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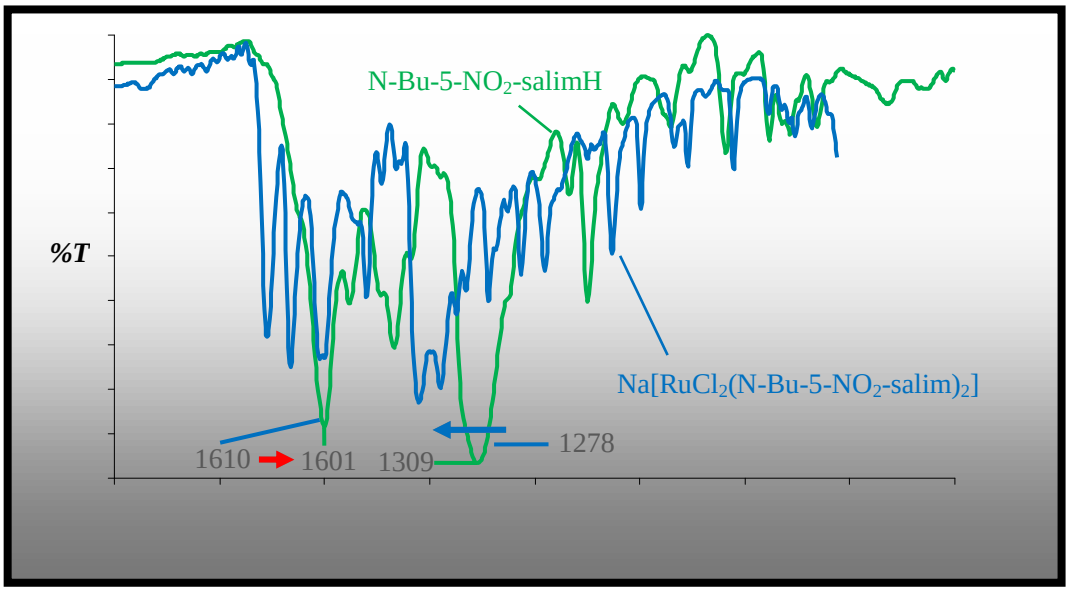

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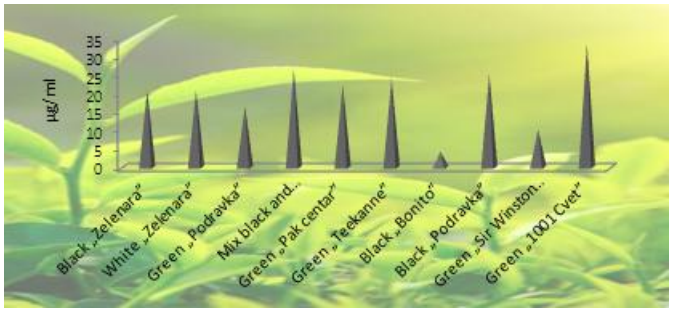
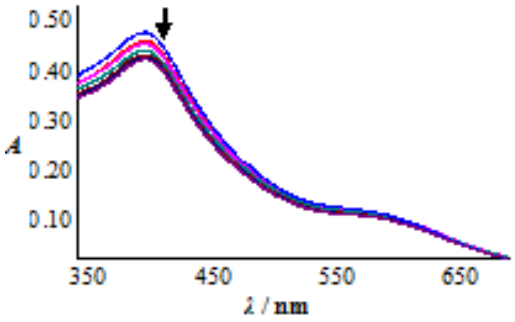
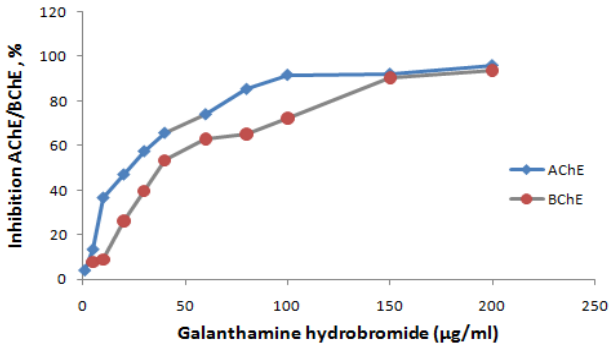
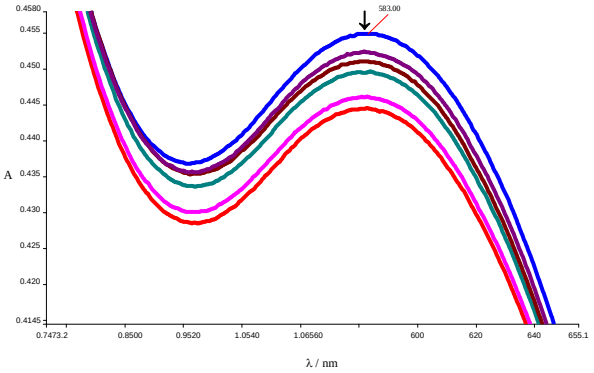
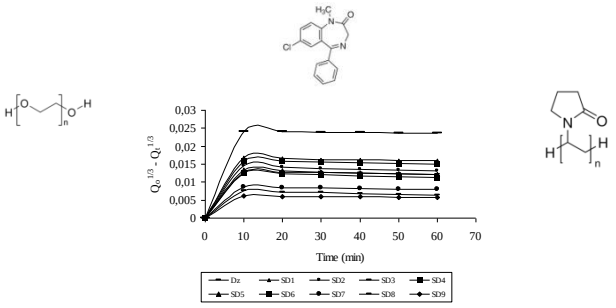
CONTENT / SADRŽAJ











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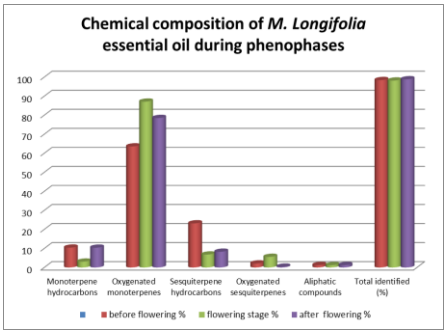
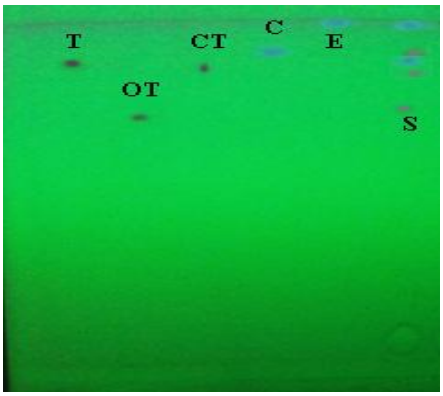
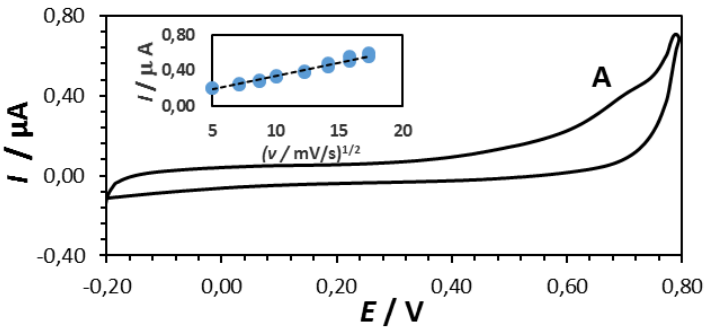
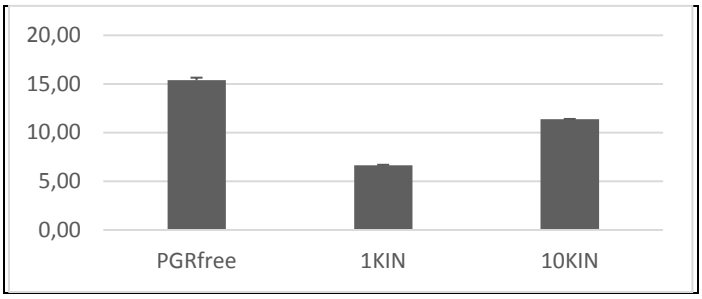
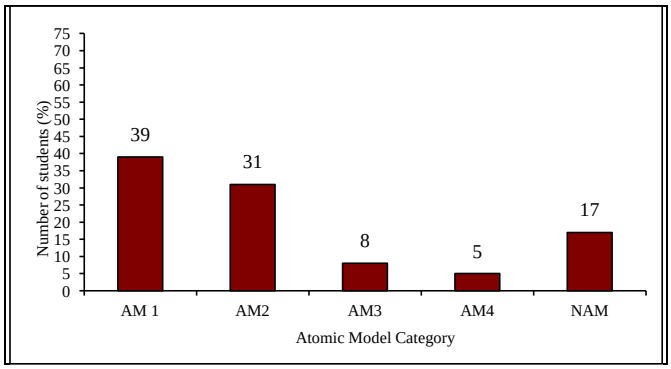


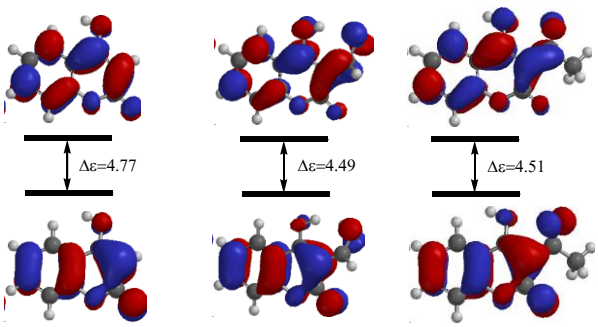








SHORT COMMUNICATIONS



Editorial

Congress of Chemists and Chemical Engineers of Bosnia and Herzegovina with International participation, organized by Society of Chemists and Technologists of Canton Sarajevo and the Faculty of Science in Sarajevo was held on October 10th-12th, 2014 in Sarajevo. There were approximately 160 delegates from academic, government and industrial environments in attendance, five invited speakers and close to 140 posters presented at this event.

The main objectives of the meeting were to foster and increase communication between scientists in academia and industry, to present emerging areas of research and to initiate new regional research collaborations. The scientific program can be viewed at http://www.pmf.unsa.ba/hemija/glasnik/index.php/current-issue

Plenary speaker, Dr. Marina Cindric from University of Zagreb, Zagreb, Croatia presented a talk on Solvent-induced transformations in feromagnetically coupled (Ni_4L_4) clusters**.** She focused on research showing synthetic procedures, interconversion scenarios of reabsorption and exchanging solvent molecules, structural and magnetic studies of a new family of Ni(II) compounds based on cubane–like clusters.

Next plenary speaker was Dr. Miloš Mladen from University of Split, Croatia. His speech focused on Investigation of antioxidative and cell signaling properties of bioactive compounds from aromatic herbs. Different methods of the isolation, fractionation and identification of chemical compounds and testing of their biological activity such as antioxidant effect and inhibition of the enzyme activity of the selected enzyme were presented.

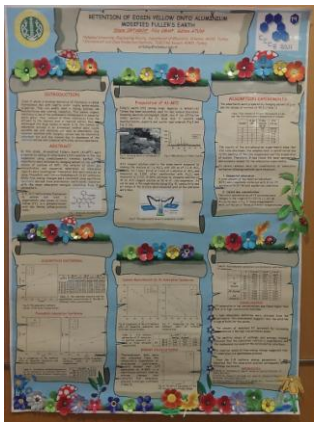
Dr. Jaćimović Željko from University of Montenegro, Podgorica, Montenegro presented talk titled "Complexes of transition metals with pyrazole derived ligands: synthesis, physico-chemical characterization and potential application" He pointed out the importance of metal complexes with pyrazolone ligands that can act as a potential anticancer agents. The second selected group of compounds was investigated in relation to the potential biological activity (fungicidal activity) on pathogenic fungi Phomopsis viticola Sacc, which is the cause of leaf spot disease of grapevine and Botryosphaeria dothidea that causes fruit rot of olives.

Plenary lecture titled Conducting polymers and their applications was presented by Dr. Toparre Levent from Middle East Technical University, Ankara, Turkey/ He presented his research o conducting polymer based electrochromic devices, solar cells, organic light emitting diodes and biosensors. Also, recent developments in The Center for Solar Energy Research and Applications, METU on the OPVs and OLEDs were discussed.

Dr. Bates W. Roderick from Nanyang Technological University, Singapore talked about Catalysis: "Awakening Affinities" for Organic Synthesis . He presented catalytic methods using a wide variety of metals to achieve mild and selective bond formation. This includes palladium, rhodium, osmium, iridium, platinum, silver and gold. In addition, methods using Lewis acidic main group metal catalysts are describe in his talk. . A selection of catalytic reactions and their applications were presented.

At the end, Congress of Chemists and Chemical Engineers of Bosnia and Herzegovina with International participation truly achieved set goals. Our next meeting, we hope, will be even better ad bigger with participants all over the world discussing ideas and new research.

Editors



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Original Scientific Paper

Determination of Caffeine in Different Comercialy Available Green and Black Teas

Salihović M., Šapčanin A.*, Pazalja M., Alispahić A., Dedić A., Ramić E.

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Abstract: Methyl derivatives of xanthine are a group of alkaloids commonly used for their effects as mild stimulants on various organ systems such as cardiovascular and central nervous system (CNS), respiratory system and skeletal muscles. The naturally occurring methyl xanthines are caffeine, theophylline and theobromine. The aim of this study was to determine content of caffeine in the green and black tea commercially available from Bosnian markets by simple, fast and economical methods. The simultaneous quantitative and qualitative determination was based on spectrophotometric UV/Vis method and thin layer chromatography (TLC) method. Content of caffeine in the green tea was in the range 33.90 to 110.73(mg/g), and in the black tea was in the range from 10.32 to 63.00 (mg/g). The highest content of caffeine was detected in the green tea of Slovenian manufacturer, and in the black tea of Croatian manufacture. Consuming a large amount of these types of tea could cause some health problems.

INTRODUCTION

Herbal teas play a vital role in maintaining human health and contributes to the improvement of human life. Herbs are considered as chemical laboratories capable of biosynthesizing a number of bio-molecules of different chemical classes. Tea is the most consumed plant-based beverage in the world. Caffeine is the most important naturally occurring xanthine derivate and it is found in many herbs, like tea leaves, coffee beans, kola nuts, cocoa beans etc. (Verma and Kumar, 2010). Caffeine is rapidly metabolized in human cells by demethylation: within 1–3 h of exposure to millimolar concentrations (Goth and Cleaver, 1976). Caffeine is a psychoactive stimulant known to increase alertness, elevate mood and give temporary energy boost thereby easing fatigue. It also increases the effectiveness of certain drugs, hence it is used with some over-the-counter drugs for the treatment of conditions such as migraine and cluster headaches (Ogah and Obebe, 2012). Some studies show that consuming caffeine causes increase in blood pressure, diuresis, increase in blood sugar, increase in gastric acid and pepsin secretion, increased plasma levels of fatty acids, cortisol and epinephrine, raised intraocular pressure and loss of calcium leading to bone loss (Klang, Wang and Meoni, 2002). Caffeine can make a person addicted to it and can have adverse effects because consumption of more than 1 g of caffeine can lead to death (Sapcanin, Uzunovic, Jancan et al. 2013). Intake of caffeine was considered safe if we consider the US Food and Drugs Administration classification of caffeine consumption. Under this classification, caffeine intake of 130 - 300 mg/day is low/moderate, and above 400 mg/day is high (Ogah and Obebe, 2012). Caffeine was first isolated from coffee in 1820 by a German chemist Friedlieb Ferdinand Runge (Lovett, 2005). Today, there are several chemical and physical methods for the determination of caffeine in tea leaves and other beverages. The most widely used methods for the determination of caffeine in beverages include various analytical techniques such as High Pressure Liquid Chromatography, Fourier Transform infrared spectroscopy, Near infrared reflectance spectrometry, Raman spectroscopy and capillary electrophoresis. However, such equipments and instruments are expensive and they are not available in most laboratories (Belay, Ture, Redi et al, 2008).

In this study, a method for determimnation of the content of caffeine in green and black teas commercially available from Bosnian markets is reported by using UV/Vis spectrophotometer, which is available in most laboratories. Purity and identification of isolated caffeine was determined by using thin layer chromatography (TLC). Moreover, methods for the determination of the content of caffeine in tea are easy, fast and cheap.

MATERIAL AND METHODS

Green and black tea, commercially available from Bosnian markets, produced by different manufacturers such as Croatian, Slovenian, Austrian, Turkish, Indian and Chinese companies were used in this study. Caffeine was purchased from Caelo and all other reagents and chemicals were purchased from Sigma-Aldrich Co. LLC. All chemicals were p.a. grade.

Isolation of Caffeine from Green and Black Tea Leaves

An accurately weighed amount of 5.0 g of tea was extracted with 100 ml of boiling distilled water. Solution was mixed for 10 minutes and then filtered. Caffeine was extracted from the primary filtrate into the separatory funnel. Extraction was done three times, each time with 20 mL of dichloromethane. Extract of caffeine was rinsed twice, each time with 20 mL of cold 6M NaOH. Furthermore, the extract was rinsed once with 20 mL of cold distilled water. Layer with dichloromethane was dried over anhydrous Na_2SO_4. The solution was evaporated to drieness (Vishnoi, 2003). The extracted caffeine was stored in a clean 10 mL volumetric flasks. The absorbance of the resulting solutions was then measured by using an UV/Vis spectrophotometer at 271 nm wavelength.

Preparation of caffeine standard solutions

Standard of caffeine was prepared by dissolving 1 mg of caffeine in 100 mL dichloromethane in a volumetric flask (100 mL). The working standard solutions (10, 20, 40, 60, 80 and 100 mg/L) were used in this study (Amos-Tautua Bamidele and Diepreye, 2014). The absorbance of each solution was measured at 271 nm wavelength. The absorbance values were then plotted against concentrations to generate a standard calibration curve. The results showed a good linear relationship between the absorbance and concentrations of the standard solutions.

Thin Layer Chromatography (TLC)

Purity of isolated caffeine was estimated by using TLC method and was compared with standards. This method can be applied for the determination of caffeine in different types of commercially available samples.. The test samples and standards were dissolved in ethanol-water (8:2 v/v) and applied to precoated TLC. The chromatographic separations were done on the silica gel F_{254}. TLC plates were developed with chloroform-acetone-methanol (1:1:1 v/v/v). The detection was performed under UV lamp at 254 nm and the evaluation of the chromatographic plate was based on processing of chromatographic images and Rf value was calculated (Harborne, 2005; Kumar, Niranjan, Chaluvaraju, 2010).

Quantitative caffeine determination

Quantitative analysis of caffeine was performed by using a Genesys UV-Vis Spectrometer, Model TM2. The λ_{max} was determined by scanning the standard solution from 200-400 nm and the obtained results gave an absorption spectrum, which was characterized by a single intensive absorption band located in the UV range at λ_{max} = 271 nm. Standard linear calibration curve was run to obtain the linear range of sample analysis, correlation factor was with accepted value (0.9916) and the standard calibration curve was linear over the range (10-100 µg/ml) caffeine with equation (y = 0.0344x + 0.2184). The quantitative amount of caffeine in samples (µg/ml) was then determined using the standard curve.

RESULTS AND DISCUSSION

The standard linear calibration curve obtained of spectrophotometric UV/Vis method for the determination of caffeine is presented in Figure 1. It showed a good linear relationship between the absorbance and concentrations of the standard solutions.

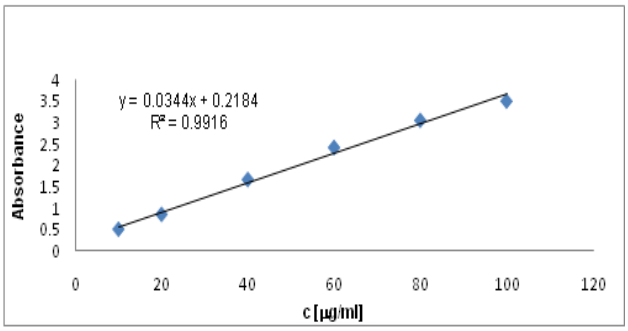


Figure 1: Calibration curves of spectrophotometric UV/Vis method for the determination of caffeine

The results obtained for caffeine in the different tea extracts are showed in Table 1.

Table 1: The content of caffeine in various tea extracts

Sample	Name	Manufacturer	µg/ml	mg/g
1	Black „*Zelenara*"	Turkey	20.74	51.80
2	White „*Zelenara*"	China	20.63	51.60
3	Green *„Podravka"*	Croatia	16.44	54.80
4	Mix black and essential oil of bergamot „*Sir Winston Tea*"	Austria	26.32	52.64
5	Green *„Pak centar"*	Bosnia	22.19	55.47
6	Green „*Teekanne*"	Austria	23.98	59.93
7	Black *„Bonito"*	India	4.13	10.32
8	Black *„Podravka"*	Croatia	25.20	63.00
9	Green „*Sir Winston Tea*"	Austria	10.17	33.90
10	Green *„1001 Cvet"*	Slovenia	33.22	110.73

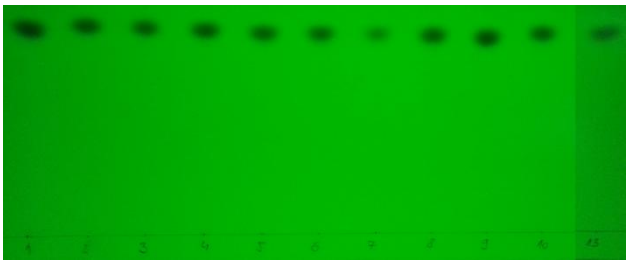
Content of caffeine in green tea was in the range 33.90 to 110.73 (mg/g), and in black tea was in the range from 10.32 to 63.00 (mg/g). The highest content of caffeine was detected in green tea from the Slovenian market, and in the black tea from Croatian market. The content of caffeine in black and green tea determined in this work is in agreement with previous works reported by Srdjenovic, Djordjevic-Milic, Grujic et al. (2008) and Bispo, Veloso, Pinheiro et al. (2002).

Identification of isolated compound by TLC and UV Spectroscopic methods.

Qualitative analysis of caffeine was performed by TLC method (Chromatogram 1.) for the determination of the caffeine in tea leaves available from Bosnian market places. UV/Vis spectroscopic study and TLC of the isolated compound were found almost similar to that of the standard caffeine Table 2. (Stahl, 2007; Harborne, 2005).

Table 2. Results of TLC and UV Spectrophotometric study of caffeine

Sample	UV-Spectra (λ_{max} in nm)	TLC Studies (Rf)
Tea leaves	271	0.79 – 0.80
Standard	271	0.80



Chromatogram 1. TLC analysis of investigated samples (1-10) and standard solution of caffeine (13)

CONCLUSION

From the results of this study it was concluded that the highest content of caffeine was detected in green tea of Slovenian manufacturer, and in the black tea of Croatian manufacturer. It was recommended that people who need caffeine restriction due to health conditions should choose products with lower caffeine contents. Since caffeine could cause some health problems, manufacturers should be required to indicate its presence and amounts on the product labels for information to consumers.

The UV/Vis spectrophotometric method employed in this study for the quantification of caffeine in tea leaves was found to be relatively fast, cheap and simple for performing. This analytical method may therefore, be recommended for the rapid quantification of caffeine in tea leaves by any educational institutions in developing countries.

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Summary/Sažetak

Derivati metil ksantina su grupa alkaloida koji se često koriste kao blagi stimulansi na različite sisteme organa, kao što su kardiovaskularni i centralni nervni sistem, respiratorni sistem i sistem skeletnih misića. Prirodni metil ksantini su kofein, teofilin i teobromin. Cilj ovog rada je određivanje kofeina u zelenim i crnim čajevima komercijalno dostupnim sa bosanskog, hrvatskog, slovenačkog, austrijskog, turskog, indijskog i kineskog tržišta, brzim i ekonomičnim metodama. Simultano kvantitativno i kvalitativno određivanje bazirano je na spektrofotometrijskoj metodi UV/Vis i tankoslojnoj hromatografiji (TLC). Sadržaj kofeina u zelenim čajevima je bio u rasponu od 33.9 do 110.73 (mg/g)., a u crnim čajevima od 10.32 do 63.00 (mg/g). Najveći sadržaj kofeina određen je u zelenom čaju sa tržišta Slovenije ,a u crnom čaju sa tržišta Hrvatske. Konzumiranje velike količine ovih čajeva može uzrokovati neke zdravstvene probleme.

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Original Scientific Paper

Spectrophotometric determination of binding constants of Ru(III) salicylideneimine complexes with CT DNA

Ljubijankić S., Zahirović A., Memišević M., Ljubijankić N., Kahrović E.*

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Abstract: The interactions of Ru(III) complexes with Schiff bases derived from salicylaldehyde and aminophenol, butylamine and naphthylamine with general formula Na[Ru(N-R-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), Na[$RuCl_2$(N-R-5-X-salim)$_2$] (R = C_4H_9, X = H, Cl, Br, NO_2) and [Ru(N-R-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br) with CT DNA, were investigated by spectroscopic titration. Experimental data show that Ru(III) complexes with salicylideneimine bind CT DNA with constants of 10^4 M^{-1}. The results indicate the influence of 5-X-substituents on K_b values.

INTRODUCTION

Ruthenium as a coordination center is interesting due to a number of reasons, especially because its complexes can act as catalysts, electron-transfer mediators or anticancer agents (Bruijnincx and Sadler, 2008; Keppler *et all* 1989) depending on a coordination environment (Bratsos *et all*, 2007). Schiff bases are organic ligands with many suitable properties that qualify them as excellent candidates for the design and development of novel metal complexes. The properties of Schiff bases, such as antibacterial, antiviral and anticancer ones are much more extensive when in metal complexes (Drozdzak *et all*, 2005; Muray *et all*, 1978). In addition, Schiff bases are able to tune the redox potentials of metal center in complex compounds. Some ruthenium complexes with salicylaldimine showed significant biological activity against some bacteria, in contrast to the less active free ligands[6] (Chittilappilly and Yusuff, 2008). For design and development of metal-based drugs (Kahrović, 2011), the activation mode and transport in biological environment is essential. There are many proofs that biologically active ruthenium compounds with anticancer properties can be activated either by hydrolysis or by reduction "*in situ*". It is generally thought that DNA is main target molecule for metal-based chemotherapeutic agents (Piggot *et all*, 2004).

The aim of this study was investigation of interaction of Ru(III) salicylaldimine complexes with CT DNA. The compounds are formulated by general formula Na[Ru(N-R-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), Na[$RuCl_2$(N-R-5-X-salim)$_2$] (R = C_4H_9, X = H, Cl, Br, NO_2) and [Ru(N-R-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br).

EXPERIMENTAL

Methods and materials

All chemicals were purchased from commercial sources and used without further purification. Calf thymus DNA (CT DNA) was obtained from Merck (Type I, highly polymerized) and was purified using phenol-chloroform-isoamyl alcohol extraction until satisfactory A_{260}/A_{280}=1.8 ratio was achieved. The stock solution of CT DNA (c = $2.21 \cdot 10^{-3}$ molL^{-1}) was prepared in Tris-HCl buffer at pH 7.4 and stored at 4 °C for 1−4 days. The concentration of

DNA was calculated based on extinction coefficient 6600 M^{-1} cm^{-1} at 260 nm wavelength (Meadows *et all*, 1993). The complexes of general formula Na[Ru(N-R-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), Na[$RuCl_2$(N-R-5-X-salim)$_2$] (R =C_4H_9, X = H, Cl, Br, NO_2) and [Ru(N-R-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br) were prepared according to published procedures (Ljubijankić *et all*, 2013; Kahrović, 2014). The purity of Ru(III) compounds was checked by infrared spectroscopy. K_b values were calculated on the basis of spectroscopic titrations of Ru(III) complexes with fixed concentrations, while increasing concentration of CT DNA. The titrations were performed in Tris-HCl buffer pH 7.42, c = 0.1 $molL^{-1}$ in presence of NaCl, c = 0.15 $molL^{-1}$. The complexes were initially dissolved in small volume of DMSO (420-1745 μL) due to insolubility in water and solutions were diluted with Tris-HCl buffer, to 2 mL giving the concentrations about 2 · 10^{-5} $molL^{-1}$. The titrations were performed by addition of 5μL-volumes of CT DNA solution, compensated in the blank, to the metal complexes solutions. The molar ratio of Ru(III) compounds and DNA were in the range of 0 - 3.85. Each titration was repeated three times.

RESULTS AND DISCUSSION

Interaction of metal complex compounds with DNA is significant initial signal about potential biological activity of a tested compound. Activity of ruthenium compounds toward DNA, as a key target for anticancer drugs, may originate from either covalent binding to DNA or non-covalent interaction. In the case of covalent interaction, the complex compounds are able to bind nucleobases, while non-covalent binding comprises electrostatic interaction of positively charged species with phosphate backbone, intercalation or groove binding. The mode of interaction depends on metal center and ligands. Schiff bases derived from 5-X-salicylaldehyde and different amines are chelating ligands, that are able to stabilize Ru(III) in solutions. Spectroscopic study of interaction of Na[Ru(N-R-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), [Ru(N-R-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br) and Na[$RuCl_2$(N-R-5-X-salim)$_2$] (R = C_4H_9, X = H, Cl, Br, NO_2) with CT DNA has been performed by titration of fixed concentration of complex compounds with increasing concentrations of calf thymus DNA (CT DNA) in the [DNA] / [complex] ratio range of 0−3.85. The binding constant, K_b were calculated using following equation (Bratsos *et all*, 2007) :

$$\frac{[DNA]}{(\varepsilon_a - \varepsilon_f)} = \frac{[DNA]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)}$$

ε_a, ε_f and ε_b represent apparent extinction coefficients for particular measurements (A_{obs} / [DNA]), free complex and completely bound form, respectively. By plotting [DNA] / ($\varepsilon_a - \varepsilon_f$) vs [DNA], K_b is obtained as the ratio of the slope and intercept.

Figures 1-3 show the titrations of the selected complexes from three series of compounds, Na[Ru(N-C_6H_4O-5-X-salim)$_2$], [Ru(N- $C_{10}H_7$-5-X-salim)$_3$] and Na[$RuCl_2$(N-C_4H_9-5-X-salim)$_2$] with CT DNA.

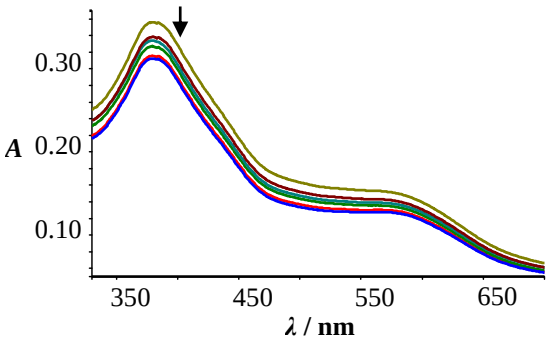


Figure 1. Spectrophotometric titration of Na[Ru(N-C_6H_4O-5-H-salim)$_2$], c = 2.21·10^{-5} $molL^{-1}$, with increasing concentration of CT DNA (stock solution, c = 2.21·10^{-3} $molL^{-1}$) in Tris-HCl buffer pH 7.42, c = 0.1 $molL^{-1}$ in the presence of NaCl, c = 0.15 $molL^{-1}$

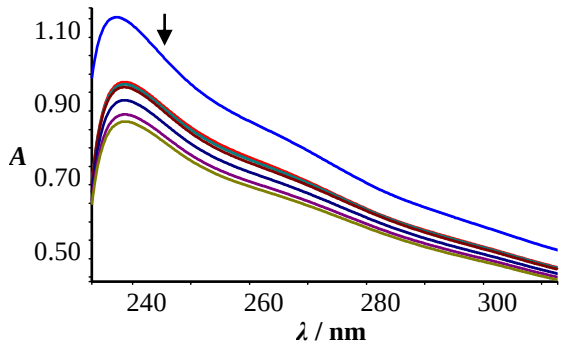


Figure 2. Spectrophotometric titration of [Ru(N-$C_{10}H_7$-5-H-salim)$_3$], c = 2.20·10^{-5} $molL^{-1}$, with increasing concentration of CT DNA (stock solution, c = 2.21·10^{-3} $molL^{-1}$) in Tris-HCl buffer pH 7.42, c = 0.1 $molL^{-1}$ in the presence of NaCl, c = 0.15 $molL^{-1}$

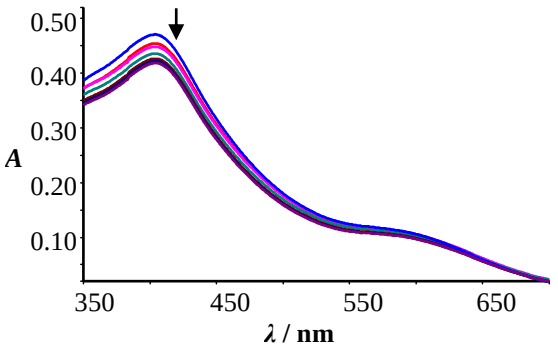
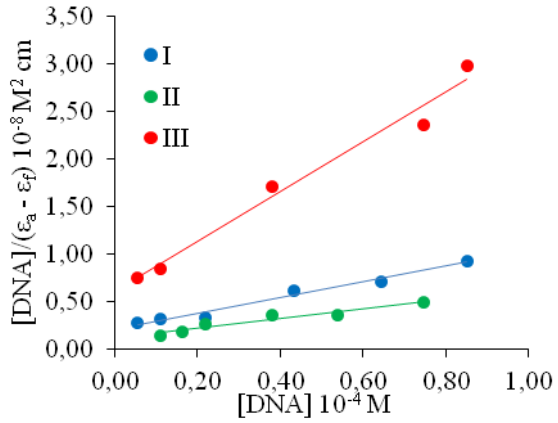


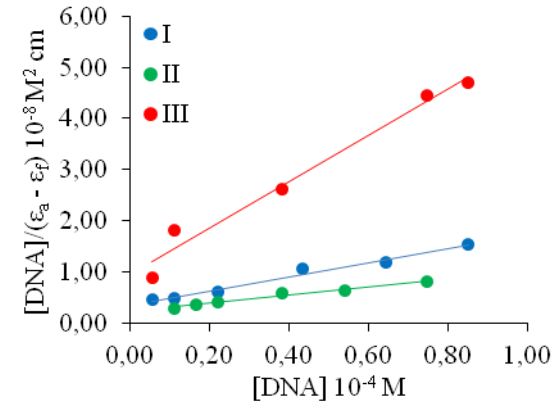
Figure 3. Spectrophotometric titration of Na[$RuCl_2$(N-C_4H_9-5-H-salim)$_2$], c = 2.19·10^{-5} $molL^{-1}$, with increasing concentration of CT DNA (stock solution, c = 2.21·10^{-3} $molL^{-1}$) in Tris-HCl buffer pH 7.42, c = 0.1 $molL^{-1}$ in the presence of NaCl, c = 0.15 $molL^{-1}$

The spectroscopic results of titrations in the regions known as LMCT (ligand → metal charge transfer) or in the region of aromatic π → π* electronic transitions showed hypochromic effect and bathochromic shifts (0-2 nm), suggesting non-covalent interaction of Ru(III) salicylideneimine complexes with DNA. Since the red shift might be an indication of the mode of binding to DNA, the lack or negligible red shift in presented measurements suggests very weak intercalative mode or rather major groove binding. Table 1. Gives all data, significant for the procedure of K_b determination, for selected $Na[Ru(N\text{-}C_6H_4O\text{-}5\text{-}H\text{-}salim)_2]$.

Table 1: Determination of binding constant of $Na[Ru(N\text{-}C_6H_4O\text{-}5\text{-}H\text{-}salim)_2]$ with CT DNA at two wavelengths with repeated measurements

λ / nm	#	V_{DNA} μL	[complex] 10^{-5} M	[DNA] / [complex]	[DNA]/($\varepsilon_a - \varepsilon_f$) 10^{-8} M^2 cm	Graphical determination of binding constant
380	I	0	2.21	0.00	-	
		5	2.20	0.25	2.74	
		10	2.20	0.50	3.10	
		20	2.19	0.99	3.35	
		40	2.17	1.96	6.15	
		60	2.15	2.91	7.13	
		80	2.13	3.85	9.31	
	II	0	2.21	0.00	-	
		10	2.20	0.50	1.32	
		15	2.20	0.74	1.84	
		20	2.19	0.99	2.58	
		35	2.17	1.72	3.53	
		50	2.15	2.44	3.58	
		70	2.13	3.38	4.91	
	III	0	2.21	0.00	-	
		5	2.20	0.25	7.55	
		10	2.20	0.50	8.48	
		35	2.17	1.72	17.2	
		70	2.14	3.38	23.6	
		80	2.13	3.85	29.9	

#	Slope 10^{-4} M cm	Intercept 10^{-8} M^2 cm	r^2	K_b / 10^4 M^{-1}
I	8.35	2.08	0.980	4.01
II	5.16	1.10	0.935	4.68
III	2.64	6.04	0.979	4.37
$K_b \pm \sigma$ / 10^4 M^{-1}				**4.35 ± 0.34**

λ / nm	#	V_{DNA} μL	[complex] 10^{-5} M	[DNA] / [complex]	[DNA]/($\varepsilon_a - \varepsilon_f$) 10^{-8} M^2 cm	Graphical determination of binding constant
581	I	0	2.21	0.00	-	
		5	2.20	0.25	4.62	
		10	2.20	0.50	4.80	
		20	2.19	0.99	6.08	
		40	2.17	1.96	10.7	
		60	2.15	2.91	12.0	
		80	2.13	3.85	15.5	
	II	0	2.21	0.00	-	
		10	2.20	0.50	2.89	
		15	2.20	0.74	3.66	
		20	2.19	0.99	4.11	
		35	2.17	1.72	5.82	
		50	2.15	2.44	6.38	
		70	2.13	3.38	8.27	
	III	0	2.21	0.00	-	
		5	2.20	0.25	8.94	
		10	2.20	0.50	18.2	
		35	2.17	1.72	26.1	
		70	2.14	3.38	44.5	
		80	2.13	3.85	47.1	

#	Slope 10^{-4} M cm	Intercept 10^{-8} M^2 cm	r^2	K_b / 10^4 M^{-1}
I	13.9	3.57	0.980	3.91
II	8.06	2.29	0.982	3.53
III	4.52	9.59	0.977	4.71
$K_b \pm \sigma$ / 10^4 M^{-1}				**4.05 ± 0.61**

The titration experiments were carried out under the same experimental conditions and obtained data were processed on the same way for all compounds. The binding constants are summarized in Table 2.

Table 2. Binding constants of complexes Na[Ru(N-R-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), Na[$RuCl_2$(N-R-5-X-salim)$_2$] (R = C_4H_9, X = H, Cl, Br, NO_2) and [Ru(N-R-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br), with CT DNA

R	X	$K_b \pm \sigma$ / M^{-1} [λ_1 / nm]	$K_b \pm \sigma$ / M^{-1} [λ_2 / nm]
C_6H_4O	H	$(4.35 \pm 0.34)\cdot10^4$ [380]	$(4.05 \pm 0.61)\cdot10^4$ [581]
	Cl	$(2.13 \pm 0.15)\cdot10^4$ [387]	$(1.70 \pm 0.24)\cdot10^4$ [560]
	Br	$(2.79 \pm 0.18)\cdot10^4$ [388]	$(2.14 \pm 0.68)\cdot10^4$ [558]
	NO_2	$(3.59 \pm 0.30)\cdot10^4$ [380]	$(2.78 \pm 0.69)\cdot10^4$ [591]
C_4H_9	H	-	$(2.20 \pm 0.28)\cdot10^4$ [403]
	Cl	$(8.52 \pm 3.30)\cdot10^3$ [349]	$(6.66 \pm 1.38)\cdot10^3$ [403]
	Br	$(4.35 \pm 1.18)\cdot10^2$ [354]	$(7.11 \pm 1.64)\cdot10^2$ [408]
	NO_2	$(8.69 \pm 0.90)\cdot10^3$ [384]	-
$C_{10}H_7$	H	$(7.40 \pm 0.43)\cdot10^4$ [237]	-
	Cl	$(4.65 \pm 0.22)\cdot10^4$ [238]	-
	Br	$(2.54 \pm 0.27)\cdot10^4$ [238]	-

The binding constants of the complex Ru(III) derived from 5-substituted salicylaldehyde and aminophenol, Na [Ru (N-R-5-X-salim)$_2$], where R = C_6H_4O, and X = H, Cl, Br, NO_2 show decreases in the series H> NO_2> Br ≈ Cl($4.35 \pm 0.34\cdot10^4$ M^{-1}) > ($3.59 \pm 0.30\cdot10^4$ M^{-1})> ($2.79 \pm 0.18\cdot10^4$ M^{-1}) ≈ ($2.13 \pm 0.15\cdot10^4$ M^{-1})

The complex where X = H has the highest binding constant. The bromo- and chloro-derivatives have close K_b values due to similar size and similar electronic effects on the electron density of the π-system of the ligand. Although K_b values differ, the influence of 5-X-substituents on salicylideneimine ligands could not be considered as substantial one under described experimental conditions.

The binding constants of Ru(III) complexes derived from 5-X-substituted salicylaldehyde and naphthylamine, [Ru(N-R-5-X-salim)$_3$] with R = $C_{10}H_7$, and X is H, Cl, Br, were determined at the wavelengths around 240 nm where intense aromatic π→ π* electronic transitions occurred since there is no significant absorptions in visible region of the spectra. The noticeable decrease of K_b values in order H ($7.40 \pm 0.43\cdot10^4$ M^{-1}) >Cl ($4.65 \pm 0.22 \cdot10^4$ M^{-1})> Br ($2.54 \pm 0.27\cdot10^4$ M^{-1}) indicates definite influence of 5-X-substituents and correlates with ability of atoms to decrease electronic density or aromatic ring.

The complex compounds of Ru(III) derived from 5-substituted salicylaldehyde and butylamine, Na[$RuCl_2$(N-R-5-X-salim)$_2$], where R = C_4H_9, and X = H, Cl, Br, NO_2 show the significant difference in K_b values changing according to the following order H ($2.20 \pm 0.28\cdot10^4$ M^{-1}) > NO_2 ($8.69 \pm 0.90)\cdot10^3$ M^{-1}≈ Cl ($8.52 \pm 3.30\cdot10^3$ M^{-1}) > Br ($4.35 \pm 1.18\cdot10^2$ M^{-1}).

CONCLUSION

The experimental results showed that interactions of Ru(III) with Schiff bases derived from 5-substituted salicylaldehydes and aminophenol or naphthylamine with CT DNA are moderate, with K_b values of order 10^4 M^{-1}, suggesting the weak intercalative mode or stronger groove binding. The binding constants correlate with the lack or minor red shift. In the case of butyl derivative, the significant deference in K_b values, from 10^{-4} to 10^{-2} M^{-1}, showed essential effect of 5-X-substituents affecting the capability and mode of non-covalent binding of Ru(III) complexes to DNA. The ability of studied Ru(III) complexes with salicylideneimine to bind DNA stimulate further investigations of this class of compounds.

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Summary / Sažetak

Interakcije Ru(III) kompleksa sa Schiff-ovim bazama izvedenim iz salicilaldehida i aminofenola, butilamina i naftilamina, opštih formula Na[Ru(NR-5-X-salim)$_2$] (R = C_6H_4O, X = H, Cl, Br, NO_2), Na[$RuCl_2$(NR-5-X-salim)$_2$] (R = C_4H_9, X = H, Cl, Br, NO_2) i [Ru(NR-5-X-salim)$_3$] (R = $C_{10}H_7$, X = H, Cl, Br) sa CT DNK, ispitivane su spektroskopskim titracijama. Eksperimentalni podaci pokazuju da se kompleksi Ru(III) sa salicilideniminima vezuju na CT DNK, sa konstantama vezivanja reda veličine 10^4 M^{-1}. Rezultati ukazuju na utjecaj 5-X-supstituenta na vrijednosti konstanti vezivanja.

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Acetylcholinesterase and butyrylcholinesterase inhibitory activity of extracts from medicinal plants

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Abstract: Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), enzymes which breakdown acetylcholine and butyrylcholine, are considered as a promising strategy for the treatment of Alzheimer's disease (AD). A potential source of AChE and BuChE inhibitors is provided by the abundance of plants in nature. In the present study, we selected five plants used in traditional medicine to treat different disorders of the central nervous system. Aqueous and methanolic extracts of sage (*Salvia officinalis* L.), arnica (*Arnica montana* L.), rue (*Ruta graveolens* L.), St. John's wort (*Hypericum perforatum* L.) and aronia (*Aronia melanocarpa* (Michx.) Elliot.) were tested for the AChE and BuChE inhibitory activity using Ellman's colorimetric method. Galanthamine hydrobromide was used as positive control. The results show that extracts from the aeiral parts of St John's wort and rue and flowers of arnica could inhibit the activity of AChE or BuChE or both. The best inhibition effect was observed using the water extract of rue, then methanolic extracts of arnica and St John's wort at concentration

INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative pathology that primarily affects the elderly population, and is estimated to account for 50-60% of dementia cases in persons over 65 years of age. The main symptoms associated with the later stages of AD involve cognitive dysfunction, primarily memory loss (Filho, Medeiros, Diniz *et al*., 2006). In mammalian brain, there are two major forms of cholinesterases, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Giacobini, 2003). The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain. Therefore, inhibition of AChE, the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD. While AChE is found in all excitable tissue, whether nerve or muscle, in most erythrocytes and in placental tissue, BuChE is present more commonly in the body including within the central and peripheral nervous system, liver and plasma. (Orhan, Kartal, Naz *et al*., 2007) The serious side effects caused by licensed drugs used to treat AD have forced researchers to investigate safer AChE or BuChE inhibitors from natural sources. Numerous plants and their constituents are reputed in traditional practices of medicine to enhance cognitive function and to alleviate other symptoms of AD, including depression (Politeo, Botica, Bilušić *et al*., 2011).

The aim of this study was to investigate a presence of possible AChE or BuChE inhibitors in few plants which traditionally used in European medicine. Selection of the

species screened in this study was based on their use as remedies for the enhance memory, central nervous system diseases, or as a source of well-known antioxidants. Aqueous and methanolic extracts of sage (*Salvia officinalis* L.), arnica (*Arnica montana* L.), rue (*Ruta graveolens* L.), St. John's wort (*Hypericum perforatum* L.) and aronia (*Aronia melanocarpa* (Michx.) Elliot.) were tested for the AChE and BuChE inhibitory activity.

EXPERIMENTAL

Plant material and chemicals

Plants were purchased from The Herbal Pharmacy-Vextra d.o.o. Mostar, Bosnia and Herzegovina. Each plant material was dried in shade at room temperature and then ground to a fine powder in a mechanic grinder. Plants and their parts used in this study are presented in Table 1.

AChE (EC 3.1.1.7) from electric eel (type VI-S), BuChE (EC 3.1.1.8) from horse-serum, acetylthiocholine iodide, butyrylthiocholine iodide, galanthamine hydrobromide, sodium dihydrogen phosphate monohydrate (NaH_2PO_4 x H_2O), disodium hydrogen phosphate (Na_2HPO_4) and methanol were purchased from Sigma-Aldrich (Germany). DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]) was purchased from Zwijndrecht (Belgium). All reagents used in the study were of analytical grade.

Extract preparation

For extraction process 6 g of dried and grinded plant material was used. Water based extraction was done by using 150 ml of re-distilled water, while the temperature of the mixture was held constant at 70°C for 2 hours. Methanol based extraction was conducted by using 120 ml of methanol with the constant temperature of 60°C of the mixture for 2 hours. Both extracts were filtered and evaporated. To keep the extracts stable the evaporation temperature was not bigger than 60°C. After evaporation extracts were kept in the fridge at 4°C. Before using the extracts for the measurements they were diluted using phosphate buffer (pH=8.0).

Microplate assay

AChE and BuChE inhibitory activity were measured using a 96-well microplate reader (IRE 96, SFRI Medical Diagnostics) based on Ellman's method (Ellman, Courtney, Andres *et al.*, 1961). The enzyme hydrolyses the substrate acetylthiocholine or butyrylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoic-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm. (Elman *et al.*, 1961, Rhee, Meent, Ingkaninan *et al.*, 2001)

In this method, total reaction volume of 220 µl consisted: 170 µl (0.1 mol/l) sodium phosphate buffer (pH 8.0), 20 µl of AChE/BuChE (0.45 U/ml), 10 µl test solution (plant extracts), 10 µl DTNB (0.03 mmol/l) and 10 µl of acetylthiocholine iodide/butyrylthiocholine iodide (final concentration of 0.68 mmol/l). The plant extracts were tested for AChE and BuChE inhibitory activity at concentrations from 100 µg/ml to 400 µg/ml. Different concentrations of dried plant extracts were prepared in phosphate buffer. Galanthamine hydrobromide was used as a AChE and BuChE positive control in a concentration range between 10 and 100 µg/ml. Appropriate amounts of buffer, extract and enzyme were incubated 15 min at 4˚C, the reaction was initiated by addition DTNB and substrate. Thereafter, the reaction mixture was incubated 30 min at 25˚C and absorbance read at 405 nm in a 96 well microtiter plate. A blank for each run consisted of 200 µl buffer, 10 µl substrate and 10 µl DTNB. Each sample was assayed in triplicate and it also included a control (C) in which buffer replaced the test solution.

Percentage of inhibition of AChE/BuChE was determined using the formula:

$$I = (C - T) / C \times 100 \qquad (1)$$

where C is the activity of enzyme without test sample and T is the activity of enzyme with test sample.

RESULTS AND DISCUSSION

In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders. The history of drug discovery has shown that plants contain active compounds that have become new sources to investigate for the pharmaceutical industry (Adewusi, Moodley and Steenkamp, 2010). In the present study, we selected five plants used in traditional medicine to treat different disorders of the central nervous system. The results on the effects of the tested herbal extracts on AChE and BuChE activity are summarized in Table 1., together with their family, plant part, solvent extract, percentage inhibition and concentration at which the enzyme is inhibited.

Data are expressed as mean with standard error. It was found out that galanthamine hydrobromide and plant extract had dose-dependent inhibitory activity. Galanthamine hydrobromide was used as a positive control, Figure 1. Galanthamine is an Amaryllidaceae alkaloid obtained from *Galanthus nivalis* L., and it is reported to be more selective for AChE than BuChE, and provides complete oral bioavailability. It is licensed in Europe for AD treatment, was well tolerated and significantly improved cognitive function when administered to AD patients (Mukherjee, Kumar, Mal *et. a*l., 2007).

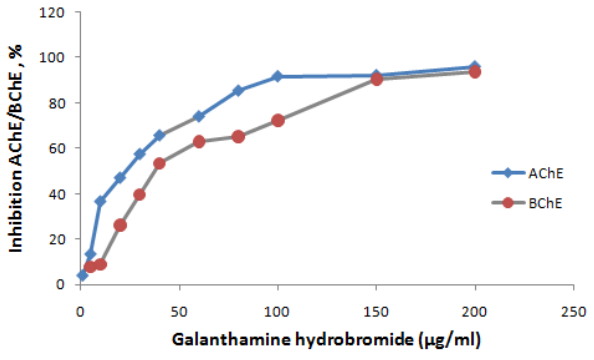

Figure1. AChE and BuChE inhibition efficiency of galanathamine hidrobromide.

Table 1. Anti-AChE and anti-BuChE acitivity of plant extracts.

Plant species	Family	Plant part analyzed	Solvent	BuChE inhibition (%)				AChE inhibition (%)			
				100 μg/ml	200 μg/ml	300 μg/ml	400 μg/ml	100 μg/ml	200 μg/ml	300 μg/ml	400 μg/ml
Arnica (*Arnica montana* L.)	Compositae	Flower	Methanol	25.4 ± 0.4	28.1 ± 0.1	32.3 ± 0.1	57.7 ± 0.1	50.7 ± 2.0	53.2 ± 2.7	60.2 ± 9.2	67.4 ± 9.3
			Water	6.4 ± 0.4	12.3 ± 0.3	18.9 ± 0.0	21,1 ± 0.5	40.7 ± 5.6	50.7 ± 3.1	53.9 ± 4.5	55.4 ± 4.1
Sage (*Salvia officinalis* L.)	Lamiaceae	Leaf	Methanol	1.4 ± 0.1	9.0 ± 0.5	34.8 ± 0.0	43,3 ± 5.4	3.8 ± 0.1	6.9 ± 0.1	9.9 ± 0.7	14.1 ± 0.4
			Water	8.0 ± 0.5	23.5 ± 0.4	27.2 ± 0.8	36.4 ± 0.6	4.4 ± 0.2	14.7 ± 0.2	17.2 ± 0.5	20.6 ± 0.6
Rue (*Ruta graveolens* L.)	Rutaceae	Herb	Methanol	9.4 ± 1.0	12.1 ± 2.3	17.0 ± 2.5	29.0 ± 2.8	30.8 ± 2.0	40.8 ± 6.4	53.8 ± 0.6	73.8 ± 5.7
			Water	19.7 ± 1.3	28.1 ± 1.1	30.9 ± 1.6	32.2 ± 1.5	55.7 ± 3.4	60.6 ± 8.2	65.7 ± 1.4	80.0 ± 0.8
St. John's wort (*Hypericum perforatum* L.)	Hypericaceae	Flower	Methanol	23.0± 0.1	37.1 ± 0.2	47.8 ± 0.7	50.5 ± 0.7	50.1 ± 0.9	58.7 ± 3.6	60.2 ± 4.2	73.5 ± 2.4
			Water	4,0± 0.1	15.4 ± 0.5	18.3 ± 0.6	19.2 ± 0.6	30.6 ± 6.9	38.5 ± 6.0	43.4 ± 8.5	52.7 ± 2.9
Aronia (*Aronia melanocarpa* (Michx.) Elliot.)	Rosacea	Fruit	Methanol	5.5 ± 0.4	13.0 ± 0.3	15.9 ± 2.2	18.9 ± 3.5	5.8 ± 2.7	8.9 ± 4.0	10.7 ± 7.5	18.3 ± 6.9
			Water	15.9 ± 1.9	18.1 ± 2.4	21.0 ± 3.4	22.6 ± 3.4	20.7 ± 4.7	55.9 ± 7.7	65.2 ± 6.1	70.2 ± 5.5

From ten investigated extracts seven of them have achieved 50% of inhibition activity for AChE and two for BuChE (Table 2). The strongest inhibition effect was detected with water extract of rue (IC_{50}=50 μg/ml) and methanolic extract of arnica (IC_{50}=75 μg/ml), following by methanolic extract of St. John's wort (IC_{50}=100 μg/ml) for AChE. Methanolic extracts of arnica (IC_{50}=389 μg/ml) and St. Jonh's wort (IC_{50}=353 μg/ml) have shown a significant inhibition effect towards BuChE.

Similar investigations were reported by Wszelaki, Kuciun and Kiss (2010). Significant inhibition effect in the fore mentioned research done by Wszelaki *et al*. was reported of AChE for hexane (IC_{50}=29 μg/ml) and methanolic (IC_{50}=43 μg/ml) extracts of the flowers of arnica (*Arnica chamissonis* Less. subs. *foliosa)*, and hexane extract (IC_{50}=34 μg/ml) of rue (*Ruta graveolens* L.) (IC_{50}=61 μg/ml) (Wszelaki *et. al*., 2010).

Table 2. The IC_{50} value of plant extracts.

Plant species	Solvent	IC_{50} (AChE) (μg/ml)	IC_{50} (BuChE) (μg/ml)
Arnica	Methanol	75	389
	Water	150	-
Sage	Methanol	-	-
	Water	-	-
Rue	Methanol	250	-
	Water	50	-
St. John's wort	Methanol	100	353
	Water	350	-
Aronia	Methanol	-	-
	Water	160	-
Galanthamine		22	24

- Not evaluated.

IC_{50} values were obtained from the dose-effect curves by linear regression.

Adsersen, Gauguin, Gudiksen, *et. al*., (2006) found AChE inhibitory activity water and methanolic extracts of rue, 0.1mg/ml caused 22 and 39%. Zheleva-Dimitrova and Balabanova (2012) reported the antioxidant and AChE inhibitory potential of methanol extract from *Arnica montana* cultivated in Bulgaria. The results demonstrated that arnica extract has strong antioxidant and AChE inhibitory activities (IC_{50}= 311 μg/ml). Many *Hypericum* species showed significant inhibition of AChE, with IC_{50} values between 0.62 and 1.79 mg dry extract/ml. The analysis indicated that chlorogenic acid, rutin, hyperoside, isoquercitrin, and quercitrin were the main compounds present in the water extracts. These compounds have strong anti- acetylcholinesterase activities, with IC_{50} values between 62 μg/ml and 196 μg/ml (Hernandez, Falé, Araújo *et al*., 2010).

In our research the lowest inhibition effect for the investigated enzymes was reported for sage exracts. Reserach done by Orhan *et al*. (2007) on *Salvia* plant family has indicated that only chloroform and petrol ether extracts have shown significant inhibitory activity on AChE. Essential oils of *Salvia lavandulaefolia,* and also some other isolated components, showed inhibition efficiency for AChE (Perry, Bollen, Perry *et. al*., 2003), while methanolic and water extracts did not show inhibition efficiency. It was also reported (Savelev, Okello and Perry, 2004) that the essential oils obtained from species of *Salvia* inhibit AChE and BuChE in a time dependent manner. The oils of *S. fruticosa* and *S. officinalis* var. *purpurea* had apparent dual cholinergic activity, namely they were active on both enzymes within the incubation time, while the duality of the oil of *S. officinalis* was less apparent.

CONCLUSION

Five medicinal plants were screened for inhibitory activity on AChE and BuChE. The results show that extracts from the aerial parts of St John's wort and rue and flowers of arnica could inhibit the activity of AChE or BuChE or both. The best inhibition effect was observed using the water extract of rue, methanolic extracts of St John's wort and arnica at concentration of 400 μg ml^{-1}. The results show that these plants could be very interesting for further isolation of acetylcholinesterase inhibitors, which are widely used in the treatment of Alzheimer's disease.

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Summary / Sažetak

Inhibicija enzima koji razgrađuju acetilkolin, acetilkolinesteraze (AChE) i butirilkolinesteraze (BuChE), smatra se obećavajućom strategijom za liječenje Alzheimerove bolesti (AD). Alzheimerova bolest je kronični neurološki poremećaj koji se očituje u smanjenju pamćenja, kognitivne disfunkcije i poremećajem ponašanja. Obilje biljaka u prirodi pruža potencijalni izvor inhibitora za AChE i BuChE. U ovom istraživanju odabrali smo pet biljaka koje se koriste u tradicionalnoj medicini za liječenje različitih poremećaja središnjeg živčanog sustava. Pomoću Ellmanove kolorimetrijske metode testiran je inhibicijski učinak vodenih i metanolnih ekstrakata kadulje (*Salvia officinalis* L.), arnike (*Arnica montana* L.), rute (*Ruta graveolens* L.), gospine trave (*Hypericum perforatum* L.) i aronije (*Aronia melanocarpa* (Michx.) Elliot.) na AChE i BuChE. Kao pozitivna kontrola korišten je galntamin hidrobromid. Rezultati ukazuju da ekstrakti nadzemnih dijelova gospine trave i rute i cvjetova arnike mogu inhibirati aktivnost AChE ili BuChE, ili oboje. Najjači inhibicijski učinak uočen je kod vodenog ekstrakta rute, potom metanolnih ekstrakata arnike i gospine trave pri koncentraciji od 400 μg ml^{-1}

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Original Scientific Paper

Synthesis, Caracterization and Interaction with CT DNA of Novel Cationic Complex Ru(III) with Indazole and Schiff Base Derived from 5-Chlorosalicylaldehyde

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Abstract: Novel cationic complex compound of Ru(III) with indazole and Schiff base derived from 5-chlorosalicylaldehyde and aniline has been synthesized. Formulation and characterization of the complex was performed using CHN analysis, MALDI-TOF/TOF mass spectrometry, FT-IR spectroscopy and UV/Visible spectrophotometry. In the octahedral environment of Ru(III), coordination of bidentate Schiff bases occurs through azomethine nitrogen and deprotonated phenolic oxygen while in indazole via nitrogen atom. The interaction of the complex with CT DNA (calf thymus DNA) was carried out under physiological conditions using spectrophotometric titration.

INTRODUCTION

In the last decades, ruthenium complexes have being in the focus of the interest, primarily because of their anticancer (Keppler *et al*, 1989), antibacterial (Kahrovic, Bektas, *et al*, 2014), catalytic (Kahrovic, 2011) or electron transfer mediated activity (Turkusic *et al*, 2012). The properties of the complex compounds depend on coordination environment. Some Ru(III) complexes with Schiff bases derived from salicylaldehyde and substituted salicylaldehyde (Kahrovic *et al*, 2010; Kahrovic, 2014) are described as moderate intercalators (Ljubijankić *et al*, 2013; Kahrović, *et al*, 2014) and electrochemical mediators for the low potential amperometric determination of ascorbic acid (Kahrovic and Turkusic, 2012) or L-cysteine (Turkusic and Kahrovic, 2012). A relatively small number of complexes containing Schiff base derived from 5-chlorosalicylaldehyde are described in literature (Blagus *et al*, 2010).

N-heterocyclic compounds, as a component of some vitamins and drugs, play a key role in many biological systems (Bayari *et al*, 2003). Two ruthenium complexes with *N*-heterocyclic ligands, imidazolium trans-imidazoledimethyl sulfoxide-tetrachlororuthenat (NAMI-A) and trans-[tetrachlorobis(1H-indazole)ruthenate(III)] (KP1019) have been reported as anticancer agents, against metastatic and colon cancers, respectively (Lakhai *et al*, 2004). Because of structural similarity to the nucleic bases, adenine and guanine, indazole is very interesting ligand.

In this study, we aimed to synthesise novel Ru(III) cationic complex with indazole and Schiff base derived from 5-chlorosalicylaldehyde and investigate interaction with CT DNA (calf thymus DNA).

EXPERIMENTAL

Materials and Physical Measurements

All chemicals were commercially available with analytical grade of purity and were used without further purification. CT DNA type 1-fibrous was purified using phenol-chloroform-isoamyl alcohol extraction method until ratio A_{260}/A_{280} = 1.8 was obtained.

Elemental analysis was obtained on a Perkin Elmer 2400 Series CHNSO/O Analyzer.

Mass spectrum was recorded by matrix-assisted laser desorption/ionization–time-of-flight MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI TOF/TOF analyzer, Applied Biosystems Inc., Foster City, CA, USA) in the positive ion reflector mode. A small amount of sample was mixed with 10 μL of MALDI matrix (DHAP (2,6-dihydroxyacetophenone); 5 mg/mL) and 1 μL was spotted on MALDI plate. The number of 1600 shots per spectrum were acquired in m/z range of 100-1000 Da (focus mass 500 Da, delay time 300 ns). As an internal standard in the positive mode,thiamine mononitrate, azithromycin 591 and 794 and B 12 were used.

The infrared spectra were recorded as KBr pellets on a Perkin Elmer spectrum BX FTIR System in the region 4000-400 cm^{-1}.

Electronic spectra, hydrolysis experiment and CT DNA binding were measured on a Perkin Elmer lambda 35 spectrophotometer. Electronic spectra were recorded in CH_2Cl_2 solution over the range 190-700 nm. Hydrolysis of Ru(III) complex was performed by adding the concentrated solution of complex in DMSO to Tris-HCl buffer solution (pH 7.4). Spectra were recorded every 10 minutes within two hours. The stock solution of CT DNA was prepared in Tris-HCl buffer pH 7.4 and stored at 4 °C up to 4 days. The concentration of DNA was determined spectrophotometrically at 260 nm using a molar extinction coefficient of 6600 M^{-1} cm^{-1} (Meadows *et al.*, 1993). Concentrated stock solution of complex was prepared by initial dissolution of complex in a small amount of DMSO and diluting with Tris-HCl buffer to the final concentration. Spectrophotometric titration of Ru(III) solution of fixed concentration with CT DNA was performed by the successive addition of DNA (in portion of 10 μL) in 2 mL of the compound solutions at pH 7.4. Each addition of DMSO, DNA or complex was compensated in the blank. The determination of the binding constant, K_b, has performed on the basis of spectrophotometric titration of complex with CT DNA by recording spectra in the range of 350 – 600 nm. The binding constant is calculated on the basis of changes in absorptions at 583 nm.

Cyclovoltammograms were recorded on an electrochemical workstation Autolab potentiostat/galvanostat (PGSTAT 12) using three electrode system: glassy carbon as working electrode, Ag/AgCl as reference and Pt-wire as counter electrode. Cyclovoltammograms were recorded in acetonitrile (MeCN) with sodium perchlorate($NaClO_4$) as supporting electrolyte in the potential range of -1.0 to 0.0 V, with scan rate 0.2 V/s and in *N*, *N*-dimethylformamide (DMF) with tetraethylammonium bromide (Et_4NBr) as suppoting electrolyte in the potential range of -1.1 to -0.5 V with scan rate 0.2 V/s .

Synthesis of the complex, [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl, Diindazole–bis-[*N*-phenyl-5-chlorosalicylideneiminato-*O,N*]ruthenium(III) chloride

Schiff base, *N*-phenyl-5-chlorosalicylideneimine, hereinafter *N*-Ph-5-Cl-salimH and starting compound, sodium dichloro-bis-[*N*-phenyl-salicylideneiminato-*N,O*]ruthenate(III), hereinafter Na[$RuCl_2$(*N*-Ph-5-Cl-salim)$_2$] were synthesized according to the published procedures (Kahrović *et al*, 2010; Ljubijankić *et al*, 2013). Starting compound was used for the synthesis of novel cationic complex without any purification. The purity of starting compound was checked by using IR spectroscopy.

Cationic complex, [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl was prepared in reaction of starting compound with indazole in molar ratio 1 : 2 in absolute ethanol. The amount of 23.6 mg (0.2 mmol) indazole in absolute ethanol (2 mL) was added to the solution of 6.55 mg (0.1 mmol) of pulverized Na[$RuCl_2$(*N*-5-Cl-salim)$_2$] in 60 mL hot absolute ethanol. Reaction mixture was refluxed at temperature 75 °C for five hours whereby the solution has changed color from dark green to dark blue. The volume of the solution was reduced under vacuum distillation to about one-quarter of initial volume. The resulting solution was kept in an ice-salt bath for five days. The green solid was filtered off and washed with water until the negative reaction for chloride ions.

Yield: 73 %.

Diindazole–bis-[*N*-phenyl-5-chlorosalicylideneiminato-*O,N*]ruthenium(III) chloride: Anal. calcd for $C_{40}H_{30}Cl_3N_6O_2Ru$: C 57.60, H 3.63, N 10.78. Found: C 55.53, H 4.51, N 9.80; MALDI TOF/TOF MS (*m*/*z*) calcd for $[C_{40}H_{30}Cl_2N_6O_2Ru]^+$, 798.0850; found, 798.0870; IR (KBr, cm^{-1}) 3444 [ν(N–H)], 1695 [ν(C=N)], 1356 m [ν(C–N)], 1076 [ν(N–N)], 669 w [ν(Ru–N), 421 w [ν(Ru–O)] UV-Vis (CH_2Cl_2) I_{max} (log e) 230 (4.63), 282 nm (4.32).

RESULTS AND DISCUSSION

Synthesis and Spectroscopic Studies

The starting complex, Na[$RuCl_2$(*N*-Ph-5-Cl-salim)$_2$] was synthesized from $RuCl_3$ and freshly prepared ligand, 5-chlorosalicilydeneimine, in absolute ethanol solution in molar ratio 1:2. The freshly prepared Na[$RuCl_2$(*N*-Ph-5-Cl-salim)$_2$] compound was used for the synthesis of novel cationic complex.

[Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl was prepared from absolute ethanol solutions containing starting compound and indazole in molar ratio 1 : 2. The synthesis of the cationic complex was carried out in relative mild conditions by replacement of two easily outgoing chloride ions in starting compound with indazole. The scheme of preparation is showed in Figure 1. The green solid is stable in air, insoluble in water, soluble in acetonitrile (MeCN), dimethyl sulphoxyde (DMSO), dimethylformamide (DMF).

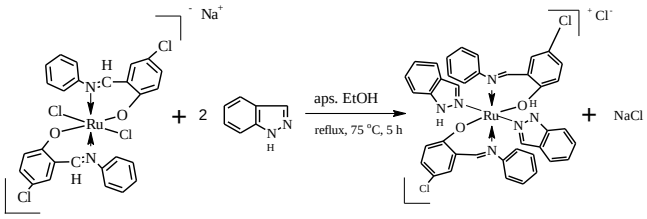

Figure 1: Synthesis of [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl

On the basis on CHN elemental analysis, mass spectra, infrared and UV/Visible spectroscopic measurements the compound formula was formulated as [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl. Mass spectra showed molecular ion (M^+) at *m/z* (100%) = 798.0870 which corresponds to $[C_{40}H_{30}Cl_2N_6O_2Ru]^+$.

The characteristic IR frequencies of starting Ru(III) compound, free indazole and [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl are given in Table 1. Coordination of indazole to Ru(III) through electronic pair on the atom nitrogen undoubtedly affects the C=N and C–N stretching vibrations which were shifted for 6 and 10 cm^{-1}, respectively, towards higher wavenumbers.

Stretching vibration of N–H bond in free indazole appears at 3410 cm^{-1}. In [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl this vibration is coupled with vibration in the starting Ru(III) compound and appears at 3444 cm^{-1}. The weak bands at 669 and 421 cm^{-1} could be attributed to Ru – N and Ru – O, respectively. Characteristic IR vibrations of starting Na[RuCl$_2$(*N*-Ph-5-Cl-salim)$_2$] are azomethine C=N, C-O phenolic , Ru-N and Ru-O which are not affected after coordination of indazole.

Infrared spectra of [Ru(*N*-Ph-5-Cl-salim)$_2$(Ind)$_2$]Cl showed that in octahedral environment of Ru(III), coordination of bidentate Schiff base occurs through azomethine nitrogen and deprotonated phenolic oxygen while in indazole via nitrogen atom (Table1).

Table 1: Characteristic vibrations (cm^{-1}) in FT-IR spectra of starting compound, cationic complex and free indazole

	Starting compound	Cationic complex	Free Indazole
ν(N–H) $_{Ind}$	-	3444	3410
ν(C=C) $_{Ind}$	-	1622	1621
ν(C=N)$_{SB}$	1607	1607	-
ν(C=N)$_{Ind}$	-	1695	1689
ν(C–N)$_{Ind}$	-	1356	1356
ν(C=O$_{Ph}$)$_{SE}$	1298	1298	-
ν(N–N) $_{Ind}$	-	1086	1076
ν(Ru–N)	668	669	-
ν(Ru–O)	419	421	-

$_{SB}$ – assigned vibrations in Schiff base; $_{Ind}$ – assigned vibrations in Indazole

Electronic spectra of Na[RuCl$_2$(*N*-Ph-5-Cl-salim)$_2$], [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl and free indazole were recorded in CH_2Cl_2. Electronic spectra of indazole has two absorption bands which could be attributed to $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions.

Weak broad absorption in the electronic spectra of starting copound centered at 609 nm in the region of d-d spin alowed transition of low spin t_{2g}^5 Ru(III) can be assigned to ($^2T_{2g}\rightarrow{}^2A_{2g}$). In the spectra of [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl this transition moves towards lower value of wavelength (574 nm). Replacement of two chloride in the starting compound with *N*-donor ligands, that split stronger crystal field than chlorides results in higher separation energies of d-atomic orbitals and moves d-d transition to higher energies. The shift of $n\rightarrow\pi^*$ transition in free indazole from 285 nm to 282 nm in [Ru(*N*-5-Cl-salim)$_2$(Ind)$_2$]Cl is an evidence of coordination of indazole through nitrogen atom.

Table 2: Characteristic absorptions in electronic spectra of Na[RuCl$_2$(*N*-Ph-5-Cl-salim)$_2$], [Ru(*N*-Ph-5-Cl-salim)$_2$(Ind)$_2$]Cl and indazole in dichlormethane

Compound	$\pi\rightarrow\pi^*$	$n\rightarrow\pi^*$	IL (SB);	d-d
Na[RuCl$_2$(*N*-Ph-5-Cl-salim)$_2$	244	sh	348	609
Indazole	250	285	-	-
[Ru(*N*-Ph-5-Cl-salim)$_2$(Ind)$_2$]Cl	230	282	sh	574

$\pi\rightarrow\pi^*$ - electronic transition of delocalized electrons of the aromatic system; $n\rightarrow\pi^*$ - electronic transitions of the atoms of azomethine group or free electron pair on the N atom of indazole with aromatic π electrons; IL (SB) – intraligand transition of whole molecule of Schiff base; d-d – transition of low spin complex; sh-shoulder.

Cyclovoltammetry

Redox potential of Ru(III) complexes is an important characteristic which determines its behavior in biological environment. It is considered that the activity of some Ru(III) complexes which are known as antitumor agents is achieved by reduction *in situ*.

Starting compound and cationic complex are insoluble in water and therefore cyclovoltammograms were recorded in non-aqueous solvents, MeCN and DMF in the presence of Et_4NBr and $NaClO_4$ as supporting electrolytes using glassy carbon electrode as working electrode. All data are given *vs* Ag/AgCl reference electrode (Table 3).

Cyclovoltammograms of [Ru(*N*-Ph-5-Cl-salim)$_2$(Ind)$_2$]Cl and Na[RuCl$_2$(*N*-Ph-5-Cl-salim)$_2$] in MeCN and DMF show defined E_{pa} anodic peaks and E_{pc} chatodic peaks. The half-wave potentials, assigned to $Ru_{(III)}/Ru_{(II)}$ couple, are quite negative and for starting compound in MeCN/Et_4NBr system is -0.832 V and for final product is -0.844 V. Formal potential, $E_{1/2}$ in DMF/$NaClO_4$ system for starting compound is -0.626 and for final product is -0.563 V. This values indicated that Ru(III) is stabilized through ON_2 ligands. The separation peaks (E_k-E_a) and ratio i_k/i_a in cyclovoltammograms in both systems indicate quasi-reversible one-electron processes.

Cyclovoltammograms recorded in MeCN/Et_4NBr showed higher peak separations and reversibility according to the DMF/$NaClO_4$ system. This is in agreement with coordination ability of solvents.

Table 3: Characteristic potentials of $Na[RuCl_2(N\text{-}Ph\text{-}5\text{-}Cl\text{-}salim)_2]$ and $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ from cyclovoltammetric measurements in different systems

	$Na[RuCl_2(N\text{-}Ph\text{-}5\text{-}Cl\text{-}salim)_2]$		$[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$	
	MeCN/Et_4NBr	DMF/$NaClO_4$	MeCN/Et_4NBr	DMF/$NaClO_4$
E_{pc} / V	-0.924	-0.858	-0.945	-0.869
E_{pa} / V	-0.740	-0.394	-0.742	-0.257
$E_{1/2}$/ V	-0.832	-0.626	-0.844	-0.563
ΔE / V	0.184	0.464	0.203	0.590

All data are given *vs* Ag/AgCl reference electrode

Interaction of cationic complex with CT DNA

Behavior of complex compounds in solution is of great importance for biological activity. The hydrolysis of $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ was monitored in Tris-HCl buffer (pH 7.4). Hydrolytic profile showed stability in the solution (Figure 2).

Spectrophotometric titration is a method of choice to study the interactions between metal complex and DNA. In this research, the titration was performed by adding increasing amount of CT DNA to the Ru(III) solution of fixed concentration keeping the [DNA] / [complex] ratio in range from 0 to 2.48. The binding constant was calculated on the basis of equation (1) (Pyle *et al*, 1989):

$$\frac{[DNA]}{[\varepsilon_a - \varepsilon_b]} = \frac{[DNA]}{[\varepsilon_b - \varepsilon_f]} + \frac{1}{Kb(\varepsilon_b - \varepsilon_f)} \quad (1)$$

where ε_a, ε_f, ε_b represent exinction coefficients for particular measurements (A_{obs} / [DNA]), free complex and completely bound form, respectively. In plots of [DNA] / ($\varepsilon_a - \varepsilon_f$) *versus* [DNA], the binding constant, K_b is given by the ratio of slope to the intercept. Experimental data for spectrophotometric titration of $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ with CT DNA are given in Table 4 and Figures 3 and 4.

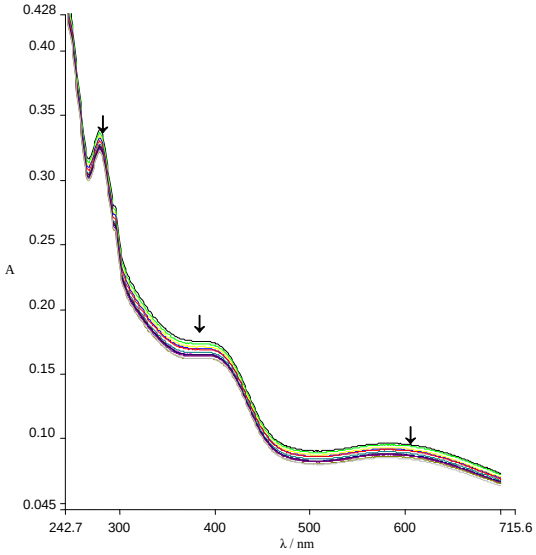

Figure 2: Hydrolysis of $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ in 0.1 M Tris-HCl buffer (pH 7.4); c = 3.36 x 10^{-5} M

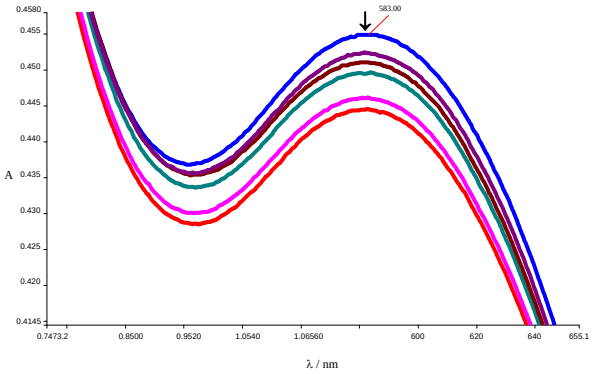

Figure 3: The spectrophotometric titration of 5.17 x 10^{-5} M $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ with increasing concentration of CT DNA (0 – 1.24 x 10^{-4} M) in 0.1 M Tris-HCl buffer (pH 7.4)

Table 4: Spectrophotometric titration of $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ with CT DNA

[complex] / 10^{-6} M	V_{DNA} / μL	[DNA] 10^{-6} M	[DNA]/ [complex]	10^{-7} [DNA]/ (ε_f-ε_a) M^2 cm
51.7	0	0	0	-
51.5	10	18.2	0.35	0.74
51.2	20	36.3	0.71	1.39
50.5	50	89.3	1.77	3.15
50.2	60	107	2.12	2.99
50.0	70	124	2.48	3.16

The binding constant (K_b) was calculated on the basis of decrease of absorptions at 583 nm. Spectral feature gives hypochromic effect, but bathochromic shift, which is characteristic for intercalation, is not evident. The calculated binding constant of 4.9 x 10^4 M^{-1} indicates non-covalent binding of complex to CT DNA which is described as major groove binding.

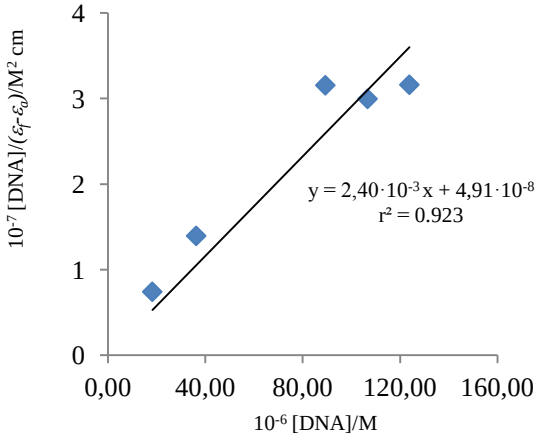

Figure 4: Graphical calculation of K_b(4.9 x 10^4 M^{-1}) on the basis of spectrophotometric titration of $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$ by CT DNA

CONCLUSION

Novel cationic complex of Ru(III) with indazole and Schiff base derived from 5-chlorosalicyladehyde and aniline was synthesized. Based on CHN elemental analysis, mass spectra, infrared and UV/visible spectroscopic measurements, the complex was formulated as $[Ru(N\text{-}5\text{-}Cl\text{-}salim)_2(Ind)_2]Cl$. In the octahedral environment of Ru(III), coordination of bidentate Schiff bases occurs through azomethine nitrogen and deprotonated phenolic oxygen while in indazole via nitrogen atom. DNA binding studie was investigated by spectrophotometric titration. The binding constant (K_b) of cationic complex was determined as 4.9×10^4 M^{-1}. According to this result novel cationic complex act as major groove binder.

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Summary / Sažetak

Sintetiziran je novi katjonski kompleksni spoj sa indazolom i Šifovom bazom izvedenom iz 5-hlorsalicilaldehida. Formulacija i karakterizacija kompleksa napravljena je na bazi CHN analize, MALDI-TOF/TOF masene spektrometrije, FT-IR spektroskopije i UV/Vidljive spektrofotometrije. U oktaedarskom okruženju Ru(III), koordinacija bidentatne Schiffove baze se ostvaruje preko azometinskog azota i deprotoniranog fenolnog kisika, dok se kod heterociklusa ostvaruje preko atoma azota. Interakcija kompleksa sa CT DNA (DNA iz timusa govečeta) provedena je u fiziološkim uslovima korištenjem spektrofotometrijske titracije.

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Kinetics and mechanism of diazepam release from solid dispersions

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Abstract: According to the biopharmaceutics classification system, diazepam belongs to the class II drugs. Inadequate dissolution rate of diazepam can be the limiting factor for its absorption rate. The aim of the present study was preparation of diazepam solid dispersions using various carriers like polyethylene glycol 2000, polyethylene glycol 4000 and polyvinylpyrrolidone K30, estimation of solubility and dissolution rate of prepared diazepam solid dispersions and comparison of these data to that of pure diazepam. The solid dispersions were prepared by solvent evaporation method. Phosphate buffer pH 6.8 was used as dissolution medium. Solid dispersions of diazepam with polymers resulted in increased solubility and dissolution rate of diazepam (highest with polyvinylpyrrolidone K30). The rate release kinetics of diazepam from the solid dispersions followed Hixson-Crowell cube root law. The correlation coefficient (r^2) values of the Hixson-Crowell's cube root model were slightly higher (0.9665 to 0.9977) when compared to the zero and first order release model. The high values of regression coefficients suggested that all formulations followed Korsmeyer-Peppas model of release kinetics. The low values of the release exponent (< 0.45) indicated that the mechanism of diazepam release from all the formulations could be described as a Fickian diffusion mechanism.

INTRODUCTION

Diazepam (Dz), as the most representative benzodiazepine, is widely used as anticonvulsant, anxiolytic, sedative agent, hypnotic, muscle relaxant and is also very useful in suppressing febrile and epileptic convulsions (Riss, Cloyd, Gates, *et al.,* 2008; Mandrioli, Mercolini, Raggi, 2008). According to biopharmaceutics classification system (BCS) of drugs, it belongs to the class II. It is almost insoluble in water, which was confirmed by the fact that the dissolution of its highest dose at pH values in range 1 - 7.4 at 37 °C required volume greater than 250 mL of water or a suitable aqueous medium. The value of the intrinsic dissolution rate of diazepam is less than 0.1 mg min^{-1} cm^{-2}, so drug dissolution would be the rate-limiting step to absorption (Hadžiabdić, Elezović, Hadžović, *et al.,* 2013; Dyas and Shah, 2007). The improvements in solubility and/or dissolution rate/bioavailability of diazepam may be achieved through the preparation of solid dispersions (SDs).

The increase in solubility/dissolution rate from solid dispersions can be attributed to one or a combination of the following factors; reduction of particle size of the drug, solubilizing effect on the drug by the water soluble carriers due to increased wettability**,** improved dispersibility of the drug particles by using various hydrophilic carriers and the possible formation of a metastable dispersion that has a greater solubility resulting in faster dissolution rate. Drug release from solid dispersions is described in several ways. Immediate release drug products allow drugs to dissolve with no intention of delaying or prolonging dissolution or absorption of the drug. Among the popular carriers used in the formation of solid dispersion are polyethylene

glycol (PEG) and polyvinylpyrrolidone (PVP) (Kumar and Vandana, 2012; Tiwari, Tiwari, Srivastava, *et al.*, 2009; Sapkal, Babhulkar, Rathi, *et al.*, 2013). In the literature, various solid dispersions of diazepam are reported for improving the dissolution of diazepam using various carriers like polymers (Cwiertnia, 2008; Rabasco, Ginesh, Fernandez, *et al.*, 1991; Verheyen, Blaton, Kingrt, *et al.*, 2002).

Diazepam was formulated as solid dispersions (solvent evaporation technique) using 1:5, 1:10 and 1:20 ratios of drug and carriers. The objective of this investigation was to examine the influence of water-soluble polymers, like polyethylene glycol 2000 (PEG 2000), polyethylene glycol 4000 (PEG 4000) and polyvinylpyrrolidone K30 (PVP K30) on solubility and dissolution rate behavior of diazepam from solid dispersions. Diazepam content, saturation solubility study and *in vitro* dissolution rate of diazepam solid dispersions were analyzed. The *in vitro* dissolution study was carried out by USP paddle method (USP-NF, 2013), using phosphate buffer with a pH of 6.8 as the dissolution medium. The increase in solubility and dissolution rate of diazepam were observed in all diazepam solid dispersions when compared to pure diazepam. In order to predict and correlate the kinetics and mechanism of diazepam release from diazepam solid dispersions using polymers, it is necessary to fit dissolution date into a suitable mathematical model. The zero order, first order, Hixson-Crowell and Korsmeyer-Peppas models were used to fit the *in vitro* diazepam release date from the solid dispersions. The correlation coefficient (r^2) was considered the main parameter for assessing the models. The diffusion coefficient (n) is indicative of transport mechanism (Gautam and Mahaveer, 2011).

EXPERIMENTAL

Materials

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) (M_r = 284.75 g mol^{-1}, F.I.S. - Fabbrica Italiana Sintetici, Italy), polyethylene glycol 2000 (M_r ~ 1800-2200 g mol^{-1}, Merck KGaA, Germany), polyethylene glycol 4000 (M_r ~ 3500-4500 g mol^{-1}, Merck KGaA, Germany), polyvinylpyrrolidone K30 (M_r ~ 40000 g mol^{-1}, BASF ChemTrade GmbH, Germany), Acidum hydrochloricum fumans, 37%, pro analysi, (Merck KgaA, Germany), Disodium hydrogen phosphate (Merck KGaA, Germany), Potassium dihydrogen phosphate (Merck KgaA, Germany), Methanol, p. a., (Merck KGaA, Germany), Ethanol, 96% v/v (Sigma-Aldrich GmbH, Germany).

Preparation of diazepam solid dispersions using polymers

Solid dispersions of diazepam were prepared by solvent evaporation technique using various polymers as carriers in 1:5 (SD1, SD4, SD7), 1:10 (SD2, SD5, SD8) and 1:20 (SD3, SD6, SD9) ratios. Diazepam and polymers were separately dissolved in a minimum amount of methanol (class 2) to get clear solution on ultrasonic bath (Sonorex Digitec DT 512 H, Bandelin, GmbH, Germany) at room temperature (at 22 $^oC \pm 2\ ^oC$). The solvent of the mixture of diazepam and polymers was removed by evaporation on Rotavapor R-205 (Buchi, Switzerland) at 40 °C. Evaporation of methanol was carried out under reduced pressure of 337 mbar, at 120 rpm/min. The samples were subjected to drying for a period of 6 hours in vacuum oven VD 23 (Binder, USA) at 40 °C ± 0.5 °C. The dried mass was sieved through sieve No. 20 (WS TylerR, Ohio, USA) (Lima, Soares-Sobrinho, Corrêa, *et al.*, 2008; Kalyanwat and Patel, 2010; van Drooge, Hinrichs, Visser, *et al.*, 2006). All SDs were kept at room temperature in screw-capped glass vials until use.

Determination of percent diazepam content in solid dispersions

Determination of content of diazepam in solid dispersions with PEG 2000, PEG 4000 and PVP K30 was carried out by weighing (Analytical balance type XS 205DU/A, Mettler Toledo GmbH, Germany) the equivalent of 0.005 mg mL^{-1} of diazepam in 0.1 mol L^{-1} hydrochloric acid. Solid dispersions of diazepam were placed in 50 mL volumetric flasks. Ethanol (10 mL) was added and samples were mixed on the magnetic stirrer (Magnetic stirrer C - MAG HP 7, IKA®, Werke GmbH, Germany) at room temperature for 20-30 minutes. The volume was filled to the mark with ethanol. After that, 1 mL of these solutions was suitably diluted with 0.1 mol L^{-1} hydrochloric acid in 100 mL volumetric flask. The samples were spectrophotometrically assayed, using Varian Cary 50, UV-VIS spectrophotometer (Varian, Inc., USA), for diazepam content at 241 nm (Calibration curve for diazepam: concentration range: 2 - 10 × 10^{-3} mg mL^{-1}, r^2 = 0.9999). To nullify the absorbance due to the presence of polymers, the apparatus was calibrated with the corresponding blank in every assay. Each preparation was tested in triplicate and the mean values were calculated (Table 1).

Determination of saturation solubility

Solubility measurements and the determination of saturation concentrations were carried out by addition of excess amounts of diazepam solid dispersions (Analytical balance type XS 205DU/A, Mettler Toledo GmbH, Germany) to phosphate buffer solution (pH meter SevenMultiTM S47-K**,** Mettler Toledo GmbH, Germany). The phosphate buffer solution is simulated intestinal fluid without enzymes, the pH value 6.8. The samples were shaken at 200 rpm/min, during 24 hours on thermostated shaking bath (BDL, Type: GFL 1083, Czech Republic), to reach equilibrium, at 37 °C ± 0.1 ^oC (USP-NF, 2013; ,US FDA, CDER, 2000; Wagh and Patel, 2010). The samples were then filtered (Cellulose acetate filter, Sartorius, Germany), suitably diluted and analyzed on Varian Cary 50 UV-VIS spectrophotometer (Varian, Inc., USA) at 230 nm wavelength (Table 2).

In vitro dissolution studies

In vitro dissolution studies were performed in phosphate buffer (pH 6.8, 900 mL) at 37 ± 0.5 °C, using dissolution tester (Dissolution Tester, Varian VanKel VK 7025, Varian, Inc., USA) type II (paddle method) (USP-NF, 2013). The agitation speed was set at 50 rpm. The samples (pure diazepam and diazepam solid dispersions) equivalent to 36 mg of diazepam were subjected to dissolution. At fixed time intervals, samples (5 mL) were

withdrawn and equal amount of fresh dissolution medium was added, pre-warmed to 37 °C. After appropriate dilution, the samples were filtered (Filters, Quality Lab Accessorius L.L.C., Varian, Inc., SAD) and spectrophotometrically assayed for diazepam content at 230 nm wavelengths using Varian Cary 50, UV-VIS spectrophotometer (Varian, Inc., USA). Experiments for dissolution studies were performed for 60 min (Table 3).

Drug release kinetics

To study the release kinetics, data obtained from *in vitro* drug release studies were plotted in various kinetic models: zero order (Equation 1) as the cumulative amount of drug released *vs.* time, first order, as log cumulative percent drug remaining *vs.* time, describing concentration dependent drug release from the system (Equation 2).

$$Q_t = Q_o - k_o \cdot t \quad (1)$$

where is
Q_t - concentration of drug dissolved in time,
Q_o - initial concentration of drug in solution,
k_o - zero order release constant,
t - time.

$$\ln Q_t = \ln Q_o - k_1 \cdot t \quad (2)$$

where is
Q_t - cumulative percent of drug remaining,
Q_o - initial concentration of drug,
k_1 - first order rate constant,
t - time (Gautam and Mahaveer, 2011; Sahoo, Chakraborti, Behera, 2012; Singh, Gupta, Kaur, 2011).

To evaluate the drug release from systems with polymer erosion/dissolution with changes in the surface area and the diameter of the particles or tablets, the data were also plotted using the Hixson-Crowell cube root law:

$$Q_o^{1/3} - Q_t^{1/3} = k_{HC} \cdot t \quad (3)$$

where is
Q_t - concentration of drug released in time t,
Q_o - initial concentration of drug in the pharmaceutical dosage form,
k_{HC} - rate constant for the Hixson-Crowell rate equation.

The equation describes the release from systems where there is a change in surface area and diameter of particles or tablets (Gautam and Mahaveer, 2011; Hixson and Crowell, 1931).

Mechanism of drug release

To study the drug release kinetics from polymeric systems the equation derived by Korsmeyer, Gurney, Doelker, et al.,(1983) was used.

$$\frac{Q_t}{Q_o} = k_{KP} \cdot t^n \quad (4)$$

where is

$\frac{Q_t}{Q_o}$ - fraction of drug released at time t,

k_{KP} - the rate constant for the Korsemeyer-Peppas,
n - the release exponent indicative of the drug release mechanism,
t - time.

If n value has the limiting values of 0.45 or less, the release mechanism follows Fickian diffusion and higher values of 0.45 to 0.89 for mass transfer follow a non-Fickian model or anomalous mechanism of drug release. The drug release follows zero-order drug release and case II transport if the n value is 0.89. For the values of n higher than 0.89, the mechanism of drug release is regarded as super case II transport (relaxation). The n value could be obtained from slop of the plot of log cumulative % of drug released *vs.* log time (Korsmeyer, Gurney, Doelker, *et al.*, 1983; Suvakanta, Padala, Lilakanta, *et al.*, 2010).

RESULTS AND DISCUSSION

Determination of percent diazepam content in solid dispersions

The content of diazepam in each preparation was assayed by UV spectroscopy. The drug content percentage in various newly prepared diazepam solid dispersions ranged from 93.33 ± 0.74% and 99.67 ± 0.45%, as reported in Table 1.

Table 1: Content of diazepam in solid dispersions with polymers

Diazepam solid dispersions	Content (%)
Dz : PEG 2000 1:5	93.33 ± 0.741
Dz : PEG 2000 1:10	99.38 ± 0.771
Dz : PEG 2000 1:20	96.00 ± 0.524
Dz : PEG 4000 1:5	98.00 ± 0.576
Dz : PEG 4000 1:10	96.00 ± 0.458
Dz : PEG 4000 1:20	96.33 ± 0.852
Dz : PVP K30 1:5	96.18 ± 0.482
Dz : PVP K30 1:10	97.33 ± 0.584
Dz : PVP K30 1:20	99.67 ± 0.450

All the solid dispersions showed the presence of high drug content and good uniformity of method employed for preparation. The method used in this study appears to be reproducible for preparation of diazepam solid dispersions.

Determination of saturation solubility

The solubility of diazepam solid dispersions in phosphate buffer at 37 ^{o}C are given in Table 2.

Table 2: Solubility of solid dispersions of diazepam with polymers (PEG 2000, PEG 4000 and PVP K30)

Solid dispersions of diazepam with polymers	A[a]	Concentration of diazepam (mg mL^{-1})	S_{SD}/S_{Dz} [b]
Dz : PEG 2000 1:5	0.559	0.0523 ± 0.72	1.13
Dz : PEG 2000 1:10	0.602	0.0572 ± 0.14	1.24
Dz : PEG 2000 1:20	0.632	0.0597 ± 0.22	1.29
Dz : PEG 4000 1:5	0.578	0.0543 ± 0.16	1.18
Dz : PEG 4000 1:10	0.585	0.0550 ± 0.20	1.19
Dz : PEG 4000 1:20	0.629	0.0593 ± 0.56	1.28
Dz : PVP K30 1:5	0.489	0.0907 ± 0.28	1.96
Dz : PVP K30 1:10	0.567	0.1067 ± 0.62	2.31
Dz : PVP K30 1:20	0.319	0.1202 ± 0.32	2.60

[a]Absorbance, [b]S_{SD}/S_{Dz} - Solubility enhancement factor calculated as the ratio of solid dispersion solubility (S_{SD}) in phosphate buffer solution versus diazepam solubility value (S_{Dz}) measured in the absence of polymer.

The solubility of diazepam is increased with increasing amount of polymers in solid dispersions (Table 2). The results of saturation solubility in phosphate buffer solution indicated that the solubility was increased by 2.60 fold with Dz : PVP K30 solid dispersion 1:20 ratio compared to solubility of pure diazepam (0.0462 ± 0.02 mg mL^{-1}, 37 ^{o}C ± 0.1 ^{o}C) (Hadžiabdić *et al.*, 2013).

Release rate studies

The *in vitro* dissolution characteristics of different types of diazepam formulations (with PEG 2000, 4000 and PVP K30) were compared with the pure drug. Dissolution profiles of diazepam and its solid dispersions are given in Table 3 as the mean concentrations of dissolved diazepam (%) for all solid dispersions (n = 3).

A plot of percent (%) diazepam released *vs.* time for the dissolution rate in accordance with zero order kinetics is shown in Figure 1, where the phosphate buffer solution pH 6.8 was used as a dissolution medium.

A linear plot of log percent (%) remaining *vs.* time from dissolution rate in accordance with first order equation is shown in Figure 2.

Table 3: Dissolution date of diazepam and diazepam solid dispersions

Time (min)	Percent diazepam release									
	Dz	SD1	SD2	SD3	SD4	SD5	SD6	SD7	SD8	SD9
10	2.60	13.15	18.75	24.50	15.35	22.52	25.37	41.95	47.48	55.22
20	2.65	13.63	20.04	24.91	15.55	23.44	26.28	42.36	48.77	55.76
30	2.74	14.31	21.22	25.04	15.98	24.63	26.98	43.22	49.81	56.34
40	2.84	14.55	21.96	25.78	16.82	25.60	28.09	44.15	51.20	56.78
50	2.90	15.08	22.55	26.08	17.12	26.02	29.25	44.65	53.04	56.86
60	2.99	15.25	23.22	26.65	17.68	26.88	29.80	45.31	53.85	57.94

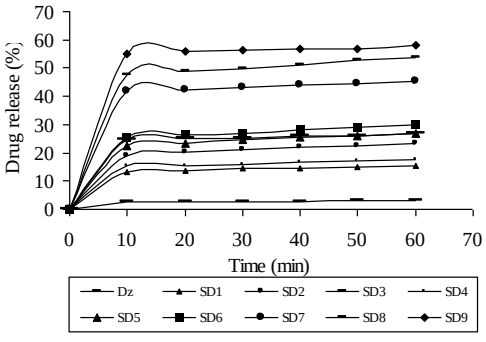

Figure 1: Zero order dissolution plots of diazepam and its solid dispersions

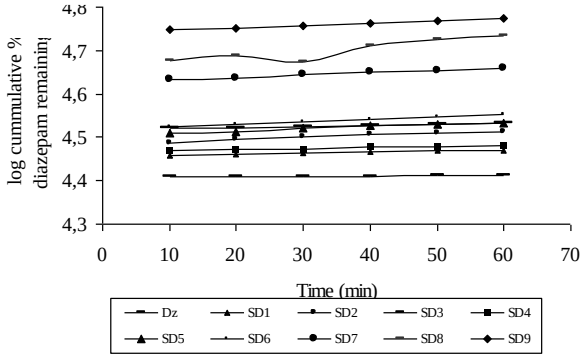

Figure 2: First order dissolution plots of diazepam and its solid dispersions

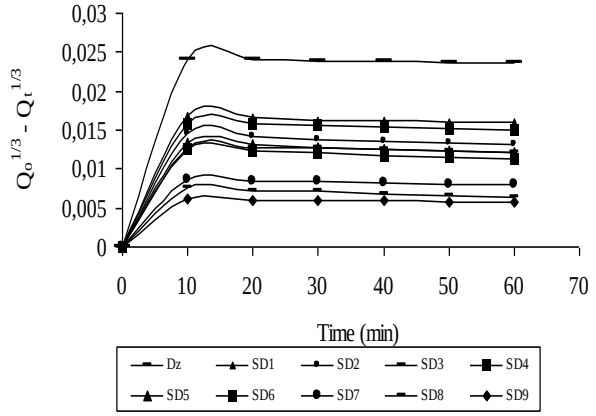

Figure 3: Hixon-Crowell cube root dissolution plots of diazepam and its dispersions

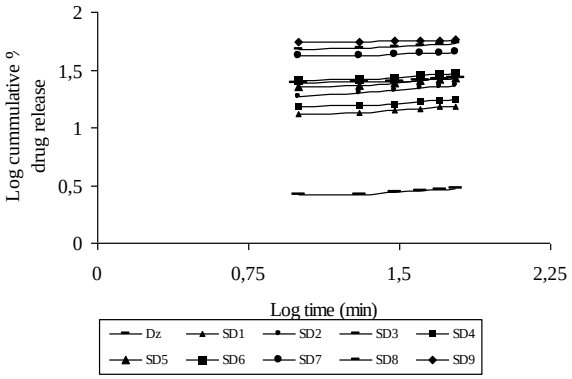

Figure 4: Korsmeyer-Peppas model of mechanism of diazepam release

The correlation coefficient (r^2) values in the analysis of dissolution data of diazepam solid dispersions as per zero order, first order, Hixson-Crowell cube root models and Korsmeyer-Peppas model are given in Table 4.

Table 4: Release kinetic and mechanism of diazepam release

Formulations	Zero order		First order		Hixon-Crowel		Korsmeyer-Peppas	
	r^2	k_o (h^{-1})	r^2	k_1 (h^{-1})	r^2	k_{HC} ($h^{-1/3}$)	r^2	n
SD1	0.9740	2.5836	0.9748	-0.0131	0.9708	-0.0011	0.9730	0.0858
SD2	0.9730	5.2403	0.9749	-0.0288	0.9667	-0.0017	0.9943	0.1196
SD3	0.9757	2.5741	0.9751	-0.0150	0.9766	-0.0007	0.8778	0.0451
SD4	0.9767	2.9452	0.9764	-0.0153	0.9776	-0.0011	0.8767	0.0800
SD5	0.9848	5.2234	0.9862	-0.0331	0.9815	-0.0015	0.9722	0.0994
SD6	0.9922	5.5074	0.9919	-0.0331	0.9925	-0.0015	0.9337	0.0931
SD7	0.9911	4.2120	0.9913	-0.0324	0.9908	-0.0008	0.9223	0.0440
SD8	0.9917	7.8792	0.9917	-0.0702	0.9977	-0.0013	0.9261	0.0708
SD9	0.9563	2.9657	0.9543	-0.0296	0.9965	-0.0005	0.8978	0.0240

Dissolution of the diazepam in pH 6.8 phosphate buffer was only 2.99% after 60 minutes. After 60 minutes, solid dispersions of PEG 2000 and PEG 4000 (1:5, 1:10 and 1:20) showed 15.25%, 23.22%, 26.65%, 17.68%, 26.88% and 29.80% diazepam released, whereas solid dispersions with PVP K30 showed 45.31%, 53.85% and 57.84% diazepam released. Dissolution profile of solid dispersions of diazepam demonstrated that drug release was faster from solid dispersions when compared to diazepam alone (Table 3 and Figure 1). Thus, the use of water soluble carrier PVP K30 resulted in greater enhancement in the dissolution rate of diazepam. The percentage of diazepam released from solid dispersions containing PVP K30 was increased as the ratio of PVP K30 increased (Figure 1).

The Hixson-Crowell cube root law describes the release from system where there is a change in surface area and diameter of the particles or tablets. An alteration in the surface area and diameter of the matrix system as well as in the diffusion path length from the matrix drug load occurs during the dissolution process. A plot of $(Q_o)^{1/3} - (Q_t)^{1/3}$ *vs.* time will be linear when dissolution occurs from monodisperse particles of uniform size. Hixson-Crowell plots of the dissolution data were found to be linear (Table 3) with all solid dispersions.

the solid dispersions is occurring from discretely suspended (monodisperse) particles. This might have also contributed to the enhanced dissolution rate of the solid dispersions.

The correlation coefficient values of the Hixson-Crowell's cube root model are found to be (r^2 = 0.967 to 0.998) slightly higher when compared to the zero and first order (Table 4) release model. Hence the release of drug from the preparations followed predominantly Hixson-Crowell cube root law compared to zero and first order kinetics.

The high values of correlation coefficient (Table 4) suggested that all formulations followed Korsmeyer-Peppas model release kinetics. The values of correlation coefficient were found to be from 0.877 to 0.994. The n values for Korsmeyer-Peppas model were 0.0204 to 0.1196, indicating Fickian release (Table 4 and Figure 4). The low values of n (< 0.45) indicated that the mechanism of drug release from all the formulations could be described as a Fickian diffusion mechanism. Fickian diffusional release occurs by the usual molecular diffusion of the drug due to the chemical potential gradient (Gautam, *et al.*, 2011).

CONCLUSION

Solvent evaporation method of preparation resulted in diazepam solid dispersions with uniform content. The determination of saturation solubility and *in vitro* dissolution study showed that the solid dispersions increased solubility and dissolution rate of diazepam. Selected hydrophilic carrier polyvinylpyrrolidone K30 resulted in greater enhancement in solubility and dissolution rate of poorly soluble diazepam when compared with other polymer dispersions. Polymer dispersion dissolution studies can provide vital information on drug release mechanisms. The *in vitro* dissolution of diazepam from these solid dispersions was found to follow Hixson-Crowell model (r^2 = 0.967 to 0.998) signifying that the drug release from the solid dispersions was erosion based, due to the decrease in surface area and diameter of particles with polymer erosion. The high values of regression coefficients (r^2 = 0.877 to 0.994), suggested that all formulations followed Korsmeyer-Peppas model of release kinetics. The low values of the release exponent (< 0.45) indicated that the mechanism of diazepam release from all formulations could be described as a Fickian diffusion mechanism. The Fickian release was possible in all the formulation indicating polymer relaxation followed by diffusion of diazepam. It can concluded that water-soluble polyvinylpyrrolidone K30 can be used as polymeric carrier and to obtain faster dissolution of diazepam in form of solid dispersion for its pharmaceutical applications.

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Summary/Sažetak

Prema biofarmaceutskom sistemu klasifikacije diazepam spada u lijekove klase II. Neadekvatna brzina otapanja diazepama može biti ograničavajući faktor za njegovu brzinu apsorpcije. Cilj ovog istraživanja je bio da se pripreme čvrste disperzije diazepama koristeći razne nosače kao što su polietilenglikol 2000, polietilenglikol 4000 i polivinilpirolidon K30, procijeni topivosti i brzina otapanja pripremljenih čvrstih disperzija diazepama i uporede podaci sa podacima čistog diazepama. Čvrste disperzije su izrađene metodom evaporacije otapala. Kao disolucijski medij korišten je fosfatni pufer pH 6.8. Čvrste disperzije diazepama s polimerima pokazuju povećanu topivost i brzinu otapanja diazepama (najviše disperzija sa polivinilpirolidonom K30). Brzina kinetike oslobađanja diazepama iz čvrstih disperzija slijedi Hixson-Crowell-ov zakon kubnog korijena. Vrijednosti koeficijenata korelacije (r^2) Hixson-Crowell-ovog modela kubnog korijena su nešto više (0.9667-0.9977) u odnosu na modele oslobađanja nultog i prvog reda. Visoke vrijednosti koeficijenata regresije sugerišu da sve formulacije slijede Korsmeyer-Peppas model kinetike oslobađanja. Niske vrijednosti eksponenta oslobađanja (< 0.45) ukazuju da se mehanizam oslobađanja diazepama iz svih formulacija može opisati Fick-ovim mehanizmom difuzije.

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Seasonal Variation in Content and Chemical Composition of Essential Oils from Leaves of *Mentha longifolia* Huds. (*Lamiaceae*)

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Abstract: The aim of the present study was to appraise variation in content and the chemical composition of essential oil from the leaves of *Mentha longifolia* as affected by harvesting season. Quantities of the essential oils were determined according to the European Pharmacopoeia 4th Ed. and chemical profiles by using a gas chromatography – mass spectrometry. The highest content of the essential oil was found plant during the flowering stage (1.9%). The main constituents of the essential oil through all the three phenophases were oxygened monoterpenes piperitone oxide (1.9-63.6%) and 1,8-cineole (5-12%), and sesquiterpenes trans-caryophyllene (3-9.3%) and germacrene D (0.16-10%). In general, the composition of the essential oil in all the three investigated phenophases was generally similar with quantitative differences. Oxygenated monoterpenes were dominant during flowering stage and after flowering stage, followed by sesquiterpene hydrocarbons. Presence of β-ocimene, γ-terpinene, and carvone were noticed only before flowering; cederole, β-cubebene and α-caryophyllene during the flowering stage; and 3-carene after flowering. Analysis of the qualitative and quantitative composition of main constituents of the essential oil in all the three investigated phenophases led to the conclusion that the piperitone oxide, as the major compound could be used as the stable chemotype marker for the taxonomy of *Mentha longifolia*.

INTRODUCTION

The genus *Mentha* L., member of the family *Lamiaceae*, consists of approximately 14-25 species, grows widely throughout the temperate regions of the world (Harley, 1972; Gobert et al., 2002). *Mentha longifolia*, member of the family Lamiaceae is a perennial bushy plant and upright, reaches height of about 1 m. Strongly aromatic, the leaves are formed in pairs opposite to each other along the square-shaped stem. *Mentha longifolia*is is used in herbal medicine and is native to the Mediterranean region and Middle East. It is mainly used for the treatment of respiratory ailments, but many other uses have been recorded. Leaves are used the most , usually for preparation of tea against coughs, colds, stomach cramps, asthma, flatulence, indigestion and headaches (Gulluce et al., 2007).

In the Lamiaceae family, essential oils are mainly produced in secretary structures known as glandular trachomes, of which there are two main kinds, peltate and capitates. The amount of essential oils produced is directly connected with the number and physiology of these structures. Essential oils are very complicated mixtures of

natural compounds at quite different concentrations (Bozin et al., 2006). Factors that determine the composition and yield of the essential oil obtained are numerous. In some instances it is difficult to segregate these factors from each other, since many are interdependent and influence each other. These variables may include seasonal and maturity variation, geographical and climatic conditions, genetic variation, growth stages, part of plant utilized and postharvest drying, storage and mode of distillation. The chemical composition of the essential oils from plants is thus subject to quantitative and qualitative variations. Biological activity which is dependent on the chemical composition is similarly subject to variation. Plant material collected at different times of the year may contain different novel compounds with other bioactivities. Examination of the published literature on the oil composition of *Mentha longifolia* reveals that it can exist in a myriad of chemical forms, as can be seen from the main constituents found in these oils (Dzamić et al., 2010). The main constituents in essential oil were piperitone oxide (13.90-50.50%), 1,8-cineole (8.18-17.80%), carvone (0.5-21.5%), beta caryophyllene (2.0-22.0%) and menthol (0.0-32.50%). Literature revealed that essential oil contents depend not only on temperature, relative humidity, but also on duration of sunshine, air movement and rainfall (Viljoen et al., 2006).

The aim of the present study was to appraise variation in content and the chemical composition of essential oil from the leaves of *Mentha longifolia* Huds. (Lamiaceae) as affected by harvesting season (from May to September). The present study describes the qualitative and quantitative composition, together with the content of essential oil of *Mentha longifolia*, native to Herzegovina, during three different phenophases (before flowering, in the flowering stage and after flowering).

EXPERIMENTAL

Chemicals and Reagents: (-)–α Thujone (≥ 96% GC) analytical standard (SigmaGermany)(No. 89231); (-)-β Pinene 99+% (Sigma Germany); R -/+) – Limonene analytical standard (Fluka Germany) (No.62118); Eucalyptol analytical standard (Fluka Germany) (No. 29210); Linalool analytical standard (Fluka Germany) (No. 51782); (+)-Carvone analytical standard (Fluka Germany) (No. 22070); Thimol standard (Fluka Germany) (No.50409); (−)-trans-Caryophyllene ≥98.5% (sum of enantiomers, GC) (Sigma Germany) (No. 22075).

Plant Material

Aerial parts of wild growing flowering plants of *Mentha longifolia* Huds. (Lamiaceae) were collected on the banks of the Jablaničko lake (Bosnia and Herzegovina) during three phenophases (before flowering, flowering and after flowering) in 2011. Voucher specimens of collected plants No. 1060/1 before flowering, 1060/2 flowering stage, 1060/3 after identity conformation by an independent expert were deposited at the Herbarium of the Department of Biology, Faculty of Sciences, University of Sarajevo.

Isolation of the Essential Oil

The leaves of *Mentha longifolia* were shade dried (15 days) at room temperature. Air-dried plants of *Menth alongifolia* were submitted to hydrodistillation according to European Pharmacopoeia 4th Ed., using Clevenger apparatus (Klaus Hofmann GmbH, Germany). The essential oil samples of each phenophase were dried over anhydrous sodium sulfate. The quantity of predistilled essential oils was determined volumetrically (Council of Europe, 2002).

Essential Oil Analysis

Qualitative and quantitative analyses of the essential oils were carried out by using gas chromatography/mass spectrometry system (GC-MS, Agilent Tecnologies series 6890N/5975B, United States of America) at electron energy of 70eV, equipped with a split-splitless injector (200°C) and a flame ionization detector (FID) (250°C). helium (1 mL/min) was used as a carrier gas. The capillary columns (HP 5MS 30m x 0.25mm; film thickness 0.25µm Agilent Tecnologies) were used. The temperature programmes were 50°C to 280°C at a rate of 10°C/min until 130°C and 130-280°C at a rate of 12°C/min, respectively with split ratio, 1:10. Co-elution and MS analysis based on the identification of the individual compounds, and the comparison of their relative retention times (RI) with those of the reference samples were performed. For the components, mostly sesquiterpenes and aliphatic compounds, for which reference substances were not available, the identification was performed by matching their retention times and mass spectra with those obtained from the authentic samples and/or The National Institute of Standards and Technology, known as the National Bureau of Standards (NIST/NBS), Wiley libraries spectra as well as with literature data (Adams, 2007).

RESULTS AND DISCUSSION

The mean oil content in the plants of *Mentha longifolia* collected in flowering stage amounted to 1.9% v/w (volume of essential oil/weight dry leaf) in dry matter. In plants collected after flowering stage the essential oil content was 1.45% v/w, while in the plants collected before flowering it was 0.49 % v/w (Table 1.).The essential oil content was in accordance with the earlier published data (Mkaddem et al., 2009; Kofidis et al. 2006).

Table 1. Essential oil content during phenophases

Phenophase	before flowering	flowering stage	after flowering
Essential oil content % v/w	0.49	1.9	1.45

The percentage composition of the essential oils during three investigated phenophases is presented in Table 2. A total of the 33, 36 and 26 chemical constituents, representing 98.42, 98.17 and 98.84% of the total content, were identified in the essential oil before flowering, in the flowering stage and after flowering, respectively. In the oil obtained from the plants collected in flowering stage the oxygenated monoterpenes were found to be the major class of substances (87.1%), followed by the sesquiterpene hydrocarbons (6.8%) and oxygenated sesquiterpenes (5.57%) (Figure 2). The oil extracted from the leaves of *Mentha longifolia* in the period before floweringcontained oxygenated monoterpenes (63.57%) and higher amount of sesquiterpene hydrocarbons (23.16%). The essential oil from the plants collected after flowering was composed mainly of monoterpenic fraction 89% (oxygenated monoterpenes 78.51% and monoterpene hydrocarbons 10.39%) (Table 2, Figure 2). In the essential oil of *Mentha longifolia* during investgated phenophases, three compounds were dominant: piperitone oxide, 1.8-cineole andgermacrene D.

Table 2. Chemical composition of *Mentha longifolia* L. essential oil during investigated phenophases

Peak No	Components	R.I.[a]	before flowering %	flowering stage %	after flowering %
	Monoterpene hydrocarbons		**10,42**	**3,06**	**10,39**
1	α-thujene	**932**	0,13		
2	α-pinene	**938**	1,06	0,78	5,89
3	sabinene	**974**	0,43	0,47	0,68
4	β-pinene	**978**	0,92	0,99	2,57
5	β-mircene	**992**	0,77	0,69	0,48
6	terpinolene	**1008**		0,07	0,35
7	limonene	**1035**		0,06	0,11
8	δ-carene	**1031**			0,31
9	*E*-β-ocimene	**1050**	6,97		
10	γ-terpinene	**1063**	0,14		
	Oxygenated monoterpenes		**63,57**	**87,1**	**78,51**
11	1,8-cineole	**1036**	5	12,03	9,3
12	*trans*-sabinene hydrate	**1098**		0,68	
13	linalool	**1099**	1,94		
14	*cis*-sabinol	**1143**		0,16	0,27
15	borneole	**1167**	0,58	0,52	0,52
16	piperitone oxide	**1170**	1,91	63,58	59,99
17	terpinen-4-ol	**1178**	0,7	0,1	
18	1-α-terpineole	**1188**	0,13	0,91	0,34
19	carvone	**1243**	52,26		
20	bornyil acetate	**1288**	0,24		0,86
21	thymol	**1291**		1,69	1,2
22	*trans*-carvyl acetate	**1342**	0,23		
23	piperitenone	**1343**		1,98	2,43
24	piperitenone oxide	**1369**	0,13	4,81	3,35
25	*cis*-jasmone	**1395**	0,45	0,64	0,25
	Sesquiterpene hydrocarbons		**23,16**	**6,79**	**8,25**
26	α-copaene	**1375**	0,26	0,19	0,12
27	β-burbonene	**1383**	1,25	0,54	0,21
28	β-cubebene	**1390**		0,48	
29	β-elemene	**1391**	0,61	0,18	0,21
30	*cis*-caryophyllene	**1405**		0,82	
31	*trans*-caryophyllene	**1419**	9,27	2,98	4,2
32	α-humulene	**1452**	0,89	0,44	
33	*allo*-aromadendrene	**1462**		0,23	
34	α-amorfene	**1485**		0,26	
35	germacrene D	**1490**	9,94	0,16	3,17
36	α-murolene	**1500**	0,14	0,11	
37	biciclogermacrene	**1501**	0,29		
38	γ-cadinene	**1514**		0,31	
39	δ-cadinene	**1523**	0,51	0,09	0,34
	Oxygenated sesquiterpenes		**2,1**	**5,57**	**0,42**
40	spathulenol	**1578**	0,14		
41	caryophyllene oxide	**1582**	0,85	4,33	0,34
42	cedrole	**1601**		0,51	
43	τ-murolol	**1651**	0,47	0,2	0,08
44	α-cadinole	**1654**	0,64	0,53	
	Aliphatic compounds		**1,27**	**1,22**	**1,27**
45	3-octanole	**991**	1,27	1,16	1,27
46	*n*-udecanole	**1370**		0,06	
	Total identified (%)		**98,42**	**98,17**	**98,84**

[a]Retention indices relative to C9-C24 *n*-alkanes on the HP-5MS column

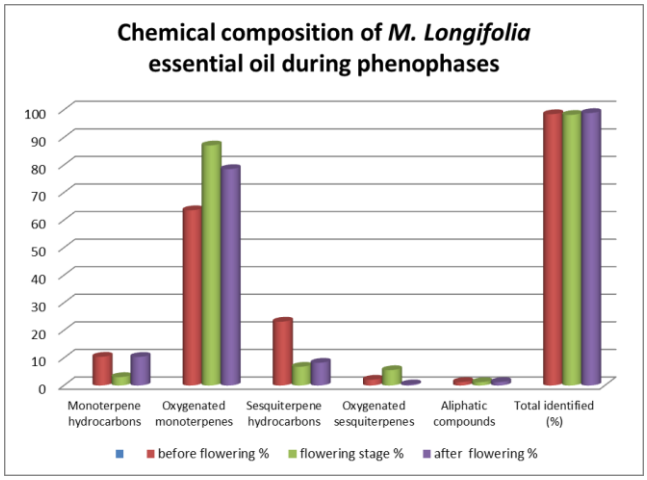

Figure 1. Major classes of substances in the investigated essential oil during three phenophases (%)

The essential oil from the plants in the flowering stage and after flowering stage revealed the high piperitone oxide content, which is in accordance with the earlier published data. The content of piperitone oxide in before flowering stage was significantly lower (1.91%), but high amount of carvone was recorded. In opposite, in samples collected in flowering stage and after flowering stage, piperitone oxide was dominant compound (63.58% and 59.99%). The following major compounds of the essential oils in all the three investigated phenophases were 1.8-cineole (5-12%), germacrene D (0.16-9.94%), trans-caryophyllene (3-9.3%), and α-pinene (0.78-5.89%). Apart from the general similarity in the main compounds, there were significant differences with respect to their quantity (Table 2). Furthermore, some qualitative differences in the chemical composition of the essential oil during investigated phenophases were also observed. In the essential oil obtained from the plants collected before flowering stage the presence of notable amount of carvone 52.26% and β-ocimene 7% was recorded. Vijoen et al. (2006) reported piperitenone oxide (15-66%) as the main compounds identified in the essential oils of piperitenone oxide chemotype of *Mentha longifolia*. Gulluce et al. (2007) reported *cis* piperitone epoxide, pulegone, piperitenone oxide, as the main components of essential oil from *Mentha longifolia*, growing in Turkey. Also Stanisavljević et al (2014) reported that dominate components in essential oils from Serbia were: piperitone (50-71%), carvone (2.9-20%), menthone (up to 17%), trans-caryophylene (4.3-5.4%), 1,8-cineole (0.8-1.3%). Samples of essential oils from Iran were characterized by a high amount of three oxygenated monoterpenescarvone (1–26%) and pulegone (7.5–31%) and piperitone oxide (4–14%) (Giti et al., 2014). Earlier data pertaining to *Mentha longifolia* essential oil point out the persistence of four chemotypes: piperitone oxide, piperitone oxide/piperitenone oxide, p-menthone/piperitone oxide and trans-dihydrocarvonechemotype (Aksita et al., 2013). The investigated essential oils, obtained from the plant material in flowering stage and after flowering stage could be categorized as piperitone oxide chemotype, but plant material collected in period before flowering stage has a specific chemical composition and could not be categorized in one of the four previously described chemotypes. The minor variations in the chemical compositions of *Mentha longifolia* essential oil across countries might be attributed to the varied agro-climatic (climatical, seasonal, geographical) conditions of the regions, isolation regimes and adaptive metabolism of plants.

CONCLUSIONS

In general, harvesting season affected the chemical composition of *Mentha longifolia* essential oils. The variation in the content of the essential oils investigated in the present study, with respect to species and harvesting season, was quantitatively significant (0.49- 1.9%). In essential oil from *Mentha longifolia*, the major variation observed was in piperitenone oxide (1.9–63.6%), 1.8 cineole (5–12%), *trans*-caryophyllene (3-9.27%) and germacrene D (0.16–9.94%). The qualitative and quantitative composition of main constituents of the essential oil in all the three investigated phenophases led to the suggestion that the piperitone oxide, as the major compound could be used as the stable chemotype marker in the *Mentha longifolia* taxonomy. Also the information observed on seasonal variation may be useful in selecting the best season for optimal yield.

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Summary/Sažetak

Cilj ovog istraživanja je utvrditi razlike u sadržaju i hemijskom sastavu eteričnog ulja iz lista vrste *Mentha longifolia* tokom vegetacijskih stadija biljke tokom sezone branja. Tokom tri fenofaze poređen je odnos sadržaja i hemijskog sastava izoliranih eteričnih ulja. Sadržaj eteričnih ulja u drogama određen je prema četvrtoj Evropskoj farmakopeji, a hemijski sastav eteričnog ulja analiziran je tehnikom gasna hromatografija – masena spectrometrija (GC-MS). Najveći sadržaj eteričnog ulja pronađen je u fazi cvjetanja biljke od 1,9%. Glavni sastojci eteričnog ulja tokom sve tri fenofaze su bili oksidovani monoterpeni piperiton oksid (1.9 – 63.6%) i 1,8-cineol (5-12%),te seskviterpeni β-kariofilen (3-9,3%) i germakren D (0,16-10%). Premda je sastav eteričnog ulja u sve tri ispitivane fenofaze kvalitativno sličan, utvrđene su kvantitativne razlike u sastavu. Oksidirani monoterpeni bili su dominantna frakcija u fazama cvjetanja i nakon cvjetanja nakon koje slijedi frakcija seskviterpenski ugljikovodonika. Prisutnost β-ocimena, γ-terpinena i karvona utvrđena je samo u fazi prije cvjetanja, dok je prisustvo cederola, β-kubebena i α-kariofilena zabilježeno sami u fazi cvjetanja, a komponenta 3-karen bila je prisutna samo nakon nakon cvjetanja. Analiza kvalitativnog i kvantitativnog sastava glavnih sastojaka eteričnog ulja u sve tri istraživane fenofaze dovelo je do zaključka da piperiton oksid, kao glavna komponenta se može se koristiti kao pouzdan hemotipski marker u taksonomiji vrste *Mentha longifolia*.

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SPE extraction and TLC Identification of Tetracycline and Fluoroquinolone in Surface Water

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Abstract: Simultaneous identification of the antibiotics tetracycline, oxytetracycline, chlortetracycline, ciprofloxacin and enrofloxacin in surface water is reported. The method is based on solid-phase extraction (SPE), separation and identification by thin-layer chromatography (TLC). TLC separation was performed on TLC silica gel 60 F254 plates using a mobile phase system water/methanol/dichloromethane (6/35/59) (v/v). The plates were previously impregnated with 10% solution EDTA pH 9,0 and dried in a horizontal position for at least two hours at room temperature and then in an oven at 105°C 30 minutes shortly before use. Antibiotics were extracted on the OASIS HLB 6cc/500 mg cartridges. Aliquots of 10 µl of the water sample and reference solutions were applied to the plate. After development the plates were air dried and the chromatograms were visualized under UV light at λ = 254 nm and λ = 366 nm. Proposed method can be applied for screening of investigated antibiotics in water samples were antibiotic concentration is equal or higher than 5 µg/ml.

INTRODUCTION

In recent years, pharmaceuticals have been detected in a wide variety of environmental samples including sewage effluent, surface waters, groundwater and drinking water at a concentration from ng/l to µg/l. They are considered as pseudo-persistent pollutants, which continually enter the environment at very low concentrations (Valverde, García, Galera *et al*., 2006).

Among different groups of pharmaceuticals, antibiotics are of special concern: large quantities are administered to humans and animals to treat diseases and infections. They are also used at sub-therapeutic levels to promote growth in livestock. After application, many of them are excreted unchanged or completely metabolized to inactive compounds, but a significant amount is excreted as active metabolites. The most prominent effect of antibiotics in the environment is the development of multi-resistant strains of bacteria (Hirsh, Hernes, Haberer *et al.* 1999, Valverde, Gil García, Galera, *et al.* 2006).

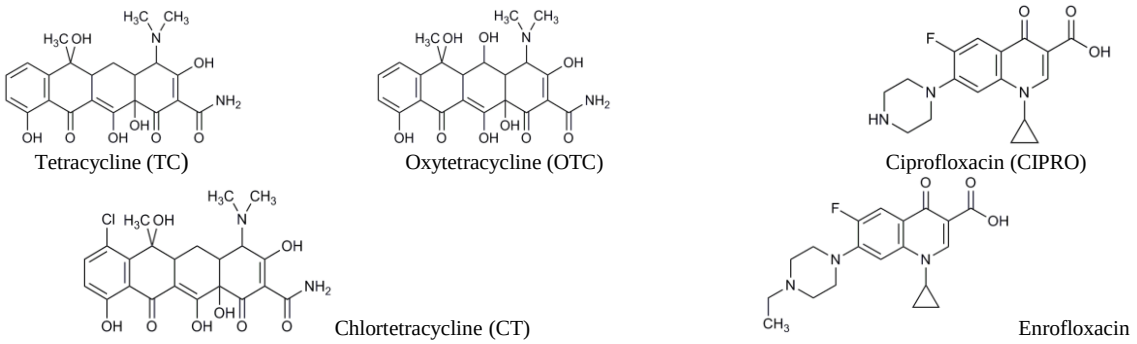
Fluoroquinolones and tetracyclines are commonly used in human and veterinary medicine and there is danger from their presence in the aquatic environment (Lindberg, Jarnheimer, Olsen *et al.,* 2005, Turiel, Bordin and Rodríguez, 2005, Brown, Kulis, Thomson *et al*., 2006). Depending on the chemical properties of the individual groups of antibiotics excretion of unchanged active compound is 10-90% (Boxal, Fogg, Kay *et al.,* 2003). Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are antibiotics of the tetracycline group frequently given to animal destined for human consumption, not only to prevent and treat certain diseases but also to accelerate growth (Stockwell and Duffy, 2012, Serrano, 2005). After medication, more than 70% of tetracycline antibiotics are excreted and released in environment in active form via urine and feces (Kuldip, Satish, Gupta *et al.* 2005). Fluoroquinolone residues can enter the environment mainly as a result of their excretion in the urine of humans and animals, as well as of aquaculture treatments (Boxal *et al.,* 2003, Turiel, Bordin and Rodríguez, 2005, Andreu, Blasco and Pico, 2007).

Numerous analytical methods are currently available to detect tetracyclines and fluoroquinolones in different samples including UV-Vis spectroscopy (Galagher and Danielson, 1995), fluorescence (Smirnova, Yu and Zhmerichkin, 2005), capillary electrophoresis (Miranda, Rodríguez and Galán-Vidal, 2009), high performance liquid chromatography (Blackwell, Holten Lützhøft, Hai Ping *et al.*, 2004) and liquid chromatography-tandem mass spectrometry (Schneider and Donoghue, 2002).

There are numerous literature data that describe the requirements for the identification and quantification of tetracyclines and fluoroquinolones in various samples using thin layer chromatography (Belal, Al-Majed and Al-Obaid, 1999, Thangadurai, Shukla and Anjaneyulu, 2002).

Separation, identification and quantification of tetracyclines and fluoroquinolones are possible on silica gel, cellulose and polyamide stationary phase. Most of the published methods requires impregnation of the TLC plate with EDTA solution before applying the samples on the plate in order to avoid the formation of the complex and hence improved separation (Feng and Dung, 2004, Naidong, Cachet, Roets *et al.*, 1990, Dong, Xie, Shuang *et al*., 1999). The aim of this study was preliminary testing of the presence of residues of tetracycline, oxytetracycline, chlortetracycline, enrofloxacine and ciprofloxacine in water samples collected from two defined localities.

Figure 1: Chemical structure of tested substances



MATERIALS AND METHODS

Acetonitrile was HPLC grade (Panreac, Italy) and the other used solvents were of p.a. grade (Merk, Germany). Tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CT), enrofloxacin (ENRO), ciprofloxacin (CIPRO) were min. 98% pure (Figure 1). Stock solutions of all antibiotics were prepared by dissolving accurate quantities of the powdered standards in 1 ml ultra pure water and then diluted with acetonitrile. Mass concentration of standard solutions was 500 μg/ml. Stock solutions were stored protected from light at 4°C. Working standard solutions were made by diluting the stock standard solutions with acetonitrile so that their concentration was 5 μg/ml. Mixtures were made by mixing 1 ml of each working standard solution. EDTA was analytical reagent grade. Water was ultra pure.

Chromatographic plates 10x20cm, Kiselgel 60 F_{254} (Merck, Darmstadt, Germany), 20x20 Silica gel H, 25 μm, binder free, Anatech, HPTLC 20x10 silica gel 60 F_{254}, (Merck, Darmstadt, Germany), were used for method optimization. For solid-phase extraction 6cc/500 mg Oasis hydrophilic-lipophilic balance (HLB) cartridges (Waters, Milford, Massachusetts) were used.

Sample Preparation

Water samples that were used as a blank were taken upstream from the place for sampling. Both, blank water and water for analysis were collected in amber glass bottles.

Prior to extraction, water used in this study was filtered through black Whatman filter to eliminate the suspended matter. The samples were stored at 4°C until SPE extraction. Before extraction, total concentration of calcium and magnesium was determined, followed by addition of the appropriate amount of 0.01 M EDTA to prevent binding of the antibiotic to the calcium and magnesium. The spiked water samples were prepared by addition of 1 ml of stock standard solution of each antibiotic to 100 mL of water. Before the extraction water samples and spiked water samples were filtered through P/N 0.2 μm 47 mm GHP membrane filter. The HLB SPE cartridges previously used for tetracycline and fluoroquinolone determination reported in literature, were used (Ternes, Bonerz and Schmidt, 2001).

Solid-Phase Extraction

The antibiotics were extracted and pre-concentrated on 6cc/500 mg (Waters) HLB cartridges on the apparatus for solid-phase extraction. Before water application, the cartridges were conditioned with 5 mL of each methanol, and water pH 3.0.

The pH of water samples and water for preconditioning and washing steps was adjusted to 3.0 with hydrochloric acid. The samples were applied to the cartridge and the flow was kept at 3 mL min^{-1}.
After extraction cartridges were washed with 2 ml of ultra pure water to remove the residue of EDTA and dried under vacuum for 5 minutes to remove water excess. Elution of the antibiotic was performed with 3 ml of acetonitrile. The filtrates were evaporated under a stream of nitrogen to a volume of 1 ml. 10 µl of the filtrate was applied on the chromatographic plate.

Thin-Layer Chromatography
Analysis was performed according to the procedure described by Dong *et al* (1999) with the modification of the mobile phase. Prior to the analysis, three different chromatographic plates 10x20cm, Kiselgel 60 F_{254}, 20x20 Silica gel H, 25 µm, binder free, HPTLC 20x10 silica gel 60 F_{254} were impregnated 24 hours with 10% EDTA solution pH 9.0 and then were dried at room temperature for 2 hours, and in oven for 30 minutes at 105°C. On the plate prepared as described previously, aliquots of 10 µl of the each working solutions, mixture, blank and water samples were applied with micropipette in the form of spot.
The plates were developed in a closed glass Camag double-trough chamber containing mobile phase water/methanol/dichloromethane (6/35/59) (v/v) with previous saturation. The system was maintained until the mobile phase ascended to a point 7 cm above initial spots. After development, the plates were air dried and the chromatograms were visualized under UV light at 254 nm and 366 nm.

Surface Water Samples Analysis
The described method was applied to the determination of tetracycline, oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin in surface water samples collected downstream from two fish farms. The volume of pre-filtered and acidified (pH 3) surface water samples was 250 ml. Before extraction, appropriate amount of 0.01 M EDTA was added to the water samples. The samples were applied to Oasis HLB cartridges. Antibiotics were eluted with 3 mL acetonitrile and filtrates were evaporated to 1 mL. Aliquot of 10 µL were applied on the TLC plate.

RESULTS AND DISSCUSION

Tetracyclines and fluoroquinolones have a very strong tendency to form complexes with trace metals in the adsorbents used, which cause lower separation performance because of spots tailing. Before use, the plates listed in section Materials and Methods, were impregnated with a 10% EDTA solution pH 9.0. Analysis was performed with several mobile phases that are described in the literature for the individual identification of tetracycline or fluoroquinolone (Dong *et al.* 1999, Wang, Chen, and Fan, 2001, Feng and Dung, 2004, Oka, Ito and Matsumoto 2000, British Pharmacopoeia, 2012).

The best simultaneous separation of all antibiotics was achieved on HPTLC plate 20x10 silica gel 60 F_{254} with a mobile phase water/methanol/dichloromethane (6/35/59 v/v/v) and these conditions are chosen for the analysis of surface water samples. (Figure 2).

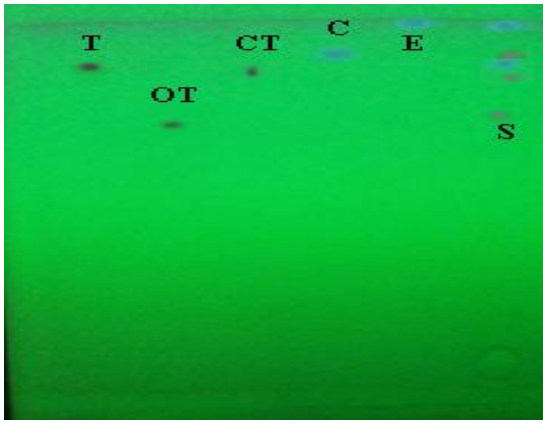


Figure 2: TLC chromatogram of antibiotic standards and mixture of standard substances (S)

Chromatographic plates were evaluated at λ=254 nm and λ=366 nm. Identification was done by comparison of Rf values of antibiotic standards and mix of standard substances (Table 1).

Table 1: Rf values of antibiotic standards and mixture of standard substances

Compds	Rf
tetracycline	0.57
oxytetracycline	0.35
chlortetracycline	0.45
ciprofloxacine	0.80
enrofloxaine	0.97
mix	0.37; 0.42; 0.56; 0.57; 0.97;

Among the five pharmaceuticals examined in this study, none were found in samples (S_1, S_2) used for analysis (Figure 3).

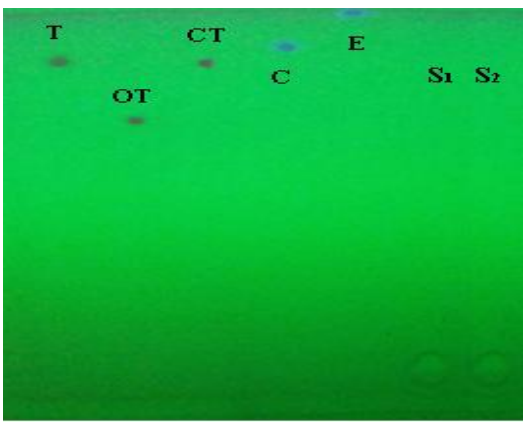


Figure 3: TLC chromatogram of antibiotic standards and water samples (S_1, S_2)

CONCLUSION

Solid-phase extraction followed by HPTLC-UV determination has been proposed for simultaneous identification (screening) of the tetracycline, oxytetracycline, chlortetracycline, enrofloxacin and ciprofloxacin. Identification were based on Rf values and UV detection. Limit of detection of proposed method was 50 ng per spot. Proposed method can be applied for screening of investigated antibiotics in water samples where antibiotic concentration is equal or higher than 5 μg/ml.

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Summary/Sažetak

Provedena je simultana identifikacija antibiotika tetraciklina, oksitetraciklina, hlortetraciklina, ciprofloksacina i enrofloksacina u površinskoj vodi. Postupak se zasnivao na ekstrakciji na čvrstim fazama (SPE), razdvajanju i identifikaciji primjenom hromatografije na tankom sloju (TLC). Razdvajanje je provedeno na HPTLC silikagel 60 F254 pločama, uz mobilnu fazu voda/metanol/dihlormetan (6/35/59) (v/v). Ploče su prethodno impregnirane sa 10%-tnom otopinom EDTA pH 9,0, sušene u horizontalnom položaju najmanje dva sata na sobnoj temperaturi, a zatim se u sušnici na 105°C 30 minuta, neposredno prije upotrebe. Antibiotici su ekstrahirani primjenom OASIS HLB 6cc/500 mg kertridža. Volumen od 10 µl uzoraka vode i standardnih otopina apliciran je na ploču. Nakon razdvajanja, ploče su osušene na zraku, a hromatogrami su vizualizirani pod UV lampom na λ=254 nm i λ=366 nm. Rezultati su pokazali da se predložena metoda može primijeniti za simultanu identifikaciju ispitivanih antibiotika u uzorcima vode, u kojima su njihove koncentracije jednake ili veće od 5 µg/ml.

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Original Scientific Paper

Electrochemical characterization of (1E)-1-N-{[4-(4-{[(E)-N-(3-aminophenyl) carboxyimidoyl] phenoxy} butoxy) phenyl] methilidene} benzene -1,3-diamine

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Abstract: Oxido-reduction properties of a newly synthesized Schiff base were investigated by cyclic voltammetry and differential pulse voltammetry. Measurements were conducted in a three electrode voltammetric cell in a non-aqueous media. Glassy carbon was used as a working electrode, platinum wire as counter electrode and non-aqueous Ag/Ag^+ electrode as a reference electrode. Inert atmosphere was acomplished by system purging with high purity argon Ar 5 (ϕ_{Ar} = 99,999 %), before each measurement. Cyclyc voltammograms revealed one oxidation peak of the investigated Schiff base ($E_{p,a}$ = 0.69 V), which increased with increasing concentration (c = 3.1 $\cdot 10^{-5}$ mol dm^{-3}-1.25 $\cdot 10^{-4}$ mol dm^{-3}) and scan rate (v = 50-300 mV/s). Differential pulse voltammetry showed one oxidation peak $E_{p,a}$ = 0,69 V, which also increased with increasing concentration.

INTRODUCTION

Symmetrical bis-Schiff bases have been widely studied due to their pronounced biological and pharmacological activity (Liang, Xia, Lei, et al., 2014), optical (Fang, Cao, Chen, et al., 2014), photochromical (Zhao, Zhao, Liu, et al., 2001), thermochromical (Minkin, Tsukanov, Dubonosov, et al., 2011) properties and other outstanding material properties. Furthermore, they can easily form different types of polydentant ligands and because of their diversified donor groups (or atoms) are suitable as chelating agents. Complex compounds of Schiff bases are considered to be a transition state between simple coordination compounds and metalloproteins (Chandra and Pundir, 2008). Recently, symmetrical bis-Schiff bases were used in design of liquid crystals (Iwan, Janeczek, Jarzabek, et al., 2009). These compounds are often used as building blocks for synthesis of liquid crystal polymers or oligomers (Iwan and Sek, 2008).

In this study, we have synthesized a novel symmetrical bis-Schiff base (1*E*)-1-N-{[4-(4-{[(*E*)-N-(2-aminophenyl) carboxyimidoyl] phenoxy} butoxy) phenyl] methylidene} benzene-1,3-diamine. The synthesized molecule is a rare example of a symmetrical bis-Schiff base with uncondensed primary amino group (there are only few known examples of similar structures (Shakir, Abbasi, Azam, et al., 2011). The compound was characterized by means of IR spectroscopy, NMR spectroscopy and TG/DSC analysis. In addition we have studied oxido-reduction properties of the synthesized compound. Preliminary information acquired from this study is very useful as indicators of potential application of the synthesized Schiff base (i.e. potentiometric sensor, organic semiconductor, liquid crystal, etc.).

EXPERIMENTAL

Chemicals and apparatus

All commercially available chemicals were of reagent grade and used as purchased from commercial sources. Dialdehyde 4-[4-(4-formylphenoxy) butoxy]benzaldehyde was prepared by previously reported method (Balić, Marković and Balić, 2013). All solvents were purchased commercially. N,N-dimethylformamide (DMF) was purchased from Fischer Chemical and lithium chloride (LiCl) from BDH Prolabo and were used without further purification.

IR spectrum was recorded on a Shimadzu FTIR 8400S spectrophotometer using the DRS 8000 attachment, in the 4000-400 cm^{-1} region. The sample was mixed with KBr (IR grade). Thermogravimetric analysis was performed using a simultaneous TGA-DSC analyser (Mettler-Toledo TGA/DSC 1). The compound was placed in aluminium pan (100 µL) and heated in nitrogen atmosphere (200 mL min^{-1}) up to 590 °C at a rate of 10 °C min^{-1}. The data collection and analysis were performed using the program package STAR^e Software 10.0 (STARe, Software 10.0 (2809), Mettler-Toledo GmbH). The ^{1}H NMR and ^{13}C NMR were recorded on NMR (300 MHz) Bruker instrument, using deuterated chloroform as solvent at NMR Laboratory of the Ruđer Bošković Institute, Zagreb.

Electrochemical experiments were performed on PalmSens potentiostat/galvanostat (PalmSens BV, Utrecht, The Netherlands) driven by PSTrace 4.2 software. A conventional three-electrode cell was used with a glassy carbon as a working electrode, non-aqueous Ag/Ag^+ (Ag wire in acetonitrile solution of 0.01 M $AgNO_3$ and 0.1 M Tetrabutylammonium perchlorate) as a reference electrode and a platinum wire as a counter electrode. The glassy carbon working electrode was polished with coarse diamond polish (1 µm, ALS, Japan) and with polishing α-Al_2O_3 (0.05µm, ALS, Japan) before each measurement. Cyclic voltammetry scan rate was 100 mVs^{-1}. The differential pulse voltammetry conditions were: scan increment 5 mV, pulse amplitude 25 mV, pulse width 70 ms and scan rate 5 mV s^{-1}.

Synthesis of the title compound

Dialdehyde, 4-[4-(4-formylphenoxy)butoxy]benzaldehyde (0.6 g, 2 mmol) was dissolved in 40 mL of methanol and 0.274 mL (2 mmol) triethylamine was added to this solution. The solution was brought to brisk reflux and 0.49 g (4.5 mmol) of m-phenylenediamine dissolved in 25 mL of methanol was gradually added. The mixture was heated at reflux temperature for 3 hours. After the reaction was completed, the resulting mixture was left at room temperature for 24 hours. The white yellow product was filtered and washed with cold ethanol and diethyl ether. Yield: 62%.

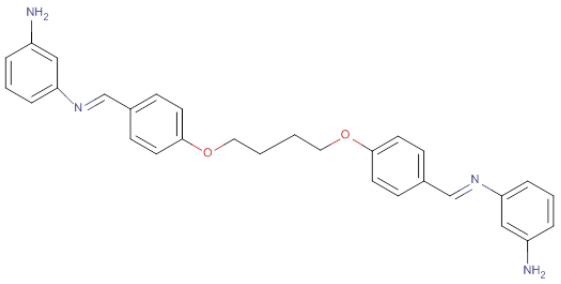

Figure 1: Structure of the title compound.

RESULTS AN DISCUSSION

Cyclic voltammetry studies

A cyclic voltammogram of the investigated Schiff base is shown in **Fig. 2.** One anodic peak is visible at a potential of 0.690 V when the potential was scanned from -0.2 V to 0.8 V vs. Ag/Ag^+ reference electrode. No reduction wave can be observed, indicating that the oxidation reaction is totally irreversible.

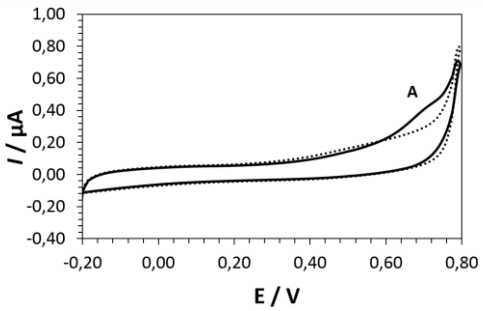

Figure 2. Cyclic voltammogram of the title compound (c = 1.1 $\cdot 10^{-4}$ mol dm^{-3}) at a glassy carbon electrode (I_c = 0.1 M LiCl in DMF). Scan rate: 100 mV/s. a) 0.1 M LiCl (supporting electrolyte), dashed line and b) title compound, solid line.

The influence of scan rate and the effect of concentration of the investigated Schiff base on anodic peak current and anodic peak potential were examined. It was observed that both anodic peak potential and anodic peak current increase with the increase in Schiff base concentration and scan rate. **Fig. 3** shows that at concentrations under $c \sim 1.1 \cdot 10^{-4}$ mol dm^{-3}anodic peak current is a linear function of the Schiff base concentration, which indicates that oxidation products are adsorbed on the glassy carbon electrode surface. At higher concentrations of Schiff base (above $c \sim 1.1 \cdot 10^{-4}$ mol dm^{-3}), the increase of peak current slows down, which could be explained by increase of interactions between molecules adsorbed on the electrode surface and by diffusion current (Zeng, Wei, Xiao, et al., 2006; Medvidović-Kosanović, Šeruga, Jakobek, et al., 2010). Oxidation peak current increases linearly with the title product concentration in the concentration range (c = $3.1 \cdot 10^{-5}$ mol dm^{-3}... $1.25 \cdot 10^{-4}$ mol dm^{-3}).

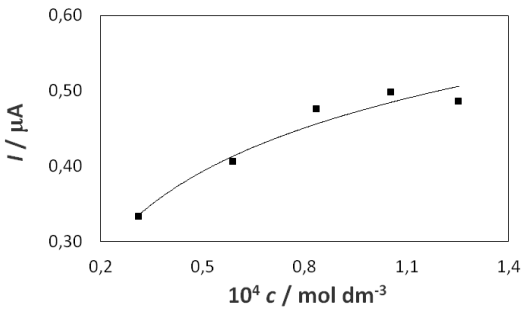

Figure 3. Anodic peak current as a function of the title compound concentration (I_c = 0.1 M LiCl in DMF). Scan rate: 100 mV/s.

Our research has shown that the Schiff base oxidation is controlled by diffusion since linear dependence (**Fig. 4a**) was found between anodic peak current and the square root of scan rate (Medvidović-Kosanović, Šeruga, Jakobek, et al., 2010; Simić, Manojlović, Šegan, et al., 2007). Logarithm of anodic peak current is also a linear function of logarithm of scan rate, with slope 0.40 (**Fig. 4b**) which also confirms that the oxidation process is diffusion controlled (Yagmur, Yilmaz, Saglikoglu, et al.,2014; Yagmur, Yilmaz, Saglikoglu, et al., 2013)

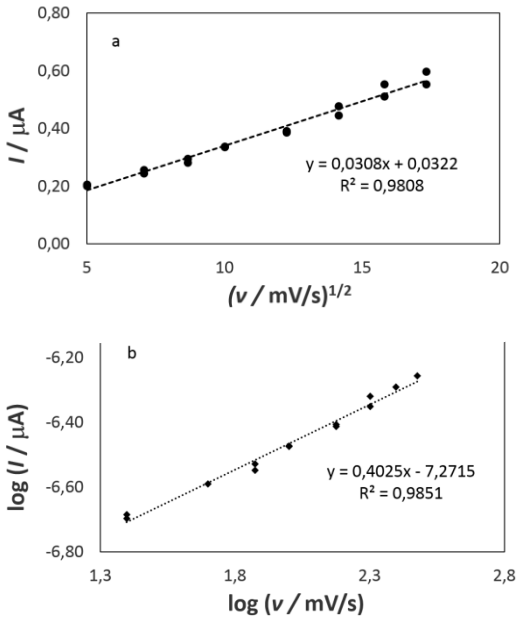

Figure 4. a) Anodic peak current, *I*, as a function of the square root of scan rate, $v^{1/2}$, b) Logarithm of anodic peak current, log *I* as a function of logarithm of scan rate, log *v*, at a glassy carbon electrode in solution of the title compound ($c = 8.4 \cdot 10^{-5}$ mol dm^{-3}, I_c = 0.1 M LiCl in DMF).

Differential pulse voltammetry studies

Differential pulse voltammogram in **Fig. 5** also reveals one oxidation peak of the investigated Schiff base at the potential of 0.690 V. The oxidation peak decreases with successive scans which confirms adsorption of the Schiff base oxidation products on the glassy carbon electrode surface.

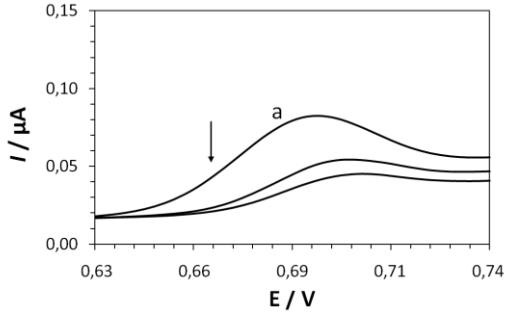

Figure 5. Differential pulse voltammogram of the title compound ($c = 1.1 \cdot 10^{-4}$mol dm^{-3}) at a glassy carbon electrode (I_c = 0.1 M LiCl in DMF). Scan rate: 5 mV/s**.** First scan (a).

Peak current and peak potential also increases with increasing Schiff base concentration (**Fig. 6**) which could be explained by kinetic limitation in the reaction between the redox sites of a glassy carbon electrode and the investigated Schiff base (Menati, Azadbakht, Taeb, et al., 2012).

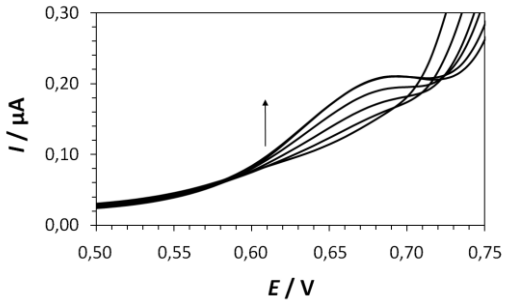

Figure 6. Differential pulse voltammograms in the solutions of the title compound with concentrations ($c = 0.7 \cdot 10^{-5}$; $0.9 \cdot 10^{-5}$; $1.1 \cdot 10^{-4}$; $1.3 \cdot 10^{-4}$; $1.5 \cdot 10^{-4}$ and $1.6 \cdot 10^{-4}$ mol dm^{-3}) at a glassy carbon electrode (I_c = 0.1 M LiCl in DMF). Scan rate: 5 mV/s

A linear relationship can be established between peak current and Schiff base concentration in the range of $0.5 \cdot 10^{-4}$ mol dm^{-3} to $1.6 \cdot 10^{-4}$ mol dm^{-3} (**Fig. 7**). A linear regression equation, $I_p = 10.969\ c + 0.4095$ with a correlation coefficient $R^2 = 0.9876$, is obtained, where I_p is the oxidation peak current and *c* is the Schiff base concentration

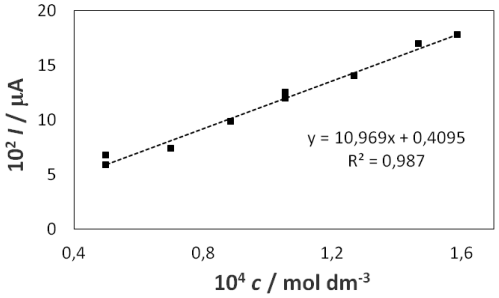

Figure 7. Anodic peak current as a function of the title compound concentration ($c = 0.5 \cdot 10^{-5}$; $0.7 \cdot 10^{-5}$; $0.9 \cdot 10^{-5}$; $1.1 \cdot 10^{-4}$; $1.3 \cdot 10^{-4}$; $1.5 \cdot 10^{-4}$ and $1.6 \cdot 10^{-4}$ mol dm^{-3}) at a glassy carbon electrode (I_c = 0.1 M LiCl in DMF). Scan rate: 5 mV/s.

CONCLUSION

The electrochemical results have shown that the oxidation of the Schiff base title compound is irreversible and controlled by diffusion at the investigated experimental conditions. Adsorption of the oxidation products on the glassy carbon electrode occurs and this process is kinetically limited. A linear relationship between peak current and Schiff base concentration in the range $0.5 \cdot 10^{-4}$ mol dm^{-3} to $1.6 \cdot 10^{-4}$ mol dm^{-3} was established.

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Summary/Sažetak

Ispitivana su oksido-redukcijska svojstva novosintetizirane Schiffove baze uporabom cikličke i diferencijalne pulsne voltametrije. Mjerenja su izvedena u troelektrodnoj ćeliji u nevodenom mediju pri sobnoj temperaturi, a inertna atmosfera je postignuta propuhivanjem sustava argonom visoke čistoće Ar 5 (ϕ_{Ar} = 99,999 %), prije svakog mjerenja. Kao radna elektroda korištena je elektroda od staklastog ugljika, protuelektroda je bila platinska žica, a kao referentna elektroda je korištena Ag/Ag^+ elektroda za nevodeni medij. Rezultati cikličke voltametrije su pokazali da se ispitivana Schiffova baza oksidira (uočen je jedan oksidacijski strujni vrh u anodnom dijelu voltamograma na potencijalu $E_{p,a}$ = 0.69 V, a visina oksidacijskog strujnog vrha raste s povećanjem koncentracije (c = $3.1 \cdot 10^{-5}$ mol dm^{-3}...$1{,}25 \cdot 10^{-4}$ mol dm^{-3}) i brzine promjene potencijala (v = 50...300 mV/s). Diferencijalnom pulsnom voltametrijom je također uočen jedan oksidacijski strujni vrh pri $E_{p,a}$ = 0.69 V, koji se povećavao s povećanjem koncentracije ispitivane Schiffove baze.

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Original Scientific Paper

Kinetin Induced Changes in Rutin content in *Knautia sarajevensis* (G. Beck) Szabó Shoot Cultures

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Abstract: *Knautia sarajevensis*, Dipsacaceae, is an endemic species found at wood margins and meadows only on mountains of Dinaric Alps. Members of this family are widely used in traditional medicine as rich sources of pharmacologically important substances. Since it is well known that flavonoid compounds are one of the main carriers of biological activities of plant extracts, the aim of this study was to investigate cytokinin effects in concentration changes of flavonoid constituents. Presence of four different flavonoid constituents was noticed: quercetin, naringenin, hesperetin and rutin in extracts of *K. sarajevenis* shoots cultivated on three *in vitro* treatments (control, 1.0 mg L^{-1} kinetin and 10.0 mg L^{-1} kinetin), but only rutin content was quantified. All extracts were prepared using dried material and 80% methanol HPLC grade. Analysis of rutin indicated that high cytokinin concentrations did induce improvement of rutin content, but these concentrations are still lower than those recorded for control treatment. Further analysis using different types and concentrations of cytokinins are necessary to establish a pattern of cytokinin induced concentration changes in content of these four investigated flavonoids in *Knautia sarajevensis*.

INTRODUCTION

Knautia sarajevensis (G. Beck) Szabó is a member of Dipsacaceae family, which is consisted from 10 genera and around 270 species, distributed through Europe, East Asia, south and central Africa (Mabberley, 2008). This is an endemic species of Bosnian Mountains (Ehrendorfer, 1980). Research regarding secondary metabolites of Dipsacaceae family included identification of active components (Rezanova *et* Naidakova, 1974; Alimbaeva *et al*., 1986; Movsumov *et al*., 2011), and only a few papers reported analysis of their bioactive properties (Hung *et al*., 2005; Chunmei *et al*., 2010). Some of the Dipsacaceae species are used in traditional Chinese medicine (Zhang *et al*., 2003), but in Europe this family is still not well investigated. From six, in Bosnia recorded Dipsacaceae genera (*Cephalaria* Schrad. Ex Roem. & Schult; *Dipsacus* L.; *Knautia* L.; *Scabiosa* L.; *Succisa* Heller; *Succisella* Beck), literature data regarding bioactive compounds are available only for a few species. Identified secondary metabolites from Dipsacaceae species beside medicinal importance are also used in chemotaxonomy. It is considered that Dipsacaceae family is characterized by loganin, loganic acid and swertosid (Jensen *et al*., 1979; Perdetzoglou *et al*., 2000; Horn *et al*., 2001).

Plant metabolism is consisted from primary (necessary for plant cell survival) and secondary metabolism (necessary for plant survival under changing environmental conditions). Production of secondary metabolites is usually very low (around 1% of dry mass), and it is dependent upon physiological and

developmental stage of the plant (Hartmann, 1996). All secondary metabolites can be classified into tri major groups: terpenes (cardiac glycosides, carotenes, sterols etc.); phenols (phenolic acids, coumarins, lignans, stilbenes, tannins and lignin) and components containing sulphur or nitrogen (alkaloids and glucosinolates) (Verpoorte, 2000). Phenols can be further grouped into simple phenols, phenolic acids, flavonoids, stilbenes, lignans and hydrolysed and condensed tannins (Shahidi *et* Ho, 2005). Flavonoids are diverse group of phenolic compounds and include flavones, flavanones, flavonols, flavanonols, isoflavons, flavanols (catechin) and anthocyanidins (Shahidi *et* Ho, 2005). Importance of secondary metabolites for humans is evident, since at least one quarter of all drugs in developed countries contain components that are directly or indirectly of plant origin. Furthermore, 11% out of 252 essential medicines are considered to be produced exclusively from flowering plants (Verpoorte, 2000). During photosynthesis and stress conditions reactive oxygen species (ROS) are formed in plant tissues. In normal conditions plant antioxidative system (AOX) scavenges radicals and protects the plant. Any kind of stress puts ratio between ROS and AOX out of equilibrium. To, stress induced, ROS accumulation plants enzymatic AOX is activated (including radical scavengers like SOD-superoxide dismutase, APX-ascorbate peroxidase, GPX-guaiacol peroxidases, GST-glutathione S-transferase etc.) and nonenzymatic antioxidative system (carotenoids and flavonoids) (Gill *et* Tuteja, 2010). Protective role of flavonoids in different biological systems is often attributed to their ability to scavenge radical electrons and chelate metal ions (Hernández *et al*., 2009). The aim of this study was to investigate effect of kinetin on rutin concentration as a bioactive compound.

EXPERIMENTAL

Plant material and *in vitro* culture: Seeds of *Knautia sarajevensis* were collected at Mt. Igman, Veliko polje, during august, 2012. Seeds were cultivated on Murashige *et* Skoog basal medium (1962; MS) containing 3% sucrose and without plant growth regulators (no PGR). Prior to cultivation seed coat and elaiosome was removed. After 30 days, seedlings were collected and cultivated on three different treatments (control – MS basal media without PGR; 1KIN – MS basal media containing 1 mg L^{-1} kinetin and 10KIN – MS basal media containing 10 mg L^{-1} kinetin). Before culture, root and apical meristem from shoots were removed in aseptic conditions. All media were autoclaved after pH adjustment to 5.7, and addition of 0.8% agar (HiMedia). All cultures were kept in growth chamber (light 2,000 lux, 70% humidity and temperature of 23 ±2°C).

Extract preparation: 80 mg of air dried plant material from each treatment was separately homogenized with addition of 80% HPLC grade methanol (SIGMA) and incubated 24 hours at 4°C, after which supernatant was collected for analysis.

Solution of standards: Flavonoids stock solutions of rutin, quercetin, naringenin and hesperitin were prepared in methanol. Solutions were kept in the dark at +4°C. Prior to injection into the HPLC system, all solutions were filtered through 0.45 μm syringe filter.

HPLC analysis: Qualitative analysis of the flavonoids rutin, quercetin, naringenin and hesperetin were done with DAD detector using wavelenght 290 nm for rutin and quercetin and at 370 nm for naringenin and hesperetin. Quantitative analysis of the rutin was done with DAD detector at 290 nm wavelennght. Analysis was performed on a column Eclipse XDB – C18 RP (4.6 mm x 250 μm, 5 μm particle size) by quaternary pump. Flow rate of mobile phase was 1 mL min^{-1}, injection volume 20 μL and the temperature of column was 35°C. Calibration curve with 5 points in the concentration range from 2.5 μg mL^{-1} to 100 μg mL^{-1} (start of the curve was set to zero) was established for rutin standard. Coefficients of correlation for all target analyses were $r^2 \geq 0.999$. The mobile phases were acetonitrile (MF - A) and 5% aqueous solution of acetic acid (MF - B) in gradient separation (0 min 5% B; 15 min 15% B; 25 min 15% B; 40 min 22% B; 70min 22% B; 80 min 25% B; 90 min 5% B; stop).

RESULTS AND DISCUSION

Analysis of rutin content in kinetin treated and control samples was performed (Figure 1). Kinetin was applied on shoot cultures of *K. sarajevensis* using solid culture. Analysis was preformed after 4 week of cultivation and presence of rutin was confirmed in all tested shoots independent upon treatment. Application of 1 mg L^{-1} kinetin induced decrease of rutin content, but application of 10 mg L^{-1} kinetin induced increase in rutin content, which was lower than in the control treatment (Figure 1). Qualitative analysis of *K. sarajevensis* shoot extracts showed the presence of selected flavonoids (quercetin, naringenin and hesperetin) in kinetin treated and control samples. Detected flavonoids were not quantified and were used only as qualitative parameters of shoot extracts.

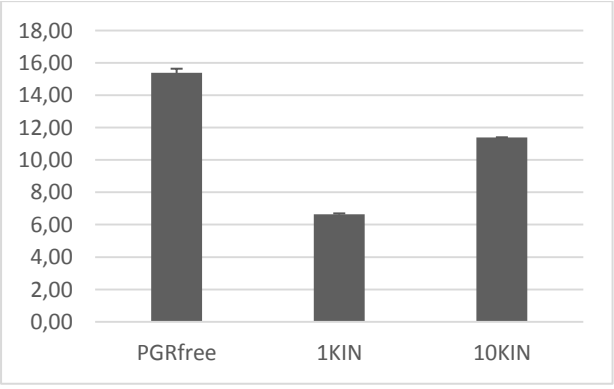

Figure 1. Content of analysed flavonoids (ng mL^{-1}) in shoot cultures of *K. sarajevensis* after kinetin treatment

PGRfree – MS basal media without PGR; 1KIN – MS basal media containing 1 mg L^{-1} kinetin and 10KIN – MS basal media containing 10 mg L^{-1} kinetin

Application of plant growth regulators can affect secondary metabolite production through effect of enzymes on phenylpropanoid synthesis (Sangwan *et al*., 2001). Plant growth regulators can stimulate flavonoid production, as recorded for BA application on *Arnica montana*, where stimulation of apigenin and luteolin production was noticed (Indu *et al*., 2013), stimulation of production of quercetin, catechin and myricetin was noticed for *Cyperus rotundus* (Krishna *et* Renu, 2013), or increased production of rutin, luteolin, quercetin and kaempferol in *Hypericum* (Pasqua *et al*., 2003; Shilpashree *et* Rai, 2009) was also detected.

Biosynthetic pathway and simulative mechanisms of flavonoid production is still not clear. The question remains does the plant growth regulators induce formation of different biosynthetic enzymes responsible for flavonoid biosynthesis (Shilpashree *et* Rai, 2009) or other mechanisms are responsible for such effect of plant growth regulators. Our research showed that application of kinetin induces changes in individual flavonoids, and this change is usually treatment dependent, but complexity of metabolic pathways that lead for such changes remain unclear.

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Summary/Sažetak

Knautia sarajevensis, Dipsacaceae, je endemična biljka koja naseljava rubna područja šuma dinarskih alpa. Pripadnici ove porodice su široko zastupljeni u tradicionalnoj medicine kao bogat izvori farmaceutski značajnih supstanici. S obzirom da je poznata činjenica da flavonoidne komponente su jedan od glavnih nositelja biološke aktivnosti biljnih ekstrakata, cilj ove studije nio je ispitati uticaj citokinina na promjene koncentracije flavonoidnih konstituenata. Utvršeno je prisustvo svačetiri ispitivana flavonoida: kvercetin, naringenin, hesperitin i rutin, u ektraktima izdanaka K. sarajevensis koji su kultivisani na tri in vitro tretmana (kontrola, 1.0 mg L-1 kinetina i 10.0 mg L-1 kinetina), ali je samo kvantifikacija izvršena za rutin. Svi ekstrakti su pripremljeni ekstrakcijom iz suhog materijala 80% metanolom HPLC čistoće. Analiza rutina pokazala je da visoke koncentracije citokinina povećavaju koncentraciju rutina, al ove koncentracije su i dalje niže nego one zabilježene za kontrolne biljke. Daljnja analiza sa većim rasponom koncentracija i različitim tipovima citokinina je neophodna za uspostavljanje obrascacitokinin induciranih promjena sadraja testitanih favonoida kod vrste K. sarajevensis.

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Orignal Scientific Paper

Knowledge of Atomic Structure and Visualization: A Research Results from Questionnaire with First-year Chemistry Students

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Abstract: This research study was conducted in order to assess the students' knowledge and ideas about basic concepts in both general chemistry and general physics courses. The research topic was knowledge of atomic structure that students already have and visualization of the atom based on that knowledge. Research examined how students' knowledge of scientific atomic theory has progressed during study year using a questionnaire as pretest and posttest. The study results showed that students' knowledge about atomic structure has predominantly descriptive and simplified character. Students have had their alternative visions of atomic structure based on their knowledge about Rutherford or Bohr model of the atom instead of the current scientific model of the atoms. That was a case even when they successfully completed the atomic structure questionnaire. Only 5% of the first-year chemistry students under this study showed an expected scientific literacy level related to the atomic theory topics after two semesters of study general chemistry and general physics. We propose different learning sequences to exceed this problem in order to help the freshmen to be prepared adequately for further more complex study. This approach is very important for the students' development of abstract thinking that is necessary for the complete scientific literacy.

INTRODUCTION

Atomic theory and atomic structure is an essential student learning topic that is an example of fundamental conceptual understanding of science as a subject matter. If it is based on the historical development and contribution of philosophers and scientists (Leucippus, Democritus, Dalton, Thomson, Rutherford, Bohr, Schrödinger and Heisenberg) it can be used as a supporting approach for helping students to better understand the abstract nature around us (Justi & Gilbert, 2000; Niaz et al., 2002; Park & Light, 2009). A main aim of science educators all around the world should be to teach their students to gain knowledge based on the scientific ideas and content of the contemporary atomic structure theory.

Description of participants and curriculum

Chemistry freshmen at Sarajevo University learn atomic structure theory according to both the *General Chemistry I* syllabus and *General Physics II* syllabus. In both mentioned courses a material is present through a lecture format. In Table 1 is given the *General Chemistry I and General Physics II* courses basic statistics.

Table 1. Course statistics

First-year Course	Academic year	Semester	Hour/ week	ECTS
General Chemistry I	2013-2014	Fall	3	5
General Physics II	2013-2014	Spring	4	4

In Table 2 is presented a list of atom structure related topics that chemistry freshmen learn. Almost the same topics relevant to this study goal are covered in the fall semester course *General Chemistry I* and in the spring semester course *General Physics II.* Both courses are done in a traditional lecture teaching format, and by more teacher-centered then student-centered approach. Average number of students in each class is around one hundred (75% as newly enrolled students and 25% of students retaking a class due to failing grade).

Table 2. List of topics included in two syllabi

Topic	General Chemistry I	General Physics II
Spectroscopy of the hydrogen atom (Lyman, Balmer, Paschen, Pfund series of lines in the spectrum of hydrogen)	-	+
Dalton's atomic theory	+	-
Leucippus -Democritus philosophy	+	+
Historical development of the model of the atom over time.	+	+
Thomson's "plum pudding" model of the atoms	+	+
Rutherford's "nuclear model" of the atoms	+	+
Bohr's "orbit" model of the atoms	+	+
Planck's theory on the quantization of light	+	+
Planck's, Heisenberg's and De-Broglie's contributions to the understanding of atomic structure	+	+
Quantum numbers and associated rules (e.g. Pauli Exclusion Principle)	+	+
Atomic orbital (s, p, d, f nomenclature)	+	+
Electron configurations	+	+
Quantum-mechanical model of the atom	+	+

Learning strategy based on the chemistry-physics knowledge integration is not present at the syllabi for "achieving students' common general physics and general chemistry courses outcomes" (Hadžibegovic & Galijašević, 2013). Aforementioned learning topics covered through numerous research goals appear to be a difficult ones for student attempts to understand the modern quantum theory (Harrison & Treagust, 2000; Taber, 2002).

Literature Background

Among first-year students of science study there are some common misconceptions about atomic theory what results in development of more negative attitudes towards chemistry and physics during the teaching-learning process (Taber, 2002; Tsaparlis & Papaphotis, 2002; Eilks, 2005, Park et al., 2009). In most physics-chemistry education research, the hybrid model of the atom used by students but different then any model of the atom as a curricular model (Justi & Gilbert, 2000) has been identified.

Several research results according to the atomic structure understanding by undergraduate students are selected. Researchers Cervellati and Perguini (1981) found some misconceptions by their Italian research participants related to the quantum-mechanical model of the atom. Some of their first-year university students understood orbitals as energy levels or as electron trajectories. Tsaparlis and Papaphotis (2002) presented in their study the research results about student difficulties related to their understanding of quantum-chemical concepts. They found that their first-year university students did not understand the electron configurations (around 6% of students gave partially correct answers). In the same research, Tsaparlis and Papaphotis identified some misconceptions by their students who showed a lack of a deep understanding of the quantum-chemical concepts (for example about atomic orbitals).

Taber (2002) in his study highlighted the research results by Cros and colleagues implying that university students showed some misconceptions about atomic models because they kept the atomic structure concepts based on planetary-type orbits „even after being taught about more sophisticed models" versus an abstract quantum-mechanical model of the atom.

Purpose

The research topic was the knowledge of atomic structure and visual models of the atom that students already have. Science teachers consider that a literate individual in science need to know what is a current scientific model of the atom after four years of school. This is an average number of years devoted to the learning science at primary and secondary school level in Bosnia and Herzegovina. In the same time, science teachers at university level have an expectation best explained as DeBoer's (2000) individual that is "well informed, cultured, literate individual" and must know after two first-year semesters of studying science that the quantum-mechanical model of the atom is a current scientific model of the atom today. The researchers' goals were to find the answers to the following research questions:

RQ1: What were the learning outcomes of two courses dealing with contemporary atomic theory?

RQ3: What misconceptions were apparent?

To answer these questions the students were given pre- and post-tests to measure their atomic structure and electron configuration knowledge and personal visualization of the atom.

METHOD

Participants

The participants were 75 first-year students (77% of female and 23% of male) at Sarajevo University in Bosnia and Herzegovina. The students were from different regions of Bosnia and Herzegovina completed different secondary school education before their university study as following:

Grammar school (75% of students);
Middle medical school (23% of students) and
Middle technical school (2% of students).

Research Instrument

For the purpose of this study we developed a test as research instrument (The Atomic Structure Questionnaire (ASQ) to measure the students' knowledge and understanding of the atomic structure and atomic models of the atom. Four learning categories were under testing focus:

(1) Atomic structure (electron configurations, shells, atomic orbitals, quantum numbers);

(2) Theoretical atomic models and the history of the atomic models (measured by the number of known atomic models mentioned by each student);

(3) Representation of an atom (measured by quality of an atom illustration according to an atomic model used);

(4) Current atomic theory (measured by the number of students' knowledge elements of quantum theory of atoms);

ASQ was created as a diagnostic test as a set of 12 questions: the first nine in the multiple choice format, one short-answer question related to the Calcium electron configuration, and last two questions as open-ended questions. The student ASQ achievements were evaluated based on 12 points in total (attached in the Appendix).

RESULTS

Quantitative results

ASQ results

The 75 chemistry freshmen among 110 of students who were taking the *General Physics II* class were tested. Students' ranking statistics is presented in Table 3 according to their ASQ correct pretest and posttest answers and values of the normalized gain.

Table 3. ASQ correct answer statistics

Question	Pretest	Posttest	<g>
Q1	53%	66%	0.277
Q2	76%	77%	0.042
Q3	79%	81%	0.095
Q4	76%	76%	0
Q5	58%	63%	0.119
Q6	77%	82%	0.217
Q7	87%	89%	0.154
Q8	93%	93%	0
Q9	85%	89%	0.267
Q10	84%	89%	0.312
Q11	5%	6%	0.011
Q12	5%	6%	0.011

Note: <g>=(posttest%-pretest%)/(100%-pretest%).

Students' knowledge evaluation related to the history of atomic models (Q1 results) and certain values of the angular momentum quantum number (Q5 results) was at the lowest student achievement in both pretest and posttest results. The students showed very weak understanding of the current quantum-mechanical model of the atom and only few of them have developed their scientific atomic theory literacy. The value of the normalized gain factor for each ASQ answer is an evidence of a weak progress after an in-class discussion session and four additional teaching hours related to the topics included in the ASQ questions, and realized as pre-post test application.

By one student was achieved both the lowest number of points (0.5) and the highest number of points (12 points) as Figure 1 shows.

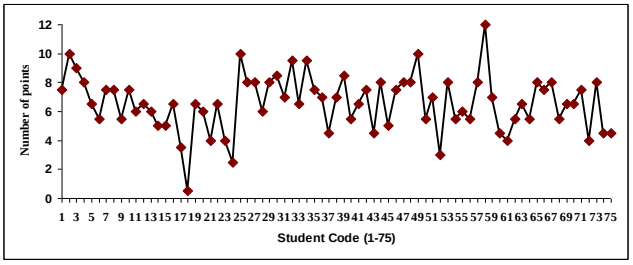

Figure 1. Distribution of the ASQ achieved pretest points by 75 students (12 points as the ASQ highest number of points).

Five categories of students according to the achieved ASQ points (Figure 2) are:

Category A (0 – 4.5 points): The lowest knowledge status.

Category B (5 – 6.5 points): Low knowledge status.

Category C (7 – 9.5 points): Medium knowledge status.

Category D (10 - 11 points): High knowledge status.

Category E (11.5 - 12 points): The highest knowledge status.

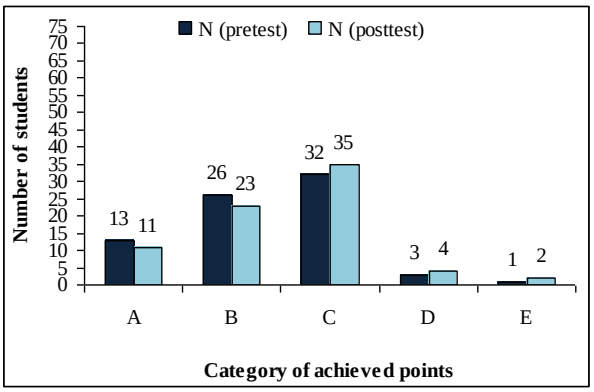

Figure 2. Distribution of the ASQ pretest/posttest points by student category.

Medium knowledge status was showed by the 32 (43%) students, but 39 (52%) students showed a low level of knowledge according to the ASQ (pretest) answers. Similar posttest data set was found presenting a weak progress and remained difficulties especially regarding to the ASQ Section C student answers. For illustration of students' results of remained medium knowledge status is an evidence of the average number of achieved 6.6 points and 7.4 related to the pretest and posttest results respectively. Probably the students under this study need better teaching-learning strategy to help them to achieve learning outcomes as correct ones related mainly the contemporary atomic theory.

Qualitative research results

A selection of pretest qualitative research results is presented here related to the students' visualization of the atom. The pretest ASQ results showed that the 62 (83%) student drawings presented students' ideas of an atom in the section C answers. According to the analysis of student illustrations of an atom four different student models of the atom were presented (Figure 3). Very similar results were found in students' posttest results.

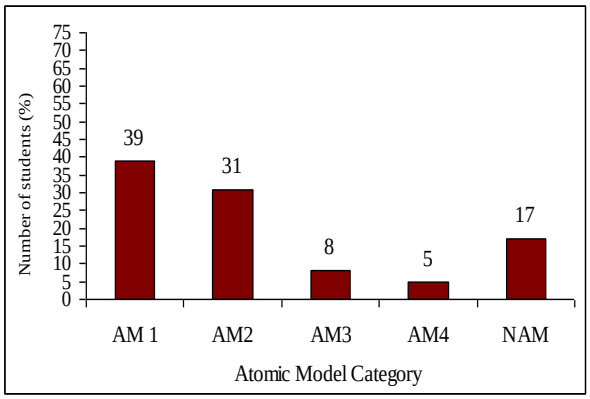

Figure 3. Atomic models presented in the student pretest Q11 illustrations.

Legend:

AM1: Rutherford model of the atom.
AM2: Bohr model of the atom.
AM3: Thompson model of the atom.
AM4: Quantum-mechanical model of the atom.
NAM: without any atomic model.

A small number of students who correctly answered the Q11 and Q12 showed students' poor knowledge about the contemporary atomic model (Table 3 & Figure 3). Only four student illustrations contained the students' written descriptions behind their illustrations as principally correct one. Did not a single student provide a proof of the contemporary model of the atom (Quantum-mechanical model of the atom). Only 5% of students showed some elements that could be considered as the AM4. Mostly, the students presented their ideas about the atom similar to Rutherford-Bohr model of the atom (around 50% of students). It is important to note that students learn about six historically important atomic models (Ancient Greek model, Dalton's model, Thomson's 'embedded mass' model, Rutherford's 'nuclear' model, Bohr's 'orbit' model, and Quantum-mechanical model) in *General Physics II* class.

Selection of student drawings of the atoms

A selection of students' pretest atom visualization according to the AM1 is presented in Figure 4. There is a sketch of an atom with a positive nucleus and perception of negative electrons in a correct shell range K, L, M, O, P and Q (Figure 4-a). Other student presented her/his idea of an atom with wrong shell names (Figure 4-b).

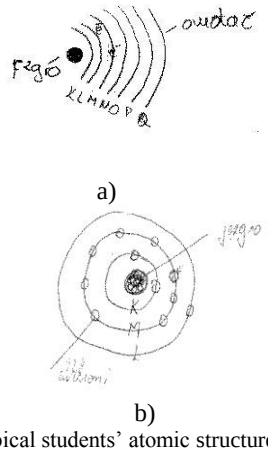

Figure 4. Two typical students' atomic structure pretest illustrations related to the Bohr's orbit model.

Figure 5 shows an AM3 illustration presenting textually Thompson's 'embedded mass' model of the atom known as "plum pudding" system.

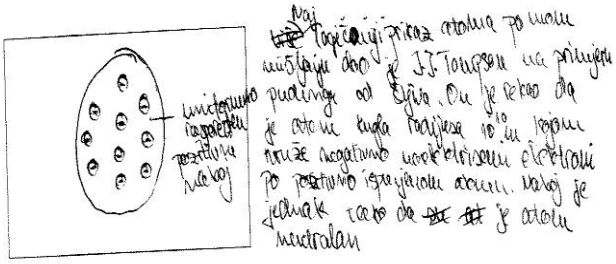

Figure 5. An AM3 pretest example.

Note: Text of student explanation translated from Bosnian into English is following:
In the frame of drawing is written: *uniformly distributed positive charge.*
The text of student's explanation translated from Bosnian into English is:
In my opinion the most logical representation of an atom gave J.J. Thompson taking an example of the plum pudding. He considered that an atom is a sphere radius 10^{-10} m where negative electrons rotate in positive filled atom. The charge is equal so that the atom is a neutral atom.

Figure 6. shows a student's sketch of an atom showing some knowledge of Quantum-mechanical model of the atom.

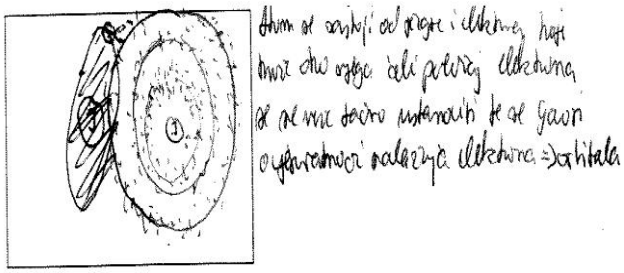

Figure 6. An AM4 pretest example.

Note: The text of student explanation allied to the illustration is translated from Bosnian into English as follows:
An atom consists of its nucleus and electrons rotate around it but electrons' positions can not be accurately identified and it is a talk about probability of electrons' position finding (a symbol for following) orbitals.

ASQ Question 12 Results

Q12: a) *Provide at least three historically significant models of the atom;* b) *Specify which known atomic model is a contemporary/current scientific model of the atom and provide your explanation.*

The pretest answers to the Q12-a are presented in Figure 7 showing the students' answers in the written format and four different categories according to the number of atomic models that they knew about.

Most of the students (42%) in the ASQ Q12-b answers stated that the Bohr model of the atom is a still valid one. Only five students (7%) answered that a current model of the atom is Quantum-mechanical model. The most important and significant is the evidence that 51% of students did not give their Q11 answers.

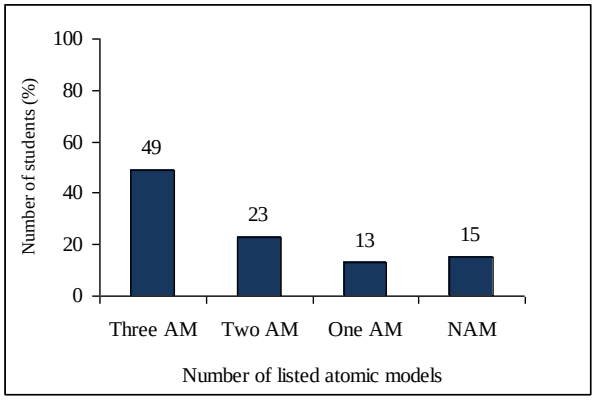

Figure 7. Percentages of student pretest answers to the Q12-a. *Notes*: AM = atomic model; NAM= without any AM.

The students gave their reflections after the ASQ implementation during an in-class discussion about atomic models and which one is a current model. In answer to RQ1 around 60% of students did not show any change according to the Bohr model of the atom as a favorite one which does not provide an accurate scientific model of the atom. Probably a main reason for such students' consideration is a powerful influence of syllabi and textbooks that give this theory a significant role in the learning process. Similar results were found when student understanding of Bohr model of the atom looking from "the point of view of the history of science" was tested (Hadžibegović & Galijašević, 2013). According to the Hadzibegovic-Galijasevic findings their research participants (58 chemistry freshmen) had beliefs that Bohr model of the atom appeared "as one the entire time valid scientific model" what is very similar to this study results.

In answer to RQ1 around 30% of the students were at medium knowledge status, but more then 50% of students showed a lower level of the atomic theory knowledge. Since these students are chemistry majors, who have chosen to study chemistry, one can find that the unexpectedly small number of students possessed higher level of knowledge according to the testing questions. A main reason could be that most of enrolled freshmen probably did not come at university with a strong chemistry-physics background. It opens a question of their adequate prior knowledge of physics and chemistry that is required for such kind of study choice.

In answer to RQ2, the students' misconceptions are similar to the findings of several researchers (e.g. Tsaparlis, 1997; Tsaparlis & Papaphotis, 2002; Nakiboglu, 2003):

1. An orbital is a shell in which electrons are placed (30 % of students).
2. Orbitals are electrons' trajectories arranged around the atomic nucleus where electrons rotate (38 % of students).
3. An orbital is an energy level of the electron (8% of students).
4. Students use the Solar System Model or a simple nucleus-electron shell model in explaining the structure of atom (around 55 % of students).
5. The electrons rotate around the nucleus like the planets around the sun (around 50 % of students).
6. Orbitals are equivalent to orbits or shells (around 35 % of students).

It is also important to note that among tested students around 25% of them did not know and probably have had difficulties to understand the meaning of quantum number (n, l, m_l, m_s). In some way, these research results showed a possible confirmation that an entry exam is reasonably needed to select future chemistry freshmen.

CONCLUSIONS

The study results of student knowledge evaluation related to atomic structure understanding are not adequate for chemistry freshmen. Only 5% of first-year chemistry students under this study showed after two semesters of study general chemistry and general physics, an expected scientific literacy level related to the atomic theory topics. As a recommendation what can be changed in both *General Chemistry I* and *General Physics II* one can consider that the instructors need to be focused on meaningful student learning to achieve a coherent student understanding of the same topics relevant for the second-year *Physical Chemistry* course content and other syllabi at higher study years. It is also important to develop a strategy to harmonize two syllabi content in a way that, knowledge needs to be gained in *General Chemistry I* into *General Physics II* using two techniques: integration and differentiation. In such way the chemistry students could much easier make links among related atomic structure concepts to overcome their misconceptions (Tsaparlis & Papaphotis, 2002).

The instructors involved in the teaching general physics and chemistry courses through the teaching-learning process need to bring performances for innovation and creativity in the classroom. They need to motivate students to be actively involved for building competitive learners as future professionals in chemistry. This approach is very important for the students' development of abstract thinking that is necessary for the complete scientific literacy.

According to Taber (2002) to identify a problem of understanding some learning content is "only one step in the process of using research to inform practice". Both chemistry/physics instructor and students need to improve

the teaching-learning process for enhancing student's scientific literacy and learning outcomes especially of quantum theory to understand the more complex context of molecular systems at higher years of study.

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Summary/Sažetak

Ova studija se odnosi na istraživanje koje je provedeno da se procijeni znanje studenata i ideje o osnovnim konceptima u oba predmeta, općoj hemiji i općoj fizici. Predmet istraživanja je bilo znanje studenata o atomskoj strukturi i njihove predstave o modelu atoma. Istraživano je znanje studenata o naučnoj teoriji o atomskoj strukturi i kako su studenti napredovali za vrijeme prve godine studija, korištenjem pred-testa, post-testa i individualnih intervjua koji su provedeni s nekoliko učesnika u istraživanju. Rezultati, prikazani u ovom radu, pokazuju da je znanje studenata o atomima i njihovoj strukturi pretežno deskriptivnog i pojednostavljenog karaktera. Studenti su imali alternativne vizije atomske strukture, koje se temelje na njihovom poznavanju Rutherfordovog i Bohrovog modela atoma, umjesto važećeg naučnog modela atoma. To je bio slučaj čak i onda kada su studenti uspješno riješili test o strukturi atoma. Kako bi se pomoglo da studenti prve godine studija hemije prevaziđu probleme razumijevanja atomske strukture predlažemo različite sekvence učenja koje bi pomogle studentima da se adekvatno pripreme za mnogo kompleksnije sadržaje studija hemije. Takav pristup je vrlo važan za razvoj apstraktnog mišljenja za cjelovitu naučnu pismenost studenata.

APPENDIX

ATOMIC STRUCTURE QUESTIONNAIRE

Section A (0.5 point for each correct answer; you should circle only one answer.)

Q1. Niels Bohr scientific contribution in 1913 was:
a) Atom discovery b) Photon discovery c) Equation $E = h\nu$ discovery
d) Discovery of the atomic theory of the electron orbits and those electrons could only have certain energies
Q2. Energy of the 535, 6 nm photon is:
a) 2, 28 $\cdot 10^{-19}$ J b) 3, 57 $\cdot 10^{-19}$ J c) 3, 71 $\cdot 10^{-19}$ J d) 5, 63 $\cdot 10^{-19}$ J
Q3. *s, p, d, f, g* are symbols to qualify atomic states with the angular momentum quantum number (*l*) values:
a) 0, 2, 4, 6, 8 b) 1, 2, 3, 4, 5 c) 0, 1, 2, 3, 4 d) $0, \pm1, \pm2, \pm3, \pm4, \pm5$
Q4. If value of the principal quantum number is 3 (*n* = 3) an impossible orbital is:
a) 3s b) 3f c) 3d d) 3p
Q5. If value of the principal quantum number is 4 (*n* = *4*) the allowed values of the angular momentum quantum number (ℓ) are:
a) 1, 2, 3 b) 1, 2, 3, 4 c) 0, 1, 2, 3, 4 d) 0, 1, 2, 3
Q6. If a value of the angular momentum quantum number is 3 (ℓ = 3), the magnetic quantum number (m_l) allowed values are:
a) 0, 1, 2 b) 0, 1, 2, 3 c) -3,-2,-1, 1, 2, 3 d) -3,-2,-1, 0, 1, 2, 3
Q7. The number of electrons in the *3d* orbital is:
a) 10 b) 2 c) 4 d) 5
Q8. The number of electrons of the $1s^2 2s^2 2p^6 3s^1$ configuration is:
a) 8 b) 6 c) 11 d) 12
Q9. The Potassium atom (K) electron configuration is:
a) $1s^2 3s^2 4s^1$ b) $1s^2 2s^2 3s^2 4s^1$ c) $1s^2 2s^2 2p^6 3s^2 3p^6 4s^1$ d) $1s^{19}$

Section B **(0.5 point)**
Q10. The Calcium (Ca) ground state electron configuration is: _____

Section C
Q11. (2 points) Sketch your idea of an atom. Explain each element of your drawings.

Your drawing of an atom

The drawing elements explanation:

Note: If student knowledge of the current atomic model is showed it brings two points.

Q12. (**2 points**) Give at least the names of three atomic models historically relevant ones and which is currently valid one.

Note: If student knowledge of the current atomic model is showed it brings one point

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Short Communication

Chemical reactivity and stability predictions of some coumarins by means of DFT calculations

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Abstract: Three synthesized coumarin derivatives were studied for their quantum-chemical properties. These compounds are commonly used as starting material for many syntheses of biologicaly active compounds. In order to explore the theoretical-experimental consistency, DFT global chemical reactivity descriptors (chemical hardness, total energy, electronic chemical potential and electrophilicity) were calculated for these compounds using standard Spartan 10 software. Complete geometry optimization was carried out by B3LYP/6-31G* level of theory. Some quantum-chemical calculations correlated well with experimental work; mechanisms of reactions with these compounds as starting material were partially explained by these calculated parameters. These calculations were once more proof that by means of mathematical models it is possible to describe chemical interactions and simulate the behavior of chemical systems – molecules and reactions.

INTRODUCTION

Density Functional Theory (DFT) has been accepted as reliable and effective approach for the computation of molecular structure, vibration frequencies and energies of chemical reactions (Beyramabadi, Morsali, 2011; Mebi, 2011; Monajjemi, Sayadian, Zare, et al., 2011; Kadhum, Al-Amiery, Shikara, et al., 2011). It provides an efficient method to include correlation energy in electronic calculations (Koch, Holthausen, 2011). In addition it constitutes a solid support to reactivity models (Pearson, 1997). Reactivity of the molecule is always governed by its electronic properties and kinetic and thermodynamic stability.

4-Hydroxycoumarin and some simple derivatives are commonly used as starting material for many chemical reactions. Great number of syntheses of biologicaly active compounds, starting from 4-hydroxycoumarin, has been described in literature. These reactions are characterized to follow mechanism of electrophilic substitution where 4-hydroxycoumarin reacts with strong electron withdrawing groups (Završnik, Špirtović-Halilović, Softić, 2011). Acetylated derivative of 4-hydroxycoumarin (3-acetyl-4-hydroxycoumarin) is involved in some reactions of nucleophilic addition (Završnik, Špirtović-Halilović, Softić, 2011; Špirtović-Halilović, Salihović, Džudžević-Čančar, et al., 2014). Also, there are references on 4-hydroxy-3-iminomethylencoumarin as a precursor undergoing hydrolysis, thus giving next precursor involved in various synthetic routes (Završnik, Bašić, Bečić, et al., 2003).

In this computational study the structural and electronic properties of the 4-hydroxycoumarin and its two 3-substituted derivatives were investigated and used to predict their relative stability and reactivity.

METHODOLOGY

Density function theory (DFT) study

In order to explore the theoretical-experimental consistency, quantum chemical calculations were performed with complete geometry optimizations using standard Spartan 10 software. Geometry optimization was carried out by B3LYP/6-31G* level of theory. The chemical reactivity descriptors calculated using DFT are: total energy (E), chemical hardness (η), electronic chemical potential (μ) and electrophilicity (ω).

Chemical hardness measures the resistance to change in the electron distribution or charge transfer and it associates with the stability and reactivity of a chemical system. On the basis of frontier molecular orbitals, chemical hardness corresponds to the gap between the HOMO and LUMO. Chemical hardness is approximated using equation 1 (Peters, Lanzilotta, Lemon, et al., 1998).

$$\eta = (E_{LUMO} - E_{HOMO})/2 \quad (1)$$

where E_{LUMO} and E_{HOMO} are the LUMO and HOMO energies. Electronic chemical potential is defined as the negative of electronegativity of a molecule and calculated using equation 2.

$$\mu = (E_{LUMO} + E_{HOMO})/2 \quad (2)$$

Physically, μ describes the escaping tendency of electrons from an equilibrium system.

Global electrophilicity index (ω), is calculated using the electronic chemical potential and chemical hardness as shown in equation 3.

$$\omega = \mu^2/2\eta \quad (3)$$

This index measures the propensity or capacity of a species to accept electrons. It is a measure of the stabilization in energy after a system accepts additional amount of electronic charge from the environment.

RESULTS AND DISCUSSION

Structural and electronic properties

DFT calculations were performed for coumarin derivatives **1**, **2** and **3**. Optimized molecular structures of the most stable form are shown in Figure 1.

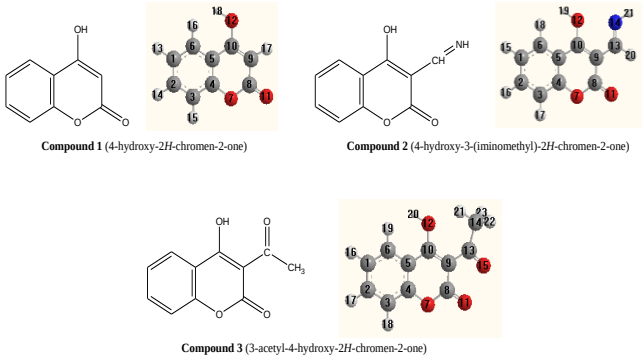

Figure 1. The 2D and 3D structures of coumarin derivatives

Molecular orbital calculations provide a detailed description of orbitals including spatial characteristics, nodal patterns and individual atom contributions. The contour plots of the frontier orbitals for the ground state are shown in Figure 2, including the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO).

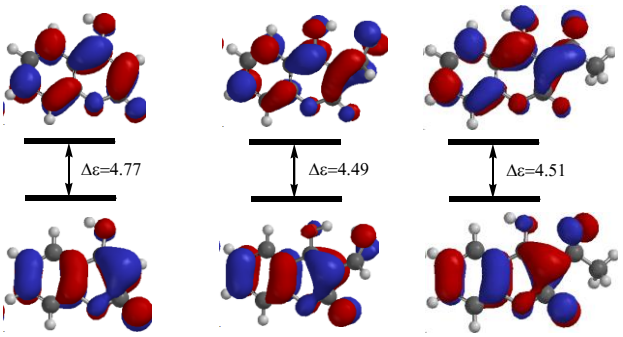

Figure 2. Frontier molecular orbitals of coumarin derivatives

Table 1. Global chemical reactivity indices of coumarin derivatives

	1	**2**	**3**
E (au)	-572.26	-665.71	-724.90
E_{HOMO} (eV)	-6.31	-6.48	-6.49
E_{LUMO} (eV)	-1.54	-1.99	-1.98
Dipole moment (debye)	8.18	7.15	8.57
logP	0.92	0.12	0.67
Energy gap (Δ) (eV)	4.77	4.49	4.51
η (eV)	2.38	2.24	2.25
μ (eV)	-3.92	-4.23	-4.23
ω (eV)	3.23	3.99	3.98

It can be seen that the energy gaps between HOMO and LUMO of compound **1** is 4.77, compound **2** is 4.49 and compound **3** is 4.51. The lower value in the HOMO and LUMO energy gap explains the eventual charge transfer interaction taking place within the molecules (Kadhum, Al-Amiery, Musa, et al., 2011). The larger the HOMO–LUMO energy gap, the harder and more stable/less reactive the molecule (Chattaraj, Maiti, 2003; Liu, 2005; Kadhum, et al., 2011).

Table 1 (row 7) contains the computed chemical hardness values for compounds **1**, **2** and **3**. The results indicate that compound **1** is harder and less reactive than compound **3** which is harder and less reactive than compound **2**. The values of μ for compounds **1**, **2** and **3** are presented in Table 1 (row 8). The trend in electronic chemical potential for coumarin derivatives is **2** > **3** > **1**.

The greater the electronic chemical potential, the less stable or more reactive is the compound. Therefore, compound **2** is more reactive than compounds **1** and **3**.

Compound **1** is the least reactive. The electrophilicity values (Table 1, row 9) for the compounds **1**, **2** and **3** are 3.23 eV, 3.99 eV and 3.98 eV. Among the compounds, compound **2** is the strongest electrophile while compound **1** is the strongest nucleophile. A good, more reactive, nucleophile is characterized by a lower value of ω and a good electrophile is characterized by a high value of ω.

Electrostatic potential charges and related quantum chemical properties

The distribution of the electronic density (electrostatic potential charges), related quantum chemical parameters [dipole moment (Table 1, row 4), HOMO/LUMO gap (Table 1, row 6) and the partition coefficients of the compounds (logP; Table 1, row 5)] were calculated for observed coumarines. These values and properties are very useful and can be used in order to evaluate chemical properties and possibilities for interaction of coumarines with biological macromolecules (receptors, enzymes). All these structural, electronic parameters and logP can be also used for a building of quantitative structure-activity relationship (QSAR) model, because all of them are closely related to pharmacokinetics (absorption, distribution, metabolism and excretion) and pharmacodynamics.

CONCLUSIONS

Obtained quantum-chemical calculations correlate well with experimental research related to coumarin compounds. Reaction mechanisms involving these compounds as precursors for many synthetic routes are highly explainable by this computational method and given results. These calculations were once more proof that by means of mathematical models it is possible to describe chemical interactions and simulate the behavior of chemical systems – molecules and reactions.

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Summary/Sažetak

Za tri sintetizirana kumarinska derivata ispitana su njihova kvantno-hemijska svojstva. Ispitivani spojevi polazne su komponente u mnogim reakcijama dobijanja biološki aktivnih spojeva.

U svrhu istraživanja teoretsko-eksperimentalne dosljednosti, DFT deskriptori globalne hemijske reaktivnosti (hemijska tvrdoća, totalna energija, elektronski hemijski potencijal i elektrofilnost) za ove supstance su izračunati koristeći standardni Spartan 10 program. Kompletna geometrijska optimizacija je urađena na B3LYP/6-31G* teorijskom nivou.

Neke kvantno-hemijske kalkulacije se dobro slažu s eksperimentalnim istraživanjima na ovim spojevima. Mehanizmi reakcija u kojima su ovi spojevi početni reaktanti mogu se djelimično objasniti rezultatima ovih proračuna.

Ovo izračunavanje je još jedan u nizu dokaza da je pomoću matematičkih modela moguće opisati hemijske interakcije i simulirati ponašanje hemijskih sistema – molekula i reakcija.

INSTRUCTIONS FOR AUTHORS

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Examples of ***reference style***:

a) Reference to a journal publication:
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d) Reference to a proceeding:
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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).

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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.

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- 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*.

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