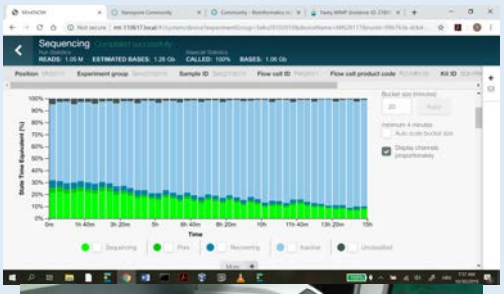
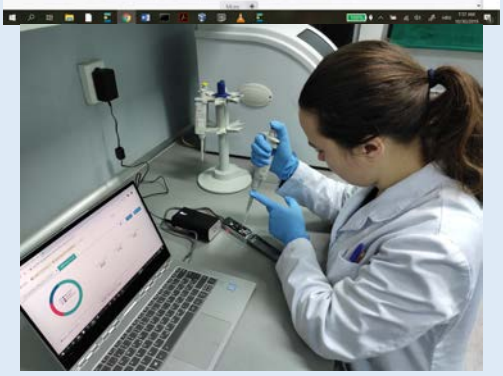
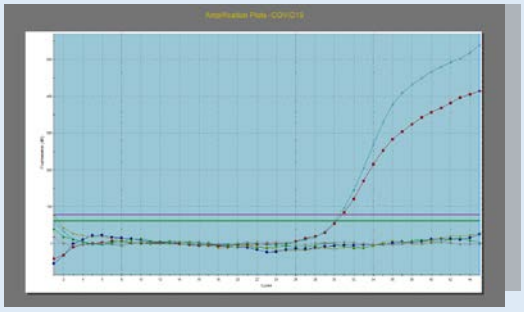
Glasnik hemičara i tehnologa Bosne i Hercegovine
Bulletin of the Chemists and Technologists of Bosnia and Herzegovina





Scientific research team of the Faculty of Veterinary Medicine, University of Sarajevo in cooperation with ANUBiH isolated "B&H" strain of COVID-19 virus



54

June, 2020.

Prirodno-matematički fakultet Sarajevo
Faculty of Science Sarajevo

Glasnik hemičara i tehnologa Bosne i Hercegovine
Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

54

June, 2020.

Prirodno-matematički fakultet Sarajevo
Faculty of Science Sarajevo

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

Zmaja od Bosne 33-35, BA-Sarajevo
Bosnia and Herzegovina
Phone: +387-33-279-918
Fax: +387-33-649-359
E-mail: glasnik@pmf.unsa.ba
glasnikhtbh@gmail.com

EDITORIAL BOARD

Faculty of Science Sarajevo
Zmaja od Bosne 33-35, BA-Sarajevo
Bosnia and Herzegovina
Phone: +387-33-279-904 (Administration)
+387-33-279-911 (Executive Editors)
Fax: +387-33-649-359

E-mail: glasnik@pmf.unsa.ba
glasnikhtbh@gmail.com

Dejan Milošević
Department of Physics, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Željko Jaćimović
Department of Chemical Technology, Faculty of Meallurgy and Technology, University of Montenegro, Podgorica, Montenegro
Ljudmila Benedik
Department of Environmental Sciences, "Jožef Stefan" Institute, Ljubljana, Slovenia
Meliha Zejnilagić-Hajrić
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Tidža Muhić-Šarac
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Sabina Gojak-Salimović
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Ismet Tahirović
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Danijela Vidic
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Mustafa Memić
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Andrea Gambaro
Department of Environmental Sciences, Informatics and Statistics, University Ca' Foscari of Venice, Venice, Italy
Institute for the Dynamics of Environmental Processes - National Research Council (CNR-IDPA), Venice, Italy
Dragana Đorđević
Centre of Chemistry - IChTM, University of Belgrade, Belgrade, Serbia
Aida Šapčanin
Department of Natural Sciences in Pharmacy, Faculty of Pharmacy, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Jože Kotnik
Department of Environmental Sciences, "Jožef Stefan" Institute, Ljubljana, Slovenia
Lucyna Samek
AGH University of Science and Technology, Faculty of Physics and Applied Computer Science, Krakow, Poland
Angela Maria Stortini
Department of Molecular Sciences and Nanosystems, University Ca' Foscari of Venice, Venice, Italy
Ivan Spanik
Institute of Analytical Chemistry, Slovak University of Technology, Bratislava, Slovakia
Mirjana Vojinović Miloradov
Department of Environmental Engineering, Faculty of Technical Sciences, University of Novi Sad, Novi Sad, Serbia
Heike Bradl
Department of Environmental Engineering, University of Applied Sciences Trier, Birkenfeld, Germany
Lea Kukoč-Modun
Department of Analytical Chemistry, Faculty of Chemistry and Technology, University of Split, Split, Croatia
Sanja Ćavar Zeljković
Central Laboratories and Research Support, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic;
Department of Genetic Resources for Vegetables Medicinal and Special Plants,Centre of the Region Haná for Biotechnological and Agricultural Research, Crop Research Institute, Olomouc, Czech Republic
Igor Jerković
Department of Organic Chemisty, Faculty of Chemistry and Technology, University of Split, Split,Croatia
Roderick W. Bates
Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technlogical University, Singapore, Singapore
Safija Herenda
Department of Chemistry, Faculty of Science, University of Sarajevo, Sarajevo, Bosnia and Herzegovina
Muna Abu-Dalo
Department of Chemistry, Jordan University of Science and Technology, Irbid, Jordan

The journal is published semiannual, and full text version of the papers published are available free of cost at http://www.pmf.unsa.ba/hemija/glasnik.

Bulletin of the Chemists and Technologists has been licensed for indexing in:

Emerging Sources Citation Index (Web of Science, Clarivate Analytics)

CAPlus (Chemical Abstracts Plus):

Academic Search Complete (EBSCO)

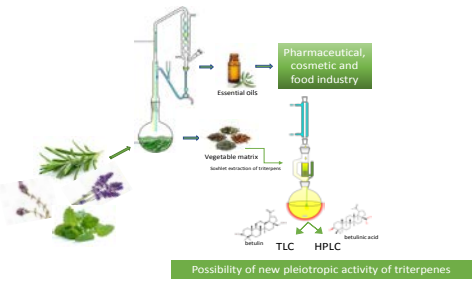
Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

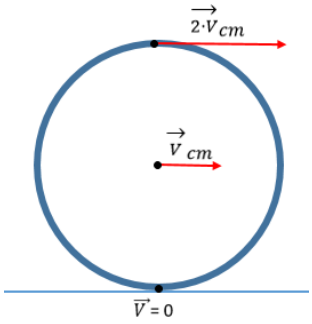
Print ISSN: 0367-4444
Online ISSN: 2232-7266

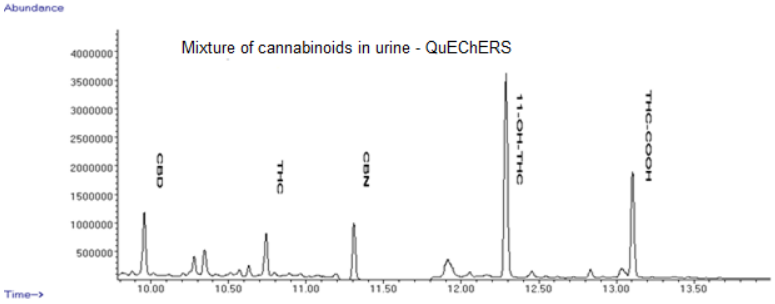
2020
Issue 54

CONTENT



	t_{inhib} (s) at different concentrations (μM)			
Phenolic acid	**50**	**100**	**200**	**250**
CA	30	60	326	491
FA	192	373	607	781
RA	58	151	1519	2107





Editorial

At the end of 2019, a new SARS-like virus was detected in China, which was later named SARS-CoV-2. This virus has since then caused hundreds of thousands of deaths linked with to the respiratory disease COVID-19. A cure or a vaccine has not yet been discovered, but in the meantime, all we can do is protect ourselves and/or others surronding us. In order to do that, testing and effective contact tracing are essential.

The most common tests used to detect the presence of COVID-19 are PCR and serologic tests. PCR tests detect the presence of an antigen, rather than the presence of the body's immune response, or antibodies. Unlike PCR tests, serologic tests detect the presence of antibodies. Different samples are needed in order to perform these tests: PCR tests usually require a nasopharyngeal swab, and serologic tests require a blood sample.

The polymerase chain reaction (PCR) is an *in vitro* method used for amplifying specific sequences of nucleic acids. This method was discovered by Kary Mullis in 1983. Using this technique, it is possible to analyse minimal traces of nucleic acids (NA).

The basic ingredients for a PCR are Taq polymerase, primers, template NA and nucleotides. These substances are combined in a tube along with cofactors needed by the enzyme, and are put through several cycles of heating and cooling that allow NA to be synthetized. The DNA which needs to be detected is the DNA of the virus-infected host cells.

The essential steps are:

1. Denaturation of the DNA strands (at 96°C),
2. Annealing of primers to their complementary sequences on the single-stranded template DNA (at 55-65°C),
3. Extension of the primers, thus synthesizing new strands of DNA (at 72°C).

A fluorescent signal is created when amplification occurs, and once the signal reaches a threshold, the test is considered positive. The technique is generally very sensitive and specific. If a PCR test is positive, the result is most likely correct (false positive results can only happen if the samples are contaminated during test processing). False negative results do not guarantee that the patient is not infected by the virus and they are most frequently the result of a wrong patient sampling (swabs not pushed far enough in the patients' nasopharynx, i.e.).

Editors

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

Original Scientific Article

54

Print ISSN: 0367-4444

DOI: 10.35666/ghtbh.2020.54.01

Online ISSN: 2232-7266

UDC: 531.19(497.6)

1-6

Phenolic Composition, Antioxidant and Antimicrobial Activity of *Cotoneaster* Medik. Species from Bosnia and Herzegovina

Mahmutović-Dizdarević, I.[a], Dizdar, M.[b], Čulum, D.[b], Vidic, D.[b], Dahija, S.[a], Jerković-Mujkić, A.[a], Bešta-Gajević, R.[a]

[a]*Department of Biology, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, Sarajevo, B&H*

[b]*Department of Chemistry, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, Sarajevo, B&H*

Article info
Received: 18/06/2019
Accepted: 07/10/2019

Keywords:
Bioactivity
Cotoneaster integerrimus Medik.
Cotoneaster tomentosus (Aiton) Lindl.
Cotoneaster horizontalis Decne

***Corresponding author:**
Irma Mahmutović-Dizdarević
E-mail: irma.contagium@gmail.com
Phone: 00 387-61-133-977

Abstract: Although the genus *Cotoneaster* Medik. includes mainly ornamental species, there are some data regarding its biological activity. The purpose of this study was to analyze the content of phenolic compounds, acetylcholinesterase inhibition, antioxidant and antimicrobial activity of methanolic extracts of leaf and bark of *C. integerrimus* Medik., *C. tomentosus* (Aiton) Lindl. and *C. horizontalis* Decne. The *C. tomentosus* leaf extract exhibited the highest content of total phenols (135.86 mg GAE/g) and flavonoids (18.17 mgQE/g), and also the most potent antioxidant activity against nonbiogenic free radicals, while the highest inhibition of acetylcholinesterase had the leaf extract of *C. horizontalis* (IC_{50} 0.34 mg/mL). All extracts showed a significant level of antibacterial and antifungal activity against tested microbial strains. The largest inhibition zones were observed against *Candida albicans* treated with *C. integerrimus* leaf extract (30.50±0.50 mm). Furthermore, *C. integerrimus* extract was the most effective in the majority of bacterial strains tested. The results indicated that methanolic extracts of the investigated *Cotoneaster* species have promising bioactive and therapeutic potentials.

INTRODUCTION

Many medicinal plants are so far recognized as valuable resources of natural antimicrobial compounds, and wide range of phytochemicals in plants have potential to inhibit microbial pathogens (Romero *et al*., 2005). These biomolecules are mainly secondary metabolites, such as: alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins (Das, Tiwari and Shrivastava, 2010). Despite the fact that numerous plant species have been used in traditional medicine for centuries to treat infectious diseases, their bioactive compounds need to be determined. Furthermore, discovering the new potential antimicrobial resources from plants overcomes the domain of folk medicine and could have a promising impact in modern phytopharmacy, especially in the case of multidrug-resistant (MDR) pathogens (Agarwal *et al*., 2016). Usage of plant products have significantly increased in last decades, since synthetically derived compounds are often expensive, and could have unfavorable health effects (Uysal *et al*., 2016). The genus *Cotoneaster* Medik. belongs to the family Rosaceae, and in Bosnia and Herzegovina is represented by two species: *C. integerrimus* Medik. and *C. tomentosus* (Aiton) Lindl. (Beck-Mannagetta, 1927; Euro+Med PlantBase). Many *Cotoneaster* species are cultivated in Europe as ornamental plants in urban areas (Fryer and Hylm, 2009), and *C. horizontalis* Decne. is one of the most famous cultivars widely used in B&H. Data suggest that *Cotoneaster* species have traditionally been used for medicinal purposes. *Cotoneaster racemiflorus* (Desf.) K. Koch is known as an aperient, expectorant and stomachic, as well as a treatment for reducing jaundice (Chopra and Nayar, 1956). Khan *et al*. (2008) isolated two bioactive compounds from this species, and these new aromatic esters were named cotonoates A and B. Furthermore, specific bioactive compounds are recognized in other *Cotoneaster* species, e.g. phenolic glycosides in *C. orbicularis* Schltdl. (El-Mousallamy *et al*., 2000). Phytoalexin named cotonefuran with bactericidal activity was primarily isolated from *C.*

lacteus W. W. Sm. (Burden *et al*., 1984), and about a decade later, confirmed as well in *C. acutifolius* Turcz., with prominent antifungal properties (Kokubun *et al*., 1995). Antimicrobial effects have also been reported in *C. nummularius* Fisch. & C. A. Mey. (Zengin *et al*., 2014) and *C. nummularioides* Pojark. (Kanaani, Sani, and Yaghooti, 2015; Siami, Sani and Branch, 2016). Data on the chemical composition and antimicrobial activity of investigated *Cotoneaster* species are scarce (Mohamed *et al*., 2012; Sokkar *et al*., 2013; Sytar *et al*., 2016; Uysal *et al*., 2016), or non-existent. The aim of this study was to investigate: phenolic compounds content, acetylcholinesterase (AChE) inhibition, antioxidant and antimicrobial activity of three *Cotoneaster* species: *C. integerrimus* Medik., *C. tomentosus* (Aiton) Lindl. and *C. horizontalis* Decne.

EXPERIMENTAL

Chemicals and plant material

All reagents and solvents were purchased commercially and were of analytical grade. The plant material (leaves and bark) of three *Cotoneaster* species*: C. integerrimus*, *C. tomentosus*, and *C. horizontalis* was collected in September 2017. Ten individuals of each species were analyzed. *Cotoneaster integerrimus* and *C. tomentosus* were collected on Mountain Ozren near Sarajevo, Bosnia and Herzegovina, while *C. horizontalis* was sampled in urban Sarajevo area. Determination of plant material was carried out in Laboratory for Plant Systematics, Department of Biology, Faculty of Science, University of Sarajevo. Voucher specimens were deposited in the Herbarium of the Faculty of Science, University of Sarajevo. Separation of plant material into leaves and bark was performed, and such separated plant material was dried in dark in ventilated room at the ambient temperature.

Preparation of extracts

Dried plant material was grounded to fine dust and then extracted for 24 hours using 80% methanol. The extracts were filtered, evaporated to dryness under reduced pressure on a rotary evaporator and dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 6 mg/mL. All extracts were stored at +4°C until use.

Determination of total phenolic content

The modified Folin-Ciocalteu method was used to determine the total phenolic content of the extracts (Singleton and Rossi, 1965). Folin-Ciocalteu reagent (1.0 mL) was reacted with 0.2 mL of diluted sample, and then 0.8 mL saturated sodium carbonate solution was added into the reaction mixture. After 30 minutes, the absorbance of the mixture was measured using the UV-Vis spectrophotometer at 765 nm. The total phenolic contents were determined from the standard curve prepared with gallic acid and the content of total phenolic compounds are expressed as gallic acid equivalents (mg GAE/g).

Determination of total flavonoid content

The Dowd method, which is based on the reaction between flavonoids and $AlCl_3$, was used to determine the total flavonoid content in the plant extracts (Dowd, 1959). The diluted extract solution (0.5 mL) was mixed with 2% $AlCl_3$ (0.5 mL). After standing for 10 minutes at room temperature the absorbance was measured at 415 nm. Total flavonoid content of the extracts was calculated from the regression equation of the quercetin calibration curve, and the results were expressed as quercetin equivalents (mg QE/g).

Acetylcholinesterase inhibition

The tests were conducted based on the Ellman´s spectrophotometric method with slight modification (Ellman *et al*., 1961). Galantamine was applied as the standard compound. In a 1.5 mL cuvette, 0.1 mL of sodium phosphate buffer (100 mM, pH 8), 0.1 mL of sample, and 0.1 mL AChE solution containing 0.54 U/mL, were mixed and allowed to incubate for 15 min at 37°C. After that, 0.1 mL of a solution of acetylcholine iodide (15 mM) and 0.5 mL of 3 mM Ellman's reagent were added and the absorbance at 405 nm was read after 5 min of the reaction. The percentage of AChE inhibition was calculated based on the absorbance value as follows:

$$\%\ Inhibition = (1 - A_t/A_0) \times 100 \qquad (1)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. The IC_{50} value was determined by non-linear regression of the log inhibitor concentration versus the percentage of inhibition.

Evaluation of antioxidant activity against DPPH$^{\bullet}$

The antioxidant activity of analyzed extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Blois, 1958). An aliquot of plant extracts (0.1 mL) was added to the DPPH$^{\bullet}$ solution (1 mL, 55 mM) and left to stand in the dark at room temperature for 30 min. After that, the absorbance of each mixture was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\%\ DPPH^{\bullet} = (1 - A_t/A_0) \times 100 \qquad (2)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. The results are expressed as mg/mL of plant extract needed to reduce DPPH radical signal by 50% (IC_{50}).

Evaluation of antioxidant activity against ABTS$^{\bullet+}$

The 2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS$^{\bullet+}$) was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to react in a dark at room temperature over night (Re *et al*., 1999). Different concentrations of the plant extracts (0.1 mL) were mixed to 1 mL ABTS$^{\bullet+}$ solution. After mixing and incubating at room temperature for 7 min, the absorbance was recorded at 734 nm. The ability to scavenge the ABTS$^{\bullet+}$ was calculated using the following equation:

$$\%\ ABTS^{\bullet+} = (1 - A_t/A_0) \times 100 \qquad (3)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. From the percentage of the scavenging activity at different tested extracts concentrations, IC_{50} values were calculated.

Antimicrobial assays

For the investigation of potential antimicrobial activity of three *Cotoneaster* species extracts, the following Gram-negative and Gram-positive bacteria, as well as fungi were tested: *Salmonella enterica* serovar Enteritidis ATCC 31194, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, Extended Spectrum Beta-Lactamase producing *E. coli* or ESBL *E. coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* or MRSA ATCC 33591, *Bacillus subtilis* ATCC 6633 and *Candida albicans* ATCC 1023. Antimicrobial effects were evaluated through the agar well diffusion method (Balouiri, Sadiki and Ibnsouda, 2016). Standard antibiotic Ampicillin (10 µg; HiMedia Laboratories Pvt.Ltd., India) and antimycotic Nystatin (100 units; Oxoid Ltd., England) were used as positive controls, while DMSO was used as solvent control. Tested microbial species were cultured overnight at 37°C, in Mueller Hinton medium and Sabouraund Glucose Agar (*Fluka* Biochemica; Buchs, Switzerland). Inoculums were diluted in sterile saline solution and adjusted to the final density of 0.5 McFarland standard (~1.5×10^8 CFU/mL), according to Wayne (2007). In order to achieve a total absorption, after the spreading of inoculums over the plates, they are left for 15 minutes at ambient temperature. Investigated extracts and controls (100 µL) were transferred into the wells of inoculated plates and incubated for 18-24 hours at 37°C, and 24-48 hours at 37°C, for bacteria and fungi respectively.

Antimicrobial activity of investigated *Cotoneaster* extracts was evaluated on the basis of inhibition zones diameter (mm), which is the result of extract diffusion in the medium and inhibition of microbial growth.

Statistical analysis

All tests were performed in three replications and the mean values ± standard deviation (SD) were calculated. Descriptive statistical analyses were carried out by Microsoft Office 2013 Excel (Microsoft Corporation, Redmond, USA). Data were further analyzed by using one-way ANOVA and *post hoc* Newman-Keuls test (STATISTICA 10; StatSoft. Inc.), at the significance level of $p<0.05$.

RESULTS AND DISCUSSION

Phenolic compounds, which are widely found as secondary metabolites in plants, are known to act as biologically active molecules. Many phenolic compounds, including flavonoids are known to have potent antiviral, anticancer, antioxidant, antibacterial or anti-inflammatory activities (Aziz *et al*., 1998; Galati and O'Brien, 2004; Kicel *et al*., 2016). The content of total phenols and flavonoids in the analyzed extracts pointed that these species are rich source of polyphenolic compounds. The amount of total phenolics in extracts ranged between 65.04 and 135.86 mg GAE/g, while the content of flavonoids ranged from 2.76 to 18.17 mg QE/g (Table 1). The highest content of total phenols and flavonoids was found in the *C. tomentosus* leaf extract,followed by the leaf extract of *C. integerrimus*, 133.54 mg GAE/g and 16.42 mg QE/g respectively, while the corresponding bark extract of *C. tomentosus* showed the lowest content of total phenolics and flavonoids. It is noticeable that among all analyzed extracts, the content of total phenolic compounds and flavonoids in all samples is higher in the leaves. Results obtained in this study strongly suggest that phenolics are important components of these plants. The one-way ANOVA and Newman-Keuls test showed statistically significant differences in phenolic and flavonoid contents among all tested extracts.

Table 1: Phenolic compounds, antioxidant activity and acetylcholinesterase inhibition of investigated extracts

Sample	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)	DPPH - IC_{50} (mg/mL)	ABTS - IC_{50} (mg/mL)	AChE - IC_{50} (mg/mL)
C. integerrimus					
LE	133.54±2.81^a	16.42±0.35^b	1.23±0.02^e	0.20±0.01^e	0.88±0.03^b
BE	80.10±0.41^d	9.38±0.27^d	2.99±0.06^b	0.73±0.02^b	n.d.
C. tomentosus					
LE	135.86±1.29^a	18.17±0.30^a	1.22±0.04^e	0.12±0.01^f	0.73±0.01^c
BE	65.04±0.82^e	2.76±0.20^f	3.73±0.02^a	0.87±0.03^a	0.35±0.01^d
C. horizontalis					
LE	93.21±1.61^b	10.55±0.51^c	2.15±0.03^d	0.38±0.01^d	0.34±0.01^d
BE	82.97±1.57^c	8.73±0.28^e	2.50±0.03^c	0.42±0.01^c	0.94±0.03^a
Positive control	-	-	0.06±0.01^f	0.01±0.00^g	0.11±0.01^e

The results are the mean ± SD (n=3). LE = leaf extract. BE = bark extract. n.d. = not detected.
Values in the same column that don't share the same letters, differ significantly at $p<0.05$ after *post hoc* Neuman-Keuls test.

The literature data on the phenolic and flavonoid content of *Cotoneaster* species are scarce and present highly variable results. The results by Uysal *et al*. (2016) showed that the total phenol content in the methanolic extract of twigs of *C. integerrimus* is 115.15 mg GAE/g, while the flavonoid content was 16.29 mg RE/g. In addition, the study of Mohamed *et al*. (2012) analysed the aerial parts of *C. horizontalis* for total phenols and flavonoids content

and obtained 14.00 mg GA/g and 6.80 mg RE/g respectively. The investigation of antioxidant activity was carried out to assess the ability of extracts of the three *Cotoneaster* species to scavenge free radicals by the ABTS and DPPH methods (Table 1). The obtained results were presented as IC_{50} values. In the DPPH method, IC_{50} values of extracts ranged from 1.22 to 3.73 mg/mL, while for the ABTS method values varied from 0.12 to 0.87 mg/mL. The best antioxidant activity showed the leaf extract of *C. tomentosus* and the lowest activity had the bark extract of the same species. Generally, extracts of leaves had better activity than bark extracts for both methods. Usually, the IC_{50} values for the DPPH method are higher than the value of ABTS (although they are based on the same reaction mechanism), due to possible steric hindrances in the case of DPPH. Also, some substances present in extracts can participate in the inactivation of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Bernatonienė *et al*., 2008). Previous studies are in lack of data on antioxidant activity of *Cotoneaster* species, and the published results showed divergence to our results (Sokkar *et al*., 2013; Kicel *et al*., 2016; Uysal *et al*., 2016). In addition, inhibition of AChE was investigated (Table 1). The obtained IC_{50} values were varied from 0.34 for the *C. horizontalis* leaf extract to 0.94 mg/mL for the corresponding bark extract. All extract showed significantly lower inhibition of AChE than galantamine (0,11±0.01) which was used as a positive control. Nevertheless, the *C. horizontalis* leaf sample showed moderate inhibitory activity (0.34±0.01) due to the presence of horizontoates A – C in this species which showed remarkable activity according to Khan *et al*. (2014).

The results regarding antimicrobial properties of the studied *Cotoneaster* leaf and bark extracts indicate that three investigated species exhibit antimicrobial activity against all microorganisms tested (Table 2).

Table 2: Zones of inhibition obtained through the agar well diffusion method

Strain / Extract	*C. integerrimus*		*C. tomentosus*		*C. horizontalis*		Positive control
	LE	BE	LE	BE	LE	BE	
S. enterica	20.12^{a}±0.71	17.24^{c}±0.63	18.01^{c}±0.21	$18.01^{c,b}$±0.53	16.82^{c}±1.50	$19.49^{b,a}$±0.86	16.03^{c}±0.07
P. aeruginosa	21.50^{a}±0.79	19.20^{b}±1.41	15.00^{d}±0.20	20.56^{b}±0.70	18.50^{c}±0.73	$18.50^{b,c}$±0.74	13.02^{e}±0.09
E. coli	$18.34^{a,b}$±0.59	19.27^{a}±0.46	12.10^{c}±0.22	18.67^{a}±0.28	16.56^{b}±2.02	18.21^{a}±0.23	8.96^{d}±0.16
ESBL *E. coli*	20.22^{a}±1.13	17.76^{b}±0.59	14.67^{d}±0.58	16.50^{c}±0.50	19.35^{a}±0.70	19.67^{a}±0.29	NI^{e}
E. faecalis	14.41^{b}±1.75	11.64^{c}±1.41	14.72^{b}±0.44	10.89^{c}±0.29	10.98^{c}±0.46	13.65^{b}±1.19	16.94^{a}±0.23
S. aureus	18.63^{b}±0.40	17.66^{c}±0.37	15.09^{e}±0.31	17.30^{c}±0.34	16.09^{d}±0.34	17.98^{c}±0.47	33.03^{a}±0.09
MRSA	$12.80^{b,c}$±1.93	14.74^{a}±0.45	$14.15^{a,b}$±0.14	$13.17^{a,b,c}$±0.50	12.22^{c}±0.23	12.03^{c}±0.50	NI^{d}
B. subtilis	20.48^{b}±0.45	18.77^{c}±0.52	19.38^{c}±0.56	19.18^{c}±0.55	17.73^{d}±0.38	20.97^{b}±0.49	47.98^{a}±0.23
C. albicans	30.50^{a}±0.50	27.66^{b}±1.33	21.83^{d}±0.35	24.36^{c}±0.71	30.40^{a}±0.53	25.04^{c}±0.83	21.12^{d}±0.24

The results are the mean ± SD (n=3). LE = leaf extract. BE = bark extract. NI = No inhibition zone.
Values in the same column that don't share the same letters, differ significantly at $p<0.05$ after *post hoc* Neuman-Keuls test.

This research showed significantly high inhibition of both Gram-positive and Gram-negative bacteria, as well as fungi, by tested extracts. Largest inhibition zones are noted in case of *C. albicans*, especially with *C. integerrimus* leaf extract (30.50±0.50 mm). This plant species caused the greatest zones of inhibition as well as in most investigated bacterial strains (Table 2). According to Uysal *et al*. (2016), methanolic extracts of *C. integerrimus* have significant antimicrobial potential and their study indicate phenolic components, especially epicatechin, responsible for antimicrobial activity. To our knowledge, this is the first report on antimicrobial properties of *C. tomentosus*. In addition to the antifungal effects observed against *C. albicans*, it is particularly interesting that *C. tomentosus* bark extract caused the greatest inhibition of *P. aeruginosa* growth (20.56±0.70 mm), which is known as a versatile pathogen with multidrug-resistance (Hirsch and Tam, 2010). *Cotoneaster horizontalis* proved to be most effective against *C. albicans* (30.40±0.52 mm), followed by *B. subtilis* (20.97±0.49 mm) and *P. aeruginosa* (20.00±0.74 mm). According to Mohamed *et al*. (2012), *C. horizontalis* is rich in polyphenols, flavonoids, hydrocarbons, phytosterols and different fatty acids, and it contain essential oil with many oxygenated compounds, as well as in phenolic acids (Mohamed *et al*., 2012; Sytar *et al*., 2016). Furthermore, this plant species has been recognized as an important source of antioxidative and anticancer compounds such as α-tocopherol and amygdalin (Sokkar *et al*., 2013). While previous studies have reported the resistance of only some Gram-negative strains to the *Cotoneaster* extracts (Zengin *et al*., 2014; Kanaani *et al*., 2015; Siami *et al*., 2016), this research showed that all investigated bacteria, including the multidrug-resistant pathogens were sensitive to the methanolic extracts of *C. integerrimus*, *C. tomentosus* and *C. horizontalis*.

CONCLUSIONS

This investigation shows that the leaf and bark extracts of three *Cotoneaster* species collected in Bosnia and Herzegovina possess significant and dose-dependent *in vitro* antioxidant activity, which positively correlate with their total phenolic content. Based on these facts, it is possible to justify the highest phenolics and flavonoid content for the *C. tomentosus* leaf extract. Different correlations were observed with AChE inhibition results. The *C. horizontalis* sample that showed an average content of total phenols and flavonoids simultaneously

shows the highest (leaves) and lowest (bark) activity by inhibition. In addition, this study shows a remarkable antimicrobial activity of the tested extracts against both Gram-positive and Gram-negative bacteria, as well as fungi. However, the compounds responsible for the bioactive properties of all three investigated *Cotoneaster* species remain unclear, so future research should be directed towards solving the detailed chemical composition and isolation of individual bioactive components.

REFERENCES

Agarwal, P., Agarwal, N., Gupta, R., Gupta, M., Sharma, B. (2016). Antibacterial activity of plants extracts against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. *Journal of Microbial and Biochemical Technology, 8*, 404-407.

Aziz, N., Farag, S., Mousa, L., Abo-Zaid, M. (1998). Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios, 93(374)*, 43-54.

Balouiri, M., Sadiki, M., Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis, 6(2)*, 71-79.

Beck-Mannagetta, G. (1927). *Flora Bosnae, Hercegovinae et regionis Novi Pazar*. Srpska Kraljevska Akademija.

Bernatonienė, J., Masteikova, R., Majienė, D., Savickas, A., Kėvelaitis, E., Bernatonienė, R., Dvoráčková, K., Civinskienė, G., Lekas, R., Vitkevičius, K. (2008). Free radical-scavenging activities of *Crataegus monogyna* extracts. *Medicina, 44(9)*, 706-712.

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature, 181(4617)*, 1199.

Burden, R. S., Kemp, M. S., Wiltshire, C. W., Owen, J. D. (1984). Isolation and structure determination of cotonefuran, an induced antifungal dibenzofuran from *Cotoneaster lactea* WW Sm. *Perkin Transactions 1,0*, 1445-1448.

Chopra, R. N., Nayar, S. L. (1956). *Glossary of Indian medicinal plants.* Council of Scientific And Industrial Research, New Delhi.

Das, K., Tiwari, R., Shrivastava, D. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agents: current methods and future trends. *Journal of Medicinal Plant Research, 4(2)*, 104-111.

Dowd, L. E. (1959). Spectrophotometric Determination of Quercetin. *Analytical Chemistry, 31(7)*, 1184-1187.

Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology, 7(2)*, 88-95.

El-Mousallamy, A. M., Hussein, S. A., Merfort, I., Nawwar, M. A. (2000). Unusual phenolic glycosides from *Cotoneaster orbicularis*. *Phytochemistry, 53(6)*, 699-704.

Euro+Med PlantBase, ww2.bgbm.org/EuroPlusMed (18/07/2019).

Fryer, J., Hylm, B. (2009) *Cotoneasters: a comprehensive guide to shrubs for flowers, fruit, and foliage.* Timber Press.

Galati, G., O'Brien, P. J. (2004). Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free Radical Biology and Medicine, 37(3)*, 287-303.

Hirsch, E. B., Tam, V. H. (2010). Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Review of Pharmacoeconomics and Outcomes Research, 10(4)*, 441-451.

Kanaani, S., Sani, A., Yaghooti, F. (2015). Antibacterial effects and chemical composition of essential oils from *Cotoneaster nummularioides* Pojark. and *Sonchus arvensis* L. leaves extracts on typical food-borne pathogens. *International Journal of Biosciences, 6(2)*, 357-365.

Khan, S., Wang, Z., Wang, R., Zhang, L. (2014). Horizontoates A–C: New cholinesterase inhibitors from *Cotoneaster horizontalis*. *Phytochemistry Letters, 10*, 204-208.

Khan, S., Yasmeen, S., Afza, N., Malik, A., Iqbal, L., Lateef, M. (2008). Cotonoates A and B, new aromatic esters from *Cotoneaster racemiflora*. *Zeitschrift für Naturforschung B, 63(10)*, 1219-1222.

Kicel, A., Michel, P., Owczarek, A., Marchelak, A., Żyżelewicz, D., Budryn, G., Oracz, J., Olszewska, M. A. (2016). Phenolic profile and antioxidant potential of leaves from selected *Cotoneaster* Medik. Species. *Molecules, 21(6)*, 688.

Kokubun, T., Harborne, J. B., Eagles, J., Waterman, P. G. (1995). Dibenzofuran phytoalexins from the sapwood of *Cotoneaster acutifolius* and five related species. *Phytochemistry, 38(1)*, 57-60.

Mohamed, S. A., Sokkar, N. M., El-Gindi, O., Zeinab, Y., Alfishawy, I. M. (2012). Phytoconstituents investigation, anti-diabetic and anti-dyslipidemic activities of *Cotoneaster horizontalis* Decne cultivated in Egypt. *Life Science Journal, 9*, 394-403.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26(9-10)*, 1231-1237.

Romero, C. D., Chopin, S. F., Buck, G., Martinez, E., Garcia, M., Bixby, L. (2005). Antibacterial properties of common herbal remedies of the southwest. *Journal of Ethnopharmacology, 99(2)*, 253-257.

Siami, M., Sani, A. M., Branch, Q. (2016). Antibacterial activity of ethanolic leaf extract from *Cotoneaster nummularioides* on food borne pathogens. *International Journal of Life Sciences, 9(2)*, 180-184.

Singleton, V. L., Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16(3)*, 144-158.

Sokkar, N., El-Gindi, O., Sayed, S., Mohamed, S., Ali, Z., Alfishawy, I. (2013). Antioxidant, anticancer and hepatoprotective activities of *Cotoneaster horizontalis* Decne extract as well as α-tocopherol and amygdalin production from *in vitro* culture. *Acta Physiologiae Plantarum, 35(8)*, 2421-2428.

Sytar, O., Hemmerich, I., Zivcak, M., Rauh, C., Brestic, M. (2016). Comparative analysis of bioactive phenolic compounds composition from 26 medicinal plants. *Saudi Journal of Biological Sciences, 25(4),* 631-641.

Uysal, A., Zengin, G., Mollica, A., Gunes, E., Locatelli, M., Yilmaz, T., Aktumsek, A. (2016). Chemical and biological insights on *Cotoneaster integerrimus*: a new (-)-epicatechin source for food and medicinal applications. *Phytomedicine, 23(10),* 979-988.

Wayne, P. (2007). *Performance standards for antimicrobial susceptibility testing.* Clinical and laboratory standards institute.

Zengin, G., Uysal, A., Gunes, E., Aktumsek, A. (2014). Survey of phytochemical composition and biological effects of three extracts from a wild plant (*Cotoneaster nummularia* Fisch. et Mey.): a potential source for functional food ingredients and drug formulations. *PLoS One, 9(11),* e11352.

Summary/Sažetak

Iako rod *Cotoneaster* Medik. obuhvata uglavnom ukrasne vrste, postoje podaci i o njihovom bioaktivnom djelovanju. Cilj ovog istraživanja bio je analizirati sadržaj fenolskih spojeva, inhibiciju acetilholinesteraze, te antioksidativno i antimikrobno djelovanje metanolnih ekstrakata lista i kore vrsta *C. integerrimus* Medik, *C. tomentosus* (Aiton) Lindl. i *C. horizontalis* Decne. Dok ekstrakt lista *C. tomentosus* ima najveći sadržaj fenolskih spojeva (135.86 mgGAE/g) i flavonoida (18.17 mgQE/g), ali i najznačajniju antioksidativnu aktivnost spram nebiogenih slobodnih radikala, ekstrakt lista *C. horizontalis* je pokazao najveću inhibitornu aktivnost AChE (IC_{50} 0.34 mg/mL). Svi ekstrakti su pokazali značajan nivo antibakterijske i antifungalne aktivnosti spram testiranih mikrobnih sojeva. Najveće zone inhibicije su uočene kod vrste *Candida albicans* tretirane ekstraktom lista *C. integerrimus* (30.50±0.50 mm). Nadalje, ekstrakt *C. integerrimus* je bio najučinkovitiji i kod većine testiranih bakterijskih sojeva. Dobijeni rezultati sugerišu da metanolni ekstrakti analiziranih vrsta roda *Cotoneaster* posjeduju obećavajući bioaktivni i terapeutski potencijal.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

Original Scientific Article

54

Print ISSN: 0367-4444

DOI: 10.35666/ghtbh.2020.54.02

Online ISSN: 2232-7266

UDC: 577.115:616.379

7-12

Serum Iron Concentration and Lipid Profile in Type 2 Diabetes Patients

Šaćira Mandal

Department of Natural Sciences in Pharmacy, Faculty of Pharmacy, University of Sarajevo

Article info
Received: 15/05/2019
Accepted: 07/10/2019

Keywords:
Type 2 diabetes
Iron
Lipid profile

***Corresponding author:**
Šaćira Mandal
Phone: *00-387- 61-614203*

Abstract: Recent studies have been showed important role of elevated iron levels in pathogenesis of *Type 2 Diabetes mellitus* (T2D) and insulin resistance. The aim of this study was to determine serum of free iron concentration in T2D patients and find out associations with lipid profile. The study included 51 participants (27 healthy control and 24 no treated diabetes patients), with ages from 45 to 65 45-65 ages and both gender. As expected, concentrations of serum iron were elevated in diabetic patients compare to healthy subjects while statistical significant difference were shown between iron levels in control group and group with good control of glycaemia ($p<0.05$). In addition, there was a significant positive correlation between free iron concentration and LDL cholesterol levels and negative significant correlation between iron concentrations with HDL cholesterol in diabetics ($p<0.05$). These findings suggest that increase serum of free iron concentrations may have an important role and influence in development of disease, especially in lipid metabolism and profile as well in risk of further complications of diabetes.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is a chronic metabolic condition characterized by elevated glucose levels due to impaired of insulin secretion and action, or both. It is also, followed by insulin resistance (IR), increase in hepatic glucose production and elevated lipids level. The pathophysiological mechanisms of the disease are not yet fully understood despite efforts and numerous studies provided. Disturbance not only in carbohydrate pathways but also im lipid and protein metabolism was included (Swaminathan, S., Fonesca, V. A., Alam, M. G., Shah, S. V., 2007; Orban *et al*., 2014; Fernández-Real J. M., McClain, D., Manco, M., 2015).

Acute and chronic complications of this disease are caused by different metabolic abnormalities in a body oxidative stress being one of them. Oxidative stress through the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been recognized as the basic cause of the underlying mechanism for the development of IR, β-cell dysfunction, impaired glucose tolerance and at the end to T2D (Backe *et al.*, 2016; Shaaban M. A., Dawod A. E., Nasr M. A., 2016). Progression of diabetes vascular dysfunction implicated by overproduction of ROS and RNS which is the consequences of sedentary lifestyle and over nutrition. Reactive oxygen and nitrogen species initiate a chain reaction leading to reduced nitric oxide availability, augmented markers of inflammation and chemical modification of lipoproteins. Alternation of functions and contribution of macronutrient and their metabolic pathways are well known but disturbance and actions of micronutrient, although having an important roles in the diabetes are still little understood. Mineral elements as a valuable micronutrient are not only integral members of the structural components of body tissues but also, they participate in various metabolic processes (Wolide *et al.*, 2017). Recent studies investigated their roles in insulin synthesis, its release and actions, along with glucose metabolism (Stechemesser *et al.*, 2017).

Iron (Fe) as an essential trace element and is one of the most important metals for almost all living organisms. Iron is involved in various biochemical and metabolic processes, including oxygen transport, synthesis of deoxyribonucleic acid, and electron transfer as well in insulin and glucose metabolism (Abbaspour N., Hurrell R., Kelishadi R., 2014; Stechemesser *et al.*, 2017).

Iron as a transition element has significant redox activity and potential detrimental effects is presented by its binding with transport or storage proteins.
Previous studies have shown association of the T2D and iron metabolism and concluded that increased iron concentrations may predicted development of disease (Simcox J.D., McClain D.A., 2013; Arija *et al.*, 2014; Orban *et al.*, 2014). Iron and diabetes were linked by two pathological conditions: hereditary hemochromatosis and thalassemia. Also, iron has an important role in pathogenesis of the disease by mediation of β-cell dysfunction and IR, as well in regulation of energy homeostasis (Fernández-Real J. M., McClain, D., Manco, M., 2015; Podmore et al., 2016; Walace D. F., 2016; Stechemesser *et al.*, 2017).

The underlying molecular mechanisms by which iron mediates these effects are incompletely understood. They include oxidative stress, changes in adipokines and alteration in cellular signal transduction. It is well known that IR and β-cell dysfunction are risk factors in genetic predispose individuals but metabolic pathways remain largely unknown.
In its free form (released by ferritin by the action of reducers that convert Fe^{3+} to Fe^{2+}), iron promotes the oxidation of biomolecules through Haber-Weiss and Fenton reactions generating of detrimental hydroxyl radicals (Figure 1).

Auto oxidation of Fe^{2+}	$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$
Fenton reaction	$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$
Haber-Weiss reaction	$H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe^{3+}} O_2 + OH^- + OH^{\bullet}$

Figure 1: Formation of free radicals catalyzing by ferric form of iron in Fenton and Haber-Weiss reactions.

These free radicals are strong pro-oxidants which cause lipid peroxidation of cellular membrane, damage the configuration of proteins and damage nucleic acids in genes. These catalytic activities of free iron lead to IR through suppressed insulin release and action followed by glucose derangements upon iron-related pathways. Oxidative stress–triggered inflammatory cytokines e.g. C reactive protein (CRP) and interleukin 6 (IL-6) may amplify and potentiate the initiated events and cause deterioration of diabetes vascular chronic complications mediate by Fe (Fernández-Real, J. M., McClain, D., Manco, M., 2015).

Inconsistent results have been reported for iron in last years and its role in pathogenesis of *diabetes mellitus* and insulin resistance. Authors emphasize that a prominent role of free iron depend of race and ethnicity of study populations as well as their nutrition habits and diet. Clinical trials are warranted to clarify the impact of dietary or pharmacological iron reduction on the development of this metabolic disorder and therefore, suppress or diminish its complications (Swaminathan, S., Fonesca, V. A., Alam, M. G., Shah, S. V., 2007; Rajpathak, S. N., Crandall, J. P., Wylie-Rosett, J., Kabat, G. C., Rohan, T. E., Hu, F. B., 2009; Sanjeevi, N., Freeland-Graves, J., Beretvas, S. N., Sachdev, P. K., 2018).
The aim of this work was to found association of free iron concentrations and lipid profile in selected, non-treated type 2 diabetes population both gender in Bosnian and Herzegovina.

EXPERIMENTAL

Subjects

This study included 51 subjects out of them 24 patients having nontreated Type 2 *diabetes mellitus* with good glycemic control (11 participants) and poor glycemic control (13 participants) and 27 normal healthy control were selected. *Diabetes mellitus* was diagnosed according to The Expert Committee on the Diagnosis and Classification of Diabetes of ADA (American Diabetes Association, 2004).
Inclusion criteria: All participants divided into four groups: Group 1-Control group (27) consisted of healthy subjects; they were free from any illness which could affect the parameters under study. Also, they were taken from general population and they were not on any medication.
Group 2 as untreated Type 2 diabetics (24); they were not on antidiabetics drugs and much older than controls, and this group further was divided on the two groups Group 2a and Group 2b as patients with good control and poor control of glycaemia, respectively. Group 2a consisted of patients (11) with good control of glycaemia i.e. glycated hemoglobin levels less than 6.5% and they were free from clinical evidence of any complication of *diabetes mellitus*.
Group 2b participants with poor control of glycaemia (13) represent by patients with glycated hemoglobin level more than 7.0% and associated with one or more diabetic complications.
Exclusion criteria: hepatitis B and C or virus infection as well patients who received antidiabetic drugs.
Study was performed in accordance to Ethical Committee and Declaration of Helsinki and informed written consent of all subjects included in the study was obtained and participation in this study was voluntary.
Diabetic patients and healthy subjects were recruited at the Clinic for Endocrinology and Diabetes, University Clinical Centre of Sarajevo, Department of Endocrinology and Internal Medicine in Sarajevo. The investigations were carried out from January to September.

Sample collection

After overnight fasting, a 5 ml of venous blood was drawn from each volunteer and serum or plasma separated within 30 min and stored at 2-8^{0}C temperature till analysis was done.

Analysis of samples

Fasting glucose was estimated by glucose oxidase-peroxidase (GODP) enzymatic method, glycated hemoglobin level measured by immunoturbidimetric method in whole blood, while lipid profile (i.e. total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides) analyses was done according standard clinical laboratory protocols and IFCC recommendations. Serum free iron concentration done by Ferrozine method and all these determinations performed by Autoanalyzator Dimension® RXL clinical-chemical system (Siemens, Germany).

Serum iron assay

The method applied was a modification of direct iron assays, using the chromophore Ferene (Smith et al., 1984). This method is a direct iron measurement using a surfactant to prevent protein precipitation. A serum blank was used to correct for differences in specimen turbidity while potential copper interference was minimized by addition of thiourea. In the serum, presence of hemolysed cells had no effect on serum iron levels and measurement. Principle of assay: Under acidic conditions (pH 4.5), iron bound to the protein transferrin is released in the presence of the reducing agent, ascorbic acid. The resulting product, Fe^{2+} (ferrous ions) forms a blue complex with 3-(2- pyridyl)-5,6-bis-2-(5-furyl sulfonic acid)-1,2,4-triazine, disodium salt (Ferene). The absorbance of the complex, measured using a dichromatic (600, 700 nm), endpoint technique, is proportional to the concentration of transferrin-bound iron in the serum. Normal concentration and reference range for iron in serum samples is 35-150 µg/dl (6-27 µmol/L).

Statistical analysis

Statistical analyses were carried out using SPSS software version 17.0 (Chicago, IL, USA). Statistical significance was set a *P* value of <0.05. Data are expressed as medians (lower–upper quartile) and nonparametric Mann-Whitney U test used for unpaired samples to estimated differences of iron, glycated hemoglobin and other biochemical variables between groups. Independent sample *t* tests were used to compare the mean values of biochemical parameters between the diabetic and control groups while Spearman's correlation coefficient was calculated to analyze the relationships between the study variables.

RESULTS AND DISCUSSION

The characteristics of type 2 diabetes patients and control subjects are presented in Table I. Values of all the measured traits were significantly different between cases and controls. The iron concentrations were significantly higher in diabetic patients compared to healthy subjects.

Table 1: Biochemical parameters in study population

Parameters	NT-T2D	Healthy control	*P* *value
Number	24	27	-
Gender (M/F)	14/10	14/13	-
Age (years)	64 (60-68)	48 (42-54)	0.000
Glucose(mmol/L)	8.20 (7.59-8.81)	5.37 (5.24-5.49)	0.000
Total cholesterol (mmol/L)	5.34 (5.02-5.65)	4.53 (4.12-4.94)	0.001
HDL cholesterol (mmol/L)	1.17 (0.89-1.44)	1.27 (1.16-1.38)	0.021
LDL cholesterol (mmol/L)	3.45 (3.11-3.78)	2.47 (2.08-2.86)	0.000
Triglygerides (mmol/L)	2.08 (1.77-2.40)	1.68 (1.28-2.08)	0.021
Glycated hemoglobin HbA1c, (%)	7.16 (6.88-7.44)	5.74 (5.57-5.90)	0.000
hs-CRP (mg/L)	3.29 (2.41-4.17)	2.07 (1.19-2.95)	0.013
Iron (µmol/L)	23.38 (14.44-32.33)	19.40 (19.52-9.95)	0.000

*NT-T2D, no treated Type 2 diabetes; HDL cholesterol, high density lipoprotein; LDL cholesterol, low density
lipoprotein; hs-CRP high-sensitive C reactive protein. Data represent medians (upper and lower values).
*Significance of difference in Mann–Whitney test.

These findings are in line with data obtained from other investigation and research studies. Numbers of studies were showed significant increase of iron concentrations in diabetic patients compared to non-diabetic subjects regardless of racial or ethnic origin. Elevated iron levels and iron overload not only increases risks for insulin resistance and Type 2 diabetes, but also, causes cardiovascular disorder (CVD) in both, non-diabetic and diabetic individuals. Recent data point out the importance of determination of iron and ferritin concentrations in diabetics, because they can be used as markers for severe hepatic IR, higher risk for vascular complications and their progression (Fernández-Real, J. M., McClain, D., Manco, M., 2015).

Earlier studies have shown a gender differences in iron concentrations of T2D patients but no significant differences were found in this study (Aregbesola et al., 2017).

A main fraction of cellular iron is bonded with proteins in the form of heme, a protoporphyrin IX and Fe^{2+} ion. Chemical activity of iron is derived from a variety of coordination complexes with organic ligands, and electron transition of reduced iron form, Fe^{2+} and oxidized form Fe^{3+} ions. The efficiency of electron transfer between two iron forms is a basic feature for many biochemical reactions and iron action as an essential element and nutrient. The major form of glycated haemoglobin in diabetic patients is haemoglobin A1c (HbA1c). In non-diabetic subjects, the HbA1c levels with 3.0% to 6.5% of the haemoglobin represent optimal glucose control while the HbA1c fraction with ≥ 6.5% is abnormally elevated haemoglobin in patients with chronic hyperglycaemia and correlates positively with glycaemic control (American Diabetes Association., 2004; International Diabetes Federation., 2006; Fernández-Real, J. M., McClain, D., Manco, M., 2015). Control of glycaemia based on glucose and glycated haemoglobin levels, shown significant differences only between controls and diabetics with good control of glycaemia (Table II).

Table 2: Mean concentration of serum iron and glycemic control in study population.

Group of participant	Number	Mean of Fe(µmol/L)	*P* value
Group 1	27	19.40	0.002
Group 2a	11	26.00	
Group 1	27	19.40	0.125
Group 2b	13	21.15	
Group 1	27	19.40	0.375
Group 2	24	26.00	
Group 2a	11	26.00	0.146
Group 2b	13	21.15	

*Group 1, healthy control subjects
Group 2, no treated diabetics
Group 2a, good control of glycaemia
Group 2b, poor control of glycaemia

Similar results of the association of serum free iron levels with glycated haemoglobin reported in studies of others (Gohel M., Sirajwala H. B., Chacko A., 2013; Misra et al., 2016).

Lipid profile referred as high levels of low-density lipoprotein cholesterol and triglycerides and low high-density lipoprotein cholesterol level correspond to dyslipidaemia. Dyslipidaemia is involved in glycaemic control and plasma lipid elevation. It is known that diabetic patients with dyslipidaemia have higher risk for macro-vascular and microvascular complications and atherosclerosis (Stechemesser et al., 2017; Wolide et al., 2017).

Correlations between serum free iron concentrations and lipid profile, presented in Table III and Table IV for control subjects and cases, respectively. In healthy subjects, there were not demonstrated significant association while in diabetic patients, positive significant correlation was showed between iron and LDL levels and negatively correlation between iron and HDL levels.

These results are in line with previously reported data. Fernández-Real and collegues found a proportional relationship between serum iron stores (ferritin) and serum glucose concentration, diastolic blood pressure, HDL cholesterol, and insulin resistance. In study of Victoria Arija and co-works demonstrated that excess of body Fe store and lipid abnormalities associated with an increased risk of T2D, CVD and other diabetic complications.

Table 3: Spearman´s correlation coefficient (rho) between serum free iron concentrations (µmol/L) and HbA1c, fasting plasma glucose, lipid profile (total cholesterol, HDL, LDL, TGs) in controls.

Parameters	Sample size	rho	*p* value
HbA1c	27	0.068	0.735
Glucose	27	0.137	0.495
TC	27	-0.081	0.688
HDL	27	0.292	0.139
LDL	27	-0.097	0.632
TGs	27	-0.252	0.205

* TC, total cholesterol
HDL cholesterol, high density lipoprotein
LDL cholesterol, low density lipoprotein
TGs, triglycerides

Table 4: Spearman´s correlation coefficient (rho) between serum free iron concentrations (µmol/L) and HbA1c, fasting plasma glucose, lipid profile (total cholesterol, HDL, LDL, TGs) in no treated Type 2 diabetics.

Parameters	Sample size	rho	*p* value
HbA1c	24	0.303	0.150
Glucose	24	0.039	0.856
TC	24	0.250	0.239
HDL	24	-0.414	0.044
LDL	24	0.438	0.037
TGs	24	-0.133	0.536

*TC, total cholesterol
HDL cholesterol, high density lipoprotein
LDL cholesterol, low density lipoprotein
TGs, triglycerides

The present work has some limitations which should be mentioned. First, a major limitation of this study is related to the relatively small number of populations' cohort, and future analysis must be done on large number of participants. Secondly, the diabetic patients were considerably older than the control group.

CONCLUSIONS

Iron elevation in diabetic patients, the major cause of increased oxidative stress, liver damage, lipid peroxidation and especially high level of LDL-C suggest it's an important role and influence in development of disease, especially in lipid metabolism and represent a main risk factor of further complications of diabetes.

REFERENCES

Abbaspour, N., Hurrell., R., Kelishadi, R. (2014). Review on iron and its importance for human health. *J Res Med Sci., 19(2),* 164-74.

American Diabetes Association. (2004). Diagnosis and classification of diabetes mellitus. *Diabetes Care, 27*, S5–S10.

Aregbesola, A., Voutilainen, S., Virtanen, J. K., Mursu, J., Tuomainen, T-P. (2017). Gender difference in type 2 diabetes and the role of body iron stores. *Annals of Clinical Biochemistry, 54(1),* 113–120.

Arija, V., Fernández-Cao, J. C., Basora, J., Bulló, M., Aranda, N., Estruch, R., Martínez-González, M. A., Salas-Salvadó, J. (2014). Excess body iron and the risk of type 2 diabetes mellitus: a nested case–control in the PREDIMED (PREvention with MEDiterranean Diet) study. *British Journal of Nutrition, 112,* 1896–1904.

Backe, M. B., Moen, I. W., Ellervik, C., Hansen, J. B., Mandrup-Poulsen, T. (2016). Iron Regulation of Pancreatic Beta-Cell Functions and Oxidative Stress. Annu Rev Nutr., 36, 241-73.

Gohel, M., Sirajwala, H. B., Chacko, A. (2013). Serum Free Iron Concentration in Patients with Type 2 Diabetes Mellitus with Good and Poor Control and Its Correlation with Glycemic Control. *International Journal of Diabetes Research, 2(2),* 33-38.

International Diabetes Federation (IDF). (2006). Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia.

Fernández-Real, J. M., McClain, D., Manco, M. (2015). Mechanisms linking glucose homeostasis and iron metabolism toward the onset and progression of type 2 diabetes. *Diabetes Care, 38,* 2169–2176.

Misra, G., Bhatter, S. K., Kumar, A., Gupta, V., Khan, M. Y. (2016). Iron profile and glycaemic control in patients with type 2 diabetes mellitus. *Med. Sci., 4(4),* 22.

Orban, E., Schwab, S., Thorand, B., Huth, C. (2014). Association of iron indices and type 2 diabetes: a meta-analysis of observational studies. *Diabetes Metab Res Rev., 30(5),* 372-94.

Podmore, C., Meidtner, K., Schulze, M. B., Scott, R. A., Ramond, A., Butterworth, A. S., Angelantonio, E. D. (2016). Association of multiple biomarkers of iron metabolism and type 2 diabetes: The EPIC-InterAct Study. *Diabetes Care, 39,* 572–581.

Rajpathak, S. N., Crandall, J. P., Wylie-Rosett, J., Kabat, G. C., Rohan, T. E., Hu, F. B. (2009). The role of iron in type 2 diabetes in humans. *Biochim Biophys Acta., 1790(7),* 671-81.

Sanjeevi, N., Freeland-Graves, J., Beretvas, S. N., Sachdev, P. K. (2018). Trace element status in type 2 diabetes: A meta-analysis. *J Clin Diagn Res., 12(5),* OE01–OE08.

Shaaban, M. A., Dawod, A. E., Nasr, M. A. (2016). Role of iron in diabetes mellitus and its complications. *Menoufia Medical Journal, 29,* 11–16.

Simcox, J. A., McClain, D. A. (2013). Iron and Diabetes Risk. *Cell Metab., 17(3),* 329-341.

Smith, F. E., Herbert, J., Gaudin, J., Hennessy, D. J., Reid, G. R. (1984). Serum iron determination using ferene triazine. *Clin Biochem., 17(5),* 306-10.

Stechemesser, L., Eder, S. K., Wagner, A., Patsch, W., Feldman, A., Strasser, M., et al. (2017). Metabolomic profiling identifies potential pathways involved in the interaction of iron homeostasis with glucose metabolism. *Molecular Metabolism, 6,* 38-47.

Swaminathan, S., Fonesca, V. A., Alam, M. G., Shah, S. V. (2007). The role of iron in diabetes and its complications. *Diabetes Care, 30(7),* 1926-1933.

Wallace, D. F. (2016). The Regulation of Iron Absorption and Homeostasis. *Clin Biochem Rev, 37(2),* 51-62.

Wolide, A. D., Zawdie, B., Alemayehu, T., Tadesse, S. (2017). Association of trace metal elements with lipid profiles in type 2 diabetes mellitus patients: a cross sectional study. *BMC Endocrine Disorders, 17,* 64.

Summary/Sažetak

Nedavne studije ukazale su važnu ulogu povišenih vrijednosti željeza u patogenezi Tip 2 *Diabetes mellitus* (T2D) i insulinske rezistencije. Cilj ove studije bio je odrediti koncentraciju slobodnog željeza u serumu kod T2D pacijenata i naći asociranost iste sa lipidnim profilom. U studiji je bio uključen 51 participant (27 zdravih kontrola i 24 netretirana dijabetičara), starosne dobi 45-65 oba spola. Kao što je očekivano, nađene su povišene vrijednosti koncentracija željeza u serumu kod dijabetičara u poređenju sa zdravim osobama dok je statistički značajna razlika pokazana između nivoa željeza u kontrolnoj grupi i grupe sa dobrom kontrolom glikermije ($p<0.05$). Nadalje, postojala je značajna pozitivna korelacija između koncentracije slobodnog željeza i LDLcholesterol nivoa i negativna signifikantna korelacija između koncentracije željeza s HDL holesterolom kod dijabetičara. Rezultati sugeriraju da povišene koncentracije serumskog željeza mogu imati važnu ulogu i uticaj na razvoj oboljenja, posebno na metabolizam lipida i lipidnog profila u riziku od budućih komplikacija dijabetesa.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

54

Print ISSN: 0367-4444
Online ISSN: 2232-7266

Original Scientific Article
DOI: 10.35666/ghtbh.2020.54.03
UDC: 581.19:582.689.2

13-18

Chromatographic Methods for the Determination of Primula Acid 1 Content in *Primulae extractum fluidum*

Dedić, M.[a], Imamović, B.[a], Bečić, E.[a], Gičević, A.[a], Žiga-Smajić, N.[b], Medanhodžić- Vuk, S.[c], Šober, M.[a]

[a] *Faculty of Pharmacy University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, B&H*

[b] *Faculty of Pharmacy University of Sarajevo, Zmaja od Bosne 8, 71000 Sarajevo, B&H*

[c] *Pharmamed doo Travnik, Dolac na Lašvi bb Travnik, B&H*

Article info
Received: 04//12/2019
Accepted: 02/06/2020

Keywords:
Primula root
Primula acid 1
HPLC
Validation

***Corresponding author:**
Gičević Armina
E-mail: armina.gicevic@ffsa.unsa.ba
Phone: +387671300067

Abstract: *Primula veris* L. (*Primulaceae*) is healing plant, whose root is officially used to treat cough associated with cold. Other reported indications are respiratory, thoracic and mediastinal disorders. These effects are result of high contents of triterpenoid saponins and phenolic glycosides. Primula acid 1 (PA 1, also primulasaponin 1) is main active component in *Primula elatior* L. This paper presents an optimized high pressure liquid chromatography (HPLC) method for the determination of primula acid 1 content in *Primulae extractum fluidum.* TLC was performed to check for the presence of the substance of interest. The determination was performed by reversed phase chromatography using C18 as a stationary phase. The mobile phase used for separation consisted of 0.2% H_3PO_4 and acetonitrile. This method was validated through different parameters. The detection limit for primula acid 1 was LD=10.41 μg/ml, and the quantification limit was LQ=34.69 μg/ml. In order to determine the content of primula acid 1, a calibration curve was constructed, and the content of primula acid 1 was calculated by the equation of the calibration curve and was 0.2793 mg per gram of extract. The results and simple preparation of sample show that HPLC is the method of choice for this type of analysis.

INTRODUCTION

According to European Pharmacopoeia, herbal drug, primula root (Primulae radix) is descibed as dried root and rhizome of *Primula veris* L. (*Primula officinalis*) and/or *Primula elatior* L. Hill*., Primulaceae* (Coran and Mulas, 2012; Eur. Ph. 7.0, 2012). *Primula veris L.* is a well-known healing plant which grows on the lawns all over Europe and Western Asia and is officially used to treat cough associated with cold. Other reported indications for primula root are respiratory, thoracic and mediastinal disorders. These pharmacological effects are the result of a high contents of triterpenoid saponins (usually between 3-10%) and phenolic glycosides, located mostly in the roots. Important saponins in *Primula veris* L. are primacrosaponin and priverosaponin B, while in *Primula elatior* L. the main active component is Primula acid 1 (PA 1, also primulasaponin 1) This acid has a triterpenoid structure which is the skeleton of oleanolic acid. The sugar component is bound to this triterpenoid aglycon (protoprimulagenine A- Fig. 1) and together form primulasaponin heteroside (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.*, 2008). The sugar chain consists of: D-glucose, D-galactose, D-glucuronic acid and L-rhamnose, and binds to the hydroxyl group of protoprimulagenin A at the C-3 atome. (Fig. 1.) (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.,* 2008). Saponins help expectoration due to their capability to dissolve bronchial mucus and can therefore be used in syrups to treat respiratory deseases. Primula extract (*Primulae extractum*) is widely used in the production of medicinal pharmaceutical formulations and herbal products. It can be used alone or in combination with thyme extract or thyme tinctura (*Thymi extractum* ili *Thymi tinctura*, *Thymi herba*) (Gruenwald, Graubaum and Busch, 2005).

Indications for the use of primula extract and its combination with thyme tincture are mostly respiratory diseases, especially when it comes to chronic cough associated with chronic bronchitis and mucus arrest, pertussis and asthma. It has been found to have the highest secretolityc properties as primula acid 1 and primula acid 2 (Coran and Mulas, 2012; Okršlar, Plaper, Kovač, *et al.*, 2007; Başbülbül, Ozmen, Biyik, *et al.*, 2008; EMEA, 2008).

The mechanism of action of primulasaponin is not yet completely clear. In literature, there is a general agreement that saponins irritate gastric mucosa locally causing a reflex to increase bronchial secretion which dilutes mucus and reduces the viscosity. Irritation of the mucous membranes in throat and respiratory tract could also cause increased bronchial secretion (EMEA, 2012).

High pressure liquid chromatography (HPLC) analysis on normal and reversed phases are most commonly used to identify and purify saponins. To achieve the best separation of saponins, HPLC on reversed phases is usually used. This technique is fast, selective and very sensitive. The separation of saponins by HPLC can be affected by different stationary and mobile phases (Negi, Singh, Pant, *et al.*, 2011; Oleszek, 2002; Zhao, Zhao, Yuan, 2012).

The aim of this paper was to develop and optimize the HPLC method for the identification and quantification of primula acid 1 and to determine the content of primula acid 1 in Primulae extractum fluidum.

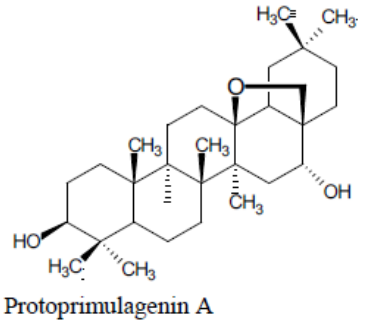


Figure 1. Aglycone part of saponin of *Primula veris* L. (protoprimulagenin A) (Wikimedia, 2019)

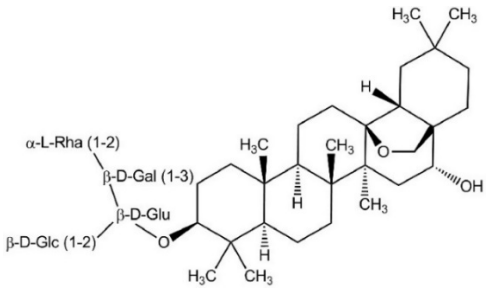


Figure 2. Structural formula of primula acid 1 (Wikimedia, 2019)

EXPERIMENTAL

Apparatus

The analysis was performed on HPLC apparatus with UV/VIS detector (HPLC system Prominence, type: 3-079, Shimadzu). The stationary phase was C18, dimensions of 250 x 4.6 mm, 5 µm Microsorb-Varian. Class-VP 7.4 software was used for signal analysis and statistical processing. Disposable filters CROMAFIL ® O-45/25- MachereyNagel, Germany; HPTLC plates 20x10 Kieselgel 60 F_{254}-Merck, USA; Microcapillaries 10 µl Camag, Swiss; UV/VIS lamp DesagaHeidelberg, Germany.

Chemicals

Primula acid 1 (≥99,9% purity)- Phytolab, Germany; Silica gel 60 GF_{254}- Merck, USA; Vanillin- Roth, Germany; Acetonitrile (HPLC grade)- Sigma Aldrich, USA; Methanol (HPLC grade)- Sigma Aldrich, USA; Sulphuric acid 96%- Lachema, Czech Republic; Ethanol absolute- Merck, USA; Purified water for HPLC.

Thin-Layer Chromatography (TLC)

Initially, screening of herbal extract was done to check for the presence of the substance of interest. Thin layer chromatography analysis was performed. For their preparation, HPTLC plates for analysis were kept overnight immersed in methanol, after which they were dried. Five samples were applied on the plate. The plate was placed in a chromatographic bath, where a mobile phase was previously prepared. When separation was completed, the plate was observed under a UV lamp at 254 nm. The plate was then sprayed with vanillin/ sulfuric acid reagent and again observed under UV lamp.

TLC conditions

HPTLC plates: 20x10 Kieselgel 60 F_{254}- Merck
Mobile phase: ethyl-acetate:formic acid:purified water (ratio 50:10:10 v/v)
Detection: UV/Vis light 254, 366 nm
Detection reagent: vanillin/sulfuric acid reagent

Sample preparation for TLC

1 ml from 5 g of liquid extract of primrose was dissolved in methanol in volumetric flask of 10 ml. 10 µl of this extracts was placed on HPTLC plate and then was separated.

Preparation of vanillin/sulfuric acid reagent

0.5 g of vanillin was dissolved in 80 ml of sulfuric acid and 20 ml of ethanol, cooled and used to visualize the TLC plates.

Chromatographic conditions

Stationary phase: column C18 (4.6 x 250 mm, 5 µm) Microsorb-Varian,
Mobile phase: 0.2% H_3PO_4 and acetonitrile (in 60:40 ratio v/v – isocratic),
Flow rate: 1.25 ml/min,
Injection volume: 20 µl,
Column temperature: 25˚C,
Detection: 195 nm.

Preparation of standard solution of primula acid 1

The stock solution of primula acid standard has been prepared at a concentration 1.0 mg/ml in methanol. For complete dissolution, solutions were placed in an ultrasonic bath for 15 minutes and then cooled. For the calibration curve, concentrations were prepared in the range of 50 to 150 µg/ml.

Preparation of *Primulae extractum fluidum* (Hagiwara, 2000).
Primulae radix 4.00 kg (v/v=0.1990)
Ethanolum (96 per centum) 8.80 kg (v/v=0.4378)
Aqua purificata 7.27 kg (v/v=0.3616)
Ammoniae solutio concentrata (10 per centum) 33.00 g (v/v=0.0016)

Total: 20.10 kg (v/v=1)

The prescribed ammounts of 96% ethanol and purified water were weighed and mixed. The ammonia solution was combined with alcohol and water. Th weighed ammount of drug (*Primulae radix*) which was previosly sieved and rinsed with cold water was poured over with 2 kg of combined solution and left to swell for 2 hours.
The drug was transferred to the percolate, the extraction solution was then poured and left to macerate for 12 hours. After maceration, percolation and filtration were performed. 5 g of liquid extract was obtained, so the drug extract ratio (DER) was 800:1. Used solvents were: ethanolum, aqua purificata and ammoniae solutio concentrata.
This prescription was used in the industrial preparation of intermediate product, from which 5 g was taken for the each analysis (HPLC and TLC), due to the concentration of primula acid in the final product (syrup).

Sample preparation of *Primulae extractum fluidum*
5 g of liquid extract of primula root (Primulae extractum fluidum, *Primula veris* L., *Primulaceae*) was dissolved in methanol in volumetric flask of 50 ml.

Contet calculation of Primula acid 1 in *Primulae extractum fluidum*
Content of Primula acid 1 in Primulae extractum fluidum can be calculated by the following formula:

$$\text{Content of Primula acid 1 } \left[{}^{\text{mg}}/_{\text{g}}\right] = \frac{X * V * 10^{-3}}{m}$$

$m_p = x * v$
m_p – mass of primula acid 1 in the sample in μg
X- concentration of primula acid 1 in the sample in μg/ml, calculated by the equation of calibration curve
V- sample volume in mililiters
m- mass of sample in grams
10^{-3} – used in formula to convert μg in mg

RESULTS AND DISCUSSION

The results of TLC analysis are shown in Figure 3. and Figure 4., where it is clearly seen the presence of primula acid 1. Rf value was 0.3.
Identification and quantification of primula acid 1 in the Primulae extractum fluidum can be proceeded by chromatographic method. Mueller et al. (2006) described the first liquid chromatographic method suitable for the characterization of bioactive compounds, i.e., saponins and phenolic glycosides, present in *Primula veris* and *Primula elatior*. On the other hand, Apel et al. (2017) developed a high performance liquid chromatography-diode array detection/mass spectrometry (HPLC/MS) method to analyze and compare the secondary metabolite profile of *Primula*.

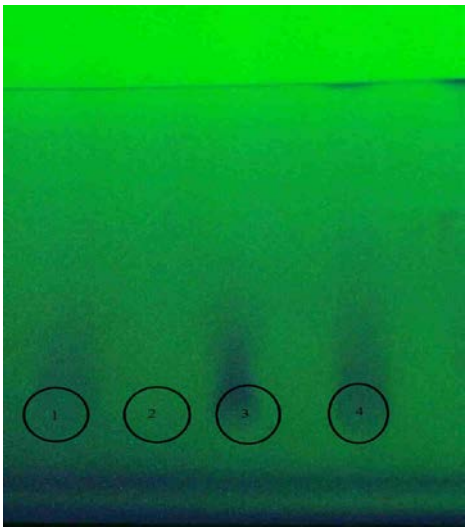


Figure 3. Chromatogram of standard of primula acid 1 (1), placebo (2), diluted sample of *Primulae extractum fluidum* (3) and sample of Primulae extractum fluidum observed at 254 nm.

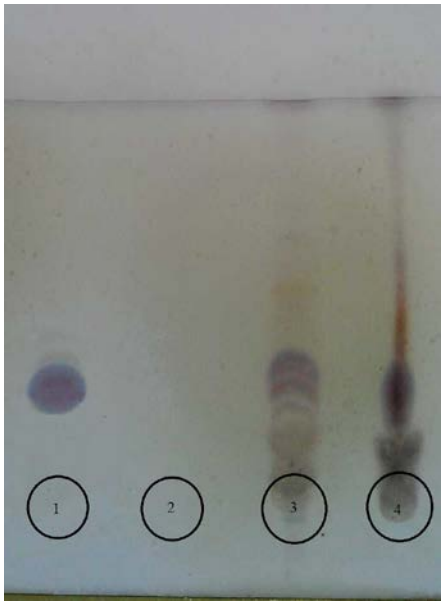


Figure 4. Chromatogram of standard of primula acid 1 (1), placebo (2), sample of *Primulae extractum fluidum* (3), sample of *Primulae extractum fluidum* (4) observed at daily light after spraying the plate with vanillin/ sulfuric acid reagent.

In this case, HPLC method was optimized and developed for identification and quantification of primula acid 1 in the *Primulae extractum fluidum.*
Validation of the analytical method was carried out by examining the following validation parameters: specificity, linearity, accuracy, repeatability, detection limit, quantification limit. (Figure 5. and Figure 6.) (ICH, 2005; Ivanović, Zečević, Malenović, 2000).
For the **selectivity** it was injected 20 μl of primrose extract (*Primulae extractum fluidum*) and 20 μl of placebo. Both solutions were recorded five times. Figure 5. and Figure 6. show a chromatogram of the primrose extract sample and a placebo chromatogram that clearly shows the retention time and area below the signal of the primula acid 1.

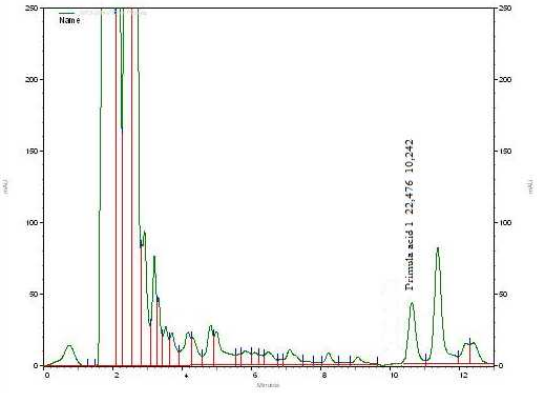


Figure 5. Chromatogram of sample of *Primulae extractum fluidum* detected at 195 nm

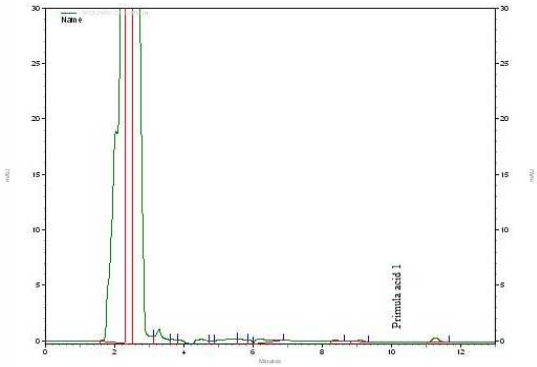


Figure 6. Chromatogram of placebo of solvents used in preparation of *Primulae extractum fluidum* detected at 195 nm

The **linearity** of the primula acid method in a wide range of concentrations of 50-150 µg/ml was determined. The calibration curves were constructed, the coefficient of correlation was calculated for primula acid R^2=0.9924, while the equation of direction was y=765.17x+1102.5 (Table 1, Figure 7.).

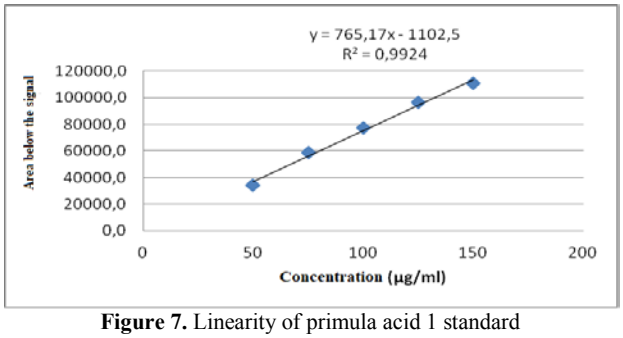


Figure 7. Linearity of primula acid 1 standard

Based on the linearity validation parameter, the values for the detection limit and quantification limit were obtained:
The detection limit was LD=10.41 µg/ml, and **the quantification limit** was LQ=34.69 µg/ml.

The **accuracy** of the test method was determined for primula acid concentrations 80; 100; 120 µg/ml representing 80, 100, 120% of the stock standard concentration. Tables 1 and 2 give the values for areas below the signal, concentration, recovery *(R%),* standard deviation (SD), relative standard deviations (RSD) and reliability coefficient (tα).

Repeatability of the test method was determined for primula acid concentrations 80; 100; 120 µg/ml representing 80, 100, 120% of the stock standard concentration. Tables 3. and Table 4 give the values for *recovery (R%)*, standard deviation (SD) and relative standard deviations (RSD%).

Intermediate precision
Intermediate precision for primula acid was also assessed, with three analysts separately performing three sample analysis in two different days (Table 5.).

Sample analysis
The chromatogram of the prepared extract solution (preparation was previously explained) can be seen in Figure 5., with associated retention times and area below the signal (10.108 minutes and area was 22 476).
The calculation shows that the solution contains 27.93 µg/ml of primula acid 1. This concentration is below quantification limit, but above detection limit, so it can be determined in the sample. Considering the extract preparation and dillution, the content of primula acid 1 per gram of extract is 0.2793 mg per gram (0.02793%) of Primulae extractum fluidum. The process of extract preparation has to be standardized so that the exact contents of the active components of primula acid coming from a different herbal material can be guaranteed by the manufacturer. These preparations are an important part of herbal medicines which have to pass strict quality control so that they could be registered on the market. This is a very fast, reliable and low-cost analysis. Due to the simple preparation of samples, this method can be used in the regular control of the content of primula acid in the final products and in semiproducts (*Primulae extractum fluidum*).

Table 1. The areas below the signals of chromatogram for different concentrations of primula acid 1 standard

Concentration (µg/ml)	P1	P2	P3	$\bar{X}$	LD (µg/ml)	LQ (µg/ml)
50	34084	34155	34061	34100.00		
75	58516	58863	58653	58677.33		
100	76919	77409	76905	77077.67	10.41	34.69
125	96372	94805	94616	95264.33		
150	111175	110100	111583	110952.67		

Table 2. Validation parameter accuracy for primula acid 1

	P1	P2	P3	$\bar{X}$	<ɣ>	SD	<R%>	RSD (%)	t_α
80% (80 µg/ml)	62194	62535	62124	62284.33	82.84	0.29	103.55	0.4	1.31
100% (100 µg/ml)	77059	77109	77907	77358.33	102.54	0.62	102.54	0.6	1.34
120% (120 µg/ml)	91859	91356	92368	91861	121.49	0.66	101.24	0.5	1.45

Table 3. Validation parameter repeatability for primula acid 1

Number of measure	**concentration 1**			**concentration 2**			**concentration 3**		
	Area	Conc. (µg/ml)	*R (%)*	Area	Conc. (µg/ml)	*R (%)*	Area	Conc. (µg/ml)	*R (%)*
1	61194	81.42	101.77	77059	102.15	102.15	91859	121.49	101.24
2	62535	83.17	103.96	77109	102.21	102.21	91356	120.83	100.69
3	62124	82.63	103.29	77907	103.26	103.26	92368	122.16	101.80
4	61931	82.38	102.97	77721	103.01	103.01	92652	122.53	102.11
5	61907	82.35	102.93	77398	102.59	102.59	91768	121.37	101.14
6	61582	81.92	102.40	77532	102.77	102.77	92579	122.43	102.03
$\bar{X}$	61878.83	82.31	102.89	77454.33	102.67	102.67	92097	121.80	101.50
SD		0.60	0.75		0.44	0.44		0.67	0.61
RSD (%)		0.7	0.73		0.4	0.43		0.6	0.60

Table 4. Validation parameter intermediate precision for primula acid 1 and (First day)

	Analyst 1/ Sample 1			**Analyst 2/ Sample 2**			**Analyst 3/ Sample 3**		
Number of measure	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*
1	91615	121.17	100.98	88984	117.73	98.11	89175	117.98	98.32
2	90586	119.83	99.86	89629	118.58	98.81	89915	118.95	99.13
3	92423	122.23	101.86	89113	117.90	98.25	90007	119.07	99.23
$\bar{X}$	91541.33	121.08	100.90	89242.00	118.07	98.39	89699.00	118.67	98.89
SD		1.20	1.00		0.45	0.37		0.60	0.50
RSD (%)		0.99	0.99		0.38	0.38		0.50	0.50

Table 5. Validation parameter intermediate precision for primula acid 1 and (Second day)

	Analyst 1/ Sample 1			**Analyst 2/ Sample 2**			**Analyst 3/ Sample 3**		
Number of measure	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*	*Area*	*Conc. (µg/ml)*	*R (%)*
1	89632	118.58	98.82	89633	118.58	98.82	90924	120.27	100.22
2	90196	119.32	99.43	88518	117.12	97.60	89733	118.71	98.93
3	89316	118.17	98.47	88082	116.56	97.13	90817	120.13	100.11
$\bar{X}$	89714.67	118.69	98.91	88744.33	117.42	97.85	90491.33	119.70	99.75
SD		0.58	0.49		1.05	0.87		0.86	0.72
RSD (%)		0.49	0.49		0.89	0.89		0.72	0.72

CONCLUSIONS

The results of HPLC analysis of *Primulae extractum fluidum* showed that the concentration of primula acid 1 was 0.2793 mg per gram (0.02793 %) of the extract. The proposed HPLC method was validated through validation parameters and all results of the statistical analysis were within the reference range recommended by the ICH guidelines. Besides that, this method proves to be very fast, reliable and low-cost. Considering all of this and the fact that this method does not require a complicated sample procedure, it can be used in the regular control of the content of primula acid 1 in the final products and in semiproducts (*Primulae extractum fluidum*).

REFERENCE

Apel, L., Kammerer, D. R., Stintzing, F. C., & Spring, O. (2017). Comparative metabolite profiling of triterpenoid saponins and flavonoids in flower color mutations of *Primula veris* L. *International journal of molecular sciences*, *18*(1), 153.

Başbülbül, G., Özmen, A., Biyik, H., Şen, Ö. (2008). Antimitotic and antibacterial effects of the *Primula veris* L. flower extracts. *Caryologia*, 61 (1), 88-91.

Coran, S. A., Mulas, S. (2012). Validated determination of primulasaponins in primula root by a high-performance-thin-layer-chromatography densitometric approach. *Journal of pharmaceutical and biomedical analysis*, 70, 647-651.

Gruenwald, J., Graubaum, H. J., & Busch, R. (2005). Efficacy and tolerability of a fixed combination of thyme and primrose root in patients with acute bronchitis. *Arzneimittelforschung, 55*(11), 669-676.

European Medicines Agency Science Medicines Health. Assessment report on *Primula veris* L. and/or *Primula elatior* (L.) Hill. Committee on Herbal Medicinal Products (HMPC) Hill, flos. 2012, London, UK.

European Medicines Agency Evaluation of Medicines for Human Use. Assessment report on *Primula veris* L., *Primula elatior* (L.) Hill, radix. 2008, London, UK.

European Pharmacopoeia 7.0, C.o. Europe, Editor. 2012, EDQM: Strasbourg, French.

Hagiwara, Y. (2000). *U.S. Patent No. 6,022,573*. Washington, DC: U.S. Patent and Trademark Office.

ICH Harmonised Tripartite Guideline, Validation of analytical procedures: text and methodology Q2(R1). 2005.

Ivanović, D., Zečević, M., Malenović, A. (2000). Analitika lekova udžbenik za laboratorijsku nastavu. Akademija štamparija, Beograd.

Müller, A., Ganzera, M., & Stuppner, H. (2006). Analysis of phenolic glycosides and saponins in *Primula elatior* and *Primula veris* (primula root) by liquid chromatography, evaporative light scattering detection and mass spectrometry. *Journal of Chromatography A*, *1112*(1-2), 218-223.

Negi, J., Pant, G., Singh, P., Rawat, M. (2011). High-performance liquid chromatography analysis of plant saponins: An update 2005-2010. *Pharmacognosy Reviews*, *5*(10), 155.

Okršlar, V., Plaper, I., Kovač, M., Erjavec, A., Obermajer, T., Rebec, A., Ravnikar, M., Žel, J. (2007). Saponins in tissue culture of *Primula veris* L. *In Vitro Cellular & Developmental Biology - Plant*, *43*(6), 644-651.

Oleszek, W. (2002). Chromatographic determination of plant saponins. *Journal of Chromatography A*, *967*(1), 147-162.

Strukturna formula primula kiseline I. Available from: <URL:https://commons.wikimedia.org/wiki/File:Primula_acid_A.PNG> pristupljeno 10.08.2019. godine.

Zhao, B., Zhao, W., Yuan, Z. (2012). Optimization of extraction method for total saponins from *Codonopsis lanceolata*. *Asian J of Traditional Medicines*, *7*(1), 14-17.

Summary/Sažetak

Primula veris L. (Primulaceae) je dobro poznata ljekovita biljka čiji se korijen u službenoj upotrebi koristi za liječenje kašlja kod prehlada. Ostale prijavljene indikacije odnose se na respiratorne, torakalne i medijastinalne poremećaje. Ovi efekti su rezultat visokog sadržaja triterpenskih saponina i fenolnih glikozida. Primula kiselina 1 (PA 1, također primulasaponin 1) je glavna aktivna komponenta u *Primula elatior* L. U ovom radu je razvijena metoda tečne hromatografije pod visokim pritiskom (HPLC) za određivanje sadržaja primula kiseline 1 u ekstraktu jagorčevine. Za utvrđivanje prisustva supstance od interesa prethodno je korištena hromatografija na tankom sloju. Određivanje je provedeno na obrnutoj fazi C18, a za razdvajanje je korištena mobilna faza 0.2% H_3PO_4 i acetonitril. Navedena metoda validirana je kroz različite parametre. Limit detekcije za primula kiselinu 1 iznosio je LD=10.41 µg/ml, a limit kvantifikacije LQ=34.69 µg/ml. Za određivanje sadržaja primula kiseline 1 konstruisana je kalibraciona kriva, te je potom na osnovu jednačine pravca određen sadržaj primula kiseline 1 u ekstraktu jagorčevine koji je iznosio 0.2793 mg po gramu ekstrakta. Rezultati i jednostavnost pripreme uzorka ukazuju na to da je HPLC metoda izbora za ovakvu vrstu analize.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

54

Original Scientific Article

Print ISSN: 0367-4444

Online ISSN: 2232-7266

DOI: 10.35666/ghtbh.2020.54.04

UDC: 581.19:582.929.4

19-26

Phytochemical Investigation and Antioxidative Capacity of Triterpenes Present in Plant Species Belonging to Lamiaceae Family

Jordamović, N.[a], Nikšić, H.[a], Muratović, S.[a], Gusić, I.[a], Korić, E.[a], Alagić, L.[b], Pašić, M.[b], Durić, K.[a]

[a]*Department of Biology, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, Sarajevo, B&H*

[b]*Bosnalijek d.d., Jukićeva 51, 71 000 Sarajevo, Bosnia and Herzegovina*

Article info
Received: 27/12/2019
Accepted: 21/04/2020

Keywords:
Triterpenes
Lamiaceae family
Antioxidative Capacity
Betulinic Acid

***Corresponding author:**
Durić Kemal
E-mail: kemal.duric@ffsa.unsa.ba
Phone: 00-387-61-213307
Fax: 00-387-33-586178

Abstract: Triterpenes are persistently associated with observed bioactivities of extracts obtained from plant material that contains these very important natural products. Many species belonging to Lamiaceae family have been used for the presence of essential oil and very little is known about the presence of the triterpene substances in this family. Qualitative and quantitative analyses of this very important substances, in the aerial parts of eight species, all belonging to Lamiaceae family, were investigated in this study. Different extracts containing triterpene substances were tested by DPPH method to evaluate their antioxidative capacity. TLC and HPLC methods, used for the analytical determination of triterpenes, showed the presence of betulin, betulinic acid, ursolic acid and oleanolic acid. Betulin (3.2 mg/g) and betulinic acid (37.1 mg/g) were the most abundant triterpene components in the hexane extracts of *Rosmarinus officinalis* L. Ursolic acid (0.14 mg/g) was the most abundant triterpene compound in the hexane extract of *Thymus pulegioides* L. All tested samples demonstrated DPPH scavenging activity in a concentration dependant manner, with a wide range of IC_{50} values from 0.4 mg/mL to 3.3 mg/mL.

INTRODUCTION

Terpenes are derived from ordinary precursors and represent the largest known group of plant secondary metabolites. Most terpenes are characteristic of the plant kingdom, but they also occur in animals, for example, sesquiterpenoid ferrohormones in insects, or diterpenes of some marine organisms. Within this group, the most important are triterpenes, from the point of view of the possibility of their industrial and therapeutic application. Triterpenes comprise about 4000 compounds that build about 40 different skeletons, with a C30 structure. Triterpenes play an important role in ecology and agronomy, in the defense against pathogens and animals, as well as in the quality of plant crops. They also play an important role in commercial use in food, cosmetics, pharmacy and industrial biotechnology sectors (Brahmkshatriya and Brahmkshatriya, 2013; Thimmappa, Geisler, Louveau, *et al.*, 2014). In terms of biological activity, the most important triterpenes are pentacyclic oleanane, ursane, lupane and tetracyclic damaran (Jäger, Trojan, Kopp, *et al.*, 2009). These include various compounds, lupane derivatives described in over 300 species of higher plants. In scientific literature, these compounds are attributed to the term "steroid triterpenes". Basically, they differ only in the oxidation state of the group bound to C-17, but the consequences of this difference with respect to their physical-chemical characteristics and pharmacological effects are very large (Dzubak, Hajduch, Vydra, *et al.*, 2006; Muffler, Leipold, Scheller, *et al.*, 2011).

Plant species from the Lamiaceae family mostly contain pentacyclic triterpenes that have anti-inflammatory, hepatoprotective, antioxidant, anticancer, antiviral, and antimicrobial activity (Dzubak, *et al.*, 2006; Shanaida, Hudz, Korzeniowska, *et al.*, 2018). Most of the

Lamiaceae species examined in this work are used to obtain essential oils, which are required in the pharmaceutical, food and cosmetic industries. Such a large production of essential oils leads to the formation of large quantities of plant residues, which contain non-volatile fractions, which can represent a good source of useful substances that are not currently used. Our aim was to investigate the presence of triterpenes in plant residues after distillation of essential oil from eight species belonging to Lamiaceae family, collected in Bosnia and Herzegovina, and to evaluate antioxidant capacity of triterpene fractions obtained with different extraction solvents.

EXPERIMENTAL

Plant material.

The aerial parts of wild plants *Rosmarinus officinalis* L. (**a**), *Salvia officinalis* L. (**b**), *Melissa officinalis* L. (**c**), *Thymus pulegioides* L. (**d**), *Lavandula officinalis* L. (**e**), *Satureja montana* L. (**f**), *Mentha piperita* L. (**g**) and *Origanum vulgare* L. (**h**), were collected at the flowering stage from May to July in Bosnia and Hercegovina in 2017. Plant material was identified by a series of comparative macroscopic, organoleptic, and TLC analyses. Plant *voucher specimens* were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Sarajevo. The raw material was dried and processed according to pharmacognostic principles. Before it was used, the plant material was stored in a dry place, in the absence of the light at room temperature.

Extraction procedure

The plant material (45.0 g), was distilled with water vapor to extract the essential oil. After steam distillation, the herbal residue was separated from water in which it was immersed, dried in an oven, in order to obtain a vegetable matrix. In order to perform quantitative extraction of triterpene compounds from the vegetable matrix, a successive extraction using three solvents of different polarity was carried out. The extraction procedure was performed in a Soxhlet apparatus for six hours. As solvents we used hexane, chloroform and methanol (each 600 ml). The extracts were concentrated under reduced pressure in a rotary evaporator.

Thin-Layer Chromatography (TLC).

In order to monitor the presence of triterpene substances in different fractions obtained by Soxhlet extraction, a TLC method was performed using pre-coated silica gel GF_{254} plates (20x20 cm, thickness 0.25 mm, Merck, Dürmsttadt). The solvent system used as eluent was benzene (Merck, Germany): ethyl acetate (Sigma-Aldrich, US): formic acid (36:12:5) (Kemika, Zagreb). Detection of triterpene substances was achieved by observation under $UV_{354/254}$, spraying with 4-anisaldehyde (Merck, Germany) - sulphuric acid (Kemika, Zagreb) and heating with a heat gun until full color development. Since the triterpene compounds are the subject of the study, the triterpene standards were used for their identification in different extracts.

High pressure liquid chromatography (HPLC)

The HPLC was used to quantifie triterpenes, using standard of betulin, betulinic acid, ursolic acid, oleanolic acid and lupeol in the extracts obtained with different solvents. This was conducted on HPLC SHIMADZU 10Avp with autosampler and spektro monitor® 3100 optical detector (LC Analytical), using a constaMetric® 3000 system for solvent release, Hyperesil ODS (Agilent Technologies) column, 4.6 x 250 mm, 5μm, mobile phase acetonitrile/aqua (700/300) acidified with ortho-phosphoric acid.

All reagents used in the experimental work were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Triterpene standards as well as investigated extracts were dissolved in acetone and used for HPLC analysis. Distilled water was used for dilution and cleaning in all analytical procedures.

1,1-Diphenyl-2-picrylhydrazyl radical-scavenging capacity (DPPH method)

The ability of phenolic derivatives to donate a hydrogen atom or an electron and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams (Brand-Williams, Cuvelier and Berset, 1995; Thaiponga, Boonprakoba, Crosbyb, *et al.,* 2006). The tested extracts were prepared as hexane solutions (16.7 mg/ml). For each fraction analyzed, a calibration relationship diagram between reduction and concentration of dilution was calculated. Sample solution (100 μL) was mixed with 1.0 mL DPPH in anhydrous ethanol (5.25×10^{-5} mol/L). Decrease in absorbance of tested mixtures was monitored every 1 minute for 30 minutes at 517 nm using *Perkin-Elmer Lambda* 25 UV/Vis spectrophotometer. Anhydrous ethanol was used to annul the absorbance at 517 nm, DPPH solution was used as blank sample, and Trolox® (Sigma-Aldrich, US) was used as a positive control. All samples were made in triplicate.

RESULTS AND DISCUSSION

Extraction of triterpene compounds

The yield obtained by successive extraction with hexane, chloroform and methanol solvents is shown in Table 1. The highest yield, calculated as the dry extract, was obtained with methanol, followed by hexane and finally chloroform. The qualitative composition of three extracts obtained by successive extractions, was obtained by TLC.

Table 1. The yield of extracts obtained from plant material (45 g) using different solvents

Herb	Yield of dry extract		
	Hexane	Chloroform	Methanol
Rosmarinus officinalis L.	3.2 g (7.0%)	4.6 g (10.3%)	8.4 g (18.6%)
Salvia officinalis L.	6.6 g (14.8%)	1.5 g (3.3%)	11.7 g (25.9%)
Melissa officinalis L.	1.9 g (4.3%)	0.8 g (1.8%)	9.3 g (20.6%)
Thymus pulegioides L.	1.1 g (2.4%)	0.6 g (1.2%)	9.2 g (20.5%)
Lavandula officinalis L.	4.5 g (10.2%)	1.6 g (3.4%)	5.7 g (12.7%)
Satureja montana L.	2.2 g (4.8%)	0.7 g (1.6%)	7.4 g (16.5%)
Mentha piperita L.	2.6 g (5.6%)	1.7 g (3.8%)	10.5 g (23.4%)
Origanum vulgare L.	2.4 g (5.4%)	0.9 g (2.1%)	14.0 g (31.0%)

The TLC conditions used in this experiment proved to be convenient since they allowed the investigated triterpene substances to be separated and identified in different extracts. Triterpene standards used in this investigation had different Rf values as follows: lupeol (Rf 0.86), betulinic acid (Rf 0.76), ursolic acid (Rf 0.66), betulin (Rf 0.70) and oleanolic acid (Rf 0.72). Although there is a small differences in Rf values between oleanolic and betulinic acid, it was possible to distinguish them, since oleanolic acid is coloured in dark violet and betulinic acid in light violet, after detection with 4-anisaldehyde - sulfuric acid. Betulin has the lowest Rf value and is coloured in violet, while lupeol has the highest Rf value and is coloured darker violet. An example of a qualitative composition of three extracts, obtained from plant material after distillation of essential oil, using TLC, was shown for Lavandulae flos. (Figure 1.).

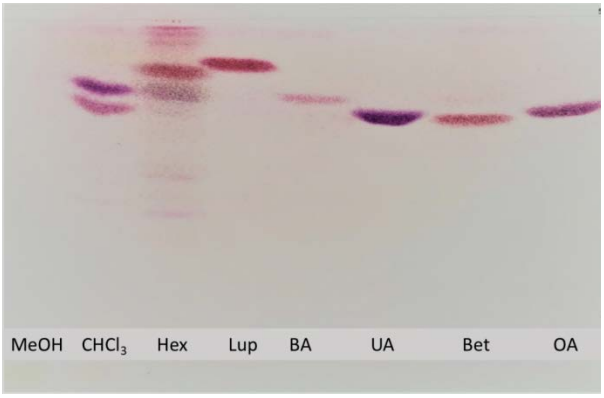


Figure 1. TLC Chromatogram of methanol (MeOH), chloroform ($CHCl_3$) and hexane (Hex) extracts of Lavandulae flos with triterpene standards: lupeol (Lup), betulinic acid (BA), ursolic acid (UA), betulin (Bet) and oleanolic acid (OA).

A fraction containing all triterpene substances could not be obtained, as different triterpenes showed different solubility in the solvents used in this investigation. The presence of triterpene substances, at any rate, in the chloroform fraction and hexane fraction were confirmed by TLC. Although TLC analysis clearly showed the absence of the investigated triterpene substances in methanol extracts, in further analysis all extracts including methanol, were subjected to HPLC in order to quantified single triterpenes by comparison with standards. The HPLC results clearly confirm the presence of triterpene substances, separated and identified by TLC method. Although the yield of methanol extract was far higher than that of hexane and chloroform extracts (Table 1), quantitative analysis showed that the latter two contained the highest amount of triterpene compounds. This indicates that the triterpene compounds are lipophilic in nature, which also determines the method of preparation of the plant materials containing them. The most common ways of preparing herbal drugs are decoct and infusion, and therefore since water is a solvent, they do not contain triterpene compounds. As a result, the use of the investigated herbs from the point of view of triterpene saponins, should involve the preparation of extracts that use lipophilic solvents.

Summarizing the results for betulin it can be concluded that the highest amount of this triterpene was found in the hexane extract of **a** (0.004 mg/ml), than hexane extract of **e** (0.002 mg/ml) followed by hexane extract of **c** (0.001 mg/ml). Chloroform extracts of **f** and **g** also contained certain amount of betulin (0.001 mg/ml) while this triterpene was not detected in the hexane extract of **h**. The highest amount of betulinic acid was detected in the hexane extract of **a** (0.05 mg/ml) and the hexane extract of **g** (0.01 mg/ml). This very important triterpene acid was detected in a very small low amount, mainly in hexane extracts of other investigated plant materials.

Interpretation of the scientific literature assign ursolic acid the role of the major bioactive principle in the plant materials with anti-inflammatory activity (Kashyap, Sharma, Tuli, *et al.*, 2016), anticancer properties (Chen, Wu, Duan, *et al.*, 2019), protective effects against cytotoxicity (Ramos-Hryb, Platt, Freitas, *et al.*, 2019) and antidiabetic activity (Wang, Zhao, Yan, *et al.*, 2019).

According to HPLC results, the highest amount of ursolic acid was detected in chloroform fractions of **e** (0.14 mg/ml) followed by **b** and **c** (009 mg/ml) and **d** (0.07 mg/ml). The content of ursolic acid was also found in hexane fractions of tested plant materials, which indicated that ursolic acid is soluble in both solvents.
After HPLC analyses of the fractions obtained from all plant materials, it has been shown that the hexane fraction of **a** was the only one containing oleanolic acid (0.001 mg/ml).

Table 2. Results of the triterpene derivatives content calculated in the hexane, chloroform and methanol extracts of individual drugs obtained by HPLC analysis.

Extract	Betulin (mg/ml) +SD	Betulinic acid (mg/ml) +SD	Ursolic acid (mg/ml) +SD	Oleanolic acid (mg/ml) +SD
a - HE	0.004 ±0	0.052 ±0.006	0.033 ±0.004	0.001 ±0
a - HL	ND	0.022 ±0.001	ND	ND
a - ME	ND	ND	ND	ND
b - HE	ND	0.002 ±0.0002	0.034 ±0.003	ND
b - HL	ND	ND	0.095 ±0.01	ND
b - ME	ND	ND	0.001 ±0	ND
c - HE	0.001 ±0.0004	0.003 ±0	0.043 ±0.005	ND
c - HL	ND	0.001 ±0.0004	0.088 ±0.008	ND
c - ME	ND	ND	ND	ND
d - HE	0.001 ±0.0005	ND	0.115 0.014	ND
d - HL	ND	ND	0.074 ±0.02	ND
d - ME	ND	0.001 ±0.0003	ND	ND
e - HE	0.002 ±0.0006	0.002 ±0	0.026 ±0.005	ND
e - HL	ND	ND	0.145 ±0.005	ND
e - ME	ND	0.001 ±0.0004	ND	ND
f - HE	ND	0.003 ±0.0003	0.062 ±0.004	ND
f - HL	0.001 ±0.0004	0.001 ±0.0005	0.035 ±0.003	ND
f - ME	ND	ND	ND	ND
g - HE	ND	0.009 ±0.001	0.020 ±0.009	ND
g - HL	0.001 ±0.0005	0.001 ±0.0003	0.013 ±0.009	ND
g - ME	ND	0.003 ±0.0005	ND	ND
h - HE	ND	0.002 ±0.0002	0.022 ±0.006	ND
h - HL	ND	ND	0.012 ±0.009	ND
h - ME	ND	ND	ND	ND

a - *Rosmarinus officinalis* L., **b** - *Salvia officinalis* L., **c** - *Melissa officinalis* L., **d** - *Thymus pulegioides* L., **e** - *Lavandula officinalis* L., **f** - *Satureja montana* L., **g** - *Mentha piperita* L., **h** - *Origanum vulgare* L., **HE** – hexane, **HL** – chroroform, **ME** – methanol, **ND**-non detected.

From the point of view of the different plant species investigated in this work, Rosmarini folium was proved to be the most important since it contains betulin, betulinic acid, ursolic acid and oleanolic acid. Figure 2 shows the HPLC chromatogram of hexane rosemary leaf extract which clearly shows the separated peaks of the tested triterpene substances, identified based on the retention times of the standard: betulinic acid (BA; Rt 15.957), oleanolic acid (OA; Rt 17.120), ursolic acid (UA; Rt 17.995) and betulin (bet; Rt 20.437). Quantification of individual triterpenes was done based on the area below the corresponding peak. The study accomplished by Andrade and coworkers corroborates our results (Andrade, Faustino, Garcia, *et al.*, 2018). Lupeol was detected neither in this material nor in the other investigated plant species.

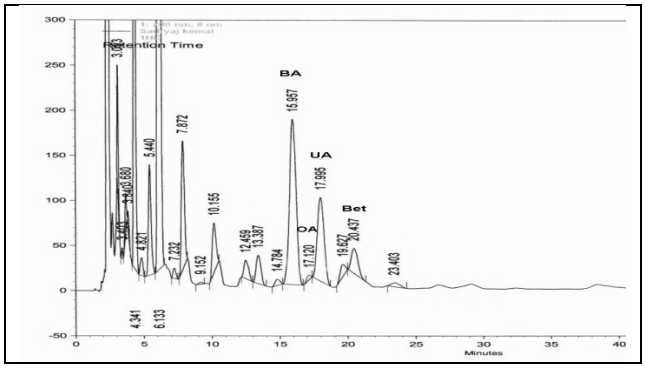


Figure 2. HPLC chromatogram of hexane extract obtained from Rosmarini folium: betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA) and betulin (Bet).

From a standpoint of the presence of certain compounds, Rosmarini folium is still the plant material (drug) of choice, because it exclusively contains betulinic acid and oleanolic acid. The plant material of choice with the highest amount of determined ursolic acid was Lavandulae flos (hexane fractions: 0.14 mg/ml) (Jäger, 2009.), followed by Thymi herba (hexane fraction: 0.11 mg/ml) (Raudone, Zymone, Raudonis, *et al.*, 2017). Since these triterpenes were found in the fractions obtained from plant material after separation of essential oil by steam distillation, the possibility of using the vegetable residue after obtaining the essential oil opens up. In this way, a huge amount of plant material that is discarded in the essential oil industry can be used to extract triterpene substances. This also mean a new pleiotropic activity and possibility of new indications for the plant species of the Lamiaceae family investigated in this work. Total amount of individual triterpenes calculated per 100 g of investigated plant material is given in Table 3.

Table 3. Overview of the total content of triterpenes in the investigated plant material

Herb	**Content of single triterpenes per 100 g of investigated plant material**			
	Betulin	Betulinic acid	Ursolic acid	Oleanolic acid
Rosmarinus officinalis L.	7.0 mg	98.0 mg	42.0 mg	1.4 mg
Salvia officinalis L.	1.5 mg	4.5 mg	169.0 mg	-
Melissa officinalis L.	2.1 mg	4.5 mg	182.0 mg	-
Thymus pulegioides L.	1.1 mg	1.1 mg	265.0 mg	-
Lavandula officinalis L.	2.8 mg	4.3 mg	230.0 mg	-
Satureja montana L.	1.4 mg	5.0 mg	126.0 mg	-
Mentha piperita L.	1.4 mg	18.2 mg	42.4mg	-
Origanum vulgare L.	-	2.8 mg	42.0 mg	

Antioxidant capacity (AC) is defined as the ability of a pure substance or complex chemical mixture to slow or prevent the oxidation of other substances when both are simultaneously exposed to free radicals that cause their oxidation. Therefore, AC represents a quantitative value of resistance toward effects of free radicals that can be expressed in different ways, and it is mostly converted into the amount of standard antioxidant, usually vitamin E per unit sample (Santos-Sánchez, Salas-Coronado, Villanueva-Cañongo, *et al.*, 2019)

Triterpenes, investigated in this work, have previously been described as substances with a strong scavenging capacity against various free radicals (Parvez, Alam, Arbab, et al., 2018; Wang, Liu, Lian, *et al.*, 2019; Yin and Chan, 2007). Moreover, oleanolic acid and ursolic acid have been indicated as proton donors and consequently with reducing power (Santiago, Dayrit, Correa, *et al.*, 2014). The total triterpene fractions obtained from different plant species of the Lamiaceae family were evaluated for their antioxidant capacity using DPPH method. The total triterpene fractions for each plant material were obtained in such a way that the chloroform and hexane fractions containing most of the triterpene saponins were mixed in equal parts. This total fraction was used for antioxidant investigation.

According to the data in Table 3, it can be said that investigated triterpene fractions have promising antioxidant capacity. In modern pharmacy, "enriched extracts" are increasingly used, these are extracts concentrated in the main active substances (in our case, these are triterpenes), aware that in addition to these substances, there are concomitant substances that contribute to the final activity. Therefore, the results obtained from the values of the antioxidant activity of the conjoint fractions are useful as they give an insight into the possibility of total antioxidant activity including the accompanying substances present in the given extracts. All of the tested extracts showed antioxidant capacity in a dose-dependent manner. The IC_{50} value ranges from 0.5 mg/ml to 3.47 mg/ml (Table 4.).

The most intense antioxidant capacity was demonstrated by the triterpene fraction obtained from rosemary leaf (IC_{50} 0.5 mg/ml). Based on the literature data, the antioxidant capacity of rosemary extracts is mainly associated with total flavonoids and phenols. The obtained antioxidant effects of the rosemary leaf triterpene fraction indicate that these compounds contribute significantly to the overall antioxidant capacity of this herbal drug (Nieto, Ros, Castillo, *et al.*, 2018)

The next most significant antioxidant capacity showed the triterpene fraction obtained from lavender flower (IC_{50} 1.6 mg/ml), followed by the triterpene fraction of thyme and mint leaf with antioxidant capacity values (IC_{50} 2.5 mg/ml). The fractions obtained from the mountain savory herb (IC_{50} 3.4 mg/ml) and oregano herb (IC_{50} 3.5 mg/ml) showed the lowest value of the antioxidant capacity. The antioxidant activity data for these fractions coincide with the lowest detected values of the triterpene substances in the above-mentioned fractions.

Table 4. Results of the analysis of antioxidant capacity of different triterpene fraction

Triterpene fraction	[Sample] (mg/ml)	(%) Antioxidant activity	IC_{50} (mg/ml)
Rosmarinus officinalis	0.7	52.82	0.5±0.06
	1.4	62.12	
	2.3	72.97	
	3.2	83.43	
Salvia officinalis	1.5	15.72	3.3±0.4
	2.8	36.97	
	3.8	63.59	
Melissa officinalis	$4.7x10^{-5}$	7.31	2.9±0.3
	1.3	25.33	
	3.8	53.235	
	4.8	62.33	
Thymus pulegioides	1.5	38.27	2.5±0.5
	2.8	52.46	
	3.8	64.32	
	4.8	76.97	
Lavandula officinalis	1.5	49.42	1.6±0.1
	2.8	58.76	
	3.8	60.85	
	4.8	67.72	
Satureja montana	1.1	25.5	3.4±0.3
	2.1	39.1	
	4.3	59.0	
Mentha piperita	0.8	13.38	2.5±0.5
	1.5	29.91	
	2.8	56.46	
Origanum vulgare	1.3	29.0	3.5±0.4
	2.3	34.1	
	3.4	50.2	
Trolox	0.012	34.67	0.018±0.002
	0.018	48.67	
	0.030	77.57	

CONCLUSION

The present study shows that the aerial parts of *Mentha piperita* L., *Thymus pulegioides* L., *Rosmarinus officinalis* L., *Origanum vulgare* L., *Salvia officinalis* L., *Satureja montana* L., *Lavandula officinalis* L. and *Melissa officinalis* L., plant species from Lamiaceae family, collected in Bosnia and Hercegovina, represent an important source of triterpenes. The presence of these pharmacologically active compounds attributes new pleiotropic properties to the investigated herbal drug and opens new opportunities for their use in official pharmacy and medicine. Successive extraction with different solvents showed that chloroform was the most suitable solvent for the extraction of investigated triterpenes. TLC and HPLC experimental conditions, used in this study, proved to be sufficiently sensitive, accurate and reproducible, from the standpoint of the qualitative and quantitative analysis of the presence of triterpene substances in different extracts. Betulin (0.004 mg/ml) and betulinic acid (0.05 mg/ml) were the most abundant triterpenoid components in the hexane extracts of *Rosmarinus officinalis* L. Ursolic acid (0.14 mg/ml) was the most abundant triterpenoid component in the hexane extract of *Thymus pulegioides* L. Oleanolic acid (0.01 mg/ml) was found only in the hexane extract of *Rosmarinus officinalis* L. Due to the different solubility of individual triterpenes in the extraction solvents used, the antioxidant activity assay was performed on fractions with total triterpene content. The best antioxidative capacity was demonstrated by the triterpene fractions from the plant species *Rosmarinus officinalis* and *Lavandula angustifilia*, which were at the same time the richest in triterpenes, indicating a direct proportionality of the antioxidant activity and content of triterpenes.

REFERENCES

Andrade, J.M., Faustino, C., Garcia, C., Ladeiras, D., Reis, C.P., Rijo, P. (2018). *Rosmarinus officinalis* L.: an update review of its phytochemistry and biological activity. *Future Science OA*, 4(4). FSO283.

Brahmkshatriya, P.P., Brahmkshatriya, P.S. (2013). Terpenes: Chemistry, Biological Role, and Therapeutic Applications. Natural Products, In: Ramawat K., Mérillon JM. (Eds.) Natural Products. Springer, Berlin, Heidelberg

Brand-Williams, W., Cuvelier, M.E., Berset, C. (1995). Use of a Free Radical Method to Evaluate Antioxidant Activity. *Lebensmittel- Wissenschaft und-Technologie*, 28, 25-30.

Chen, H., Wu, X., Duan, Y., Zhi, D., Zou, M., Zhao, Z., Zhang, X., Yang, X., Jianying Zhang, J. (2019). Ursolic acid isolated from Isodon excisoides induces apoptosis and inhibits invasion of GBC SD gallbladder carcinoma cells. *Oncology Letters, 18*, 1467-1474.

Dzubak, P. Hajduch, M., Vydra, D., Hustova, A., Kvasnica, M., Biedermann, D., Markova, L., Urban, M., Serak, J. (2006). Pharmacological activities of natural triterpenoids and their therapeutic implications. *Natural Products Reports, 23*(3), 394-411.

Jäger, S., Trojan, H., Kopp, T., Laszczyk, M., Scheffler, A. (2009). Pentacyclic Triterpene Distribution in Various Plants – Rich Sources for a New Group of Multi-Potent Plant Extracts. *Molecules, 14*(6), 2016-2031.

Kashyap, D., Sharma, A., Tuli, H.S., Punia, S., Sharma A.K. (2016). Ursolic Acid and Oleanolic Acid: Pentacyclic Terpenoids with Promising Anti-Inflammatory Activities. *Recent Patents Inflammation Allergy & Drug Discovery*, 10(1), 21-33.

Muffler, K., Leipold, D., Scheller, M., Haas, C., Steingroewer, J., Bley, T., Ulber, R. (2011). Biotransformation of triterpenes. *Process Biochemistry*, *46*(1), 1-15.

Nieto, G., Ros, G., Castillo, J. (2018). Antioxidant and Antimicrobial Properties of Rosemary (*Rosmarinus officinalis*, L.): A Review. *Medicines, 5(98)*, 1-13.

Parvez, M. K., Alam, P., Arbab, A.H., Al-Dosari, M. S., Alhowiriny, T. A., Alqasoumi, S.I. (2018). Analysis of antioxidative and antiviral biomarkers β-amyrin, β-sitosterol, lupeol, ursolic acid in *Guiera senegalensis* leaves extract by validated HPTLC methods. *Saudi Pharmaceutical Journal, 26*(5), 685-693.

Ramos-Hryb, A.B., Platt, N., Freitas, A.E., Heinrich, I.A., López, M.G., Leal, R.B., Kaster, M.P., Rodrigues, A.L.S. (2019). Protective Effects of Ursolic Acid against Cytotoxicity Induced by Corticosterone: Role of Protein Kinases. *Neurochemical Research, 44*, 2843-2855.

Raudone, L., Zymone, K., Raudonis, R., Vainoriene, R., Motiekaityte, V., Janulis, V. (2017). Phenological changes in triterpenic and phenolic composition of *Thymus* L. species. *Industrial Crops and Products, 109*, 445-451.

Santiago, A.L., Dayrit, K.C., Correa, P.C.B., Mayor, A.B.R. (2014). Comparison of antioxidant and free radical scavenging activity of triterpenes α-amyrin, oleanolic acid and ursolic acid. *Journal of Natural Products, 7*, 29-36.

Santos-Sánchez, N.F., Salas-Coronado, R., Villanueva-Cañongo, C., Hernández-Carlos, B. (2019). Antioxidant Compounds and Their Antioxidant Mechanism. In: Emad Shalaby, (Ed.) *Antioxidants*, IntechOpen. DOI: 10.5772/intechopen.85270

Shanaida, M., Hudz, N., Korzeniowska, K., Wieczorek, P. (2018). Antioxidant activity of essential oils obtained from aerial part of some Lamiaceae species. *International Journal Green Pharmacy, 12*(3), 200-2004.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, H.D. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis, 9*, 669-675.

Thimmappa, R., Geisler, K., Louveau, T., O'Maille, P., Osbourn A. (2014). Triterpene Biosynthesis in Plants. *Annual Review of Plant Biology, 65*, 225-257.

Wang, C., Liu, X., Lian, C., Ke, J., Liu, J. (2019). Triterpenes and Aromatic Meroterpenoids with Antioxidant Activity and Neuroprotective Effects from Ganoderma lucidum. *Molecules, 24*(23), 4353.

Wang, J., Zhao, J., Yan, Y., Liu, D. Wang, C., Wang, H. (2019). Inhibition of glycosidase by ursolic acid: *in vitro*, *in vivo* and *in silico* study. *Journal of the Science of Food and Agriculture, 100*(3), 986-994.

Yin, M.C., Chan, K.C. (2007). Nonenzymatic Antioxidative and Antiglycative Effects of Oleanolic Acid and Ursolic Acid. *Journal of Agricultural and Food Chemistry, 55*(17), 7177-7181

Summary/Sažetak

Triterpeni se konstantno povezuju s uočenim bioaktivnostima ekstrakata dobivenih iz biljnog materijala koji sadrže ove vrlo važne prirodne supstance. Mnoge vrste iz porodice Lamiaceae se koriste radi prisustva eteričnih ulja, a vrlo malo se zna o sadržaju triterpenskih supstanci u njima. U ovom radu provedena je kvalitativna i kvantitativna analiza ovih vrlo važnih supstanci, u nadzemnim dijelovima osam vrsta koje pripadaju porodici Lamiaceae. Dobiveni ekstrakti koji su sadržavali triterpenske supstance, testirani su DPPH metodom, kako bi se utvrdio njihov antioksidativni kapacitet**.** TLC i HPLC metode, korištene za analizu triterpena, pokazale su prisutnost betulina, betulinske kiseline, ursolne kiseline i oleanolne kiseline. Betulin (3.2 mg/g) i betulinska kiselina (37.1 mg/g) bili su najzastupljenije triterpenske komponente u heksanskom ekstraktu lista ruzmarina. Ursolna kiselina (0.14 mg/g) bila je najzastupljenija triterpenska supstanca u heksanskom ekstraktu lista timjana. Korištenjem metode DPPH, svi ispitivani uzorci pokazali su antioksidativnu aktivnost na koncentracijom ovisan način, sa širokim rasponom IC_{50} vrijednosti od 0.4 mg/ml do 3.3 mg/ml.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

54

Original Scientific Article

Print ISSN: 0367-4444

Online ISSN: 2232-7266

DOI: 10.35666/ghtbh.2020.54.05

UDC: 541.1:[547.587:66.097.8]

27-30

Investigation of the Antioxidant Synergisms and Antagonisms among Caffeic, Ferulic and Rosmarinic Acids using the Briggs-Rauscher Reaction Method

Gojak-Salimović, S., Ramić, S.

Department of Chemistry, Faculty of Science, University of Sarajevo, Zmaja od Bosne 33-35, Sarajevo, B&H

Article info
Received: 28/10/2019
Accepted: 02/06/2020

Keywords:
Briggs-Rauscher reaction
phenolic acids
inhibition time
synergism
antagonism

***Corresponding author:**
Sabina Gojak-Salimović
E-mail: sgojak@gmail.com

Abstract: Phenolic acids have been attracting huge attention over recent years due to their prominent antioxidant activity and potential health benefits. In this study, the antioxidant activity of caffeic, ferulic, and rosmarinic acids was evaluated using Briggs-Rauscher reaction method. In addition to single phenolic acids at different concentrations (50, 100, 200 and 250 μM), equimolar mixtures of two phenolic acids and all three phenolic acids were tested. The best ability to inhibit oscillations, i.e. the highest antioxidant activity showed rosmarinic acid at a concentration of 250 μM. Inhibition time of the Briggs-Rauscher oscillating reaction mixture was obtained experimentally for the different combinations of phenolic acids and compared with theoretical values calculated by adding up the effects of phenolic acids analyzed individually. The most of tested phenolic acids mixtures showed a difference in the antioxidant activity when compared to individual values of their constituents. The highest synergistic effect showed a mixture of caffeic acid and ferulic acid at a concentration of 250 μM while the highest antagonistic effect showed the mixture of caffeic acid, ferulic acid and rosmarinic acid at same concentration.

INTRODUCTION

Phenolic acids have been attracting huge attention over recent years due to their potential biological properties which makes them interesting to the food and pharmaceutical industries. They are widespread in all plant foods and are therefore an integral part of the human diet (Ota et al., 2011; Saxena et al., 2012). Phenolic acids have been associated with color, nutritional, organoleptic and antioxidant properties of foods. The food industry has investigated effect of phenolic acids on the fruit maturation, prevention of enzymatic browning and their roles as food preservatives (Robbins, 2003). Most of phenolic acids have a beneficial effect on health, or an active role in the treatment of a disease (Mota et al., 2008; Saxena et al., 2012).

Ferulic acid (FA) has received much attention among all phenolic acids (Karamać et al., 2005). It is the most abundant hydroxycinnamic acid in plants (Mota et al., 2008). It is found in many fruits and vegetables, seeds of rice, wheat and oats, coffee and olive oil (Rice-Evans et al., 1996; Mota et al., 2008; Kadoma and Fujisawa, 2008). Ferulic acid has significant health benefits and it serves as a precursor in the manufacture of useful aromatic compounds (Mota et al., 2008).

Caffeic acid (CA) is one of the most prominent naturally occurring hydroxycinnamic acids. It is found in many fruits, vegetables, seasonings, beverages and olive oil. Cafeic acid is highly predominant in sunflower seeds and greatly affects the solubility of plant proteins (Rice-Evans et al., 1996; Mota et al., 2008). It selectively blocks the biosynthesis of leukotrienes, components involved in immunoregulation diseases, asthma and allergic reactions (Robbins, 2003).

Rosmarinic acid (RA) is one of the more abundant ester of caffeic acid occurring in several members of the Lamiaceae family plants. Rosmarinic acid has antioxidant and anti-inflammatory effects and used to treat upper respiratory and allergic symptoms (Stansbury, 2014).

Each phenolic acid has a different antioxidant activity depending on its structure, number of aromatic and hydroxyl groups and their distribution in the structure. The phenolic acids in plant foods exist in the free, esterified, glycosidic, and insoluble-bound forms

(Karamać et al., 2005). However, interactions among phenolic acids may promote changes in overall antioxidant activity, which is difficult to predict on the basis of their individual antioxidant activities. Although the antioxidant activity of individual phenolic acids is widely described, information about their interactions and potential additive, synergistic or antagonistic effects is lacking (Peyrat-Maillard et al., 2003; Pinelo et al., 2004; Palafox-Carlos et al., 2012; Hajimehdipoor et al., 2014; Skroza et al., 2015; Sonam and Guleria, 2017).

In this study, the antioxidant activity of individual phenolic acids (caffeic acid, ferulic acid and rosmarinic acid) and equimolar mixtures of two or three phenolic acids were investigated using Briggs-Rauscher reaction method. The Briggs-Rauscher reaction method is very applicable method for measuring the activity of antioxidants because it works at pH which is similar to pH value of the fluids in the human stomach. Antioxidants added to an active oscillating Briggs-Rauscher reaction mixture cause an immediate cessation of the oscillations. After inhibition time the oscillations restart again. The inhibition time linearly depends on the type and amount of the added antioxidant. Better antioxidant results with longer inhibition time (Cervellati et al., 2001; Cervellati et al., 2002; Höner and Cervellati, 2002). It is easy to use Briggs-Rauscher reaction method for determine the potential synergistic, antagonistic or additive effects between antioxidants in the mixtures (Milos and Makota, 2012).

EXPERIMENTAL

Reagents

All used reagents were of analytical grade. Potassium iodate, sulfuric acid, hydrogen peroxide, and ethanol were obtained from Semikem (Sarajevo, BiH), malonic acid, manganese(II) sulfate monohydrate and starch were obtained from Merck (Darmastadt, Germany), caffeic acid, ferulic acid, and rosmarinic acid were obtained from Sigma (St. Louis, USA).

Preparation of the solutions of phenolic acids

A stock solutions of caffeic, ferulic and rosmarinic acids, 1000 μM, were prepared daily by dissolving pure phenolic acid in 2 mL of ethanol and diluting with distilled water to 100 mL. Other solutions of phenolic acids in the concentrations of 50, 100, 200 and 250 μM were obtained by diluting the stock solution with the respective volumes of distilled water. Thus prepared solutions were used for the preparation of equimolar mixtures of two and three phenolic acids.

The Briggs-Rauscher reaction method for the determination of antioxidant activity

The antioxidant activity of individual phenolic acids and equimolar mixtures of two and three phenolic acids mixture have been evaluated using the Briggs-Rauscher reaction method described by Cervellati et al. (2001), with small modifications. In our study, three stock solutions (A, B and C) were prepared daily: solution A: 0.2 M potassium iodate and 0.08 M sulfuric acid; solution B: 0.15 M malonic acid, 0.02 M manganese(II) sulfate monohydrate and 0.03% starch; solution C: solution of hydrogen peroxide in distilled water concentration of 15%. The Briggs-Rauscher reaction mixture was prepared by mixing 10 mL of each stock solution (A, B and C). Kinetics of oscillating reactions were observed potentiometrically by recording the potential of the Briggs-Reaction reaction mixture using a platinum wire electrode as working electrode and Ag/AgCl electrode as reference electrode at 25±0.5°C. After the third oscillation, 1 mL solution of phenolic acid at corresponding concentration (or the mixture of two or three phenolic acids) was added to an active oscillating Briggs-Rauscher reaction mixture. The total antioxidant activity of the corresponding phenolic acids or their mixture was expressed as the inhibition time (t_{inhib}). The addition of 1 mL of distilled water, without phenolic acids does not interrupt the oscillations.

RESULTS AND DISCUSSION

Antioxidant activity of caffeic, ferulic and rosmarinic acids tested individualy

In this study, the antioxidant activities of caffeic, ferulic and rosmarinic acids were evaluated at different concentrations (50, 100, 200 and 250 μM) using the described Briggs-Rauscher reaction method. The obtained results are presented in Table 1. The longest inhibition time was detected with rosmarinic acid at a concentration of 250 μM (2107 s), and the lowest with caffeic acid at a concentration of 50 μM (30 s).

Table 1: Inhibitory effects of individual phenolic acids at different concentrations

Phenolic acid	t_{inhib} (s) at different concentrations (μM)			
	50	100	200	250
CA	30	60	326	491
FA	192	373	607	781
RA	58	151	1519	2107

*CA - caffeic acid, FA - ferulic acid, RA - rosmarinic acid

The inhibition time increased with higher concentration, and linearity was found in an tested concentration range of phenolic acid added. The parameters of the linearity are presented in Table 2.

Table 2: Parameters of linearity ($t_{inhib} = m$(antioxidant) + q) and R-squared values

Phenolic acid	m (μM^{-1} s)	q (s)	R^2
CA	2.376	-129.6	0.965
FA	2.824	64.65	0.993
RA	10.93	-681.1	0.965

*CA - caffeic acid, FA - ferulic acid, RA - rosmarinic acid

The obtained results confirmed that the antioxidant activity of the tested phenolic acids vary depending of their structure and concentration. The antioxidant activity decreased in the following order: rosmarinic acid > ferulic acid > caffeic acid, similar to the results in previous studies (Cervellati et al., 2001; Cervellati et al., 2002) obtained using Briggs-Rauscher reaction method.

Effect of combination phenolic acids on the total antioxidant activity

The obtained results for the inhibition time of the mixture of two and three combination phenolic acids are presented in Table 3. The results for the inhibition time ranged from 51 s for the mixture of rosmarinic acid and caffeic acid at a concentration of 50 μM to 1958 s for their combination at a concentration of 250 μM. To conclude about the antioxidant interactions between caffeic acid, ferulic acid and rosmarinic acid their values of the inhibition time were compared with the values obtained by mixing them in different combinations. The experimentally values for the different mixtures were compared with theoretical values calculated by adding up the effects of phenolic acids analyzed individually. Any significant difference values obtained from these comparisons indicate an sinergistic, antogonistic or additive effect.

Table 3: Inhibitory effects of the mixture of phenolic acids at different concentrations

Mixtures	t_{inhib} (s) at different concentrations (μM)			
	50	**100**	**200**	**250**
CA+FA	113 (111)	353 (216)	430 (466)	1881 (636)
FA+RA	126 (125)	**366 (262)**	**1241 (1063)**	1845 (1444)
RA+CA	51 (44)	102 (105)	699 (922)	**1958 (1299)**
CA+FA+RA	**193 (93)**	279 (195)	1206 (817)	679 (1126)

*CA - caffeic acid, FA - ferulic acid, RA - rosmarinic acid; The values in parentheses are the sum of antioxidant activities of individual phenolic acids.

The obtained results indicate that most of tested mixtures showed a difference in antioxidant activity when compared to their theoretical values. The highest antagonistic effect was found for mixture of rosmarinic and caffeic acid at a concentration of 200 μM, while the highest synergistic effect showed a mixture of caffeic acid and ferulic acid at a concentration of 250 μM. The mixtures of caffeic acid and ferulic acid, ferulic acid and rosmarinic acid at a concentration of 50 μM, and mixture of rosmarinic acid and caffeic acid at a concentration of 100 μM showed additive effect. The mixtures of three phenolic acids showed synergistic effect at concentrations of 50, 100 and 200 μM, while same combination mixture at concentration of 250 μM showed the high antagonism. In our previous study (Aljović and Gojak-Salimović, 2017) the antioxidant activity was investigated for two-component and three-component mixtures of ferulic acid, homovanillic acid and vanillic acid using Briggs-Rauscher reaction method. Most of the tested combinations of phenolic acids showed a sinergistic effect.

Peyrat-Maillard et al. (2003) investigated mixture effects between phenolic antioxidants in equimolar binary systems on the protection of linoleic acid from 2,2′-azobis (2-amidinopropane) dihydrochloride-induced oxidation. The slight synergy was observed between rosmarinic acid and caffeic acid. Hajimehdipoor et al. (2014) investigated synergistic antioxidant effects of binary and ternary combinations of methanolic solutions of caffeic acid, gallic acid, rosmarinic acid, chlorogenic acid, rutin and quercetin using FRAP method. The synergistic effect (37.5%) showed the combination of caffeic acid (600 μM) and rosmarinic acid (150 μM).

CONCLUSIONS

The obtained results support the fact that antioxidant activity of the phenolic acid mixtures cannot be predicted from the antioxidant activity for individual phenolic acids. Interaction of investigated phenolic acids (caffeic acid, ferulic acid and rosmarinic acid) in the mixtures depend on the concentrations of phenolic acids present. The most of tested phenolic acids mixtures showed a difference in antioxidant activity when compared to individual values of their constituents. Our future investigation will be focused on the potential synergistic or antagonistic effects among other phenolic acids using Briggs-Rauscher reaction method.

REFERENCES

Aljović, I., Gojak-Salimović, S. (2017). Evaluation of the antioxidant activity of ferulic, homovanillic and vanillic acids using the Briggs-Rauscher oscillating reaction method, *Bulletin of the Chemists and Technologists of Bosnia and Herzegovina/Glasnik hemičara i tehnologa Bosne i Hercegovine*, 49, 35-38.

Cervellati, R., Höner, K., Furrow, S.D., Neddens, C., Costa, S. (2001). The Briggs-Rauscher reaction as a test to measure the activity of antioxidants, *Helvetica Chimica Acta*, 84(12), 3533-3547.

Cervellati, R., Renzulli, C., Guerra, M.C., Speroni, E. (2002). Evaluation of antioxidant activity of some natural polyphenolic compounds using the Briggs-Rauscher reaction method, *Journal of Agricultural and Food Chemistry*, 50(26), 7504-7509.

Hajimehdipoor, H., Shahrestani, R., Shekarchi, M. (2014). Investigating the synergistic antioxidant effects of some flavonoid and phenolic compounds, *Research Journal of Pharmacognosy*, 1(3), 35-40.

Höner, K., Cervellati, R. (2002). Measurements of the antioxidant capacity of fruits and vegetables using the BR reaction method, *European Food Research and Technolology*, 215(5), 437-442.

Kadoma, Y., Fujisawa, S. (2008). A comparative study of the radical-scavenging activity of the phenolcarboxylic acids: caffeic acid, *p*-coumaric acid, chlorogenic acid and ferulic acid, with or without 2-mercaptoethanol, a thiol, using the induction period method, *Molecules*, 13, 2488-2499.

Karamać, M., Kosińska, A., Pegg, R.B. (2005). Comparison of radical-scavenging activities for selected phenolic acids, *Polish Journal of Food and Nutrition Sciences*, 14/55(2), 165-170.

Milos, M., Makota, D. (2012). Investigation of antioxidant synergisms and antagonisms among thymol, carvacrol, thymoquinone and *p*-cymene in a model system using the Briggs-Rauscher oscillating reaction, *Food Chemistry*, 131(1), 296-299.

Mota, F.L., Queimada, A.J., Pinho, S.P., Macedo, E.A. (2008). Aqueous solubility of some natural phenolic compounds, *Industrial Engineering Chemistry Research*, 47, 5182-5189.

Ota, A., Abramović, H., Abram, V., Poklar-Ulrih, N. (2011). Interactions of *p*-coumaric, caffeic and ferulic acids and their styrenes with model lipid membranes, *Food Chemistry*, 24(4), 1256-1261.

Palafox-Carlos, H., Gil-Chávez, J., Sotelo-Mundo, R.R., Namiesnik, J., Gorinstein, S., González-Aguilar, G.A. (2012). Antioxidant interactions between major phenolic compounds found in 'Ataulfo' mango pulp: chlorogenic, gallic, protocatechuic and vanillic acids, *Molecules*, 17, 12657-12664.

Peyrat-Maillard, M.N., Cuvelier, M.E., Berset, C. (2003). Antioxidant activity of phenolic compounds in 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidation: Synergistic and antagonistic effects, *The Journal of the American Oil Chemists' Society***,** 80(10), 1007-1012.

Pinelo, M., Monzocco, L., Nunez, M.H., Nicoli, M.C. (2004). Interaction among phenols in food fortification: negative synergism on antioxidant capacity, *Journal of Agriculture and Food Chemistry*, 52(5), 1177-1180.

Rice-Evans, C.A., Miller, N.J., Paganga, G. (1996). Structure-antioxidant activity relationship of flavonoids and phenolic acids, *Free Radical Biology and Medicine*, 20(7), 933-956.

Robbins, R.J. (2003). Phenolic acids in food: An overview of analytical methodology, *Journal of Agricultural and Food Chemistry*, 51(10), 2866-2887.

Saxena, M., Saxena, J., Pradhan, A. (2012). Flavonoids and phenolic acids as antioxidants in plants and human health, *Journal of Pharmaceutical Sciences Review and Research*, 16(2), 130-134.

Skroza, D., Generalić Mekinić, I., Svilović, S., Šimat, V., Katalinić, V. (2015). Investigation of the potential synergistic effect of resveratrol with other phenolic compounds: A case of binary phenolic mixtures, *Journal of Food Composition and Analysis*, 38, 13-18.

Sonam, K.S., Guleria, S. (2017). Synergistic antioxidant activity of natural products, *Annals of Pharmacology and Pharmaceutics*, 2(8), 1086.

Stansbury, J. (2014). Rosmarinic acid as novel agent in the treatment of allergies and asthma, *Journal of Restorative Medicine*, 3(1), 121-126.

Summary/Sažetak

Fenolske kiseline posljednjih godina privlače veliku pažnju zbog njihove značajne antioksidacijske aktivnosti i potencijalnih benefita po ljudsko zdravlje. U ovom istraživanju, antioksidacijska aktivnost kafene kiseline, ferulinske kiseline i ruzmarinske kiseline ispitivana je primjenom Briggs-Rauscher oscilirajuće reakcije. Osim pojedinačnih fenolskih kiselina pri različitim koncentracijama (50, 100, 200 i 250 μM), testirane su ekvimolarne smjese dviju fenolskih kiselina i sve tri fenolske kiseline. Najbolju sposobnost inhibicije oscilacija, tj. najveću antioksidacijsku aktivnost pokazala je ruzmarinska kiselina pri koncentraciji od 250 μM. Vrijeme inhibicije Briggs-Rauscher reakcione smjese dobijeno eksperimentalno za različite kombinacije fenolskih kiselina poređeno je s teorijskim vrijednostima izračunatim zbrajanjem efekata dvije ili tri fenolske kiseline pojedinačno analizirane. Većina ispitivanih smjesa fenolskih kiselina pokazala je određen stepen odstupanja u antioksidacijskoj aktivnosti u usporedbi s induvidualnim vrijednostima njihovih sastojaka. Najveći sinergijski efekat pokazala je smjesa kafene kiseline i ferulinske kiseline pri koncentraciji od 250 μM, dok je najveći antagonizam pokazala smjesa kafene kiseline, ferulinske kiseline i ruzmarinske kiseline pri istoj koncentraciji.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

54

Original Scientific Article

Print ISSN: 0367-4444

Online ISSN: 2232-7266

DOI: 10.35666/ghtbh.2020.54.06

UDC: 371.315:54-057.875.1/.2(497.5)

31-36

Gender differences in chemical engineering students identified in understanding of rolling motion

Andrej Vidak

Faculty of Chemical Engineering and Technology, University of Zagreb, Savska cesta 16, 10000 Zagreb, Croatia

Article info
Received: 31/10/2019
Accepted: 21/04/2020

Keywords:
Work-energy principle
Rolling motion
Gender differences
Visualization

Corresponding author:
Andrej Vidak
E-mail: avidak@fkit.hr
Phone: +385-1-4597-106

Abstract: In this research paper we presented the results of exploration of gender differences in conceptual understanding of rolling motion (velocities and work-energy principle). For this purpose, we have selected nine conceptual items and conducted experiment with 184 first year students at the Faculty of Chemical Engineering and Technology, University of Zagreb. Results show that male students significantly outperformed female students. We detected particularly large differences on items that tests knowledge of the rolling phenomena. Results of our research can help teachers to create lessons that are adapted to general student population.

INTRODUCTION

The goal of every teacher at the university level should be to transfer the knowledge about a particular phenomenon to his/her students as effectively as possible. Many factors affect the effectiveness of the teaching and learning process and the teacher's task is to adopt his/his lectures to the target group of students. Motivated by the fact that majority of students at the Faculty of Chemical Engineering and Technology in Zagreb are females, we decided to conduct a study in which we are comparing gender scores from test that includes phenomena from mechanics i.e. rolling motion (velocity and work-energy principle). We focused our research to the phenomena that includes rolling of rigid bodies.

We have chosen these phenomena from mechanics because this knowledge is the basis for understanding other fields of physics as well as other scientific disciplines in curricula for chemical engineering students.

Furthermore, in this field of physics, it is necessary for students to develop visuospatial skills i.e. have ability to visuals the rotation in order to successfully solve conceptual problems.

Lohman defines spatial ability as "the ability to generate, retain, retrieve and transform well-structured visual images" (Lohman 1996, p. 112). There are many studies that emphasize importance of visuospatial ability and its relation to mathematical conceptualization, problem-solving skills, creative and higher order thinking skills in science and mathematics, design and graphical representation skills in engineering and technology (Maeda and Yoon, 2013).

Furthermore, studies have shown that male students outperform female students in solving visuospatial tasks (Voyer and Saunders, 2004; Peters, 2005).

Visuospatial skills are especially important in tasks that require to visualization of 3D-objectsrotation. It has been shown that male students are outperforming female students in the tasks related to visualization of three-dimensional rotation ability (Linn and Petersen, 1985; Voyer, Voyer and Bryden, 1995; Maeda and Yoon, 2013).

Therefore, study from Fisher, Schult and Hell presented evidence that female students have higher achievement motivation which results in a better secondary school grades. (Fischer, Schult and Hell, 2013)

From a biological point of view, the causes of such differences can relate to functional, morphological, hormonal or genetic differences in the brains of males and females (Jordan et al., 2002; Koscik et al., 2009; Hausmann et al., 2000; Thomas and Kail, 1991).

From a sociological point of view, gender differences may exist because boys are often engaged in activities that help them to resolve visuospatial tasks. It is well known fact that they are more likely to play video games, certain sports, and more often play with building toys such as Lego cubes (Cherney, 2008; Ginn and Pickens, 2005; Deno, 1995).

In order to accurately solve our conceptual tasks related to rotation and energy, students should use visualization skills. In our example of body rotation, we should

emphasize that students need to visualize what is happening with the points on the edge of the wheel that are in contact with other ground material in the process of rigid body rotation.

Furthermore, earlier studies have shown that students have many misconceptions related to this phenomena. It is often the case that students haven't developed an understanding of wheel velocity in contact with the ground, as well as some seemingly unintuitive aspects of applying mechanical energy conservation law in the context of rolling. Other students also believed that, for a very large static friction coefficient, the body on the slope would not even move at inclination angles near 90 degrees (Rimoldini and Singh, 2005). Results from other study that was using simulations and physical experiments showed that many students do not understand how to identify the exact direction of the velocity of a point on the edge of a rolling body (De Ambrosis, Malgieri, Mascheretti, and Onorato, 2015).

Generally, it is well known that many student misconceptions are rooted in the daily student experiences (Reiner et al., 2000).

Aim of the present study

Our goal in this study is to investigate gender differences in understanding the velocity and work-energy principle in the rolling motion example. This type of research is very importnat because it provides us with feedback that is useful in creation of lectures that are more effective for teaching introductory courses at the Faculty of Chemical Engineering and Technology.

METHODOLOGY

Research design

To answer our research question we conducted a survey research study were students had lectures and recitation sessions in their natural learning environment. Covered topics were about velocities and work-energy principle in rolling motion example. Students had 20 minutes to solve the nine questions test.

Participants

Our study was conducted in academic year 2017./2018. The number of 184 first year students at the Faculty of Chemical Engineering and Technology, University of Zagreb (Croatia) was included in the research project Student population mostly consist of 19 year old first year students that were enrolled in introductory physics course. Gender analysis showed that our sample consisted of 74 % female and 26 % male students.

Curriculum and teaching treatment

We conducted research in standard teaching processes. Before the conceptual test students participated in standard traditional lectures and seminars about velocities and work-energy principle in the example of rolling motion. In the curriculum of the introductory physics course, the phenomena of rolling is taught in the first semester of the first year at the Faculty of Chemical Engineering and Technology in Zagreb. Traditional seminars are based on summarizing and applying the most important principles and facts that students encountered earlier in their lectures. We can characterize this introductory physics course as standard introductory physics course for scientists and engineers in Croatia.

Assessment instruments

To enable effective assessment of student differences we decided to create a conceptual diagnostic test that consisted of nine multiple-choice and open ended items. In the test we have used most common student misconceptions as distractors. We provided short description of the conceptual test items in the Table 1.

Table 1: Short description of the conceptual test items

Item 1	Item 2	Item 3	Item 4	Item 5
Determine linear velocity vectors at points on the wheel that is rotating around the center.	Determine linear velocity vectors at points on the wheel that is rolling down an incline.	How slipping of the car wheel's influences magnitudes of linear velocities of points on the wheel.	How the motion trajectory of the point on the cylinder edge looks like?	Comparetravelled distances of points on the bottom, center and top of the the cylinder.
Multiple-choice	Multiple-choice	Multiple-choice	Open-ended	Multiple-choice
Item 6	Item 7	Item 8	Item 9	
What is the direction of linear velocity at point on the wheel's edge.	Writing an equation for the linear velocity at point on the wheel's edge.	Influence of inclination on the motion of the cylinder.	What is the maximum height that ball will reach during its motion in a hard material surface groove?	
Multiple-choice	Open-ended	Multiple-choice	Multiple-choice	

RESULTS

In order to detect between-gender differences we used analysis of variance (ANOVA) on the given nine items test. Results of ANOVA revealed that there was a statistically significant difference between male and female students on the selected test items, F (1, 182)=7.098, $p<0.01$, partial $\eta^2=0.038$.

In the following lines, we will focus our research on in-depth exploration of the four multiple-choice test items on which we discovered the biggest gender differences.

Table 2 provides a concise overview of students' achievement on four multiple choice test items.

Table 2: Summarized overview of correct answers average proportion for male and female students. Standard deviations are provided in parentheses.

	Item 3	Item 6	Item 8	Item 9
Male students	0.64 (0.48)	0.52 (0.50)	0.60 (0.49)	0.41 (0.49)
Female students	0.52 (0.50)	0.43 (0.49)	0.51 (0.50)	0.36 (0.48)

From the answers provided in the Table 2 it is evident that male students have better results than their female colleagues on all four test items. On the Item 3 we discovered most prominent difference (12%).

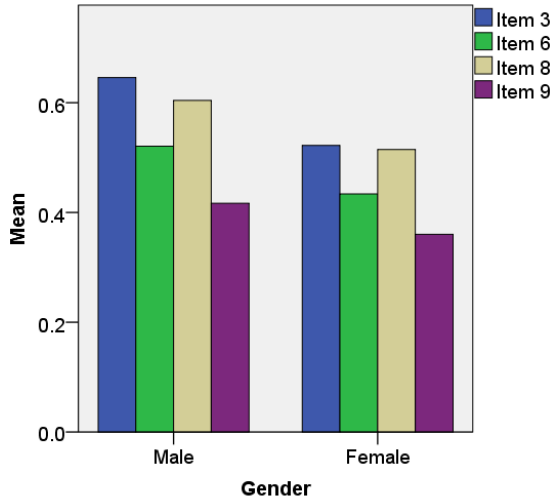


Figure 1: Mean value of the correct answers for the conceptual test items 3, 6, 8 and 9.

From Figure 1 we can see that the between-gender differences in correct answers mean value are pronounced for all four items.

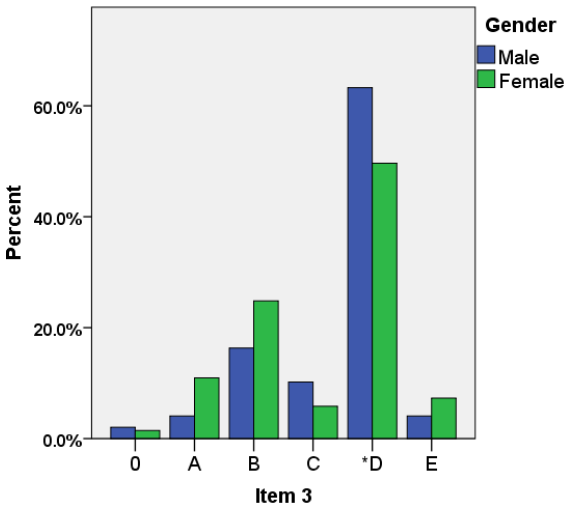


Figure 2: Percentage of chosen answers for the male and female students on the Item 3. Correct answer is labeled with asterisk. Zero indicates percentage of students that didn't choose any answer.

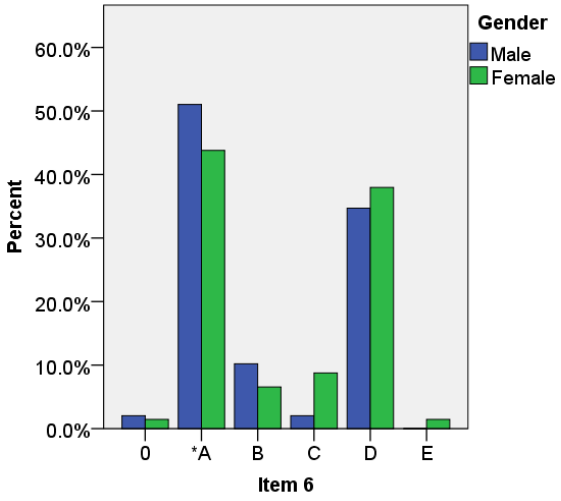


Figure 3: Percentage of chosen answers for the male and female students on the Item 6. Correct answer is labeled with asterisk. Zero indicates percentage of students that didn't choose any answer.

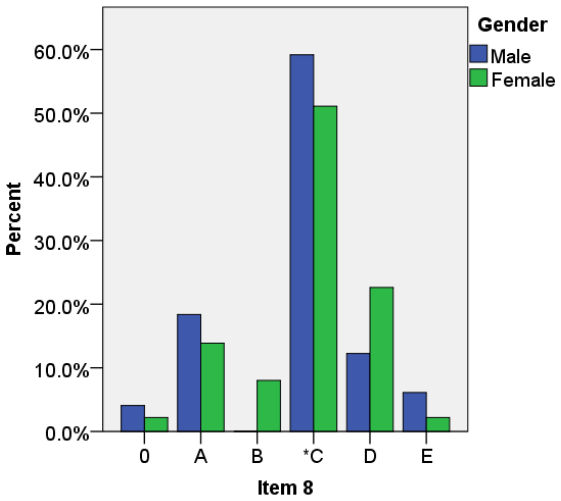


Figure 4: Percentage of chosen answers for the male and female students on the Item 8. Correct answer is labeled with asterisk. Zero indicates percentage of students that didn't choose any answer.

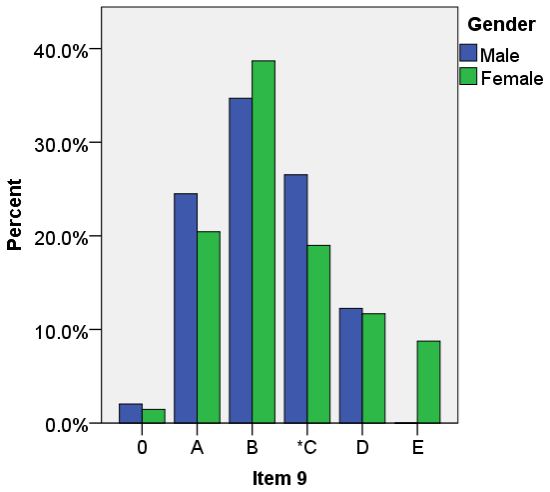


Figure 5: Percentage of chosen answers for the male and female students on the Item 9. Correct answer is labeled with asterisk. Zero indicates percentage of students that didn't choose any answer.

From the given figures, we received information about mean values and chosen answers on the items 3, 6 and 9 for the male and female students.

DISCUSSION

We should firstly mention that overall achievement in our sample was low on all four items but we can see from data provided in Table 2 that male students outperformed female students. However, we cannot say that these results are very surprising because previous research has shown that the concepts covered in our experiment have not been effectively addressed within traditional teaching. In the study from Rimoldini and Singh, none of the 16 students in the study did not explain the velocity at the top and bottom of the wheel relative to the ground, and they discover many difficulties with the application of the vwork-energy concepts (Rimoldini and Singh, 2005). Furthermore, low efficiency of traditional seminars is in line with Kim and Pak's research where they found that many students fail to solve conceptual problems even after they have completed more than 1000 traditional problems (Kim and Pak, 2002).

Most prominent between-gender differences on individual items

In this section, we will discuss the score differences between genders for the selected items.
In Item 3 students were required to reason how slipping of the car wheel's influence magnitudes of linear velocities of points on the bottom, center, and top. Visualizing the rolling process of the wheel could have facilitated creation of student mental models that were more compatible with scientifically acceptable knowledge. Scores of male students are 12% higher than female students.
In Item 6 students were shown a wheel of radius R that is rolling without slipping along the horizontal plane and they needed to find direction of linear velocity at point on the wheel's edge. Correct visualization and summing of the vectors resulted in accurate answer. Male students outperformed female students by 9%.
In Item 8 students were shown cylinder on an incline and they needed to visualize what will happen if the coefficient of static friction between the cylinder and the inclined plane amounts to zero. In order to answer this question correctly, students needed to visualize what happens with cylinder motion when it is in contact with the surface and static friction coefficient is changing. When the coefficient of friction decreases the cylinder is starting to slip and in the case of a completely smooth surface, it only slide. On this item male students scored 11 % higher than their female colleagues.
In Item 9 students needed to visualize how steel ball rolls down a groove to conclude the maximum height that will ball reach during its motion in a hard material groove. Ball is in contact with surface and students need to visualize collisions of the ball surface with the groove surface. Mechanical energy of the ball is transforming into elastic potential energy. Male students have 5% higher scores then their female colleagues.
According to the above results we can conclude that our research supports earlier studies where male students outperformed female students on visuospatial tasks (Voyer and Saunders, 2004; Peters, 2005; Maeda and Yoon, 2013).

Students' misconceptions and conceptual change

Next, we will discuss misconceptions that we identified for four test items.
In Item 3 students were required to reason about the influence of slipping on the velocity of the selected points on the wheel.
From Figure 2 we can see that the most common wrong answer for Item 3 was answer B (male students - 16%, female students – 46%). Incorrect answer B reflects the idea that the magnitude of linear velocity for all points on the wheel decreases when slipping occurs. A given example shows that male students have less often chose this misconception. It is possible that male students visualized better to make their mental models more compatible with scientifically acceptable knowledge.
In Item 6 students were required to reason about the direction of linear velocity of a point on the wheel.
From Figure 3 we can see that many students in this context apply rotation model, which is evident from the fact that they chose answer D (male students – 34%, female students - 38%). According to this answer, the velocity of the point at the wheel edge has the direction of the tangent at that point while the correct answer is the direction of the tangent to the trajectory point. This misconception in rolling motion is obtained by combining parts related to rotation and translational motion which has been identified in previous research (De Ambrosis, Malgieri, Mascheretti, and Onorato, 2015; Rimoldini and Singh, 2005). Male students have recognized accuracy of this motion in greater proportion.
Items 8 and 9 are addressing the role of static friction and mechanical energy in the rolling motion.
From Figure 4 it is obvious that the most common wrong answer for Item 8 was answer D (male students - 12%, female students - 22%). This answer reflects the

misconception that the cylinder will roll on an incline (no slipping) for all angles of inclination. Our data is in accordance with the results of De Ambrosis, Malgieri, Mascheretti, and Onorato (2015). In fact, their study found that 40% of students did not recognize how the kinetic force of friction creates a transition from sliding to rotational motion of the ball, and 42% of students answered that the ball simply cannot slide on an incline without friction. It is no surprise that male students outperformed female students because in this item visualization is a key that unlocks correct answer.

From Figure 5 we can see that the most frequent wrong answer for Item 9 was answer B (male students – 34%, female students - 38%). It is evident from the results of answer B that more than 30% of students in both groups had a misconception that the effect of static friction results in the loss of mechanical energy of the rolling object. This result showed that students have generally realized that there is a static friction force, but they often believe that a static friction force does a negative work on the ball which leads to energy losses. Furthermore, most students realized that the work performed by the static friction force was equal to zero, but some students failed to recognize that the losses of mechanical energy due to micro-collisions were not negligible in this case.

Presented results are showing that male students outperformed female students on tasks that involve rolling which could be probably explained that male students can more successfully develop certain visual mental models of rolling which facilitate solving of chosen tasks (Nersessian, 2008; Greca and Moreira, 2000; Maeda and Yoon, 2013).

Both genders achieved lower scores on tasks that involve work-energy concept. The reason of lower scores could be more complex visualization of this phenomena which stimulated higher intrinsic load and negatively affected scores of both genders (Sorden, 2005).

Finally, it should be noted that the findings of our study are limited to the contexts of introductory courses in physics for scientists and engineers at the university level. Main limitation of our study is related to the relatively small number of test items which can be justified by using narrow field of measurement i.e. rolling and work-energy principle.

CONCLUSION

In this study, we have investigated the difference in achieved scores between male and female students on answers related to the rolling and work-energy principle. We have found that male students outperformed female students when solving these problems. The most prominent differences were on the tasks that required visuospatial reasoning. It is useful to emphasize that among visuospatial skills is also the ability of intuitively choosing an appropriate system of reference i.e. to choose between the laboratory system and the system where the center of wheel is at rest. Presented results support earlier findings that students in traditional introductory physics courses fail to develop a deep understanding of velocity at the top and the bottom of the rolling body, as well as the application of the laws of conservation of mechanical energy in the context of body rolling. Furthermore, these results supports past findings in which male students outperformed female students in the tasks related to three-dimensional mental rotation ability and visuospatial tasks (Voyer, Voyer and Bryden, 1995; Maeda and Yoon, 2013). Based on the findings from our and other studies we strongly believe that it would be beneficial for female student population if teachers put an emphasis on visualization and enrich their lectures with visually abundant models that could improve visuospatial skills. In future research related to understanding of rolling and work-energy principle it would be useful to conduct a mixed research design that will allow us further explore the content and structure of students' mental models (Creswell and Clark, 2011).

REFERENCES

Cherney, I. D. (2008). Mom, let me play more computer games: they improve my mental rotation ability. Sex Roles, 59, 776–786.

Creswell, J. W. & Clark, V.L.P. (2011). Designing and Conducting Mixed Methods Research (2nd ed.). California: Sage Publications.

De Ambrosis, A., Malgieri, M., Mascheretti, P., & Onorato, P. (2015). Investigating the role of sliding friction in rolling motion: a teaching sequence based on experiments and simulations. European Journal of Physics, 36(3), 035020.

Deno, J. (1995). The relationship of previous experiences to spatial visualization ability. Engineering Design Graphics Journal, 59, 5–17.

Fischer, F., Schult, J., & Hell, B. (2013). Sex differences in secondary school success: Why female students perform better. European journal of psychology of education, 28(2), 529-543.

Ginn, S. R., & Pickens, S. J. (2005). Relationships between spatial activities and scores on the mental rotation test as a function of sex. Perceptual and Motor Skills, 100, 877–881.

Greca, I. M., & Moreira, M. A. (2000). Mental models, conceptual models, and modelling. International journal of science education, 22(1), 1-11.

Jordan, K., Wüstenberg, T., Heinze, H.-J., Peters, M., & Jäncke, L. (2002). Women and men exhibit different cortical activation patterns during mental rotation tasks. Neuropsychologia, 40, 2397–2408.29-543.

Kim, E., & Pak, S. J. (2002). Students do not overcome conceptual difficulties after solving 1000 traditional problems. American Journal of Physics, 70(7), 759-765.

Koscik, T., O'Leary, D., Moser, D. J., Andreasen, N. C., & Nopoulos, P. (2009). Sex differences in parietal lobe morphology: relationship to mental rotation performance. Brain and Cognition, 69, 451–459.

Lohman, D. F. (1996). Spatial ability and G. In I. Dennis & P. Tapsfield (Eds.), Human abilities: their nature and measurement (pp. 97–116). Hillsdale, NJ: Erlbaum.

Maeda, Y., & Yoon, S. Y. (2013). A meta-analysis on gender differences in mental rotation ability measured by the Purdue spatial visualization tests: Visualization of rotations (PSVT: R). Educational Psychology Review, 25(1), 69-94.

Nersessian, N. J. (2008). Creating Scientific Concepts. London: The MIT Press.

Peters, M. (2005). Sex differences and the factor of time in solving Vandenberg and Kuse mental rotation problems. Brain and Cognition, 57, 176–184.

Reiner, M., Slotta, J. D., Chi, M. T., & Resnick, L. B. (2000). Naive physics reasoning: A commitment to substance-based conceptions. Cognition and instruction, 18(1), 1-34.

Rimoldini, L. G., & Singh, C. (2005). Student understanding of rotational and rolling motion concepts. Physical Review Special Topics-Physics Education Research, 1(1), 010102.

Sorden, S. D. (2005). A cognitive approach to instructional design for multimedia learning. Informing Science, 8.

Thomas, H., & Kail, R. (1991). Sex differences in speed of mental rotation and the X-linked genetic hypothesis. Intelligence, 15, 17–32.

Voyer, D., Voyer, S., & Bryden, M. P. (1995). Magnitude of sex differences in spatial abilities: a meta-analysis and consideration of critical variables. Psychological bulletin, 117(2), 250.

Voyer, D., & Saunders, K. A. (2004). Gender differences on the mental rotations test: a factor analysis. Acta Psychologica, 117, 74–94.

Linn, M. C., & Petersen, A. C. (1985). Emergence and characterization of sex differences in spatial ability: A meta-analysis. Child development, 1479-1498.

Summary/Sažetak

U ovom istraživanju fokusirali smo se na provjeru spolnih razlika u konceptualnom razumijevanju kotrljanja (brzine i principi povezani s radom i energijom. U tu svrhu odabrali smo devet konceptualnih zadataka i proveli istraživanje na 184 studenata prve godine na Fakultetu kemijskog Inženjerstva i tehnologije, Sveučilišta u Zagrebu. Rezultati pokazuju da su studenti značajno nadmašili studentice. Osobito značajne razlike otkrili smo u zadacima koji testiraju znanje o brzinama kod kotrljanja. Rezultati našeg istraživanja mogu pomoći nastavnicima u stvaranju lekcija prilagođenih općoj studentskoj populaciji.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2020

54

37-44

Original Scientific Article

Print ISSN: 0367-4444

Online ISSN: 2232-7266

DOI: 10.35666/ghtbh.2020.54.07

UDC: 615.9:616.63

Modified QuEChERS extraction and GC-MS analysis of selected cannabinoids from human urine

Korać, N.[a,b], Vidic, D.[b,*], Sutlović, D.[c,d]

[a]*Institute of Occupational Health Sarajevo, Laboratory for Toxicology Studies and Sanitary Work Environment, Bulevar Meše Selimovića 2, Sarajevo, Bosnia and Herzegovina*

[b]*University of Sarajevo, Faculty of Science, Department of Chemistry, Zmaja od Bosne 33-35, Sarajevo, B&H*

[c]*University of Split, University Department for Health Studies, Ruđera Boškovića 35, 21 000 Split, Croatia*

[d]*Department of Toxicology and Pharmacogenetics; Department of Forensic Medicine, University of Split School of Medicine, Šoltanska 2, 21 000 Split, Croatia*

Article info
Received: 05/02/2020
Accepted: 17/05/2020

Keywords:
Quechers Extraction
Cannabinoids
THC
GC-MS
Urine

Corresponding author
Danijela Vidic
Phone: +387 33 279 899
E mail: danijela.vidic@gmail.com

Abstract: The aim of this work is to apply a modified QuEChERS method to extract cannabinoids from urine, using a mixture of salts for extraction in an appropriate ratio instead of commercially available cartridges. The analysis was performed on blank urine to which a known concentration of tetrahydocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD), 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) and 11-nor-9-carboxy-Δ^9-tetrahydrocannnabinol (THC-COOH) was added. Six solvents, as well as four solvent mixtures, were tested for extraction and the solvent mixture acetonitrile: dichloromethane (1:3) was selected for which the best recovery factor was obtained. Derivatization of all samples was done with MSTFA (N-trimethylsilyl-N-methyl trifluoroacetamide) + 1% TMCS (2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)-acetamide, chlorotrimethylsilane) at room temperature. The prepared extracts were analyzed by a coupled system, gas chromatography-mass spectrometry (GC-MS) in full-scan mode. Peaks of selected cannabinoids are well separated indicating that there was no interference with the selected analytes. The results were calculated from a calibration curve ranging from LOQ to 1000 ng/mL for selected cannabinoids with a correlation factor over 0.998. The LOD and LOQ for THC are (3.0 ng/mL; 9.0 ng/mL), for CBN (5.0 ng/mL; 18.0 ng/mL) for CBD (5.0 ng/mL, 16.0 ng/mL), for THC-OH (2.6 ng/mL; 8.7 ng/mL) and for THC-COOH (5.0 ng/mL; 15.0 ng/mL). The recovery factor was recorded in the range of 79.40% for THC-COOH to 94.86% for CBN.
The modified QuEChERS extraction method can be used for routine analysis of selected cannabinoids. This method was successfully applied on real samples and thirty urine samples were analyzed.

INTRODUCTION

Cannabinoids are commonly present in *Cannabis sativa* L., and include compounds of which Δ9-tetrahydrocannabinol (THC) is the most psychologically active component. These psychoactive constituents are responsible for most of the pharmacological effects. Cannabis also known as marijuana has number of health

benefits for humans, including treating glaucoma, controlling epileptic seizures, and stopping cancer spreading, (Zhang, Wang, Mi, *et al.*, 2016). Analysis of THC often includes the simultaneously analyses of other cannabinoids and its metabolites.

THC is a lipophilic compound and is widely distributed in the body. From the gastrointestinal tract, THC is absorbed, but absorption is slow and irregular. It is oxidised to the active metabolite THC-OH, which is further oxidized to the inactive metabolite THC-COOH. Up to almost a quarter of the dose is excreted in the urine in 3 days, mainly as THC-COOH in free and conjugated form (Moffat, Osselton, Widdop, *et al.*, 2011).

Recently, the increasing use of marijuana for medicinal purpose has led to increased interest in the determination of cannabinoids in biological matrixes. Urine is a sample of choice for these analyses, because its simplicity and security of sampling and after metabolic process, the concentration of THC metabolites is higher in urine than in blood. Cannabinoids and their metabolites are in the form of conjugates, so they need to be hydrolyzed by enzymatic (Fuchs, Miljanić, Katić, *et al.*, 2019) or alkaline hydrolysis (Battista, Sergi, Montesano, *et al.*, 2014).

Many analytical techniques are available for determination of cannabinoids such as thin layer chromatography (Galand, Ernouf, Montigny, *et al.*, 2004), radioimunoassay (Clatworthy, Oon, Smith, *et al.*, 1990), HPLC method (Scheidweiler, Desrosiers, and Huestis, 2012, Aizpurua-Olaizola, Zarandona, Ortiz, *et al.*, 2016), and GC-MS method (Nestić, Babić, Pavlović, *et al.*, 2013, Heinl, Lerch and Erdmann, 2016).

The coupled system GC-MS ensures the necessary selectivity and sensitivity to confirm positive results induced by screening methods or by the quantification process needed in clinical studies (Kemp, Abukhalaf, Manno, *et al.*, 1995).The analysis of cannabinoids in biological fluids is a challenging issue as it is very important to determine low concentrations for toxicology as well as for clinical use. Numerous analytical methods have been applied to analyze THC, CBD and CBN in urine samples, but the extraction procedure is expensive, (Raharjo and Verpoorte, 2004).

Many studies have investigated methods of extraction and determination of cannabinoids in body fluids, (Citti, Braghiroli, Vandelli, *et al.*, 2018, Aizpurua-Olaizola, Zarandona, Ortiz, *et al.*, 2017, Ramesh, Manjula, Bijargi, *et al.*, 2015).

Legalization of marijuana has been increasingly talked about, increasing the need to find methods for determining the lowest concentrations of cannabinoids in biological samples, both for medical purposes and for determining the concentration of these compounds in case of marijuana abuse. Numerous extraction methods have been developed for the determination of cannabinoids in body fluids. The aim of this work was to determine the concentration of tetrahydrocannabinol (THC), and its metabolites, 11-nor-9-carboxy-Δ9-tetrahydrocannnabinol (THC-COOH) and 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH), as well as cannabinol (CBN), and cannabidiol (CBD) in human urine of patients on methadone therapy and in urine samples subjected to analysis as samples obtained from Laboratory for Toxicology Studies and Sanitary Work Environment, Institute of Occupational Health Sarajevo.

EXPERIMENTAL

Chemicals and reagents

All standards, THC, THC-OH, THC-COOH, CBN and CBD were purchased from Lipomed (Switzerland). Working solutions were prepared in methanol. The derivatization reagent was MSTFA+ 1% silicate derivative TMCS (N-Methyl-N-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane), ≥99% from Sigma-Aldrich, Deisenhofen, Germany, while all solvents (hexane, methanol, hydrochloric acid, ethyl acetate, dichloromethane, methyl *tert*-butyl ether, chloroform, acetonitrile, glacial acetic acid) were of HPLC-purity and obtained from Sigma-Aldrich, Seelze, Germany. The solid salts used for QuEChERS extraction were magnesium sulfate, sodium chloride, trisodium citrate dihydrate, and disodium hydrogen sesquihydrate, sodium tungstate and diatomaceous earth *purchased from Sigma-Aldrich, Gemany.* Immunochromatographic test, Syva-RapidTestd.a.u. 10 were obtained from Dade Behring (Leusden, Netherlands) and Detox-tubes A from (Varian).

Sample collection and pre-treatment

Human urine samples (blank), which were used for the optimization and validation of the analytical method, were obtained from volunteers, healthy people not subjected to drugs or any pharmacological treatment.

Two milliliters of urine sample was pipetted into a glass test tube (15 mL). Volume of 200 □L 6 mol/L NaOH was added in urine, for hydrolysis for 30 min at room temperature (25°C). Samples of urine had a high pH, so 400 μL of concentrated acetic acid was added to neutralise and the pH value was adjusted using the acetate buffer (pH 4) to the total volume of 5 mL. The urine sample was briefly vortexed and then centrifuged at 2600 rpm for 10 min.

A fully optimized and validated method was applied to the urine samples of drug-positive patients who underwent methadone therapy at Institute for Addiction Disorders of Canton Sarajevo, and samples obtained from Laboratory for Toxicology Studies and Sanitary Work Environment.

First, all samples were tested on the presence of cannabinoids by immunochromatographic test, and only positive samples were stored at -20°C until further analysis.

Extraction with QuEChERS salts – modified

The extraction procedure was used according to the optimization results. Optimization of the procedure was performed with samples of blank urine (2 mL) spiked with 50 ng/mL of each standard, THC, CBD, CBN, THC-OH and THC-COOH.

The QuEChERS procedure was modified using mixture of salts in a 15 mL glass test tube. Content of 1.3 g of mixture was as follows, 0.4 g of $MgSO_4$; 0.1 g NaCl; 0.1

g of $C_6H_5Na_3O_7x2H_2O$ and 0.05 g of $C_{12}H_{18}Na_4O_{17}$. Ratio of salts in mixture for extraction was $MgSO_4$: NaCl : $C_6H_5Na_3O_7x2H_2O$: $C_{12}H_{18}Na_4O_{17}$ (4:1:1:0.5).
Solid salts and 3 mL of a solvent mixture of acetonitrile: dichloromethane (1:3) were placed in test tube for extraction. Aliquot of 5mL of prepared urine sample was added and shake gently for about 1 minute manually. Mixing was continued on a stir on the roller for 10 minutes, and finally centrifuged for 10 min at 3000 rpm.
The top organic layer was separate into a vial and evaporated to dryness under a stream of nitrogen flow at room temperature.
The derivatization was done with 30 µL MSTFA +1% TMCS, vortexed for 5 s and allowed to stand at 25ºC for 30 min (Nadulski, Sporkert, Schnelle, *et al.,* 2005) to accomplish silylation. The extract was transferred to 250 µL vial. An aliquot of 1 µL of the prepared extract was injected into the GC-MS system.

GC-MS analysis

The GC-MS analysis was performed on GC-MS Agilent Technologies. Inc. GC7890A; MS 5975C and Autosampler 7983. Chromatographic separation was achieved using a capillary column HP–5MS 30 m x 0.25 mm ID, 250 µm film thickness. The analyses were performed using simultaneous MS Scan (scan range 40–600 Da) and Single Ion Recording. Mass spectrometer mode: electron ionization (EI) conditions (70 eV). Asample volume of 1 µL was injected in splitless mode. Injector temperature was set to 250°C. Ultrapure - grade helium was used as the carrier gas at a flow rate 1.5 mL/min.
Initial oven temperature was 100°C held for 1 min, raised to 175°C at 30°C/min, and then to 310°C at 12°C/min and held for 10.25 min (Alves, Agonia, Cravo, *et al.,* 2017, Angeli, Casati, Ravelli, *et al.,* 2018). The total run time was 25 min. Each analyte was identified according to their retention time and three characteristic ions (Table 1). The most abundant ion was used for quantification and the second and third ions were used for the confirmation. Samples were analyzed with GC-MS using the full-scan mode. The readings were compared with the Wiley Library of Mass Spectra of Designer Drugs (Rösner, Junge, Westphal, *et al.,* 2015) and the free database of the Scientific Working Group for the Analysis of Seized Drugs SWGDRUG-3, (Comittee 2001).

Method validation
The analytical parameters tested within the validation of the method were: selectivity, linearity, examination of extraction steps, accuracy, *limit of detection* (LOD) and *limit of quantification* (LOQ) and measuring range (Peters, Drummer and Musshoff, 2007, Shah, Midha, Findlay, *et al.,* 2000). Due to the lack of deuterated cannabinoids as internal standards, validation was performed using the standard addition method (Sutlović, *et al.,* 2011). For validation process, standard solutions of THC, CBD, CBN, THC-OH and THC-COOH in methanol and in blank urine, as well as urine samples (blank urine, positive and negative urine on THC content, CBD, CBN, THC-OH and THC-COOH) were used.

The selectivity was examined by extracting ten urine samples collected from ten different individuals who had not consumed cannabis preparations or other drugs. The resulting extracts were derivatized and analyzed by the GC-MS method. There were no interfering signals at retention times in the tested samples where peaks of interest were expected.

The linearity of the method and the measurement range were determined by the analysis of standard solutions of CBD, THC and CBN, THC-OH and THC-COOH prepared in the blank urine. For this purpose, blank urine samples were spiked with increasing concentrations of CBD, THC and CBN, THC-OH and THC-COOH, and subjected to GC-MS analysis after the extraction procedure was performed. Calibration curves were constructed based on the peak area of CBD, THC, CBN, THC-OH and THC-COOH as analytes, with respect to the corresponding concentration. All measurements were made in triplicate.

The effect of the extraction steps on the determination of CBD, THC and CBN, THC-OH and THC-COOH was investigated by comparative analysis of standard solutions of increasing concentrations (eight concentration points) of the non-extracted test analytes and standard solutions which were added to blank urine samples and extracted.

The accuracy and precision of the method was determined by dosing a standard solution at a concentration of 50 ng/mL for CBD, THC, CBN, THC-OH and THC-COOH in seven (7) different blank urine samples to include matrix influence. Accuracy was calculated as (mean concentration - nominal concentration)/(nominal concentrationx100), while precision was calculated as - the relative standard deviation (RSD, %).

Limit of detection (LOD) and Limit of quantification (LOQ)
Determination of the limit of detection (LOD) and limit of quantification (LOQ) was performed on the basis of the standard deviation of the peak area of the urine and the slope of the direction obtained by analysing five blank urine analysis in triplicate, soldered with increasing concentrations of standard solutions CBD, THC, CBN, THC-OH and THC-COOH. The formulas used for the calculation were: LOD=3*SD/b LOQ=10*SD/b (SD – standard deviation, b-slope).The standard deviation of the CBD, THC, CBN, THC-OH and THC-COOH peak area was calculated based on linear regression.

Measuring range
The measuring range (determination range) covered by this method was the range from the quantification limit shown to the highest value on the calibration curve of 1000 ng/mL.

RESULTS AND DISCUSSION

In order to develop a sensitive and reliable method, it is important to obtain an efficient simple and inexpensive procedure of extraction. The aim of this work was to establish procedure for extraction of cannabinoids as a simple, sensitive, inexpensive and reliable method, using a prepared mixture of salts instead of commercial cartridges. Urine is the preferred sample for drug abuse testing because drugs and metabolites are present in higher concentration in urine than in plasma and because urine can easily be sampled.

In order to find the best conditions for extraction, optimisation was done with solvents, composition of salts mixtures for extraction, pH values and condition of derivatization.

Six solvents; hexane, ethyl acetate, methyl *tert*-butyl ether, acetonitrile, chloroform, dichloromethane, and four mixtures of solvents, hexane : ethylacetate (8:2), ethylacetate : hexane : acetic acid (49:49:2), ethylacetate : dichloromethane (1:3), acetonitrile : dichloromethane (1:3) were used. It was concluded that a mixture of acetonitrile : dichloromethane (1:3) gave best recovery for THC and its metabolites and this mixture is selected for further analyses. The mixture of hexane : ethylacetate (8:2) is often used for extraction (Abraham, Lowe, Pirnay, *et al.*, 2007), but using this mixture recovery was 80%. Also, a mixture of chlorophorm : ethylacetate (6:4) showed good results (Nestić, Babić, Pavlović, *et al.*, 2013).

The optimisation was done according to the total mass of QuEChERS salt used but in the same ratio, (4:1:1:0.5).

The best results were with a mass of 1.3 g, while half of that mass was not sufficient for complete extraction, and twice of that mass was not suitable for extraction the process. Gas chromatogram showed that the use of half of the prepared mass of mixture of salt response for cannabinoids was lower and the extraction was insufficient. In addition to QuEChERS salt, sodium tungstate and diatomaceous earth were used for the extraction. The results of the analysis using sodium tungstate gave similar results to QuEChERS salts, while the extracts with diatomaceous earth were cloudy, colored, and there were many interferences on the chromatogram near the peaks of interest.

The extraction procedure was carried out with three pH values (3, 4, 5) using acetate buffer. Optimal pH value is important for preparation the sample for analysis, especially for the simultaneous analysis of analytes with different characteristics.

The best results were achieved with pH 4. Cannabinoids cannot be directly analyzed with GC without derivatization. After optimization, the best conditions for derivatization were found as follows, addition of a derivatization reagent, MSTFA + 1%TMCS, at room temperature for 30 min. Similar results were obtained using temperature of 75°C and time of derivatization of 10 min, but because of simplicity, conditions at room temperature were chosen.

Under specified chromatographic conditions, the retention times of standards prepared in blank urine corresponded to the retention times of standards prepared in methanol, shown in Table 1 with characteristic ions, ions in bold are target ions, while the others were confirmative ions.

Table 1.Retention times and characteristic ions of selected cannabinoids

Analyte	Retention time (min)	Ions (m/z)		
CBD	9.98	**390.3**	301.2	458.4
THC	10.75	**386.3**	303.2	371.3
CBN	11.30	**367.3**	310.2	382.3
THC-OH	12 30	**474.3**	459.2	371.3
THC-COOH	13.35	**371.3**	473.3	488.3

Figure 1 shows GC chromatogram of blank urine and GC chromatogram of blank urine spiked with all analytes. Peaks of selected cannabinoids are well separated and can be easily and reproducibly integrated because the other peaks do not influence the height and area, which indicates that there are no interferences of the extraction process. All analytes were sufficiently separated to allow simultaneous determination of THC, its metabolites as well as, CBD and CBN.

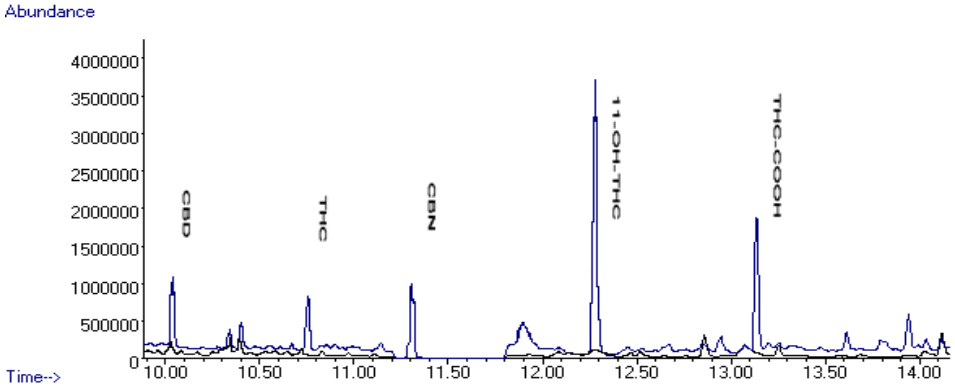


Figure 1. GC chromatograms of blank urine (black line) and mixture of canabinoids in human urine (blue line)

Calibration was performed over a wide range of concentration for all analytes and the obtained calibration parameters are presented in Table 2. These samples were then prepared in triplicate according to the procedure described above.

The developed method was linear over the range from LOQ to 1000.0 ng/mL for CBD and THC, and the other three analytes CBD, THC-OH and THC-COOH was linear from LOQ to 500.0 ng/mL. For CBD, THC, CBN, THC-OH and THC-COOH, LOD was 5.0, 3.0, 5.0, 2.6, 4.5 ng/mL, respectively. LOQ was in range from 8.7 ng/mL for THC-OH to 18.0 ng/mL for CBN (Table 2). Linearity was determined using linear regression analysis. The correlation coefficient (R^2) exceeded 0.998 thus confirming the linearity of the method.

Table 2. Calibration parameters for determination of cannabinoids

Analyt	Linearity range (ng/mL)	Equation of calibration curve	R^2	LOD (ng/mL)	LOQ (ng/mL)
CBD	16.0-1000	y = 965942x-2760	0.9999	5.0	16.0
THC	9.0-1000	y = 942573x-11427	0.9998	3.0	9.0
CBN	18.0-500	y = 3E+07x-17697	0.9998	5.0	18.0
THC-OH	8.7-500	y = 215980x-15096	0.9979	2.6	8.7
THC-COOH	15.0-500	y = 3E+07x-27881	0.9984	4.5	15.0

The accuracy and precision of the method was determined by dosing a standard solution of CBD, THC and CBN, THC-OH and THC-COOH in seven different blank urine samples to avoid matrix influence.

Accuracy values ranged from -5.2% for CBN, to -20.6% for THC-COOH, (Table 3). The method is precise; % precision is in a range from 0.941 for THC to 1.239 for THC-COOH.

The mean average recovery value was obtained for different matrices from 91.00% for CBD, to 79.40% for THC-COOH. The effect of the matrix is negligible, except slightly for THC-COOH.

For analysis in clinical and forensic toxicology, the acceptance criteria for precision are (15% R.S.D., 20% R.S.D. near LLOQ), and for accuracy, (within15% of the accepted reference value, within 20% near LLOQ), have been widely accepted in bioanalysis, (Peters, Drummer and Musshoff, 2007).

Table 3. Validation parameters – precision, accuracy, recovery of the method

#	ɤ (ng/mL)	CBD ɤ(ng/mL)	THC ɤ(ng/mL)	CBN ɤ(ng/mL)	THC-OH ɤ(ng/mL)	THC-COOH ɤ(ng/mL)
1.	50.0	46.0	40.0	48.0	41.4	39.8
2.	50.0	45.0	40.0	48.0	41.2	40.2
3.	50.0	46.0	41.0	48.0	40.9	39.7
4.	50.0	45.0	40.0	47.0	41.2	39.1
5.	50.0	45.0	40.0	47.0	41.0	39.0
6.	50.0	45.0	40.0	47.0	39.7	40.0
7.	50.0	45.0	40.0	47.0	41.3	40.4
	Average	45.3	40.1	47.4	40.9	39.7
	SD	0.5	0.4	0.5	0.5	0.5
	Precision (%)	**1.077**	**0.941**	**1.127**	**1.239**	**1.197**
	Recovery (%)	**91.00**	**80.29**	**94.86**	**81.88**	**79.40**
	Accuracy (%)	**-9.4**	**-19.8**	**-5.2**	**-18.2**	**-20.6**

Following the validation of the method, 30 urine samples received at the Laboratory for Toxicology Studies and Sanitary Work Environment, which had previously been positive on THC by drug strips, were analyzed, and results are presented in Table 4.

Table 4. Content of cannabinoids in positive urine samples extracted by modified QuEChERS method

Sample	γ(ng /mL)				
	CBN	THC	CBD	THC-OH	THC-COOH
1.	n.d.	**20.0**	<LOQ	<LOQ	<LOQ
2.	n.d.	**70.0**	<LOQ	**9.5**	n.d.
3.	n.d.	<LOQ	n.d.	n.d.	<LOQ
4.	<LOQ	**81.0**	<LOQ	n.d.	**20.0**
5.	n.d.	n.d.	<LOQ	n.d.	**36.0**
6.	n.d.	n.d.	n.d.	n.d.	<LOQ
7.	n.d.	n.d.	n.d.	n.d.	**58.0**
8.	n.d.	**38.0**	<LOQ	n.d.	<LOQ
9.	<LOQ	**65.0**	n.d.	n.d.	**19.0**
10.	n.d.	<LOQ	<LOQ	<LOQ	**54.0**
11.	n.d.	**9.7**	n.d.	n.d.	**25.1**
12.	n.d.	**12.5**	<LOQ	n.d.	**38.0**
13.	n.d.	**63.0**	n.d.	<LOQ	**141.0**
14.	n.d.	**22.3**	<LOQ	n.d.	**18.5**
15.	n.d.	<LOQ	n.d.	n.d.	**39.0**
16.	n.d.	n.d.	n.d.	n.d.	**90.0**
17.	n.d.	**14.2**	n.d.	n.d.	<LOQ
18.	n.d.	<LOQ	n.d.	n.d.	<LOQ
19.	n.d.	**20.0**	n.d.	n.d.	**36.0**
20.	n.d.	<LOQ	n.d.	**10.2**	**51.9**
21.	n.d.	**13.8**	<LOQ	**16.4**	**26.4**
22.	n.d.	**17.0**	n.d.	n.d.	**32.0**
23.	n.d.	**35.0**	n.d.	**9.0**	**25.7**
24.	n.d.	<LOQ	n.d.	**29.2**	**168.0**
25.	n.d.	n.d.	n.d.	n.d.	**40.3**
26.	n.d.	<LOQ	n.d.	<LOQ	**35.0**
27.	n.d.	n.d.	<LOQ	n.d.	<LOQ
28.	n.d.	<LOQ	n.d.	<LOQ	**37.8**
29.	n.d.	n.d.	n.d.	n.d.	**34.0**
30.	n.d.	<LOQ	n.d.	**35.1**	**53.0**

n.d.- not detected; <LOQ - lower than limit of quantification

For the analyzed urine samples, it is not known how often these people consumed cannabis, whether they consumed it occasionally or were chronic addicts, in what form and amount they consumed, or when they last consumed it.

Analysis of positive urine samples showed that in fourteen of the thirty samples, THC was quantified, in nine samples the concentration was lower than LOQ, while in seven samples THC was not detected.

The inactive metabolite THC-COOH was found in twenty two samples at a concentration higher than LOQ (9 ng/mL). THC-COOH was detected in seven samples at a concentration lower than LOQ. In one sample, THC-COOH was not detected, but the concentration of THC in this sample was high, so it could be assumed that this person had recently consumed cannabis, so that it was not metabolized.

The concentration of the active metabolite THC-OH was quantified in six samples with a concentration higher than

LOQ (8.7 ng/mL), while in nineteen samples was not detected. Rarely, CBD and CBN are suitable analytes for proving cannabis consumption in human matrices, if CBD and CBN are positive, which undoubtedly indicates recent cannabis consumption, (Citti, Braghiroli, Vandelli, *et al.*, 2018). Usually, the high concentration of CBD in *Cannabis* means that these samples were of good quality for medicinal purpose. In analysed samples CBD was not quantified, but in ten samples was detected at a concentration lower than LOQ. CBN was detected in two samples but at a concentration lower than LOQ.

Figure 2 shows the chromatogram of sample 13, where THC and THC-COOH were quantified, while THC-OH was detected.

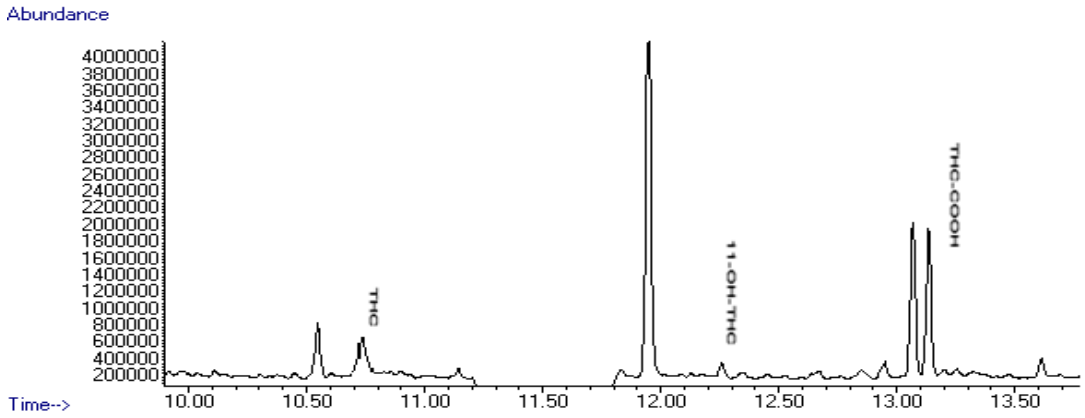


Figure 2. GC chromatogram of sample 13

CONCLUSIONS

A validated method of extraction and simultaneous determination of cannabinoids using a mixture of solid salts instead of the commercially available cartridges for QuEChERS extraction was described. The developed analytical method is a simple, fast, accurate and economical alternative to expensive cartridges, as well as the LLE method, in the analysis of cannabinoids and their metabolites. After extraction, the chromatograms of the obtained extracts indicate that there was no interference with the selected analytes. Acceptable parameters for characterization of analysis, such as LOD, LOQ, accuracy, were obtained and the method was successfully applied to the real samples.

The results of the analysis indicate the adequacy of the method for determining THC metabolites as well, which is of particular interest for determining the pharmacokinetic parameters of THC.

Moreover, since marijuana, beside THC, contains other cannabinoids, this method is suitable for the determination of certain cannabinoid compounds in the same sample, which can be significant in determination of marijuana medical use.

The method is completely acceptable for the needs of toxicological analysis and can be applied for routine analysis of THC, CBN, CND, THC-OH, THC-COOH from human urine using the GC-MS method.

REFERENCES

Abraham, T. T., Lowe, R. H., Pirnay, S. O., Darwin, W. D., Huestis, M. A. (2007). Simultaneous GC-EI-MS Determination of Δ9-tetrahydrocannabinol, 11-hydroxy-Δ9-tetrahydrocannabinol, and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in human urine following tandem enzyme-alkaline hydrolysis. *Journal of analytical toxicology*. *31(8)*, 477-485.

Aizpurua-Olaizola, O., Zarandona, I., Ortiz, L., Navarro. P., Etxebarria, N., Usobiaga, A. (2017).Simultaneous quantification of major cannabinoids and metabolites in human urine and plasma by HPLC-MS/MS and enzyme-alkaline hydrolysis. *Drug testing and analysis*. *9(4)*, 626-633.

Alves, E. A., Agonia, A.S., Cravo, S. M., Afonso, C. M., Pereira Netto, A., de Lourdes Bastos, M., F. Carvalho, Dinis-Oliveira, R. J. (2017).GC-MS method for the analysis of thirteen opioids, cocaine and cocaethylene in whole blood based on a modified quechers extraction. *Current Pharmaceutical Analysis*. *13(3)*, 215-223.

Angeli, I., Casati, S., Ravelli, A., Minoli, M., Orioli, M. (2018). A novel single-step GC–MS/MS method for cannabinoids and 11-OH-THC metabolite analysis in hair. *Journal of pharmaceutical and biomedical analysis*. *155*, 1-6.

Battista, N., Sergi, M., Montesano, C., Napoletano, S., Compagnone, D., Maccarrone, M. (2014). Analytical approaches for the determination of phytocannabinoids and endocannabinoids in human matrices. *Drug testing and analysis. 6(1-2)*, 7-16.

Citti, C., Braghiroli, D., Vandelli, M. A., Cannazza, G. (2018). Pharmaceutical and biomedical analysis of cannabinoids: a critical review. *Journal of pharmaceutical and biomedical analysis*. *147*, 565-579.

Citti, C., Braghiroli, D., Vandelli, M. A.,Cannazza, G. (2018). Pharmaceutical and biomedical analysis of cannabinoids: A critical review. *Journal of pharmaceutical and biomedical analysis*, *147*, 565-579.

Clatworthy, A. J., Oon, M. C. H., Smith, R. N., Whitehouse, M. J. (1990). Gas chromatographic-mass spectrometric confirmation of radioimmunoassay results for cannabinoids in blood and urine. *Forensic science international*. *46(3)*, 219-230.

Comittee, S. W. G. D. R. U. G. (2001). Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), Recommendations for: Education and Training. Quality Assurance, Methods of Analysis, US Department of Justice, Drug Enforcement Administration/Executive Office of the President, Office of National Drug Control Policy, Counterdrug Technology Assessment Center.

Fuchs, N., Miljanić, A., Katić, A., Brajenović, N., Micek, V., Fuchs, R., Karačonji, I. B. (2019). Optimisation of a gas chromatography-mass spectrometry method for the simultaneous

determination of tetrahydrocannabinol and its metabolites in rat urine. *Archives of Industrial Hygiene and Toxicology*. *70(4)*, 325-331.

Galand, N., Ernouf, D., Montigny, F., Dollet, J., Pothier, J. (2004). Separation and identification of cannabis components by different planar chromatography techniques (TLC, AMD, OPLC). *Journal of chromatographic science*. *42(3)*, 130-134.

Heinl, S., Lerch, O., Erdmann, F. (2016). Automated GC–MS Determination of Δ9-Tetrahydrocannabinol, Cannabinol and Cannabidiol in Hair. *Journal of analytical toxicology*. *40(7)*, 498-503.

Kemp, P. M., Abukhalaf, I. K., Manno, J. E., Manno, B. R., Alford, D. D., Abusada, G. A. (1995). Cannabinoids in humans. I. Analysis of Δ9-tetrahydrocannabinol and six metabolites in plasma and urine using GC-MS. *Journal of analytical toxicology*. *19(5)*, 285-291.

Moffat, A. C., Osselton, M. D., Widdop, B., Watts, J. (2011). *Clarke's analysis of drugs and poisons* (Vol. 3). London: Pharmaceutical press.

Nadulski, T., Sporkert, F., Schnelle, M., Stadelmann, A. M., Roser, P., Schefter, T., Pragst, F. (2005). Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *Journal of analytical toxicology*. *29(8)*, 782-789.

Nestić, M., Babić, S., Pavlović, D. M., Sutlović, D. (2013). Molecularly imprinted solid phase extraction for simultaneous determination of Δ9-tetrahydrocannabinol and its main metabolites by gas chromatography–mass spectrometry in urine samples. *Forensic science international*. *231(1-3)*, 317-324.

Peters, F. T., Drummer, O. H., Musshoff, F. (2007) Validation of New Methods, *Forensic science international*. *165*, 216–224.

Raharjo, T. J., Verpoorte, R. (2004). Methods for the analysis of cannabinoids in biological materials: a review. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*. *15(2)*, 79-94.

Ramesh, B., Manjula, N., Bijargi, S. R., Sarma, V. U. M., Devi, P. S. (2015). Comparison of conventional and supported liquid extraction methods for the determination of sitagliptin and simvastatin in rat plasma by LC–ESI–MS/MS. *Journal of pharmaceutical analysis*. *5(3)*, 161-168.

Rösner, P., Junge, T., Westphal, F., Fritschi, G. (2015). Mass Spectra of Designer Drugs 2015. Wiley-VCH.

Scheidweiler, K. B., Desrosiers, N. A., Huestis, M. A. (2012). Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. *Clinica chimica acta*. *413(23-24)*, 1839-1847.

Shah, V. P., Midha, K. K., Findlay, J. W., Hill, H. M., Hulse, J. D., McGilveray, I. J., McKay, G., Miller, K. J., Patnaik, R. N., Powell, M. L., Tonelli, A., Viswanathan, C. T., Yacobi, A. (2000). Bioanalytical method validation-a revisit with a decade of progress. *Pharmaceutical research*, *17(12)*, 1551-1557.

Sutlović, D. i suradnici (2011). *Osnove forenzične toksikologije*. Split, Redak.

Zhang, W., Wang, J., Mi, Z., Su, J., You, X., Keceli,G., Wang, Y., Cao, R., Lai, H. C. (2016). Extraction and Analysis of Tetrahydrocannabinol. A Cannabis Compound in Oral Fluid. *International journal of biology*. *9(1)*, 30-35.

Summary/Sažetak

Cilj ovog rada jeste primjena modificirane QuEChERS metode za ekstrakciju kanabinoida iz humanog urina, koristeći smjesu soli za ekstrakciju u odgovarajućem omjeru umjesto komercijalno dostupnih kertridža. Analiza je vršena na blank urinu u koji je dodata poznata koncentracija tetrahidokanabinola (THC), kanabinola (CBN) i kanabidiola (CBD), kao i metabolita THC-a, 11-hidroksi-Δ^9-tetrahidrokanabinol (THC-OH) i 11-nor-9-karboksi-Δ^9-tetrahidrokanabinol (THC-COOH). Testirano je šest rastvarača, kao i četiri smjese rastvarača, za ekstrakciju, a kao najefikasnija odabrana je smjesa rastvarača acetonitril : dihlormetan (1:3) za koju je dobiven najbolji *recovery* factor.

Derivatizacija svih uzoraka je urađena sa MSTFA + 1% TMCS na sobnoj temperaturi. Dobiveni ekstrakti su analizirani vezanim sistemom gasna hromatografija-masena spektrometrija (GC-MS) uz *full-scan* mod. Pikovi odabranih kanabinoida su dobro razdvojeni što pokazuje da nema interferenci sa izabranim analitima. Rezultati su izračunati iz kalibracione krive u rasponu od LOQ do 1000 ng/mL za izabrane kanabinoide, sa korelacionim faktorom preko 0.998. Vrijednosti LOD i LOQ za THC su (3.0 ng/mL; 9.0 ng/mL), za CBN (5.0 ng/mL; 18.0 ng/mL) za CBD (5.0 ng/mL; 16.0 ng/mL), za THC-OH (2.6 ng/mL; 8.7 ng/mL) i za THC-COOH (5.0 ng/mL; 15.0 ng/mL). Zabilježen je *recovery* factor u rasponu od 79.40% za THC-COOH do 94.86% za CBN. Modificirana QuEChERS metoda ekstrakcije se može koristiti za rutinsku analizu izabranih kanabinoida. Metoda je uspješno primjenjena na realne uzorke, analizirano je trideset uzoraka urina.

INSTRUCTIONS FOR AUTHORS

GENERAL INFORMATION

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina (Glasnik hemičara i tehnologa Bosne i Hercegovine) is a semiannual international journal publishing papers from all fields of chemistry and related disciplines.

Categories of Contributions

1. *Original Scientific Papers* – (about 10 typewritten pages) report original research which has not been published previously, except in a preliminary form. The paper should contain all the necessary information to enable reproducibility of the described work.
2. *Short Communications* – (about 5 typewritten pages) describing work that may be of a preliminary nature but which merits immediate publication.
3. *Notes* – (about 3 typewritten pages) report unpublished results of short, but complete, original research or describe original laboratory techniques.
4. *Reviews* – (about 30 typewritten pages) present a concise and critical survey of a specific research area. Generally, these are prepared by the invitation of the Editor.
5. *Book and Web Site Reviews* – (about 2 typewritten pages).
6. *Extended Abstracts* – (about 2 typewritten pages) of Lectures given at international meetings.
7. *Technical Papers* – (about 10 typewritten pages) report on applications of an already described innovation. Typically, technical articles are not based on new experiments.

Reviewing the Manuscript

All contributors are evaluated according to the criteria of originality and quality of their scientific content, and only those deemed worthy will be accepted for publication. To facilitate the reviewing process, authors are encouraged to suggest three persons competent to review their manuscript. Such suggestions will be taken into consideration but not always accepted.

The Editor-In-Chief and Editors have the right to decline formal review of a manuscript when it is deemed that the manuscript is:

1. on a topic outside the scope of the Journal;
2. lacking technical merit;
3. of insufficient novelty for a wide international readership;
4. fragmentary and providing marginally incremental results; or
5. is poorly written.

Proofs

When a manuscript is ready for printing, the corresponding author will receive a PDF-formatted manuscript for proof reading, which should be returned to the journal within one week. Failure to do so will be taken as the authors are in agreement with any alteration which may have occurred during the preparation of the manuscript.

Copyright

Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations.

Professional Ethics and Publication Policy

The journal expects the Editors, Referees and authors to adhere to the well-known standards of professional ethics. Authors are responsible for the factual accuracy of their contributions. Submission of the paper commits the author not to submit the same material elsewhere. Referees should act promptly. If certain circumstances preclude prompt attention to the manuscript at the time it is received, the non-received manuscript should be returned immediately to the Editor or the Referee should contact the Editor for possible delay of the report submission date. The Editor accepts full responsibility for his decisions on the manuscripts.

PREPARATION AND SUBMISSION OF MANUSCRIPT

Cover Letter

Manuscripts must be accompanied by a cover letter in which the type of the submitted manuscript. It should contain:

1. full name(s) of the author(s),
2. mailing address (address, phone and fax numbers, e-mail) of the author to whom correspondence should be addressed,
3. title of the paper (concise, without any abbreviations),
4. type of contribution,
5. a statement that the article is original and is currently not under consideration by any other journal or any other medium, including preprints, electronic journals and computer databases in the public domain, and
6. the names, full affiliation (department, institution, city and country), and
7. e-mail addresses of three potential Referees.

Contributors from Bosnia and Herzegovina should provide the name and full affiliation of at least one Referee from abroad.

Authors are fully encouraged to use ***Cover Letter Template*****.**

Manuscript preparation

The submitted articles must be prepared with Word for Windows. Manuscripts should be typed in English (either standard British or American English, but consistent throughout) with 1.5 spacing (12 points Times New Roman; Greek letters in the character font Symbol) in A4 format leaving 2.5 cm for margins. Authors are fully encouraged to use ***Manuscript Template***.

All contributions should be written in a style that addresses a wider audience than papers in more specialized journals. Manuscripts with grammar or vocabulary deficiencies are disadvantaged during the scientific review process and, even if accepted, may be returned to the author to be rewritten in idiomatic English. The authors are requested to seek the assistance of competent English language expert, if necessary, to ensure their English is of a reasonable standard. The journal maintains its policy and takes the liberty of correcting the English of manuscripts scientifically accepted for publication.

Tables and figures and/or schemes should not be embedded in the manuscript but their position in the text indicated. In electronic version (Word.doc document) tables and figures and/or schemes should follow the text, each on a separate page. Please number all pages of the manuscript including separate lists of references, tables and figures with their captions.

IUPAC and International Union of Biochemistry and Molecular Biology recommendations for the naming of compounds should be followed.

SI units, or other permissible units, should be employed. The designation of physical quantities should be in Times New Roman font. In text, graphs, and tables, brackets should be used to separate the designation of a physical quantity from the unit. Please do not use the axes of graphs for additional explanations; these should be mentioned in the figure captions and/or the manuscript (example: "pressure at the inlet of the system, kPa" should be avoided).

Percents and *per mills*, although not being units in the same sense as the units of dimensioned quantities, can be treated as such. Unit symbols should never be modified (for instance: w/w %, vol.%, mol.%) but the quantity measured has to be named, *e.g.* mass fraction, w=95 %; amount (mole) fraction, x=20 %.

Latin words, as well as the names of species, should be in *italic*, as for example: *i.e., e.g., in vivo, ibid, Artemisia annua* L., *etc*. The branching of organic compound should also be indicated in *italic*, for example, *n*-butanol, *tert*-butanol, *etc*.

Decimal numbers must have decimal points and not commas in the text (except in the Bosnian/Croatian/Serbian abstract), tables and axis labels in graphical presentations of results. Thousands are separated, if at all, by a comma and not a point.

Structure of the Manuscript

The manuscript must contain, each on a separate page, the title page, abstract in English, (abstract in Bosnian/Croatian/Serbian), graphical abstract (optional), main text,

list of references, tables (each table separately), illustrations (each separately), and legends to illustrations (all on the same page).

1. ***Title page*** must contain: the title of the paper (bold letters), full name(s) of the author(s), full mailing addresses of all authors (italic), keywords (up to 6), the phone and fax numbers and the e-mail address of the corresponding author.
2. A one-paragraph ***abstract*** written of 150–200 words in an impersonal form indicating the aims of the work, the main results and conclusions should be given and clearly set off from the text. Domestic authors should also submit, on a separate page, a Summary/Sažetak. For authors outside Bosnia and Herzegovina, the Editorial Board will provide a Bosnian/Croatian/Serbian translation of their English abstract.
3. Authors are encouraged to submit a ***graphical abstract*** that describes the subject matter of the paper. It should contain the title of the paper, full name(s) of the author(s), and graphic that should be no larger than 11 cm wide by 5 cm tall. Authors are fully encouraged to use ***Graphical Abstract Template.***
4. ***Main text*** should have the following form:
   - ***Introduction***should include the aim of the research and a concise description of background information and related studies directly connected to the paper.
   - ***Experimental*** section should give the purity and source of all employed materials, as well as details of the instruments used. The employed methods should be described in sufficient detail to enable experienced persons to repeat them. Standard procedures should be referenced and only modifications described in detail.
   - ***Results and Discussion***should include concisely presented results and their significance discussed and compared to relevant literature data. The results and discussion may be combined or kept separate.
   - The inclusion of a ***Conclusion***section, which briefly summarizes the principal conclusions, is highly recommended.
   - ***Acknowledgement*** (optional).
   - Please ensure that every ***reference*** cited in the text is also present in the reference list (and *vice versa*). Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either "Unpublished results" or "Personal communication" Citation of a reference as "in press" implies that the item has been accepted for publication. As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. No more than 30 references should be cited in your manuscript.

     In the text refer to the author's name (without initials) and year of publication (e.g. "Steventon, Donald and Gladden (1994) studied the effects..." or "...similar to values reported by others (Anderson, Douglas, Morrison, *et al.*, 1990)..."). Type the names of the first three authors at first citation. At subsequent citations use

first author *et al.* The list of references should be arranged alphabetically by authors' names and should be as full as possible, listing all authors, the full title of articles and journals, publisher and year.

Examples of ***reference style***:

a) Reference to a journal publication:
Warren, J. J., Tronic, T. A., Mayer, J. M. (2010). Termochemistry of proton-coupled electron transfer reagents and its implications. *Chemical Reviews*, *110 (12)*, 6961-7001.

b) Reference to a book:
Corey, E. J., Kurti, L. (2010). *Enantioselective chemical synthesis*. (1st Ed.) Direct Book Publishing, LLC.

c) Reference to a chapter in an edited book:
Moody, J. R., Beck II, C. M. (1997). Sample preparation in analytical chemistry. In Setlle, F. A. (Ed.), *Handbook of instrumental techniques for analytical chemistry*. (p.p. 55-72). Prentice Hall.

d) Reference to a proceeding:
Seliskar, C. J., Heineman, W.R., Shi, Y., Slaterbeck, A.F., Aryal, S., Ridgway, T.H., Nevin, J.H. (1997). *New spectroelectrochemical sensor*, in Proceedings of 37th Conference of Analytical Chemistry in Energy and Technology, Gatlinburg, Tenesee, USA, p.p. 8-11.

e) Patents:
Healey, P.J., Wright, S.M., Viltro, L.J., (2004).*Method and apparatus for the selection of oral care chemistry*, The Procter & Gamble Company Intellectual Property Division, (No.US 2004/0018475 A1).

f) Chemical Abstracts:
Habeger, C. F., Linhart, R. V., Adair, J. H. (1995). Adhesion to model surfaces in a flow through system. *Chemical Abstracts,* CA 124:25135.

g) Standards:
ISO 4790:1992. (2008). *Glass-to-glass sealings - Determination of stresses*.

h) Websites:
Chemical Abstract Service, www.cas.org, (18/12/2010).

- ***Tables*** are part of the text but must be given on separate pages, together with their captions. The tables should be numbered consequently in Latin numbers. Quantities should be separated from units by brackets. Footnotes to tables, in size 10 font, are to be indicated consequently (line-by-line) in superscript letters. Tables should be prepared with the aid of the Word table function, without vertical lines. Table columns must not be formatted using multiple spaces. Table rows must not be formatted using Carriage returns (enter key; ↵ key). Tables should not be incorporated as graphical objects.
- ***Figures and/or Schemes*** (in high resolution) should follow the captions, each on a separate page of the manuscript. High resolution illustrations in TIF or EPS format (JPG format is acceptable for colour and greyscale photos, only) must be uploaded as a separate archived (.zip or .rar) file.

Figures and/or Schemes should be prepared according to the artwork instructions.

- ***Mathematical and chemical equations*** must be numbered, Arabic numbers, consecutively in parenthesis at the end of the line. All equations should be embedded in the text except when they contain graphical elements (tables, figures, schemes and formulae). Complex equations (fractions, inegrals, matrix…) should be prepared with the aid of the Word Equation editor.

Artwork Instructions

Journal accepts only TIF or EPS formats, as well as JPEG format (only for colour and greyscale photographs) for electronic artwork and graphic files. MS files (Word, PowerPoint, Excel, Visio) are NOT acceptable. Generally, scanned instrument data sheets should be avoided. Authors are responsible for the quality of their submitted artwork.

Image quality: keep figures as simple as possible for clarity - avoid unnecessary complexity, colouring and excessive detail. Images should be of sufficient quality for the printed version, i.e. 300 dpi minimum.

Image size: illustrations should be submitted at its *final size* (8 cm for single column width or 17 cm for double column width) so that neither reduction nor enlargement is required.

Photographs: please provide either high quality digital images (250 dpi resolution) or original prints. Computer print-outs or photocopies will not reproduce well enough for publication. Colour photographs rarely reproduce satisfactorily in black and white.

The facility exist for color reproduction, however the inclusion of color photographs in a paper must be agreed with Editor in advance.

Reporting analytical and spectral data

The following is the recommended style for analytical and spectral data presentation:

1. ***Melting and boiling points***:
   mp 163–165°C (lit. 166°C)
   mp 180°C dec.
   bp 98°C
   Abbreviations: mp, melting point; bp, boiling point; lit., literature value; dec, decomposition.
2. ***Specific Rotation***:
   $[a]^{23}_{D}$ –222 (*c* 0.35, MeOH).
   Abbreviations: a, specific rotation; D, the sodium D line or wavelength of light used for determination; the superscript number, temperature (°C) at which the determination was made; In parentheses: *c* stands for concentration; the number following *c* is the concentration in grams per 100 mL; followed by the solvent name or formula.

3. ***NMR Spectroscopy***:
   ^{1}H NMR (500 MHz, DMSO-d_6) d 0.85 (s, 3H, CH_3), 1.28–1.65 (m, 8H, 4´CH_2), 4.36–4.55 (m, 2H, H-1 and H-2), 7.41 (d, *J* 8.2 Hz, 1H, ArH), 7.76 (dd, *J* 6.0, 8.2 Hz, 1H, H-1'), 8.09 (br s, 1H, NH).
   ^{13}C NMR (125 MHz, $CDCl_3$) d 12.0, 14.4, 23.7, 26.0, 30.2, 32.5, 40.6 (C-3), 47.4 (C-2'), 79.9, 82.1, 120.0 (C-7), 123.7 (C-5), 126.2 (C-4).
   Abbreviations: d, chemical shift in parts per million (ppm) downfield from the standard; *J*, coupling constant in hertz; multiplicities s, singlet; d, doublet; t, triplet; q, quartet; and br, broadened. Detailed peak assignments should not be made unless these are supported by definitive experiments such as isotopic labelling, DEPT, or two-dimensional NMR experiments.
4. ***IR Spectroscopy***:
   IR (KBr) n 3236, 2957, 2924, 1666, 1528, 1348, 1097, 743 cm^{-1}.
   Abbreviation: n, wavenumber of maximum absorption peaks in reciprocal centimetres.
5. ***Mass Spectrometry***:
   MS *m/z* (relative intensity): 305 (M^+H, 100), 128 (25).
   HRMS–FAB (*m/z*): $[M+H]^+$calcd for $C_{21}H_{38}N_4O_6$, 442.2791; found, 442.2782.
   Abbreviations: *m/z*, mass-to-charge ratio; M, molecular weight of the molecule itself; M^+, molecular ion; HRMS, high-resolution mass spectrometry; FAB, fast atom bombardment.
6. ***UV–Visible Spectroscopy***:
   UV (CH_3OH) l_{max} (log e) 220 (3.10), 425 nm (3.26).
   Abbreviations: l_{max}, wavelength of maximum absorption in nanometres; e, extinction coefficient.
7. ***Quantitative analysis***:
   Anal.calcd for $C_{17}H_{24}N_2O_3$: C 67.08, H 7.95, N 9.20. Found: C 66.82, H 7.83, N 9.16.All values are given in percentages.
8. ***Enzymes and catalytic proteins relevant data:***
   Papers reporting enzymes and catalytic proteins relevant data should include the identity of the enzymes/proteins, preparation and criteria of purity, assay conditions, methodology, activity, and any other information relevant to judging the reproducibility of the results[1]. For more details check Beilstein Institut/STRENDA (standards for reporting enzymology data) commission Web site (http://www.strenda.org/documents.html).

[1] For all other data presentation not mentioned above please contact Editor for instructions.

Submission Checklist

The following list will be useful during the final checking of an article prior to sending it to the journal
for review:

- E-mail address for corresponding author,
- Full postal address,
- Telephone and fax numbers,
- All figure captions,
- All tables (including title, description, footnotes),
- Manuscript has been "spellchecked" and "grammar-checked",
- References are in the correct format for the journal,
- All references mentioned in the Reference list are cited in the text, and *vice versa*.

Submissions

Submissions should be directed to the Editor by e-mail: ***glasnik@pmf.unsa.ba***, or ***glasnikhtbh@gmail.com***. All manuscripts will be acknowledged on receipt (by e-mail) and given a reference number, which should be quoted in all subsequent correspondence.

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

Zmaja od Bosne 33-35, BA-Sarajevo
Bosnia and Herzegovina
Phone: +387-33-279-918
Fax: +387-33-649-359
E-mail: glasnik@pmf.unsa.ba
glasnikhtbh@gmail.com

Sponsors

Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

Print ISSN: 0367-4444
Online ISSN: 2232-7266

Zmaja od Bosne 33-35, BA-Sarajevo
Bosnia and Herzegovina
Phone: +387-33-279-918
Fax: +387-33-649-359
E-mail: glasnik@pmf.unsa.ba
glasnikhtbh@gmail.com

www.elektroprivreda.ba/stranica/te-kakanj

FMON
Federalno ministarstvo obrazovanja i nauke

www.fmon.gov.ba

Ministarstvo za obrazovanje, nauku i mlade Kantona Sarajevo

https://mon.ks.gov.ba