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Evaluation of Cytotoxicity and Genotoxicity of *Micromeria pulegium* (Rochel) Benth Extract in Human Lymphocytes and Gr-M Melanoma Cells *in vitro*

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Abstract

Micromeria pulegium (Rochel) Benth is an endemic species of Lamiaceae family that includes plants frequently used in culinary and folk medicine. As cytotoxic potential of some species of *Micromeria* genus has been confirmed, this study aimed to test unknown antiproliferative and genotoxic potential of aqueous leaf extract of *M. pulegium*, endemic bh. species, in normal (human lymphocytes) and cancer (human melanoma GR-M) cells. The results of study would aid the burden of evidence required for the establishment of conservation program of small populations of native *M. pulegium* or promote its controlled micropropagation and/or cultivation. Cytokinesis-block micronucleus cytome assay was applied to human lymphocyte cultures, while trypan blue exclusion assay was used for evaluation of cytotoxicity in human GR-M melanoma cells. No genotoxic effects were detected in human lymphocyte *in vitro* up to the concentration of 0.2 mg/ml but cell viability in human GR-M melanoma cell line cultures treated with 0.3 mg/ml of *Micromeria* aqueous extract was significantly reduced.

Introduction

The number of registered cancer patients constantly increases. Despite the level of investments in pharmaceutical research and development, the number of new drugs still remains low. High costs of drugs development are largely connected to strict regulations in the field (Munos, 2009). However, the search for new active compounds continues. There is special interest for the products from biological sources as their use in traditional medicine dates from ancient times (Cragg et al., 1997). Sakarkar & Deshmukh, 2011 estimate that 80% percent of worldwide population primarily relies on traditional medicine (Sakarkar & Deshmukh, 2011). The estimates are similar for developed countries (WHO, 2011). Despite, the available data reflect that general knowledge of biological potential of natural

resources is not at the high level. In the year 2000, chemical composition was known for only about 15% of plants while the biological activity was described for not more that 6% of total known plants. Accordingly, research in that field is important and even recommended (WHO, 2005).

Species of Lamiaceae family are widely used as aromatic culinary supplements and also as medicinal plants due to the recognized and proved bioactive properties of their secondary metabolites, such as essential oils or terpenoid and phenolic compounds. Among around 30 species of Lamiaceae in bh. flora (Redžić, 2010; Ferrier et al., 2015), there is a respective number of endemic species, such as *Micromeria pulegium* (Rochel) Benth (syn. *Clinopodium pulegium*). *Micromeria* genus (tribe Mentheae, and subfamily Nepetoideae) with 130 polymorphic flowering herbs, subshrubs, and shrubs (Slavkovska et al., 2005), growing in rocky ground from the Micronesian-Mediterranean region to southern Africa, India and China, was described by Bentham (1829). *M. pulegium* is also distributed in S.W. Romania and E. Serbia, inhabiting rocky ground 1000 to 1200 m above sea level. The species is a perennial herb with erect straight stems and ovate, obtuse to acute, more or less crenate-dentate leaves. The leaves are densely punctated on the abaxial side. The bracts are linear. The verticillasters have 10-40 flowers. The calyx is 13-veined, shortly pubescent, sparsely hairy in the throat, the teeth are 1/3-1/2 the length of the tube, linear-lanceolate, equal. The corolla is white or lilac in color (Chater & Guinea, 1972). Many authors have shown that plants of *Micromeria* genus contain aboundant amounts of essential oils, with pulegone, isomenthone and menthone as the most dominant (Vladimir-Knežević et al., 2001; Medine et al., 2004; Duru et al., 2004; Slavkovska et. al., 2005; Telci & Ceylan 2007; Šavikin et al., 2010; Radulović & Blagojević 2012; Karousou et al., 2012). However, *M. pulegium* is rarely investigated species regarding its chemical composition but also medical, antiproliferative and genotoxic significance.

Significance of *Micromeria* genus in traditional medicine has been linked to the heart disorders, headache, wound healing (Formisano et al., 2014) as well as anti-inflammatory, antimicrobial (Ali-Shtayeh et al., 1997) and antioxidative effects (Couladis et al., 2003; Güllüce et al., 2004). In Algeria, the place of *Micromeria* genus origins, its leaf decoct is frequently used for treatment of wound, stomach ache, colds, fevers and even as condiment (Benomari et al., 2016). Antiproliferative potential of *Micromeria fruticosa* (L.) Druce *ssp. serpyllifolia* (Bieb) PH Davis, in concentration of 200 μg/ml in human glioblastoma cell line, justifies research of anticancer activity of *Micromeria* extracts (Koc et al., 2017). However, identification of unwanted effects may reduce wide consumption of endemic species that often grow in small populations. As *M. pulegium* is rich in pulegone, known for its bio-insecticide and a bio-pesticide potential (Stojičić et al., 2017), we hypothesized its cytogenic and genotoxic activity. In addition, there is only limited number of genotoxicity studies with pulegrone (NTP, 2011).

Accordingly, this research was conducted in order to test cytotoxic and genotoxic activity of *M. pulegium* leaf aqueous extract *in vitro* in normal human lymphocyte cultures as well as cytotoxic effects in human melanoma GR-M cultures, as these data lacking and may influence future utilization of *M. pulegium*.

Materials and methods

Plant collection and extract preparation

Whole plants were collected in Višegrad area [(E) 19° 17′ 16.69″; (N) 43° 46′ 56.66″] during the flowering season of the year 2015. Voucher specimen is deposited in Herbarium of the National Museum of Bosnia and Herzegovina (Voucher No.: *M. pulegium* (Rochel) Benth. - (SARA):0051546).

Upon collection, plants were air dried and kept in paper begs at room temperature. The crude methanol extracts were prepared by maceration of plant leaves (100 g) in 80% methanol (10 mL) (Alanis et al., 2005). After 24-hour incubation at 4°C, extracts were centrifuged at 2000 rpm for 15 minutes. Supernatants were transferred in clean tubes, evaporated to dryness in vacuum and then dissolved in sterile distilled water to stock concentration (20 mg/mL).

Cytokinesis-block micronucleus cytome assay in human lymphocytes culture

In human lymphocyte cultures, cytotoxic and genotoxic potential of plant extract was tested by cytokinesis-block micronucleus cytome assay (CBMN-Cyt assay) according to OECD Guideline for the Testing of Chemicals (2014). Peripheral blood samples for lymphocytes cultivation were taken into Li-heparin vacutainers, from three healthy, non-smoking male volunteers who signed informed consent forms. Duplicates of whole blood cultures were set up in 15-mL sterile, plastic tubes with a conical bottom (Isolab GmbH, Wertheim Germany), containing 5 mL of PB-MAX Karyotyping Medium (GIBCO-Life Technologies, Grand Island, NY, USA) and 400 µl of peripheral blood. Cells were harvested 72 hours after cultivation at 37ºC. Extract was added after initial 24 hours of cultivation to the final concentrations of 0.01, 0.05, 0.1 and 0.2 mg/mL. For each blood sample negative control containing water and positive control with mytomicine C (Sigma-Aldrich Co., St Louis, MO, USA) (0.25 µg/mL) were set up as well. Cytochalasin B (Sigma-Aldrich Co., St Louis, MO, USA) in final concentration of 4.5 µg/mL was added at the beginning of the 45th hour of cultivation in order to block cytokinesis enabling microscopic observation of cytotoxicity and genotoxicity markers. After 72 hours of cultivation, cultures were briefly treated with 0.56% KCl, fixed and dropped on labelled microscope slides that were air-dried and stained in 5% Giemsa for 7 minutes. Mononuclear, binuclear, trinuclear, quadrinuclear as well as apoptotic and necrotic cells were registered in the total number of at least 500 counted cells per each treatment and control. Cytotoxic potential of tested extract was evaluated by calculating the nuclear division index (NDI) and nuclear division cytoxicity index (NDCI). Micronuclei, nuclear buds and nucleoplasmic bridges were scored in 2000 binuclear (BN) cells per each treatment, according to the established criteria (Fenech 2000, Fenech et al., 2003, Fenech, 2007). Slides were analyzed on Olympus BX51 microscope (Tokyo, Japan) independently by two experienced analysts.

Results of cytokinesis – block, micronucleus cytome assay were evaluated with one-way analysis of variance (ANOVA), followed by Newman - Keuls multiple comparison using MedCalc for Windows, version 16.8.4 (MedCalc Software, Ostend, Belgium). Differences were considered significant if $p<0,05$.

Cytotoxicity evaluation in GR-M melanoma cell line

The Human Caucasian melanoma cell line (GR-M), obtained from Culture Collections, Public Health England, UK (Cat. No. 95032301) was grown in RPMI 1640 medium supplemented with L-glutamine, 10% of fetal bovine serum (FBS) as well as 100 units/ml of penicillin and 100 mg/ml of streptomycin. T-25 flasks (NUNC, Rochester, NY, USA) with vented caps were used as a culture dishes and cultures were maintained in CO_2 incubator (EC 160, Nuve) at 37ºC in a 5% CO_2 atmosphere with 95% humidity.

Trypan blue exclusion assay was performed 72 hours after subcultivation of melanoma cell cultures. Cytotoxic potential of *Micromeria* agueous leaf extract in GR-M melanoma cell line was tested in final concentrations of 0.1; 0.2 and 0.3 mg/ml and treatment duration of 24 hours. Each treatment was carried out in triplicates (Strober, 2001). Cells were harvested by trypsinization, diluted in trypan blue and counted in hemocytometar. The cell viability (%) was determined as (no. of viable cells/total no. of viable + non-viable cells) x 100. Simple linear regression and one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests in WINKS 4.5 Professional software (TexaSoft, Cedar Hill, Texas, USA) were used for statistical analysis and pairwise comparisons. Statistical significance threshold was fixed at 0.05.

Results and Discussion

M. pulegium is *Lamiaceae* species that is widely used in traditional medicine. Its distribution range is limited and includes rather small natural populations (Stojičić et al., 2017). Therefore, the utilization of this species from natural sources should be continued in sustainable manner. As many species of *Lamiaceae*, plants of *Micromeria* genus contain various essential oils, dominantely pulegone,

isomenthone and menthone (Vladimir-Knežević et al., 2001; Medine et al., 2004; Duru et al., 2004; Slavkovska et al., 2005; Telci & Ceylan, 2007; Šavikin et al., 2010; Radulović & Blagojević, 2012; Karousou et al., 2012). However, it has been shown that chemical composition, or more precisely the amount of essential oils, depends on environmental factors (Kokkini et al., 2004; Lakušić et al., 2011; Lakušić et al., 2013) and differs in various developmental stages of the plant (Slavkovska et al., 2013). Due to the bio-insecticide and bio-pesticide activity (Stojičić et al., 2017) and limited data about pulgone genotoxicity, we aimed to test genotoxic and cytotoxic effects of *M. pulegium* aqueous leaf extracts that is the most often used form of traditional plant preparation.

Micromeria extract genotoxicity and cytotoxicity in human lymphocytes in vitro

Genotoxicity of substance was evaluated in cytokinesis blocked human lymphocytes by scoring micronuclei (MN), nuclear buds (NB) and nucleoplasmic bridges (NPB) in total of 1000 binuclear cells (BN) per treatment. Cytostatic and cytotoxic effects, expressed as nuclear division indexes (NDI and NDCI) were calculated after scoring of mono-, bi-, tri- and tetranuclear cells as well as apoptosis and necrosis in at least 500 cells per treatment. Results are shown in Table 1.

Table 1. CBMN-cyt assay in human lymphocytes treated with *M. pulegium* aqueous extract

Treatment (mg/ml)	Sample	MN	NB	NPB	NDI	NDCI
Negative control (H_2O)	1.	10	6	1	1.90	1.89
	2.	5	4	2	1.27	1.27
	3.	19	10	0	1.42	1.41
	Xav	11.33	6.67	1.00	1.53	1.52
	SD	7.09	3.06	1.00	0.33	0.33
0.01	1.	20	9	3	1.59	1.59
	2.	7	4	0	1.22	1.22
	3.	5	7	2	1.58	1.54
	Xav	10.67	6.67	1.67	1.46	1.45
	SD	8.14	2.52	1.53	0.21	0.20
0.05	1.	27	7	1	1.54	1.54
	2.	9	7	1	1.24	1.24
	3.	10	8	1	1.72	1.70
	Xav	15.33	7.33	1.00	1.50	1.49
	SD	10.12	0.58	0.00	0.24	0.24
0.1	1.	27	11	1	1.61	1.60
	2.	7	4	2	1.28	1.27
	3.	9	9	1	1.54	1.52
	Xav	14.33	8	1.33	1.47	1.46
	SD	11.02	3.61	0.58	0.17	0.17
0.2	1.	19	11	5	1.47	1.47
	2.	10	4	2	1.33	1.32
	3.	4	9	3	1.62	1.60
	Xav	11	8	3.33	1.47	1.46
	SD	7.55	3.61	1.53	0.15	0.14
Positive control (mytC)	1.	591	90	19	1.12	1.11
	2.	275	39	12	1.19	1.17
	3.	697	83	23	1.18	1.18
	Xav	521*	70.67*	18*	1.16	1.15
	SD	219.54	27.65	5.57	0.04	0.04

MN – micronuclei; NB – nuclear buds; NPB – nucleoplasmic bridges; NDI – nuclear division index; NDCI – nuclear division cytotoxicity index.
*p<0.001

One-way ANOVA showed no significant deviation in MN, NB and NPB frequencies among treatments while significant increases in frequencies of all genotoxicity markers were evidenced only in

positive control ($p<0.001$), as expected. Calculated values of nuclear division did not significantly differ nor against positive control.

Results of trypan blue assay in GR-M human melanoma cell line

Since genotoxicity analysis revealed no genotoxic effects in lymphocytes cultures in concentrations of aquous leaf extract of *M. pulegium* reaching 0.2 mg/ml, another treatment with higher concentration was introduced in cytotoxicity test in GR-M melanoma cell line (0.3 mg/ml). The conducted trypan blue assay in human GR-M melanoma cell line revealed an increase in frequency of non-viable cells in the treated cultures. Also, a general dose-dependent decrease in the total number of cells was observed. Calculated viability index consequently reflects dose-dependent cytotoxicity of aqueous leaf extract of *M. pulegium*. The results are summarized in Table 2.

Tabela 2. Cytotoxicity of *M. pulegium* aqueous extract in human GR-M melanoma

Treatmant/ conc. (mg/ml)		Replicate I		Replicate II		Replicate III		% Cell viability (Xav)
		V	N	V	N	V	N	
Control	Σ	347	15	1515	29	1108	31	97.085
	viability %	95.856		98.122		97.278		
0.1	Σ	189	11	185	5	199	12	95.394
	viability %	94.500		97.368		94.313		
0.2	Σ	178	16	166	12	173	18	91.862
	viability %	91.753		93.258		90.576		
0.3	Σ	18	3	20	9	18	9	73.782
	viability %	85.714		68.965		66.667		

ANOVA revealed significant decrease in GR-M cells viability in the cultures treated with 0.3 mg/ml extract ($p=0.002$). Simple linear regression showed significant association between *M. pulegium* extract concentration and GR-M cell viability ($p=0.002$) (Figure 1).

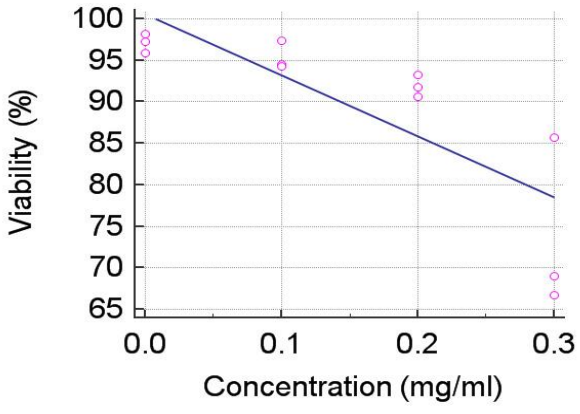

Figure 1. Cell viability of GR-M melanoma cells treated with *M. pulegium* aqueous extract

Previous studies of mutagenicity of pulegone, a monoterpene ketone, present in the leaves and terminal inflorescence of several members of the mint family *Lamiaceae*, show controversial results (Andersen & Jensen, 1984; NTP, 2011). Studies of pulegone using *Drosophila melanogaster* somatic mutation and recombination test, show weak mutagenicity, while a sample of pennyroyal oil, reported to contain 75.7% of pulegone, was not mutagenic in this assay (Franzios et al., 1997). Pulegone did not increase formation of micronuclei in peripheral blood erythrocytes of B6C3F1 mice at doses up to 150 mg/kg bw per day by gavages for 3 months (NTP, 2011). Menthone that can be easily converted into isomenthone has been identified as mutagenic in Ames test (Anderson & Jensen, 1984) and is genotoxic in fruit flies (Franzios et al., 1997). Nevertheless, the extracts and aqueous fraction of *M. biflora* (Buch.-Ham. ex D.Don) Benth showed dose-dependent cytotoxic activity in brine shrimp cytotoxic assay (Rauf et al., 2017).

Conclusions

Significant cytotoxic effect of *M. pulegium* aqueous extract in concentration of 0.3 mg/ml on human GR-M melanoma cell line and the absence of genotoxic

effects in healthy cells in lower concentrations, present promising basis for further research of *Micromeria* species extracts as potential anticancer drugs. However, the utilization of endemic species must be taken into consideration and models of sustainable use should be developed.

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