Preliminary *in vitro* Activity and Correlation with Chemical Composition of *Momordica charantia*, *Vaccinium myrtillus* and *Vaccinium vitis-idaea* after Enzymatic Extraction Process

ANDREA ERDELYI POP[1], ADRIAN VAMANU[2], OCTAVIAN VASILE POP[1], EMANUEL VAMANU[2]*

[1] Hypericum Impex, 3 Gutinului Str., 430414, Baia Sprie, Maramures, Romania

[2] University of Agronomical Sciences and Veterinary Medicine Bucharest, Faculty of Biotechnology, 59 Marasti Blvd, 011464, Bucharest, Romania

Nutritional imbalances are an increasingly common health problem, for example, diabetes is manifested by a low tolerance to blood glucose level, and natural supplements are an alternative to allopathic medication. The aim of this research was to obtain some products from Momordica charantia (fruits), Vaccinium myrtillus (fruit and leaves) and Vaccinium vitis-idaea (fruits and leaves) by means of an enzymatic extraction process. The physico - chemical composition, the antioxidant and the hypoglycaemic activity of the spray-dried extracts were determined. The biological activity was evaluated by the total antioxidant activity, DPPH scavenging activity and reducing power. Extracts showed high inhibitory capacity of α-amylase, for example 74±2.00% for the Momordica charantia (fruits) extract, at 20 mg/mL. The extracts, however, showed a reduced ability to inhibit the activity of α-glucosidase. The results demonstrated that products of V. myrtillus remain some of the most complex nutritional supplements due to a different phytochemical composition.

Keywords: antioxidant, hypoglycaemic, Vaccinium myrtillus, Vaccinium vitis-idaea, Momordica charantia

The current lifestyle and the processed food with high amounts of fat and sugar lead to an increasing incidence of diabetes, mainly type II diabetes, which is occurring more and more in population of younger ages. The pathology of diabetes is manifested by increased blood glucose levels and is the result of a dysfunction in the insulin secretion [1]. Although there is reluctance in using herbs to prevent and reduce dependence of allopathic medication, the numerous *in vitro* and *in vivo* results registered have demonstrated the hypoglycaemic effect. Herbs that are known to have such an effect come particularly from the Indo-Asian part of the world. *In vivo* studies on humans are a few, but those performed in laboratory have demonstrated clear effects associated with low toxicity products [2].

According to recent studies, flavonoid components express the hypoglycaemic effect, in the detriment of other classes of biologically active components. In the folk medicine of Romania, *Urtica dioica*, a common plant has been reported to show hypoglycaemic activity. Mainly, rutin and catechin are the compounds that express this effect and are directly associated with an antioxidant effect too. Oxidative stress, mainly, is the cause of decreased tolerance of human body at a high concentration of glucose in blood [3]. Although the results of herbal products with hypoglycaemic effects are still diminished, it is likely that the entire phenolic component will play an important role as this metabolic dysfunction determines the increase of the amount of superoxide anion. This is mainly inhibited by the action of the entire phenolic fraction which is involved in antioxidant and anti-inflammatory actions [4]. The purpose of this study was to demonstrate a relationship between the chemical composition (the biologically active component) and the antioxidant and hypoglycaemic potential of the extracts of *Momordica charantia* (fruits), *Vaccinium myrtillus* (fruit and leaves) and *Vaccinium vitis-idaea* (fruits and leaves) using various *in vitro* methods.

Experimental part

Samples. M. charantia (fruits – E1), *V. myrtillus* (fruit – E2 and leaves – E3) and *V. vitis-idaea* (fruits – E3 and leaves – E5) were dried and previously obtained from nature. Only the clean, organically certified samples were used. In the freshly harvested bitter cucumber, maximum length of 10–15 cm in raw status, maximum quantity of bitter substance is present. In this study, the bitter cucumber is used in dry form. Blueberries and forest cranberries (fruits) were harvested fresh when they were very ripe. Leaves were harvested in spring when they were very young before entering the fruiting cycle. Fruits were used raw or frozen at -25°C, and leaves were used that were only naturally dried.

Obtaining dried extracts

Samples were ground with a mechanical mill until a fine powder was obtained. This powder together with demineralized water was placed in a mechanical shaker with palaces (Hypericum Impex, Romania). They were mixed and the extraction enzyme was added (Rapidase FC). It was maintained at a constant temperature of 25–30°C, *p*H 4.5–5 for 3–4 h followed by pressing in a mechanic or hydraulic press. The resulted substance (the dry residue) was introduced in a steam infusion (own design). The aqueous extract resulted from this stage was mixed with the one obtained in the cold procedure by enzymatic procedures and dried in a Spray-Dryer (GEA Niro, Danemarca). Maltodextrin was used as atomization medium.

The same protocol was used for blueberries and cranberries and in addition to Rapidase FC, one more

* *email: email@emanuelvamanu.ro; Tel.: (+40) 0742218 240*

enzyme Rapidase Smart Color for the cold extraction was used with a maximum performance of the tannins and colour from fruits. The process takes approximately 7 h at a temperature of approximately 40°C. Both enzymes have maximum activity at these temperatures.

Evaluation of hypoglycaemic activity

Two *in vitro* enzymatic methods, namely, α-amylase and α-glucosidase inhibitory assay (A.F. Olympikon, Arad, Romania) have been used, which included dilutions between 1 and 20 mg/mL for analysis. Both methods used acarbose as standard.

The hypoglycaemic effect was determined by absorbance at 540 nm for α-amylase activity and at 405 nm for α - glucosidase activity. The inhibitory activity was calculated as percentage of inhibition [5, 6, 7].

Evaluation of antioxidant activity

In vitro methods have been used, methods that included for analysis dilutions between 0.2 and 1 mg/mL: total antioxidant activity, DPPH scavenging activity and reducing power.

For total antioxidant activity (TAA) a phospho-molybdenum method was used. TAA was calculated using the formula: TAA (%) = $[(A_C - A_S)/A_C] \times 100$, where A_C is the absorbance of the control at 765 nm, and A_S is the absorbance of the sample at 765 nm detected using a Helios λ spectrophotometer (Thermo Fisher Scientific Inc., USA) [8, 9]. DPPH scavenging activity was determined at 517 nm. The decrease of DPPH solution absorbance in presence of sample (25 μg/mL) means an increase of DPPH antiradical activity. DPPH scavenging activity was calculated using the formula: DPPH scavenging activity (%) = $[(A_C - A_S)/A_C] \times 100$, where A_C is the absorbance of the control, and A_S is the absorbance of the sample [10, 11, 12]. Reducing power was determined by the measurement of formation of Perl's Prussian blue at 700 nm [13]. For all tests, ascorbic acid was used as standard.

Determination of bioactive phytocompounds

Total phenolic and flavonoid content

Calculation of the total phenolic content used Folin-Ciocalteu reagent (Sigma Aldrich Chemical Co., Germany), and calculation of the total flavonoid content used the aluminium chloride colourimetric method [14].

The determination of polyphenol carboxylic acids and flavones was performed by HPLC (ELITE – LaChrom with DAD detector) and was presented in a previous study [10, 15].

Statistical analysis

All parameters for antioxidant and hypoglycaemic activities were assessed in triplicate, and results were expressed as a mean ± standard deviation (SD) of three observations. Mean values and SDs were calculated using the EXCEL programme from the Microsoft Office 2010 package (Microsoft®; Microsoft Corp., Redmond, WA, USA). Statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, California, USA) with a significance level of $p < 0.05$. Correlation analysis (R^2) was carried out using the Microsoft EXCEL (Microsoft®; Microsoft Corp.) [14, 15].

Results and discussions

Antioxidant Effect

DPPH Scavenging Activity

The ability to scavenge DPPH radicals is one of the most recommended methods to determine the antioxidant properties of the product. The biologically active molecules scavenge the DPPH radicals by the hydrogen atom [16]. Figure 1 shows the capacity of the five extracts to scavenge DPPH radicals compared with ascorbic acid as a standard. Thus, at a concentration of maximum 20 mg/mL, ascending order of DPPH scavenging activity was: *M. charantia* (fruits) < *V. myrtillus* (fruit) < *V. vitis-idaea* (fruits) < *V. vitis-idaea* (leaves) < *V. myrtillus* (leaves). It was found that the extract of leaves of *V. vitis-idaea* had a similar activity to that of the ascorbic acid (as standard), at the same maximum concentration of the sample. The fruits of *M. charantia* showed reduced activity whose EC_{50} value exceeded 75 mg/mL. These results were in contrast with the lyophilized aqueous extract, whose inhibition of the DPPH radical exceeded 50%, at a minimum concentration of 0.2 mg/mL [17].

Ferric Reducing Power

Determination of the reducing power is a direct indicator of the antioxidant capacity of an extract. According to the method used, the reaction mixture coloured in different shades of green and blue in proportion to the reducing power of the extracts atomized. Proportional with the quantity of biologically active compounds (with antioxidant potential) the atomized reduced the complex Fe^{3+} to Fe^{2+} and transformation was measured spectrophotometrically at 700 nm [18]. The results showed that the power to reduce extracts increased with the increase of the concentration of the samples. At the concentration of 20 mg/mL, the ascending order was: *M. charantia* < *V. vitis-idaea* (fruits) < *V. myrtillus* (fruit) H" *V. vitis-idaea* (leaves) < *V. myrtillus* (leaves). Differences registered between extracts were of maximum 5% and with 10% lower compared to the ascorbic acid at a concentration of 1 mg/mL (data not shown). According to studies conducted on certain biologically active extracts (herbal or medicinal mushrooms), the increase of the reduction power is

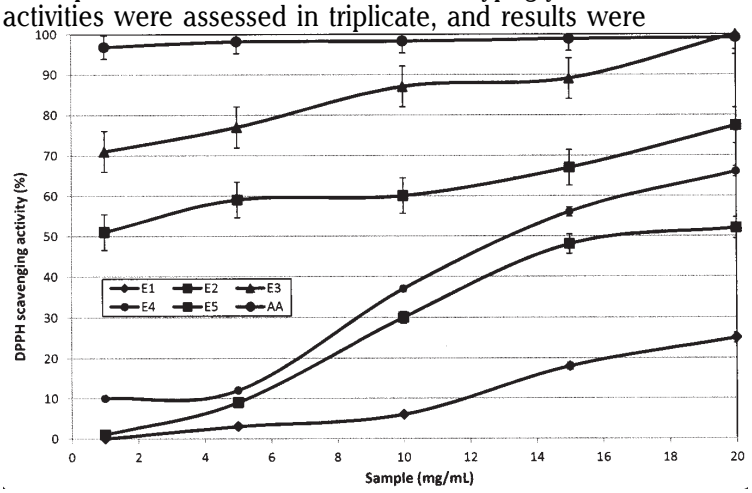

Fig. 1. DPPH scavenging activity of spray-dried extracts after enzymatic extraction process

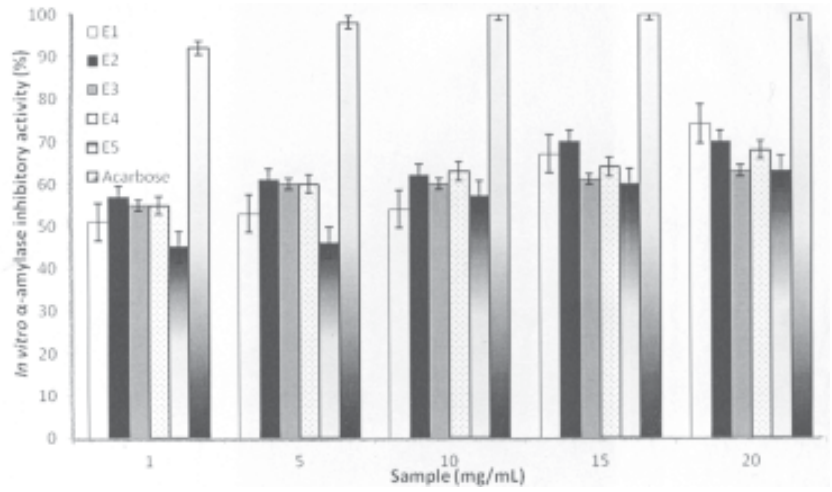

Fig. 2. In vitro α-amylase inhibitory activity of spray-dried extracts after enzymatic extraction process

proportional to the increase of the stabilization power and inhibition of the generation of free radicals [19].

Total Antioxidant Activity

Depending on the amount of antioxidant molecules, the phosphomolybdate ion reduction and formation of the green phosphate/MoV complex [8] take place. *V. myrtillus* (fruit and leaves) and *V. vitis-idaea* (leaves) showed the highest values of the total antioxidant activity, determining at a concentration of 20 mg/mL an average value of over 13.64 equivalents μg ascorbic acid/g extract (data not shown). For this parameter, *M. charantia* (fruits) presented approximately 15% lower values, which indicated a much higher correlation than with the other methods. Compared to other studies, the results from our study can be considered similar in terms of this parameter, but the literature data are few because the extraction process is different. All these studies have demonstrated that the expressed value is mainly influenced by the content of flavonoids [20].

Hypoglycaemic effect

M. charantia is known in alternative medicine as having a strong hypoglycaemic effect, which is associated with pronounced bitter taste. Thus, α-amylase and α-glucosidase inhibition assay were used for the evaluation *in vitro* hypoglycaemic properties of the extracts obtained after the enzymatic extraction process. Inhibition of the activity of such enzymes will reduce, related to persons in the risk groups, the level of glucose absorbed [21]. Acarbose was used as a standard (fig. 2). Extracts of leaves showed the lowest inhibitory activity on α-amylase with an EC_{50} value between 5 and 10 mg/mL. Otherwise, extracts had EC_{50} values below 1 mg/mL. At maximum concentration of 20 mg/mL, the extract of M. charantia (fruits) had the maximum inhibitory value of 74 ± 2.00%, which was lower by an average of 23% compared to acarbose (as standard).

In contrast, extracts have shown in vitro lower inhibition of α-glucosidase activity with an EC_{50} value that exceeded 20 mg/mL. *V. myrtillus* (fruit) showed maximum inhibitory activity at 20 mg/mL (38 ± 1.50%) with approximately 35% higher than that with the *M. charantia* (fruits) and with the leaves of the same plant (data not show). These values are lower than other hydro alcoholic extracts of *Citrus medica* L., for example [4]. These studies show that depending on the maturity stages of the fruit, the biological activity may be improved mainly by using it early during the harvest period.

Phytochemical composition

The phenolic composition analysis performed at 350 and 370 nm was similar to the previous studies and is presented in table 1. Compared to these studies, the number of compounds identified was lower; however, this type of extractive process resulted in a much higher quantity [22]. Differences registered between the compounds identified and total quantities show that there are other compounds that have not been determined within the study. Thus, a single phenolic compound was identified, namely, chlorogenic acid (fig. 3 – peak 1), and four other types of flavonoids.

Flavonoids identified are represented by a*glycone and glycoside flavonoids. Rutin is present only in the extract of V. myrtillus* (fruit) (fig. 3 – peak 2). Hyperoside, shown in the same figure (peak 3) is found between 0.006 ± 0.001 and 0.14 ± 0.008 mg/g extract. Quercetin was determined in all the extracts being a widely spread compound in herbal extracts and extracts from medicinal mushrooms. A similar quantity was determined in extracts of *V. myrtillus* (leaves) and *V. vitis-idaea* (leaves), which was at least 10 times higher than in the fruits of the same plant (fig. 4 – peak 1). Moreover, kaempferol was identified exclusively

Compound	*Momordica charantia* (fruits)	*Vaccinium myrtillus* (fruit)	*Vaccinium myrtillus* (leaves)	*Vaccinium vitis-idaea* (fruits)	*Vaccinium vitis-idaea* (leaves)
Total phenols (mg/g)	0.0918±0.02	0.885±0.003	7.23±0.1	0.022±0.005	0.189±0.006
Chlorogenic Acid (mg/g)	-	0.0325±0.001	1.075±0.001	-	0.0287±0.0001
Total flavonoids (mg/g)	0.5	4.75±0.04	3.40±0.01	0.59±0.01	0.83±0.06
Aglycone flavonoids					
Quercetin (mg/g)	0.0005	0.0311±0.001	0.3239±0.02	0.0167	0.3427±0.005
Kaempferol (mg/g)	-	-	0.001±0.0005	-	0.002±0.0004
Glycosidic flavonoids					
Rutin (mg/g)	-	0.03±0.006	-	-	-
Hyperoside (mg/g)	-	0.14±0.008	0.06±0.002	-	0.006±0.001

Table 1
COMPOUNDS WITH ANTIOXIDANT EFFECTS

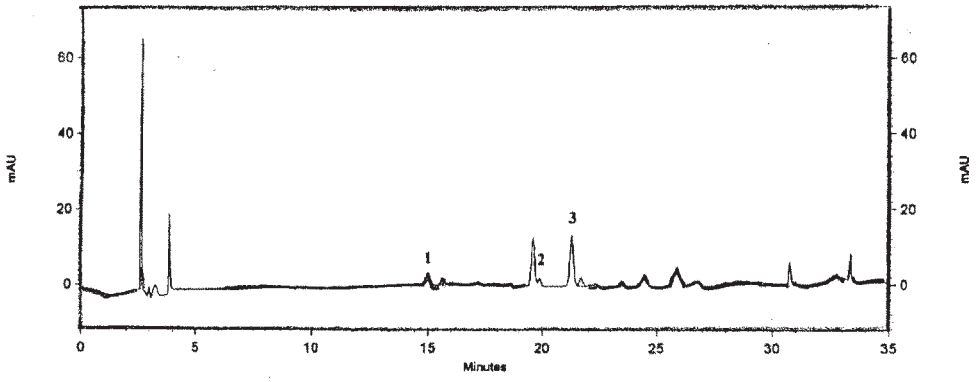

Fig. 3. The phenolic profile of the extract of *Vaccinium myrtillus* (fruit) at 350 nm

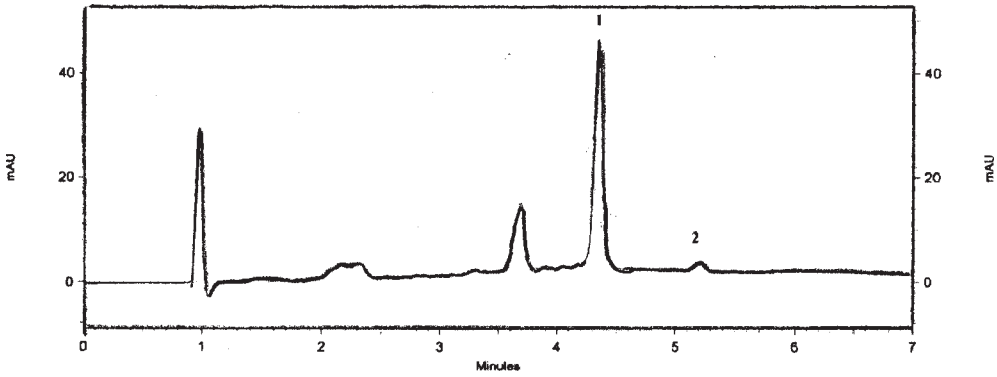

Fig. 4. Phenolic profile of the extract of *Vaccinium myrtillus* (leaves) at 370 nm

in the extracts of the two plants (fig. 4 – peak 2) with small differences between samples [22].

Total phenolic content was expressed as gallic acid equivalent in mg/g extracts and is shown in table 1. Results showed an average phenolic content; all five enzymatic extracts showing phenolic compounds. *V. myrtillus* (fruit and leaves) had the highest values of these compounds ($p < 0.05$). The extract of leaves of *V. myrtillus* had a content of approximately 7 times higher than that of the fruit. Low values (below 0.1 mg/g extract) were determined in extracts of the fruits of *V. vitis-idaea* and *M. charantia* [23].

Compared with other studies that use methods of enzymatic extraction, significant differences have been noticed ($p < 0.05$) between extracts. It can be interpreted according to such studies that the hydrolysis capacity of the cellular wall (even between fruits and leaves from the same plant) is different. This type of extraction based on the use of enzymes is more adequate to degradation of polysaccharides present in leaves, while the peel of the fruits is less susceptible to such treatment. Thus, the leaves of the two plants contain more structural types of flavonoids that will directly express the antioxidant and hypoglycaemic potential. Among flavonoids identified, quercetin is the one that majorly influences the antioxidant effect, which is also confirmed in previous studies [24]. Also, according to previous studies, quercetin is responsible for the inhibitory action on α-amylase [20].

As in other previous studies, no direct correlation could be identified between the hypoglycaemic activity of the phenols and flavonoid content. The extract of *V. myrtillus* (fruits), by the content of rutin, significantly contributed to the inhibitory activity of α-glucosidase, R^2 showed values of over 0.7. Otherwise, extract analysed with high phenolic content showed the highest antioxidant and hypoglycaemic activity. The antioxidant activity had a maximum correlation, for leaves extracts (over 0.9), while fruit extracts showed at least an average correlation degree with the two biological activities analysed. As in previous studies, the extraction processes result in complex phytochemical products that determine a sole biological response [25].

In conclusion, the study presented preliminary phytochemical, antioxidant and hypoglycaemic analysis of an enzymatic extractive process. Thus, *in vitro* studies have demonstrated that products made of *V. myrtillus* remain some of the most complex supplements with various biological activities, regardless of the technology applied. These preliminary studies must be followed by *in vivo* studies to conform, mainly, the capacity to reduce the uptake of glucose. Also, it must be identified which bioactive compound directly intervenes in these physiological mechanisms. Further study is required by conducting an optimization step of the extractive process.

Acknowledgement: This research was financed by means of the doctoral programme of the USAMV Bucharest, Romania. The chromatographic analysis was made within the Department for Physical-Chemical Analysis and Quality Control, under the coordination of Chemist, Sultana Nita, PhD and financed by SC Hypericum SRL.

References

1. TAMIL I.G., DINESHKUMAR B., NANDHAKUMAR M., SENTHILKUMAR M., MITRA A., Indian J. Pharmacol., **42,** 2010, p. 280
2. HAMDAN I.I., AFIFI F.U., J. Ethnopharmacol., **93**, 2004, p. 117
3. COMAN C., RUGINĂ O.D., SOCACIU C., Not. Bot. Horti. Agrobo., **40**, 2012, p. 314
4. MENICHINI F., LOIZZO M.R., BONESI M., CONFORTI F., DE LUCA D., STATTI G.A., DE CINDIO B., MENICHINI F., TUNDIS R., Food Chem. Toxicol., **49**, 2011, p. 1549
5. AHMAD Z., ZAMHURI K.F., YAACOB A., SIONG C.H., SELVARAJAH M., ISMAIL A., HAKIM M.N, Molecules, **17**, 2012, p. 9631
6. OBOH G., ADEMOSUN A.O., ADEMILUYI A.O., OMOJOKUN O.S., NWANNA E.E., LONGE K.O., Path. Res. Int., Article ID 549287, 2014, p. 1
7. OLUBOMEHIN O.O., ABO K.A., AJAIYEOBA E.O., J. Ethnopharmacol., **146**, 2013, p. 811.
8. JAN S., KHAN M.R., RASHID U., BOKHARI J., Osong Public Health Res. Perspect, **4**, 2013, p. 246
9. VAMANU E., NITA S., Rev. Chim.(Bucharest), **65**, no. 3, 2014, p. 372
10. SHIHABUDEEN H.M.S., PRISCILLA D.H., THIRUMURUGAN K., Nutr. Metab., 46, 2011, p. 1

11. KOKILAM G., VASUKI S., SAJITHA N., J. Appl. Pharm. Sci., 3, 2013, p. 99
12. GUO T., WEI L., SUN J., HOU C., FAN L., Food Chem., **127**, 2011, p. 1634
13.ZHU K.X., LIAN C.X., GUO X.N., PENG W., ZHOU H.M., Food Chem., **126**, 2011, p. 1122
14. VAMANU E., NITA S., Food Sci. Biotechnol., **23**, 2014, p. 1483
15. VAMANU E., PELINESCU D., AVRAM I., NITA S., BioMed Res. Int., Article ID 289821, 2013, p. 1
16. MA L., CHEN H., ZHU W., WANG Z., Food Res. Int., **50**, 2013, p. 633
17. KUBOLA J., SIRIAMORNPUN S., Food Chem., **110**, 2008, p. 881
18.ZHU K.X., LIAN C.X., GUO X.N., PENG W., ZHOU H.M., Food Chem., **126**, 2011, p. 1122
19. BARROS L., FERREIRA M.J., QUEIRO´S B., FERREIRA I.C.F.R., BAPTISTA P., Food Chem., **103**, 2007, p. 413
20. AKTUMSEK A., ZENGIN G., GULER G.O., CAKMAK Y.S., DURAN A., Food Chem. Toxicol., **49**, 2011, p. 2914
21. MENICHINI F., LOIZZO M.R., BONESI M., CONFORTI F., DE LUCA D., STATTI G.A., DE CINDIO B., MENICHINI F., TUNDIS R., Food Chem. Toxicol., **49**, 2011, p. 1549
22. MARTINS N., BARROS L., SANTOS-BUELGA C., SILVA S., HENRIQUES M., FERREIRA I.C.F.R., Food Chem., **167**, 2015, p. 131
23. HASSAN L.E.A., AHAMED M.B.K., MAJID A.S.A., BAHARETHA H.M., MUSLIM N.S., NASSAR Z.D., MAJID A.M.S.A., BMC Complement. Alt. Med., **14**, 2014 p. 1
24. NAVARRO-GONZÁLEZ I., GARCÍA-VALVERDE V., GARCÍA-ALONSO J., PERIAGO M.J., Food Res. Int., **44**, 2011, p. 1528
25. GHASEMI K., GHASEMI Y., EBRAHIMZADEH M.A., Pak. J. Pharm. Sci., **22**, 2009, p. 277

Manuscript received: 28.01.2015