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

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Received: April 24, 2019
Accepted: November 11, 2019
Published on-line: December 15, 2019
Published: December 25, 2019

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 (EC₅₀ 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.

Key words: cell growth; essential oil profile; polyphenolics; rosemary

http://dx.doi.org/10.5937/leksir1939044M

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, isolation), 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, antioxidant properties of rosemary in in vitro tumor cell models (Slamečková 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 anti-
oxidative properties on one side, and their effect as prooxi-
dants 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-depently 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 chemotherapy as well as other compounds of plant origin through synergetic 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 synergetic action of rosemary extract ingredients. As it is de-
scribed by some authors (Ivanova et al., 2014a; Plouzek et al., 1999), chemo-sensitising is the effect when biologically active molecule increases the activity of chemotherapy 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 moi-
eties in both DNA strands, interruption that is highly similar to those of ionizing radiation (Povirk, 1996).

Having in mind the antitumor potential of rosemary con-
stituents and a lack of data concerning this medicinal plant collected from the North Adriatic region, this study was con-
ducted 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 antiprolifera-
tive activity of these compounds, alone or in combination with bleomycin against human cell lines - one non-transformed hu-
man cell line MRC-5 and three malignant MCF-7 (breast ade-
3. MATERIALS AND METHODS

2.1. Plant material

Aerial parts of the tested wild growing Rosmarinus offici-
nalis were collected at Mali Kijec-Omišalj, Krk Island (Croa-
tia), located in the Northern Adriatic (GPS coordinates 32°59'59.8"N 14°8'15.44"E) in early June right before full blos-
som. Voucher specimen was confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Fac-
ulty of Sciences, University of Novi Sad, Serbia (Voucher N 2-1527). Plants were air dried in shade, and after that pulver-
ized in a mortar with pestle and a coffee mill and used for

2.2. Determination of individual essential oil components

using GC-MS analysis

The essential oil was isolated by hydrodistillation method us-
ing n-hexane as collecting solvent, according to European Phar-
macopoeia (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 x 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 (Agil-
ent 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% aque-
ous 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%
(v/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 replicas)
was centrifuged for 10 min at 1118 x g. Each supernatant was
derived using the Chromafil AO-20/25 polyamide filter
produced by Machery-Nagel (Düren, Germany) and trans-
ferred to a vial prior to injection into the HPLC (high perfor-
mane 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 Gem-
ini 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 isotro-
cic mixture for 5 min, followed by a linear gradient from 30%
to 90% B for 5 min, and then an isotropic 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
Finnigan, San Jose, CA) instrument with electrospray interface
(ESI) operating in negative ion mode. The analyses were car-
ried 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 compar-
ning 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 calcu-
lated from peak areas of the sample and the corresponding
standards and expressed in mg 100 g⁻¹ dry weight (DW). For

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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 Evolutions 220 (Thermo Scientiﬁc, San Jose, USA). TPC was determined by Folin-Ciocalteu method (Mikulic-Petkovsek et al., 2013). Tubes with 8.4 mL H₂O, 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% Na₂CO₃ 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, effect on growth and culture of the cell lines
For the estimation of cell growth activity, one non-transformed human line MRC-5 (fetal lung ﬁbroblast, ECACC 84101801) and three human malignant transformed cell lines: MCF-7 (breast adenocarcinoma ECACC No. 86012803), HeLa (cervix epitheloid carcinoma, 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 cm² ﬂasks at 37 °C in the atmosphere of 5% CO₂ 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 5x10³ cells per well for MRC-5 and MCF-7, 4 x 10³ cells for HeLa and 6x10³ cells for HT-29 in a volume of 180 µL, and pre-incubated in complete 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 cm² ﬂasks at 37 °C in the atmosphere of 5% CO₂ 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 5x10³ cells per well for MRC-5 and MCF-7, 4 x 10³ cells for HeLa and 6x10³ 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 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 the percent of the control (ˇCetojevi´c Simin et al., 2015). Based on concentration-cell growth curves, EC₅₀ values (concentration that inhibit cell growth by 50%) were determined using CalcuSyn Version 1.1 (Mike Hayball, Copyright Biosoft, 1996). Using EC₅₀ values obtained in a non-tumor cell line and in the respective tumor cell line, non-tumor/tumor EC₅₀ 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 (ˇCetojevi´c 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

<table>
<thead>
<tr>
<th>Compound name</th>
<th>RI</th>
<th>%sm/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-Thujene</td>
<td>932</td>
<td>0.31</td>
</tr>
<tr>
<td>a-Pinene</td>
<td>940</td>
<td>13.41</td>
</tr>
<tr>
<td>Camphene</td>
<td>955</td>
<td>4.36</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>983</td>
<td>3.95</td>
</tr>
<tr>
<td>Myrcene</td>
<td>992</td>
<td>5.64</td>
</tr>
<tr>
<td>a-Phellandrene</td>
<td>1009</td>
<td>0.43</td>
</tr>
<tr>
<td>Carene 3-delta</td>
<td>1014</td>
<td>1.63</td>
</tr>
<tr>
<td>a-Terpine 2</td>
<td>1020</td>
<td>0.46</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>1028</td>
<td>0.70</td>
</tr>
<tr>
<td>1,8-Cineole</td>
<td>1035</td>
<td>32.99</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>1064</td>
<td>0.79</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>1090</td>
<td>1.02</td>
</tr>
<tr>
<td>Linalool</td>
<td>1100</td>
<td>1.43</td>
</tr>
<tr>
<td>Camphor</td>
<td>1144</td>
<td>15.55</td>
</tr>
<tr>
<td>Borneol</td>
<td>1167</td>
<td>3.17</td>
</tr>
<tr>
<td>Terpinen 4-ol</td>
<td>1179</td>
<td>0.97</td>
</tr>
<tr>
<td>a-Terpine 5</td>
<td>1190</td>
<td>2.54</td>
</tr>
<tr>
<td>Verbenone</td>
<td>1206</td>
<td>1.88</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>1287</td>
<td>2.60</td>
</tr>
<tr>
<td>Caryophyllene Z</td>
<td>1406</td>
<td>3.08</td>
</tr>
<tr>
<td>a-Humulene</td>
<td>1456</td>
<td>0.59</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>1582</td>
<td>0.97</td>
</tr>
<tr>
<td>Total identified compounds</td>
<td>/</td>
<td>98.47</td>
</tr>
</tbody>
</table>

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-cineole (32.9%) and camphor (15.5%) were the dominant compounds. A considerable amount of some other monoterpenes compounds was also identified: α-pinene (13.4%), myrcene (5.6%), camphene (4.3%), β-pinene (3.9%), and bornene (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 hydroxycinamic 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). Besides these, low amounts of lignin midioresinol 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 3).

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 EC₅₀ values between 344.3 and 461.5 µg mL⁻¹ (Table 4). 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 Rosemary effect was HeLa (EC₅₀ 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 (EC₅₀, 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 (1.5-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 a-pinene have been related to antioxidant activities (Sedighi et al., 2015), whereas antiproliferative activity has been ascribed to 1,8-cineole 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, flavonoids 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-dio-methylisorosmanol, carnosic acid and 12-O-methylcarnosic acid are main phenolic diterpenes found in leaves of rosemary grown in the Mediterranean region. However, their investigations concluded that content of phenolic diterpenes 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 (Tounékti 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 (Yiseli-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)

<table>
<thead>
<tr>
<th>Rt (min)</th>
<th>[M-H] (m/z)</th>
<th>MS² [M-H] (m/z)</th>
<th>Tentative identification</th>
<th>mg per 100 g⁻¹ DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.2</td>
<td>341</td>
<td>179, 281, 251</td>
<td>caffeoyl-hexoside derivative 1</td>
<td>2.24±0.09</td>
</tr>
<tr>
<td>13.2</td>
<td>341</td>
<td>281, 179, 179</td>
<td>caffeoyl-hexoside derivative 2</td>
<td>7.97±1.10</td>
</tr>
<tr>
<td>15.0</td>
<td>353</td>
<td>179, 131, 136</td>
<td>4-O-cafeoylquinic acid derivative 1</td>
<td>0.15±0.00</td>
</tr>
<tr>
<td>16.4</td>
<td>353</td>
<td>179, 131, 136</td>
<td>4-O-cafeoylquinic acid derivative 2</td>
<td>0.29±0.00</td>
</tr>
<tr>
<td>19.4</td>
<td>371</td>
<td>353</td>
<td>caffeic acid derivative</td>
<td>0.91±0.00</td>
</tr>
<tr>
<td>26.4</td>
<td>677</td>
<td>515, 353, 319, 509, 191</td>
<td>1,3,5-tri-O-cafeoylquinic acid</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>15.0</td>
<td>387</td>
<td>207</td>
<td>medioresinol derivative 1</td>
<td>0.12±0.00</td>
</tr>
<tr>
<td>15.1</td>
<td>387</td>
<td>207</td>
<td>medioresinol derivative 2</td>
<td>0.24±0.00</td>
</tr>
<tr>
<td>19.4</td>
<td>387</td>
<td>207</td>
<td>medioresinol derivative 3</td>
<td>0.50±0.00</td>
</tr>
<tr>
<td>26.4</td>
<td>677</td>
<td>515, 353, 319, 509, 191</td>
<td>1,3,5-tri-O-cafeoylquinic acid</td>
<td>0.94±0.00</td>
</tr>
<tr>
<td>21.6</td>
<td>521</td>
<td>353, 161, 197</td>
<td>rosmarinic acid derivative 1</td>
<td>59.85±2.31</td>
</tr>
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<td>161, 197, 225</td>
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<td>0.85±0.01</td>
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<tr>
<td>34.5</td>
<td>359</td>
<td>207</td>
<td>medioresinol derivative 3</td>
<td>0.24±0.00</td>
</tr>
<tr>
<td>35.4</td>
<td>359</td>
<td>207</td>
<td>medioresinol derivative 4</td>
<td>0.50±0.00</td>
</tr>
<tr>
<td>35.5</td>
<td>359</td>
<td>207</td>
<td>medioresinol derivative 5</td>
<td>0.50±0.00</td>
</tr>
<tr>
<td>33.8</td>
<td>345</td>
<td>301, 283</td>
<td>epirosmanol derivative 1</td>
<td>3.34±0.06</td>
</tr>
<tr>
<td>33.9</td>
<td>345</td>
<td>301, 283</td>
<td>epirosmanol derivative 2</td>
<td>3.34±0.06</td>
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</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Rt (min)</th>
<th>[M-H] (m/z)</th>
<th>MS² [M-H] (m/z)</th>
<th>Tentative identification</th>
<th>mg per 100 g⁻¹ DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.9</td>
<td>593</td>
<td>503, 491</td>
<td>apigenin-6,8-C-diglucoside (vicenin II)</td>
<td>1.63±0.02</td>
</tr>
<tr>
<td>23.0</td>
<td>597</td>
<td>447, 285</td>
<td>luteolin-rhamnoglucoside (luteolin-7-rutinoside)</td>
<td>16.17±0.12</td>
</tr>
<tr>
<td>24.2</td>
<td>461</td>
<td>285</td>
<td>luteolin-3-glucuronide 1</td>
<td>16.47±0.14</td>
</tr>
<tr>
<td>25.3</td>
<td>461</td>
<td>285</td>
<td>luteolin-3-glucuronide 2</td>
<td>13.55±0.09</td>
</tr>
<tr>
<td>27.6</td>
<td>461</td>
<td>285</td>
<td>luteolin-3-glucuronide 3</td>
<td>85.28±0.19</td>
</tr>
<tr>
<td>17.2</td>
<td>503</td>
<td>285, 399</td>
<td>luteolin-3-O-(O-acetyl)-β-D-glucuronide derivative 1</td>
<td>12.02±0.07</td>
</tr>
<tr>
<td>30.8</td>
<td>503</td>
<td>285, 399</td>
<td>luteolin-3-O-(O-acetyl)-β-D-glucuronide derivative 2</td>
<td>44.73±0.21</td>
</tr>
<tr>
<td>31.8</td>
<td>503</td>
<td>285, 399</td>
<td>luteolin-3-O-(O-acetyl)-β-D-glucuronide derivative 3</td>
<td>62.81±1.30</td>
</tr>
<tr>
<td>32.1</td>
<td>503</td>
<td>285, 443</td>
<td>luteolin-3-O-(O-acetyl)-β-D-glucuronide derivative 4</td>
<td>49.84±1.24</td>
</tr>
<tr>
<td>31.5</td>
<td>607</td>
<td>299</td>
<td>diosmetin-8-C-rhamnosyl-7-O-glucoside (diosmin)</td>
<td>20.02±0.14</td>
</tr>
<tr>
<td>25.6</td>
<td>609</td>
<td>447, 285</td>
<td>kaempferol-3,7-di-O-glucoside</td>
<td>8.27±0.02</td>
</tr>
<tr>
<td>22.0</td>
<td>593</td>
<td>285</td>
<td>kaempferol-3-O-rutinoside</td>
<td>5.14±0.01</td>
</tr>
<tr>
<td>12.8</td>
<td>477</td>
<td>315</td>
<td>isorhamnetin hexoside</td>
<td>5.95±0.05</td>
</tr>
<tr>
<td>23.5</td>
<td>477</td>
<td>315, 301</td>
<td>isorhamnetin-3-O-hexoside derivative 1</td>
<td>31.37±1.10</td>
</tr>
<tr>
<td>25.7</td>
<td>477</td>
<td>315, 301</td>
<td>isorhamnetin-3-O-hexoside derivative 2</td>
<td>2.32±0.04</td>
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<tr>
<td>24.8</td>
<td>491</td>
<td>315, 300</td>
<td>isorhamnetin-3-O-glucuronide</td>
<td>2.72±0.09</td>
</tr>
<tr>
<td>25.9</td>
<td>639</td>
<td>477, 315, 300</td>
<td>isorhamnetin-3,7-diglucoside</td>
<td>10.81±1.12</td>
</tr>
<tr>
<td>22.7</td>
<td>623</td>
<td>315, 299, 477</td>
<td>isorhamnetin-3-O-rhamnosyl hexoside derivative 1</td>
<td>3.94±0.07</td>
</tr>
<tr>
<td>27.1</td>
<td>623</td>
<td>315, 300</td>
<td>isorhamnetin-3-O-rhamnosyl hexoside derivative 2</td>
<td>5.00±0.06</td>
</tr>
<tr>
<td>27.8</td>
<td>623</td>
<td>315, 300</td>
<td>isorhamnetin-3-O-rhamnosyl hexoside derivative 3</td>
<td>6.09±0.07</td>
</tr>
<tr>
<td>19.9</td>
<td>609</td>
<td>301</td>
<td>quercetin-3-rhamnosyl hexoside derivative 1</td>
<td>4.99±0.03</td>
</tr>
<tr>
<td>24.5</td>
<td>609</td>
<td>301</td>
<td>quercetin-3-rhamnosyl hexoside derivative 2</td>
<td>16.47±0.09</td>
</tr>
<tr>
<td>20.5</td>
<td>463</td>
<td>301</td>
<td>quercetin-3-O-glucoside</td>
<td>8.72±0.13</td>
</tr>
<tr>
<td>23.9</td>
<td>625</td>
<td>301, 463</td>
<td>quercetin-dihexoside</td>
<td>16.47±0.17</td>
</tr>
<tr>
<td>27.8</td>
<td>653</td>
<td>315, 300</td>
<td>quercetin-diglucuronide</td>
<td>8.78±0.07</td>
</tr>
</tbody>
</table>

Effects. 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.

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-
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 different origins in Iran and comparison with other countries, Food and Chemical Toxicology 80: 215–222.

Table 4. EC50 values (µg ml⁻¹) and non-tumor/tumor EC50 ratios (NT/T) in human cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>R⁴</th>
<th>R + BLM</th>
<th>BLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-5</td>
<td>344.32</td>
<td>170.81</td>
<td>80.3</td>
</tr>
<tr>
<td>MCF-7</td>
<td>407.91</td>
<td>58.6</td>
<td>119.81</td>
</tr>
<tr>
<td>HeLa</td>
<td>385.15</td>
<td>162.85</td>
<td>77.2</td>
</tr>
<tr>
<td>HT-29</td>
<td>461.54</td>
<td>291.27</td>
<td>165.31</td>
</tr>
<tr>
<td>MCF-7 (NT/T)</td>
<td>0.84</td>
<td>2.91</td>
<td>0.67</td>
</tr>
<tr>
<td>HeLa (NT/T)</td>
<td>0.89</td>
<td>1.05</td>
<td>1.04</td>
</tr>
<tr>
<td>HT-29 (NT/T)</td>
<td>0.75</td>
<td>0.59</td>
<td>0.49</td>
</tr>
</tbody>
</table>

⁴ EC50 values for effect of rosemary extract (R) and bleomycin were established for bleomycin (BLM) concentration of 100 µg ml⁻¹.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant number 175056).

REFERENCES


P. Furlas, Strasbourg.


