Novel insights to the anti-proliferative activity of rosemary (*Rosmarinus officinalis* L.) co-treatment

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The aim of this study was to characterize volatile and non-volatile compounds of rosemary from the North Adriatic region and to determine its antiproliferative activity, alone or in combination with radiomimetic bleomycin (BLM) on three malignant and one non-transformed human cell lines. Chemical analysis of the volatile compounds revealed the presence of monoterpenes (93.8%), among which 1.8-cineol (32.9%) and camphor (15.5%) were the dominant compounds. Also, obtained results showed that the major polyphenolic constituents in rosemary extract were phenolic acids (rosmarinic acid and its derivatives up to 69.2 mg 100 g^{-1}), as well as flavones and flavonols in the following order: luteolin>isorhamnetin>quercetin>kaempferol>apigenin. Cell growth tests showed that rosemary extract alone exerted moderate antiproliferative activity, as well as a synergistic antiproliferative effect with bleomycin (EC$_{50}$ 344.3-461.5 μg mL^{-1} and 58.6-292 μg mL^{-1}, respectively). The anti-tumor effect of rosemary extract in combination with BLM was much stronger, compared to BLM itself on the breast cancer cells. Through its proposed sensitizing effect, rosemary extract, in combination with the standard chemotherapeutics, could be used for the investigations of possible therapeutic modalities.

1. INTRODUCTION

Among numerous spices and medicinal plants which are considered a promising source of natural products and various bioactivities, *Rosmarinus officinalis* L. has been frequently reported for its antiproliferative activity. In rosemary extracts numerous components have been found and their content depends on geographical origin (e.g. humidity, salinity, insolation), cultivar and extraction methods. These ingredients have a variety of extremely useful medicinal properties such as anti-inflammatory, anti-allergic, anti-irritant, antibacterial, antifungal, antioxidant and the most important for this investigation, anticancer (Leal et al., 2003; Kabouche et al., 2005; Peng et al., 2005).

Primary bioactive principles of rosemary are: rosmarinic acid, flavonoids and phenolic diterpens (carnosic acid and carnosol) (Petiwala and Johnson, 2015). Essential oil also has remarkable chemopreventive and anticancerogenic properties through mechanisms such as antioxidant, antimutagenic, antiproliferative and immunostimulatory mechanism of volatile constituents (Ali et al., 2015).

Biological activity of rosemary and its constituents was investigated in numerous *in vitro* and *in vivo* studies using tumor models. Antiproliferative and colony forming abilities of rosemary were observed on many various cell lines including leukemia (Cheung and Tai, 2007; Okumura et al., 2012), prostate (Petiwala et al., 2014) and ovarian cancer (Tai et al., 2012). Also, a number of studies regarding apoptosis and cell death showed that treatment with rosemary extracts had significantly reduced viability in various cell lines (Tai et al., 2012; González-Vallinas et al., 2013; 2014). Furthermore, antioxidative properties of rosemary in *in vitro* tumor cell models (Slameňová et al., 2002; Alexandrov et al., 2006; Cheung and Tai, 2007), as well as *in vivo* studies in experimental animals with induced cancer were confirmed (Sancheti and Goyal, 2006).

Flavones present in rosemary are potent antiproliferative com-

pounds whose activity is based on balance between their antioxidative properties on one side, and their effect as prooxidants and mitochondrial toxic agents on the other (Haddad et al., 2006).

Also, caffeic and rosmarinic acid possess multiple biological properties such as anti-inflammatory, antimicrobial, cardioprotective and antitumor (Prasad et al., 2011; Bittner Fialová et al., 2019). Recent *in vivo* studies showed that rosmarinic acid dose-dependently suppresses growth of pancreatic cancer cells from xenograft nude mice (Han et al., 2019). Also, it is known that rosemary constituents potentiate the effectiveness of conventional chemotherapeutics as well as other compounds of plant origin through synergistic influence (Lewandowska et al., 2014). Based on their investigations on a panel of colon cells, Borrás-Linares et al. (2015) put forward a theory about the synergistic action of rosemary extract ingredients. As it is described by some authors (Ivanova et al., 2014a; Plouzek et al., 1999), chemo-sensitising is the effect when biologically active molecule increases the activity of chemotherapeutic in tumour cells. Therefore, we included radiomimetic antitumor drug bleomycin in treatment of cell lines with rosemary extract. Namely, bleomycin, as free radical-based DNA damaging agent, induces a double-strand breaks on deoxyribose moieties in both DNA strands, interruption that is highly similar to those of ionizing radiation (Povirk, 1996).

Having in mind the antitumor potential of rosemary constituents and a lack of data concerning this medicinal plant collected from the North Adriatic region, this study was conducted to characterize volatile and non-volatile compounds of rosemary harvested from the region of the Krk island (North Adriatic). Secondarily, our aim was to evaluate antiproliferative activity of these compounds, alone or in combination with bleomycin against human cell lines - one non-transformed human cell line MRC-5 and three malignant MCF-7 (breast adenocarcinoma), HeLa (cervix epitheloid carcinoma) and HT-29 (colon adenocarcinoma) cell lines.

2. MATERIALS AND METHODS

2.1. Plant material

Aerial parts of the tested wild growing *Rosmarinus officinalis* were collected at Mali Kijec-Omišalj, Krk Island (Croatia), located in the Northern Adriatic (GPS coordinates $32°59'59.8''N 148°15'44.3''E$) in early June right before full blossoming. Voucher specimen was confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia (Voucher N 2-1527). Plants were air dried in shade, and after that pulverized in a mortar with pestle and a coffee mill and used for further analyses.

2.2. Determination of individual essential oil components using GC-MS analysis

The essential oil was isolated by hydrodistillation method using *n*-hexane as collecting solvent, according to European Pharmacopoeia (Ph.Eur.8.0, 2013). The obtained hexane extract was dried over anhydrous sodium sulphate and decanted. Hexane was evaporated under reduced pressure and the oil yield was measured.

Gas chromatography-mass spectrometry (GC-MS) analysis was carried out using Agilent 5975C Series GC-MSD system (7890A GC and 5975C inert MSD), equipped with a HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.50 µm). One µL of diluted essential oil (100 times in *n*-heptane) was injected in split mode (10:1), and inlet temperature was held at 250 °C. Helium was used as carrier gas in constant flow mode at 1 mL min⁻¹. The oven temperature was programmed as follows: 70 °C increased to 104 °C (2 °C min⁻¹) which was

held for 2 min, then to 180 °C (2 °C min⁻¹ without holding), and then to 200 °C (4 °C min⁻¹) which was held for 10 min. Ion source was operated at 70 EV, and mass spectra were acquired in scan mode in the 50-550 *m/z* range. Essential oil components were identified by comparing their retention indices and mass spectra with those published by Adams (2007) and with Wiley and NIST/NBS mass spectral libraries. A mixture of *n*-alkanes from *n*-octane (C8) to eicosane (C20) was used for calculation of Kovats retention indices (KI). ChemStation software (Agilent Technologies) was used for data analysis, and curves used for experimental estimation of KI were plotted and drawn using SciDaVis software.

2.3. Preparation of rosemary extract

Extract of *R. officinalis* was prepared from 10 g of milled herba extracted using maceration technique with 50 mL of 80% aqueous methanol, during 24 h at 4 °C. After filtration, the solvent was evaporated at 40 °C and concentrated to dryness under vacuum. Obtained dried extract was used for analyses of polyphenolic compounds and cell growth activity tests.

2.4. Extraction and determination of phenolic compounds using HPLC-DAD-MS analysis

Dried extract was dissolved in 80% methanol containing 1% (*w/v*) 2.6-di-tert-butyl-4-methylphenol (BHT) to prevent oxidation in a cooled ultrasonic bath for 1 h (final concentration of the extract was 0.2 g mL⁻¹). Obtained extract (in six replications) was centrifuged for 10 min at 1118 x g. Each supernatant was filtered through the Chromafil AO-20/25 polyamide filter produced by Macherey-Nagel (Düren, Germany) and transferred to a vial prior to injection into the HPLC (high performance liquid chromatography) system. The solutions were kept at -80 °C until further analysis. Phenolic compounds were analyzed on a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, USA) with a diode array detector at 280 nm (cinnamic acid derivatives and flavanols) and 350 nm (flavonols and flavones). Spectra of the compounds were recorded between 200 and 600 nm. The column was a Gemini C18 (150 × 4.6 mm, 3 µm; Phenomenex, Torrance, USA) operated at 25 °C. The elution solvents were aqueous 0.1% formic acid in twice distilled water (A) and 0.1% formic acid in acetonitrile (B). Samples were eluted according to the linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20% to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30% to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions (Wang et al., 2002). The injection volume was 20 µL and flow rate was 0.6 mL min⁻¹.

Polyphenolic compounds were identified by an HPLC-Finnigan MS detector and an LCQ Deca XP MAX (Thermo Finigan, San Jose, CA) instrument with electrospray interface (ESI) operating in negative ion mode. The analyses were carried out using full scan data-dependent MSn scanning from *m/z* 110 to 1500. Column and chromatographic conditions were identical to those used for the HPLC-DAD analyses. The injection volume was 10 µL and the flow rate 0.6 mL min⁻¹. The capillary temperature was 250 °C, the sheath gas was 60 units and auxiliary gas 15 units; the source voltage was 3 kV and normalized collision energy was between 20-35%. Spectral data were elaborated using the Excalibur software (Thermo Scientific).

The identification of compounds was confirmed by comparing retention times and their spectra, as well as by adding the standard solution to the sample and by fragmentation (Tables 2 and 3). Concentrations of phenolic compounds were calculated from peak areas of the sample and the corresponding standards and expressed in mg 100 g⁻¹ dry weight (DW). For

compounds lacking standards, quantification was carried out using compounds with structural similarities.

2.5. Total Phenolic Compounds (TPC) Assay

Quantification of TPC in rosemary extract (prepared using the same way as the one for HPLC analysis, but without BHT) was performed using spectrophotometer UV/Visible Evolution 220 (Thermo Scientific, San Jose, USA). TPC was determined by Folin-Ciocalteu method (Mikulic-Petkovsek et al., 2013). Tubes with 8.4 mL H_2O, 0.5 mL 33% Folin-Ciocalteu phenol reagent and 0.1 mL extract (except in blank) were vortexed and after 3-6 min 1 mL of 20% Na_2CO_3 was added. Absorbance was recorded at λ=765 nm after 1h of incubation at room temperature. TPC was expressed as gallic acid equivalents (GAE) in mg g^{-1} dry weight (DW).

2.6. Cytotoxic activity of rosemary extract, growth and culture of the cell lines

For the estimation of cell growth activity, one non-transformed human cell line MRC-5 (fetal lung fibroblast, ECACC 84101801) and three human malignant transformed cell lines: MCF-7 (breast adenocarcinoma ECACC No. 86012803), HeLa (cervix epitheloidcarcinoma, ECACC No. 93021013) and HT-29 (colon adenocarcinoma, ECACC No. 91072201) were used. The cell lines were grown and maintained in Dulbecco's Modified Eagle's Medium - DMEM (Sigma-Aldrich, USA) medium supplemented with Fetal Calf Serum - FCS (10%), penicillin (100 Units mL^{-1}) and streptomycin (100 µg mL^{-1}), being referred to as complete medium. The cells were cultured in 25 cm^2 flasks at 37 °C in the atmosphere of 5% CO_2 and high humidity, and sub-cultured twice a week. A single cell suspension was obtained using 0.1% trypsin with 0.04% EDTA. Cell growth was evaluated by the colorimetric sulforhodamine B (SRB) assay (Rubinstein et al., 1990). Cell lines were plated into 96-well microtiter plates (Sarstedt, Newton, USA) at different seeding density of 5×10^3 cells per well for MRC-5 and MCF-7.4×10^3 cells for HeLa and 6×10^3 cells for HT-29 in a volume of 180 µL, and pre-incubated in complete medium supplemented with 5% FCS, at 37 °C for 24 h.

For the evaluation of the cell growth activity, dry extract of *Rosmarinus officinalis* was diluted in mixture of DMSO and 0.9% NaCl to obtain final concentrations in range of 500-1000 µg mL^{-1} while in control cell group, mixture of DMSO and 0.9% NaCl were added (DMSO concentration was under 0.2%).

For the co-treatment we have chosen antitumor drug BLM. For investigations of co-effect of rosemary extract and BLM on cell lines growth, the chosen final concentration of BLM was 100 µg mL^{-1}. This concentration of BLM was chosen because higher concentrations of BLM can induce very high frequency of micronuclei (MN), which was difficult to score accurately. Serial dilutions of rosemary extract and BLM (20 µL $well^{-1}$) were added to achieve required final concentrations. Microplates were then incubated at 37 °C for an additional 48 h. Colour development was measured using Multiscan Ascent (Labsystems; Helsinki, Finland) photometer at 540 nm against 620 nm as background. Results of cell growth activity were expressed as mean ± SD of three independent experiments, performed in quadruplicate.

The effect on cell growth was expressed as a percent of the control and calculated as: (At/Ac)×100 (%), where At is the absorbance of the test sample and Ac is the absorbance of the control (Četojević Simin et al., 2015). Based on concentration-cell growth curves, EC_{50} values (concentration that inhibit cell growth by 50%) were determined using CalcuSyn Version 1.1 (Mike Hayball, Copyright Biosoft, 1996). Using EC_{50} values obtained in a non-tumor cell line and in the respective tumor cell line, non-tumor/tumor EC_{50} ratios (NT/T) were calculated for extract, combination of extract and drug. NT/T ratio shows the efficiency of tested material effect on tumour cells in comparison to healthy ones (Četojević Simin et al., 2015).

2.7. Statistical analysis

Values of biochemical parameters and the cell growth activity were expressed as means ± standard error of determinations made in triplicates and quadruplicate, respectively. Results were tested by ANOVA followed by comparisons of means by the Duncan's test (P<0.05). Statistical analyses were performed using STATISTICA for Windows version 13 (Dell Software) and CalcuSyn Version 1.1 (Mike Hayball, Copyright Biosoft, 1996).

3. RESULTS

Plant material tested in this study belongs to Spanish type of rosemary according to International Standard of rosemary essential oil (ISO 1342, 2012). The yield of essential oil in the above-ground parts of examined specimen (1.9% of rosemary dry weight) permit the assignment of this species to oil-rich representatives of the Lamiaceae family. Bearing in mind that the yield of the hydrodistillation is much lower compared to steam distillation, the yield of our sample was much higher in comparison to the Algerian rosemary (0.4%), but lower in comparison to the Iranian sample (2.6%) (Boutekedjiret et al., 2003; Jamshidi et al., 2009).

Table 1. Essential oil composition of the rosemary herba

Compound name	RI	%m/m
α-Thujene	932	0.31
α-Pinene	940	13.41
Camphene	955	4.36
β-Pinene	983	3.95
Myrcene	992	5.64
α-Phellandrene	1009	0.43
Carene 3-delta	1014	1.63
α-Terpinene	1020	0.46
p-Cymene	1028	0.70
1,8-Cineole	1035	32.99
γ-Terpinene	1064	0.79
Terpinolene	1090	1.02
Linalool	1100	1.43
Camphor	1144	15.55
Borneol	1167	3.17
Terpinen-4-ol	1179	0.97
α-Terpineol	1190	2.54
Verbenone	1206	1.88
Bornyl acetate	1287	2.60
Caryophyllene Z	1406	3.08
α-Humulene	1456	0.59
Caryophyllene oxide	1582	0.97
Total identified compounds	/	98.47

The composition of the essential oil is summarized in Table 1. The percentage of identified components in the oil sample

was 98.47%. Of the 22 components detected, all of them were identified in the oil in amount higher than 0.1%. The sample contained predominantly monoterpenes (93.8%), in which 1.8-cineol (32.9%) and camphor (15.5%) were the dominant compounds. A considerable amount of some other monoterpene compounds was also identified: α-pinene (13.4%), myrcene (5.6%), camphene (4.3%), β-pinene (3.9%), and borneol (3.2%). Relative portion of sesquiterpenes was low (4.6%) with the caryophyllene Z as the dominant component (3.1%).

The major polyphenolic constituents in investigated rosemary herba were flavones, flavonols and phenolic acids. As it is shown in Table 2, most of the phenolic acids were hydroxycinnamic acids derivatives (caffeic and quinic acid derivatives). Caffeic acid ester and rosmarinic acid derivatives were present in the highest amount (69.2 mg 100 g^{-1}).

A scale according to the content of flavones and flavonols can be organised in the following order: luteolin>isorhamnetin>quercetin>kaempferol>apigenin (Table 3). Beside these, low amounts of lignin medioresinol derivatives (0.4 mg 100 g-1), as well as phenolic terpene epirosmanol (0.9 mg 100 g^{-1}) isomers were also detected (Table 2). According to our results TPC in investigated extract was 6.3 g GA 100 g^{-1}, whereas the most abundant were luteolin-3-glucuronide derivatives (Table 2).

The antitumor activity of rosemary extract without and with BLM was evaluated *in vitro* by the colorimetric sulforhodamine B (SRB) assay using human non-tumor MRC-5 and three tumor cell lines MCF-7, HeLa and HT-29 (Figure 1). Rosemary extract showed moderate activity regarding the cell growth inhibition with EC$_{50}$ values between 344.3 and 461.5 µg mL^{-1} (Table **??**). There was no selective antiproliferative activity of rosemary extract towards tumor cells in comparison to non-tumor ones (NT/T ratio for all cancer cell line was lower than 1). The most susceptible tumor cell line regarding R effect was HeLa (EC$_{50}$ 385.2 µg mL^{-1}), then MCF-7, while the lowest antitumor activity rosemary showed on HT-29. Tested cell lines proved to have the same susceptibility. Concentration-dependent effect was achieved experimentally, by applying different concentrations of rosemary and BLM. The antiproliferative activity at co-treatment was higher from those with BLM only in MCF-7 cells (EC$_{50}$, rosemary+BLM : BLM = 58.9 : 119.8) (Table 4).

Antitumor activity of rosemary extract in combination with BLM was higher in comparison to effect of extract solely on all cell lines. Among tumour cells, most susceptible were MCF-7 (7-fold more than for rosemary extract), then HeLa cells (2.3-fold more than for rosemary extract), while HT-29 cells were the least susceptible (1.58-fold more than for rosemary extract). NT/T ratio was 1.0 for HeLa and 2.9 for MCF-7 cells, pointing to moderate selective response of rosemary extract in combination with BLM regarding the breast adenocarcinoma tumour type.

4. DISCUSSION

Regarding the investigations about combined administration of natural compounds and antineoplastic drugs, there are comprehensive possible benefits of such therapy. They might be reflected through lowering the dosage of conventional medicines and consequently decreasing the toxicity of antineoplastic drugs on healthy cells followed by higher cytotoxic effects toward cancer cells. Authentic samples of essential oil of rosemary harvested in coastal areas of California, Corsica, Croatia or Haute Provence differ significantly in their respective content of cineole, camphor, bornyl acetate and verbenone. Samples with low camphor and high verbenone contents are referred to as "rosemary verbenone" (California, Corsica) (Schnaubelt, 2011). This oil has been recognized for its specific mucolytic properties and its usefulness for skin

care formulas. The Haute Provence variety with high cineole and high camphor content is used in aromatherapy as well as for its expectorant and anti-infectious effects. Rosemary essential oil from the islands of the Adriatic coast of Croatia has a composition squarely in the middle, with a camphor content of approximately 10%. Our result for camphor content (15.5%) is somewhat above this, presumably because the Krk Island is located in the North of the Adriatic Sea and very close to mountainous mainland, with strong cold winds during winter and spring. It is known that camphor and α-pinene have been related to antioxidant activities (Sedighi et al., 2015), whereas antiproliferative activity has been ascribed to 1,8-cineol even for antiproliferative effect, leading to apoptosis in the leukemia cell (Kladniew et al., 2014).

The content and composition of phenolic compounds is very important due to their antitumor activity. For example, flavones act against cancer through antioxidant reactions in cells (Ren et al., 2003). Furthermore, their role in inhibition of angiogenesis, promotion and differentiation, as well as in apoptosis is well defined (Ren et al., 2003; Ivanova et al., 2014b). Also, flavonoids can play sensitizing role in malignant cells exposed to conventional chemotherapeutics through modifying the molecular events that control the cell growth, differentiation and programmed cell death (Ivanova et al., 2014b). Among the flavons and flavonols in the examined extract, the most common are luteolin-3-glucuronide derivatives. More recent studies highlight luteolin as a compound with strong selective anticancer activity that potentiates with dose increase (Goodarzi et al., 2018; Seydi et al., 2018).

Some authors (Munné-Bosch et al., 2000) reported that isorosmanol, rosmanol, carnosol, 11,12-di-O-methylisorosmanol, carnosic acid and 12-O-methylcarnosic acid are main phenolic diterpens found in leaves of rosemary grown in the Mediterranean region. However, their investigations concluded that content of phenolic diterpens varies during the vegetation season, as well as due to some abiotic factors, such as: relative water content of the leaf, high solar radiation and temperature. Rosemary plants subjected to enhanced water deficit, salinity, intense light, and heat stress seem to have lower carnosic acid concentrations (Tounekti and Munné-Bosch, 2012), which could explain the deficit of these compounds in our samples. Extract used in this study, as opposed to the low concentration of carnosic acid, is characterized with presence of other biologically active components, among which mostly flavons, as well as caffeic and rosmarinic acid derivatives.

Previous study showed that rosmarinic acid has low cytotoxic effect on cell lines and low effect on the cell viability (Yesil-Celiktas et al., 2010). Caffeic acid initiates the fibrosarcoma cancer cell death by decreasing cell proliferation, increasing intracellular reactive oxygen species (ROS), alteration in mitochondrial membrane potential, lipid peroxidation, DNA damage and apoptosis (Prasad et al., 2011). Also, caffeic acid phenethyl ester has a possibility to induce apoptosis via Fas signal activation in human breast cancer MCF-7 cells (Watabe et al., 2004). It is known that rosmarinic acid blocks the proliferation induced by tumor necrosis factor-alpha or platelet-derived growth factor (PDGF). These effects occur at both the G0–G1 and G1–S phases of cell division (Makino et al., 2000). Present investigation confirmed that methanol extract of rosemary, with proven predominant flavons and derivatives of caffeic and rosmarinic acid, led to a moderate inhibition of cell growth for all investigated cell lines, with cervix carcinoma cell line as the most susceptible to the activity of the extract, and colon carcinoma cell line as the least susceptible.

The study of Borrás-Linares et al. (2015) on colon adenocarcinoma HT-29 and SW480 cells investigated the comparative antiproliferative and cytotoxic feature of rosemary and its fractions with review on their potential synergistic ef-

Table 2. Characterisation and content of phenolic acids and other polyphenols in rosemary herba (results are presented as mean ± standard deviation)

Rt (min)	[M-H]⁻ (*m/z*)	MS² [M-H]⁻ (*m/z*)	Tentative identification	mg per 100 g⁻¹ DW
12.2	341	179, 281, 251	caffeoyl-hexoside derivative 1	2.24±0.09
13.2	341	281, 251, 179	caffeoyl-hexoside derivative 2	7.97±1.10
15.0	353	179, 173, 191, 136	4-*O*-caffeoylquinic acid derivative 1	0.15±0.00
16.4	353	179, 173, 191, 136	4-*O*-caffeoylquinic acid derivative 2	0.29±0.00
19.4	371	353	caffeic acid derivative	0.91±0.00
26.4	677	515, 353, 329,509, 191	1,3,5-tri-*O*-caffeoylquinic acid	0.94±0.00
15.0	387	207	medioresinol derivative 1	0.12±0.00
15.1	387	207	medioresinol derivative 2	0.24±0.00
36.1	359	161, 197, 225	rosmarinic acid derivative 1	59.85±2.31
34.5	359	161, 197, 314	rosmarinic acid derivative 2	0.85±0.01
35.4	359	161, 197, 314	rosmarinic acid derivative 3	0.50±0.00
35.5	359	161, 197, 314	rosmarinic acid derivative 4	2.98±0.51
25.4	549	387, 489	rosmarinic acid derivative 5	3.94±1.13
21.6	521	359, 161, 197	rosmarinic acid hexoside	1.05±0.07
32.7	299	137	hydrobenzoic acid hexoside	4.62±0.07
33.8	345	301, 283	epirosmanol derivative 1	3.54±0.06
33.9	345	301, 283	epirosmanol derivative 2	1.86±0.03

Table 3. Characterisation and content of flavones and flavonols in rosemary herba (results are presented as mean ± standard deviation)

Rt (min)	[M-H]⁻ (*m/z*)	MS² [M-H]⁻ (*m/z*)	Tentative identification	mg per 100 g⁻¹ DW
16.9	593	503, 491	apigenin-6,8-C-diglucoside (vicenin II)	1.63±0.02
23.0	597	447, 285	luteolin-rhamnoglucoside (luteolin-7-rutinoside)	16.17±0.12
24.2	461	285	luteolin-3-glucuronide 1	16.47±0.14
25.3	461	285	luteolin-3-glucuronide 2	13.55±0.09
27.6	461	285	luteolin-3-glucuronide 3	85.28±0.19
17.2	503	285, 399	luteolin-3-*O*-(*O*-acetyl)-β-D-glucuronide derivative 1	12.02±0.07
30.8	503	285, 399	luteolin-3-*O*-(*O*-acetyl)-β-D-glucuronide derivative 2	44.73±0.21
31.8	503	285, 399	luteolin-3-*O*-(*O*-acetyl)-β-D-glucuronide derivative 3	62.81±1.30
32.1	503	285, 443	luteolin-3-*O*-(*O*-acetyl)-β-D-glucuronide derivative 4	49.84±1.24
31.5	607	299	diosmetin-8-C-rhamnosyl-7-*O*-glucoside (diosmin)	20.02±0.14
25.6	609	447, 285	kaempferol-3,7-di-*O*-glucoside	8.27±0.02
22.0	593	285	kaempferol-3-*O*-rutinoside	5.14±0.01
12.8	477	315	isorhamnetin hexoside	5.95±0.05
23.5	477	315, 301	isorhamnetin-3-*O*-hexoside derivative 1	31.37±1.10
25.7	477	315, 301	isorhamnetin-3-*O*-hexoside derivative 2	2.32±0.04
24.8	491	315, 300	isorhamnetin-3-*O*-glucuronide	2.72±0.09
25.9	639	477, 315, 300	isorhamnetin-3,7-diglucoside	10.81±1.12
22.7	623	315, 299, 477	isorhamnetin-3-*O*-rhamnosyl hexoside derivative 1	3.94±0.07
27.1	623	315, 300	isorhamnetin-3-*O*-rhamnosyl hexoside derivative 2	3.50±0.06
27.8	623	315, 300	isorhamnetin-3-*O*-rhamnosyl hexoside derivative 3	6.09±0.07
19.9	609	301	quercetin-3-rhamnosyl hexoside derivative 1	4.99±0.03
24.5	609	301	quercetin-3- rhamnosyl hexoside derivative 2	16.47±0.09
20.5	463	301	quercetin-3-*O*-glucoside	8.72±0.13
23.9	625	301, 463	quercetin-dihexoside	16.47±0.17
27.8	653	315, 300	quercetin-diglucuronide	8.78±0.07

fects. The phenomenon of synergism was also investigated by Lewandowska et al. (2014), who presented a review of studies which confirms that rosemary constituents potentiate the effectiveness of conventional chemotherapeutics, as well as other compounds of plant origin, on MCF7 and HT29 cell lines. Previous data indicate that low-toxic conventional synthetic antioxidants, as well as antioxidant constituents from rosemary and other plants may have important role in process of sensitization of irradiated and chemotherapeutic-treated tumor cells (Plouzek et al., 1999; Berdowska et al., 2013; Ivanova et al., 2014b). Berdowska et al. (2013) confirmed the antiproliferative activity of polyphenol plant extract components on adriamycin resistant MCF-7 cells through MTT test. It is interesting that tested polyphenolics exhibited more beneficial
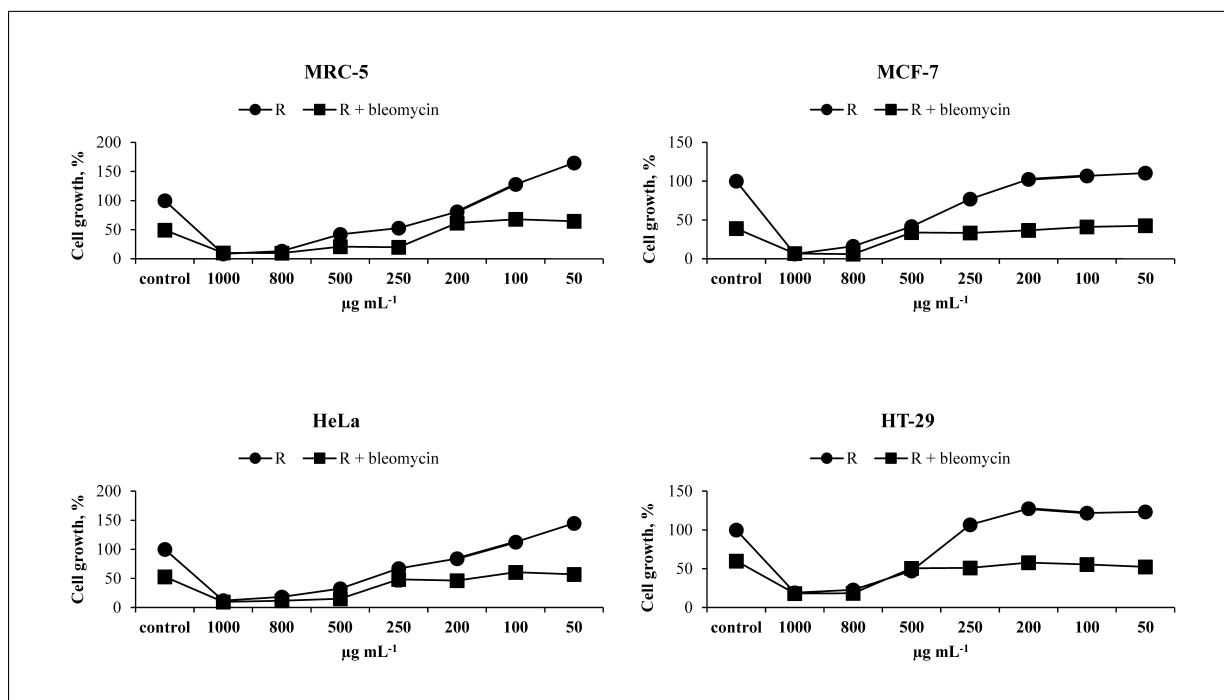
Fig. 1. Effect of rosemary with and without bleomycin (100 μg mL^{-1}) on growth of cell lines at 48h treatment. Results are shown as mean ± standard deviation of three independent experiments, performed in quadruplicate.
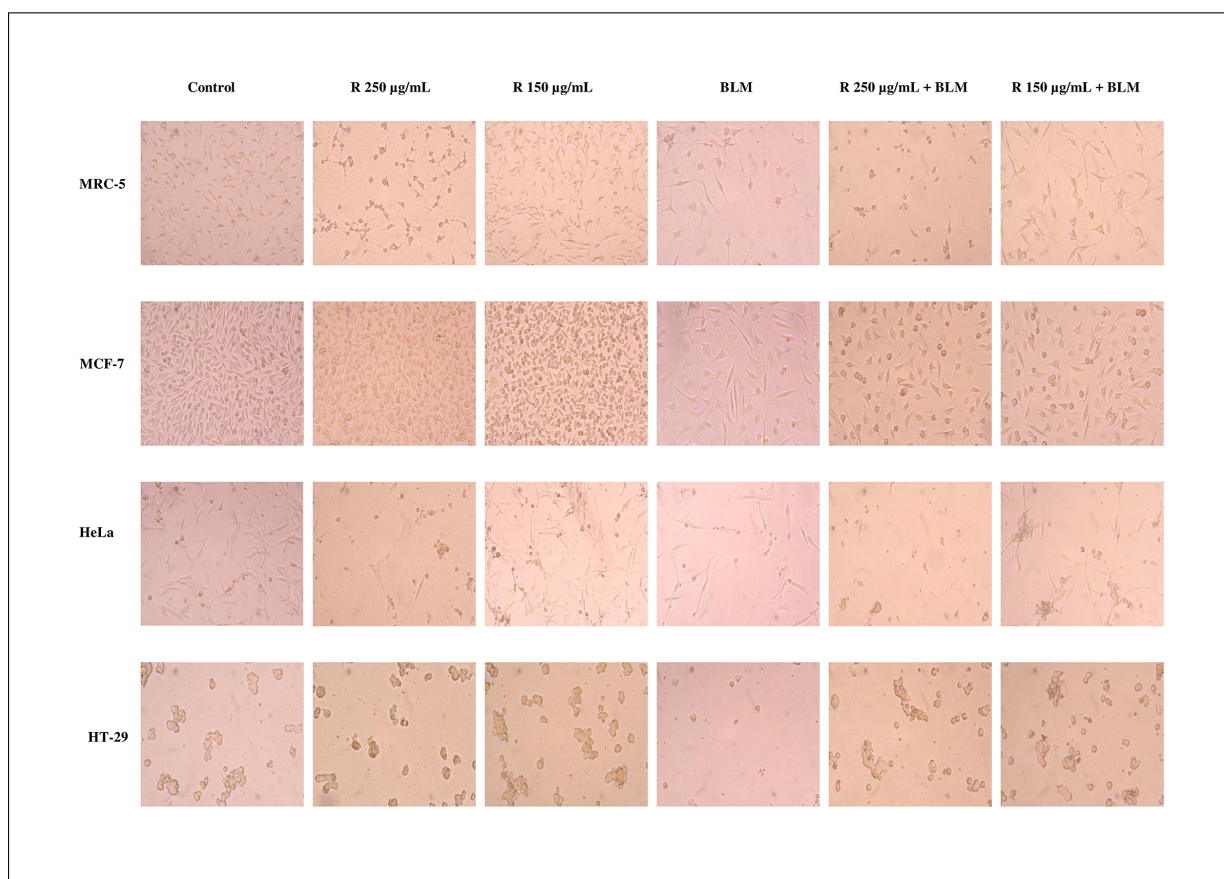

Fig. 2. Appearance of cells treated by rosemary with and without bleomycin (100 μg mL^{-1}) during 48h treatment, (magnification 400x). R-rosemary extract, BLM-bleomycin

properties when they were applied in the form of extracts comprising their mixtures than individually (Berdowska et al., 2013).

CONCLUSION

Results of this study showed that the major polyphenolic constituents in investigated rosemary extract were phenolic acids (rosmarinic acid and its derivatives), as well as flavones and flavonols in the following order: luteolin>isorhamnetin>quercetin>kaempferol>apigenin. Regard-

Table 4. EC$_{50}$ values (μg ml^{-1}) and non-tumor/tumor EC$_{50}$ ratios (NT/T) in human cell lines.

Cell line	R[a]	R + BLM	BLM
MRC-5	344.32	170.81	80.3
MCF-7	407.91	58.6	119.81
HeLa	385.15	162.85	77.2
HT-29	461.54	291.27	165.31
MCF-7 (NT/T)	0.84	2.91	0.67
HeLa (NT/T)	0.89	1.05	1.04
HT-29 (NT/T)	0.75	0.59	0.49

[a] EC$_{50}$ values for effect of rosemary extract (R) and bleomycin were established for bleomycin (BML) concentration of 100 μg ml^{-1}

ing the antiproliferative activity, we confirmed that treatment with rosemary extract led to moderate inhibition of cell growth for all investigated cell lines, where the most susceptible to rosemary effect was cervix carcinoma cell line, and the least susceptible was colon carcinoma cell line. The antiproliferative activity at co-treatment was higher from those with BLM in breast cancer cells alone. This combination also potentiates the antiproliferative effect of rosemary extract in all investigated cancer cell lines, with 7 times increased effect on breast cancer cells compared to the effect of rosemary extract alone. Co-treatment with BLM causes moderate selectivity to breast and cervical cancer cells compared to healthy cells.

To the best of our knowledge, this study represents the first report on the antiproliferative effects of combination of rosemary extract and antineoplastic drug bleomycin, in spite of their wide single use and numerous studies on the subject. Considering these conclusions and our present results, we suggest the usage of rosemary extract in combination with the standard chemotherapeutics, for further studious investigations on a variety of human cancer cell lines.

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