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The Antioxidant Analysis of Selected Types of Climbing Plants with Therapeutic Effect

Original Paper

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Abstract Climbing plants with therapeutic effects are relatively little studied group of medicinal species. In the collection of climbing species planted in 2019 in the Botanical Garden of the Slovak University of Agriculture (SUA) in Nitra, we observed the growth and potential food, horticulture and healing use. For the antioxidant analysis, 7 species from 152 plants of 13 genera and 22 species were selected. The highest antiradical activity was recorded in the species *Vigna sinensis*, where reached average measured values achieved up to 73.79%. However, the related species *Vigna unguiculata* achieved only 26% of antioxidant activity. Of the fruits, the highest activity was *Momordica charantia* in immature state (73%). Other fruits showed very low antiradical activity

Keywords *Antioxidant activity – climbing plants – fruits – medicinal species – therapeutic effect*

INTRODUCTION

In 2019, the Botanical Garden of SUA in Nitra planted a collection of creeping medicinal plants in order to monitor their growth and potential food, horticulture and healing use. All the species were grown from seed obtained as a gift and seed exchanged through the international network *Index seminum*.

The use of climbing medicinal plants is very wide. In addition to their therapeutic function, they are used as decorative liana in outdoor expositions (*Basella* L., *Lablab* Adans., *Ipomoea* L.), but also as food (*Phaseolus* L., *Cyclanthera* Schrad., *Vigna* Savi), oil source (*Lagenaria* Ser.), as washing sponges (*Luffa* Mill.), soap replacement (*Vigna* Savi), vessels and musical instruments (*Lagenaria* Ser.) and other. Vertical vegetation is realized in current plantations either as planting of plants vertically on various constructions or as planting of climbing plants using natural height growth. For food purposes, fruits and seeds are processed in the form of canned products, various sauces or dried. The medicinal effects of these plants are used primarily in folk medicine, but in some cases, also in the pharmaceutical industry.

The selected species are a diverse group of plants in terms of botany. *Cyclanthera pedata* (L.) Schrad, *Diplocyclos palmatus* (L.) C. Jeffrey, *Luffa acutangula* (L.) Roxb., *Momordica charantia* L. and *Trichosanthes cucumerina* L. belong to family *Cucurbitaceae*. From family *Leguminosae* are *Vigna unguiculata* (L.) Walp. and *Vigna sinensis* (L.) Savi ex Hassk.[23]

Cyclanthera pedata (L.) Schrad, native to the Andes, are largely used in South America for their anti-inflammatory, hypoglycaemic and hypocholesterolemic properties.[16] Plant chemicals of fruits: phenols, peptin, galacturonic acid, picrin, lipoproteins, flavonoids, glycosides, mucilage, alkaloids, lipids, tannins, terpenes, resins, carbohydrates, sterols, scoparin, vitamins, vitexin, and minerals.[24]

Diplocyclos palmatus (L.) C. Jeffrey is an annual climber with bright red fruit and is reported to be highly medicinal.[11] The chloroform and methanolic extracts of aerial parts of this species contained alkaloids, flavonoids, tannins, saponins, diterpenes, triterpenoids, glycosides and steroids.[4]

Luffa acutangula (L.) Roxb. is a perennial climber native to southern and western India. It is commonly known as ridge gourd, sponge gourd or angled lufa.[10] In Ayurveda, fruits and seeds of *L. acutangula* used to treat jaundice, biliousness, bronchitis and asthma.[12]

L. acutangula has been shown to possess CNS depressant activity,[15] in vitro antioxidant activity[3] and larvicidal activity.[17]

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Momordica charantia L. contains biologically active chemicals that include glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids.[19] Some clinical trials have also confirmed the hypoglycaemic action.[14]

Trichosanthes cucumerina L. has been traditionally used as anthelmintic and antipyretic remedies. The root has been used to treat bronchitis and heart disease.[18] The plant is a good source of bioactive compounds such as carotenoids, flavonoids, and phenolic acids and this makes it a suitable antioxidant source.[1]

Vigna unguiculata (L.) Walp. is native to tropical Africa, where it still grows wild. It is a prostrate or climbing leguminous plant producing narrow, straight or slightly curved pods, 8–100 cm long, but not exceeding 1 cm in width.[7] It is a good source of phenolic compounds[2] with the potential to protect against chronic diseases including cancer.[13]

Vigna sinensis (L.) Savi ex Hassk. (syn.: *Vigna unguiculata* (L.) Walp.) contain bioactive compounds such as vitamins, carotenoids and phenolic compounds.[8] The seeds contain phenolic compounds in various forms: hydroxybenzoic and hydroxycinnamic acids, both free and bound to other molecules, as organic acids, in esterified forms.[8,22,9] Significant variability in seed protein and some of the essential amino acids has been identified in cowpea accessions.[21]

MATERIAL AND METHODS

The assortment of climbing plants with therapeutic effects was obtained in the form of seeds by gift and seed exchange through the international network *Index seminum*. All the analysed species were planted in the Botanical Garden of SUA in 2019. Because most of the species included in the collection originate from tropical and subtropical areas, the grown under protected environment of foil in spring time was suggested. The selected assortment also tolerates outdoor cultivation in our climatic conditions. Regarding the increase in production, but especially sufficient maturation of fruits, analysed plants were planted in a greenhouse. For the antioxidant analysis, the fruits of the following species were selected: *Cyclanthera pedata* (L.) Schrad, *Diplocyclos palmatus* (L.) C. Jeffrey, *Luffa acutangula* (L.) Roxb., *Momordica charantia* L., *Trichosanthes cucumerina* L. var. *anguina*, and ground vine seeds, particularly *Vigna sinensis* (L.) Savi ex Hassk. and *Vigna unguiculata* (L.) Walp. ssp. *sesquipedalis 'Red Noodl,'* In the case of *Trichosanthes* and *Momordica*, we harvested the unripe fruits, the ripened fruits and the tegument. The species *Cyclantera* and *Luffa* were taken in consumer maturity, in the immature fruits, the species *Diplocyclos* in full maturity. *Vigna* seeds were harvested in both cases after maturation. The harvesting of fruits and seeds took place on 3rd September 2019. The reason for harvesting unripe fruits in some species was their traditional green consumption. For experimental evaluation, 1 g per aqueous solution and 1 g per methanol solution were taken from ground and crushed fruits and seeds.

Antiradical activity was determined by the modified DPPH method of[5] using a Genesys 20 model 4001/4 spectrophotometer. In principle, it is a reduction of the 2.2-diphenyl-1-picrylhydrazyl (DPPH) stable radical in methanol solution in the presence of antioxidants. This translates into a decrease in absorbance at 515 nm in the dark. The decrease is recorded after 10 minutes of reaction. For the measurement, we used a basic solution of 25 mg DPPH in 100 ml methanol. This is diluted in a ratio of 10 ml to 100 ml of methanol per working solution and this is used to measure the antioxidant activity. 3.9 ml of DPPH solution is pipetted into the cuvette and the absorbance value corresponding to the initial concentration of DPPH solution as A_0 is recorded. Add 0.1 ml of the solution/extract of interest, which is stirred in the cuvette by moving the stirrer upwards and immediately starting the measurement of the dependence of A_t, where t = 10 (10 minutes). The efficiency of the extracts is calculated according to the mathematical formula: % inhibition = $[(A_0 - A_{10}) / A_0] \times 100$ (where A_0 is absorbance without extract and A_{10} is absorbance with extract). Inhibition of DPPH radicals is determined as a percentage and expresses how much DPPH radical is the monitored component capable of removing at a given time. The value of antioxidant activity (A_A) was subsequently classified according to the values obtained as: high inhibition > 70 %, average 40–70 % and low < 40 %.

RESULTS AND DISCUSSION

For the antioxidant analysis, 7 species from collection of 22 planted species were selected.

Water and methanol solutions prepared by mixing 1 g of crushed or chopped fruits and seeds with 25 ml of water and 25 ml of methanol were used to analyse the antiradical activity. The values obtained (Table 1) show a low degree of variability in methanol solution in species *Vigna sinensis*, *Vigna unguiculata*, *Momordica charantia* (immature fruit) and *Diplocyclos palmatus*. In water solution, *Vigna sinensis* has shown lowest degrees of variability. Others species has shown moderate degrees of variability (excepting *Cyclanthera pedata* – this one has extremely high coefficient of variation). For all varieties in aqueous solution, we have documented on average lower antioxidant activity compared to methanol solution. The highest values of antioxidant activity were found in *Vigna sinensis* seeds. On average, this is up to 73.79% for methanol solution and 70.06% for aqueous solution. Compared to a similar species of *V. unguiculata*, the measured values were higher by approximately 49% in both solutions. In Nigeria,[6] they compared the ability to absorb free radicals between different forms of *Vigna unguiculata*. The result revealed that *V. unguiculata* had the average free radical scavenging ability (5.5–30 %.). These are comparable results to our measurements. In fruits, the highest activity was found in methanol solution in *Momordica* immature fruits (up to 73 % on average). In contrast, the aqueous solution of *Momordica charantia* showed very low antioxidant activity,

Table 1. The antioxidant activity of selected climbing plants.

Solution	Species	n	min	max	x	s	sx	V%
water	*Cyclanthera pedata* (ripe fruit)	3	0,54	3,87	1,88	1,76	1,02	93,62
	Diplocyclos palmatus (ripe fruit)	3	6,68	10,26	8,35	1,80	1,04	21,57
	Momordica charantia (immature fruit)	3	2,25	3,11	2,58	0,46	0,27	17,94
	Momordica charantia (ripe fruit)	3	3,07	4,93	3,74	1,03	0,60	27,64
	Momordica charantia (seed coat)	3	8,34	9,89	9,27	0,82	0,47	8,82
	Trichosanthes cucumerina (ripe fruit)	3	1,33	2,38	1,76	0,55	0,32	31,36
	Trichosanthes cucumerina (immature fruit)	3	1,17	2,81	2,05	0,83	0,48	40,48
	Trichosanthes cucumerina (seed coat)	3	5,01	6,23	5,58	0,61	0,35	10,92
	Luffa acutangula (immature fruit)	3	3,23	3,66	3,46	0,22	0,13	6,38
	Vigna sinensis (seed)	3	69,91	70,25	70,06	0,17	0,10	0,25
	Vigna unguiculata ´Red Noodle´ (seed)	3	16,67	21,14	19,39	2,39	1,38	12,34
Solution	**Species**	**n**	**min**	**max**	**x**	**s**	**sx**	**V%**
methanol	*Cyclanthera pedata* (ripe fruit)	3	2,17	4,96	3,61	1,40	0,81	38,73
	Diplocyclos palmatus (ripe fruit)	3	14,69	15,22	14,93	0,26	0,15	1,77
	Momordica charantia (immature fruit)	3	2,88	5,33	4,47	1,38	0,80	30,89
	Momordica charantia (ripe fruit)	3	72,62	73,98	73,41	0,71	0,41	0,96
	Momordica charantia (seed coat)	3	2,70	4,44	3,56	0,87	0,50	24,42
	Trichosanthes cucumerina (ripe fruit)	3	2,77	3,59	3,07	0,45	0,26	14,78
	Trichosanthes cucumerina (immature fruit)	3	2,05	3,51	2,60	0,80	0,46	30,61
	Trichosanthes cucumerina (seed coat)	3	3,94	6,11	4,71	1,21	0,70	25,63
	Luffa acutangula (immature fruit)	3	7,03	9,02	7,86	1,03	0,60	13,15
	Vigna sinensis (seed)	3	73,04	75,07	73,79	1,11	0,64	1,50
	Vigna unguiculata ´Red Noodle´ (seed)	3	25,16	26,65	25,93	0,74	0,43	2,87

Explanations: n-number of observations, min/max – minimum and maximum value measured in the file (%), x – arithmetic mean of the set, s – standard deviation, V% - coefficient of variation (%).

only 3.74 % on average. In a study[20] from Taiwan, stronger DPPH radical scavenging activity was demonstrated in aqueous solution than in ethanol solution. However, dry fruits were used in this study.

In other samples, we found only very low antioxidant activity, on average below 10 %.

In the statistical evaluation, the high coefficient of variation of *Cyclantehra pedata* is interesting, when we found an extremely high degree of variability (93.62%) in aqueous solution. For the methanol solution, the coefficient of variation of this species was moderately high – 38.73%.

Surprising values were achieved by *Trichosanthes*, where in all variants only a low ability to bind radicals was found. It is worth mentioning, however, the high proportion of water in fruits of this kind, which apparently contributed to the measured values. In the future, it will be interesting to compare the achieved results with dried fruits, where we anticipate higher values of antiradical activity. In *Trichosanthes cucumrina*, there is even a difference in antioxidant activity between morphotypes[1] within this species.

CONCLUSION

Chosen species for antioxidant analysis were very different in results. Of the 7 analysed species, *Vigna sinensis* seeds showed the highest antioxidant activity. In fruits, the samples were based on fresh fruits. This had, probably a great effect on the results of antioxidant activity.

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Preparation and evaluation of an oral mucoadhesive gel containing nystatin-loaded alginate microparticles

Original Paper

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Abstract Nystatin is an antifungal agent used for prophylaxis and treatment of candidiasis, especially oral mycosis. Efficacy of nystatin conventional dosage forms is limited by the short residence time and bitter taste of the drug. This research aims at designing an optimized formulation of oral mucoadhesive gel of nystatin-loaded alginate microparticles, which can be retained in the mouth. Sodium alginate solution containing nystatin was added to the solution of calcium chloride under stirring. Microparticles containing nystatin were incorporated into the Carbopol gel. Size, loading, and release profile and mucoadhesion were investigated. The most suitable microparticles with particle size of < 250 µm were prepared with alginate concentration of 1%(w/v), calcium chloride of 1%(w/v), drug:polymer concentration 1%, and ratio of alginate solution:calcium chloride of 1:10. This formulation showed 49.1% drug loading and 98.2% encapsulation efficiency. Carbopol 934 gel provided optimal mucoadhesive properties. Release profile proved a burst release, which can be attributed to the surface associated drug, followed by a slower sustained release phase for all microparticles. The developed system with ability to adhere to the oral mucosa has great appeal for treatment of localized infections and can mask bitter taste of the drug and be retained in the mouth for long periods.

Keywords *Alginate – Carbopol – Microparticle – Mucoadhesion – Nystatin*

INTRODUCTION

Oral aphthous and oral lichen planus are two common oral mucosal diseases that are diagnosed by very painful and recurrent ulcers disturbing the normal activities of the oral mucosa and the patients' quality of life. These two chronic inflammations are mainly presented in immunocompromised patients and are often associated with oral mycosis. Therefore, drug therapy regimens of these inflammatory diseases mostly contain an antifungal agent (Conklin and Blasberg 1991, Ujević, Lugović-Mihić et al. 2013). Nystatin (Nys) belongs to the polyene antifungal antibiotics, which is produced by Streptomyces noursei strains and is effective against fungal infection unresponsive to azoles like Candida species. Nys interferes with the synthesis of fungal cell membrane and is widely used for treatment of susceptible fungal infections, especially oral mucosal infections (Lacy, Armstrong et al. 2004, Sean and Paul 2009, Sawant and Khan 2017). Conventional pharmaceutical dosage forms are not very effective for treatment of oral mucosal pathologies, because drugs are removed from the site of action by swallowing or salivary clearance (Roque, Castro et al. 2018). For Nys, there is an extra problem for systemic therapy. Nys is a large water-insoluble molecule that cannot penetrate the intestinal mucosa, and the drug therapy with Nys is limited to topical effects (Croy and Kwon 2004, Martín, Calpena et al. 2015). Therefore, designing a formulation that could keep the drug in the oral cavity for longer times seems a potentially successful approach for delivery of Nys. One of these formulations is mucoadhesive drug delivery systems, which utilize polymers that can be quickly and strongly bonded to the oral mucosa (Sudhakar, Kuotsu et al. 2006, Netsomboon and Bernkop-Schnürch 2016). Mucoadhesive dosage forms can be formulated in the form of multiparticulate systems to provide a more uniform and controlled mode of drug delivery (Madhav and Kala 2011). Alginate is a polysaccharide polymer with very interesting properties. Besides its biocompatibility and mucoadhesion properties, alginate forms gels and particles in the presence of cations such as Ca^{2+}. This very simple and rapid

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gelation reaction (without use of any harsh solvents or pH) makes alginate a good choice for delivery of biological agents and cells (Takka 1999, Tafaghodi, Tabasi et al. 2006). Therefore, the aim of the present study was to develop and evaluate drug-loaded alginate microparticles to provide a multiunit drug delivery system. This part is the most challenging part as the drug molecule is very large and insoluble. To further enhance the mucoadhesion properties, the particles were introduced into the carbomer gel to provide a mucoadhesive oral dosage form that could be retained in the mouth. The formulation then was characterized in terms of particle size, drug loading, encapsulation efficiency, in vitro release profile, measurement of mucoadhesion, and physicochemical properties using DSC and FT-IR.

MATERIALS AND METHODS

Materials

Nys was purchased from Raha. Pharmaceutical Company (Isfahan, Iran). Sodium alginate medium viscosity and sodium citrate was obtained from Sigma (USA); **Carbopol** 934 (cp934), calcium chloride, sodium lauryl sulfate (SLS), and thriethylamine were purchased from Merck (Germany). All other chemicals and solvents were obtained from certified sources.

Preparation of alginate microparticles

The formulation of Nys-loaded alginate microparticles was based on the simple and well-known direct ionic gelation method. Alginate solution was prepared by dissolving sodium alginate in de-ionized water. Briefly, Nys powder was suspended thoroughly into aqueous solution of sodium alginate under magnetic stirrer, and then, the resulting solution containing Nys was extruded from a syringe into aqueous solution of $CaCl_2$. Alginate microparticles were filtered, collected, and washed using distilled water and dried at room temperature (Heng, Chan et al. 2003).

Preparation of mucoadhesive gel of nystatin containing alginate microparticles

The oral gel was prepared using cp934. Alginate microparticles containing Nys were incorporated into a 1% w/v cp934 gel under magnetic stirrer. For preparation of gel, 1 g cp934 was dissolved in water, and then, by adding triethylamine dropwise, the viscosity of the gel was modified, and the final pH was set to 5.5 ± 0.3.

Experimental design

To determine the effects of different parameters and choosing the most optimum formulation, experimental design was performed with four different factors at two levels. The corresponding runs and designed formulations are given in Table 1.

Particle size analysis

The particle size was evaluated by sieving method. A series of standard sieves were used to sieve the resulting particles, and the optimum alginate microparticles were considered the ones with particle size less than 250 µm. These microparticles were passed from sieve with mesh 60.

Determination of drug loading and encapsulation efficiency

4 mg of alginate microparticles containing Nys were added to sodium citrate solution 1% (w/v) under magnetic stirring until complete dissolution of microparticles. Nys extraction was done by centrifugation at 5,000 rpm for 30 min, and separation of the settled drug from the supernatant. Nys was dissolved by adding sodium lauryl sulfate (SLS) solution 1% (w/v), and then, the solution was analyzed by a UV spectrophotometer. The percentage of drug loading and encapsulation efficiency were determined based on the following equations:

Equation 1. Drug loading % $= \frac{\text{weight of drug in microparticles}}{\text{total weight of microparticles}} * 100$

Equation 2. Encapsulation efficiency % $= \frac{\text{weight of drug loaded in microparticles}}{\text{initial weight of the drug used}} * 100$

Fourier transform infrared spectroscopy measurement

Fourier transform infrared (FT-IR) spectroscopy was performed using FT-IR spectrophotometer (Bruker FTIR-Vertex 70, USA). Spectra of pure drug, sodium alginate, and alginate microparticles containing Nys were obtained using KBr pellet method at a wave number region of 400–4,000 cm^{-1}.

Differential scanning calorimetry

Thermal properties of Nys, sodium alginate, blank alginate microparticles, and physical mixture of Nys and blank microparticles were analyzed by differential scanning calorimetry (DSC). A known amount of each was weighed and placed in sealed aluminum pans. Heating was performed under a flow of nitrogen gas at a rate of 5°C/min in the temperature range 25–300°C using DSC 302 apparatus (BAHR Thermoanalyse GmbH, Germany). A blank aluminum pan was used as the reference.

Measurement of mucoadhesive force

There are various methods for measuring the force of adhesion of mucoadhesive products; most of them are based on measuring the force required to detach the product from a smooth surface.

This research employed a homely designed apparatus, which consisted of a fixed base, a jack, a digital scale fixed to a metal

Table 1: Run parameters for two – level four factorial experimental design

Experiments	Alginate solution conc.(w/v%)	$CaCl_2$ solution conc. (w/v%)	Alginate:$CaCl_2$ ratio	Drug:polymer ratio
1	1	1	1:5	1:1
2	1	1	1:10	1:1
3	1	2	1:5	1:1
4	1	2	1:10	1:1
5	0.5	1	1:10	1:1
6	0.5	1	1:5	1:1
7	1	1	1:5	1:5
8	1	1	1:5	1:5
9	1	2	1:10	1:5
10	1	2	1:5	1:5
11	0.5	2	1:5	1:5
12	0.5	1	1:5	1:5
13	0.5	1	1:10	1:5
14	0.5	2	1:10	1:1
15	0.5	2	1:5	1:1
16	0.5	2	1:10	1:5

frame, and two pieces of smooth glasses with the product placed between them. Once the product is placed between the two surfaces, by turning a knob and lowering the movable part a certain shear stress is created, which is shown by the digital scale (Mohammadi-Samani, Bahri-Najafi et al. 2005).
A biological substrate (sheep's oral mucosa) with a cross section of 4 cm^2 was used for examining mucoadhesion of the product. The biological mucosa was applied on one of the glass surfaces. Half a gram of cp934 gel containing alginate microparticles was also put between the two glass surfaces in contact with the mucosa. The jack was lifted to make the digital scale show a certain value. Consequently, the jack was lowered slowly, and the maximum value shown by the scale when the two glass surfaces detached was considered as the gel's adhesion strength. The force was calculated in g/cm^2 by dividing the value into the cross-section area. The adhesion determination process was repeated five times, and the average was reported in g/cm^2.

Drug release studies

USP dissolution apparatus II (Erweka, Germany) was used for studying drug release. 250 ml of buffer solution containing 1% w/v SLS solution in water was poured into the apparatus vessels, and the temperature and rotation speed were set to 37±0.5°C and 50 rpm, respectively. Consequently, three 3 cm-diameter petri dishes were filled equally (5.3 g) with cp934 gel containing alginate microparticles. Each petri dish was fixed individually at the bottom of the dissolution vessels, and samples were taken at 10, 20, 30, and 60 min. At each interval, 5 ml of the solution of each vessel was taken and replaced by 5 ml of fresh buffer. Absorption of the samples was measured by a UV spectrophotometer at 306 nm against buffer with 1% SLS as the blank solution. The amount of released drug was calculated using the standard curve equation. Average of the amount of drug released was calculated and converted into drug release percentage.

To validate the quantification method, the test was also carried out on drug loaded alginate microparticles without gel base and drug-free alginate microparticles, and absorption of each sample was recorded using a spectrophotometer at a wavelength of 306 nm.

RESULTS AND DISCUSSION

Mucoadhesive drug delivery systems have opened new horizons in drug delivery, as they can increase retention time of the dosage form in the place and hence improve the therapeutic efficacy. By merging multi-unit dosage forms and mucoadhesion, a controlled release drug delivery system with improved patient compliance could be achieved. These dosage forms can attach to mucosa, stay in the site, and release an acceptable amount of drug content at a given time. These are slow-releasing systems, and if properly designed, can release the drug regularly and controlled (Helliwell 1993, Lee and Chien 1996). Alginate microparticles are one of these multi-unit systems that can be readily prepared without need to harsh conditions or toxic solvents (Alipour, Montaseri et al. 2016).

Preparation of microparticles

Sodium alginate, a sodium salt of alginic acid, is soluble in water and is crosslinked with divalent or polyvalent cations such as calcium ion, to form an insoluble network (Østberg, Lund et al. 1994). Being biodegradable and biocompatible polymer, with low toxicity and immunogenicity and high availability and affordability, alginate microparticles could be an attractive choice for development of controlled release drug delivery systems. Indeed, because of mucoadhesive properties, alginate microparticles could stick to the mucosa for prolonged period of time and have been exploited for the site-specific drug delivery to mucosa (Tafaghodi, Tabasi et al. 2006, Laffleur, Shahnaz et al. 2013).

Table 2. Effect of different parameters on size of microparticles

Experiment	Alginate (w/v%)	$Cacl_2$ (w/v%)	Alginate to $cacl_2$ ratio	drug to polymer ratio	Particle size (µm)
1	1	1	1:10	1:1	<250
2	0.5	1	1:10	1:1	710
3	1	2	1:10	1:1	296
4	1	1	1:5	1:1	450
5	1	1	1:10	1:5	710

Alginate microparticles were prepared using the ionic gelation method. To this end, the drug was added to an aqueous solution of sodium alginate under stirring with a magnetic stirrer. Then, the mixture of drug and sodium alginate was added to the aqueous solution of calcium chloride using an insulin syringe under stirring with the magnetic stirrer. Alginate microparticles were formed through ionic interaction between positive ions of calcium and negative carboxylic ions of alginate polymer.

The impact of parameters effective on particle size is shown in Table 2. As presented in the table, using 0.5% (w/v) sodium alginate did not yield small size microparticles. 2% (w/v) calcium chloride resulted in a high content of calcium ions in the system, leading to binding of a high number of calcium ions to carboxyl groups of glucuronic acid in the structure of sodium alginate. Then, the system viscosity was increased, which resulted in large size microparticles. By reducing the volume of calcium chloride solution to 5 ml, more microparticles were accumulated and bound to each other, and hence, bigger microparticles were obtained. A drug to polymer ratio of 1:5 did not result in small size microparticles. The finest microparticles were obtained from 1% (w/v) alginate and 1% (w/v) calcium chloride, alginate to calcium chloride ratio of 1:10, and drug to polymer ratio of 1:1. They were smaller than 250 µm in size, and all passed through mesh no. 60. Previous studies on alginate microparticles also reported similar results. Tafaghodi et al. observed that increasing alginate concentration to more than 2% (w/v) results in increased particle size (Tafaghodi, Tabasi et al. 2006). There are many reports on the optimum concentration of calcium chloride solution to prepare fine particles. Heng et al. showed that high concentrations of calcium ions would raise the viscosity of the system and size of the particles (Heng, Chan et al. 2003).

Drug loading and encapsulation efficiency

The loaded drug was measured through a simple and direct method. After washing the microparticles deposited by centrifugation with 1% (w/v) solution of sodium citrate, the sample was diluted with an appropriate amount of SLS 1% (w/v). Sodium citrate can detach calcium ions from calcium alginate due to its ability to create complex between citrate and calcium ions and therefore increase the solubility of alginate. There are many controversies in choosing an optimum concentration for calcium chloride to achieve high drug loading efficiency (Østberg, Lund et al. 1994, El-Kamel, Al-Gohary et al. 2003). Calcium chloride solution 1% (w/v) was used in this study, which was suitable for loading the drug and drug loading efficiency.

The results of the amount of drug loaded in alginate microparticles and encapsulation efficiency were 49.1 and 98.2%, respectively, which is quite high for loading such an insoluble large molecule in a hydrophilic carrier.

FT-IR results

The infrared spectra of Nys, alginate and Nys-loaded alginate microparticles are presented in Figure 1. IR spectrum of Nys showed a strong absorption frequency at the wavenumber of about 3,410 cm^{-1}, which is related to stretching vibrations of hydrogen bonds. While absorption frequency of 2,934 cm^{-1} is due to asymmetric stretching vibration of methylene groups and absorption frequency of 1,706 cm^{-1} to stretching vibration of ester carbonyl and carboxylic acid groups. Absorption frequencies of 1,556 cm^{-1} and 1,844 cm^{-1} are related to CH=CH double bonds, absorption frequency of 1,400 cm^{-1} to flexural vibrations of C-H group, and 1,070 cm^{-1} to hydroxyl groups. IR spectroscopy of alginate showed a strong absorption frequency in 3,075 cm^{-1}, which is related to the primary amino group, and 1,423 cm^{-1} and 1 624 cm^{-1} to asymmetric and symmetric stretching vibration of the carboxyl group, respectively. IR spectroscopy of Nys-loaded alginate microparticles showed a high frequency absorption at 3,275 cm^{-1}, which is related to stretching vibrations of hydrogen bonds in C=O, C-C-O, and O-C-C groups (Martín-Villena, Fernández-Campos et al. 2013). These spectra prove that Nys has been successfully loaded to alginate microparticles, and the main peaks of drugs could be seen in the spectrum of the particles.

DSC results

The results of DSC analysis for Nys, alginate, physical mixture of alginate and Nys, drug-free alginate microparticles, and Nys-loaded alginate microparticles are presented in Figure 2. Nys melting peak was seen at 160.3°C, above which the drug was degraded. Alginate melting peak was appeared at

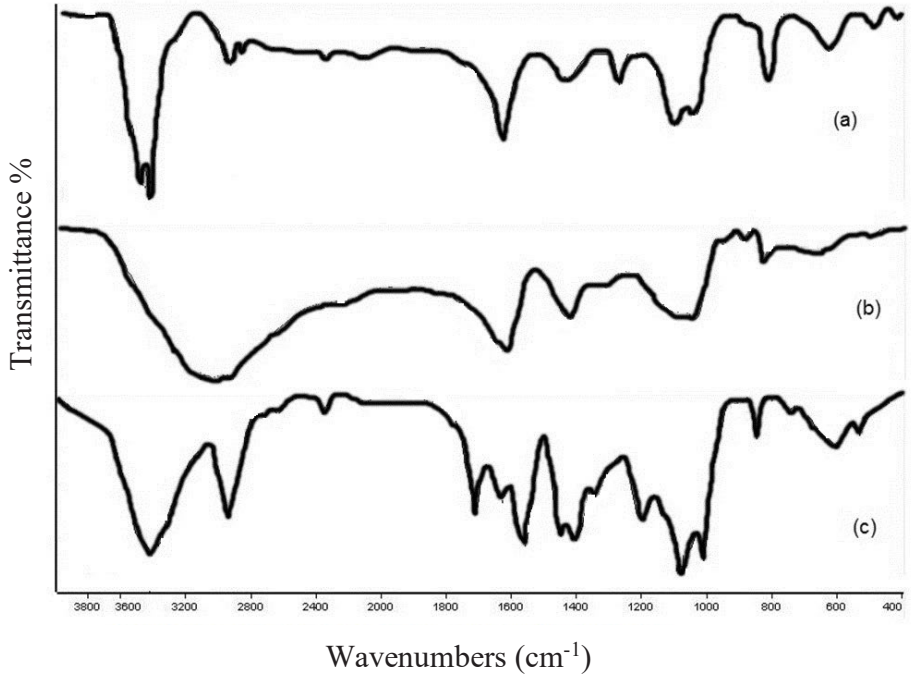

Figure 1: FT-IR spectra of (a) Nys-loaded alginate microparticulate, (b) alginate, and (c) Nys.

227.1°C. Melting peaks of physical mixture of alginate and Nys were observed at 169.6°C and 228.4°C, which is the proof of compatibility. The polymer peak in the blank alginate microparticles was seen at 151.3°C; therefore, the polymer was not degraded. Two peaks were appeared at 145°C and 168.6 °C for Nys-loaded alginate microparticles. The peak at 168.6°C can be related to free Nys on the microparticles surface. The DSC results are almost similar to the study of Martín-Villena et al. (Martín-Villena, Fernández-Campos et al. 2013).

Table 3: Adhesion strength of cp934 gel with and without alginate microparticles (n=6)

Formulation	Adhesion strength (mean ± SD) g/cm^2
cp934 gel without alginate particles	1.88 ± 0.04
cp934 gel containing alginate particles	1.94 ± 0.15

Measurement of mucoadhesive force

Pharmaceutical mucoadhesive systems are used mainly to achieve two goals: first, slow and delayed release of active substance of formulation, which can lead to a uniform and appropriate plasma concentration of systemic products, and second, to locally focus the drug molecules of interest with a maximum absorption peak and hence a maximum effect. This property is applied in both topical and systemic products.

The results of measurement of mucoadhesion capability of gel 1% (w/v) of cp934 containing microparticles and the gel of cp934 without alginate microparticles are shown in Table 3. In this study, the mucoadhesive polymer cp934 was used to prepare the mucoadhesive gel. This synthetic high molecular weight polymer is derived from acrylic acid and contains 56 to 68% carboxyl groups. The water absorption capacity of cp934 is low; thus, it has weak hygroscopicity, but a high tensile strength. The binding mechanism of poly-carboxylic acids such as cp934 to mucin is performed through swelling of the polymer in water, which results in interacting of polymer chains with superficial mucus (Llabot, Manzo et al. 2004). We used a cp934 solution 1% (w/v), because the system integrity was acceptable with this concentration and the system was low porous. According to other studies, use of high concentrations of carbapol934 creates a strong acidity in the mouth, resulting in significant adverse effects in the mucosa (Llabot, Palma et al. 2007, Llabot, Palma et al. 2007).

Drug release study

The profiles of drug release from gel-free and gel-based alginate microparticles are shown in Figure 3. This formulation showed an initial burst release, because the release environment contains 250 ml SLS solution 1% (w/v) in water, which can affect the structure of alginate, leading to rapid release of the drug. SLS 1% (w/v) solution in water was used in this study for the release medium to establish sink conditions, because the drug is easily dissolved in SLS, leading to a concentration gradient, while no concentration gradient is provided in case of low solubility, and hence, precipitation of the drug may happen. As depicted in the figure, drug release from alginate microparticles was completed in 20 min, but it

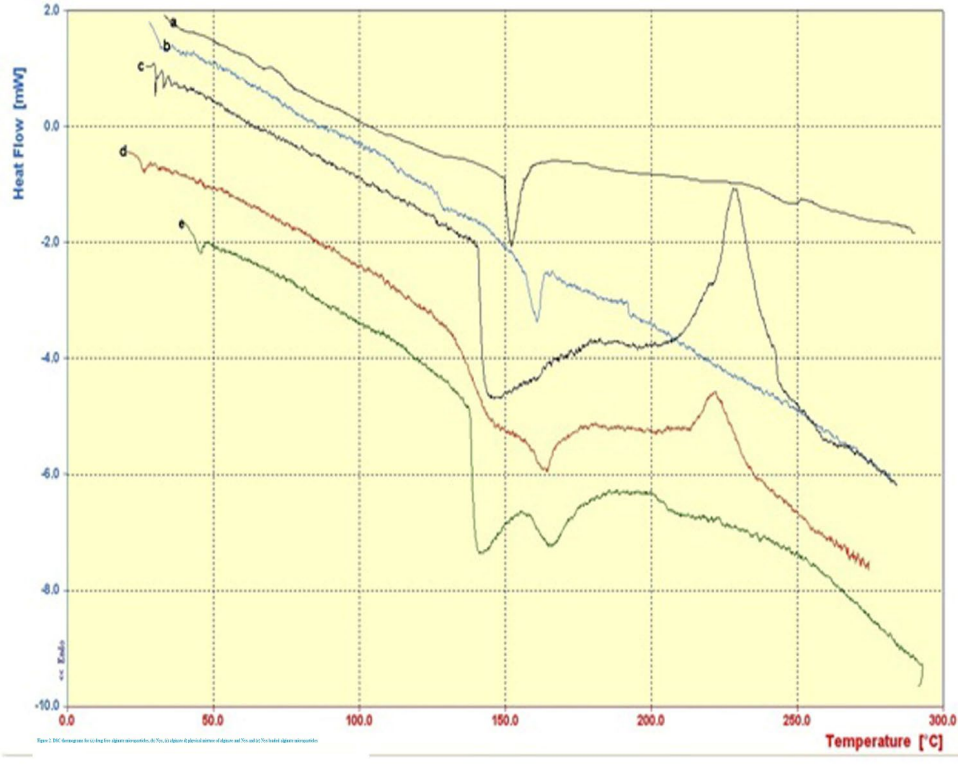

Figure 2: DSC thermograms for (a) drug-free alginate microparticles, (b) Nys, (c) alginate, (d) physical mixture of alginate and Nys, and (e) Nys-loaded alginate microparticles

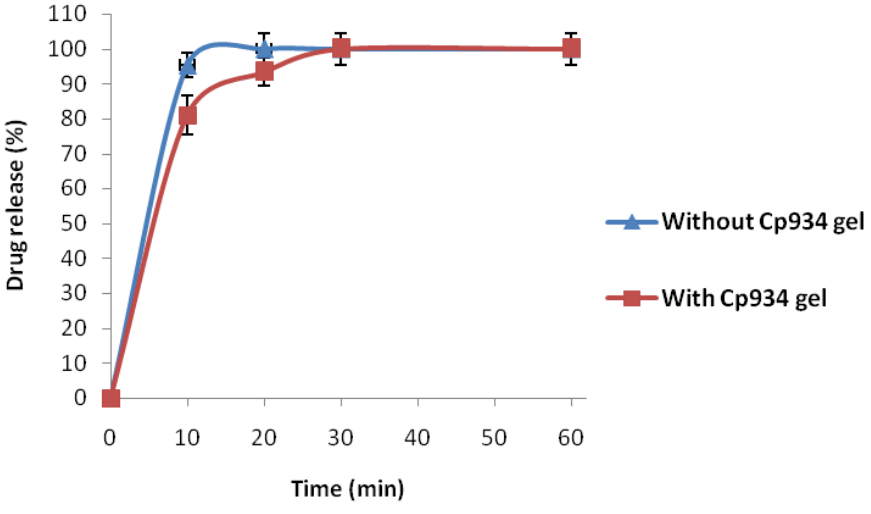

Figure 3. Release profiles of Nys from alginate microparticles with and without Carbopol gel

was continued until 30 min when the particles were loaded in cp934 gel, which is not statistically different ($P > 0.05$).

As alginate is a hydrophilic polymer and the drug is dissolved in SLS easily and quickly, it results in the maximum solubility of Nys in the release medium. It should be noted that the large molecule of the drug may be adsorbed on the microparticles surface, leading to the burst release.

Distilled water has been used for the release study of Nys-loaded alginate microparticles by Martín-Villena *et al.* As Nys is not dissolved in water, no burst release was reported (Martín-Villena, Fernández-Campos et al. 2013). Other studies on the release behavior of Nys have also used distilled water for the release environment and reported a slow drug release (Llabot and Manzo 2002).

CONCLUSION

A new and stable mucoadhesive gel of Nys-loaded alginate microparticles with desired size and high drug loading efficiency was developed by ionic gelation method. The size of the particles was dependent on different factors, including concentration of alginate and calcium solutions and their ratios, as well as to drug to polymer ratio. Carbopol base of the gel makes it very strong mucoadhesive system. Ability of this system to adhere to the oral mucosa has great appeal for the treatment of localized oral infections. In addition, the dosage form can mask the bitter taste of drug and retain it in the mouth for long periods.

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Functionality evaluation of co-processed excipients as diluents in tablets manufactured by wet granulation

Original Paper

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Abstract Diluents are essential components of a tablet formulation. The type of diluent used in a formulation influences the quality of tablets produced from that formulation. The aim of this study was to evaluate the tableting properties of co-processed excipients (C-PEs) incorporated as diluents in tablet formulation by wet granulation. Metronidazole tablets were prepared by wet granulation incorporating different diluents that were either single component excipients (SCEs) (lactose and microcrystalline cellulose) or C-PEs (Ludipress®, StarLac®, Prosolv® and AVICEL®HFE). The granules obtained for each formulation were evaluated for particle size analysis, flow properties and compression properties. Tablets weighing 500 mg were compressed from the metronidazole granules on a Single Station Tablet Press using a 12 mm punch and die tooling system. The tablets were kept for 24 h post-production, and the properties of weight uniformity, thickness, tensile strength, friability, disintegration time and dissolution profile evaluated subsequently. Results of granule properties showed that variations in parameters evaluated was as a result of differences in the type and composition of diluent used in formulation. Compactibility and tabletability profile of metronidazole granules revealed a better performance with granules processed with C-PE based diluents compared to SCE-based diluents. Tablets formulated with C-PEs as diluents were uniform in tablet weight, disintegrated faster and yielded a faster drug release compared to tablet formulations containing SCEs as diluent. This study reveals the performance advantage of C-PEs as diluents in tablets manufactured by wet granulation and highlights the importance of rational selection of excipients during tablet formulation.

Keywords *Diluents – co-processed excipients – tablets – wet granulation*

INTRODUCTION

Tablets constitute about 80–90% of dosage forms commonly prescribed by medical practitioners for therapeutic management of disease conditions. It is a solid dosage form that exercises advantages over other dosage forms because of its ease of administration, stability profile and amenable to large-scale production. Tablet making involves the addition of a category of substances known traditionally as excipients. Though commonly referred to as the inactive components of a tablet formulation, they are functional in nature because they contribute significantly to the processing, stability, manufacturability and performance of tablets as a dosage form. Depending on the functionality, excipients may be classified as diluents, disintegrants, binders, lubricants, glidants, etc. They are usually added in stated amounts to facilitate the development of a robust tablet product.

Most tablet formulations are prepared by wet granulation and require the addition of all the listed excipients. One critical excipient that is incorporated in tablet formulations by wet granulation is the diluent. Diluents also known as fillers or bulking agents are routinely added in formulation development to increase the bulk of the formulation and to bind other excipients with the active pharmaceutical ingredient (API). The use of diluents becomes more significant in tablet formulations of low-dose API where the diluent occupies a larger proportion of the formulation. Hence, the properties of diluents become extremely important and can significantly influence the final product property. Studies have been carried out to characterise the mechanical properties of widely used pharmaceutical diluent powders to understand how their mechanical properties impact on the manufacturing performance and properties of the final product (Zhang et al., 2017). These diluent powders are more

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or less single component excipients (SCEs) like lactose, starch, mannitol, microcrystalline cellulose (MCC), which have been used in tablet formulation by wet granulation. However, due to the limited functionality of SCEs in tablet formulation, attention has shifted to the development of novel excipients with improved functionality.

Co-processing as a particle engineering technique has been used to develop novel excipients with improved functionality. This technique involves the combination of two or more excipients in optimal proportions at the sub-particle level using a defined method that promotes synergy of properties in composite particles while minimising the limitations of the constituent excipient in the final product. Some of the functionality improvement recorded with co-processed excipients (C-PEs) includes enhanced flowability, compressibility, dilution potential and low lubricant sensitivity and/or moisture sensitivity (Nachaegari and Bansal, 2004). Many of these C-PEs have been designed originally as filler-binders in direct compression formulation (Wang et al., 2015), as superdisintegrating agents for orodispersible tablets (Daraghmeh et al., 2015) and as drug-release retardants in controlled release formulations (Choudhari et al., 2018). No study has reported the use of C-PEs as diluents in wet granulated tablet formulations.

Considering the improved tableting profile of C-PEs, changing the diluent type in a wet granulated tablet formulation from an SCE to a C-PE is likely to result in better tableting properties of the drug product. Hence, the aim of this study is to evaluate the tableting properties of C-PEs as diluents in a wet granulated formulation of metronidazole tablets. Two samples each of lactose-based C-PEs (Ludipress®, StarLac®) and MCC-based C-PEs (Prosolv®, AVICEL®HFE) were evaluated as diluents in comparison to their SCEs (lactose, MCC).

MATERIALS AND METHODS

Materials

Metronidazole (CDH laboratory Chemicals, India), lactose (DFE Pharma, Germany), maize starch (Burgoyne Burbidge & Co. India, Mumbai), Acacia (Kerry Ingredients and flavours Ltd, Ireland), StarLac® (Roquette Pharma, France), Ludipress® (BASF SE, Germany), MCC, Prosolv®, sodium stearyl fumarate (JRS Pharma, Germany), Avicel®HFE-102 (FMC BioPolymer AS, Norway), colloidal silicon dioxide (Evonik Industries, Germany), distilled water.

Preparation of Metronidazole Granules

Metronidazole granules were prepared by wet granulation according to the formulation given in Table 1. A powder blend of metronidazole (20 g), lactose (19.5 g) and maize starch (5 g) was obtained by low shear mixing in a mortar with a pestle (Abdallah et al., 2014). Binder solution was prepared containing acacia (10%$^{w}/_{w}$) and incorporated to mass the powder mix. The wet powder mass obtained was screened through a 1.6 mm sieve to generate granules. The granules formed were allowed to dry for 20 min in the oven, screened through a 1 mm sieve and then drying completed in the hot-air oven (Gallenkamp BS, England) at 40ºC for 2 h.

Characterisation of Metronidazole Granules

Moisture Content

Residual moisture content of each granule formulation was determined using the gravimetric method (Olayemi et al., 2008). A sample of granules (1 g) for each formulation was dried to constant weight in an oven at 105°C for a period of 1 h. The percentage moisture content was calculated using Eq. 1:

$$\%\ \text{moisture content} = \frac{initial\ weight - final\ weight}{inial\ weight} \times 100\% \quad (1)$$

Particle Size Analysis

Particle size analysis was carried out using the sieving method as described by Ohwoavworhua and Adelakun (2010). Test sieves ranging from 500 µm to 75 µm aperture size were arranged in a descending order. A sample of granules (20 g) was placed in the top sieve, and the setup shaken for 10 min at an amplitude of 1 mm. The weight of granules retained on each sieve was determined and a plot of cumulative weight (undersize) against sieve size was drawn to extrapolate the median particle diameter (d_{50}).

True Density

True density for each granule formulation was determined using the liquid displacement method (Olayemi et al., 2008). The empty weight of a pycnometer bottle was obtained, filled with xylene, and its weight determined. A sample of the granules (2 g) was transferred into the bottle and the excess liquid wiped off. The new weight was determined, and Eq. 2 was used to compute true density having obtained the weight of xylene displaced by the sample:

$$\rho_T = \frac{weight\ of\ sample \times \rho\ of\ xylene\ (0.864)}{weight\ of\ xylene\ displaced\ by\ sample} \quad (2)$$

Angle of Repose

Angle of repose for each granule formulation was measured using the method described by Pilpel (1965). The angle of repose was calculated using Eq. 4, and a mean of three replicates was reported:

$$\text{Tan}\ \theta = \frac{h}{r} \quad (3)$$

where *h* is the height of the cone and *r* is the radius of the cone base.

Table 1. Formula for preparing metronidazole granules and tablets (n = 100 tablets).

	Formulations					
Ingredients	**I**	**II**	**III**	**IV**	**V**	**VI**
Metronidazole (40%)	20.00	20.00	20.00	20.00	20.00	20.00
Diluent (39%)	19.50	19.50	19.50	19.50	19.50	19.50
Maize starch (10%)	5.00	5.00	5.00	5.00	5.00	5.00
Acacia (10%)	5.00	5.00	5.00	5.00	5.00	5.00
CSD (0.5%)	0.25	0.25	0.25	0.25	0.25	0.25
SSF (0.5%)	0.25	0.25	0.25	0.25	0.25	0.25
Total (g)	**50.00**	**50.00**	**50.00**	**50.00**	**50.00**	**50.00**

Diluents (I – lactose, II – Ludipress®, III – StarLac®, IV – MCC, V – Prosolv®, VI – AVICEL®HFE), CSD – colloidal silicon dioxide, SSF – sodium stearyl fumarate

Bulk and Tapped Densities

Bulk density (BD) and tapped density (TD) were calculated using Eqs. 4 and 5, respectively, according to the method described by USP (2012). The values obtained for BD and TD were used to compute Carr's index (CI) and Hausner's ratio (HR) using Eqs. 6 and 7, respectively:

$$BD = \frac{\text{weight of granules}}{\text{Bulk volume}} \quad (4)$$

$$TD = \frac{\text{weight of granules}}{\text{Tapped volume}} \quad (5)$$

$$CI = \frac{TD - BD}{TD} \times 100 \quad (6)$$

$$HR = \frac{TD}{BD} \quad (7)$$

Compaction Studies

Compacts weighing 500 mg were compressed at pressures ranging from 40 MPa to 250 MPa on a Hydraulic Carver Press. The compacts were kept for 24 h after compression to allow for elastic recovery. The weight, thickness and crushing strength of each compact was measured, and the parameters of tensile strength and porosity were computed for each compact. Plots of porosity against compression pressure (compressibility), tensile strength against porosity (compactibility) and tensile strength against compression pressure (tabletability) were generated for each granule formulation. The plots were generated with mean values without including standard deviation.

Tableting

Metronidazole tablets were prepared by compressing the granules lubricated with corresponding quantities of CSD and SSF (Table 1) on a Single Station Tablet Press (Type EKO, Erweka, Germany) using 12 mm flat-faced punches at 10 kN. Tablets weighing ~500 mg were obtained and kept for 24 h to allow for elastic recovery before evaluation of tablet properties.

Evaluation of Tablet Properties

Tablet properties for each formulation were evaluated according to BP requirements (2013). The weight of 20 tablets selected at random from each formulation were obtained, and the mean and standard deviations were calculated to ascertain uniformity in weight of tablets for each formulation. The thickness and crushing strength of 10 randomly sampled tablets from each formulation were measured using the Digital Vernier Calliper and Monsanto Hardness Tester, respectively. The mean and standard deviation for each parameter were recorded. The corresponding tensile strength (TS) was calculated for each formulation using the following equation (Fell and Newton, 1970):

$$Ts = \frac{2F}{\pi dt} \quad (8)$$

where F is the crushing strength, d and t are the diameter and thickness, respectively.

Ten tablets from each formulation were randomly selected, accurately weighed, placed in the chamber of Digital Friability Test Apparatus 903 (Environmental and Scientific Instruments CO., India) and allowed to rotate at 25 rpm for 4 min. Tablets were removed from the chamber, gently cleaned from dust particles and accurately weighed. Friability was calculated in terms of percentage weight loss using Eq. 9:

$$Weight\ loss\ (\%) = \frac{W_i - W_f}{W_i} \times 100\ \% \quad (9)$$

where w_i and w_f are the weights of the tablets before and after friability test, respectively.

The time taken for six tablets from each formulation to disintegrate was determined using the BJ-3 Disintegration

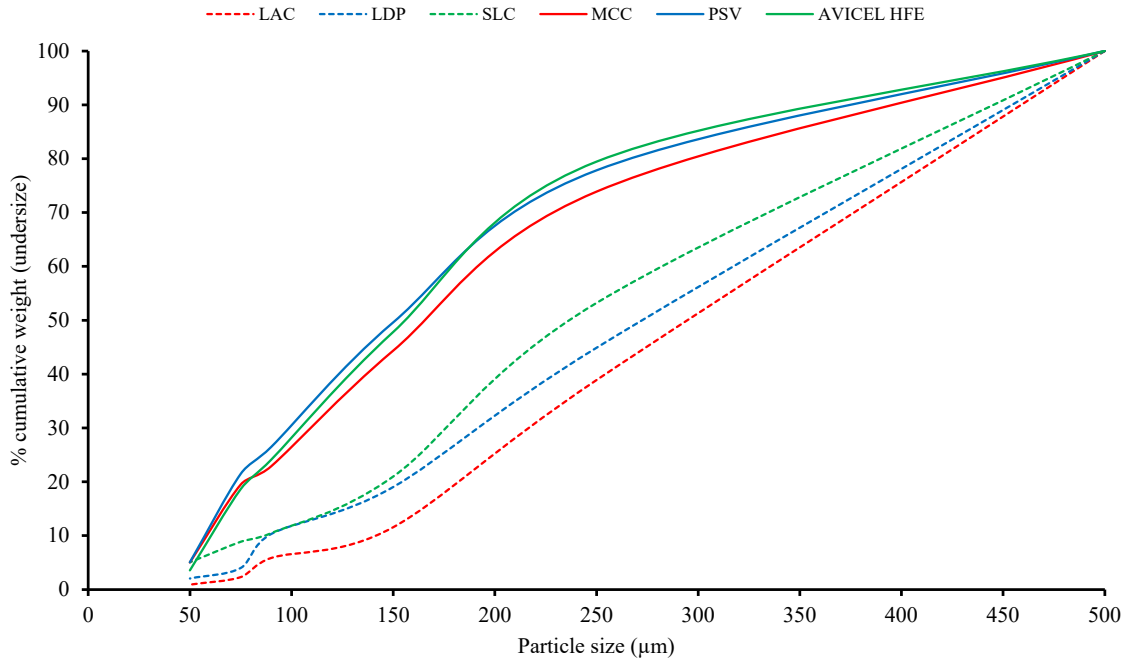

Figure 1. Particle size distribution of LAC (I), LDP (II), SLC (III), MCC (IV), PSV (V), & AVICELÂ®HFE (VI).

Tester (Ningbo Hinotek Instrument Co., Ltd, China). The experiment was conducted in a controlled temperature environment of 37 ± 0.5 °C using distilled water as the medium for disintegration. The mean and standard deviation of six replicates were computed and recorded for each formulation. *In vitro* dissolution studies were conducted according to USP specifications (USP, 2011) using the Tablet Dissolution Test Apparatus (Model 912, Electronics India, Kamli – 173220, Parwanoo, Himachal Pradesh, India). One tablet was placed in a basket submerged in a dissolution chamber containing 900 mL of 0.1 N HCl maintained at 37 °C. The basket was allowed to rotate at 100 rpm, and 5 mL samples were withdrawn after 5, 10, 15, 20, 30, 45 and 60 min of drug release. The dissolution medium was replaced with equal volume of 0.1 N HCl after each withdrawal. The samples collected were filtered and sufficiently diluted with 0.1 N HCl before taking the absorbance readings at 277 nm using the ultraviolet (UV) spectrophotometer (UV-1800 Spectrophotometer, Shimadzu Corporation, USA). The amount of drug released (%) was calculated based on the equation, $y = 0.0395x + 0.1314$, $r^2 = 0.999$, derived from the calibration curve of metronidazole and a plot of drug released (%) against time was generated for the six tablet formulations. The plot was generated with mean values without including standard deviation.

RESULTS AND DISCUSSION

Tablet production by wet granulation involves the addition of a diluent at the formulation stage. The goal of this study was to evaluate the performance of C-PE as diluents in tablets manufactured by wet granulation in comparison to SCE.

Physical properties of granules prepared using the different diluents consisting of C-PE and SCE materials are summarised in Table 2. Particle size (d_{50}) of granules ranged from 150 µm to 290 µm as extrapolated from the particle size distribution curve (Fig. 1). Lactose-based formulations (I–III) possessed larger particle sizes relative to MCC-based formulations. This may be attributed to the low agglomerating effect of MCC-based excipients when used in wet granulation. It was generally observed that the formulations containing C-PEs as diluents (II, III, V, VI) had lower particle size compared to formulations containing SCEs as diluents (I and IV). This may be linked to the primary particle sizes of C-PEs, which are relatively smaller having been produced by spray drying (Dong et al., 2018).

Angle of repose values for all granule formulations did not exceed 30°, implying that the granules possessed good flow properties (Osei-Yeboah et al., 2014). Generally, tablet formulations prepared by wet granulation are expected to flow well as a result of the agglomeration occurring during granulation leading to particle size enlargement that enhances flow. This is a quality attribute that granules must possess to ensure uniformity in content and weight of tablets during production (Osamura et al., 2018). BD and TD ranged from 0.41 to 0.55 g/cm^3 and 0.56 to 0.67 g/cm^3, respectively, with lactose-based formulations recording higher values for both parameters. This may not be unrelated to the fluffy and fibrous nature of MCC that results in a higher bulk volume when

Table 2. Physical properties of metronidazole granules prepared with different diluents.

	Formulations with diluents					
Parameters	**I**	**II**	**III**	**IV**	**V**	**VI**
Particle size (d_{50}, µm)	290.00	270.00	235.00	165.00	150.00	155.00
Angle of repose (°)	30.03 ± 0.20	29.63 ± 0.50	28.58 ± 1.87	30.25 ± 0.61	27.52 ± 0.32	28.27 ± 0.56
Bulk density (g/cm^3)	0.55 ± 0.01	0.54 ± 0.02	0.48 ± 0.01	0.43 ± 0.03	0.43 ± 0.03	0.41 ± 0.01
Tapped density (g/cm^3)	0.66 ± 0.02	0.67 ± 0.00	0.60 ± 0.01	0.62 ± 0.07	0.58 ± 0.01	0.56 ± 0.02
True density (g/cm^3)	1.46	1.44	1.54	1.52	1.52	1.54
Carr's Index (%)	16.44 ± 0.32	18.86 ± 3.10	19.28 ± 0.33	30.95 ± 3.37	26.51 ± 2.92	25.79 ± 1.83
Hausner's ratio	1.20 ± 0.00	1.23 ± 0.05	1.24 ± 0.01	1.45 ± 0.07	1.36 ± 0.05	1.35 ± 0.03
Moisture content (%)	5.00	5.00	5.00	7.00	9.00	8.00

I – lactose, II – Ludipress®, III – StarLac®, IV – MCC, V – Prosolv®, VI – AVICEL® HFE

loosely packed resulting in low BD and TD (Chaerunisaa et al., 2019). With respect to CI and HR, MCC-based formulations (IV–VI) were found to be more compressible. Higher values of CI and HR imply better compressibility, while lower values of the same indices correspond to better flowability. The lower particle size of MCC-based formulations tends to promote a greater degree of packing during consolidation of the granules because of significant reduction in powder porosity and lower volume occupied by the granules, which translates to high values of CI and HR.

True density values did not vary significantly across the different granule formulations, though it was observed that granulations formulated with lactose-based diluents (I–III) had relatively lower values in comparison to MCC-based formulations. True density is a measure of intermolecular distance between bonding surfaces and becomes critical in estimating the compaction behaviour of the material. Materials having higher true density correspond to a greater propensity for plastic deformation during compression, leading to tablets of higher tensile strength (Sun, 2011). Moisture content of granules ranged from 5% to 9%, with MCC-based granules having higher values relative to those of lactose-based granules. Slight differences in moisture content may be ascribed to the hygroscopicity of MCC (Sun, 2016). Generally, it was observed that there were variations in granule properties as a result of the type of diluent used in the formulation. In addition, the properties of the granules were also influenced by whether the diluent was either an SCE or C-PE material.

The compressibility–tabletability–compactibility (CTC) profile of the various granule formulation is illustrated in Figs. 2–4. The compressibility plot (Fig. 2) denotes the effect of compression pressure on the porosity of compacts. For all the granule formulations (I–VI), the porosity of compacts was found to decrease with increasing compaction pressure. Compressibility describes the ability of a material to undergo volume reduction when pressure is applied and is usually indicated by a decrease in volume or porosity and a corresponding increase in relative density or solid fraction (Joiris et al., 1998; Upadhyay et al., 2013). For the lactose-based formulations (Fig. 2a), the extent of reduction in porosity did not appear to differ widely across the three granule formulations (I–III). However, it was observed with MCC-based formulations (Fig. 2b) that the extent of porosity reduction differed across the three granule formulations (IV–VI) with MCC granules having the highest degree of compressibility. At 250 MPa, a porosity less than 0.02 was obtained for MCC as compared to 0.04 for AVICEL®HFE and 0.05 for PSV. This may be attributed to the deformation behaviour of MCC, which undergoes extensive plastic deformation during compression, leading to an increased bonding area that promotes compressibility. Hence, in terms of compressibility, MCC, an SCE material had a superior profile compared to C-PE materials. The compressibility of any material is usually determined by many factors like mechanical and particle properties (Sun, 2011).

The compactibility plot displayed as Fig. 3 graphically illustrates the effect of porosity on the tensile strength of compacts. Compactibility refers to the ability of a powder to form coherent compacts under the effect of densification (Sonnergaard, 2006). It describes the relationship between tablet microstructure (porosity) and tensile strength (Sun, 2017; Zeitler & Rades, 2017) For all granule formulations (I–VI), tensile strength of compacts increased with a decrease in porosity. The compactibility profile (Fig. 3a) showed that at a porosity of 0.05, higher tensile strength values were obtained for formulations containing C-PE as diluents (II and III) when compared to the formulation containing SCE as diluent (I). A similar occurrence was observed with MCC-based formulations (Fig. 3b), as higher tensile strength values were obtained at a porosity of 0.05 for formulations containing C-PEs as diluents (V and VI) compared to the formulation containing SCE as diluent (IV). This implies that the compactibility profile of CPEs as diluents was superior to that of SCE material as diluent. Higher compactibility suggests higher inter-particulate bonding. This has been

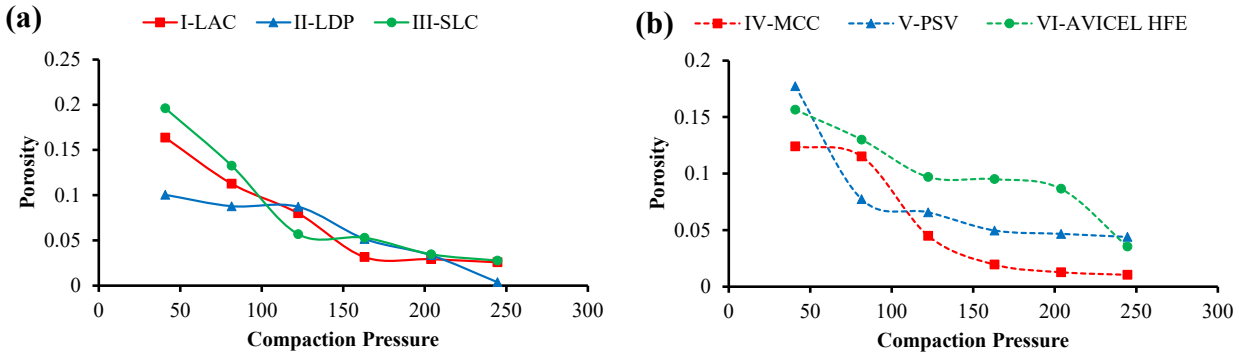

Figure2. Compressibility plot of (a) Lactose-based formulations, (b) MCC-based formulations.

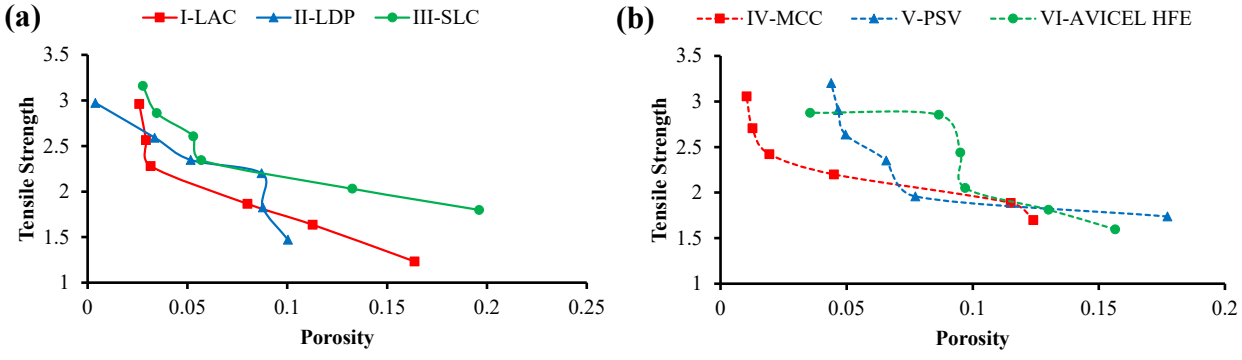

Figure 3. Compactibility plot of (a) Lactose-based formulations, (b) MCC-based formulations.

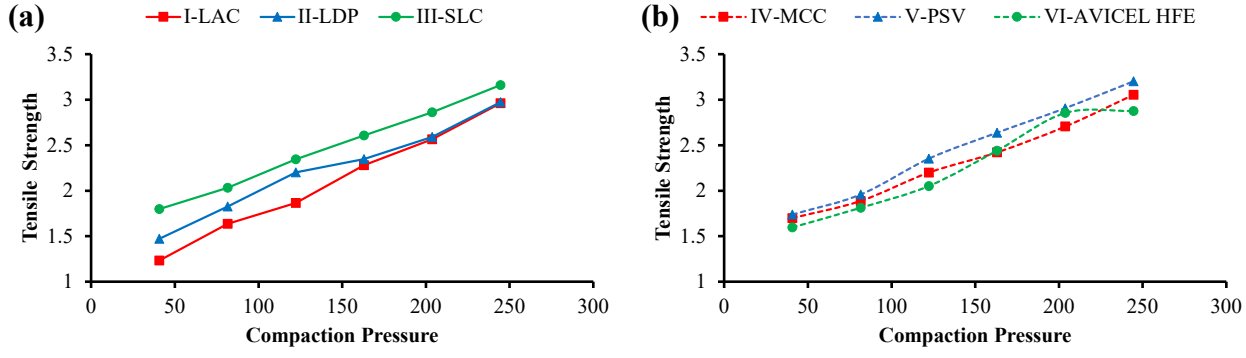

Figure 4. Tabletability plot of (a) Lactose-based formulations, (b) MCC-based formulations.

attributed to an increase in interfacial adhesion occurring during compaction, leading to an increase in bonding strength per unit area giving rise to tablets of higher tensile strength at low porosity. Many composite excipients demonstrate an improvement in compactibility as a result of engineering of particle properties during co-processing. This explains why C-PEs performed better as diluents with respect to compactibility when compared to SCEs.

The tabletability profile of all six granule formulations (I–VI) is presented as Fig.4. The plot correlates the relationship between compression pressure and tensile strength of compacts. All the granule formulations displayed an increase in tensile strength with increase in compaction pressure. Formulations containing C-PE as diluents (II, III, V and VI) produced tablets with relatively higher tensile strength across the entire range of compaction pressure in comparison to formulations containing SCE as

Table 3. Physical properties of metronidazole tablets prepared using different diluents.

	Formulations with diluents					
Parameters	**I**	**II**	**III**	**IV**	**V**	**VI**
Mean weight (mg)	503.10 ± 26.62	507.00 ± 11.41	501.80 ± 13.40	511.90 ± 15.21	509.20 ± 12.84	506.00 ± 9.36
Thickness (mm)	4.02 ± 0.13	4.33 ± 0.06	4.38 ± 0.02	4.02 ± 0.05	4.27 ± 0.06	4.08 ± 0.02
Tensile strength (MPa)	0.78 ± 0.12	0.67 ± 0.30	0.57 ± 0.05	1.19 ± 0.11	0.77 ± 0.04	1.20 ± 0.23
Friability (%)	1.20	1.95	2.00	0.78	1.1	0.69
Disintegration time (min)	14.01 ± 1.57	10.99 ± 1.39	10.96 ± 1.08	25.40 ± 0.34	5.77 ± 2.37	0.32 ± 0.06
$T_{80\%}$ (min)	18.00	15.00	14.00	30.00	4.00	35.00

I – lactose, II – Ludipress®, III – StarLac®, IV – MCC, V – Prosolv®, VI – AVICEL® HFE

diluent (I and IV). This implies that C-PE materials used as diluents provided a greater bonding strength per unit surface area, with minimal elastic recovery during decompression stage of tableting, resulting in tablets with relatively higher tensile strength. Improved tabletability profile of C-PEs has been attributed to the material properties of C-PE that combines a plastic deforming excipient and brittle material into the composite structure of the C-PE, thereby reducing the tendency for significant elastic recovery upon ejection of tablet (Nachaegari & Bansal, 2004). Substantial elastic recovery occurring after ejection leads to the breakage of bonds formed during tableting, thereby lowering the tensile strength upon relaxation of tablet. This explains why granule formulations consisting of SCE as diluent returned tablets of lower tensile strength under the effect of compaction pressure. Hence, tabletability is described as the capacity of a powdered material to be transformed into a tablet of specified strength (tensile strength) under the effect of compaction pressure (Joiris et al., 1998; Yadav et al., 2017).

The physical properties of metronidazole tablets prepared using the different diluents is summarised in Table 3. Average tablet weight ranged from 503.1 mg to 511.9 mg. Formulations containing C-PE as diluents (II, III, V and VI) produced tablets with a uniform distribution in tablet weight compared to formulations containing SCE as diluents (I, IV). This implies that the use of C-PE as diluents improved the flow of granules during tableting, resulting in the formation of tablets of narrow distribution in weight. Thickness of tablets did not differ significantly across the various formulations ranging from 4.02 mm to 4.33 mm and could be attributed to the compressibility of the granules that generated the tablets. Tablet hardness was characterised using tensile strength, and values were ranked in the following order, VI > IV > I > V > II > III, with formulations containing C-PE as diluents returning lower tensile strength values compared to formulations containing SCE as diluents. The lowering of tensile strength of tablets formulated with C-PE as diluents may be associated with the inability of the diluents to exert their effect as directly compressible excipients in tablets formulated by wet granulation. Formulation studies have shown that directly compressible excipients tend to lose their compressibility when incorporated in tablet formulations by wet granulation (Nguyen et al., 2015). Tablet friability parameter was evaluated to measure the weakness of the tablet. Many manufacturers recommend a limit of 1% as tablet friability (Adjei et al., 2017). Only two tablet formulations (IV and VI) passed the friability test. The lactose-based formulations (I–III) did not pass the friability test possibly due to the brittle nature of the diluents used in the formulations. It was generally observed that MCC-based formulations passed the friability test possibly due to the mechanical property of the diluents. MCC-based diluents are known to undergo extensive plastic deformation during tableting, resulting in increased bonding surface area and inter-particulate bonding that produces tablets with higher tensile strength (Edge et al., 2000). Tablet friability of all the formulations corresponded to the tensile strength of tablets. Tablets having lower tensile strength gave rise to high friability and vice versa.

According to BP requirements, immediate release tablets are expected to disintegrate within 15 min. All the tablet formulations passed the disintegration test, except for formulation IV containing MCC as diluent, which failed the disintegration test. MCC is commonly used as a directly compressible excipient because of its exceptional binding properties and prolongs disintegration time when used in concentrations exceeding 15% in tablet formulations (Apeji et al., 2011). It was observed that tablets formulated with C-PE as diluents (II, III, V and VI) disintegrated faster when compared to tablets containing SCE as diluents (I and VI). This has been attributed to the composition of the C-PE, which usually contains an excipient with excellent disintegrant properties like mannitol, starch and crospovidone found in AVICEL®HFE (VI), StarLac (III) and LDP (II), respectively. In addition, the high friability of the tablets must have contributed to rapid disintegration by promoting rapid uptake of water due to high porosity of the tablet created by the relatively high friability.

The rate and extent of drug release from metronidazole tablets was illustrated in Figs. 5 and 6. Drug-release profile

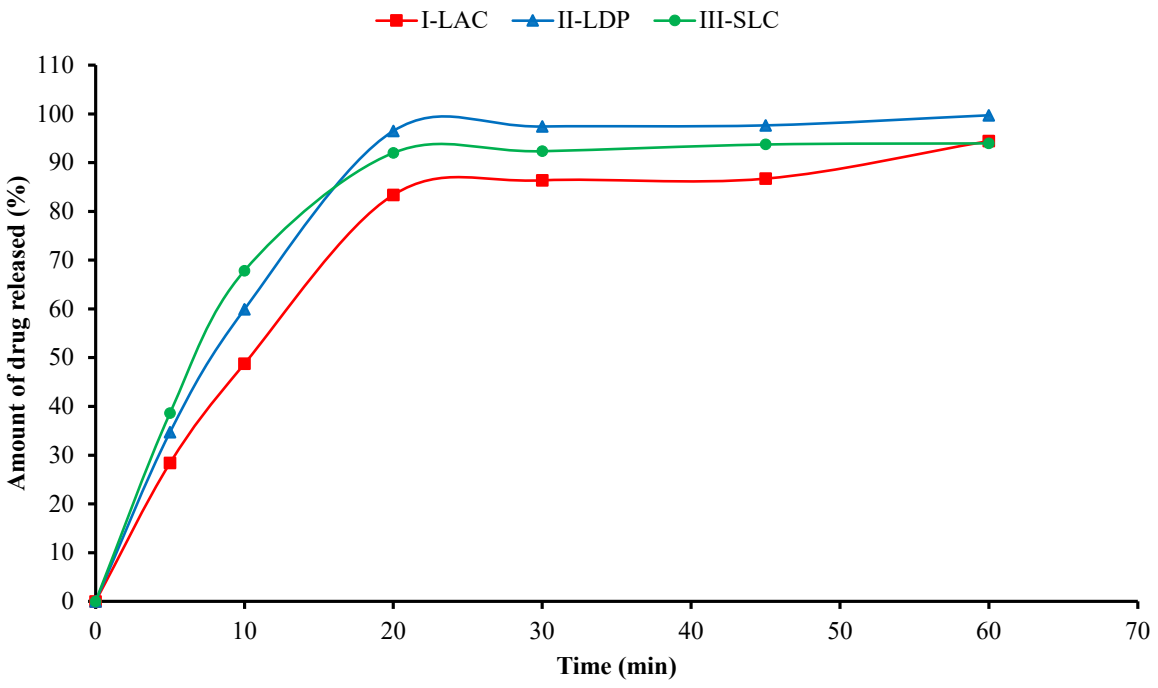

Figure 5. Drug-release profile of Lactose-based formulations.

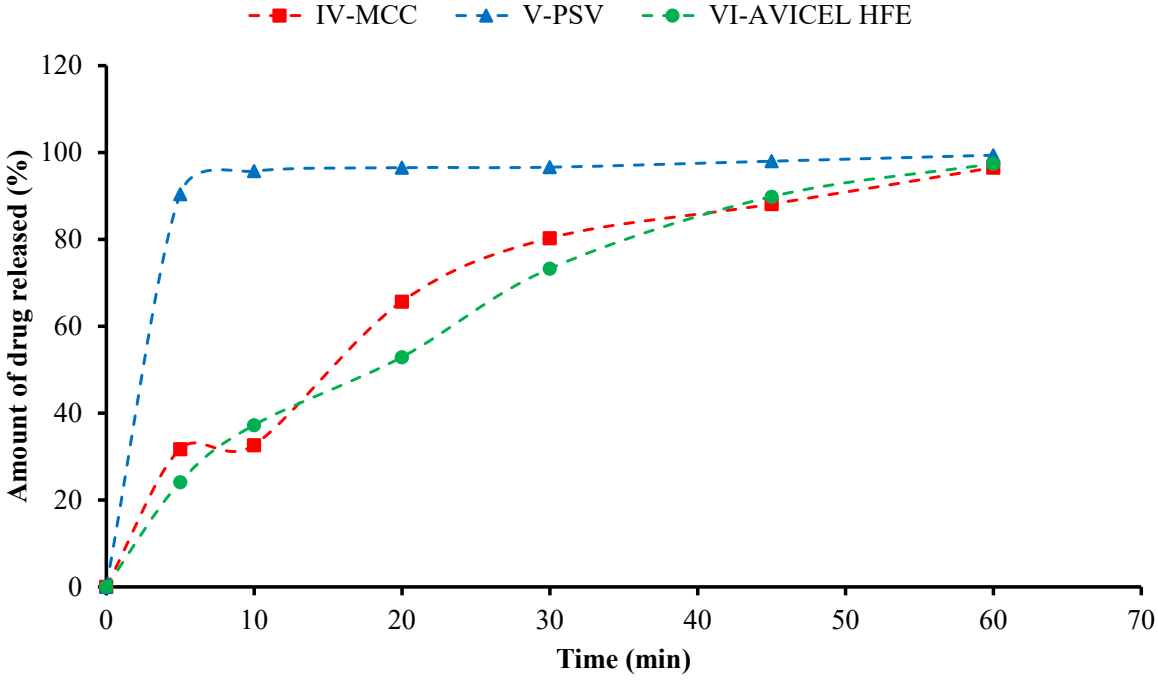

Figure 6. Drug-release profile of MCC-based formulations.

for lactose-based formulations (Fig. 5) shows that the rate and extent of drug release was faster with tablets formulated with C-PE as diluents (II and III) compared to drug release from tablets containing SCE as diluent (I).

A similar pattern was observed for the MCC-based formulations (Fig. 6) where tablets formulated with C-PE as diluent (V) gave a faster and more extensive drug release. The rate and extent of drug release for all tablet formulations corresponded with the time it took for the tablets to disintegrate, as presented in Table 3. However, it was not so for tablets formulated with AVICEL®HFE as diluent as the rapid disintegration time did not correspond to faster drug release possibly because of compact crystal structure of mannitol present in AVICEL®HFE, which may have impeded drug solubility (Daraghmeh et al., 2010). Nonetheless, this tablet formulation passed the dissolution test, which was within the USP limits (not less than 85% of the labelled amount of metronidazole should dissolve in 60 mins). All other formulations prepared with different diluents passed the dissolution test.

CONCLUSION

The aim of the study was to evaluate the tableting properties of C-PEs used as diluents in tablets formulated by wet granulation in comparison to SCEs. The properties of the granules prepared using various diluents differed on the basis of the type of diluent (lactose-based or MCC-based) and composition of the diluent (C-PE or SCE). With respect to the compaction properties of granules, C-PE-based formulations performed better in terms of compactibility and tabletability when compared to SCE-based formulations. The tableting properties of the various tablet formulations revealed the superiority of C-PE-based formulations in tablet weight uniformity, disintegration and drug-release profile of the corresponding tablet formulation. This study, therefore, summarises the performance advantages of incorporating C-PEs as diluents in tablets manufactured by wet granulation and underscores the necessity for rational selection of excipients during tablet formulation.

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Study of the Qualitative Composition and Quantitative Content of Parthenolide in the Feverfew (Tanacetum Parthenium) Herb Cultivated In Ukraine

Original Paper

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Abstract Feverfew is a well-known medicinal plant as a source of the sesquiterpene lactones, the main one is a parthenolide (about 85%). Owing to the parthenolide, feverfew herb has anti-inflammatory activity and it is widely used for prevention of migraine. The aim was to study the qualitative composition and quantitative content of the parthenolide in the samples of the feverfew herb cultivated in Ukraine and to study the influence of environmental factors on the accumulation of parthenolide.
Methods. The identification in the samples of the feverfew herb by the thin-layer chromatography method was carried out on silica gel 60 F254 plates, Merck. For the quantitative determination of the parthenolide, a high-performance liquid chromatography ProStar equipped with a diode-matrix detector, were used.
Results. The chromatographic profile of the samples of the feverfew herb was studied using a TLC method. In the chromatograms of test solutions from all samples, the blue zones were detected at the level of parthenolide. The quantitative content of parthenolide ranged between 0.16% and 0.39%. The highest content was observed in the sample from the Central region of Ukraine. The data obtained indicate that the optimal conditions for parthenolide accumulation in feverfew herb are as follows: black soils, solar insolation not higher than 5.25 kWh/m2/day, average air temperature from +20°C to +25°C. All the calculated validation parameters of the parthenolide quantitative determination method meet the necessary acceptance criteria.
Conclusion. The study of the qualitative composition and quantitative content of parthenolide in the samples of feverfew herb cultivated in different regions of Ukraine has been conducted. According to the methodology of Eur.Ph. and SPhU, a parthenolide was identified in all the samples. HPLC method was used to determine the quantitative content of parthenolide in the samples studied. The optimal conditions for parthenolide accumulation in feverfew herb were established.

Keywords *Feverfew – parthenolide – thin-layer chromatography – high-performance liquid chromatography – environmental conditions*

INTRODUCTION

A search for new biologically active substances (BAS) is an urgent task for pharmaceutical science. Recently, there has been a growing interest in a group of natural compounds called sesquiterpene lactones. Sesquiterpene lactones cause a wide range of biological effects such as antibacterial, anti-inflammatory, cytostatic, fungicidal. Chemically, they are classified according to the degree of carbon skeleton cyclization. The most common among them include monocyclic ones, namely germacranolides, xantanolides, and elemanolides, as well as bicyclic ones, namely eudesmanolides, guaianolides, and pseudoguaianolides. Among the above-mentioned sesquiterpene γ-lactones, it is monocyclic germacranolides that are more often found in plants (more than 600 compounds of this class are known) (Konovalova et al., 2008). Methods for extraction of sesquiterpene lactones from plants are quite diverse due to their physical and chemical characteristics, as well as their location in the plants. Sesquiterpene lactones are most often extracted from leaves and flowers of the plants, and less often from roots and bark (Erasto et al., 2006; Youn et al., 2012). Polar and nonpolar solvents are used for extraction. Ethanol, methanol, chloroform, diethyl ether, and various mixtures thereof can act as extractants.

Sesquiterpene lactones attract the attention of scientists in many countries as they are found in plant sources of such families as *Asteraceae, Amaranthaceae, Apiaceae, Lauraceae, Magnoliaceae,* and *Lamiaceae*, as well as in some species of fungi and mosses. One of the sources of this BAS group is a

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representative of the Asteraceae family, feverfew (*Tanacetum parthenium* (L.) Schultz Bip.). It is a perennial herb, also known as *Chrysanthemum parthenium* (L.) Bernh., *Leucanthemum parthenium* (L.) Gren and Gordon, *Pyrethrum parthenium* (L.) Smith, *Matricaria eximia* Hort., *Matricaria parthenium* (L.) Smith. It is widely cultivated in Europe, North America, and Ukraine as an ornamental and honey-bearing plant, but it has found a wide use in medicine abroad as an anti-inflammatory, antipyretic, and cytostatic agent (Pareek et al., 2011). The main indications for the use of feverfew herb are inflammatory diseases of the connective tissue, gynaecological diseases, and migraines. The biological effect of this plant is mainly due to sesquiterpene lactones, about 30 of which are present in it. Among the sesquiterpene lactones of feverfew, about 85% is parthenolide that belongs to the class of germacranolides. Other sesquiterpene lactones are found in fewer amounts, they are chrysanthemum, artecanin, artemorin, balchanin, canin, 10-epicanin, epoxyartemorin, santamarin, epoxysantamarin, and so on (Konovalova et al., 2008).

The accumulation of BAS is influenced by environmental factors, first of all, air temperature, precipitation, solar insolation, and soil composition. A separate important factor for the harvesting of medicinal plant raw materials with the highest BAS content is the vegetation phase. According to the literature, the vast majority of medicinal plant raw material is harvested during the period of mass flowering (Zolotaykina et al., 2016; Feduraev et al., 2019). Accumulation of parthenolide has been previously studied by foreign researchers. For example, Awang V.C. has studied the accumulation of parthenolide in a mixture of leaves and flowering tops and separately in the leaves and flowering tops of feverfew by HPLC. The content of parthenolide in the mixture of leaves and flowering tops was 0.18 %, 0.09% in leaves, and 0.27 % in flowering tops (Awang et al., 1991). Hendricks H.[8] has analysed the content of parthenolide accumulation in feverfew herb during the vegetation season. The content of parthenolide ranged from 0.29 % to 0.92 % and the highest content was observed at the beginning of flowering (Hendricks et al., 1997). According to the research of Avula B., the content of parthenolide in feverfew herb collected in different regions ranged from 0.25% to 0.28 % (Avula et al., 2006).

Monograph 'Tanaceti parthenii herba" is presented in the European, American, American herbal, and British Pharmacopoeias, as well as in the State Pharmacopoeia of Ukraine (SPhU) (Eur.Ph. 9th, 2013; Br.Ph. III, 2009; AHPh, 2011; USP 30th, 2007; SPhU II, 2014). The SPhU monograph on feverfew herb is fully harmonized with the European Pharmacopoeia (Eur.Ph.), but studies of domestic raw materials for compliance with the requirements of this monograph have not been conducted yet. According to the monograph, the identity and quality of medicinal plant raw materials are determined by morphological and anatomical features, qualitative composition, and quantitative content of sesquiterpene lactone, parthenolide.

The purpose of the study was to study the qualitative composition and quantitative content of parthenolide in the samples of feverfew cultivated in Ukraine, as well as to study the influence of environmental factors on the accumulation of parthenolide.

MATERIALS AND METHODS

The objects of the study were sample batches of feverfew herb collected in Kharkiv (Rs 868), Zhytomyr (Rs 864), Cherkasy (Rs 865), Sumy (Rs 866), Dnipropetrovsk (Rs 867), Poltava (Rs 869), and Kyiv (Rs 870) regions during the mass flowering period in 2018. Samples for analysis were prepared by collecting and combining three individual plants as one sample from a specific area.

The batches of medicinal plant raw materials are registered in the State Enterprise 'Ukrainian Scientific Pharmacopoeia Centre for Quality of Medicines.'

Identification of parthenolide was performed by TLC using the method given in 'Feverfew' monograph of the European Pharmacopoeia (Eur.Ph.) and 'Feverfew' monograph of the SPhU 2.0 (Eur.Ph. 9th, 2013, SPhU II, 2014). The studies were performed using chromatography plates TLC silica gel 60 F254 (Merck).

For identification and quantitative determination, a standard sample of parthenolide by Sigma-Aldrich, Germany (lot no. 1002453389, content ≥ 98%) was used. For identification of parthenolide in feverfew herb, a reference solution was prepared at a concentration of 1 mg/mL in methanol. The test solution was prepared by mixing 1 g of the powdered raw material (355-μm sieve) in 20 ml of methanol and heating it in a water bath at 60 °C for 15 minutes. The resulting solution was cooled, filtered, and evaporated under reduced pressure, after which the remainder was dissolved in 2 mL of methanol. A 15:85 acetone/toluene mixture was used as mobile phase. The solutions for analysis were applied in strips 10 mm long and the volume of 20 mL. For development, the chromatography plate was sprayed with a solution of 5 g/L of vanillin in a mixture of 20 volumes of ethanol and 80 volumes of sulfuric acid.

Determination of the quantitative content of parthenolide in feverfew herb was performed using a high-performance liquid chromatograph ProStar, equipped with a diode-matrix detector PDA 330 (Varian, the USA), according to the method described in 'Feverfew' monographs of the Eur.Ph. and SPhU 2.0 (Eur.Ph. 9th, 2013, SPhU II, 2014). To determine the content of parthenolide, a column Purospher® STAR RT-18e, Hibar (250 x 4.0 mm, particle size 5 μm) by Merck was used. Mobile phase: a mixture of acetonitrile and water (40 : 60), elution type: isocratic. Separation was performed at a flow rate of 1 mL/min. The injection volume was 20 μl. The detection was performed at a wavelength of 220 nm. Acetonitrile and methanol by Sigma-Aldrich (Germany) with 'gradient grade, for HPLC' qualification and water for chromatography were used for analysis.

Table 1. Results of quantitative determination of parthenolide in batches of feverfew herb.

Batch No.	The region of collection of raw materials	Requirements, %	$X_{avr} \pm_{\Delta} X$, %	Criterion satisfaction
Rs 864	Zhytomyr region	≥ 0.2	0.22 ± 0.01	Satisfied
Rs 865	Cherkasy region		0.16 ± 0.06	No satisfied
Rs 866	Sumy region		0.27 ± 0.11	Satisfied
Rs 867	Dnipropetrovsk region		0.26 ± 0.09	Satisfied
Rs 868	Kharkiv region		0.22 ± 0.02	Satisfied
Rs 869	Poltava region		0.24 ± 0.04	Satisfied
Rs 870	Kyiv region		0.39 ± 0.10	Satisfied

Note. X_{avr} – average value; $_{\Delta}X$ – half-wight of confidence interval

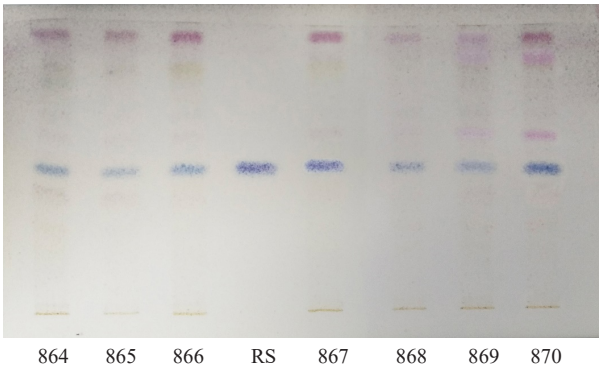

Figure. 1. TLC of feverfew herb batches and parthenolide reference sample (RS).

Test solution. About 50 g of the tested raw material (355-μm sieve) was powdered. 1.00 g of powdered raw material after homogenization was placed into a flask. After adding 40 mL of methanol, it was heated in a water bath at a temperature of 60°C for 10 minutes, then it was cooled and filtered. The filter was washed with 15 mL of methanol R. 40 mL of methanol was added to the remainder, the procedure was repeated, the filtrates and washings were combined, and were evaporated to dryness under reduced pressure. The residue was dissolved in methanol R and the volume of the solution was made up to 20.0 mL with the same solvent. 10.0 mL of the resulting solution was made up to 50.0 mL with mobile phase.

Reference solution. 5.0 mg of parthenolide RS was dissolved in methanol and the volume of the solution was made up to 10.0 mL with the same solvent. 2.0 mL of the resulting solution was made up to 50.0 mL with mobile phase.

Before chromatography, solutions were filtered through membrane filters with a pore size of no more than 0.45 μm.

The content (*X*) of parthenolide, in per cent, is calculated by the following formula:

$$X = \frac{A_1 * m_2 * 40}{A_2 * m_1},$$

where:

A_1 is the area of parthenolide peak in the chromatogram of the test solution;

A_2 is the area of parthenolide peak in the chromatogram of the reference solution;

m_1 is the exact weight of the raw material used for the preparation of the test solution, in g;

m_2 is the weight of parthenolide in the reference solution, in g.

RESULTS AND DISCUSSION

A method of thin-layer chromatography was used to identify parthenolide in the samples of feverfew herb cultivated in different regions of Ukraine. The results of the study are shown in Figure 1.

As seen from the chromatogram presented, in the central part of the chromatogram, a blue zone is identified at the level of the main zone in the chromatogram of parthenolide reference sample in all the studied samples of the herb. The colour intensity of the identified zones of the corresponding compounds is approximately the same.

During the quantitative determination of parthenolide in the studied samples by HPLC, it is found that the retention time of parthenolide under the described conditions is approximately 16.3 minutes. Typical chromatograms of the reference solution and the test solution are shown in Fig. 2 and 3.

The results of quantitative determination of parthenolide in samples of feverfew herb are shown in Table 1.

To confirm the reliability of the obtained analysis results of parthenolide quantitative content in the samples of feverfew

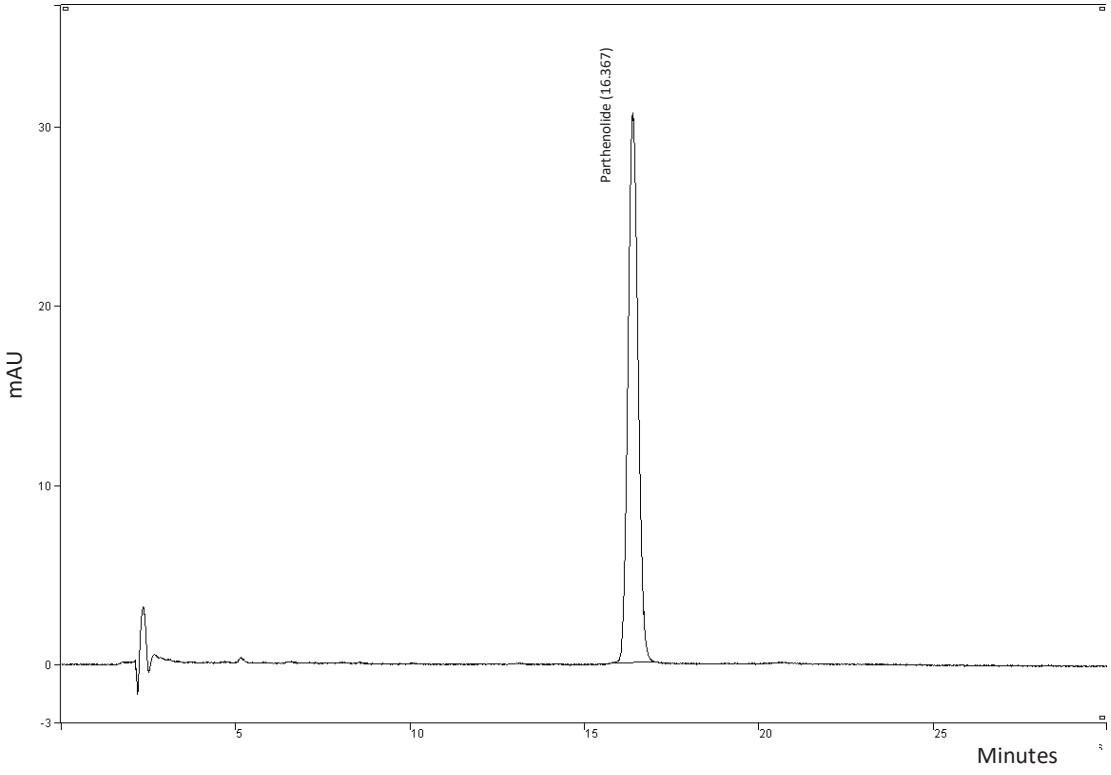

Figure 2. Chromatogram of parthenolide reference solution.

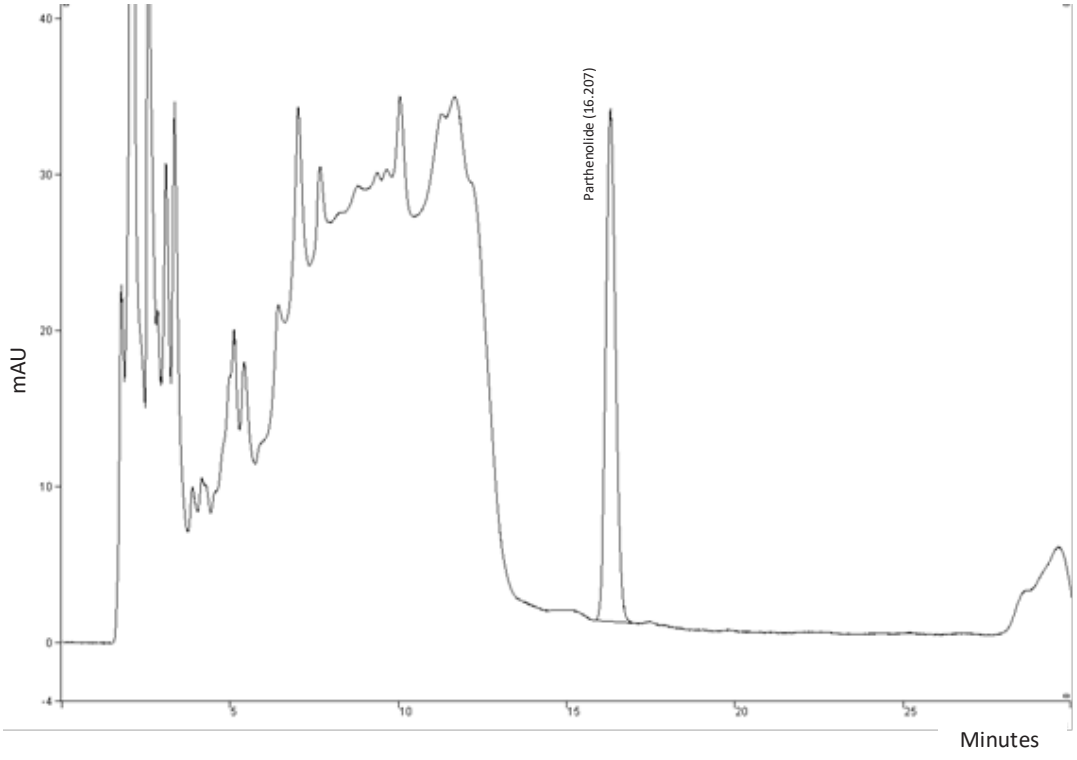

Figure 3. Chromatogram of the test solution.

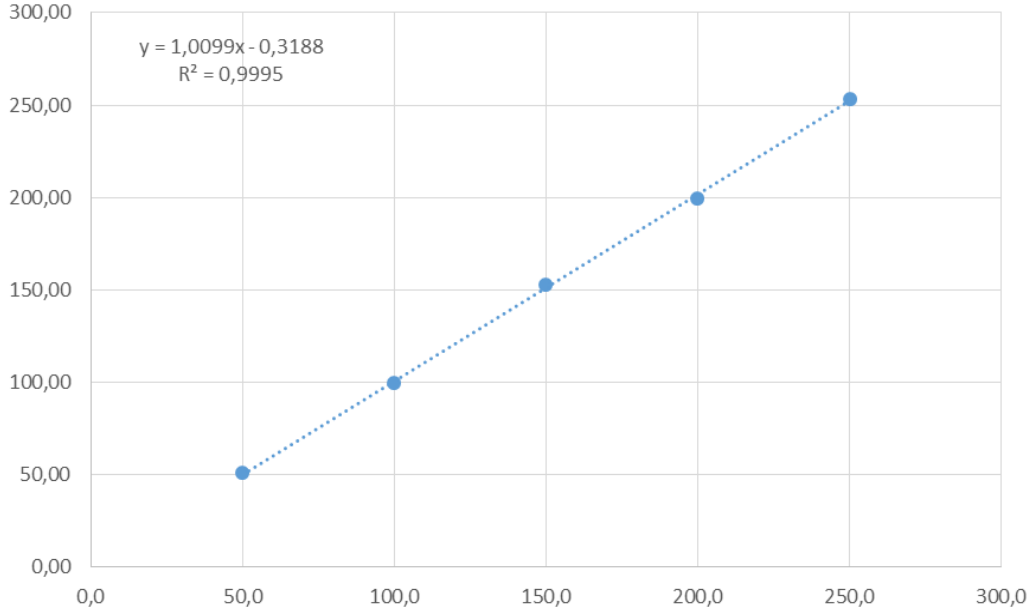

*Figure 4. Graph of linear dependence Yi = b * Xi + a for parthenolide reference solution.*

Table 2. The linearity parameters of the quantitative determination method.

Parameter	Requirements	Received value	Criterion satisfaction
$\lvert a \rvert$	≤ 4.096	0.32	Satisfied
S_0	≤ 2.72	2.57	Satisfied
r	> 0.9821	0.9998	Satisfied

herb, we verified the quantitative determination method according to the following parameters: linearity, accuracy, and precision.

The method of quantitative determination should be linear within the range of application, which should overlap the possible values of the active substance concentrations. In accordance with the requirements of the SPhU, the range of application of the quantitative determination method for parthenolide should be between 50 and 250% compared to the content claimed (0.01–0.05 mg/ml).

To confirm the linearity of the method, 5 model solutions of parthenolide were prepared, the concentration of which varied evenly within the range of application (a step of 50%).

Fig. 4 shows a graph of the linear dependence of parthenolide peak area on the actual concentration of the solution, constructed in normalized coordinates.

The acceptance criteria are calculated in accordance with the requirements of SPhU 2.0 and are shown in Table 2.

The results obtained confirm that the method for quantitative determination of parthenolide in the concentration range between 50% and 250% is linear.

To calculate accuracy and precision, the overall uncertainty of the analysis method was determined. According to the requirements of SPhU 2.0 for quantitative determination (one-way normalization 'at least'), the maximum allowable uncertainty of the analysis method is max $\Delta_{AS} \leq 6.4\%$. The accuracy and precision criteria were determined in accordance with the requirements of SPhU 2.0 monograph 5.3.N. 2. 'Validation of analytical methods and tests.' The results are shown in Table 3.

The method for determining parthenolide meets the criteria for acceptance of the validation indicator 'accuracy' according to two criteria. The method is characterized by sufficient convergence, since the determined value of the relative confidence interval of the parameter (2.83%) is less than the critical value for the convergence of results (6.4%) and meets the criteria for acceptance of the validation indicator 'precision.'

According to the purpose, the next step was to study the influence of environmental factors on the accumulation of parthenolide in the domestic samples of feverfew raw materials. Climatic and edaphic conditions of the regions during the growing season are shown in Table 4.

According to the results of quantitative determination of parthenolide samples of feverfew herb grown in Ukraine, the highest content was observed in the sample from the Central region of Ukraine, namely Kyiv region, and was 0.39 %, which is probably due to soil composition and moderate environmental conditions. For example, in Kyiv region, black soils predominate, as well as during the growing season

Table 3. The results of the evaluation of the accuracy and precision of the HPLC method.

Parameter	Index	Criterion		Criterion satisfaction
		Requirements for statistical insignificance	Requirements for practical insignificance	
	0.79	≤ 1.26%	≤ 2.048%	Satisfied
ΔZ	2.83	≤ 6.4%		Satisfied

Table 4. Climatic and edaphic conditions of the regions during the growing season of feverfew herb

The region of collection of raw materials	Soil	Solar insolation, kWh/m2/day	Air temperature, °C	Precipitation, mm
Zhytomyr region	black	3.10–5.21	+21–+26	55–85
Cherkasy region	sod-gley / sod-podzolic	3.99–5.54	+20–+25	52–73
Sumy region	black	3.12–5.24	+22–+33	50–75
Dnipropetrovsk region	black	3.11–5.22	+22–+30	51–74
Kharkiv region	black	3.12–5.25	+19–+24	55–85
Poltava region	black	3.13–5.23	+21–+26	50–69
Kyiv region	black	3.12–5.25	+20–+25	50–70

of feverfew herb, solar insolation indicators ranged from 3.12 to 5.25 kWh/m2/day, the air temperature ranged from +20°C to +25°C, and the amount of precipitation ranged from 50 to 70 mm. The sample from Sumy region had a parthenolide content 1.4 times less than the sample from Kyiv region, and was 0.27%, which could be affected by higher air temperature, namely between +22°C and +33°C, and more precipitation: 50 to 75 mm. Approximately at this level, the content of parthenolide was observed in the sample from Dnipropetrovsk region, 0.26%. In the samples from Kharkiv and Zhytomyr regions, the content of parthenolide was observed at the same level of 0.22%, which was 1.7 times less than in the sample from Kyiv region. During the vegetation season, there was an increased amount of precipitation in these regions, namely 55 to 85 mm. The lowest parthenolide content was determined in the sample from Cherkasy region, which may be associated with sod-gley and sod-podzolic soils in this region, as well as the highest solar insolation index, namely 3.99 to 5.54 kWh/m2/day. The data obtained indicate that the optimal conditions for parthenolide accumulation in feverfew herb are as follows: black soils, solar insolation not higher than 5.25 kWh/m2/day, average air temperature from +20°C to +25°C, and average precipitation not more than 70 mm. Compliance with these conditions during cultivation may contribute to a higher content of parthenolide in feverfew herb.

CONCLUSIONS

For the first time, the study of the qualitative composition and quantitative content of parthenolide in the samples of feverfew herb cultivated in different regions of Ukraine has been conducted. According to the methodology of Eur. Ph. and SPhU, a sesquiterpene lactone parthenolide was identified in all raw material samples. HPLC method was used to determine the quantitative content of parthenolide in the samples studied. The quantitative content of parthenolide ranged between 0.16% and 0.39%. The obtained results of the analysis of 6 batches of raw materials indicate compliance with the requirements of the Eur.Ph. and SPhU according to parameters 'Identification C' and 'Assay.' A batch of herbs harvested in Cherkasy region (batch Rs 865) did not meet the requirements of the monograph 'Feverfew' as for indicator 'Assay,' which may be due to the natural and climatic conditions during its cultivation.

All the calculated validation parameters of the parthenolide quantitative determination method meet the necessary acceptance criteria.

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Synthesis, antimicrobial and antiradical activity of (3-alkoxymethyl-4-hydroxyphenyl)propan-1-ones, intermediates of biologically active compounds and activity comparison with 3-(alkoxymethyl)-4-(alkylamino-2-hydroxypropoxyphenyl)alkanones type of beta blockers

Original Paper

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Abstract A homologous series of (3-alkoxymethyl-4-hydroxyphenyl)propan-1-ones was prepared by the reaction of (3-chloromethyl-4-hydroxyphenyl)propan-1-ones with the corresponding alcohols (methanol – decan-1-ol, propan-2-ol, 2-methylpropan-1-ol, 3-methylbutan-1-ol, cyclopentanol, benzylalcohol) in the presence of sodium hydrogen carbonate. The composition of the synthesised compounds was elucidated by IR, UV and ^{1}H-NMR and ^{13}C-NMR spectra. Selected compounds were tested against human pathogens: gram-positive bacterium *Staphylococcus aureus* (CNCTC Mau 29/58), gram-negative bacterium *Escherichia coli* (CNCTC 377/79) and yeast *Candida albicans* (CCM 8186). Their antimicrobial activities were expressed as minimum inhibitory concentrations. Antioxidant activity was determined using DPPH and $ABTS^{\cdot+}$ methods. It could be shown that both biological activities, antimicrobial and antioxidant, were lower in comparison with the (2*RS*)-bis [3-(4-acetyl-2-propoxymethyl)phenoxy-2-hydroxypropyl]isopropylammonium fumarate type of beta blockers.

Keywords *substituted phenols – antimicrobial activity – antioxidant activity – antiradical activity*

INTRODUCTION

The introduction of a phenolic group into drug molecules confers upon them a reactive functionality with acidic nature. Phenols can form chelates with metal ions (Hider et al., 2001; Fernandez et al., 2002) and are able to bind to basic functional groups of proteins, hence their broad spectrum of biological activities, such as antimicrobial (Taguri et al., 2004; Taguri et al., 2006; Cueva et al., 2010; Park et al., 2001) and antioxidative activity (Sroka & Cisowski, 2003; Bendary et al., 2013).

The bioactivity of phenols and their toxicity are both influenced by the number of phenolic groups and their relative position (Calliste et al., 2001; Amouar et al., 2009; Kadoma et al., 2010).

Fujisawa (Fujisawa et al., 2004) studied antioxidative activity of 2-methoxy- and 2-*tert*-butylphenols having up to three substituents on the aromatic ring. It could be shown that antioxidative activity decreases as a result of exchange of the methoxymethyl group against a methyl group. Derivatives with a *tert*-butyl group exhibited marked increase in antioxidative activity.

Kadoma et al. (2008) and Kadoma et al. (2009) investigated, besides antioxidative properties, also the cytotoxicity of 2- or 2,6-*tert*-butylphenols and 2-methoxyphenols in several cancer cell lines. Both antioxidative and cytotoxic activities followed similar structure–activity relationship. The introduction of a *tert*-butyl group as well as dimerisation led to a distinct increase in cytotoxicity.

The structurally similar eugenol (2-methoxy-4-(prop-2-en-1-yl)phenol) exhibits antibacterial (Devi et al., 2010), antifungal (Morcia et al., 2012; Abbaszadeh et al., 2014), antioxidant

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(Fujisawa et al., 2002), local anaesthetic (Park et al., 2001) and anti-depressant activities (Irie et al., 2004).

Herein, we report a two-stage synthesis of the target compounds starting from 4-hydroxyphenylpropan-1-one (Table 1 and Figure 1). In the first step, the electrophilic substitution reaction of 4-hydroxyphenylpropan-1-one with paraformaldehyde and hydrochloric acid yields 1-[3-(chloromethyl)-4-hydroxy-phenyl]propan-1-one. Nucleophilic substitution of this intermediate by corresponding alcohols leads to 1-[-3-(alkoxymethyl)-4-hydroxyphenyl]propan-1-ones, where the substituent can be an aliphatic alkyl with chain length C_1–C_{10}, cyclopentyl and phenylmethyl (benzyl).

Many of these substances find application as intermediates in the synthesis of biologically active compounds of the aryloxyaminopropanol type with beta adrenoceptor blocking, antiarrhythmic and anticonvulsive activity (Čižmáriková et al., 1985; Čižmáriková et al., 1986; Čižmáriková et al., 2003).

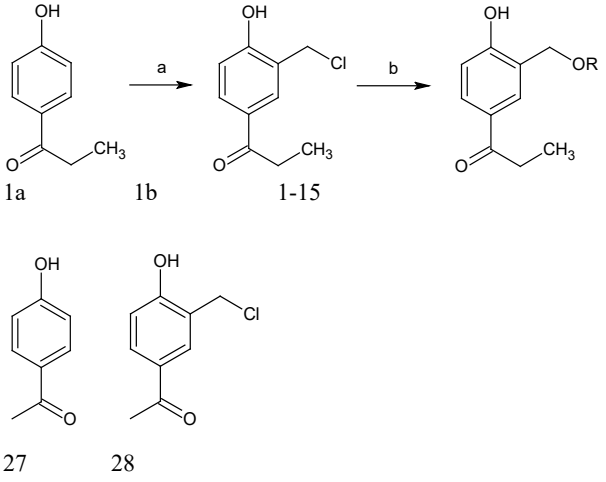


Figure 1. Synthetic route for derivatives of propiophenone and chemical structures of derivatives of acetophenone.

EXPERIMENTAL

The melting points were determined using a Kofler micro hot stage and were quoted uncorrected. Elemental analysis was carried out on a FLASH 2000 (Thermo Scientific) analyser, and the results were within 0.3% of the theoretical values.

The purity of newly prepared compounds was assessed by TLC using Silufol® UV 254 (Merck) sheets with the mobile phase cyclohexane/ethyl acetate (8:2 v/v). UV spectra were recorded on the spectrometer Hewlett-Packard 8452 in methanol. IR spectra were measured using FTIR IMPACT 400 D (Nicolet) 6700. ^{1}H-NMR were recorded on Varian Gemini 2000 spectrometer operating at 3,000 MHz for protons.

Synthesis

(3-Chloromethyl-4-hydroxyphenyl)propan-1-one (1b) (Čižmáriková et al., 1991)

To a sulfonation flask setup with mechanical stirring contact thermometer and powder funnel, 0.15 mol of 4-hydroxyphenylpropan-1-one (1a) and 90 cm^3 of concentrated HCl were added. The temperature was subsequently maintained, the mixture was stirred and the reaction was allowed to proceed for 4.5 h. Following the precipitation, the solid product was collected using suction filtration, washed with water and crystallised from benzene or ethyl acetate. M.p. 132-5°C, yield 75% (da Re & Verlichi, 1956) m.p. 133-6°C, yield 57%).

(3-Alkoxymethyl-4-hydroxyphenyl)propan-1-one (1-15) (Čižmáriková et al., 1991)

To a sulfonation flask setup with mechanical stirring, reflux condenser and contact thermometer, 0.12 mol (chloro-4-hydroxyphenyl)propan-1-one and 100 cm^3 of dried corresponding alcohol were added. The temperature was raised to 40–50°C, and 19.2 g (0.23 mol) of sodium hydrogen carbonate was added gradually during 1 h. The products were crystallised from heptane.

(2RS)- bis[3-(4-acetyl-2-propoxymethyl)phenoxy-2-hydroxypropyl]isopropylammonium fumarate

0.57 mol chloromethyloxirane was gradually added to a solution of 0.55 mol (3-alkoxymethyl-4-hydroxyphenyl) ethanone in 0.59 mol potassium hydroxide dissolved in 50 cm^3 water. The stirred mixture was left to react at room temperature for 24 h, the product was extracted with diethyl ether or chloroform, the extract was washed with 5% sodium hydroxide and water. The organic layer was dried with magnesium sulphate, and the solvent was evaporated. The residue formed by 4-(2,3-epoxypropoxy)-3-(alkoxymethyl) ethanone (cca. 60% yield) was dissolved without previous purification in ethanol or propan-1-ol (50 cm^3) and reacted with isopropylamine (10 cm^3). The mixture was kept at 30°C for 3 h and then under reflux for 4 h. The solvent and unreacted isopropylamine was removed under reduced pressure, the residue was diluted with water (25 cm^3) and the base was extracted to diethyl ether. The extract was dried with potassium carbonate. Addition of an ethereal solution of fumaric acid resulted in separation of the salt, which was crystallised from an appropriate solvent.

EP1 1-(3-methoxymethyl-4-hydroxyphenyl)propan-1-one (1)

$C_{11}H_{14}O_3$ M_r 194.23, Anal. calcd. %C 68.02, %H 7.27 found %C 68.30, %H 7.20. Yield: 63%, R_F 0.53, Mp. 78–80°C (heptane), IR (cm^{-1}) 3,236 (νOH$_{asoc.}$), 1,653 (νC=O), 1,596 (νC=C), 1,274 (νCOC), UV (CH_3OH, λ in nm, ε in m^2.mol^{-1}) λ_{max} 222 nm (log ε_1 3.24), 273 (log ε_2 3.25)

^{1}H-NMR ($CDCl_3$): 1.18–1.23 (t, 3H, $COCH_2CH_3$), 2.90–2.99 (q, 2H, $COCH_2CH_3$), 3.47 (s, 3H, CH_2OCH_3), 4.72 (s, 2H, Ar-CH_2O), 6.89–6.92 (d, 1H, CH_{AR}^6), 7.70–7.71 (d, 1H, CH_{AR}^3), 7.83–7.87 (dd, 1H, CH_{AR}^5), 8.16 (s, 1H, ArOH)

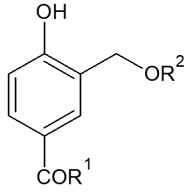


Table 1. Overview of the studied 1-[3-(alkoxymethyl)-4-hydroxyphenyl]alkanones.

Substance Number	Description	R1	R2
1	EP1	CH_2CH_3	CH_3
2	EP2	CH_2CH_3	CH_2CH_3
3	EP3	CH_2CH_3	$(CH_2)_2CH_3$
4	EP3i	CH_2CH_3	$CH(CH_3)_2$
5	EP4n	CH_2CH_3	$(CH_2)_3CH_3$
6	EP4i	CH_2CH_3	$CH_2CH(CH_3)_2$
7	EP5n	CH_2CH_3	$(CH_2)_4CH_3$
8	EP5i	CH_2CH_3	$(CH_2)_2CH(CH_3)_2$
9	EP6n	CH_2CH_3	$(CH_2)_5CH_3$
10	EP7n	CH_2CH_3	$(CH_2)_6CH_3$
11	EP8n	CH_2CH_3	$(CH_2)_7CH_3$
12	EP9n	CH_2CH_3	$(CH_2)_8CH_3$
13	EP10n	CH_2CH_3	$(CH_2)_9CH_3$
14	EP5c	CH_2CH_3	Cyclopentyl
15	EPbenz	CH_2CH_3	CH_2phenyl
16	EA1	CH_3	CH_3
17	EA2	CH_3	CH_2CH_3
18	EA3n	CH_3	$CH_2CH_2CH_3$
19	EA3i	CH_3	$CH(CH_3)_2$
20	EA4n	CH_3	$(CH_2)_3CH_3$
21	EA5n	CH_3	$(CH_2)_4CH_3$
22	EA7n	CH_3	$(CH_2)_6CH_3$
23	EA8n	CH_3	$(CH_2)_7CH_3$
24	EA9n	CH_3	$(CH_2)_8CH_3$
25	EA5c	CH_3	Cyclopentyl
26	EAbenz	CH_3	CH_2phenyl
27	4-OHacet		
28	EAch		

^{13}C-NMR ($CDCl_3$): 8.45 ($COCH_2CH_3$), 31.34 ($COCH_2CH_3$), 58.49 (CH_2OCH_3), 73.97 (Ar-CH_2O), 116.39 (C_{AR}^6), 121.90 (C_{AR}^3), 128.53 (C_{AR}^2), 129.32 (C_{AR}^5), 130.19 (C_{AR}^4), 160.60 (C_{AR}^1), 199.43 (CO)

EP2 1-(3-ethoxymethyl-4-hydroxyphenyl)propan-1-one (2) $C_{12}H_{16}O_3$ M_r 208.26, Anal. calcd. %C 69.21, %H 7.74 found % C69.01, %H 7.93. Yield: 57%, R_F 0.52, Mp. 65–67°C (heptane), IR (cm^{-1}) 3,236 ($\nu OH_{asoc.}$), 1,653 (νC=O), 1,596 (νC=C), 1,274 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 201 nm (log ε_1 3.29), 216 (log ε_2 3.26), 264 (log ε_3 3.22)

^{1}H-NMR ($CDCl_3$): 1.17–1.21 (t, 3H, $COCH_2CH_3$), 1.22–1.26 (t, 3H, CH_{3alk}^2), 2.92–2.95 (q, 2H, $COCH_2CH_3$), 3.59–3.67 (q, 2H, $CH_{2\,alk}^1$), 4.76 (s, 2H, Ar-CH_2O), 6.89–6.92 (d, 1H, CH_{AR}^6), 7.69–7.70 (d, 1H, CH_{AR}^3), 7.82–7.85 (dd, 1H, CH_{AR}^5), 8.39 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.08 ($COCH_2CH_3$), 15.60 (C_{alk}^2), 32.28 ($COCH_2CH_3$), 67.25 (Ar-CH_2O), 68.60 (C_{alk}^1), 115.89 (C_{AR}^6), 126.33

(C_{AR}^{2}), 129.89 (C_{AR}^{5}), 130.91 (C_{AR}^{4}), 131.12 (C_{AR}^{3}), 161.53 (C_{AR}^{1}), 202.23 (CO)

EP3n 1-(4-hydroxy-3-propoxymethylphenyl)propan-1-one (3)

$C_{13}H_{18}O_3$ M_R=222.29 Anal. calcd. %C 70.24 %H 8.16 found %C 70.54, %H 8.32. Yield: 65%, R_F 0.55 (Mp. 61–63°C (heptane), IR (cm^{-1}), 3,248 ($\nu OH_{asoc.}$), 1,686 ($\nu C=O$), 1,602 ($\nu C=C$), 1,272 (νCOC), UV (CH_3OH, λ in nm ε in $m^2.mol^{-1}$); λ_{max} 227 nm (log ε_1 3.01), 264 (log ε_2 3.132), 277 (log ε_3 3.10)

^{1}H-NMR ($CDCl_3$): 0.93–0.98 (t, 3H, $CH_{3\ alk}^{3}$), 1.13–1.18 (t, 3H, $COCH_2CH_3$), 1.61–1.68 (m, 2H, $CH_{3\ alk}^{2}$), 2.92–3.00 (q, 2H, $COCH_2CH_3$), 3.51–3.55 (t, 2H, $CH_{3\ alk}^{1}$), 4.75 (s, 2H, Ar-CH_2O), 6.82–6.85 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{3}), 7.82–7.91 (dd, 1H, CH_{AR}^{5}), 8.43 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.08 ($COCH_2CH_3$), 11.12 (C_{alk}^{3}), 24.05 (C_{alk}^{2}), 32.28 ($COCH_2CH_3$), 68.74 (Ar-CH_2O), 73.62 (C_{alk}^{1}), 115.87 (C_{AR}^{6}), 126.41 (C_{AR}^{2}), 129.86 (C_{AR}^{5}), 130.85 (C_{AR}^{4}), 131.02 (C_{AR}^{3}), 161.74 (C_{AR}^{1}), 199.81(CO)

EP3i 1-(4-hydroxy-3-isopropoxymethylphenyl)propan-1-one (4)

$C_{13}H_{18}O_3$ M_r 222.29 Anal. calcd. % C 70.24% H 8.16 found % C 70.54, % H 8.32. Yield: 65%, R_F 0.55, Mp. 61-63°C (heptane), IR (cm^{-1}), 3,248 ($\nu OH_{asoc.}$), 1,686 ($\nu C=O$), 1,602 ($\nu C=C$), 1,272 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 226 nm (log ε_1 3.14), 263 (log ε_2 3.16), 276 (log ε_3 3.12)

^{1}H-NMR: 1.17–1.23 (t, 3H, $COCH_2CH_3$), 1.25–1.27 (d, 6H, $CH(CH_3)_2$), 2.89–2.97 (q, 2H, $COCH_2CH_3$), 3.74–3.82 (m, 1H, CH), 4.76 (s, 2H, Ar-CH_2O), 6.88–6.91 (d, 1H, CH_{AR}^{6}), 7.68–7.69 (d, 1H, CH_{AR}^{3}), 7.81–7.84 (dd, 1H, CH_{AR}^{5}), 8.57 (s,1H, ArOH)

^{13}C-NMR: 9.07 (COCH2CH3), 22.55 ($CH(CH_3)_2$), 32.23 ($COCH_2CH_3$), 66.25 (Ar-CH_2O), 73.04 (CH), 115.80 (C_{AR}^{6}), 126.70 (C_{AR}^{3}), 129.82 (C_{AR}^{4}), 130,72 (C_{AR}^{5}), 130.90 (C_{AR}^{2}), 161.39 (C_{AR}^{1}), 202.11 (CO)

EP4n 1-(3-butyloxymethyl-4-hydroxyphenyl)propan-1-one (5)

$C_{14}H_{20}O_3$ M_r=236.36, Anal. calcd. %C 71.16 %H 8.53 found %C 71.40, %H 8.52. Yield: 69%, R_F 0.57, Mp. 68–70°C (heptane), IR (cm^{-1}), 3,340 ($\nu OH_{asoc.}$), 1,684 ($\nu C=O$), 1,600 ($\nu C=C$), 1,270 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 201 (log ε_1 3.23), 217 (log ε_2 3.22), 264 (log ε_3 3.13); 277 (log ε_4 3.20)

^{1}H-NMR ($CDCl_3$): 0.91–0.96 (t, 3H, $CH_{3\ alk}^{4}$), 1.13–1.18 (t, 3H, $COCH_2CH_3$), 1.41–1.46 (m, 2H, $CH_{2\ alk}^{3}$), 1.56–1.64 (m, 2H, $CH_{2\ alk}^{2}$), 2.92–3.00 (m, 2H, $COCH_2CH_3$), 3.52–3.57 (t, 2H, CH_{2alk}^{1}), 4.54 (s, 2H, Ar-CH_2O), 6.81–6.86 (d, 1H, CH_{AR}^{6}), 7.80–7.86 (d, 1H, CH_{AR}^{3}), 7.95–7.98 (dd, 1H, CH_{AR}^{5}), 8.39 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.10 ($COCH_2CH_3$), 14.39 (C^{alk4}), 20.55 (C_{alk}^{3}), 32.25 (C_{alk}^{2}), 33.02 ($COCH_2CH_3$), 68.78 (Ar-CH_2O), 71.67 (C_{alk}^{1}), 115.88 (C_{AR}^{6}), 126.41 (C_{AR}^{2}), 129.87 (C_{AR}^{5}), 130.85 (C_{AR}^{4}), 131.04 (C_{AR}^{3}), 161.51 (C_{AR}^{1}), 202.21 (CO)

EP4i 1-(4-hydroxy-3-isobutyloxymethylphenyl)propan-1-one (6)

$C_{14}H_{20}O_3$ M_r 236.36 Anal. calcd. %C 71.97 %H 8.86 found %C 72.30, %H 8.70. Yield: 62%, R_F 0.62, Mp. 52–54°C (heptane), IR (cm^{-1}) 3344 ($\nu OH_{asoc.}$), 1656 ($\nu C=O$), 1592 ($\nu C=C$), 1277 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 203 (log ε_1 3.26), 222 (log ε_2 3.28), 274 (log ε_3 3.20)

^{1}H-NMR ($CDCl_3$): 0.94–0.96 (d, 6H, $CH(CH_3)_2$), 1.19–1.21 (t, 3H, $COCH_2CH_3$), 1.90–1.99 (m, 1H, $CH(CH_3)_2$), 2.92–2.67 (q, 2H, $COCH_2CH_3$), 4.75 (s, 2H, Ar-CH_2O), 6.89–6.90 (d, 1H, CH_{AR}^{6}), 7.68–7.69 (d, 1H, CH_{AR}^{3}), 7.82–7.92 (dd, 1H, CH_{AR}^{3}), 8.38 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.11 ($COCH_2CH_3$), 19.91 ($CH(CH_3)_2$), 29.80 ($CH(CH_3)_2$), 32.29 ($COCH_2CH_3$), 68.91 (Ar-CH_2O), 78.84 (CH_2-CH), 115.86 (C_{AR}^{6}), 126.52 (C_{AR}^{2}), 129.87 (C_{AR}^{5}), 130.79 (C_{AR}^{4}), 130.92 (C_{AR}^{3}), 161.47 (C_{AR}^{1}), 202.25 (CO)

EP5n 1-(4-hydroxy-3-pentyloxymethylphenyl)propan-1-one (7)

$C_{15}H_{22}O_3$ M_r 250.34, Anal. calcd. % C 71.97 %H 8.86 found %C 72.20, %H 8.60. Yield: 68%, R_F 0.60, Mp. 30–32°C (heptane), IR (cm^{-1}) 3340 ($\nu OH_{asoc.}$), 1684 ($\nu C=O$), 1604 ($\nu C=C$), 1272 (νCOC). UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 202 (log ε_1 2.90), 222 nm (log ε_2 3.25), 274 (log ε_3 3.21)

^{1}H-NMR ($CDCl_3$): 0.88–0.93 (t, 3H, $CH_{3\ alk}^{5}$), 1.18–1.23 (t, 3H, $COCH_2CH_3$), 1.32–1.35 (m, 4H, $CH_{2\ alk}^{3,4}$), 1.61–1.70 (m, 2H, $CH_{2\ alk}^{2}$), 2.90–2.97 (q, 2H, $COCH_2CH_3$), 3.54–3.58 (t, 2H, $CH_{2\ alk}^{1}$), 4.75 (s, 2H, Ar-CH_2O), 6.89–6.92 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{3}), 7.82–7.90 (dd, 1H, CH_{AR}^{5}), 8.41(s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.08 ($COCH_2CH_3$), 14.57 ($C_{al}k^{5}$), 23.70 (C_{alk}^{4}), 29.65 (C_{alk}^{3}), 30.57 (C_{alk}^{2}), 32.26 ($COCH_2CH_3$), 68.76 (Ar-CH_2O), 71.95 (Calk^1), 115.85 (C_{AR}^{6}), 126.37 (C_{AR}^{2}), 129.82 (C_{AR}^{5}), 130.80 (C_{AR}^{4}), 130.98 (C_{AR}^{3}), 161.46 (C_{AR}^{1}), 202.11 (CO)

EP5i 1-(4-hydroxy-3-isopentyloxymethylphenyl)propan-1-one (8)

$C_{15}H_{22}O_3$ M_r 250.34, Anal. calcd. % C 71.97 %H 8.86 found %C 72.30, %H 8.70. Yield: 63%, R_F 0.58, Mp. 68–70°C (heptane), IR (cm^{-1}), 3,342 ($\nu OH_{asoc.}$), 1,683 ($\nu C=O$), 1,604 ($\nu C=C$), 1,273 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 204 (log ε_1 3.18), 223 nm (log ε_2 3.15), 274 (log ε_3 3.23)

^{1}H-NMR ($CDCl_3$): 0.88–0.90 (d, 6H, $CH(CH_3)_2$), 1.18–1.20 (t, 3H, $COCH_2CH_3$), 1.51–1.56 (m, 1H, $CH(CH_3)_2$), 2.92–2.95 (q, 2H, $COCH_2CH_3$), 4.75 (s, 2H, Ar-CH_2O), 6.89–6.92 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{3}), 7.82–7.86(dd, 1H, CH_{AR}^{5}), 8.40 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.27 ($COCH_2CH_3$), 19.91 ($CH(CH_3)_2$), 29.80 ($CH(CH_3)_2$), 32.13 ($COCH_2CH_3$), 70.49 (Ar-CH_2O), 77.82 (CH_2-CH), 117.15 (C_{AR}^{6}), 123.03 (C_{AR}^{2}), 129.11 (C_{AR}^{5}), 129.97 (C_{AR}^{4}), 130.86 (C_{AR}^{3}), 161.50 (C_{AR}^{1}), 200.35 (CO)

EP6n 1-(3-hexyloxymethyl-4-hydroxyphenyl)propan-1-one (9)

$C_{16}H_{24}O_3$ M_r 264.36, Anal. calcd. %C 72.69 %H 9.15 found %C 72.77, %H 8.90. Yield: 60%, R_F 0.61, Mp. 51–53°C (heptane), IR (cm^{-1}), 3,340 ($\nu OH_{asoc.}$), 1,684 ($\nu C=O$), 1,600 ($\nu C=C$), 1,272 (νCOC). UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$) λ_{max} 202 (3.26), 216 nm (log ε_1 3.25), 264 (log ε_2 3.24)

^{1}H-NMR ($CDCl_3$): 0.89–0.93 (m, 3H, $CH_{3\ alk}^{6}$), 1.13–1.17 (t, 3H, $COCH_2CH_3$), 1.28–1.40 (m, 8H, $CH_{2\ alk}^{3,4,5,}$), 1,58–1,62 (m, 2H, $CH_{2\ alk}^{2}$), 2.94–3.01 (q, 2H, $COCH_2CH_3$), 3.50–3.53 (t, 2H, $CH_{2\ alk}^{1}$), 4.73 (s, 2H, Ar-CH_2O), 6.82–6.84 (d, 1H, CH_{AR}^{6}), 7.78–7.81 (d, 1H, CH_{AR}^{3}), 7.85–7.88 (dd, 1H, CH_{AR}^{5}), 8.39 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.13 ($COCH_2CH_3$), 14.56 (C_{alk}^{6}), 23.82 (C_{alk}^{5}), 27.40 (C_{alk}^{4}), 30.40 (Calk^3), 30.90 (C_{alk}^{5}), 32.32 (C_{alk}^{2}), 33.15 ($COCH_2CH_3$), 68.75 (Ar-CH_2O), 71.93 (C_{alk}^{1}), 115.90 (C_{AR}^{6}), 126.42 (C_{AR}^{2}), 129.83 (C_{AR}^{5}), 130.85 (C_{AR}^{4}), 131.08 (C_{AR}^{3}), 161.60 (C_{AR}^{1}), 202.20 (CO)

EP7n 1-(3-heptyloxymetyl-4-hydroxyphenyl)propan-1-one (10)

$C_{17}H_{26}O_3$ M_r 278.19, Anal. calcd. %C 73.35 %H 9.41 found %C 73.10, %H 9.20. Yield: 57%, R_F 0.56, Mp. 47-49°C (heptane), IR (cm^{-1}), 3,352 ($\nu OH_{asoc.}$), 1,686 ($\nu C=O$), 1,602 ($\nu C=C$), 1,272 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 202 (3.30), 222 nm (log ε_1 3.31), 264 (log ε_2 3.24)

^{1}H-NMR ($CDCl_3$): 0.86–0.90 (m, 3H, CH_3 alk^7), 1.14–1.19 (t, 3H, $COCH_2CH_3$), 1.29–1.41 (m, 8H, $CH_{2\ alk}^{3,4,5,6}$), 1.59–1.64 (m, 2H, $CH_{2\ alk}^{2}$), 2.90–2.97 (q, 2H, $COCH_2CH_3$), 3.54–3.58 (t, 2H, $CH_{2\ alk}^{1}$), 4.76 (s, 2H, Ar-CH_2O), 6.89–6.93 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{5}), 7.83–7.86 (dd, 1H, CH_{AR}^{5}), 8.38 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.11 ($COCH_2CH_3$), 14.59 (C_{alk}7), 23.84 (C_{alk}^{6}), 27.42 (C_{alk}^{4}), 30.41 (Calk^3), 30.91 (C_{alk}^{5}), 32.30 (C_{alk}^{2}), 33.17 ($COCH_2CH_3$), 68.75 (Ar-CH_2O), 71.93 (C_{alk}^{1}), 115.92 (C_{AR}^{6}), 126.44 (C_{AR}^{2}), 129.85 (C_{AR}^{5}), 130.87 (C_{AR}^{4}), 131.09 (C_{AR}^{3}), 161.61 (C_{AR}^{1}), 202.23 (CO)

EP8n 1-(4-hydroxy-3-octyloxymetylphenyl)propan-1-one (11)

$C_{18}H_{28}O_3$ M_r 292.45, Anal. calcd. %C 73.93 %H 9.65 found %C 73.7 0, %H 9.45. Yield: 56%, R_F 0.57, Mp. 54-57°C (heptane), IR (cm^{-1}), 3,352 ($\nu OH_{asoc.}$), 1,686 ($\nu C=O$), 1,600 ($\nu C=C$), 1,272 (νCOC). UV (CH_3OH λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 206 (3.26), 222 nm (log ε_1 3.29), 274 (log ε_2 3.25)

^{1}H-NMR ($CDCl_3$): 0.86–0.90 (t, 3H, $CH_{3\ alk}^{8}$), 1.18–1.206 (t, 3H, $COCH_2CH_3$), 1.21–1.28 (m, 10H, $CH_{2\ alk}^{3-7}$), 1.60–1.65 (m, 2H, CH_{2alk}^{2}), 2.90–2.98 (m, 2H, $COCH_2CH_3$), 3.54–3.58 (t, 2H, CH_{2alk}^{1}), 4.75 (s, 2H, Ar-CH_2O), 6.89–6.92 (d, 1H, CH_{AR}^{6}), 7.69–7.92 (d, 1H, CH_{AR}^{3}), 7.83–7.91 (dd, 1H, CH_{AR}^{5}), 8.40 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.09 ($COCH_2CH_3$), 14.57 (C^{alk8}), 23.86 (C_{alk}^{7}), 27.45 (C_{alk}^{6}), 30.57 (C_{alk}^{5}), 30.67 (C_{alk}^{4}), 30.88 (C_{alk}^{3}), 32.29 (C_{alk}^{2}) 33.14 ($COCH_2CH_3$), 68.73 (Ar-CH_2O), 71.91 (C_{alk}^{1}), 115.88 (C_{AR}^{6}), 126.40 (C_{AR}^{2}), 129.86 (C_{AR}^{5}), 130.82 (C_{AR}^{4}), 131.07 (C_{AR}^{3}), 161.51 (C_{AR}^{1}), 202.17 (CO)

EP9n 1-(4-hydroxy-3-nonyloxymethylphenyl)propan-1-one (12)

$C_{19}H_{30}O_3$ M_r 306.45, Anal. calcd. %C 74.47 %H 9.87 found %C 74.10 %H 9.50. Yield: 67%, R_F 0.60, Mp. 59-61°C (heptane), IR (cm^{-1}), 3,343 ($\nu OH_{asoc.}$), 1,671 ($\nu C=O$), 1,600 ($\nu C=C$), 1,275 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 206 (3.23), 222 nm (log ε_1 3.26), 275 (log ε_2 3.23)

^{1}H-NMR ($CDCl_3$): 0.86–0.92 (t, 3H, $CH_{3\ alk}^{9}$), 1.18–1.21 (t, 3H, $COCH_2CH_3$), 1.24–1.27 (m, 12H, $CH_{2\ alk}^{3-8}$), 1.57–1.64 (m, 2H, $CH_{2\ alk}^{2}$), 2.92–3.00 (m, 2H, $COCH_2CH_3$), 3.51–3.55 (t, 2H, $CH_{2\ alk}^{1}$), 4.74 (s, 2H, Ar-CH_2O), 6.81–6.86 (d, 1H, CH_{AR}^{6}), 7.79–7.83 (d, 1H, CH_{AR}^{5}), 7.84–7.86 (dd, 1H, CH_{AR}^{5}), 8.38 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.10 ($COCH_2CH_3$), 14.61 (C_{alk}^{9}), 23.88 (C_{alk}^{8}), 27.45 (C_{alk}^{7}), 30.57 (C_{alk}^{6}), 30.72 (C_{alk}^{5}), 30.87 (C_{alk}^{4}), 30.89 (C_{alk}^{3}), 32.29 (C_{alk}^{2}), 33.20 ($COCH_2CH_3$), 68.75 (Ar-CH_2O), 71.93 (C_{alk}^{1}), 115.89 (C_{AR}^{6}), 126.40 (C_{AR}^{2}), 129.85 (C_{AR}^{5}), 130.81 (C_{AR}^{4}), 131.03 (C_{AR}^{3}), 161.50 (C_{AR}^{1}), 202.12 (CO)

EP10n 1-(3-decyloxymethyl-4-hydroxyphenyl)propan-1-one (13)

$C_{20}H_{32}O_3$ M_r 320.48, Anal. calcd. %C 74.96 %H 10.06 found %C 74.80 %H 9.90. Yield: 62%, R_F 0.62, Mp. 52-54°C (heptane), IR (cm^{-1}), 3,343 ($\nu OH_{asoc.}$), 1,670 ($\nu C=O$), 1,602 ($\nu C=C$), 1,272 (νCOC), UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 222 (log ε_1 3.26), 275 (log ε_2 3.23)

^{1}H-NMR ($CDCl_3$): 0.86–0.91 (t, 3H, $CH_{3\ alk}^{10}$), 1.13–1.18 (t, 3H, $COCH_2CH_3$), 1.26–1.40 (m, 12H, $CH_{2\ alk}^{3-9}$), 1.57–1.64 (m, 2H, CH_{2alk}^{2}), 2.88–3.00 (m, 2H, $COCH_2CH_3$), 3.51–3.55 (t, 2H, $CH_{2\ alk}^{1}$), 4.54 (s, 2H, Ar-CH_2O), 6.83–6.87 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{3}), 7.74–7.86 (dd, 1H, CH_{AR}^{5}), 8.40 (s,1H, ArOH)

^{13}C-NMR ($CDCl_3$): 9.10 ($COCH_2CH_3$), 14.62 (C_{alk}^{10}) 23.89 (C_{alk}^{9}), 27.46 (C_{alk}^{8}), 30.62 (C_{alk}^{7}, 30.67 (C_{alk}^{6}), 30.81 (C_{alk}^{5}), 30.86 (C_{alk}^{4}), 30.89 (C_{alk}^{3}), 32.29 (C_{alk}^{2}), 33.22 ($COCH_2CH_3$), 68.74 (Ar-CH_2O), 71.93 (C_{alk}^{1}), 115.88 (C_{AR}^{6}), 126.40 (C_{AR}^{2}), 129.84 (C_{AR}^{5}), 130.80 (C_{AR}^{4}), 131.02 (C_{AR}^{3}), 161.49 (C_{AR}^{1}), 202.17 (CO)

EP5c 1-(4-hydroxy-3-cyclopentyloxymethylphenyl) propan-1-one (14)

$C_{15}H_{20}O_3$ M_r 248.32, Anal. calcd. %C 72.55 %H 8.12 found %C 72.30, %H 8.30, Yield: 62%, R_F 0.62 Mp. 68-69°C (heptane), IR (cm^{-1}), 3,255 ($\nu OH_{asoc.}$), 1,657 ($\nu C=O$), 1,601 ($\nu C=C$), 1,250 (νCOC). UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 203 (log ε_1 3.17), λ_{max} 222 (log ε_1 3.20), 274 (log ε_3 3.19)

^{1}H-NMR ($CDCl_3$): 1.19–1.22 (t, 3H, $COCH_2CH_3$), 1.59; 1.74–1.79 (m, m, 2H, 6H, $CH_2^{alk2,3,4,5}$), 2.91–2.96 (q, 2H, $COCH_2CH_3$), 4.08 (m, 1H, CH^{alk1}), 4.73 (s, 2H, Ar-CH_2O), 6.89–6.91 (d, 1H, CH_{AR}^{6}), 7.69–7.70 (d, 1H, CH_{AR}^{3}), 7.82–7.85 (dd, 1H, CH_{AR}^{5}), 8.54 (s, 1H, Ar-OH)

^{13}C-NMR ($CDCl_3$): 9.09 ($COCH_2CH_3$), 24.66 ($C_{alk}^{3,4}$), 32.28 ($COCH_2CH_3$), 33.37 (Calk^{2,5}), 66.82 (Ar-CH_2O), 82.96 (C_{alk}^{1}), 115.82 (C_{AR}^{6}), 126.70 (C_{AR}2), 129.87 (C_{AR}^{5}), 130.77 (C_{AR}^{4}), 131.03 (CAR^3), 161.47 (C_{AR}^{1}), 202.23 (CO)

EPbenzyl 1-(3-phenylmethoxymethyl-4-hydroxyphenyl) propan-1-one (15)

$C_{17}H_{18}O_3$ M_r 270, Anal. calcd. %C 75.53 %H 6.71 found %C 75.40, %H 6.50. Yield: 62%, R_F 0.62, Mp. 52-54°C (heptane), IR (cm^{-1}), 3,361 ($\nu OH_{asoc.}$), 1,667 ($\nu C=O$), 1,593 ($\nu C=C$), 1,278 (νCOC). UV (CH_3OH, λ in nm, ε in $m^2.mol^{-1}$); λ_{max} 206 (log ε_1 3.39), λ_{max} 219 (log ε_2 3.29), 274 (log ε_3 3.19)

^{1}H-NMR **(**$CDCl_3$**):** 1.19–1.22 (t, 3H, $COCH_2CH_3$), 2.90–2.96 (q, 2H, $COCH_2CH_3$), 4.62 (s, 2H, CH_2-phenyl), 4.79 (s, 2H, Ar-CH_2O), 6.92–6.94 (d, 1H, $CH_{AR}{}^6$), 7.33–7.40 (m, 5H, phenyl), 7.70–7.71 (d, 1H, $CH_{AR}{}^3$), 7.85–7.87 (dd, 1H, $CH_{AR}{}^5$), 8.16 (s, 1H, Ar-OH)

^{13}C-NMR ($CDCl_3$): 8.94 ($COCH_2CH_3$), 32.13 ($COCH_2CH_3$), 68.11 (Ar-CH_2O), 73.60 (CH_2-phenyl), 115.75 ($C_{AR}{}^6$), 126.07 ($C_{phenyl}{}^4$), 128.72 ($C_{AR}{}^2$), 128.96 ($C_{phenyl}{}^{2,6}$), 129.39 ($C_{phenyl}{}^{3,5}$), 129.73 ($C_{AR}{}^5$), 130.78 ($C_{AR}{}^4$), 131.01 ($C_{AR}{}^3$), 139.55 ($C_{phenyl}{}^1$), 161.36 ($C_{AR}{}^1$), 202.06 (CO)

ANTIMICROBIAL ACTIVITY

Antimicrobial activity of prepared (3-alkoxymethyl-4-hydroxyphenyl)propan-1-ones was evaluated *in vitro* and expressed as the minimum inhibitory concentration (MIC). It was determined using the standard broth dilution method (Valentová et al., 2018). The following strains of gram-positive, gram-negative bacteria and a yeast pathogen were selected for the experiments: *Staphylococcus aureus* CNCTC Mau 29/58, *Escherichia coli* CNCTC 377/79 and *Candida albicans* CCM 8186, respectively. Tested bacterial strains were purchased from Czech National Collection of Type Cultures (Prague, Czech Republic); yeast was obtained from Czech Collection of Microorganisms (Brno, Czech Republic). For the sake of comparison, antibacterial activity of the antibiotic ciprofloxacin **(**1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid) was also evaluated.

ANTIOXIDATIVE ACTIVITY

DPPH assay (Brand-Williams et al., 1995)

The evaluation of antioxidative capacity by this method is based on the redox reaction of the tested compounds with the stable radical 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH). The solution of this radical is purple coloured with maximum absorption at 517 nm. In the course of the reduction of DPPH, the solution changes its colour from purple to yellow, resulting in corresponding shift in UV-VIS spectrum. The lower the measured absorption, the higher the antioxidative capacity of the tested compound.

A solution of DPPH in methanol was prepared, in the concentration 44 μg/ml (112 μM). Subsequently, solution of the tested sample in methanol in the concentration 10^{-2} mol. dm^{-3} or 10^{-3} mol. dm^{-3} was prepared. For the spectrophotometric assay, 270 mL of the DPPH solution and 30 mL of tested compound solution or standard were mixed, and the absorbance using a microplate reader was determined at 517 nm at 5 min after mixing. The absorbance at each time point was corrected for the absorbance of a DPPH blank. Three parallel measurements were made for each sample. Trolox was used as a standard for measured antioxidant activity of the target compounds.

ABTS assay (Re et al., 1999)

Antiradical activity was measured as % inhibition of $ABTS^{+}$. Aqueous solutions of ABTS (7.7 μg/ml, 14 mM) and $K_2S_2O_8$ (1.32 mg/ml, 4.9 mM) were prepared. These two solutions were mixed in a 1:1 vol. ratio and allowed to stand for 24 h in the refrigerator. The spectrophotometric measurement was carried out using a 96-well plate reader. Each well on the microplate was filled with 60 μl of sample solution (10^{-2} or 10^{-3} $mol.dm^{-3}$, respectively), and 240 μl of ABTS solution. Absorbance was assessed spectrophotometrically at 734 nm at 5 min after mixing. For each sample, three parallel measurements were made. During the reaction, the colourless 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid is oxidised by potassium peroxydisulfate, yielding the stable blue-green $ABTS^{\bullet+}$ radical. The addition of antioxidants leads to reduction of the $ABTS^{\bullet+}$ radical and discolouration of the solution.

DISCUSSION

The aim of the presented research was the synthesis of (3-alkoxymethyl-4-hydroxyphenyl)propan-1-ones (Table 1), screening of antimicrobial and antioxidant activities of selected products, as well as comparison of the results with the previously prepared (3-alkoxymethyl-4-hydroxyphenyl) ethanones and (2*RS*)-bis [3-(4-acetyl-2-propoxymethyl) phenoxy-2-hydroxypropyl]isopropylammonium fumarate with beta-blocking activity.

The compounds 1–15 (Table 1) were synthesised by an established two-step procedure from 4-hydroxyphenylpropan-1-one (Čižmáriková et al., 1991). During the first stage, (3-chloromethyl-4-hydroxyphenyl)propan-1-one was prepared in 57% yield via electrophilic substitution reaction. This intermediate reacts in the second step of the synthesis with the respective alcohol in the presence of $NaHCO_3$ to give (3-alkoxymethyl-4-hydroxyphenyl)propan-1-ones in 60% yield. The products are white solids with mp between 30 and 70°C. Their purity was checked by TLC on silica, the mobile phase consisting of cyclohexane/ethyl acetate in 8:2 v/v ratio. The structure of the final (3-alkoxymethyl-4-hydroxyphenyl) propan-1-ones 1-15 was confirmed by spectral analysis. The following bands could be assigned in the infrared spectra: 3,236–3,352 cm^{-1} ($\nu OH_{asoc.}$), 1,653–1,686 cm^{-1} ($\nu C=O$), 1,596–1,604 cm^{-1} ($\nu C=C$) and 1,270–1,275 cm^{-1} (νCOC). In the ^{1}H and ^{13}C-NMR spectra, the signals of the aromatic ring, the propanoyl and the alkoxymethyl groups were identified. Two or four bands can be seen in the UV spectra, corresponding to π-π* transitions at 202–206, 216–227, 264 and 274–277 nm.

Table 2. Antimicrobial activity of 1-[3-(alkoxymethyl)-4-hydroxyphenyl]alkanones.

Substance Number	Description	MIC (mmol/L) E.coli	MIC (mmol/L) S. aureus
2	EP2	5.01	5.01
3	EP3n	2.46	n
6	EP4i	1.06	n
9	EP6n	0.38	n
12	EP9n	1.43	1.43
13	EP10n	0.77	0.13
14	EP5c	1.29	0.65
15	EPbenz	1.25	0.31
1a	4OHPr	n	n
18	EA4n	0.31	n
20	EA7n	0.94	n
21	EA8n	0.09	n
22	EA5c	4.11	2.05
28	EAch	0.53	n

n = non-measurable values, compounds not active against Candida albicans

Table 3. Antioxidant activities of 1-[3-(alkoxymethyl)-4-hydroxyphenyl]alkanones.

Substance Number	Description	Inhibition DPPH [%]±SD	Inhibition ABTS [%]±SD
1	EP1	n	3.9 ±0.04
2	EP2	2.5±0.1	8.5±0.4
3	EP3n	0.6±0.01	11.9±0.4
4	EP3i	3.7±0.02	28.1±0.3
6	EP4i	2.4±0.7	10.2±0.2
7	EP5n	1.8±1.3	1.3 ±0.4*
8	EP5i	12.6±0.6	5.8±0.7*
11	EP8n	6.2±0.7	n
13	EP10n	6.3±2.1	n
14	EP5c	3.3±0.03	25.0±1.6
15	EPbenzyl	3.3±0.9	1.5 ±0.3*
16	EA1	n	6.1±0.2
17	EA2	n	1.4±1.0
18	EA3n	n	6.1±0.7
19	EA3i	n	7.5±0.2
20	EA4n	n	1.5± 1.0
21	EA5n	3.9±0.8	24.8±0.8
22	EA7n	n	0.9±0.2*
23	EA8n	n	n
24	EA9n	3.9±0.7	n
25	EA5c	n	8.4±0.7
26	EAbenzyl	1.59±1.4	16.7±0.7
27	4-OHacet	n	0.9±0.01
28	EAch	4.32±2.5	8.2±0.4*

**concentration 10^{-3} mol.dm^{-3} n = non-measurable values*

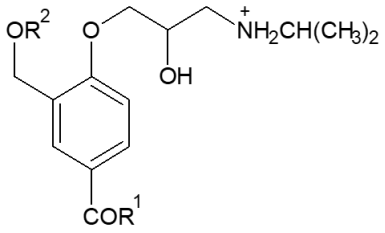
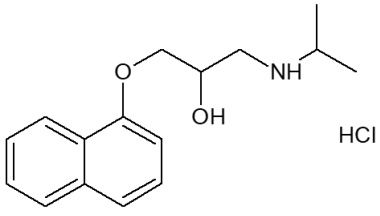


Table 4. Antioxidative activity of (2RS)-bis [3-(4-acetyl-2-alkoxymethyl)phenoxy-2-hydroxypropyl]isopropylammonium fumarate exhibiting beta-blocking activity.

Description	R1	R2	Inhibition DPPH [%]±SD	Inhibition ABTS [%]±SD
FA23i	CH_3	CH_2CH_3	N	99.0±1.3
FA5n3i	CH_3	$(CH_2)_4CH_3$	N	48.5±0.8
FA5i3i	CH_3	$CH_2CH_2CH(CH_3)_2$	N	67.2±1.4
FA7n3i	CH_3	$(CH_2)_6CH_3$	0.4±0.04	52.2±1.3
FA8n3i	CH_3	$(CH_2)_7CH_3$	N	89.8±3.7
FA9n3i	CH_3	$(CH_2)_8CH_3$	N	86.7±5.3
FAB3i	CH_3	CH_2phenyl	1.6±0.04	n
Propranolol			4.6±1.9	97.5±2.3

n = non-measurable values



propranolol

Table 5. Antimicrobial activity of (2RS)- bis [3-(4-propionyl-2-alkoxymethyl)phenoxy-2-hydroxypropyl]isopropylammonium fumarate.

Description	R1	R2	MIC(mmol/L) *E. coli*	MIC(mmol/L) *S. aureus*	MIC(mmol/L) *C. albicans*
FpP4n3i	CH_3CH_2	$(CH_2)_3CH_3$	N	0.61	n
FpP5n3i	CH_3CH_2	$(CH_2)_4CH_3$	N	0.35	n
FpP6n3i	CH_3CH_2	$(CH_2)_5CH_3$	0.34	0.08	0.23
FpP7n3i	CH_3CH_2	$(CH_2)_6CH_3$	0.22	0.03	0.07
FpP9n3i	CH_3CH_2	$(CH_2)_8CH_3$	0.20	0.01	0.01
FpA5n3i	CH_3	$(CH_2)_4CH_3$	N	0.61	n
FpA6n3i	CH_3	$(CH_2)_5CH_3$	0.35	0.08	0.24
FpA7n3i	CH_3	$(CH_2)_7CH_3$	0.23	0.07	0.23
FpA8n3i	CH_3	$(CH_2)_7CH_3$	0.22	0.03	0.08
FpA9n3i	CH_3	$(CH_2)_8CH_3$	0.21	0.01	0.02
ciprofloxacin			3.10^{-4}	$6.89.10^{-4}$	n

n = non-measurable values

The compounds 16–26 (Table 1) derived from 4-hydroxyphenylethanones are described in (Čižmáriková et al., 2002).

Antimicrobial activity of selected final products (Table 2) was tested against gram-negative bacterium (*Escherichia coli*), gram-positive bacterium (*Staphylococcus aureus*) and human fungal pathogen (*Candida albicans*).

The comparison between the tested compounds showed that maximum activity against *E. coli* can be found in the propanone derivative with the hexyloxymethyl-side chain (EP6n, MIC 0.38 mmol/L). Ethanone derivative with the butoxymethyl group (EA4n, MIC 0.31 mmol/L) showed similar activity. Comparison of activities of ethanone (EA5c) and propanone (EP5c) derivatives with the cyclopentyloxymethyl moiety revealed that the activity of the propanone derivative was higher (MIC 1.29 mmol/L) than the activity of the ethanone derivative (MIC 4.11 mmol/L). Prolongation of the alkyl chain caused an increase in activity from ethoxymethyl up to nonyloxymethyl (EP2 through EP9n).

Similar observation was made also with *S. aureus*. The highest effect was shown by the compound with decyloxymethyl substituent (EP10n, MIC 0.13 mmol/L). The compounds EP2 and EP10n exerted comparable activity both in *E. coli* and *S. aureus*. None of the tested compounds exhibited substantial activity against *C. albicans*.

Published data suggest that compounds with one or several phenolic hydroxyls act as radical scavengers and exert antioxidative activity. Hence, they impede oxidative stress, a condition that can be the main cause of numerous diseases, especially those of the cardiovascular system. The antioxidative activities of the prepared compounds were evaluated using methods based on DPPH (1,1-diphenyl-2-picrylhydrazyl) and $ABTS^{\cdot+}$ (2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid). The DPPH method involves the reaction between the antioxidant and the stable radical DPPH•, which acts as acceptor of hydrogen. The solution of this radical has intensely purple hue, caused by an unpaired electron of the hydrazyl group. Its reaction with the antioxidant yields the reduced form DPPH-H, and the solution discolours in the course of this reaction. The degree of antioxidant activity is determined from the decrease of absorbency of the solution at 517 nm wavelength. The ABTS method employs oxidation of the colourless 2,2´ azinobis(3-ethylbenzothiazoline-6-sulfonic acid) by potassium peroxydisulfate, yielding the stable blue-green radical-cation $ABTS^{\bullet+}$. The addition of antioxidants to such a solution leads to reduction of the $ABTS^{\bullet+}$ radical and discolouration of the solution.

The antioxidant activity of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) used as standard was determined along with the activities of investigated products. The values of antioxidative activities (Table 3) determined by the DPPH method were generally lower than those provided by the ABTS methods, in some cases, even below the detection threshold. The antioxidative activities of the products were in the range 0.6–4.3%, with the exception of the substance EP5i, in which case, it was 12.6%. The ABTS method provided activities in the range 0.9–28.1%, the highest activity being found in the substance EP3i with isopropoxymethyl substituent (28.1%). Neither of the methods provides a clear dependency between the activity and the length of the alkyl substituent.

The majority of the compounds in the group of 3-alkoxymethyl-4-hydroxyphenylethanones (16–26) did not show any activity detectable by the DPPH method. The highest activity provided by the ABTS method was found in the compound with pentyloxymethyl substituent (EA5n, 24.8%).

Low ABTS value was shown also by the parent structure 4-hydroxyphenylethanone. 3-Chloromethyl-4-hydroxyphenylethanones exerted higher activity both by the DPPH (4.4%) and ABTS (8.2%) methods (Table 3).

The activities correspond to the values for antioxidative activities of phenols reported in the literature. Compounds with only one hydroxy group in the molecule exhibit only minor antioxidant effects, markedly higher values being shown by compounds with two or more hydroxy groups (Sroka & Cisowski, 2003).

Several previously reported compounds with beta-adrenolytic effect (Čižmáriková et al., 1985; 1986; 2003) were tested for comparison. The compounds exerted marked anti-isoprenaline activity with negative chronotropic, dromotropic and inotropic effects. Optimum antiarrhythmic and anticonvulsive activity was found in derivatives with methoxymethyl and propoxymethyl groups. To assess the antioxidant activity, (2*RS*)-bis[3-(4-acetyl-2-propoxymethyl) phenoxy-2-hydroxypropyl]isopropylammonium fumarate with varying length of the alkoxymethyl chain was selected. The ABTS method was found to be more convenient compared to the DPPH assay. The antioxidative (Table 4) activities acquired by the ABTS method were in the range 52.2–99.0%, the highest effect being observed in ethoxymethyl (FA23i, 99.0%) and octyloxymethyl (FA8n3i, 89.8%) derivatives (Table 4). Similarly to the investigated substances, the antioxidative activity of the standard compound propranolol appraised by the ABTS method (4.6%) was higher in comparison with DPPH.

In some cases (EP5, EPbenzyl, EA7, EAchlormet), the antioxidant activities were assessed at a lower concentration (10^{-3} mol.dm^{-3}). (2*RS*)-bis[3-(4-acetyl-2-propoxymethyl) phenoxy-2-hydroxypropyl]isopropylammonium fumarate was more active than the phenolic derivatives, suggesting potentiation of antioxidative activity as a result of introduction of isopropylaminopropanol moiety into the molecule. The published results implicate correlation between the antioxidant activity, connected to reduction in oxidative stress and therapeutic effect in cardiovascular disease.

The antimicrobial activity (Table 5) of selected (2*RS*)-bis[3-(4-acetyl-2-propoxymethyl)phenoxy-2-hydroxypropyl] isopropylammonium fumarate in tested microbial strains was higher in comparison with the intermediate etanones and

propanones. This increase in activity stems from the elevated lipophilicity connected to prolongation of the alkoxymethyl chain. In both series, the highest effect on all tested strains was observed in compounds with nonyloxymethyl substituent in the side chain. In comparison with the standard ciprofloxacin, the antimicrobial activities were inferior in both groups.
Considering both types of bioactivity, the antimicrobial effects (in *S. aureus* and *E. coli*) and the antioxidant activity (by ABTS assay), were both higher in comparison with the (2*RS*)-bis [3-(4-acetyl-2-propoxymethyl)phenoxy-2-hydroxypropyl] isopropylammonium fumarate with beta-blocking activity.

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Toxicity of primulic acid 1 against a daphnid species *Simocephalus expinosus* s.l.

Original Paper

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Abstract Primulic acid 1 is the main saponin present in *Primula elatior*. The present study describes the isolation of this amphiphilic compound from primula root. It was performed by ultrasonic maceration, reverse-phase column chromatography and crystallization. Investigations of its physicochemical property are represented by the determination of critical micelle concentration (cmc). The cmc value of the amphiphile was 9.4×10^{-5} mol·dm^{-3}. The evaluation of environmental toxicity was performed on a daphnid species *Simpocephalus expinosus* s.l., which was very sensitive to primulic acids 1. The results from acute immobilisation test show that the tested compound has half maximal effective concentration after 24 hours (EC_{50}-24 h) equal to 6.9 mg·l^{-1}. Saponin can be classified as a toxic compound for aquatic organisms.

Keywords *Critical micelle concentration – daphnid – ecotoxicology – extraction – primula root*

INTRODUCTION

Primulic acid 1 (primulasaponin 1 or saponin PS4) is the main triterpene saponin from primula roots. This drug is officinal according to the tenth edition of the European Pharmacopoeia (Ph. Eur. 10.0, 2019). The source of the drug is *Primula veris* or *Primula elatior*. Both plants are native in Europe and temperate Asia. The main indication for primula root is the treatment of cough. Laboratory studies showed that this drug increased the production of airway secretions, making phlegm less thick and easier to expel. However, no clinical studies were made with the remedy containing primula root only (EMA, 2016).

Chemical structure of primulic acid 1 is depicted in Figure 1. Saponin is composed of two main parts, the hydrophilic and the hydrophobic part. The structure is characteristic for amphiphilic compounds. The lipophilic part consists of a pentacyclic triterpene moiety. The hydrophilic part is represented by saccharides. Galactose, glucose and rhamnose are neutral aldohexoses. However, glucuronic acid is acidic monosaccharide. It can be ionized and transformed from a non-ionic amphiphile to its anionic form. Ionisation of primulic acid 1 improves the solubility of saponin in aqueous media (Böttger et al., 2012).

Increasing use of medicines and nutritional supplements is related to higher environmental risks. Many drugs are produced and used in tonne quantities. They are introduced into the environment and are considered as contaminants of emerging concern. These drugs are responsible for hazards and toxicity towards aquatic and terrestrial living systems (Kar et al., 2020). The influence of pollutants towards environment is evaluated on many bacterial, plant and animal models. One of the aquatic organisms highly recommended for the short-term toxicity is *Daphnia* sp. The application of this organism in the tests is regulated by the EC directive No. 1907/2006 of the European parliament and of the council (Regulation (EC) No. 1907/2006).

The aim of the present study is to provide a simple method for the isolation of primulic acid 1 from primula root and the evaluation of its toxicity towards environment. The evaluation

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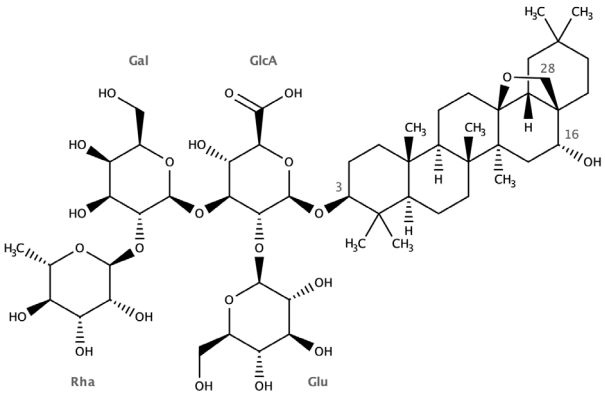

Figure 1. Chemical structure of primulic acid 1.

was performed on an aquatic organism obtained from nature. It is represented by *Simocephalus expinosus* s.l.

MATERIAL AND METHODS

Isolation of primulic acid 1

Primulic acid 1 was isolated from *Primula elatior*. The plant was collected in Považská Bystrica, Slovakia (WGS84: 49.093457° N; 18.465616° E) on March 8, 2018. Powdered root (20 g) was extracted with methanol (1 × 75 ml, 2 × 50 ml). Extraction was performed in ultrasonic bath at r.t. for 10 minutes. Solutions were filtered and 175 ml of 0.5 % solution of formic acid in water was added. The solution was transferred in the column which was filled with 55 g of MCI gel CHP20P. The column was eluted gradiently with a solution consisting of 0.5% formic acid in water and methanol (50:50 → 0:100, V/V). Fractions containing primulic acid 1 were collected and evaporated. The dry compound (690 mg) was dissolved in methanol (10 ml) and diethyl ether (100 ml) was subsequently added. The precipitate was filtered and dried in vacuum. The amount of isolated primulic acid 1 was 624 mg. NMR spectra of the investigated compounds are in accordance with the published data (Siems et al., 1998).

Determination of critical micelle concentration of primulic acid 1

The equilibrium surface tension measurements were carried out according to the procedure described previously (Lukáč et al., 2020). The Wilhelmy plate technique was used for the determination of the solvent surface tension values. Measurements were performed with a Krüss 100 MK2 tensiometer. The samples were prepared by dissolving primulic acid 1 in ISO water. The stock solutions were prepared in a volumetric flask. The measurements were performed at 25 ± 0.1°C. The measurements of equilibrium surface tension were taken repeatedly. The values were recorded every 360 s. The measurement was stopped if the difference between the values of two successive measurements was less than 5 $\times 10^{-5}$ N·m^{-1}. The break point of the linear parts of the surface tension vs. log c curve was used for the determination of the critical micelle concentration (cmc).

Acute immobilisation test

Acute immobilisation test was performed according to the OECD guideline No. 202 (OECD, 2004). *Simocephalus expinosus* s.l. was used as the investigated organism. Parthenogenetic females were obtained from nature (Lukáč, 2019). Daphnids were determined microscopically (Hudec, 2009). Experiments were performed on juveniles, aged less than 24 hours. ISO water was used as the solvent for cultivation. Potassium dichromate was used as a standard for the determination of viability of daphnids. The dissolved oxygen concentration at the end of the test was determined by the Winkler method. The immobilisation experiments were performed in 30 ml vials. The vials were filled with 10 ml solutions containing 5 juveniles each. The testing was performed in pure ISO solution and at five various concentrations of $K_2Cr_2O_7$ and primulic acid 1 solutions. Each concentration for any compound was tested on 20 juveniles (4 × 5 juveniles). The temperature during the tests was kept at 21 ± 1°C. A 16-hour light and 8-hour dark cycles were applied. The duration of the test was 24 hours. The activity was expressed as the half maximal effective concentration after 24 hours (EC_{50}-24 h).

RESULTS AND DISCUSSION

The investigated compound, primulic acid 1, was isolated from the roots of *Primula elatior*. We applied an improved method of isolation of the saponin. Extraction was performed by methanol. The crude extract was purified using reverse phase MCI gel CHP20P. Additionally, the saponin was crystalized. The amount of isolated primulic acid 1 was 3.1 %. The method applying highly porous styrene-divinylbenzene polymer resin instead of C18-silica (Çalis and Yürüker, 1992; Siems et al. 1998) is sufficient for the purification of primulic acid 1. Structure of primulic acid 1 was confirmed by NMR spectroscopy. Its HSQC spectrum is depicted in Fig. 2. C-H interactions of acetal moieties present in four saccharides are visible in the bottom left corner of Fig. 2. The signals come from rhamnose (δ_H = 5.28 ppm; δ_C = 100.7 ppm), galactose (δ_H = 5.16 ppm; δ_C = 99.5 ppm), glucose (δ_H = 4.85 ppm obscured by solvent; δ_C = 101.2 ppm) and glucuronic acids (δ_H = 4.50 ppm; δ_C = 104.4 ppm). The presence of glucuronic acid is also confirmed by a characteristic carbon signal of carboxylic group in ^{13}C NMR (δ_C = 170.7 ppm). The signals depicted in the middle of Fig. 2 mainly arise from C-H interactions of sugars. Only four signals represented C-H interaction observed in aglycone. They are assigned in Fig. 2 by the number of carbons. On the other side, the signals displayed in the top right corner of Fig. 2 represent mainly C-H interactions of aglycone. Only signal with δ_H = 1.27 ppm and δ_C = 16.4 ppm represents atoms of the methyl group of rhamnose.

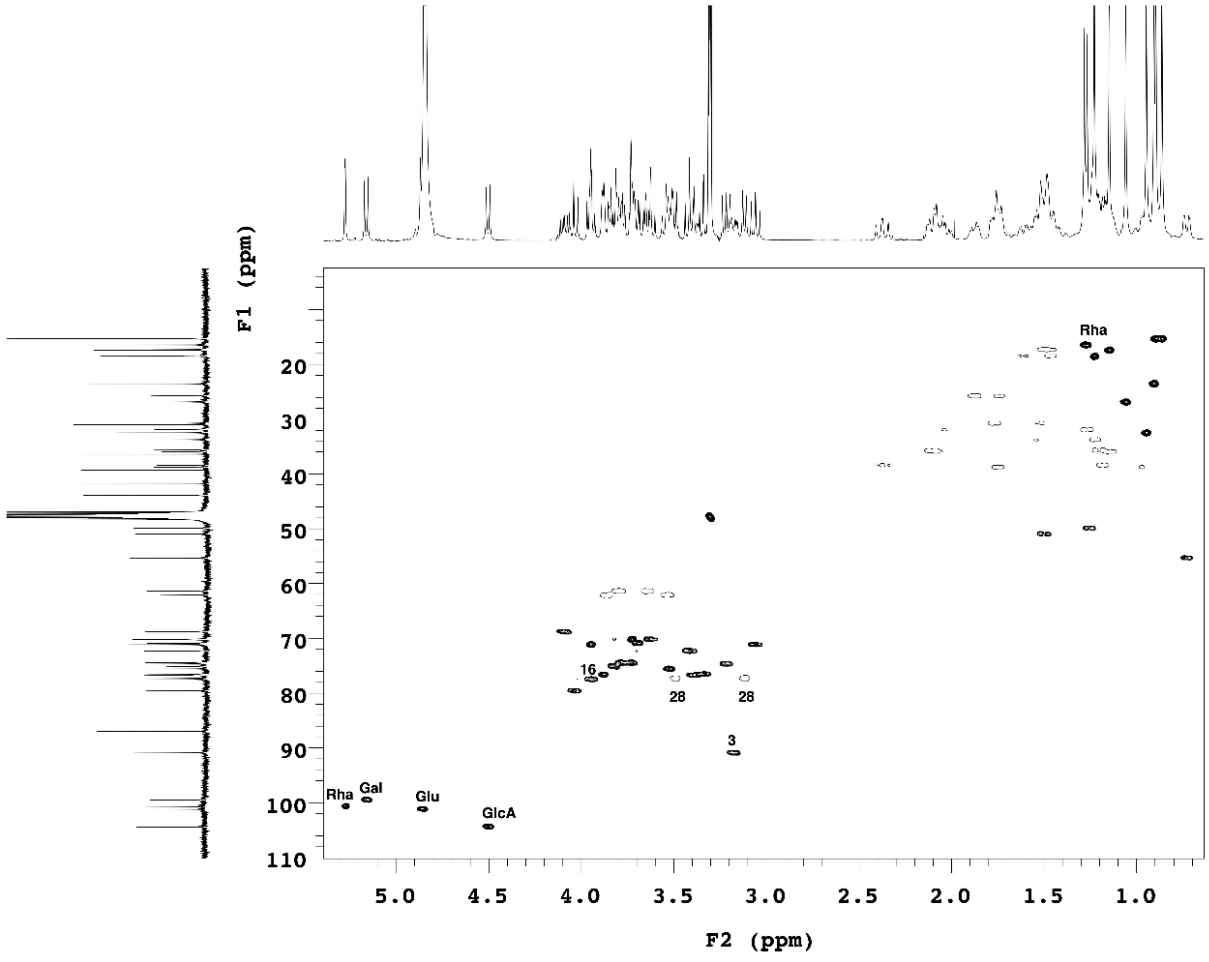

Figure 2. HSQC spectrum of primulic acid 1.

Critical micelle concentration of primulic acid 1 was obtained from surface tension measurements. The measurements were performed in ISO water. The solution contained several salts beside sodium hydrogen carbonate. This salt could react with primulic acid 1 and it caused ionisation of glucuronic acid. This resulted in the formation of anionic amphiphilic compounds – sodium primulate 1. The cmc value of the amphiphile was 9.4×10^{-5} mol·dm^{-3} (Fig. 3), which is in good agreement with the published data. Böttger et al., (2012) measured cmc of primulic acid in phosphate-buffered saline (PBS) solution. His value of cmc in PBS was 9.0×10^{-5} mol·dm^{-3}.

The acute immobilisation test was performed on *Simocephalus expinosus* s.l. It is a crustacean from family Daphnidae like *Daphnia magna*, daphnids which is the most commonly used in the ecotoxicological tests. Both daphnids have similar environmental requirements. They can live in small periodic waters and they can be found at a single location (Hudec, 1989). Our results showed that *S. expinosus* was sensitive to the standard $K_2Cr_2O_7$ in a similar way as *D. magna*. The EC_{50}-24 h for *S. expinosus* was 1.77 mg·l^{-1}. It is in the range of 0.6 – 2.1 mg·l^{-1} which was determined for *D. magna* (OECD, 2004). Therefore, *S. expinosus* can be considered a suitable animal model for acute immobilisation tests.

The results from acute immobilisation test show that primulic acid 1 has EC_{50}-24 h = 6.9 mg·l^{-1} (Fig. 3). According to the Globally Harmonized System of Classification and Labelling of Chemicals (United Nations, 2019), the compound should be classified as a toxic for aquatic organisms. The toxicity of primulic acid 1 is related to its low cmc. Saponin solubilises membranes and causes leakage of intracellular constituents. Primulic acid 1 has high haemolytic activity (25000 – 29000; Steiner and Holtzem, 1955) and membrane toxicity (IC_{50} = 58 μM; Böttger et al., 2012). Its value of membrane toxicity is lower than the cmc. It means that solubilisation of membranes occurs at a premicellar concentration. Formation of micelles is not required for this process. Böttger et al., (2012) also studied general cytotoxicity of primulic acid on human urinary bladder epithelial carcinoma cells (ECV-304). They obtained lower value (IC_{50} = 13 μM) for this activity than that of membrane toxicity. It means that cells are affected by primulic acid 1 at a concentration smaller than the concentration causing membrane disruption. Integrity

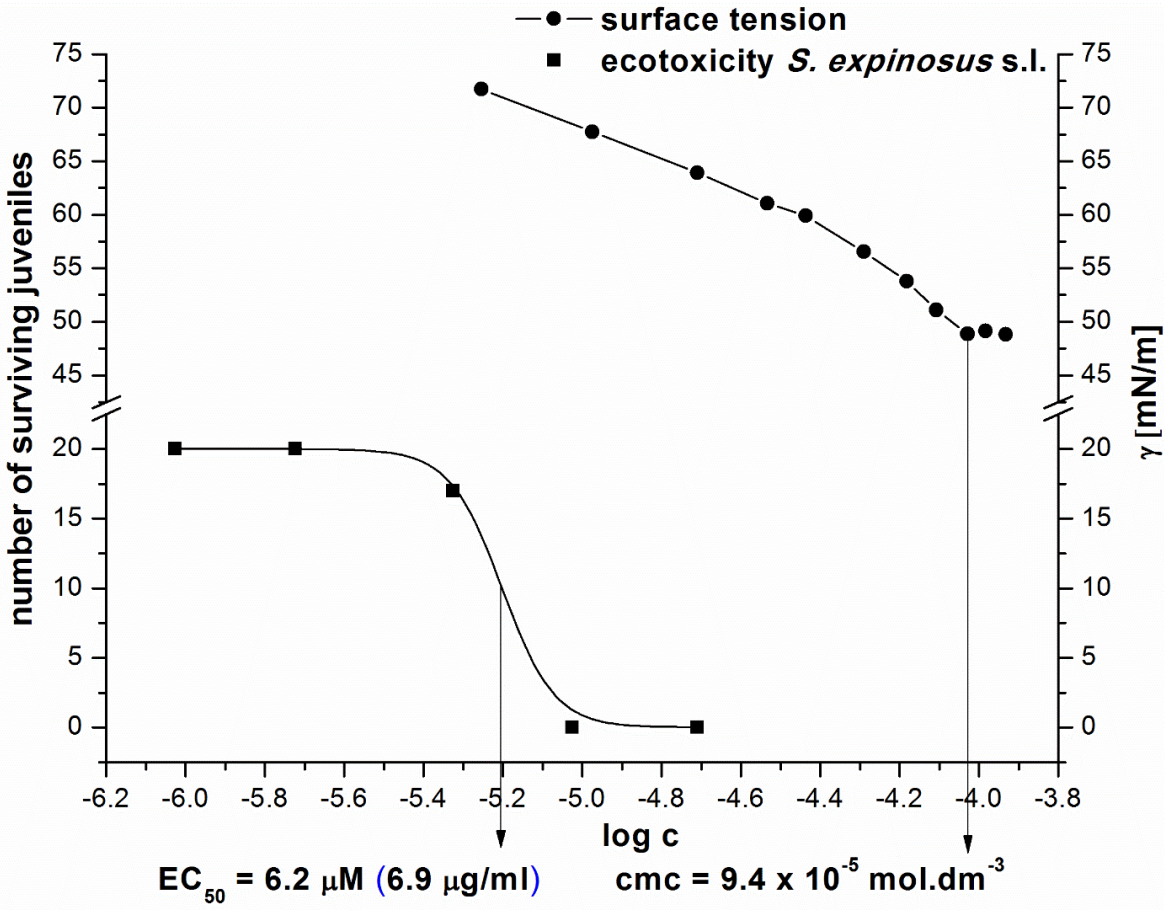

Figure 3. Plot of the number of surviving juveniles of Simocephalus expinosus vs. log concentration of primulic acid 1 (squares) and plot of surface tension vs. log concentration of primulic acid 1 (full circles).

of plasmatic membrane or biochemical processes of cells are held at a concentration that was four and half time smaller than the leakage of intracellular constituents from the cells. The toxicity on the cells corresponds with the toxicity on animals. *S. expinosus* is sensitive to primulic acid 1 at a half of the concentration determined by Böttger et al., (2012) for ECV-304. The snail *Biomphalaria glabrata*, responsible for the spread of intestinal schistosomiasis disease is also highly sensitive to primulic acid 1. Its molluscicidal concentration is 7.2 μM (Hostettmann et al., 1982).

CONCLUSION

The present study provides a simple method of isolation of primulic acid 1 from the roots of *Primula elatior*. The procedure utilises ultrasonic maceration of powdered root in methanol, reverse phase chromatography of the extract on highly porous styrene-divinylbenzene polymer resin and crystallization of saponin. The influence of primulic acid 1 on the environment was evaluated by the acute immobilisation test performed with *Simocephalus expinosus* s.l. The test showed that the saponin can be classified as toxic for aquatic organisms. The toxicity of primulic acid 1 (EC_{50}-24 h) is lower than its value of cmc. We assume the action at the subcellular level and not just solubilisation of a plasmatic membrane which is typical for amphiphilic compounds. Our results show that medicaments containing primula acid 1 or extracts from primula root should be also evaluated in relation to the environment.

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