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Anti-proliferative and anti-inflammatory activity of triterpene extracts from plant species belonging to Lamiaceae family

[Actividad antiproliferativa y antinflamatoria de los extractos de triterpenos de plantas pertenecientes a la familia Lamiaceae]

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Abstract: Triterpenes are very important secondary metabolites with wide structural diversity and significant role in pharmacy and medicine. In the present research, a comparative study of pharamacological activities of the triterpene fractions obtained from several plant species belonging to Lamiaceae family, was carried out. In-vitro anti-proliferative activity was performed using a standard proliferation assay based on tetrazolium salts. In vitro anti-inflammatory activity of triterpene fractions was determined by an assay of inhibition of albumin denaturation. In general, the triterpene fractions obtained from plant species belonging to Lamiaceae family showed a strong anti-proliferative activity and anti-inflammatory activity. The triterpene fraction of Rosmarini folium showed the strongest anti-proliferative activity (GI_{50} range from 4 to 37 µg/ml) and the strongest anti-inflammatory activity in the range from 57.27% to 80.69%. This comparative study provides scientific evidence to support the traditional use of Lamiacae plant species for medical purposes as anti-inflammatory and anti-proliferative medicines.

Keywords: Anti-proliferative; Anti-inflammatory; Triterpenes; Lamiaceae family; *Rosmarinus officinalis*

Resumen: Los triterpenos son metabolitos secundarios muy importantes, con una amplia diversidad estructural y un rol significativo en la farmacia y la medicina. En esta investigación, se realizó un estudio comparativo de las actividades farmacológicas de las fracciones de triterpenos obtenidas de varias especies de plantas pertenecientes a la familia Lamiaceae. La actividad antiproliferativa *in vitro* se realizó mediante un ensayo estándar de proliferación basado en sales de tetrazolio. Se determinó la actividad antinflamatoria de las fracciones de triterpenos mediante un ensayo de inhibición de desnaturalización de la albúmina. En general, las fracciones de triterpenos obtenidas de las plantas pertenecientes a la familia Lamiaceae mostraron una actividad antiproliferativa y antinflamatoria fuerte. La fracción de triterpeno de Folium Rosmarini mostró la actividad antiproliferativa más fuerte (rango GI_{50} entre 4 y 37 µg/mL) y la más fuerte actividad antinflamatoria en el rango de 57,27% a 80,69%. Este estudio comparativo provée evidencia científica para apoyar el uso tradicional de especies de plantas Lamiaceae para usos médicos como medicinas antinflamatorios y antiproliferativas.

Palabras clave: Antiproliferativo; Antinflamatorio; Triterpenos; Familia Lamiaceae; *Rosmarinus officinalis*

INTRODUCTION

Numerous natural products and phytochemicals have been investigated for different pharmacological properties in order to serve as a potential source for new therapeutic indications (Bijauliya *et al*., 2017). The pharmacological activities of phytochemicals can be explained by the presence of various compounds such as tannins, saponins, flavonoids, alkaloids, triterpenes, and other constituents that act as inhibitors and mediators of molecular responses (de Almeida et al., 2015). Triterpenes are secondary metabolites with wide structural diversity and significant role in ecology, agronomy, cosmetics and pharmaceutical industry (Priyanka *et al*., 2013; Thimmappa *et al*., 2014).

In recent years, triterpenes have aroused great interest due to their pharmacological properties and they have been marked as promising agents with great therapeutic potential (Nazaruk *et al*., 2015). In terms of pharmacological activity, the most common investigations regard pentacyclic oleans, ursans, lupanes and tetracyclic damarns (Muffler *et al*., 2011). Plant species from the *Lamiaceae* family have been used to treat different disorders and mainly contain pentacyclic triterpenes that possess anti-inflammatory, hepatoprotective, antioxidant, anticancer, antiviral and, antimicrobial activity (Dzubak *et al*., 2006; Manning & Goldblatt, 2012; Shanaida *et al*., 2018).

Betulin, and its derivative betulinic acid, are a naturally occurring lupane-type pentacyclic triterpenes, widely distributed in plants which have been investigated in terms of their anti-inflammatory, anticancer and antiviral activity (Hordyjewska *et al*., 2018; Hordyjewska *et al*., 2019). It is stated that the anti-inflammatory activity of betulinic acid is related to its ability to inhibit enzymes involved in the biosynthesis of leukotrienes, specifically 5-lipoxygenase (Nworu & Akah, 2015; Zhang *et al*., 2016). The use of betulinic acid, as anticancer compound, is based on the fact that cells require adequate levels of polyamine for their normal growth, and that their growth can be prevented with drugs that affect the enzymatic synthesis of polyamine (Bildziukevich *et al*., 2015). The main role in this synthesis is played by the enzyme ornithine decarboxylase. Betulinic acid is known as an ornithine decarboxylase inhibitor (Ajumeera *et al*., 2018) and it is able to prevent the activation of both nuclear factor kappa B (NFκB) and the translocation of its nucleus, preventing cell proliferation and activation of the extrinsic apoptosis pathway in cancer cells (TNF-α dependent apoptosis) (Takada & Aggarwal, 2003; Zhang *et al*., 2016). Betulinic acid also showed specific cytotoxic activities against the colon cancer cell line (HCT 116), with minimal cytotoxic effect on normal cell columns (CCD-18Co) (Aisha *et al*., 2012; Potze *et al*., 2016; Soica *et al*., 2017).

Oleanolic acid and lupeol also belong to the group of triterpenes and they have been identified in many plant species (Sporn & Suh, 2000). In nature, oleanolic acid is found on its own in the form of crystals or bound to a different sugar groups forming triterpenoid saponins, glycosides (Sultana & Ata, 2008; Pollier & Goossens, 2012). Lupeol as a pentacyclic triterpene is found free in nature (Patočka, 2003; Saleem, 2009). Anticancer activity of oleanolic acid have been noticed on various tumor types such as gliomas (Guo *et al*., 2013), breast cancer (Liu *et al*., 2014), osteosarcoma (Zhou *et al*., 2011), hepatocellular carcinomas (Wang *et al*., 2013) and lung cancer (Way *et al*., 2014). The main mechanisms of its anticancer activity is blockage of cell cycle, induction of apoptosis and inhibition of metastasis (Wei *et al*., 2012; Guo *et al*., 2013). Furthermore, oleanolic acid exhibits its anti-inflammatory activity by inhibiting C3 convertase, reducing prostaglandin production (PGE2), and by inhibiting activity of cyclooxygenase 2 (COX2) (Paszel-Jaworska *et al*., 2014; Fajemiroye *et al*., 2015). Anti-inflammatory activity is also exhibited by lupeol (Nguemfo *et al*., 2008). Different *in vivo* studies have revealed that lupeol and its derivates possess stronger anti-inflammatory activity than the most commonly used nonsteroidal anti-inflammatory drug indomethacin (Lima *et al*., 2007; Sudhahar *et al*., 2008; Zhu *et al*., 2016; Meera *et al*., 2017; Kangsamaksin *et al*., 2017). Ursolic acid, a triterpene, also inhibits the growth of different cancers cells by its cytotoxic effect and induction of apoptosis on cancer cells (Bijauliya *et al*., 2017).

The present comparative study aims to investigate anti-inflammatory and antiproliferative *in vitro* activity of triterpene fractions from the selected plant species from *Lamiaceae* family. Plant species of the *Lamiaceae* family examined in this study are *Mentha piperita* L., *Thymus pulegioides* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., *Lavandula officinalis* L. *and Melissa officinalis* L. In

this comparative study, we investigated the following triterpenes: betulin, betulinic acid, lupeol, ursolic acid and oleanolic acid.

MATERIALS AND METHODS

Sample collection

The aerial parts of wild plants *Rosmarinus officinalis* L., *Salvia officinalis* L., *Melissa officinalis* L., *Thymus pulegioides* L., *Lavandula officinalis* L. and *Mentha piperita* L. were collected at the flowering stage from May to July 2017 in the Central and South part of Bosnia and Herzegovina. The plant material was identified through a series of comparative macroscopic, microscopic, organoleptic and TLC analyses. The plant material was stored in a dry location in the absence of light at room temperature. The sample of plant species was kindly identified and confirmed by prof. dr Samir Đug from the Faculty of Science in Sarajevo. The *voucher specimens* were deposited in the Herbarium of the Faculty of Pharmacy, University of Sarajevo. The *voucher specimens* are *Rosmarini folium* 0119, *Salviae folium* 0219, *Melissae folium* 0319, *Thymi herba* 0419, *Lavandulae flos* 0519, *Menthae piperitae folium* 0619.

Extraction and tested triterpene fractions

Plant marterial (45g), *Menthae piperitae folium* (sieve 2), *Thymi herba* (sieve 3), *Rosmarini folium* (sieve 2), *Salviae folium* (sieve 2), *Lavandulae flos* (sieve 3) and *Melissae folium* (sieve 2), was subjected to hydro distillation for two hours with 400 mL distilled water to remove the essential oil. After distillation, the herbal residue was separated from water and dried in oven at 50°C. In order to complete the separation of the compound of interest, the residual material underwent to successive extractions with 600 mL of n-hexane, chloroform and methanol in a Soxhlet's apparatus. The extraction with each solvent lasted for six hours. In this way, from the resulting three separate organic phases, after having removed the solvent under reduced pressure, we obtained three extracts, hexane, chloroform and methanol (Jordamović *et al*., 2020).

Qualitative and quantitative analysis of the presence of triterpenes of potential pharmacological importance in the three different extracts, was performed using thin layer chromatography (TLC), and high-pressure liquid chromatography (HPLC). Thin-layer chromatoraphy. In order to monitor the presence of triterpenes in different extracts, TLC was performed using preocoated silica gel GF254 plates (20x20 cm, thickness 0.25 mm, Merck, Dürmsttadt). The solvent system used as eluent was benzene: ethyl acetate: formic acid (36:12:5) (Merck, Germany). Detection of triterpene substances was achieved by observation under UV 366/254, spraying with 4-anisaldehyde (Merck, Germany) - sulphuric acid (Kemika, Zagreb) and heating in owen 10 minutes at 110ºC. Since the triterpene compounds are the subject of the study, the triterpene standards were used for their identification in the extracts.

High pressure liquid chromatography. This was conducted on HPLC Shimadzu 10 Avp with autosampler and spektro monitor® 3100 optical detector (LDC Analytical), using a constaMetric® 3000 system for solvent release, Hyperesil ODS (Agilent Technologies) column, 4.6 x 250 mm, 5 μm, mobile phase acetonitrile/aqua (700/300) acidified with ortho-phosphoric acid.

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

For every plant species, fractions with the highest content in triterpenes were used for further investigation of anti-inflammatory and anti-proliferative activity. Some plant species showed the presence of triterpenes in different fractions. Those fractions were combined and tested for pharmacological activity.

The following samples were used in testing pharmacological activity: a) *Rosmarini folium* - hexane fraction; b) *Salviae folium* - hexane and chloroform fractions combined (1:1); c) *Melissae folium* - hexane and chloroform fractions combined (1:1); d) *Thymi herba* - hexane fraction; e) *Lavandulae flos* - hexane and chloroform fractions combined (1:1); f) *Menthae piperitae folium* -hexane fraction.

Methods and materials, used during the extraction of triterpenes from the plant species, were described and published in our previous study (Jordamović *et al*., 2020).

Cell lines and chemicals

Human breast adenocarcinoma (MCF-7), acute lymphoblastic leukemia (MOLT-4), human epidermal keratinocyte (HaCaT) and cancer of human

colon (HC-T116) cell lines were obtained from the American Tissue Culture Collection. Doxorubicin (543.52 g/mol), dimethylsulfoxide (DMSO) and all other chemicals were obtained from Sigma-Aldrich Co. (Germany).

In vitro anti-proliferative assay

In vitro antiproliferative activity was performed using a MTT cell proliferation assay. A total of six triterpene fractions were examined on three tumor cell lines [human breast adenocarcinoma (MCF-7), acute lymphoblastic leukemia (MOLT-4) and, human colon (HC-T116) cell line] and one non-tumor cell line [human epidermal keratinocyte (HaCaT) cell line]. Stock solutions of tested triterpene fractions were dissolved in DMSO and prepared at concentration of 200 mg/ml. However, the extracts were not completely dissolved in DMSO and precipitated upon dilution in cellular (aqueous) medium. Doxorubicin (543.52 g/mol), was used as a positive control in the final concentration of the stock solution of 1 mM.

The assay was performed according to a slightly modified method of National Cancer Institute (NCI) *in vitro* testing of new substances (Mosmann, 1983; Skehan *et al*., 1990; Boyd & Paull, 1995). Cellular lines were cultured on RPMI 1640 (Sigma) nutrient medium with the addition of 10% fetal calf serum (FBS), penicillin and streptomycin at 37°C, 5% CO_2 and 100% moisture. Cell lines were planted on plastic plates with 96 wells (so-called "zero" day) in concentrations of 1.25×10^4 IU/mL (HCT116), 1.5×10^4 IU/ml (HaCaT), 2.25×10^4 IU/ml (MCF- 7) and 7.5×10^4 IU/mL (MOLT-4), depending on the doubling time of the cell number specific to each cell line. Tested triterpene fractions were added at concentrations of 1 μg/mL, 5 μg/mL, 10 μg/mL, 50 μg/mL, 100 μg/mL, 500 μg/mL or 1000 μg/mL and incubated for a 72 hours. Dilutions were prepared fresh in cell culture medium, RPMI 1640 on the day of testing. After 72 hours of incubation, cell growth was assessed using a tetrazolium salt-based proliferation assay (MTT). The MTT test is a metabolic test that determines cell survival by measuring the activity of the enzyme dehydrogenase. Tetrazolium salts are cleaved into formazan by the succinate-tetrazolium reductase system. Dehydrogenases reduce the yellow, water-soluble compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan purple crystals which are insoluble in water. In order to measure and quantify resulting compounds, the crystals were dissolved in DMSO.

Quantification was performed by the spectrophotometric method using enzyme-linked immunosorbent assay (ELISA) strains and the obtained values are directly proportional to the number of living cells. An increase in the number of living cells results in an increase in total metabolic activity, which leads to the appearance of more intense staining. The percentage of growth (PG) of cells of a particular cell line is calculated according to one of the following two equations:

$$\textbf{[1] If } (A_{test} - A_{start\ time}) \geq 0 \textbf{ then: } PG = 100 \times (A_{test} - A_{start\ time})/(A_{control} - A_{start\ time})$$

$$\textbf{[2] If } (A_{test} - A_{start\ time}) < 0 \textbf{ onda: } PG = 100 \times (A_{test} - A_{start\ time})/A_{start\ time}$$

$$A_{start\ time} \textbf{ = mean value of absorbance before cell exposure}$$

$$A_{test} \textbf{ = mean value of absorbance after 72 hours in treated cells}$$

$$A_{control}\ A \textbf{ = mean value of absorbance after 72 hours of non-exposed cells}$$

The results are presented as the ratio of the concentration of the tested triterpene fractions and the percentage of living cells (concentration-response graphs). A negative percentage indicates cytotoxicity following drug treatment where -100% shows no cells survived the treatment at the specific drug concentration. Furthermore, obtained results are also presented as GI_{50}, which represents the concentration of compounds required to inhibit 50% growth. The GI_{50} values for each compound are calculated from

dose-response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the respective reference value (e.g. 50 for GI_{50}). Therefore, a "real" value for any of the response parameters is obtained only if at least one of the tested drug concentrations falls above, and likewise at least one falls below the respective reference value. If, however, for a given cell line all of the tested concentrations produce PG's exceeding the respective reference level of effect, then the lowest tested concentration is assigned as the default value. Each result is the mean value obtained from three separate measurements.

In vitro anti-inflammatory assay

In vitro anti-inflammatory activity of triterpene fractions was determined by inhibition of albumin denaturation assay according to Mizushima and Kobayashi (Mizushima & Kobayashi, 1968) with certain modifications.

All solutions of the tested triterpene fractions (0.05 mL) as well as all solutions of the acetylsalicylic acid standard (0.05 mL) were mixed with a 0.45 mL of 0.5% bovine albumin serum (BAS) solution. The following range of sample concentrations was tested: 50, 100, 150, 200 and 250 µg/mL. All samples were incubated at 37ºC for 20 minutes and then incubated again at 57ºC for another 3 minutes. After cooling, 2.5 mL of phosphate buffer was added to all samples and the absorbance was measured on a spectrophotometer at 660 nm. Control represents the 100% of protein denaturation. The results were compared with the standard of acetylsalicylic acid (Mizushima & Kobayashi, 1968; Rashmi *et al*., 2011; Sathe *et al*., 2011).

Anti-inflammatory activity was expressed as the percentage of inhibition of protein denaturation and calculated by using the following formula:

$$\textbf{Inhibition (\%)} = 100 \times (1 - A_{sample}/A_{control})$$

Each result is the mean value obtained from three separate measurements.

We used 0.5% bovine albumin serum (SGA), 500 mg of bovine albumin serum was dissolved in 100 mL of water. Phosphate buffer (ph 6.3) - 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4), and 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) were dissolved in 800 mL of water. The pH was adjusted to 6.3 with 1 N HCl and then made up to 1000 mL with water. Test extract solution (0.5 mL) consists of 0.45 mL (SGA) (0.5%) and 0.05 mL of solution of tested triterpene fractions of different concentrations. Standard solution (0.5 mL) consists of 0.45 mL (SGA) (0.5%) and 0.05 mL of acetylsalicylic acid solution of various concentrations. Solution of triterpene standards consists of 0.45 mL (SGA) (0.5%) and 0.05 mL mixture of triterpene standards of betulin, betulinic acid, oleanolic acid and ursolic acid solutions of various concentrations.

RESULTS

HPLC analysis

Results of the HPLC analysis are shown in the Table No. 1.

Table No. 1
Results of the trierpenes content calculated in the hexane, chloroform and methanol extracts obtained by HPLC analysis

Extract	Betulin (mg/mL) ± SD	Betulinic acid (mg/mL) ± SD	Ursolic acid (mg/mL) ± SD	Oleanolic acid (mg/mL) ± SD
a - HE	0.004 ±0	0.052 ±0.006	0.033 ±0.004	0.001 ±0
a - HL	ND	0.022 ±0.001	ND	ND
a - ME	ND	ND	ND	ND
b - HE	ND	0.002 ±0.0002	0.034 ±0.003	ND
b - HL	ND	ND	0.095 ±0.01	ND
b - ME	ND	ND	0.001 ±0	ND
c - HE	0.001 ±0.0004	0.003 ±0	0.043 ±0.005	ND
c - HL	ND	0.001 ±0.0004	0.088 ±0.008	ND

c - ME	ND	ND	ND	ND
d - HE	0.001 ±0.0005	ND	0.115 0.014	ND
d - HL	ND	ND	0.074 ±0.02	ND
d - ME	ND	0.001 ±0.0003	ND	ND
e - HE	0.002 ±0.0006	0.002 ±0	0.026 ±0.005	ND
e - HL	ND	ND	0.145 ±0.005	ND
e - ME	ND	0.001 ±0.0004	ND	ND
f - HE	ND	0.009 ±0.001	0.020 ±0.009	ND
f - HL	0.001 ±0.0005	0.001 ±0.0003	0.013 ±0.009	ND
f - ME	ND	0.003 ±0.0005	ND	ND

a - *Rosmarini folium*, **b** - *Salviae folium*, **c** - *Melissae folium*, **d** - *Thymi herba*, **e** *Lavandulae flos*, **f** - *Menthae piperitae folium*, **HE** – hexane, **HL** – chroroform, **ME** – methanol, **ND**-non detected.

In vitro anti-proliferative activity

The results of *in vitro* anti-proliferative assay of triterpene fractions of plant species from the *Lamiaceae* family shown differential activity towards tested cell lines (Table No. 2 and Figure No. 1 to Figure No. 7).

Table No. 2
Results of anti-proliferative activity GI_{50} of tested triterpene fractions

GI_{50}* (µg/mL)				
	Cell lines			
Tested triterpene fractions	MOLT-4	MCF-7	HCT116	HaCaT
Rosmarini folium	4±1	29±1	19±17	37±13
Salviae folium	6±1	52±8	52±3	53±1
Melissae folium	66±6	225±120	123±50	168±60
Thymi herba	10±4	457±118	243±59	274±144
Lavandulae flos	3±2	68±13	41±8	71±7
Menthae piperitae folium	64±11	200±77	110±43	96±3
Doxorubicin	0.001±0.0002 (2 ± 0.1 nM)	0.02±0.01 (30 ± 10 nM)	0.02±0.01 (30 ± 10 nM)	0.002±0.001 (4 ± 1 nM)

***GI_{50}: the concentration of compound required to inhibit 50% cell growth, after 72 hours of incubation**

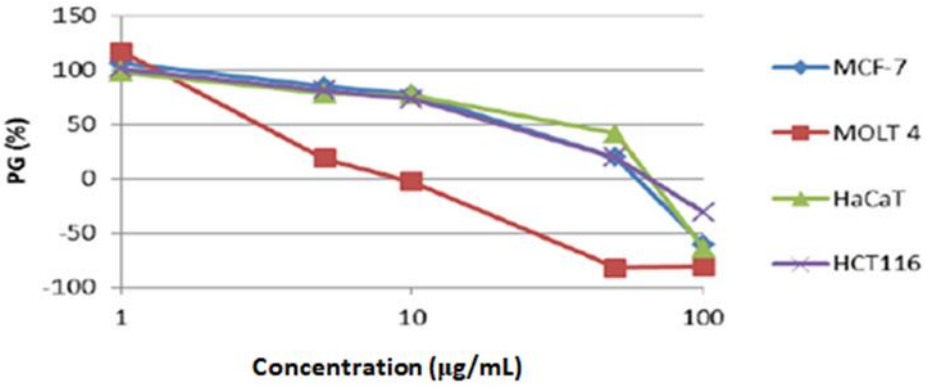

Figure No. 1
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro*, after 72 hours of incubation. Triterpene fraction of *Rosmarini folium*, presented as concentration-response profiles

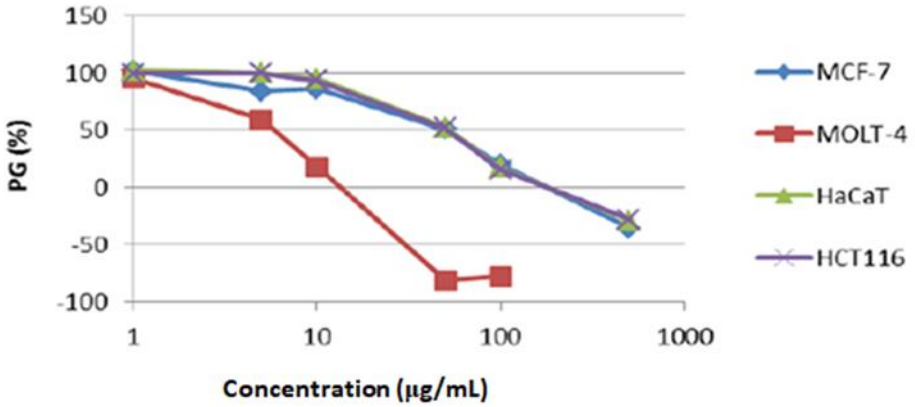

Figure No. 2
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro*, after 72 hours of incubation. Triterpene fraction of *Salviae folium*, presented as concentration-response profiles

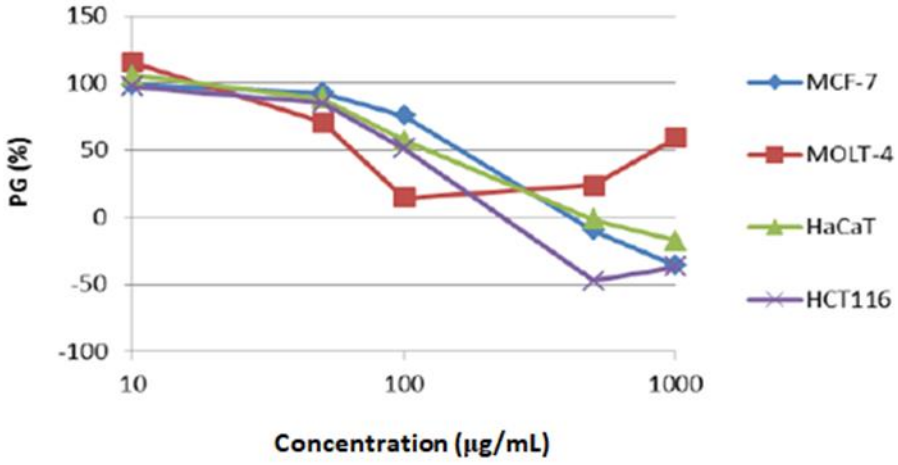

Figure No. 3
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro,* after 72 hours of incubation. Triterpene fraction of *Melissae folium*, presented as concentration-response profiles

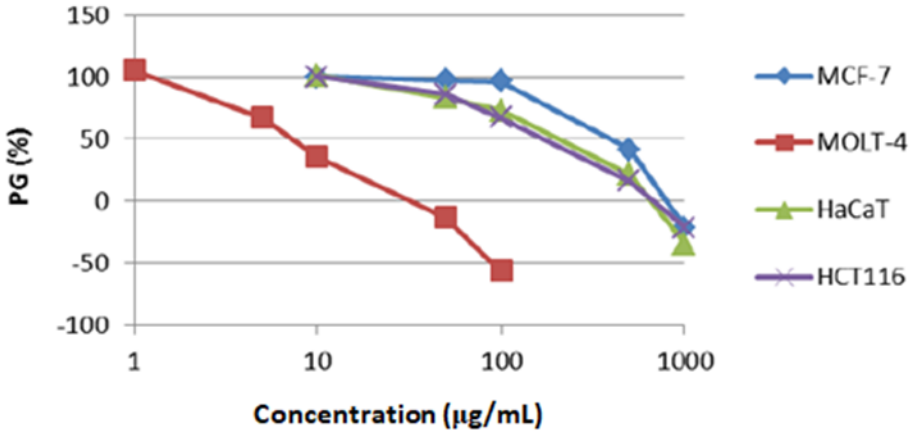

Figure No. 4
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro,* after 72 hours of incubation. Triterpene fraction of *Thymi herba*, presented as concentration-response profiles.

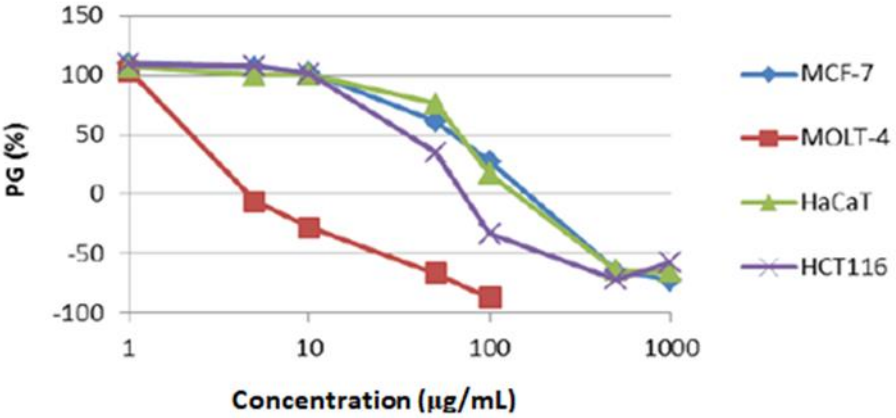

Figure No. 5
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro,* after 72 hours of incubation. Triterpene fraction of *Lavandulae flos*, presented as concentration-response profiles

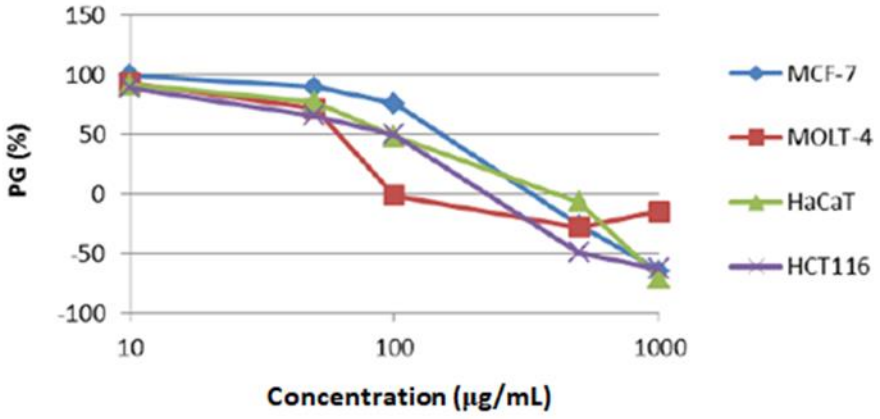

Figure No. 6
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro,* after 72 hours of incubation. Triterpene fraction of *Menthae piperitae folium*, presented as concentration-response profiles

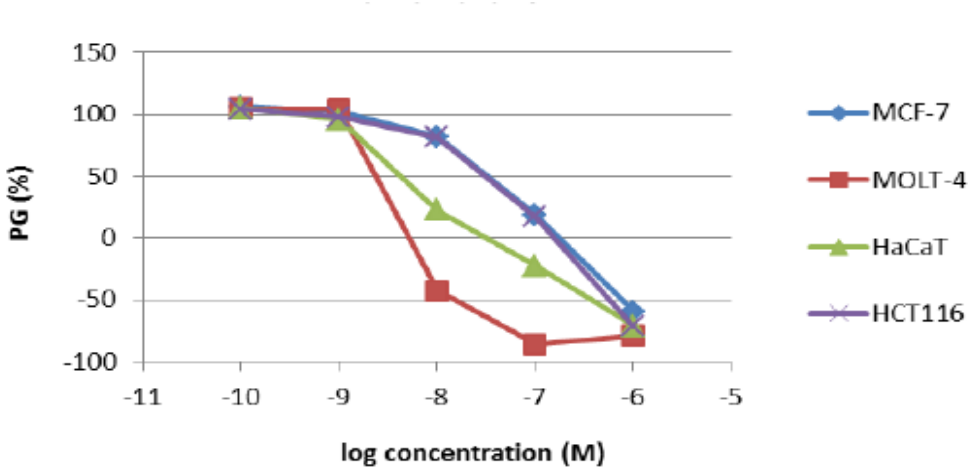

Figure No. 7
Growth inhibition of MOLT-4, MCF-7, HCT116 and HaCaT cells *in vitro,* after 72 hours of incubation. Doxorubicin, presented as concentration-response profiles

In vitro anti-inflammatory activity

The results of *in vitro* anti-inflammatory activity of triterpene fractions of plant species from the *Lamiaceae* family are shown in the Table No. 3 and Chart No. 1.

Table No. 3
Results of anti-inflammatory activity of tritepene fractions

	Anti-inflammatory activity (% inhibition)				
Tested samples	50 µg/mL	100 µg/mL	150 µg/mL	200 µg/mL	250 µg/mL
Acetylsalicylic acid	67,42	73,2	76,99	79,43	82,42
Solution of triterpen standards	59,45	61,68	70,34	74,9	76,08
Triterpene fractions					
Rosmarini folium	57,27	58,74	61,81	62,21	80,69
Salviae folium	29,35	37,38	46,59	53,84	54,2
Melissae folium	48,27	59,99	62,04	63,75	71,54
Thymi herba	25,54	32,56	37,58	47,01	50,24
Lavandulae flos	27,85	28	32,4	45,59	46,22
Menthae piperitae folium	27,64	37,86	41,17	51,13	54,46

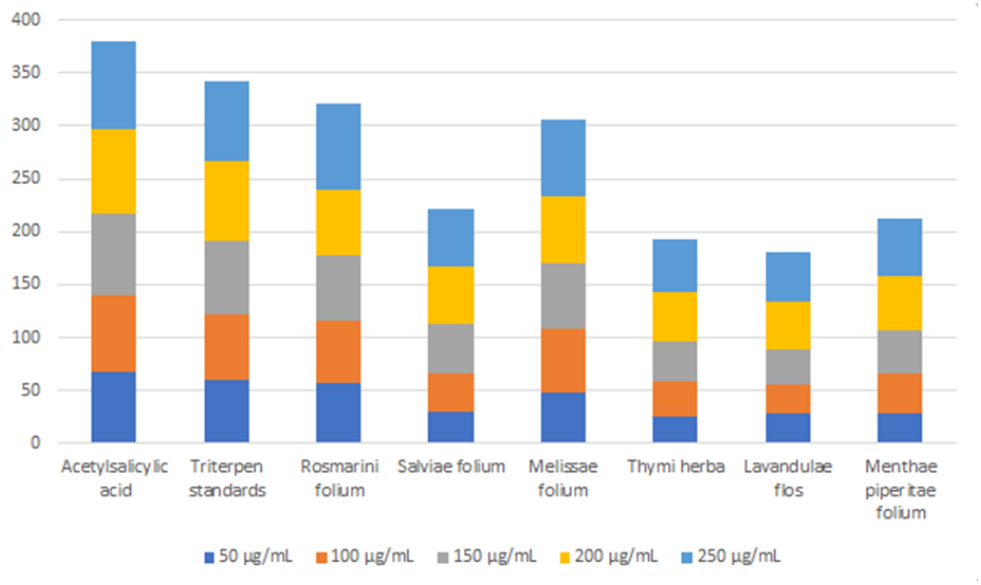

Chart No. 1
Overall results of the anti-inflammatory activity of the tested tritepene fractions

DISCUSSION

The tested triterpene fractions showed different anti-proliferative effects on three tumor and one non-tumor line. All triterpene fractions exhibited a cytotoxic effect on the MOLT-4 cell line. Triterpene fractions of *Melissae folium*, *Thymi herba* and *Menthae piperitae folium* moderately inhibited cell growth. The triterpene fractions of *Rosmarini folium*, *Salviae folium* and *Lavandulae flos* showed strong anti-proliferative activity. The triterpene fraction of *Rosmarini folium* most effectively inhibited the growth of all cells (GI_{50} 4 - 37 µg/mL). In general, the non-tumor line of human keratinocytes - HaCaT was equally sensitive to the examined triterpene fractions as to tumor cells.

The strongest antiproliferative activity of the triterpene fractions was shown on the MOLT-4 cell line with a GI_{50} range of 3 to 66 µg/mL (concentration that inhibits 50% cell growth 72 hours after the addition of test substances). Following the MOLT-4 cell line, weaker anti-proliferative activity was shown by triterpene fractions on colon cancer cell cultures (HCT116), where the GI_{50} ranged from 19 to 243 µg/mL, and the weakest anti-proliferative activity was shown in cell culture of adenocarcinoma (MCF-7) with a GI_{50} of 29 to 457 µg/mL. The triterpene fractions demonstrated a weaker anti-proliferative effect on the culture of HaCaT cells (human immortalized keratinocytes) with a GI_{50} range from 37 to 274 µg/mL. As a positive control, the classic antitumor drug doxorubicin was used, which had the most efficient anti-proliferative effect on the examined cell lines.

According to the results of our study, the triterpene fraction of *Rosmarini folium* had the strongest anti-proliferative efficacy, which can be attributed to the highest total content of triterpenes. Similar findings were reported in a study by Pérez-Sánchez *et al*. (2018). Various studies have shown the anti-proliferative activity of betulinic acid on cancer cells (Kontogianni *et al*., 2013). In a review by Sharifi-Rad *et al*. (2020), the cytotoxic effect of rosemary extract in the treatment of cancer has been attributed to carnosic acid, monoterpenes and triterpenes (betulinic and ursolic acid) and their synergistic action, which suggests that anticancer activity is manifested by a combination of two activities, cytotoxic and antioxidant. It has been shown that betulinic acid exert low toxicity towards the healthy cells and tissues, and at the same time its anti-proliferative activity has been confirmed in cancer cell cultures MGC-803, PC3, A549, MCF-7, NIH3T3, SGC-7901, HepG-2, LNCaP, DU- 145, SK-MEL-2, SK-OV-3, HCT15 and XF498. Betulinic acid and its analogues exert their anti-proliferative activity by apoptosis of cancer cells intrinsically by

acting on the permeability of the mitochondrial membrane, increasing the release of cytochrome c (Zhang *et al*., 2016; Soica *at al*., 2017). The triterpene fraction of *Rosmarini folium*, according to our phytochemical analysis (Jordamović *et al*., 2020), contains the largest amount of triterpenes of betulinic acid, betulin and oleanolic acid.

A slightly weaker anti-proliferative activity was shown by the triterpene fraction of *Lavandulae flos*, followed by the triterpene fraction of *Salviae folium*. In this case, these are triterpene fractions which, in addition to the triterpene fraction of *Rosmarini folium*, had the highest content of triterpenes. In these two triterpene fractions, ursolic acid is present in a larger amount, and it contributes to significant anti-proliferative activity. According to Banerjee et al. ursolic acid exerts its anti-proliferative effect via the Akt/mTOR and MAPK pathways leading to inhibition of cancer cell proliferation, reducing its size and inducing apoptosis of breast cancer cells. Furthermore, ursolic acid stops the growth of breast cancer cell cultures (MCF-7, MCF-7/ADR and MDA-MB-231) via mTOR, c-Jun N-terminal kinase (JNK) and Akt signaling pathway, inducing apoptosis via the mitochondrial pathway, extrinsic receptor pathway death and suppression of FoxM1 protein expression (Banerjee *et al*., 2019).

The results of *in vitro* anti-inflammatory activity of the tested triterpene fractions demonstrated a strong linearly dose-dependent activity. The strongest anti-inflammatory activity (80.69%) was shown by the triterpene fraction of *Rosmarini folium*, at a concentration of 250 μg/mL. The range of anti-inflammatory activity of the triterpene fraction of *Rosmarini folium* ranges from 57.27% at a concentration of 50 μg/mL to 80.69%, at the concentration mentioned above. This anti-inflammatory effect of the triterpene fraction of *Rosmarini folium* can be attributed to the highest content of triterpenes (Jordamović *et al*., 2020). The percentage inhibition of the triterpene fraction of *Melissae folium* range from 48.27% (50 μg/mL) to 71.54% (250 μg/mL). At a concentration of 50 μg/mL the triterpene fraction of *Melissae folium* has ~10% lower activity than the triterpene fraction of *Rosmarini folium*, while higher concentrations were approximately with similar activities. This is followed by, in terms of anti-inflammatory activity, triterpene fractions of *Salviae folium* and *Menthae piperitae folium*, which have almost identical activity. The range of activities ranged from 29.35% and 27.64% at a concentration of 50 μg/mL to activities of 54.2% and 54.46% at a concentration of 250 μg/mL, respectively.

Acetylsalicylic acid, a standard anti-inflammatory drug, showed a maximum inhibition of 82.42% at a concentration of 250 μg/mL compared to the control group. The anti-inflammatory drug has proven dose-dependent efficacy in the ability to thermally inhibit protein denaturation (Mizushima & Kobayashi, 1968). Similar results were observed in this study with a percentage of inhibition of 67.42%, 73.2%, 76.99%, 79.43% and 82.42% at concentrations of 50 μg/mL, 100 μg/mL, 150 μg/mL, 200 μg/mL and 250 μg/mL.

The standard solution of triterpenes, betulin, betulinic acid, ursolic acid, oleanolic acid and lupeol, showed strong anti-inflammatory activity, similar to the anti-inflammatory drug acetylsalicylic acid. Therefore, the presence of these bioactive compounds in the triterpene fractions of plant species of the *Lamiaceae* family contributes to their anti-inflammatory activity.

According to the results obtained in this study, extracts containing higher concentrations of betulinic acid and betulin, demonstrated a stronger anti-inflammatory activity.

The study by Abdulbary *et al*. (2018), of the alcoholic extract of the rosemary leaf also demonstrated anti-inflammatory activity, however they attributed this activity to glycosides, tannins, alkaloids and phenolic compounds, as they did not investigated triterpenes.

CONCLUSIONS

From the general perspective of elucidating *in vitro* anti-proliferative and anti-inflammatory activity of triterpene fractions of plant species belonging to the *Lamiaceae* family it should be noted promising results**.**

The present observations lead to the conclusion that triterpenes content in the tested plant extracts play a significant rool in anti-inflammatory and anti-proliferative activity. Phytochemical analysis of tested triterpene fractions, proved the presence of betulinic acid, betulin, oleanolic acid and ursolic acid, compounds which demonstrated anti-inflamatory and anti-proliferative activity that atribute new pleiotropic properties to the investigated triterpene fractions and opens new opportunities for

their use. Successive extraction with different solvents showed that the chloroform was the most suitable solvent for extraction of investigated triterpenes.

The best anti-inflamatory and anti-proliferative activity showed triterpene fractions from *Rosmarini folium* and *Lavandulae flos*, which were at the same time the richest in the triterpenes, indicating that activity is direct proportional to the content of teriterpenes.

These studies provide scientific evidence to support the traditional use of these *Lamiacae* plant species for medical purposes and point to promising potential for the development of new anti-inflammatory and anti-proliferative medicines.

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