Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *82* (2) 141–150 (2017)
JSCS–4954
UDC 582*Umbilicaria crustulosa*: 633.879.6:
547.284.3:577–188:615.279:612.112
Original scientific paper

Biological activities of *Umbilicaria crustulosa* (Ach.) Frey acetone extract

IVANA ZLATANOVIĆ[1]*, MIROSLAVA STANKOVIĆ[2], VESNA STANKOV JOVANOVIĆ[1], VIOLETA MITIĆ[1], IVANA ZRNZEVIĆ[1], ALEKSANDRA ĐORĐEVIĆ[1], and GORDANA STOJANOVIĆ[1]

[1]*Department of Chemistry, Faculty of Science and Mathematics, University of Niš, Višegradska 33, 18000 Niš, Serbia and* [2] *Nuclear Facilities of Serbia, Mike Petrovića Alasa 12–14, 11351 Vinča, Serbia*

(Received 19 October, revised 16 December, accepted 21 December 2016)

Abstract: This paper reports for the first time the effect of an acetone extract of *Umbilicaria crustulosa* on the micronucleus distribution of human lymphocytes, and on the cholinesterase activity and antioxidant activity by the cupric ion reducing antioxidant capacity (CUPRAC) method. Additionally, the total phenolic compounds (TPC) and the antioxidant properties were estimated *via* DPPH, ABTS and TRP assays. Moreover, the antibacterial activity against two Gram-positive and three Gram-negative bacteria were determined. Acetone extract of *U. crustulosa* at concentration of 1 and 2 μg mL^{-1} decreased a frequency of micronuclei (MN) by 10.8 and 16.8 %, respectively, acting more or slightly less than the synthetic protector amifostine (AMF, WR-2721, 11.4 %, at concentration of 1 μg mL^{-1}). The tested extract did not inhibit cholinesterase activity nor did it exhibit activity toward the examined bacteria. The extract reduced the concentration of DPPH and ABTS radicals by 88.7 and 96.2 %, respectively. Values for total reducing power (TRP) and cupric reducing capacity (CUPRAC) were 0.6197±0.0166 μg ascorbic acid equivalents (AAE) per mg of dry extract, and 19.7641±1.6546 μg trolox equivalents (TE) per mg of dry extract, respectively. The total phenol content was 350.4188 ±14.587 μg gallic acid equivalents (GAE) per mg of dry extract. The results of the present study showed that *U. crustulosa* acetone extract is a promising candidate for *in vivo* experiments considering its antioxidant activity and protective effect on human lymphocytes.

Keywords: micronucleus test; antioxidant activity; cholinesterase inhibition; antimicrobial activity; chemical composition.

* Corresponding author. E-mail: ivana.z.988@gmail.com
doi: 10.2298/JSC161019014Z

INTRODUCTION

Lichens of the genus *Umbilicaria* are generally known as rock tripe. These species grow on siliceous rocks usually in the Alpine vegetation zone and the Northern hemisphere.[1] Some of these species are used as food and represent a part of folk medicine.[2,3] *Umbilicaria crustulosa* (Ach.) Frey is known as a crusty novel lichen. Serina and Arroyo found two *U. crustulosa* chemotypes in samples growing in Spain: gyrophoric acid and crustinic acid chemotype. Beside crustinic and gyrophoric acids, *Umbilicaria* species are characterized by the presence of lecanoric, hiascic, ovoic, umbilicaric, salazinic and norstictic acids.[3,4]

The presence of phenolic groups in the structure of the constituents of *U. crustulosa* extracts results in their antioxidant activity.[3,5] Antioxidants could prevent oxidation of biomolecules in food as well as in human cells. Industrial antioxidants are mostly synthetic components which could have negative impacts on human health.[6] Natural antioxidants are very important source of protection against free radicals, which could be related to many disease such as Alzheimer's disease, atherosclerosis, emphysema and many forms of cancer.[7,8] A decrease in the amount of acetylcholine in the brain is the hallmark of Alzheimer's disease. Some synthetic cholinesterase inhibitors are used for the treatment of disease but an alternative approach could be the utilization of natural anticholinesterases.[9] According to published data, *Hypogimnia physodes* extracts and lichens secondary metabolites have an impact on micronucleus (MN) expression in peripheral blood lymphocytes.[10,11] Micronuclei result from lesions/adducts at the level of DNA or chromosomes, or at the level of proteins directly or indirectly involved in chromosome segregation. The cytokinesis-block micronucleus technique enables micronuclei to be specifically scored in cells that have completed nuclear division and is, therefore, not influenced by variations in cell division kinetics, and it has been shown to be a sensitive and reliable index of chromosome damage.[12]

Taking into account the above-mentioned biological activities of the lichens, the aim of present study was to evaluate for the first time effect of *U. crustulosa* acetone extract on the micronucleus distribution in human lymphocytes, and the effect on cholinesterase and antioxidant activity by the CUPRAC method.

Additionally, total phenolic compounds (TPC), the antioxidant properties estimated *via* 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging radical capacity, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity and TRP (total reduction power) assays as well as the antibacterial activity against two Gram-positive (*Bacillus subtilis* subsp. *spizizenii* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) and three Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017) were determined.

EXPERIMENTAL

Lichen material

The lichen *Umbilicaria crustulosa* (Ach.) Frey (syn. *Gyrophora depressa* (Ach.) Schaer, *Omphalodiscus crustulosus* (Ach.) Schol, *Gyrophora crustulosa* (Ach.), *Gyrophora spodochroa* var. *crustulosa* (Ach.) Arnold, *Gyrophoropsis crustulosa* (Ach.) Räsänen) was collected in May 2015, from the peak of Mount Babin zub (1650 m above sea level, coordinates 43° 23′ N, 22° 40′ E; Stara planina Mountain, Serbia) from the population growing on rock habitat (red sandstone, silicates). A voucher specimen has been deposited in the Herbarium collection at the Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš under the acquisition number 9373. The lichen material was air-dried without exposure to direct sunlight for 10 days and stored at ambient temperature (25±2 °C) prior to further treatment.

Extraction

Extracts were prepared in triplicate according to the following procedure: powdered lichen material (10 g) was first subjected to ultrasound-assisted extraction with acetone (50 mL; 30 min in an ultrasound bath, type UZK 8; manufactured by Maget, Bela Palanka, Serbia. Subsequently, the extract was left in the dark (at room temperature) for an additional five days. Dry residues of the extracts were obtained using a rotary evaporator with the water bath set at 40 °C. The extract yield was 7.4±0.5 %.

HPLC analysis

HPLC analysis was performed on an Agilent, Zorbax Eclipse XDB-C18, 5 μm, 4.6 mm×150 mm column using an Agilent 1200 series liquid chromatograph (equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a quaternary pump, an online vacuum degasser, auto sampler and a thermostated column compartment. The mobile phase, methanol–water–formic acid (80:20:0.2 volume ratios), was pumped at a flow rate of 0.5 mL min^{-1}, the injection volume was 5 μL (concentration 10 mg of the dry extract per 1 mL of acetone), at 25 °C. The spectra were acquired in the range 190–400 nm and the chromatogram plotted at 254 nm. Identification was conducted using retention times and UV spectra.

Cytokinesis-block micronucleus assay (CBMN)

The cytokinesis-block micronucleus assay was performed as previously described.[10,12] Cell culture lymphocytes were treated with 1.0, 2.0 and 3.0 μg mL^{-1} of the examined extracts. Amifostine, WR-2721 (98 % *S*-[2-[(3-aminopropyl)amino]ethyl]phosphothioate; Marligen Biosciences) at a concentration of 1 μg mL^{-1} was used as a positive control. Three experiments were performed for each sample. The results are expressed as the means ± standard deviation (*SD*).

The statistical analysis was performed using Origin software package version 7.0. The statistical significance of difference between the data pairs was evaluated by analysis of variance (one-way ANOVA) followed by the Tukey test. Statistical difference was considered significant at $p < 0.01$ and $p < 0.05$.

Total phenolic content and antioxidant activity

Determination of the total phenolic content (TPC) and four antioxidant assays, *i.e.*, DPPH and ABTS scavenging radical capacity, CUPRAC (cupric reducing antioxidant capacity) and TRP (total reducing power), were performed as previously described.[13,14] All spectrophotometric assays were conducted on a Perkin Elmer lambda 15 double beam UV/Vis spectrophotometer. For all the above-mentioned experiments, the concentration of the sample

solution was 15 mg of extract per mL of methanol. All analyses were performed in triplicate. The results are presented as mean ± standard deviation (*SD*).

Cholinesterase activity

Assessment of the effect of the extract on cholinesterase activity was performed as previously described.[15] The activity was measured spectrophotometrically using a Konelab 20 analyzer (Thermo Fisher Scientific, Helsinki, Finland) with thermostated flow cells, length 7 mm (at a wavelength 405 nm). The sample concentration was 10 mg of dry extract per 1 mL of DMSO. A solution of neostigmine bromide at a concentration of 200 μg mL^{-1} was used as the reference standard.

Antimicrobial activity

Antimicrobial activity was evaluated against two Gram-positive (*Bacillus subtilis* subsp. *spizizenii* ATCC 6633 and *Staphylococcus aureus* ATCC 6538) and three Gram-negative (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017) bacteria. Analysis was performed according to the National Committee for Clinical Laboratory Standards (NCCLS).[16] Each test was performed in triplicate. The sample concentration was 1 mg per disk (diameter 12 mm). Streptomycin and chloramphenicol were used as positive controls, at a concentration of 10 mg and 30 mg per disk, respectively.

RESULTS AND DISCUSSION

HPLC analysis

The HPLC chromatogram of the acetone extract of *U. crustulosa* and the UV spectra of the identified constituents are presented in Fig. 1.

Chemical composition and structures of the constituents are given in Table I. The most abundant component of the acetone extract of *U. crustulosa* is the tridepside gyrophoric acid and this sample could be classified as a gyrophoric acid chemotype.[4]

The major components of the extract were the didepside lecanoric acid and the tridepside gyrophoric acid, comprised of two and three, respectively, orsellinic acid units joined with ester bonds at the *para*-positions. Crustinic acid is an exception, as it has the ester bonded at the *meta*-position of the third ring. No direct evidence is available on the biosynthesis of *meta*-depsides. The co-occurrence and interconversion of the depsides *meta*- and *para*-scrobiculin, which exist in dynamic equilibrium, in *Lobaria scrobiculata* led to the hypothesis that *meta*-depsides are formed from *para*-depsides *via* an intramolecular rearrangement after hydroxylation of the carbon at the *meta*-position of the B-ring.[17]

Cytokinesis-block micronucleus assay (CBMN)

The extract was tested for *in vitro* protective effect on chromosome aberrations in peripheral human lymphocytes using the cytochalasin-B blocked MN assay at concentrations of 1.0, 2.0 and 3.0 μg mL^{-1}. The frequencies and distribution of MN in human lymphocytes were scored. The statistical significance of difference between the data pairs was evaluated by analysis of variance (One-way ANOVA) followed by the Tukey test. The results are presented in Table II.

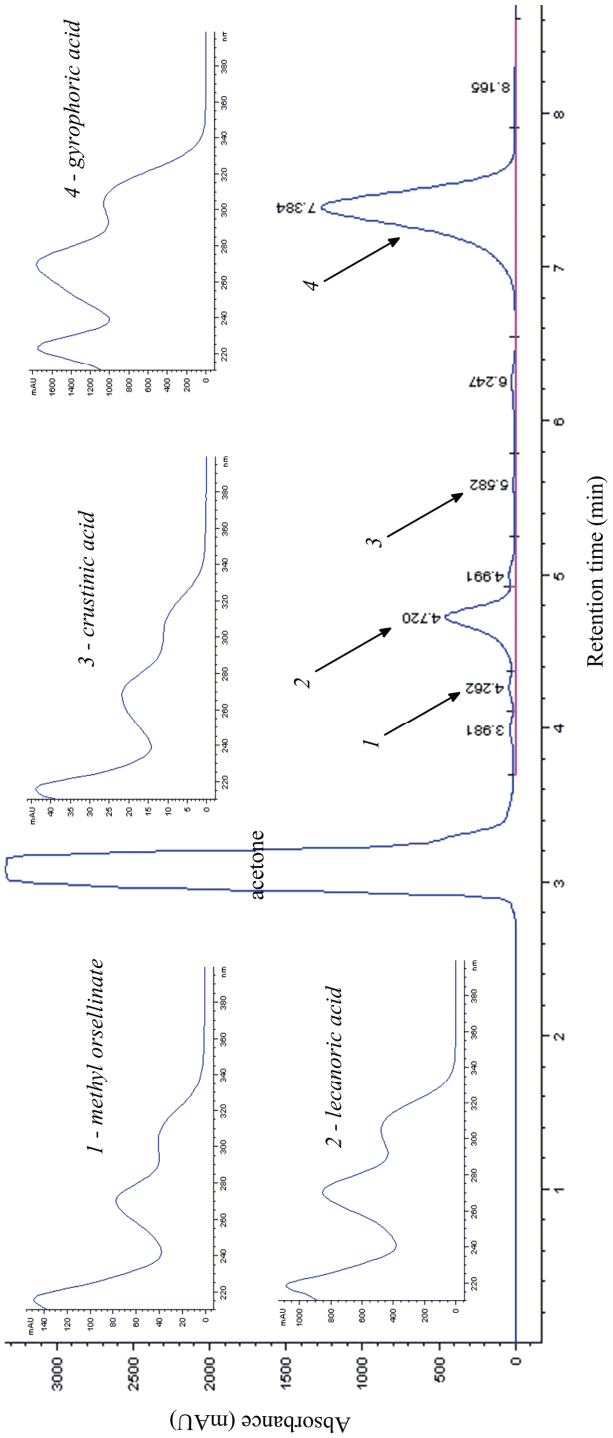


Fig. 1. HPLC Chromatogram of *U. crustulosa* acetone extract and UV spectra of identified constituents.

TABLE I. Composition (as percentages of the total peak area of the HPLC chromatograms recorded at 254 nm) and structures of the constituents of the *U. crustulosa* extract

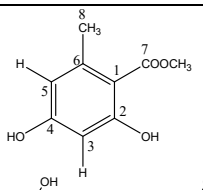
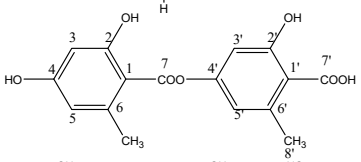
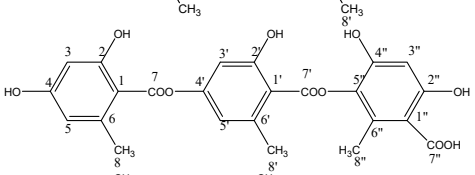
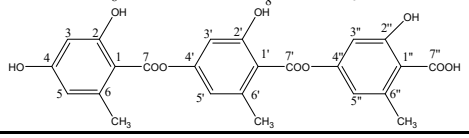
Component number	Cmpd.	Structure	*Rt* / min	HPLC peak area, %
1	Methyl orsellinate		4.262	2.1
2	Lecanoric acid		4.720	23.5
3	Crustinic acid		5.582	0.1
4	Gyrophoric acid		7.384	68.6

TABLE II. Incidence of MN, cytokinesis-block proliferation index, distribution of MN per cells (mean ± *SD*) and frequency of MN in cell cultures of human lymphocytes treated with different concentration of *Umbilicaria crustulosa* acetone extract (AE); MN/1000 Bn cells – incidence of micronuclei in 1000 binucleated cells; % Bn cells with micronuclei; MN/Bn cells – incidence of micronuclei in binucleated cells; CBPI – cytokinesis-block proliferation index; Frequency of MN – incidence of MN present as % from control groups in cell cultures of human lymphocytes treated with different concentration of extracts; a* – statistically significant difference from control at $p < 0.05$

Concentrations	MN/1000 Bn cells	Bn cells with MN, %	MN/Bn Cells	*CBPI*	Frequency of MN rel. to control, %
Untreated cells	25.83±0.58	2.06±0.12	1.25±0.06	1.63±0.03	100.00
Amifostin (1 μg mL^{-1})	22.89±0.68^{a*}	1.87±0.06	1.22±0.06	1.62±0.04	88.62
AE (1 μg mL^{-1})	23.05±1.06	1.89±0.08	1.22±0.06	1.68±0.06	89.24
AE (2 μg mL^{-1})	21.60±1.68	1.62±0.13	1.34±0.13	1.63±0.01	83.62
AE (3 μg mL^{-1})	24.24±1.21	2.06±0.12	1.18±0.04	1.63±0.02	93.84

The cell cultures treated with amifostine (radioprotectant, previously known as WR-2721) at a concentration of 1 μg mL^{-1} resulted in a decrease of the MN frequency of 11.4 % compared to the control cell cultures (statistically significant, $p < 0.05$). The *U. crustulosa* extract at a concentration of 2 μg mL^{-1} gave a

decrease in the MN frequency of 16.38 %, which was higher than that with amifostine, while at a concentration of 1 μg mL^{-1}, the effect was 10.8 %, which is slightly less than the effect of amifostine. The *U. crustulosa* extract at a concentration of 3 μg mL^{-1} was less effective (6.2 %) than amifostine.

Since the number of micronuclei serves as an indicator of DNA damage, these results indicate that the examined extract at a concentration of 2 μg mL^{-1} protects DNA. Previous results of *H. physodes* extracts suggested a similar conclusion. A diethyl ether extract of the lichen *H. physodes* at a concentration of 1 μg mL^{-1} caused a significant decrease in the frequency of micronuclei, 17.4 %, while it was less effective at a concentration of 2 μg mL^{-1}.[11]

The effect of examined extracts on cell proliferation was investigated by determining the cytokinesis-block proliferation index (*CBPI*), calculated as $[(M_I + 2M_{II} + 3(M_{III} + M_{IV})]/N$, where $M_{I–IV}$ represent the number of cells with 1 to 4 nuclei, respectively, and N is the number of cells scored.[18] The comparable *CBPI* values of extracts, amifostine WR-2721 and untreated cells have confirmed absence of the impact on the cell proliferation. This fact is important for validity of the results because MN expression is dependent on cell division.

The total phenolic content and antioxidant activity

TPC analysis showed a high content of phenolic components in the acetone extract of *U. crustulosa*, 350.4188±14.587 μg GAE mg^{-1} due to the presence of the tridepside gyrophoric acid and the didepside lecanoric acid (total 92.0 % of the sample). It was not possible to compare the present results expressed as galic acid equivalents (GAE) with those of Kosanic *et al.* because they expressed the amount of total phenolic compounds as the pyrocatechol equivalent.[19]

The assessment of the DPPH and ABTS scavenging activity showed that extract reduced the concentration of DPPH and ABTS radicals by 88.7 and 96.2 %, respectively, which is consistent with previously published results. Namely, Kosanic *et al.* found that the scavenging activity of a methanol extract of *U. crustulosa* was 79.85 %, while Buçukoglu *et al*. found that the values of the DPPH scavenging activity of gyrophoric and leconaric acids were 50.96 and 32.48 %, respectively.[19,20]

The value of the total reducing power ability (TRP, ability of antioxidants to reduce hexacyanidoferrate (III) to hexacyanidoferrate (II), which leads to an increase in the absorbance of the reaction mixtures) was 0.6197±0.0166 mg ascorbic acid equivalents (AAE) per mg dry extract weight. Again, it was not possible to compare the obtained results with those previously published due to the different expression of the results (absorbance *vs*. AAE).[19]

The CUPRAC assay has some advantages compared to the other experiments since the reagent is stable, accessible, low-cost, and the reaction (reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous

form (Cu(I)–Nc)) is realized at nearly physiological pH. The result obtained for the *U. crustulosa* acetone extracts (19.7641±0.0166 mg trolox equivalents (TE) per mg dry extract) is close to the highest CUPRAC capacity of mushrooms (21.738–4.164 mg TE per mg dw).[13]

Cholinesterase activity

Results obtained from the screening of the interaction of the extract with cholinesterase from pooled human serum showed that *U. crustulosa* possess a weak activation effect on the cholinesterase activity. In the conducted experiment, neostigmin bromide (commercial cholinesterase inhibitor) inhibited cholinesterase to extent of 96.6 % while *U. crustulosa* extract manifested weak activation effect on cholinesterase to an extent of 1.6 %.

Antibacterial activity

The results of the antibacterial assays against two Gram-positive *(B. subtilis* subsp. *spizizenii* ATCC 6633 and *S. aureus* ATCC 6538) and three Gram-negative bacteria (*E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027 and *S. abony* NCTC 6017) showed that the acetone extract of *U. crustulosa* had no activity against the tested bacteria at a concentration of 1 mg per disk. These results were not entirely in accordance with those previously published. Namely, Rankovic *et al*. and Kosanic *et al*. reported no activity toward *Escherichia coli, Botrytis cinerea and Candida albicans* but moderate antimicrobial activity of methanol and acetone extracts of *U. crustulosa* to several bacteria and fungi, such as*: Bacillus mycoides, Bacillus subtilis, Enterobacter cloaceae, Klebsiella pneumoniae, Staphylococcus aureus, Aspergillus flavus, Aspergillus fumigatus, Fusarium oxysporum, Mucor mucedo*, *Paecilomyces variotii , Penicillium purpurescens, Penicillium verrucosum***,** *Saccharomyces cerevisiae, Trichoderma harzianum.*[2,19] Bucukoglu *et al.* and Candan *et al.* found that gyrophoric acid was not effective against *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Salmonela typhimurium*.[20,21] Since gyrophoric acid participated 68.5 % to the extract composition, the results obtained in the present study could be explained by its inactivity.

CONCLUSION

The results of present study show that *U. crustulosa* acetone extract is a promising candidate for *in vivo* experiments considering antioxidant activity and protective effect on human lymphocytes, which could not be said for its antibacterial and anti-cholinesterase activity.

Acknowledgement. The authors acknowledge the Ministry of Education, Science and Technological Development of the Republic of Serbia for the financial support (Grant No. 172047).

ИЗВОД

БИОЛОШКА АКТИВНОСТ АЦЕТОНСКОГ ЕКСТРАКТА ЛИШАЈА *Umbilicaria crustulosa* (ACH.) FREY

ИВАНА ЗЛАТАНОВИЋ[1], МИРОСЛАВА СТАНКОВИЋ[2], ВЕСНА СТАНКОВ ЈОВАНОВИЋ[1], ВИОЛЕТА МИТИЋ[1], ИВАНА ЗРНЗЕВИЋ[1], АЛЕКСАНДРА ЂОРЂЕВИЋ[1] и ГОРДАНА СТОЈАНОВИЋ[1]

[1]*Департман за хемију, Природно–математички факултет, Универзитет у Нишу, Вишеградска 33, 18 000 Ниш и* [2]*Институт за нуклеарне науке Винча, Мике Петровића Аласа 12–14, 11351 Винча*

У овом раду је по први пут испитан утицај ацетонског екстракта лишаја *Umbilicaria crustulosa* (Ach.) Frey на дистрибуцију микронуклеуса у људским лимфоцитима, холинестеразну активност и антиоксидативну активност коришћењем CUPRAC методе. Укупни садржај фенола, антиоксидативна својства (процењена коришћењем DPPH, ABTS и TRP тестова), као и антибактеријска активност према две грам-позитивне и три грам-негативне бактерије су такође одређени. Ацетонски екстракт лишаја *U. crustulosa* у концентрацији од 1 и 2 μg mL^{-1} смањује фреквенцију микронуклеуса (МН) за 10,8 и 11,4 %, редом, делујући јаче или нешто слабије од комерцијалног заштитног агенса амифостина (AMF, WR-2721), 11,4 % у концентрацији 1 μg mL^{-1}). Испитивани екстракт није показао инхибицију активности холинестеразе, као ни активност према тестираним бактеријама. Екстракт смањује концентрацију DPPH и ABTS радикала за 88,7 и 96,2 %, редом. Вредности укупне редукционе моћи (TRP) и купрак-редукционог капацитета (CUPRAC) су 0,6197±0,0166 mg еквивалента аскорбинске киселине (AAE) по mg сувог екстракта и 19,7641±1,6546 mg тролокс еквивалента (TE) по mg сувог екстракта, редом. Укупни садржај фенола је 350,4188±14,587 mg еквивалената галне киселине по mg сувог екстракта. Резултати испитивања су показали да је *U. crustulosa* обећавајући кандидат за *in vivo* испитивања у погледу антиоксидативне активности и заштитног ефекта на људске лимфоците.

(Примљено 19. октобра, ревидирано 16. децембра, прихваћено 21. децембра 2016)

REFERENCES

1. E. Frey, *Ber. Schweiz. Bot. Ges*. **46** (1997) 412
2. B. Rankovic, M. Misic, S. Sukdolak, *Biologia* **64** (2009) 53
3. N. Manojlovic, P. Vasiljevic, P. Z. Maskovic, M. Juskovic, G. Bogdanovic-Dusanovic, *Evidence Based Complementary Altern. Med.* **2012** (2012), Article ID 452431
4. E. Serina, R. Arroyo, *Bryologist* **99** (1996) 335
5. M. E. Hidalgo, E. Fernandez, W. Quilhot, *Phytochemistry* **37** (1994) 1585
6. F. Shahidi, U. N. Anasundra, R. Amarowicz, *Food Res. Int.* **27** (1994) 489
7. K. Guddadarangavvanahally, R. Jayaprakasha, *Z. Naturforsch., C: J. Biosci.* **55** (2000) 1018
8. K. Molnar, E. Farakas, *Z. Naturforsch., C: J. Biosci.* **65** (2010) 157
9. E. Giacobini, *Drugs that target cholinesterase*, in *Cognitive Enhancing Drugs,* J. J. Buccafusco, Ed., Springer, Basel, 2004, p. 11
10. I. Stojanović, M. Stanković, O. Jovanović, G. Petrović, A. Šmelcerović, G. Stojanović, *Nat. Prod. Commun.* **8** (2013) 109
11. G. Stojanovic, M. Stankovic, I. Stojanovic, I. Palic, V. Milovanovic, S. Rancic, *Nat. Prod. Commun.* **9** (2014): 503
12. M. Fench, A. A. Morley, *Mutat. Res.* **285** (1993) 35
13. M. Dimitrijevic, V. Stankov Jovanovic, J. Cvetkovic, T. Mihajilov-Krstev, G. Stojanović, V. Mitic, *Anal. Methods* **7** (2015) 4181

14. R. Re, N. Pellegrini, A. Proreggente, A. Pannala, M. Yang, *Free Radical Biol. Med.* **26** (1996) 1231
15. V. Stankov Jovanovic, M. Ilic, V. Mitic, T. Mihajilov-Krstev, S. Simonovic, S. Nikolic--Mandic, J. C. Tabet, R. B. Cole, *J. Pharm. Biomed. Anal.* **111** (2015) 78
16. NCCLS (National Committee for Clinical Laboratory Standards), *Performance Standards for Antimicrobial Disk Susceptibility Test*, 6th ed., Approved Standard, (1997) M2-A6
17. J. A. Elix, K. L. Gaul, *Aust. J. Chem.* **39** (1986) 613
18. J. Surralles, N. Xamena, A. Creus, J. Catalan, H. Norppa, R. Marcos, *Mutat. Res*. **341** (1995) 169
19. M. Kosanic, S. Šeklic, M. Markovic, B. Rankovic, *Dig. J. Nanomater. Biostruct.* **9** (2014) 273
20. Z. T. Buçukoglu, S. Albayrak, G. Halici, Y. Tay, *J. Food Process. Preserv.* **37** (2013) 1103
21. M. Candan, M. Yilmaz, T. Tay, M. Kivanç, H. Turk*, Z. Naturforsch., C: J. Biosci.* **61** (2006) 319.