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ORIGINAL ARTICLE

Antioxidative and antiproliferative effects of *Satureja montana* L. extracts

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Summary

Purpose: *To study in vitro the antioxidative effect of 6 Satureja montana L. extracts on free radicals and their antiproliferative effect on human tumor cell lines.*

Materials and methods: *The antioxidative effect of extracts on 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical was investigated by electron spin resonance (ESR) spectroscopy. Cell growth effect was measured by sulforhodamine B colorimetric assay on HeLa (human cervix epidermoid carcinoma), HT-29 (human colon adenocarcinoma), and MCF-7 (human breast adenocarcinoma) cell lines. IC_{50} values were calculated from the concentration response curves following 48 h exposure time.*

Results: *The antioxidative activity of extracts increased dose-dependently at mass concentrations ranging from 0.05 to 0.3 mg/ml, and decreased in the following order: n-butanol > methanol > water > ethyl acetate > petroleum ether. All extracts effected cell growth but in a different way, depending on the extract dose and cell line. Extracts exhibited antiproliferative effect on HeLa cell line with IC_{50} values ranging from 0.41 to 0.84 mg/ml except petroleum ether (IC_{50} >1 mg/ml). Petroleum ether and chloroform extracts stimulated proliferation of HeLa cells within a concentration range from 0.0625 to 0.125 mg/ml. No extract reduced MCF-7 cells growth by 50% even at the concentration of 1 mg/ml. Only petroleum ether and chloroform extracts induced significant growth inhibition of HT-29 cells (IC_{50} was approximately 0.74 mg/ml for both extracts). Strong stimulation of HT-29 proliferation was observed within a concentration range from 0.0625 to 0.25 mg/ml for petroleum ether, n-butanol and chloroform extract, and from 0.0625 to 0.5 mg/ml for methanol and water extracts, respectively.*

Conclusion: *The obtained results indicated that Satureja montana L. extracts are strong antioxidants in vitro. ESR data demonstrated that n-butanol, methanol and water Satureja montana L. extracts possess high antioxidative activity. Chloroform extract did not show any antioxidative activity. Satureja montana L. extracts selectively inhibited the growth of human tumor cells.*

Key words: antioxidative activity, antiproliferative activity, electron spin resonance, *Satureja montana* L., tumor cell lines

Introduction

Satureja montana L. is a hardy, woody perennial, native to Europe and North Africa with only limited use. The green leaves and herbaceous sections of stems from both species are used fresh and dried as flavoring agents in seasonings, stews, meat dishes, poultry, sausages, and vegetables. As a medicinal plant, it has been traditionally used as a stimulant, stomachic, carminative, expectorant, antidiarrheic and aphrodisiac. The essential oil has demonstrated antimicrobial and antidiarrheic activity because of the phenols in the oil. *Satureja montana* L. has been used in the treatment of cancer [1-3].

Numerous investigations have proved that medicinal herbs contain diverse classes of compounds such as polyphenols, tocopherols, alkaloids, tannins, carotenoids, terpenoids, etc. [4,5]. Among them, phenolic acids and flavonoids are particularly attractive as they

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are known to exhibit various beneficial pharmacological properties such as vasoprotective, anticarcinogenic, antineoplastic, antiviral, anti-inflammatory, as well as antiallergic and antiproliferative activity on tumor cells [1-3].

Our previous investigations showed that different *Satureja montana* L. extracts possess high total phenolic and flavonoid contents. We continued our investigation with the goal to define the antioxidative and antiproliferative activities of these extracts.

Materials and methods

Experimental material

Methanol, ethyl acetate, chloroform, *n*-butanol and petroleum ether were obtained from "Zorka", abac (Serbia). DPPH was purchased from Sigma Chemical Co. (USA). These chemicals were of analytical reagent grade. Other used chemicals and solvents were of the highest analytical grade.

Plant material

Satureja montana L. was collected in August 2002 from the region of Zlatibor. Voucher specimen of the collected plant was confirmed and deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Medicine, University of Novi Sad.

Preparation of extracts

Dried plant of *Satureja montana* L. (10 g) was extracted with 70% methanol (2X500 ml) at room temperature for 2X24 h. Part of obtained extract (20% v/v) was evaporated to dryness under reduced pressure and used further as methanol extract. The rest of the extract (80% v/v) was concentrated under reduced pressure. After removing methanol, the extract was successively treated with petroleum ether (2X20 ml), chloroform (2X20 ml), ethyl acetate (2X20 ml) and *n*-butanol (2X20 ml). The petroleum ether, chloroform, ethyl acetate, *n*-butanol and water extract were evaporated to dryness under reduced pressure. The yields of extracts were: methanol, m = 0.22±0.010 g; petroleum ether, m=0.05±0.0023 g; chloroform, m= 0.04±0.002 g; ethyl acetate, m=0.07±0.0031 g; *n*-butanol, m=0.19±0.009 g, and water, m = 1.49 ± 0.072 g.

DPPH radical assay

Blank probe was obtained by mixing 600 µl 0.4 mM methanolic solution of DPPH and 200 µl of methanol. A volume of X µl of 1% methanolic solution of the investigated extract was added to a mixture of (200 X) µl of methanol and 600 µl of 0.4 mM methanolic solution of DPPH radical (probe). The range of the investigated extract concentrations was 0.05-0.30 mg/ml. After that, the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT.

The ESR spectra were recorded on an ESR spectrometer Bruker 300E (Rheinstetten, Germany) under the following conditions: modulation frequency 100 kHz, modulation amplitude 0.256 G, receiver gain $2X10^4$, time constant 40.96 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23° C. The antioxidative activity (AA) value of the extract was defined as: $AA=100\cdot(h_o- h_x)/h_o$ [%], where h_o and h_x are the height of the second peak in the ESR spectrum of DPPH radicals of the blank and the probe, respectively.

Cell lines

The 3 human tumor cell lines used in the study were: HeLa (cervix epithelioid carcinoma), MCF-7 (breast adenocarcinoma, estrogen receptor-positive) and HT-29 (colon adenocarcinoma). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, UK) with 4.5% glucose, supplemented with 10% heat inactivated fetal calf serum (FCS, NIVNS, Serbia and Montenegro), and antibiotics: 100 IU/ml of penicillin and 100 mg/ml of streptomycin (ICN Galenika, Serbia and Montenegro). The cells were subcultured twice a week and a single cell suspension was obtained using 0.5% trypsin (Serva). All cell lines were cultured in 25 cm^2 flasks (Costar) at 37° C in atmosphere of 5% CO_2 and 100% humidity. Exponentially growing cells were used throughout the assay. The cell density (number of cells per unit volume) and the percentage of viable cells were defined as previously described [6].

Sulforhodamine B (SRB) assay

Cell growth was evaluated by colorimetric SRB assay according to Skehan et al. [7]. The SRB dye ($C_{27}H_{29}N_2O_7S_2Na$) binds to protein basic amino acid residues under mildly acidic conditions, providing sensitive index of cellular protein content. Tumor cells were harvested and plated into 96-well microtiter plates (Corning) at seeding density of 3 X 10^3 cells per well, in a volume of 180 ml, and preincubated in complete medium at 37° C for 24 h. Extracts were added to all

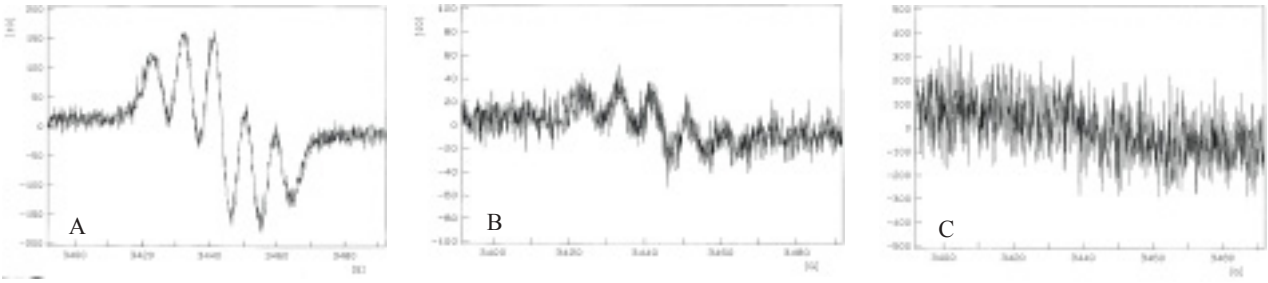

Figure 1. ESR spectra of the DPPH radicals: (A) in the absence of extracts (blank); (B) in the presence of 0.15 mg/ml of *n*-butanol extract; (C) in the presence of 0.3 mg/ml of *n*-butanol extract of *Satureja montana* L.

the wells (20 ml/well), except control, to achieve the required final concentrations (0.0625-1 mg/ml). Microplates were incubated at 37° C for 48 h in atmosphere of 5% CO_2 and 100% humidity. After incubation, the cells were fixed (50% TCA, 1h, +4° C), washed 5 times with tap water and dried in air. Cells were stained with 0.4% SRB (100 ml/ well) for 30 min at room temperature. Plates were washed 4 times with 1% acetic acid to remove unbound dye, and dried in air. Protein-bound dye was extracted with 10 mM TRIS base (200 ml/ well). Absorbance (A) was measured on a microplate reader (Multiscan Ascent, Labsystems) at 540/ 620 nm. Effect on cell growth was expressed as a percent of a control, calculated as: (At/Ac) X 100 [%], where At and Ac are the absorbance of the test sample and the control, respectively.

IC_{50} values defined as the dose of compound that inhibits 50% of cell growth were interpolated from concentration response curves following 72 h exposure time.

Statistical analysis

Data were expressed as mean ± standard deviation of quadruplicate and evaluated using two-tailed Student's t- test. The level of statistical significance was 95% ($p < 0.05$).

Results

DPPH radical antioxidative activity of extracts

The ESR spectra of DPPH radicals in the blank and in probes were characterized by their 5 lines of relative intensities 1:2:3:2:1 and hyperfine splitting constant $a_N = 9.03$ G (Figure 1).

Generally, the AA of methanol, ethyl acetate, *n*-butanol and water extracts increased dose-dependently at mass concentrations ranging from 0.05 to 0.30 mg/ ml (Figure 2). The 3 extracts, *n*-butanol, methanol and

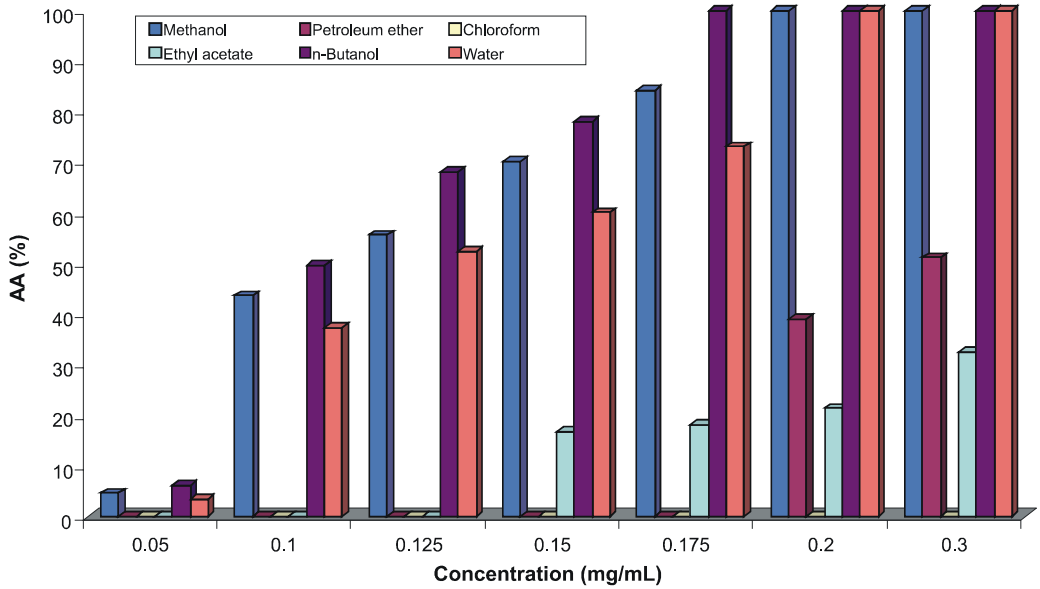

Figure 2. The influence of different concentrations of *Satureja montana* L. extracts on DPPH radicals.

water, exerted excellent antioxidative effect on DPPH radical (at the concentrations 0.20 and 0.30 mg/ml, AA=100%). Ethyl acetate extract present at concentrations range 0.05 - 0.15 mg/ml did not exert any antioxidative effect, while higher concentrations (0.15 - 0.3 mg/ml) of this extract showed better antioxidative activity (AA=16.88 - 32.5%). Also, petroleum ether extract possessed antioxidative activity only at the higher investigated concentrations 0.20 and 0.30 mg/ml (AA = 39.06% and 51%, respectively). Chloroform extracts did not show any antioxidative activity.

Antiproliferative activity on human tumor cell lines

All extracts effected cell growth but in a different way, depending on the extract dose and cell line

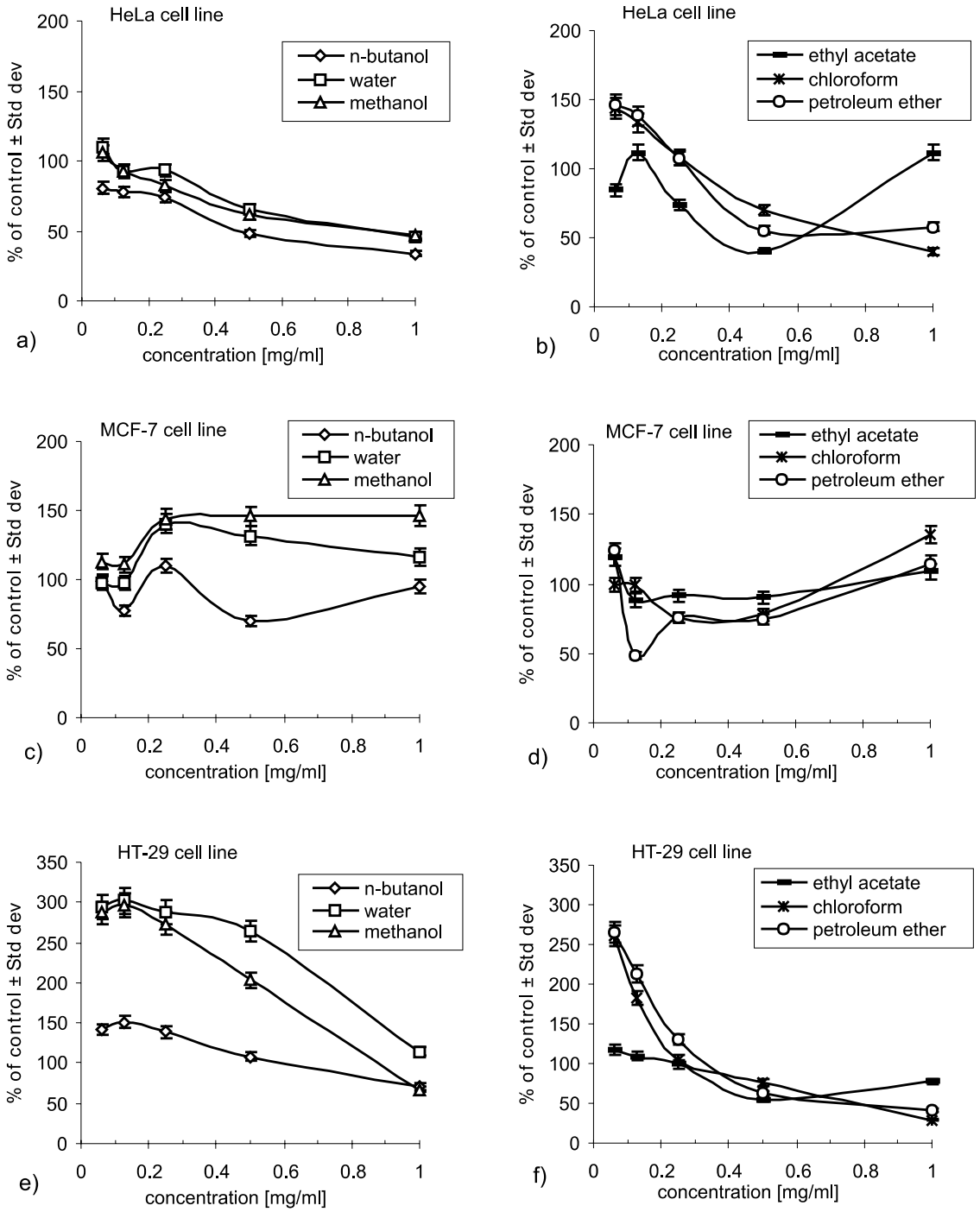

Figure 3. Growth response of HeLa (a, b), MCF-7 (c, d), and HT-29 cells (e, f) to *Satureja montana* L. extracts. Cells were incubated with various concentrations of extracts for 48 h. Growth inhibition was measured by SRB assay. Data are the means ± SD of quadruplicate.

Table 1. IC50 values of 6 *Satureja montana* L. extracts

Cell line	*IC50 [mg/ml]*					
	n-butanol	*methanol*	*water*	*ethyl acetate*	*chloroform*	*petroleum ether*
HeLa	0.46	0.83	0.84	0.41	0.78	>1
HT-29	>1	>1	>1	>1	0.74	0.74
MCF-7	>1	>1	>1	>1	>1	>1

Cells were exposed to *Satureja montana* L. extracts (concentration range from 0.0625 to 1 mg/ml) for 48 hours

(Figure 3). Extracts exhibited antiproliferative effect on HeLa cell line with IC_{50} values ranging from 0.41 to 0.84 mg/ml, excluding petroleum ether with IC_{50} >1 mg/ml (Table 1; Figure 3a-b; Figure 4a-c). Petroleum ether and chloroform extracts significantly stimulated proliferation of HeLa cells within a concentration range from 0.0625 ($p < 0.01$) to 0.125 mg/ml ($p < 0.05$) (Figure 3b). No extract inhibited the growth of MCF-7 cells even at the highest concentration (1 mg/ml) (Figure 3c-d; Figure 4g-i). Only petroleum ether and chloroform extracts induced significant growth inhibition of HT-29 cells (IC_{50} was approximately 0.74 mg/ml for both extracts; $p < 0.05$) (Table1; Figure 3f; Figure 4f). Strong stimulation of HT-29 proliferation was observed within a concentration range from 0.0625 to 0.125 mg/ml for petroleum ether ($p < 0.05$), *n*-butanol and chloroform extract ($p < 0.05$), and from 0.0625 to 0.5 mg/ml for methanol ($p < 0.01$) and water extracts ($p < 0.01$),

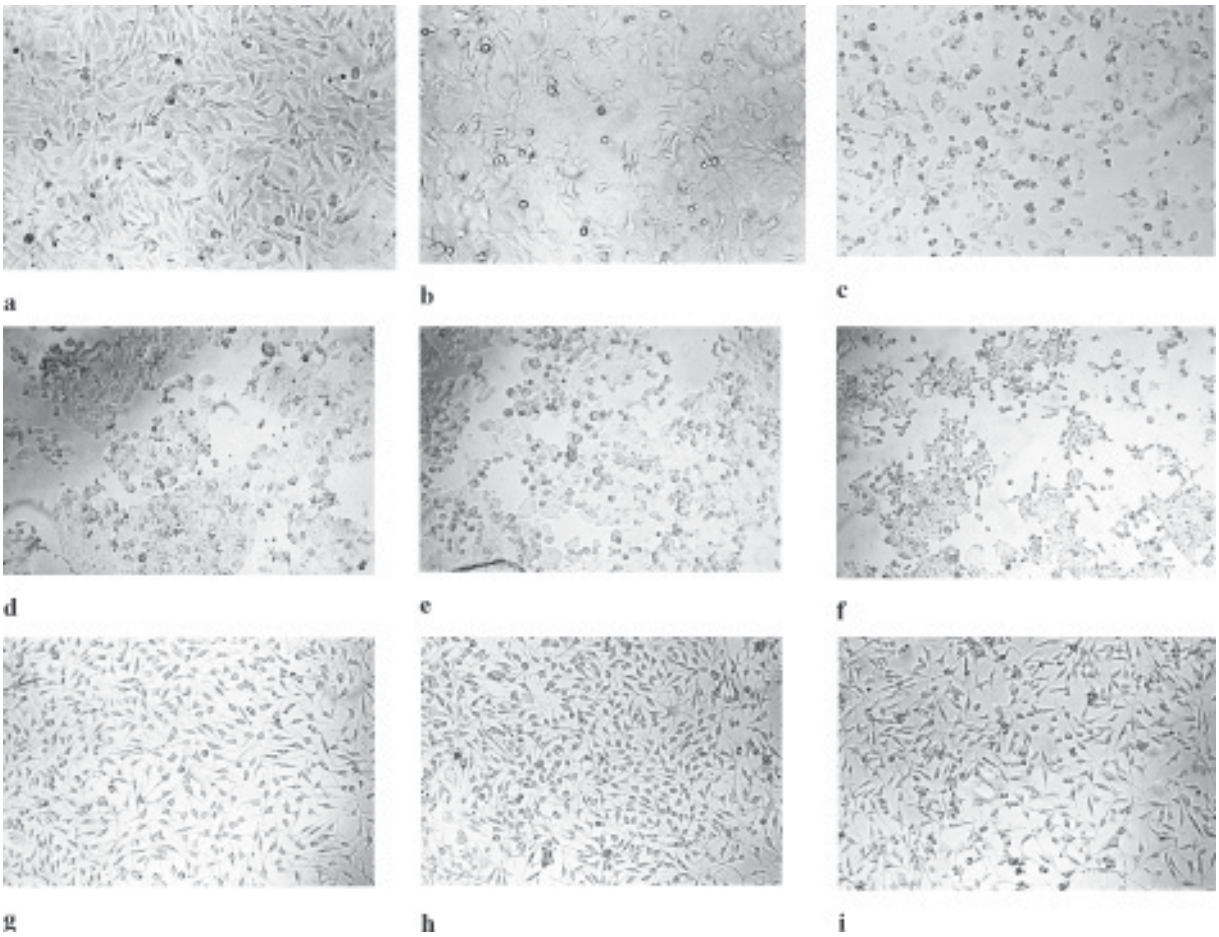

Figure 4. Antiproliferative effect of *Satureja Montana L.* extracts after 48 h of exposure: HeLa cell line (a: control; b: petroleum ether, 0.25mg/ml; c: ethyl acetate, 0.25mg/ml); HT-29 cell line (d: control; e: methanol, 0.25 mg/ml; f: petroleum ether, 0.25 mg/ml); MCF-7 cell line (g: control; h: *n*-butanol, 0.25mg/ml; i: methanol, 0.25mg/ ml). Reichert-Jung inverted microscope. Magnification 10X10.

respectively (Figure 3e-f; Figure 4e). Antiproliferative activity of extracts based on IC_{50} values decreased in the following order: ethyl acetate> *n*-butanol> chloroform> petroleum ether> methanol> water.

Discussion

In this study we demonstrated for the first time the growth inhibition activity of *Satureja montana* L. extracts against human cervix epidermoid HeLa and human colon adenocarcinoma HT-29 tumor cells, and their growth stimulation activity on human breast adenocarcinoma MCF-7 cell line. The AA of extracts, documented with DPPH assay, was most prominent by *n*-butanol extract that, also, strongly inhibited the growth of HeLa cells.

Free radicals, which are formed by exogenous chemicals or endogenous metabolic processes in the human body or in food, are capable of oxidizing biomolecules, resulting in cell death and tissue damage [8,9]. Natural phytochemicals such as phenolic compounds found in numerous herbs, commonly involve an aromatic ring as part of the molecular structure, with one or more hydroxyl groups.

Our previous investigations showed that different *Satureja montana* L. extracts possess high total phenolic and flavonoid contents. Hydroxyl derivatives of benzoic acid (protocatechuic and vanillic), cinnamic acid (caffeic, coumaric, ferulic and syringic) and flavonoids (catechin and epicatechin) were identified by HPLC (unpublished data). The AA of extracts can therefore be attributed to the hydrogen-donating ability and direct scavenging activity of the active constituents [10,11]. The antioxidative efficiency based on this mechanism is typical for different phenolic acids and flavonoids whose presence has been proved in *Satureja montana* L. The DPPH AA of extracts increased dose-dependently at mass concentrations ranging from 0.05 to 0.30 mg/ml. The AA of extracts was changed in the following order: *n*-butanol > methanol > water > ethyl acetate > petroleum ether.

Antiproliferative and antioxidative effects of *Satureja montana* L. extracts were achieved using extracts at a range of mass concentrations. However, AA was achieved by concentration range that mostly induced either small growth inhibition or growth stimulation of cells.

The growth inhibition activity of *Satureja montana* L. extracts was evaluated on 3 histologically different human cancer cell lines. Among them, HeLa cells were found to be the most sensitive to all extracts except petroleum ether extract. The growth of human adenocarcinoma HT-29 cells was inhibited by extracts only at concentrations above 0.7 mg/ml. Cell growth stimulation of HeLa and HT-29 cells at concentrations below 0.4 mg/ml is in accordance with reported biphasic effect of plant extracts [12,13].

MCF-7 cells responded by growth stimulation to all extracts at almost the whole range of extract concentrations. No extract reduced MCF-7 cells growth by 50% even at the concentration of 1 mg/ml. As the MCF-7 cells are estrogen receptor-positive, it could be proposed that, at least, some extracts might contain phytoestrogens or growth-stimulating factors. It is known that genistein, a natural isoflavone phytoestrogen, stimulates the growth of estrogen-dependent MCF-7 cells at low concentrations, but higher concentrations have opposite effect [12,13]. The precise composition of *Satureja montana* L. extracts has not been available so far. Therefore, explanation of the possible mechanism of cell growth stimulation through estrogen receptors could not be reliably neither proved nor excluded.

Low level of free oxygen species is necessary for promoting the cell proliferation, and the redox alterations play a significant role in a signal transduction pathway important for cell growth regulation [14,15]. Therefore, it is reasonable to propose that *Satureja montana* L. extracts, as strong antioxidants, might influence cell redox state leading to decreased cell proliferation.

In conclusion, the obtained results indicate that *Satureja montana* L. extracts are strong antioxidants *in vitro* and that they can selectively inhibit the growth of human tumor cells. However, the antiproliferative as well as growth stimulation mechanisms of the extracts require further targeted investigations.

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References

1. Kuhnau J. The flavonoids. A class of semi-essential food components: Their role in human nutrition. Wld Rev Nutr Diet 1976; 24: 117-191.
2. Middleton E, Kandaswami C. Effects of flavonoids on immune and inflammatory functions. Biochem Pharmacol 1992; 43: 1167-1179.
3. Carr AC, Zhu BZ, Frei B. Potential antiatherogenic mechanism of ascorbate (Vitamin C) and alpha-tocopherol (Vitamin E). Circ Res 2000; 87: 349-354.

4. Yoshida T, Mori K, Hatano T et al. Studies on inhibition mechanism of autoxidation by tannins and flavonoids. Radical-scavenging effects of tannins and related polyphenols on DPPH radical. Chem Pharm Bull 1989; 37: 1919-1921.
5. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity in fruits and total phenolic in selected fruits, vegetables, and grain products. J Sci Food Agric 1998; 46: 4113-4117.
6. Bogdanović G, Raletić-Savić J, Marković N. In vitro assays for antitumor-drug screening on human tumor cell lines: Dye exclusion test and colorimetric cytotoxicity assays. Archive Oncol 1994; 2:181-184.
7. Skehan P, Storeng R, Scudiero D et al. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 1990; 82: 1107-1122.
8. Halliwell B, Gutteridge JM (eds). Free radicals in biology and medicine. Oxford University Press: Oxford, 1999, pp 22-81.
9. Milić BLj, Djilas SM, Čanadanović-Brunet JM, Milić NB. Radicali liberi in biologia, medicina e nutrizione. In: Capasso F, De Pasquale R, Grandolini G, Mascolo N (eds): Farmacognosia. Springer: Berlin, 2000, pp 449-462.
10. Djilas SM, Canadanovic-Brunet J, Cetkovic GS, Tumbas VT. Antioxidative activity of some herbs and spices- review of ESR studies. In: Belton PS, Gill AM, Webb GA, Rutledge D (eds): Magnetic Resonance in Food Science. The Royal Society of Chemistry, Cambridge, UK, 2003, pp 110-120.
11. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. Lebensm-Wiss U-Technol 1995; 28: 25-30.
12. Fioravanti L, Cappalletti V, Miodini P, Ronchi E, Brivio M, Di Fronzo G. Genistein in the control of breast cancer cell growth: insights into the mechanism of action in vitro. Cancer Lett 1998; 130: 143-152.
13. Constantinou AI, Kamath N, Murley JS. Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. Eur J Cancer 1998; 34: 1927-1934.
14. Burdon RH, Gill V. Cellularly generated active oxygen species and HeLa cell proliferation. Free Radical Res Commun 1993; 19: 203-213.
15. Wei YH, Lee HC. Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. Exper Biol Med (Maywood) 2002; 227:671-682.