and Ecology Volume 31, Issue 2, February 2003, Pages 115–127.

(12) ROLSON W; OSINSKA E; PIORO-JABRUCKA, E; GRABOWSKA A. Morphological and Chemical Variability of Wild Populations of Bilberry (Vaccinium myrtillus L.) Polish Journal of Environmental Studies Volume: 20 Pages: 237 - 243 Date: 2011.
(13) BENZIE IFF, WACHTEL-GALOR S, editors. Boca Raton (FL) Herbal Medicine: Biomolecular and Clinical Aspects. 2nd edition. CRC Press; 2011.
(14) ISTUDOR VIORICA– Farmacognozie Fitochimie Fitoterapie, Vol II, III – Editura Medicala, Bucuresti 2001.
(15) FARNSWORTH NR, ed. NAPRALERT database. Chicago, IL, University of Illinois at Chicago, 9 February 2001 production (an online database available directly through the University of Illinois at Chicago or through the Scientifi c and Technical Network (STN) of Chemical Abstracts Services).
(16) WHO MONOGRAPHS ON SELECTED MEDICINAL PLANTS, Volume 3, ISBN 978 92 4 154702 4.
(17) WAGNER H., BLADT S., (1996) - Plant Drug Analysis, Second Edition, Springer.
(18) ROMANIAN PHARMACOPOEIA, X, 1993.
(19) SANCHEZ-MORENO, C. LARRAURI, J.A., SAURA-CALIXTO, F., (1998) A procedure to measure the antiradical efficiency of polyphenols. Journal of Agricultural adn Food Chemistry 76, 270-276.
(20) PRIETO P., PINEDA M., AQUILAR M., Spectrophotometric qantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. //Analytical Biochemistry, 1999, p. 337-341.
(21) KAREL KROFTAL, ALEXANDR MIKYŠKA, AND DANUŠA HAŠKOVÁ - Antioxidant Characteristics of Hops and Hop Products JOURNAL OF THE INSTITUTE OF BREWING, VOL. 114, NO. 2, 2008.
(22) DRAGANA M. VUČIĆ, MIROSLAV R. PETKOVIĆ, BRANKA B. RODIĆ-GRABOVAC, OLGICA D.STEFANOVIĆ, SAVA M. VASIĆ AND LJILJANA R. Čomić Antibacterial and antioxidant activities of bilberry (Vaccinium myrtillus L.) in vitro - Advanced Research Journal of Microbiology, Vol. 1 (4), pp. 067-073, December, 2013.