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## Assessment of polyphenol content, *in vitro* antioxidant, antimicrobial and toxic potentials of wild growing and cultured rue

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### Summary

*Ruta graveolens* L. (rue) is an edible medicinal plant that is traditionally used in various countries. This study aimed to investigate and compare the phenolic content, antioxidant capacity, antibacterial and cytotoxic activities of the methanolic and ethanolic extracts of wild growing and cultured rue. The total phenolic content of the tested extracts varied from 57.90 to 166.91 mg of catechin equivalent (CE)/g of extract and the total flavonoid content from 4.18 to 26.87 mg of rutin equivalent (Ru)/g of extract. All the tested samples exhibited significant antioxidant potential in DPPH radicals scavenging and lipid peroxidation inhibition assays (comparable with activity of rutin in the same test systems), antimicrobial activity determined by microdilution method (particularly against Gram (+) bacteria strains) and ability to induce inhibition of HeLa cells growth and proliferation (up to 71.81 %). In addition, rue-treated HeLa cells showed various morphological changes after 72 h of incubation with rue extracts. Extracts from wild growing rue with the highest polyphenol, tannin and flavonoid contents demonstrated the strongest activities in all tested systems. The present study also emphasized the fact that the rue leaves and herb should be harvested at the beginning of blossoming stage in order to achieve the maximal level of secondary metabolites and optimal pharmacological effects.

### Introduction

*Ruta graveolens* L. (Rutaceae), rue, is a shrub-like herbaceous perennial aromatic plant that is native to Mediterranean region (TOWNSEND, 1968). It is cultivated in the gardens all over the world, though preferably grows in Mediterranean climates.

Rue is highly used in the traditional medicine in various countries to treat a variety of ailments, ranging from absence of menstruation to rheumatism and various mental conditions (PDR, 2000; TUCAKOV, 1997). Due to the lack of sufficient evidence of the efficacy of the drug in the proposed indications and the unfavorable ratio of benefit to risk, the Commission E gave the drug, rue leaves and herb, a negative rating. Rue can cause contact dermatitis, phototoxic reactions, severe liver and kidney damage, while serious undesirable effects can occur even after administration of therapeutic doses (BLUMENTHAL, 1998; FRITZ WEISS and FINTELMANN, 2000).

This edible plant has been part of East Asian diets for many years and has dual function as food and medicine (YANG et al., 2006). In the European Union, *R. graveolens* herb may be used as spice and flavoring agent in certain food products as for instance baked goods, frozen dairy products, soft candy or non-alcoholic beverages. *R. graveolens* and its essential oil have been approved for Generally Recognized as Safe status by the United States Food and Drug Administration and may be added to human food as flavoring agents (up to 2000 and 10 000 µg/kg, respectively). Rue herb and oil are also allowed in the United States in animal feedstuffs at the same levels (EMA, 1999).

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The plant contains active compounds like flavonoids, alkaloids, coumarin derivatives, lignans and essential oils (PDR, 2000). The drug (rue herb and/or leaves) is antimicrobial, abortifacient, and photosensitizing. As the current information shows, it expresses pharmacological functions including anti-inflammatory, analgesic, antian-drogenic, antihyperlipidemic, antihyperglycemic, xantine oxidase inhibition and anticancer activities, among others (ASGARPANAH and KHOSHKAM, 2012; YANG et al., 2006).

VITKOVA and PHILIPOV (1999) conducted a comparative phytochemical study of rue with material from natural Bulgarian populations of the species and from cultivated specimens. Their conclusion was that cultivation of rue revealed changes in the second metabolite composition.

However, as far as our literature survey could ascertain, our study is the first comparative study of effects of wild growing and cultured *Ruta graveolens*. The focus of our research was to explore and compare antimicrobial and antioxidant activity alongside phenolic contents of ethanolic and methanolic extracts of plants collected from two different localities (rocky slopes and private garden). Additionally, the antiproliferative/cytotoxic activity of these extracts on the human cervix adenocarcinoma HeLa S3 cell line was included in this study, due to the fact that rue is well-known traditional uterine remedy (PDR, 2000; TASIĆ et al., 2004; TUCAKOV, 1997). Therefore the study sought to elucidate the impact of plant growth conditions and geographical location on these properties.

### Material and methods

#### General

Plant materials were collected from *Ruta graveolens* L. (Rutaceae) in 2007 at two different locations: 1) Sićevačka gorge, Sićevo (E Serbia), cca. 590 m a.s.l., rocky and bushy slopes at the limestone ground; GPS Coordinates: 43°20'34.60"N; 22°6'30.79"E; 2) Niš (Novo selo), (SE Serbia), cca. 180 m a.s.l., cultivated in the garden; GPS Coordinates: 43°19'3.39"N; 21°48'44.41"E. The aerial parts of the wild growing and cultivated plants were collected at the beginning of the flowering season and the aerial parts of the wild growing plants were also collected at the end of the flowering season. The plant was identified and authenticated by the taxonomist Dr. Bojan Zlatković. The voucher herbarium specimens (accession number 3106 HFF) have been deposited in the Herbarium collection of the Faculty of Pharmacy, University of Belgrade.

The plant material was reduced to a fine powder and extracted with ethanol (70 %, v/v) or methanol (80 %, v/v) by percolation, as described in European Pharmacopoeia 6.0.<sup>10</sup> (Ph. Eur. 6.0, 2007). Ethanolic and methanolic extracts of wild growing plants collected at the beginning (RE and RM) and at the end of the flowering season (R2E and R2M), and of cultured plants (RDE and RDM) were obtained after evaporation to the dryness under reduced pressure below 40 °C.

The reference chemicals such as rutin and catechin used for calibration curves were purchased from Sigma-Aldrich (St. Louis, USA),

and cisplatin as commercial cytostatic from Pfizer (Australia). All other chemicals, reagents and solvents used were of analytical grade.

Spectrophotometric measurements were performed using Evolution 60 Thermo scientific spectrophotometer (Fisher Scientific, UK) and Multiskan Ascent No354 (ThermoLabsystems, Finland) ELISA microplate reader. Incuterm Raypa®trade (Catalonia, Spain) and Jouan, EG 110IR (Saint Herblain, France) were used for incubation and Vibramix 30 (Tehtnica, Slovenia) for shaking of microplates. Phenotype changes of HeLa cells were evaluated by invert microscope (Observer Z1, Carl Zeiss, Germany).

All results are presented as mean  $\pm$  standard deviation. Data obtained by MTT assay after HeLa cells treatment with rue extracts were compared to control (untreated cells) using paired sample t-test. Statistical analysis was performed with commercial statistical software package. Values of  $p < 0.01$  and  $p < 0.001$  were accepted for statistical significance and marked on figures as \* and \*\*, respectively.

### Determination of constituents

#### Determination of total flavonoids

The content of total flavonoids was determined spectrophotometrically using aluminium chloride (LAMAISON and CARNAT, 1990). Quantification was done on the basis of a standard curve with rutin and results were expressed as mg rutin (Ru)/g of dry extract.

#### Determination of total polyphenols

The total phenolic content of extracts was estimated using the Folin-Ciocalteu colorimetric procedure (MAKKAR et al., 2000). Quantification was performed using a standard curve with (+)-catechin (concentration span 0.1-1 mg/ml). The results were expressed as catechin equivalents (mg CE/g extract) per g of sample.

#### Determination of total tannins

Total tannins content was determined by the same Folin-Ciocalteu procedure after removal of tannins by their adsorption on insoluble matrix – polyvinylpyrrolidone (MAKKAR et al., 2000). The assay was carried out with clear supernatant and results were expressed as mg CE/g of sample.

### Determination of antioxidant capacity

#### DPPH assay

The free radical scavenging activities were determined using a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical (CUENDET et al., 1997). Various concentrations of samples were mixed with methanol or ethanol solution of DPPH $\cdot$  (0.05mM), vigorously shaken and left in the dark at room temperature for 30 min. Inhibition of DPPH free radical in percent was calculated according to:

$$\% \text{ DPPH} = (A_b - A_s / A_b) \times 100,$$

where  $A_b$  is the absorbance of the control reaction (containing ethanol or methanol instead of test solution), and  $A_s$  is the absorbance of the sample.

Dose response curves were constructed and DPPH results calculated as the concentration of sample required for scavenging 50 % of the free radical (IC<sub>50</sub>). Concentrations are expressed in mg/ml. Rutin was used as reference compound.

#### Inhibition of lipid peroxidation

Inhibition of lipid peroxidation in  $\beta$ -carotene-linoleic acid assay was determined according to the slightly modified method described by

KOLEVA et al. (2002). In brief, 200  $\mu$ l of freshly prepared  $\beta$ -carotene-linoleic acid emulsion were added to 20  $\mu$ l of sample (different concentrations of ethanol extracts in ethanol, 70 % v/v, or methanol extracts in methanol, 80 %, v/v) in each well of 96-well microtitration plate. Samples were prepared in triplicate for each concentration used. The plate was shaken on a microplate shaker and read in microplate reader using a 450 nm filter immediately ( $t = 0$  min) and after 120 min ( $t = 120$  min) of incubation at 55 °C. The percentage inhibition of  $\beta$ -carotene bleaching by the samples was calculated according to formula (BAROS et al, 2007):

$$\% \text{ inhibition} = (A_{120} / A_0) \times 100$$

where  $A_{120}$  is the absorbance of the sample at  $t = 120$  min and  $A_0$  is the absorbance of the sample at  $t = 0$  min.

IC<sub>50</sub> value, which denotes the concentration of sample that is required for decreasing the  $\beta$ -carotene bleaching at 50 %, was calculated and expressed in mg/ml. Rutin was used as reference compound.

### Assessment of antimicrobial activity

For the antibacterial bioassays six bacterial strains were used, three Gram-positive: *Bacillus cereus* (ATCC 10876), *Listeria monocytogenes* (ATCC 15313) and *Staphylococcus aureus* (ATCC 6538), and three Gram-negative: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Salmonella enteritidis* (ATCC 13076). *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404) were used for antifungal estimation. All microorganisms were from the American Type Culture Collection (ATCC).

The bacterial and fungal inoculates were made up from overnight broth cultures. Suspensions with microorganisms were adjusted to 0.5 McFarland standard turbidity according to consensus standard of the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Determination of MIC (minimum inhibitory concentration) and MBC/MFC (minimum bactericidal/fungicidal concentration) was carried out by micro-well dilution method according to the NCCLS (2003). Serial doubling dilutions of the tested extracts were prepared in the 96-well microtiter plates in inoculated nutrient broth. The final volume was 100  $\mu$ l and the final bacterial concentration was  $2 \times 10^6$  CFU/ml in each well and  $2 \times 10^5$  of spores for fungal strains. The plates were incubated for 24 h at 37 °C for bacteria and 48 h at 25 °C for fungi. Microbial growth was determined by adding 20  $\mu$ l of 0.5 % triphenyl tetrazolium chloride (TTC) aqueous solution (SARTORATTO et al., 2004). The minimal concentration where there was no visible growth was defined as minimal inhibitory concentration (MIC). For MBC/MFC determination broth was taken from each well and inoculated into Mueller Hinton agar at 37 °C for 24 h for bacteria or in malt extract agar at 25 °C for 48 h for fungal strains. MBC/MFC was defined as the lowest concentration of extract which had killed 99.9 % of microorganism cells (NCCLS, 2003).

Chloramphenicol, streptomycin, and nystatin were used as positive control for Gram-positive bacteria, Gram-negative bacteria and fungal strains, respectively. A 10 % solution of dimethyl sulfoxide (DMSO) in water, used for extracts dissolving, was used as a negative control.

### Determination of cell viability and proliferation

Viability and proliferation of HeLa S3 cells (human cervical adenocarcinoma cell line) after treatment with ethanolic and methanolic extracts of cultured and wild growing rue (both collected on the beginning of flowering period) were determined by MTT test (ISO, 1998; KOSTIĆ et al., 2008).

Dry extracts were dissolved in DMEM (Dulbecco's Modified Eagle's Minimal Essential Medium, PAA Laboratories GmbH) enriched with L-glutamine, 100 IU/ml penicillin, 100 GU/ml streptomycin and 10 %

fetal calf serum (Fetal Calf Serum, Gibco, United Kingdom) to the concentration of 1 mg/ml (stock solutions), followed by filtration through syringe filters (pore diameter 0.2 µm, Millipore, Bedford, MA, USA). Further, stock solutions were diluted in previously described culture media. Final concentrations of the extracts were 0 (drug-free control), 0.1, 1, 10, 100 and 1000 µg/ml. For viability testing  $1 \times 10^5$  cells in 0.1 ml of extracts were seeded in sterile 96-well plate and incubated in a humidified incubator at 37 °C in an atmosphere of 5 % CO<sub>2</sub> for 24 hours. For proliferation testing  $1 \times 10^4$  cells in 0.1 ml of extracts were seeded in sterile 96-well plate and incubated in a humidified incubator at 37 °C in an atmosphere of 5 % CO<sub>2</sub> for 72 hours. The medium in which cells were incubated was drawn at the end of the incubation and the cells were washed with 0.1 ml of PBS (phosphate buffered saline) and 0.02 ml of MTT was added. After 4 hour incubation at 37 °C, the formazan crystals were dissolved with 0.1 ml of isopropanol. Spectrophotometric measurement of the intensity of MTT reduction was carried out at optical density of 540 nm on the multi-channel photometer. To get inhibition of cell viability (%), which was the parameter of cytotoxic effects, and inhibition of cell proliferation (%), which was the parameter of cytostatic effects, A (absorbance at 570 nm) of a sample with cells grown in the presence of various concentrations of the extracts was subtracted and divided with control optical density (the A of control cells grown only in culture medium), and multiplied by 100. Cisplatin was used as a positive control. MTT test was performed through three independent experiments in triplicates. HeLa cells morphological characteristics were observed after 72 h incubation with extracts under the invert microscope.

## Results and Discussion

### Phenolic content

Since food phenolics from spices may augment the body's source of natural antioxidants, their consumption could offer protection against chronic diseases caused by free radicals and could also augment cellular defenses against oxidative damage (OTUNOLA et al., 2013). Also, the putative therapeutic effects of many traditional medicines have been ascribed to phenolics in particular due to their antioxidant activity. In order to compare wild growing and cultivated rue, the first approach was to quantify total phenolic contents in methanolic and ethanolic extracts alongside with their antioxidant activity. Considerable amounts of phenolic compounds were found

in rue extracts (Tab. 1). The total phenolic content varied from 57.90 to 166.91 mg of catechin equivalent (CE)/g of extract and the total flavonoid content from 4.18 to 27.72 mg of rutin equivalent (Ru)/g of extract. The tannin fraction represents more than half of the total polyphenolic compounds (from 50.45 % in RDE to 61.71 % in RM).

It was established that rutin content was influenced by the climatic condition. According to VITKOVA and PHILIPOV (1999), the above-ground parts of *R. graveolens* from experimental plots in Bulgaria manifested a slightly enhanced quantity of rutin as compared to those from the natural populations. Our results indicated three to four fold lower content of rutin in extracts obtained from cultivated compared to wild growing plants. Also, we agreed with colleagues in conclusions that the above-ground parts of the plants had the highest rutin content at the beginning of blossoming and that quantity of tannins manifested a decrease in cultivated plants.

We emphasize that extraction yields were notably higher for extracts obtained from wild growing rue. Namely, extraction yields for ethanolic and methanolic extracts of wild growing plants collected at the beginning (RE and RM) and at the end of the flowering season (R2E and R2M), and of cultured plants (RDE and RDM) were 27.3, 23.3, 23.95, 19.45, 13.2 and 14.2 % (w/w), respectively.

### Antioxidant activity

Antioxidant activity was determined through two complementary test systems: DPPH free radical scavenging and inhibition of the lipid peroxidation in β-carotene-linoleic acid system. Results are presented in Tab. 1. According to DPPH assay, our samples possess notable and quite similar anti-radical activity with IC<sub>50</sub> range from 36.36±1.20 µg/ml (R2M) to 59.49±4.31 µg/ml (RDE). All extracts also showed considerable, although less pronounced activity against lipid peroxidation. IC<sub>50</sub> values in β-carotene-linoleic acid test system varied in wider extent: from 31.06±2.30 µg/ml (R2M) to 185.55±1.86 µg/ml (RDE). Methanolic extract of wild rue collected at the end of flowering season (R2M) exhibited the strongest antioxidant activity. Its antioxidant effects are comparable and in the β-carotene-linoleic acid test system even stronger than antioxidant activity of rutin. Ethanolic extract of wild rue collected at the beginning of flowering season (RE) also exhibited significant activities in DPPH and β-carotene-linoleic acid tests with IC<sub>50</sub> values 36.41±1.37 µg/ml and 70.81±2.40 µg/ml, respectively.

**Tab. 1:** Phenolic contents of rue extracts and IC<sub>50</sub> values of *Ruta graveolens* extracts and rutin in antioxidant assays.

Extract	Total polyphenols (mg CE/g)	Tannins (mg CE/g)	Flavonoids (mg Ru/g)	Radical scavenging activity IC <sub>50</sub> (µg/ml)	Inhibition of lipid peroxidation IC <sub>50</sub> (µg/ml)
RM	154.04±3.53	95.06±1.05	26.87±0.36	40.65±2.53	93.95±3.83
R2M	87.55±1.84	49.89±3.16	23.58±0.16	36.36±1.20	31.06±2.30
RDM	57.90±1.24	29.36±0.12	4.18±0.09	45.08±1.40	105.62±3.47
RE	166.91±2.62	102.69±1.64	27.72±0.15	36.41±1.37	70.81±2.40
R2E	95.87±2.34	53.55±1.96	26.77±0.27	36.43±2.01	91.63±6.91
RDE	71.72±2.35	36.18±2.62	7.10±0.07	59.49±4.31	185.55±1.86
Rutin				2.68±0.25	33.29±1.49

RM – methanolic extract of wild growing plants collected at the beginning of the flowering season

R2M – methanolic extract of wild growing plants collected at the end of the flowering season

RDM – methanolic extract of cultured plants collected at the beginning of the flowering season

RE – ethanolic extract of wild growing plants collected at the beginning of the flowering season

R2E – ethanolic extract of wild growing plants collected at the end of the flowering season

RDE – ethanolic extract of cultured plants collected at the beginning of the flowering season

Among the tested samples, extracts obtained from cultivated plants (RDM and RDE) have the strikingly lower total polyphenols, tannins and flavonoids values. Thus, cultivation of the rue revealed changes in the second metabolite composition. Accordingly, as we expected, these extracts showed lower antioxidant activity in both applied tests. These differences were determined by the ecological factors of the environmental characteristic of the respective locations and the plants capability for adaptation to the new conditions. The climatic and the soil conditions in the area of Sićevo George rise to considerable spring and summer water shortages. It is known that drought is a stress factor for plants and entails intensification of phenolic biosynthesis which apparently is equally valid for *R. graveolens* (KITIĆ, 2006; TAVARINI and ANGELINI, 2013). Significant influence of environmental conditions on the phenolic compounds and consequently to antioxidant capacity of various fruits and medicinal plants have been well described, indicating the important role played by location, UV radiation, temperature, water stress and mineral nutrient availability in determining antioxidant capacity (TAVARINI and ANGELINI, 2013). The lower flavonoid, total polyphenolic and tannin content in cultivated plants from Novo Selo could be explained with the greater precipitation in the region during the period of investigation, type of soils and the high level of underground water and water supply in garden conditions.

#### Antimicrobial activity

The antimicrobial activity of methanolic and ethanolic extracts of *R. graveolens* collected at the beginning of flowering season was tested against selected microorganisms. The samples exhibited moderate antimicrobial activity against most of the bacterial and fungal strains tested. Inhibitory effects on the growth of Gram-positive bacteria were more potent (Tab. 2.).

Methanolic rue extracts were active in inhibiting of the growth of all the Gram-positive bacteria and *P. aeruginosa* (MIC 25 or lower than 25 mg/ml) except low activity of RDM against strains of *S. aureus* (MIC 100 mg/ml). The anti-microbial activity of rue methanolic ex-

tract against *P. aeruginosa* has already been reported and attributed to presence of alkaloids, flavonoids, terpenoids, steroids and tannins (BENAZIR et al., 2011). According to IVANOVA et al. (2005) methanolic extract of cultivated rue and its fractions showed no activity against the Gram-negative strain *E. coli* and the fungus *C. albicans* in disk diffusion method. Same samples demonstrated a good antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes* and *Bacillus subtilis* and no inhibition of *Corynebacterium diphtheriae*. Our results for RM and RDM antimicrobial activity string along these findings.

For ethanolic extract of wild rue (RE), the most prominent effect was achieved on *Listeria monocytogenes* with equal concentrations of MIC and MBC, 6.25 mg/ml. The lowest inhibitory activity was noticed for *S. enteritidis* (50 mg/ml), while MBC for all tested Gram-negative bacterial species was not reached at the highest concentration applied (100 mg/ml). RE exhibited the same antifungal activity on *C. albicans* and *A. niger* (MIC=MBC 25 mg/ml).

The major classes of secondary metabolites identified in rue herb were alkaloids, flavonoids, coumarin derivatives, lignans, terpenoids, primary and secondary alcohols, quinines, fatty acids, steroids and other minor components (BENAZIR et al., 2011; PDR, 2000). Besides methoxypsoralens, that are generally cytotoxic and have been widely reported to be fungitoxic, quinoline and quinolone alkaloids are important to the rue plant in defense against plant pathogens (OLIVA et al., 2003).

As referent antimicrobial drugs, streptomycin, chloramphenicol and nystatin exhibited obviously higher antimicrobial activity than rue extracts which is additionally substantiated with obtained MIC/MBC values. However, extracts of wild rue showed a better antimicrobial activity than cultivated rue extracts and the most active of all tested samples was ethanolic extract of wild rue.

#### Influence on HeLa cell viability and proliferation

The MTT test has been widely used as rapid and sensitive method for screening anticancer drugs as well as for the assessment of cytotoxic

**Tab. 2:** Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values for *Ruta graveolens* extracts and positive controls.

Microorganism	RM MIC/MBC mg/ml/mg/ml	RDM MIC/MBC mg/ml/mg/ml	RE MIC/ MBC mg/ml/mg/ml	RDE MIC/MBC mg/ml/mg/ml	Positive control* MIC/ MBC µg/ml/µg/ml
Gram-positive bacteria					
<i>Staphylococcus aureus</i>	25 / >100	100 / >100	12.5 / 25	50 / 100	1/8
<i>Bacillus cereus</i>	12.5 / 100	25 / >100	6.25 / 25	25 / 50	4/16
<i>Listeria monocytogenes</i>	12.5 / 100	25 / >100	6.25 / 6.25	50 / >100	8/16
Gram-negative bacteria					
<i>Pseudomonas aeruginosa</i>	12.5 / 25	12.5 / 12.5	6.25 / >100	50 / 100	8/8
<i>Salmonella enteritidis</i>	>100 / >100	100 / >100	50 / >100	12.5 / >100	4/4
<i>Escherichia coli</i>	100 / >100	>100 / >100	12.5 / >100	100 / 100	16/16
Fungal strains					
<i>Candida albicans</i>	100 / 100	25 / >100	25 / 25	50 / 100	8/16
<i>Aspergillus niger</i>	100 / 100	100 / >100	25 / 25	50 / 100	16/16

\*positive control: streptomycin for Gram-positive bacteria, chloramphenicol for Gram-negative bacteria and nystatin for fungal strains

RM – methanolic extract of wild growing plants collected at the beginning of the flowering season

R2M – methanolic extract of wild growing plants collected at the end of the flowering season

RDM – methanolic extract of cultivated plants collected at the beginning of the flowering season

RE – ethanolic extract of wild growing plants collected at the beginning of the flowering season

R2E – ethanolic extract of wild growing plants collected at the end of the flowering season

RDE – ethanolic extract of cultivated plants collected at the beginning of the flowering season

and cytostatic effect of different samples (KOSTIĆ et al., 2008). This colorimetric assay is based on the uptake and the reduction by mitochondrial succinate dehydrogenase of the yellow water soluble substrate MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) to an insoluble purple formazan product. Process depends both on the number of cells present and on mitochondrial activity per cell (CHIBA et al. 1998).

Cells that were used are adherent epithelial cells, and the possible toxic effect of the rue extracts was evaluated through the possible change of their characteristics. Reduction of adherent phenotype was sign of the toxic effect. The round forms of living cells was considered as non-adherent cells. Impact of different concentration of rue extracts on HeLa cells morphology was shown on Fig. 1. In control culture, HeLa cells (Fig. 1d) expressed typical morphology of adherent cells, and no apoptotic changes were observed. In contrast, treatment with rue extracts for 72 h had dramatic effects on cell proliferation as well as cell morphology. Upon rue extracts exposure, the proliferation of HeLa cells decreased remarkably also, most of the cells were rounded, reduced in size and detached from the substratum of the culture dish. In addition, cells exposed to the extract revealed typical apoptotic morphology (Fig. 1c). Decreased cell proliferation and reduction of adherent cells in presence of tested extracts were observed. Morphological changes in higher level were observed in cells that were incubated in a medium containing higher concentrations of all types of extracts. In the lowest applied concentration phenotypic changes were also present as phyllopodic and thread-like extensions and changes of cell shape.

The HeLa cell viability and proliferation were significantly reduced by rue extract treatment. The impacts of different concentrations of methanolic and ethanolic extracts of *R. graveolens* collected at the beginning of flowering season on HeLa cells after 24 h of incubation

and after 72 h of incubation (as % of inhibition) were presented on Fig. 2 and 3, respectively. Since longer exposure to rue extracts leads to stronger inhibition of HeLa cells growth and proliferation, these effects are time-dependent.

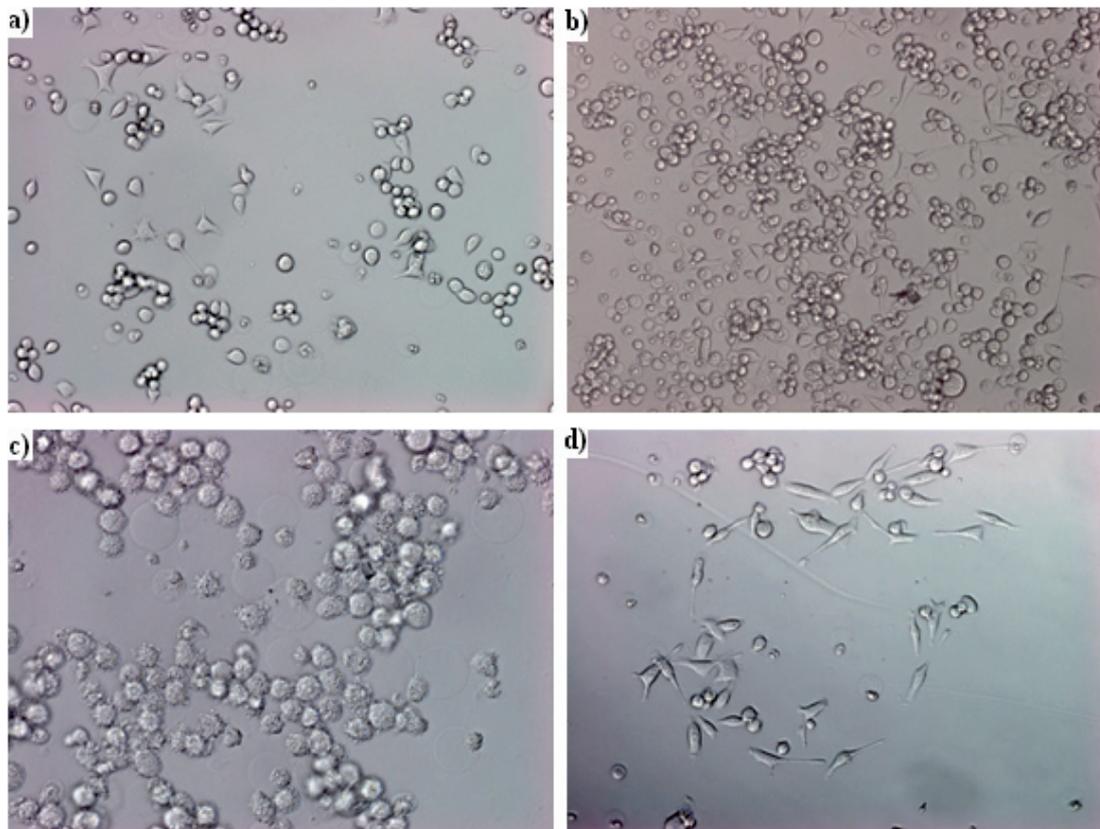
Viability of HeLa cells in presence of extracts (except lowest concentrations of ethanolic extract of cultured rue) was lower than viability of untreated control. RE showed maximal cytotoxic effect in this assay (inhibition of cell viability was 38.98 %).

All investigated samples (except lowest concentrations of ethanolic extracts of wild growing plants and of cultured plants collected both at the beginning of the flowering season) showed statistically significant ( $p < 0.001$ ) inhibition of HeLa cells proliferation after 72 h of incubation. Maximal inhibition ( $71.81 \pm 3.81$  %) was measured in culture with greatest concentration of wild rue ethanolic extract. There are no statistically significant differences between effects of 4 investigated extracts. Our results indicate notable *in vitro* antiproliferative activity of *R. graveolens* in HeLa cells (Fig. 3).

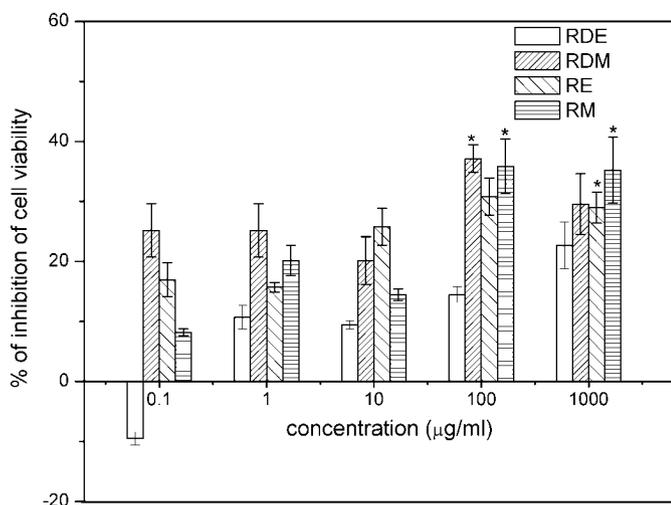
Cells viability and proliferation were not inhibited in a strictly dose-dependent manner (Fig. 2, Fig. 3), although strongest inhibition was found at the highest rue concentrations applied. HeLa cell line proved to be more sensitive to rue extracts obtained from wild growing plants.

PREETHI et al. (2006) stated that prooxidant activity of high concentrations of *R. graveolens* extract may be responsible for cytotoxic action and its ability to produce tumor reduction. Our study confirmed only a strong antioxidant activity in tested concentration span of rue extracts. Bearing in mind that oxidative stress is commonly connected to cell death (FULDA et al., 2010), antioxidant activity of rue extracts anyway contributes to their overall effect on HeLa cell growth and viability.

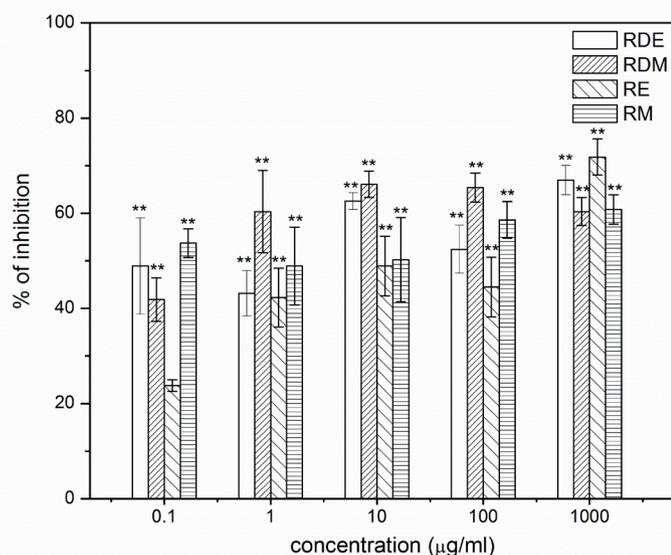
The present study, on one hand, brings forward the new data regard-



**Fig. 1:** Phenotypic changes of HeLa cells in a) 0.1 µg/ml RE – ethanolic extract of wild growing plants, b) 10 µg/ml RDE – ethanolic extract of cultured plants, c) 1000 µg/ml RE – ethanolic extract of wild growing plants, and d) untreated cells (control).



**Fig. 2:** The influence of different rue samples on HeLa cell viability (% of inhibition of cell viability) after 24 h of incubation. Differences of the effects of different *Ruta graveolens* extracts (RM – methanolic extract of wild growing plants, RDM – methanolic extract of cultured plants, RE – ethanolic extract of wild growing plants, RDE – ethanolic extract of cultured plants) in different concentrations to untreated control were checked using t-test, significant differences  $p < 0.01$  being marked with (\*).



**Fig. 3:** Cytostatic activity of different *Ruta graveolens* samples (RM – methanolic extract of wild growing plants, RDM – methanolic extract of cultured plants, RE – ethanolic extract of wild growing plants, RDE – ethanolic extract of cultured plants) on HeLa cells (% of inhibition) after 72 h of incubation. Differences of the effects of different rue extracts in different concentrations to untreated control were checked using t-test, significant differences  $p < 0.001$  being marked with (\*\*).

ing the phenolic content, antioxidant, antimicrobial and cytotoxic activities of *Ruta graveolens* considering the fact that rue is still frequently used in many parts of the world either as a spice or as a medicinal plant. On the other hand, the comparative study of wild growing and cultivated plants extracts confirmed the effects of different soil and climatic conditions on their final pharmacological effect.

Our observation that ethanolic extract of wild rue collected at the beginning of flowering season, containing the highest polyphenol, tannin and flavonoid contents, demonstrated the marked activities in

all tested systems, emphasizing the fact that the rue leaves and herb should be harvested at the beginning of blossoming stage and that wild growing plants present rich source of secondary metabolites.

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