A comparative study on the biological activity of essential oil and total hydro-alcoholic extract of *Satureja hortensis* L.

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Abstract. *Satureja hortensis* L. presents an increased interest due to its chemical composition, abundant in monoterpenes, aglyconic and glycosylates flavonoids, and phenolic acids, leading to important biological activity. The present study compared the biological activity of volatile oil (VO) and total hydro-alcoholic extract (TE) of *Satureja hortensis* L. in terms of: i) antioxidant activity; ii) antimicrobial activity; and iii) viability, migration and proliferation on two healthy cell lines (keratinocytes-HaCaT and fibroblasts-1BR3) and two melanoma cell lines (human-A375 and murine-B164A5). Antioxidant activity of VO and TE showed maximal values around 72%. Antimicrobial screening highlighted the inhibitory capacity of VO against all seven tested bacteria strains, with the most pronounced effect against *S. aureus* and *C. albicans*, while TE exerted only a slight activity against three bacteria strains. VO showed greater efficacy than TE on both tumor cell lines (A375 and B164A5), the activity of the compounds was higher when low concentrations were used (5, 10 and 25 μM) while at high concentrations (50 and 100 μM) the percentages of viability were increased.

Introduction

Satureja hortensis L. is a plant known and used as remedy for more than 20 centuries. The name was assigned by the Roman writer Pliny, being derived from the word 'satyr' (the Latin name satureia) which describes a creature from ancient mythology (half man, half goat) and the legend says that savories belonged to him (1-3). This perennial plant belongs to an important family of aromatic and medicinal herbs, Lamiaceae that includes more than 200 genera widespread in Europe (the southern and eastern regions), Asia (the western region), South America and some Spanish islands (3,4). *Satureja hortensis* L., known as summer savory, is native of Europe, especially from the Balkan regions and its leaves and stems are currently utilized as tea, spice or flavoring agent (2).

The increased interest towards this plant is due to its chemical composition which affords important biological activity. The main classes of compounds identified, are: volatile compounds (carvacrol, thymol, *o*-cymene, (+)-*4*-carene, *cis*-terpinene, citronellol, geraniol, limonene, linalool, myrcene, *p*-cymene, α-pinene and γ-terpinene), phenolic acids (rosmarinic acid, caffeic acid and gallic acid), flavonoids and associated compounds (apigenin, quercetin, naringenin, and their glycosides) and other compounds (e.g., enzymes) (3,4). The main structures are presented in Fig. 1.

Satureja hortensis L. was used in folk medicine to treat various disorders, such as cramps, muscle pains, stomach, intestinal, and infectious diseases (3,5). The modern techniques applied to study the effects of biologically active substances from its composition revealed a plethora of beneficial activities, such as antimicrobial, antioxidant, cytotoxic, insecticidal, fumigant toxicity, insect repellant, antinociceptive/analgesic, antileishmanial, anti-inflammatory, antidiarrheal, antispasmodic, matrix metalloproteinase inhibitory activity, inhibition on blood platelet adhesion, aggregation and secretion, effect on immune system and on rhinosinusitis (3). Taking into account the great interest for the treatment and the prophylaxis of different pathologies, the plant material should be meticulously selected from verified and certified sources due to the fact that along with the bioactive compounds, it can also contain toxic compounds (e.g., heavy metals) (6).

Skin cancers are malignancies most often developed by people with lighter skin and the incidence is steadily increasing, especially due to uncontrolled exposure to ultraviolet radia-

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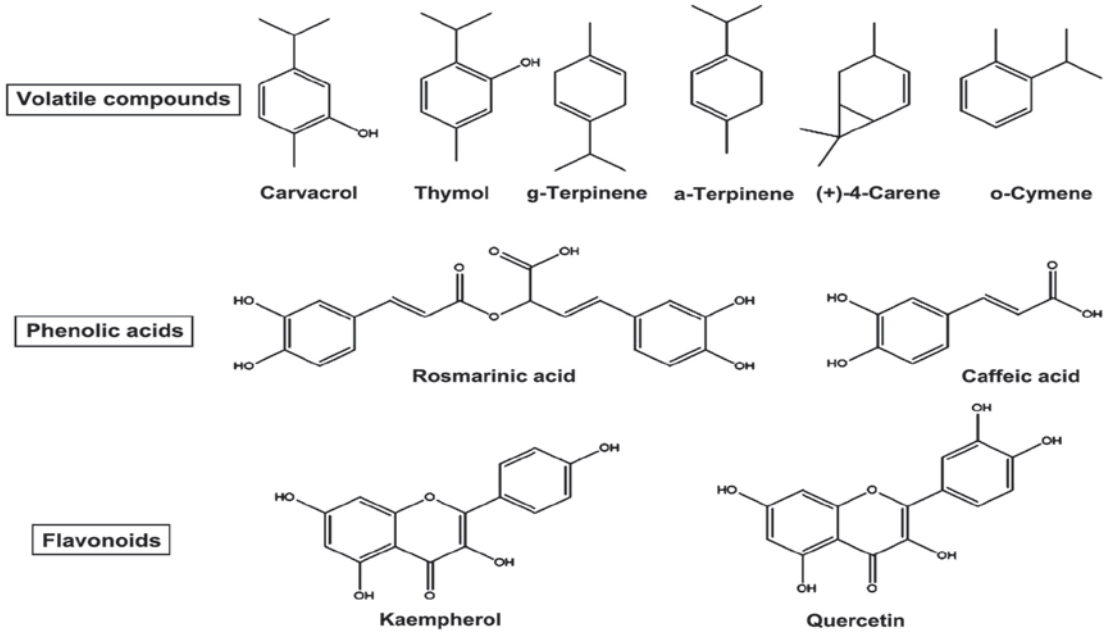

Figure 1. Representative examples of the main classes of compounds detected in *Satureja hortensis* L.

tion, recognized carcinogens (7). Skin cancers are divided into two major classes: non-melanoma cancers, the most common types diagnosed and melanoma cancers, the most aggressive with the lowest life expectancy after diagnosis (8,9).

The present study was aimed to characterize the biological activity of essential/volatile oil (VO) in comparison with the total hydro-alcoholic extract (TE) in terms of *in vitro* experiments, namely: antioxidant activity (AOA), antibacterial and antifungal activity, and cytotoxicity on two normal cell lines (HaCaT, immortalized human keratinocytes; 1BR3, human skin fibroblasts) and two melanoma cell lines (A375, human melanoma; B16 melanoma 4A5, mouse melanoma) (Fig. 2).

Materials and methods

Plant material and reagents. Satureja hortensis L. (summer savory) from spontaneous flora was collected in Timis County (western region of Romania) during growing season of the year 2017. Botanical identification of the plant was realized by Professor Diana Antal, at the Department of Pharmaceutical Botany, Faculty of Pharmacy, 'Victor Babes' University of Medicine and Pharmacy (Timisoara, Romania) and a voucher specimen (no. CD_004) is deposited at the Herbarium of the Faculty. The plants were harvested at the time when the volatile oil content was at the maximum percentage regarding the volatile compounds of interest, namely at full flowering stage, and were dried in oven at 42˚C. Before processing, the plant material was crushed using an analytical laboratory mill (A 11 basic Analytical Mill; IKA Werke, Staufen, Germany). All standard compounds, reagents and solvents used for LC-MS analysis were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The reagents used for antioxidant activity (AOA) assessment were ethanol 96% (v/v) from Chemical Company S.A. (Iasi, Romania), 2,2-diphenyl-1-picrylhydrazyl (DPPH) from Sigma Aldrich; Merck KGaA and ascorbic acid from Lach-Ner, Ltd. (Neratovice, Czech Republic).

Extraction procedures. Plant material (aerial parts, 250 g) carefully selected and dried was subjected to hydro distillation method for three hours, using an all glass Clevenger-type apparatus according to the well-known methods described in the literature (10). The final product obtained was dried and stored at -20˚C until further analysis.

In order to obtain summer savory total TE Soxhlet extraction method was employed. Aerial parts (50 g), crushed and homogenized, were placed in the Soxhlet apparatus (Solvent Extractor™, SER 148 Series; Velp Scientifica, Usmate, Italy) and 400 ml of 70% EtOH were used to sequentially extraction for 48 h. The final extract was filtered through filter paper, the solvent was removed by a rotary evaporator (Heidolph Hei-VAP Advantage Rotary Evaporator package) under vacuum, the pellet being lyophilized and stored in a dark glass tube at -20˚C until further analysis.

Chemical composition of VO and TE.
GC-MS analysis. The chemical characterization of essential oil was realized by using a gas-chromatograph equipment with mass spectrometer (GS/MS)-QP2010 Plus (Shimadzu, Tokyo, Japan) with a capillary column with the characteristics: DB-WAX, 30 m length x 0.32 mm x 1 μm. The carrier gas used was helium with a flow rate of 1 ml/min. The program used for the compounds separation was: start at 40˚C and increased with a rate of 5˚C/min to 250˚C and hold for 5 min. Injector and ion source temperatures were 250 and 220˚C, respectively. The injection volume was 1 μl at a split ratio of 1:50. The NIST 02 database (webbook.nist.gov), integrated in the device software was used to identify volatile compounds. The percentages of individual components were calculated based on GC peak areas without using correction factors. The

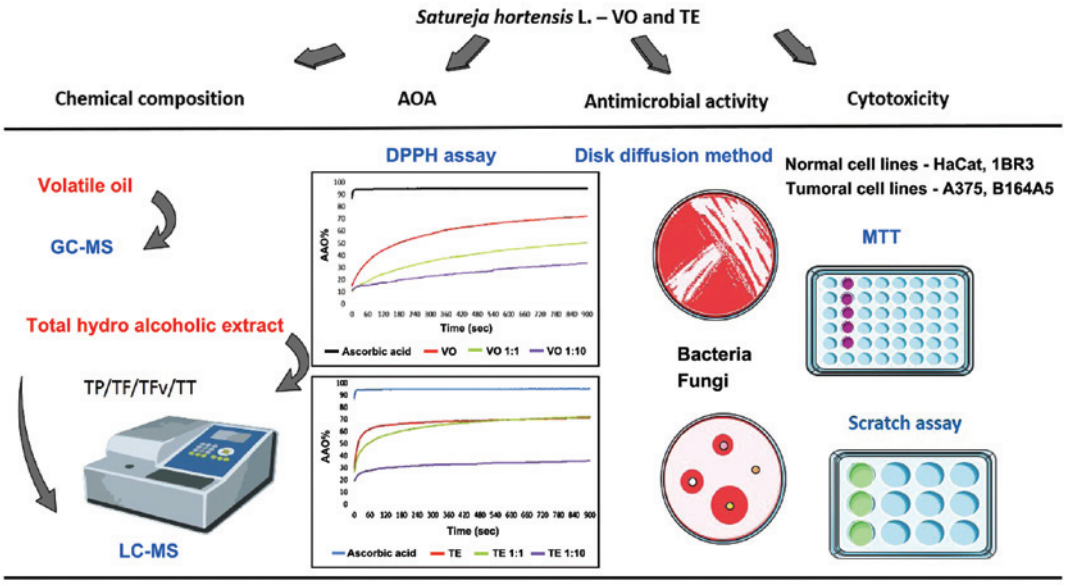

Figure 2. Schematic overview of the techniques applied to assess the biological activity of *Satureja hortensis* L. essential/VO in comparison with the TE. VO, volatile oil; TE, total hydro-alcoholic extract; AOA, antioxidant activity; TP, total polyphenols, TF, total flavonoids, TFv, total flavonols; TT, total codensed tannins; HaCaT, human immortalized keratinocytes; 1BR3, skin normal fibroblast; A375, human melanoma; B164A5, mouse melanoma.

linear retention indices (LRI) were determined under the same operating conditions in relation to a homologous series of n-alkanes (C8-C24) according to Van den Dool and Kratz (11):

$$LRI = 100 \times n + \frac{100 \times (t_x - t_n)}{t_{n+1} - t_n}$$

where t_n and t_{n+1} are the retention times of the reference n-alkane hydrocarbons eluting immediately before and after chemical compound 'X' and t_x is the retention time of compound 'X'.

Total polyphenols, flavonoids, flavonols, and tannins. The total phenols (TP) evaluation was realized using Folin-Ciocalteu method as described in the literature (12). Samples (0.5 ml) were treated with 1.25 ml Folin-Ciocalteu reagent (Sigma-Aldrich; Merck KGaA) diluted 1:10 with water and incubated for 5 min at room temperature. After the addition of 1 ml sodium carbonate 60 g/l the samples were heated for 30 min at 50˚C and then the sample absorbance was measured at 750 nm using an UV-VIS spectrophotometer (Specord 205; Analytik Jena AG, Jena, Germany). The calibration curve was obtained using gallic acid (GA) (Sigma-Aldrich; Merck KGaA) as positive control, in concentration of 0.03-1 mg/ml. The results were expressed in mg GAE/g dry material (DM).

Flavonoids/flavonols (TF/TFv) content was evaluated by using classical test (Al colorimetric analysis) according to method described in the literature, dilution 1:1, at room temperature (13). Briefly, for flavonoid content 500 μl of extract was treated with 500 μl $AlCl_3$ solution 2% (incubation for 30 min) and absorbance values measured at 417 nm. For flavonols content, same volume of extract was mixed with $AlCl_3$ (2%) and CH_3COONa (5%) (incubation for 3 h) and absorbance values measured at 445 nm. An Secomam UviLine 9400 Spectrophotometer (Kisker Biotech GmbH and Co., KG, Steinfurt, Germany) was used for all determinations and rutin was utilized as reference standard while the data are expressed as rutin equivalents (REs).

The total condensed tannin (TT) was determined by the vanillin test (14) slightly modified: Extract (500 μl) was treated for several minutes, in an ice bath, with 1.5 ml vanillin solution (1% in dilute sulphuric acid) (incubation for 15 min) and the absorbance was read at 500 nm on UviLine 9400 Spectrophotometer. The TT was expressed as milligrams of (+)-catechin equivalents (mg CE/g extract). All experiments were performed in triplicate.

LC-MS analysis. Quantification of individual phenolic compounds was performed using a Shimadzu chromatograph equipped with SPD-10A UV and LC-MS 2010 detectors (Schimadzu, Tokyo, Japan), and EC 150/2 Nucleodur C18 Gravity SB (MACHEREY-NAGEL GmbH & Co., KG, Düren, Germany), 150 x 2 mm x 5 μm column. Chromatographic conditions were as follows: mobile phase A, water acidified with formic acid at pH 3.0. Mobile phase B, acetonitrile acidified with formic acid at pH 3.0, gradient program: 0.01-20 min, 5% phase B; 20.01-50 min, 5-40% phase B; 5-55 min, 40-95% phase B; and 55-60 min, 95% phase B. Solvent flow rate of 0.2 ml/min, temperature at 20˚C. The monitoring wavelengths were 280 and 320 nm. The calibration curves were performed in the range of 20-50 μg/ml. The limit of quantification (LOQ), representing the lowest concentration for which S/N ≥5, was 0.3 μg/ml. Determinations were performed in duplicate. All reagents and solvents used were analytical grade chemicals. Standards were purchased from Sigma-Aldrich; Merck KGaA.

Antioxidant activity. The DPPH assay was applied to estimate the radical-scavenging ability of the tested samples. Briefly, 500 μl of test sample was diluted with 2 ml hydro-alcohol mixture (ethanol 50%) and 500 μl of DPPH 1 mM was added. The absorbance was continuously measured at 516 nm with a T70 UV/VIS Spectrophotometer (PG Instruments Ltd., Leicestershire, UK) for 900 sec to observe the changes in the values of AOA. The antioxidant activity recorded was

compared in each case to that of ascorbic acid, used as positive control. The percent of AOA activity (%) of each sample was calculated according to the formula used in our previous studies (13,15).

Antimicrobial activity

Disc diffusion assay. Extracts of *Satureja hortensis* L. were tested for antimicrobial activity against *Staphylococcus aureus* (ATCC 25923™), *Bacillus cereus* (ATCC 8035™), *Escherichia coli* (ATCC 25922™), *Pseudomonas aeruginosa* (ATCC 27853™), *Shigella flexneri* (ATCC 12022™), *Salmonella typhimurium* (ATCC 14028™), *Streptococcus pyogenes* (ATCC 19615™) and for antifungal activity against *Candida albicans* (ATCC 10231™) and *Candida parapsilosis* (ATCC 22019™) (all from American Type Culture Collection, Manassas, VA, USA) using the Disk diffusion method for susceptibility testing, according to the Standard Rules for Antimicrobial Susceptibility Testing using Impregnated Disks.

In vitro testing was performed in plates, and microtablets with Gentamicin (for the antimicrobian activity) and Nystatin (for the antifungal activity) were used as positive controls. Commercial Gentamicin discs (10 mg, ref. E110712; BioMaxima, Lublin, Poland) and Nystatin (100 mg - ref. SD 025; Himedia, Mumbai, Maharashtra, India), alongside filter papers impregnated with a water:ethanol mixture (as negative controls) and filter papers impregnated with a known quantity of samples were assessed. A 10^{-2} dilution of the fresh fungi cultures and a 10^{-3} fresh bacteria cultures were used to perform the assay, an inoculum equivalent to a 0.5 McFarland standard. The Petri plates, prepared by a method previously described (16,17) were seeded and the respective specimens were treated with the VO (10 μl/disk) and TE (250 μg/disk), respectively and were incubated at 30˚C for fungi and 37˚C in case of bacteria, for 24-48 h. Tests were performed in triplicate.

In vitro cytotoxicity

Cell lines and specific reagents. The cell lines used in this study: HaCaT, immortalized human keratinocytes (cat. no. 300493; CLS Cell Lines Service GmbH, Eppelheim, Germany), 1BR3, human skin fibroblasts (cat. no. 90011801; European Collection of Authenticated Cell Cultures, Salisbury, UK), A375, human melanoma cells (ATCC® CRL-1619™), and B16 melanoma 4A5 cell line from mouse (ECACC; cat. no. 94042254) were acquired as frozen items and stored in liquid nitrogen until the experiment began. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The reagents used for cell culture: Dulbecco's modified Eagle's medium (DMEM) was provided from ATTC and trypsin/EDTA solution, phosphate-buffered saline (PBS), penicillin/streptomycin mixture, fetal calf serum (FCS) and Trypan blue solution were supplied by Sigma-Aldrich; Merck KGaA.

Cell culture. Viability and migration assay. The impact of the samples on cell viability was tested on all four cell lines: HaCaT, 1BR3, human (A375) and murine (B164A5) melanoma cells. HaCaT, A375 and B164A5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS and antibiotic mixture (100 U/ml penicillin and 100 μg/ml streptomycin) and for 1BR3 cells was used as culture medium the Eagle's Minimum Essential Medium (EMEM) supplemented with 15% FBS. The culture plates were incubated at 37˚C in a humidified atmosphere with 5% CO_2, and passaged every day. Cell counting was performed with Countess™ II Automated Cell Counter (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in Trypan blue presence.

MTT assay is a colorimetric assay which was performed for cell viability evaluation. The viability percentage is directly proportional with the mitochondrial reduction of viable cells which will convert MTT to formazan via succinic dehydrogenase activity. Briefly, 10^4 cells/well were plated onto a 96-well plate in 200 μl media and incubated until 90% confluence was reached. The cells were stimulated with 100 μl media containing 5, 10, 25, 50, and 100 μM (for VO based on the molecular weight of γ-terpinene and for TE based on the molecular weight of rosmarinic acid) of test samples. A volume of 10 μl MTT/well was added at 24 h post stimulation and the mitochondrial reduction of the tetrazolium salt (MTT) to formazan was determined after a 4 h contact time. The concentration of formazan was measured at 570 nm wavelength, via spectrophotometry with a microplate reader (xMark™ Microplate; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The migratory character of the tumor cells used in this study was examined by applying scratch assay technique. In brief, $2x10^5$ cells/well were seeded in 12-well plates in specific culture medium and when the confluence was appropriate (85-90%) a gap was drawn in the middle of the well with a 10 μl tip. The capacity of the cells to migrate and fill the

Table I. Chemical constituents of *Satureja hortensis* L. volatile oil (VO).

Compounds	LRI reported in literature (18)	LRI	Concentration (%)
α-Thujene	1022-1027	1024	3.695
β-Pinene	1105-1108	1107	1.374
β-Myrcene	1160	1159	3.931
(+)-*4*-Carene	1149-1157	1148	6.086
D-Limonene	1196-1199	1198	0.558
β-Phellandrene	1195-1212	1207	0.361
γ-Terpinene	1243	1245	37.862
o-Cymene	1268	1267	15.113
α-Thujone	1433-1438	1437	0.546
Camphor	1490-1518	1511	0.521
β-Caryophyllene	1597-1618	1602	1.496
Bicyclo[5.1.0]octane, *8*-(*1*-methylethylidene)	-	1698	0.274
Anethole	1817-1819	1817	0.420
Octanoic acid	2056-2084	2067	0.304
Thymol	2162-2169	2168	13.491
Isothymol	2179-2225	2183	0.645
Carvacrol	2189	2188	13.225
Total			99.902

Table II. Total content of different bioactive classes from *Satureja hortensis* L. total hydro alcoholic extract (TE) determined by spectrophotometric method.

Sample	Extraction yield (%)	TP (mg GAE/g DM)	TF (mg RE/g DM)	TFv (mg RE/g DM)	TT (mg CE/g DM)
TE	28.4	164.75±2.47	24.04±1.26	6.65±0.41	16.23±0.94

gap was monitored for 24 h by acquiring images at different time-points, namely 0, 3 and 24 h using an Optika Microscopes Optikam Pro Cool 5 and Optika View (Optika, Ponteranica, Italy).

Statistical analysis. The GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA) was employed for the description and performance of the data. One-way ANOVA followed by Tukey's multiple comparisons test was used to determine the statistical differences between the various experimental and control groups ($P<0.05$, $P<0.01$, and $P<0.0001$). The results were expressed as the mean ± standard deviation (SD).

Results

Chemical composition of VO and TE. The chemical composition of Satureja *hortensis* L. VO is presented in Table I. A number of 18 compounds were identified in total, and the main compounds were: γ-terpinene (37.862%), *o*-cymene (15.113%), thymol (13.491%), carvacrol (13.225%), (+)-*4*-carene (6.086%), β-myrcene (3.931%), α-thujene (3.695%), β-caryophyllene (1.496%), β-pinene (1.374%), isothymol (0.645%), D-limonene (0.558%), α-thujone (0.546%) and camphor (0.521%).

The resulting hydro alcoholic extract of summer savory was evaluated for total phenols, flavonoids, flavonols, and condensed tannins contents with the help of spectrophotometric methods and the results are presented in the Table II.

Individual quantitative analysis of polyphenols, revealed the chemical composition found in the total hydro alcoholic extract of *Satureja hortensis* L., quercetin and kaempherol being the major compounds. Moreover, a number of phenolic acids (caffeic, gallic, rosmarinic, coumaric, ferullic, and protocatechuic), stilbenoid (resveratrol), flavonoid aglycones (epicatechin), and glycosides (rutin) were identified in the extract. The identity of the compounds was certified by LC-MS by comparison with standards. The polyphenols identified in composition of summer savory TE are presented in Table III.

Table III. The main polyphenols of *Satureja hortensis* L. total extract (TE).

Compounds	Retention time	m/z	Concentration (μg/g)
Gallic acid	4.8	169	28.39
Protocatechuic acid	10.8	153	8.3
Caffeic acid	21.9	179	29.12
Epicathechin	22.7	289	171.65
Coumaric acid	24.4	163	4.15
Ferullic acid	24.7	193	29.14
Rutin	25.7	609	187.45
Rosmarinic acid	28.8	359	121.05
Resveratrol	31.9	227	44.00
Quercetin	32.1	301	480.04
Kaempherol	34.9	285	3518.99

Antioxidant activity. The Fig. 3 presents the AOA of the summer savory VO and TE which proved to possess a high activity compared to the one of positive control used, ascorbic acid. For the evaluation, three samples from each type were analyzed: VO crude sample, VO 1:1 dilution and VO 1:10 dilution and TE crude sample, TE 1:1 dilution and TE 1:10 dilution, respectively. VO samples showed a steady increase throughout the entire time period. Thus, the AOA values after the first 300 sec were: VO, 57.35%; VO 1:1, 34.98%; and VO 1:10, 22.83% while at the end of period (900 sec) the values were: VO, 72.12%; VO 1:1, 50.37%; and VO 1:10, 33.73%. In the case of TE samples, data recorded revealed AOA as follows: after the first 300 sec TE, 67.86%; TE 1:1, 64.56%; and TE 1:10, 32.42% while at the end of period assessed (900 sec) TE, 71.38%; TE 1:1, 72.19%; and TE 1:10, 35.77%. The graphs prove that TE and TE 1:1 possesses the highest increase, especially in the first seconds followed by VO.

Antimicrobial activity. In Table IV are presented the data obtained after the antimicrobial activity evaluation. The results showed that VO, used in concentration of 10 μl/disk, exhibits an antibacterial effect against both Gram positive and Gram negative bacteria, respectively and also an antifungal effect. The most pronounced antibacterial effect was against Gram positive bacteria *S. aureus* (16 mm) and the weakest against Gram negative bacteria *S. flexneri* and Gram positive bacteria *S. pyogenes* (8 mm) while the antifungal effect was recorded only against *C. albicans* (10 mm). TE, used in concentration of 250 μg/disk, exhibited only a slight effect against *S. flexneri, S. typhimurium* and *S. pyogenes* while no antifungal effect was recorded.

Cell viability. In order to assess the effects induced by VO and TE on A375 and B16 melanoma 4A5 cell viability, different concentrations (5, 10, 25, 50 and 100 μM) were tested in a 24 h exposure. The results revealed that: i) VO induced a significant decrease of A375 living cells percentage at low concentrations, ii) the effect was dose-dependent, and iii) the concentrations lower than 25 μM were more toxic for the cells (Fig. 4). The IC_{50} value was 22.27 μM, which proves the potent inhibitory effect of VO on A375 cells viability. The evaluation of TE revealed a reduced viability percentage of A375 cells, but much lower compared to VO (IC_{50} value for TE was 40.38 μM). The viability rate (%) of A375 cells decreased at

Table IV. Antimicrobial and antifungal activities of the volatile oil and total hydro alcoholic extract from *Satureja hortensis* L. by Disk Diffusion method, expressed as diameter (mm) of inhibition zone (mean ± SD) including the disc diameter (6 mm).

Bacteria and fungi	VO (mm)	TE (mm)	Positive control (antibiotic, mm)	Negative control (solvent)
Bacteria				
S. aureus, ATCC 25923(+)	16±0.58	-	15	-
E. coli, ATCC 25922(-)	9±0.50	-	12	-
P. aeruginosa, ATCC 27853(-)	10±0.61	-	15	-
B. cereus, ATCC 8035(+)	12±0.71	-	15	-
S. flexneri, ATCC 12022(-)	8±0.36	8±0.21	13	-
S. typhimurium, ATCC 14028(-)	11±0.41	8±0.26	12	-
S. pyogenes, ATCC 19615(+)	8±0.35	7±0.25	21	-
Fungi				
C. albicans, ATCC 10231	10±0.54	-	12	-
C. parapsilosis, ATCC 22019	-	-	13	-

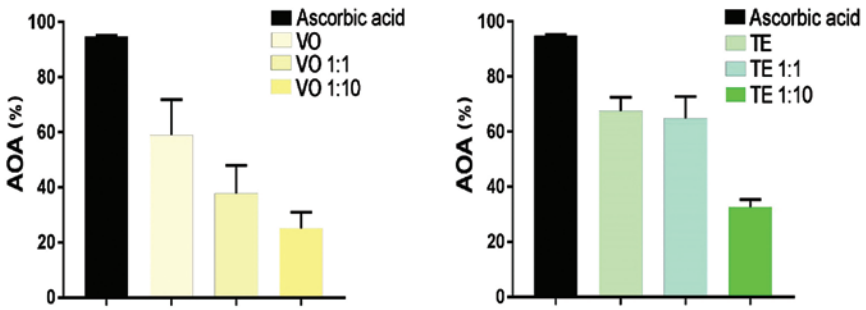

Figure 3. Antioxidant activity of *Satureja hortensis* L. volatile oil (VO) and total hydro alcoholic extract (TE) obtained from aerial parts after the first 300 sec.

concentrations lower than 25 μM, whereas the highest concentration tested of 100 μM did not affect the cells (91.22±0.27% viable cells) (Fig. 4).

The B164A5 cells also seemed to be sensitive to VO effect (IC_{50}, 34.16 μM). The media percentages of viable cells at low concentrations of 5 and 10 μM have been reduced, but not in the same manner as in the case of A375 cells (5 μM: 60.55±1.28 vs. 36.18±1.51 and 10 μM: 63.26±1.83 vs. 42.14±2.06, respectively). TE induced a lower decrease of living cell percentage in the case of B164A5 cells as compared to the one determined for A375 cells, the calculated IC_{50} value being 204.4 μM with a non-significantly reduced percentage of viable cells at 100 μM (94.46±0.87) (Fig. 4).

On normal human keratinocyte and fibroblasts, the concentrations of VO and TE used did not show a significant toxicity, whereas a potent dose-dependent stimulatory effect was observed (Fig. 5). In the case of keratinocytes, TE had a low cytotoxic effect on cell viability at the lowest concentrations (~82% at 5 μM and ~86% at 10 μM, viable cells), while volatile oil exerted a weak stimulating effect at the highest concentrations used. Human fibroblasts were affected in terms of viability, only by small sample concentrations, for both VO (~93% at 5 μM) and TE (~90% at 5 μM), respectively whereas highest concentrations of VO utilized presented a strong stimulatory effect (~120% at 50 μM and ~116% at 100 μM).

Cell morphology. Since the test compounds (VO and TE), exhibited a significant cytotoxic effect against tumor cells A375 and B164A5, the impact of these compounds on cell morphology was monitored by light microscopy (Optikam Pro Cool 5; Optika Microscopes, Ponteranica, Italy). In the case of A375, the control cells (unstimulated) displayed a normal epithelial morphology, with spindle and cobblestone shapes, strongly bound and adherent to culture plate, and highly confluent after 24 h. The A375 cells stimulated with VO and TE for 24 h did not present differences in terms of morphology, still a reduced confluence and loose bonds was detected between the cells as compared to control cells, and several cells were floating in the culture medium (Fig. 6). B164A5 control cells presented a healthy fibroblastic-like morphology, with polygonal shape, increased adherence to culture plate and confluence at 24 h. The VO and TE stimulation led to several changes in cell shape, becoming shrunken with a reduced confluence tendency and started to detach from the culture plate (Fig. 6), results that confirm the data recorded for cell viability tests.

Cell migration and proliferation. Based on the results obtained for the cell viability test, the effects of VO and TE on cell migration were assessed by using only the concentrations that did not induce a significant cell death of the cells (25 μM). The

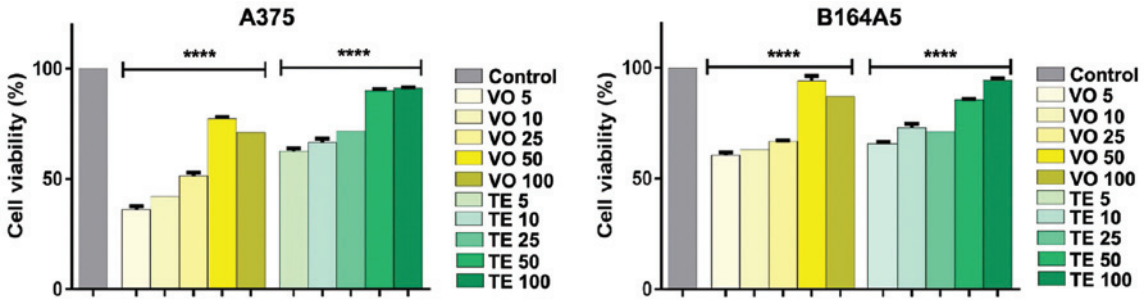

Figure 4. Viability percentage of A375-human melanoma and B16 melanoma 4A5 cells at 24 h post-stimulation with samples (5, 10, 25, 50 and 100 μM). The results are expressed as cell viability percentage (%) normalized to control cells. ****P<0.0001 compared to control group.

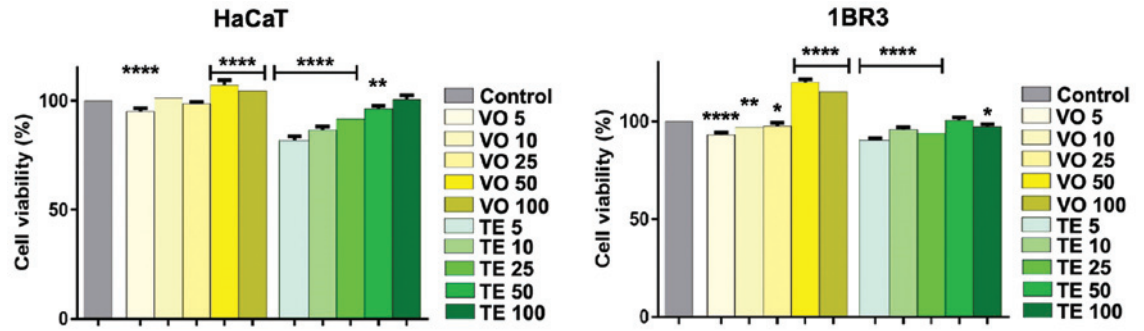

Figure 5. Viability percentage of HaCaT immortalized human keratinocytes and 1BR3 human skin cells at 24 h post-stimulation with samples (5, 10, 25, 50 and 100 μM). The results are expressed as cell viability percentage (%) normalized to control cells. *P<0.05, **P<0.01, ****P<0.0001 compared to control group.

effect of the VO and TE on cell migration and proliferation was evaluated by the means of scratch assay, a wound healing type technique. After the scratches were drawn (when the confluence of the cells was higher than 85%), the cells were stimulated for 24 h. Images were taken at three different time-points, namely 0, 3, and 24 h in order to study the impact of the VO and TE on cell migration and proliferation.

The A375 control cells presented migratory capacity by covering the wound area after 24 h but in the case of cells treated with VO and TE an inhibition of the cell migration process was observed, more pronounced after 24 h (Fig. 7). Similar effects were detected in the case of B164A5 cells, but the effect exerted by VO was more pronounced (Fig. 8).

The VO did not affect the normal keratinocyte and fibroblast migration after 3 h, neither after 24 h, moreover, the sample had a stimulatory effect on cell proliferation. The cells were abundant on the plate and well attached. The TE induced a similar effect on HaCaT cell migration and proliferation as that described for VO: a stimulatory effect, results that were in agreement with the data recorded for the cytotoxicity test.

Discussion

A variety of factors can influence the chemical composition of *Satureja hortensis* L. essential oils including environmental factors, extraction and isolation procedures, and storage conditions (19,20). To obtain the essential oil, the hydro distillation method was chosen due to the reduced time of obtaining, the facile way of preparation and the increased content of volatile compounds of interest. Sefdikon *et al* (19) noted that drying the aerial parts in the oven at 45˚C and application of the above method represents the proper alternative for obtaining the desired yields (19). Studies regarding the chemical composition of essential oils obtained from the aerial parts of the plant harvested from different regions of the world showed that the main compounds are carvacrol, γ-terpinene, *p*-cymene, α-terpinene and myrcene (19,21,22). However, it should be mentioned that there is a lack of data in the literature regarding the chemical composition in terms of the region, cultivation conditions and environmental factors. Some authors mentioned that the percentages of carvacrol and terpinene are highly influenced by the climate, especially by ultraviolet radiation so they can lead to an increase in carvacrol content and a decrease in terpinene (21,23). The data obtained in the present study is partly consistent with the literature, specifying that the percentages of the mentioned compounds decreased in the following order γ-terpinene (~38%) > *o*-cymene (~15%) > thymol (~13.5%) > carvacrol (~13%) as detailed in Table I.

As in the case of essential oil, the chemical composition of the extract varies depending on a number of factors, such as plant origin, harvest period, geographic and climatic factors, and extraction method. Chkhikvishvili *et al* (24) identified in the ethanolic extract of *Satureja hortensis* L. by HPLC analysis, a series of compounds: rosmarinic and ferulic acids were the major compounds, and caffeic, *p*-coumaric acids, catechin, epicatechin, luteolin, apigenin, rutin, hesperidin, and apigenin-7-glucoside were also detected (24). By applying Soxhlet extraction method and using EtOH, 96% as solvent, an extract concentrated in rosmarinic acid and quercetin was obtained, with lower concentrations of other polyphenols such as apigenin, kaempherol, luteolin, rutin, *p*-coumaric acid and chlorogenic acid (25).

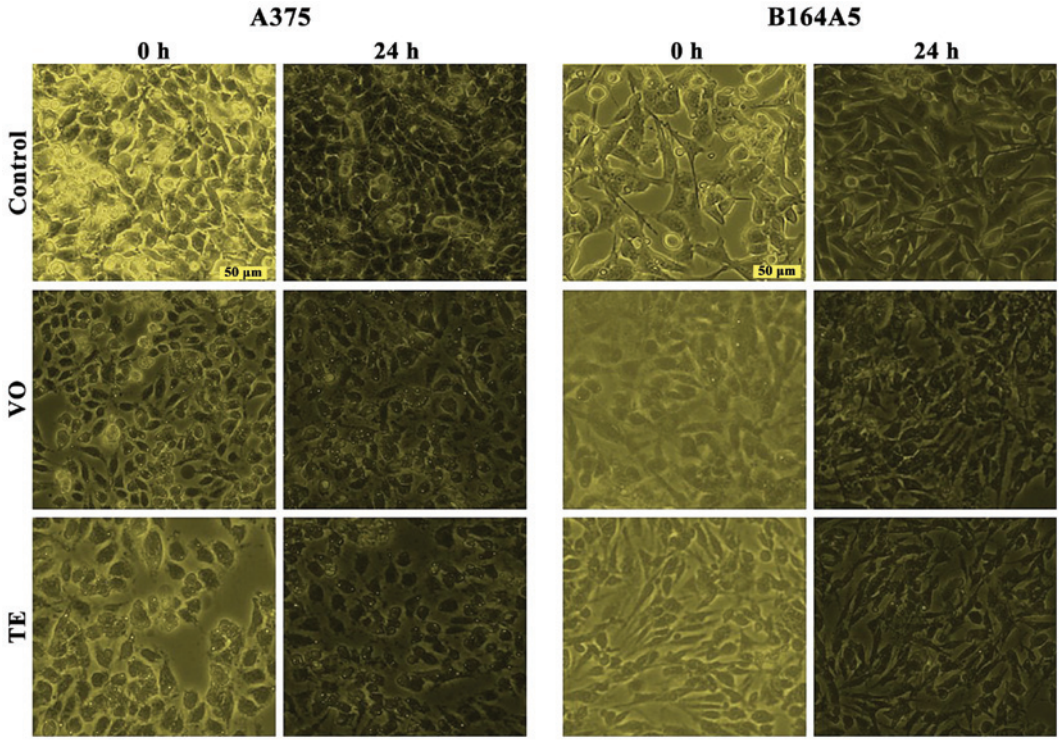

Figure 6. Morphological aspect of control A375 and B164A5 cells stimulated with *Satureja hortensis* L. volatile oil and total hydro alcoholic extract for 24 h at a concentration of 25 μM. Images were obtained by light microscopy at x20 magnification. Scale bar, 50 μm.

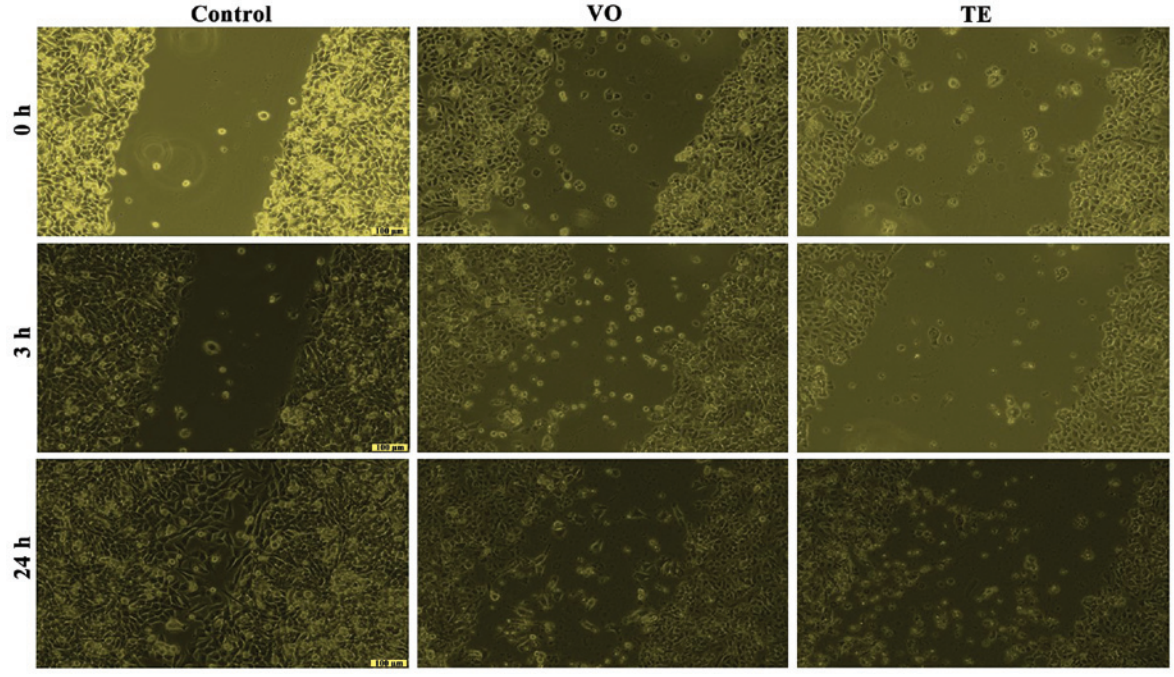

Figure 7. The effect of *Satureja hortensis* L. VO and TE on A375 cell migration and proliferation after stimulation with a concentration of 25 μM at different time-points (0, 3, and 24 h) post-stimulation. Images were obtained by phase contrast microscopy at x10 magnification. Scale bar, 100 μm.

A number of causes, including a disorganized lifestyle, an inadequate diet or particular conditions developed by foreign stimuli lead to the formation of reactive oxygen species. Thus, peroxide (ROO•), hydroxyl (HO•), nitric oxide (NO•), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and other free radicals are produced *in vivo* by partial reduction of O_2 through mitochondrial respiration/oxidative phosphorylation as a protection mechanism. Increased production of these species most often lead to alterations in the DNA, proteins and lipids, with serious consequences on the body in terms of cellular aging, mutagenicity, carcinogenicity, and other (26).

Antioxidant compounds possess the ability to react in different conditions transferring electrons, binding metal ions, activating enzymes, reducing radicals and inhibiting oxidases,

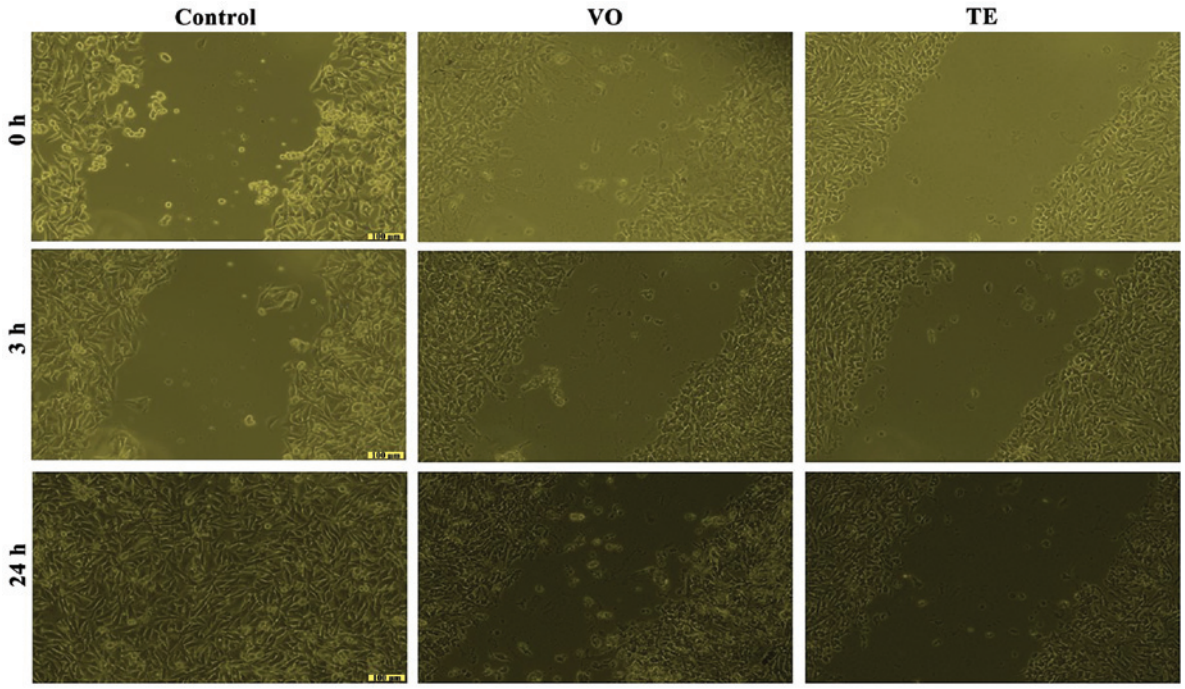

Figure 8. The effect of *Satureja hortensis* L. VO and TE on B164A5 cell migration and proliferation after stimulation with a concentration of 25 μM at different time-points (0, 3, and 24 h) post-stimulation. Images were obtained by phase contrast microscopy at x10 magnification. Scale bar, 100 μm.

all of these leading to the neutralization of the reactive oxygen species (27). Two types of antioxidant compounds have been described, endogenous antioxidants naturally produced in the body and exogenous antioxidants which are found in different sources of natural origin (e.g., fruits, vegetables and plants). Antioxidant properties of VO and TE, were found to be significant; therefore, due to the rich content in compounds with antioxidant properties, which possess an increased number of OH groups capable of participating in the reactions mentioned above, their properties are explored in the study of certain pathologies.

Considering the antimicrobial activity, volatile oils exert some actions on cell membranes, such as interference and destabilization, with repercussions on the phospholipid bilayer and alteration of enzyme activity (28). The inhibitory effect against bacteria and fungi of summer savory VO can be attributed to the increased content of biologically active compounds of the monoterpenes class, especially terpinene, thymol and carvacrol (5). Some studies reported that thymol and carvacrol possess an increased activity against bacterial and fungal strains, while γ-terpinene and *p*-cymene are active against fungal strains (29,30). Other data mention that thymol has an important inhibitory activity against *S. aureus*, carvacrol and *p*-cymene against *E. coli* and γ-terpinene against *S. aureus* and *C. albicans* (5). The volatile oil of *Satureja hortensis* L. possesses a wide antimicrobial spectrum, against both bacteria and fungi (25 bacteria, 8 fungi, and 1 yeast species) (31). Mihajilov *et al* (32) proved the activity against *E. coli, S. typhimurium, S. aureus, L. monocytogenes, P. putida* strain isolated from meat (32). In the present study both VO and TE were tested; VO showed activity against tested bacteria and fungi, with the indication that *S. aureus* was the most sensitive while TE exerts only a slight effect against three bacteria. These aspects are in agreement with the hypothesis that only the volatile oil has in its composition more antimicrobial compounds than the extracts (31).

Data related to the activity of different natural compounds, volatile oil or extract obtained from different medicinal plants have captured the attention in recent years and are extensively studied and tested in various cancer pathologies (33,34). Extracts from *Satureja hortensis* L. on melanoma cells are scarce and also briefly described. In one study, Stanojković *et al* (35) tested the methanol extract of *Satureja hortensis* L. and observed a strong antitumor activity against Fem-x human malignant melanoma cells with an IC_{50} of 39.66±2.71 μg/ml (35). It was also proved that the extract highly inhibited the K562 cell viability at low concentrations of 10 μg/ml (IC_{50}, 52 μg/ml), and in case of Jurkat cell line the IC_{50} value was 66.7 μg/ml (36). Another study showed that *Satureja hortensis* L. and its rosmarinic acid-rich fraction are able to protect Jurkat cells against oxidative stress caused by H_2O_2 (24). In the case of Hep2c, human cervix carcinoma, RD, human rhabdomyosarcoma and L2OB, murine fibroblast cell lines, the calculated inhibitory concentrations were in the ranges of 13.23-35.29, 18.43-31.03 and 20.51-34.09 μg/ml, respectively for ethanolic summer savory extracts (25).

Multiple studies were focused on the chemical compounds found in the composition of volatile oil and extract. Common active principles of *S. hortensis* L. such as α-pinene, γ-terpenene, caryophyllene and others, presented antiproliferative activity against K562 cells (IC_{50} value between 98-329 μM/ml) (37). Consequently, carvacrol exerted cytotoxic activity against breast cancer (MCF-7), skin cancer (SK-ML-2), colon cancer (HCT-15) and pancreatic cancer (MIAPaCa-2) (38); kaempherol presented antiproliferative activity (IC_{50}, 20 μM) against human melanoma (A375) after 48 h incubation (39) and showed various suppressive effects on melanoma A375SM cells, at a concentration of 20 μM (40); quercetin slightly decreased

the survival of human melanoma cells (A375 and A2058) in a time- and dose-dependent manner with IC_{50} values of 99.6 and 118.1 μM after 48 h treatment and the viability of murine melanoma cells (B16F10) decreased in a dose-dependent manner (41,42); quercetin and other compounds from its class, such as epigallocatechin, kaempherol, myricetin and luteolin showed inhibitory actions on HGF-stimulated melanoma cell migration and invasion. These findings denoted that these compounds shared similar activities and may be taken into account in melanoma treatment and prevention (42).

The novelty of this study consists in the biological assessment of essential oil and total hydro-alcoholic of *Satureja hortensis* L. both as individuals and in comparative terms. VO and TE of summer savory possessed different antimicrobial activity against tested bacteria and fungi. VO proved to be active against all tested bacteria, especially against *S. aureus* while TE showed poor activity against three of the tested bacteria (*S. flexneri, S. typhimurium* and *S. pyogenes*) with no activity on fungi. The antitumor activity against melanoma cells was present, and no toxic effects on healthy cell lines tested (immortalized human keratinocytes and human skin fibroblast) was recorded. VO showed a greater efficacy as compared to TE on human melanoma cells (A375) while in the case of murine melanoma cells (B164A5) the impact on cell viability was similar. The activity of the compounds tested was higher at low concentrations whereas at high concentrations the percentages of viability were increased. Further *in vitro* and *in vivo* studies are necessary in order to elucidate the anticancer mechanisms of activity, and for a better understanding of the role played by the biological active compounds.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

RAP, IPi, CAD, HTS and DV made a major contribution to the conceptualization, validation, writing, reviewing and editing of the manuscript. IPo and EA obtained, analyzed and interpreted the data regarding volatile oil and extracts. CGF, DC and CD performed *in vitro* assays (analysis and interpretation). DV and VL contributed to the writing of the manuscript, methodology and project resources. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that there is no competing of interests.

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