

EFFECTS OF ROYAL JELLY ON ENERGY STATUS AND EXPRESSION OF APOPTOSIS AND BIOTRANSFORMATION GENES IN NORMAL FIBROBLAST AND COLON CANCER CELLS

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ABSTRACT. Royal jelly is natural bee product, traditionally used in medicine for antitumor, anti-inflammatory, antioxidant, antibiotic and many other beneficial properties. The aim of this study was to determine biological effects of royal jelly samples originating from Serbia on normal human fibroblast (MRC-5) and colorectal cancer (HCT-116 and SW-480) cells. MTT cell viability assay was used to determine cytotoxic activity, and NBT test was used for determination of superoxide anion radical concentration. Parameters of cell energy status were determined using LDH and ATP colorimetric methods. Relative expression of mRNA of apoptosis and biotransformation genes was monitored by qPCR method. Royal jelly affected cell viability, caused oxidative stress appearance and elevated parameters of energy status in cancer cell lines. The relative expression of genes whose proteins are included in biotransformation of xenobiotics were changed with notable suppression of *CYP1A1*, while increased expression of apoptosis genes was noted in tested cell lines. Royal jelly demonstrated cell selective effect and could be prospective in anticancer therapy.

Keywords: apoptosis, LDH, royal jelly, superoxide anion radical, ATP, colorectal cancer.

INTRODUCTION

Colorectal cancer is major public health problem and among the most commonly diagnosed type of cancer around the world, counting more than a million cases detected on an annual basis (NASRALLAH and EL-SIBAI, 2014). Apoptosis is programmed cell death, an essential genetically controlled process, which regulates tissue homeostasis and elimination of genetically damaged cells. Tumor progression is result of successful avoidance of apoptosis, where altered expression of genes included in apoptotic pathway contribute to inhibition of cell death and enhance cell proliferation. Bcl-2 protein family is responsible for regulation of apoptosis, consisting of pro- and anti-apoptotic proteins and therefore has been targeted for antitumor therapy (BAIG *et al.*, 2016). This process is depended on energy (ELMORE, 2007). Modified energy status in cancer cells is characterized by elevated rate of aerobic glycolysis.

Enzyme lactate dehydrogenase (LDH) catalyzes conversion of pyruvate to lactate, final step in anaerobic glycolysis, enabling generation of sufficient amount of energy (ATP molecules), required for increased proliferation of cancer cells, as well as apoptotic processes (VALVONA *et al.*, 2016). Reactive oxygen species (ROS), such as superoxide anion, are generated during metabolic processes in cells. Mild ROS levels have significant impact on many cell processes including cell proliferation, activation of specific stress-induced signaling pathways, while accumulation of ROS can induce apoptotic pathway (LEE and KANG, 2013; KAMOGASHIRA *et al.*, 2015; PAGES *et al.*, 2015). Elevated ROS generation can also depend on genes and proteins included in biotransformation of xenobiotics (HRYCAY and BANDIERA, 2015). Mechanism of biotransformation of most drugs used in anticancer therapy depends on variety of enzymes expressed in cancer cells, such as cytochrome P450 (CYP family) 1A1, glutathione S-transferase P1 (GSTP1), multidrug resistance protein 2 (MRP-2). Genes that encode those proteins could be targeted in anticancer therapy (TAMASI *et al.*, 2011; SODANI *et al.*, 2012).

Considering the fact that cancer is multifactorial disease, dietary habits and nutrition quality are factors of significant influence in colorectal cancerogenesis (KAUR *et al.*, 2009). Currently, major attention has been focused on natural products as part of daily diet intake, with a positive influence on reducing the risk of cancer (KARADENIZ *et al.*, 2011; NABAS *et al.*, 2014). Many studies have recently been oriented toward pharmacological properties of bee products, which are well known both in traditional and modern medicine (NAKAJIMA *et al.*, 2009; PASUPULETI *et al.*, 2017).

Royal jelly (RJ) is one of the most attractive natural products, secreted by the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera* L.). It is a milky white secretion with a sharp smell and fruity taste, an essential food for both the queen bee and her larvae (SUZUKI *et al.*, 2008; RAMADAN and AL-GHAMDI, 2012; SHIRZAD *et al.*, 2013). Composition of RJ includes many important compounds such as proteins, lipids, essential amino acids, minerals, vitamins, sugars, nucleic acids, enzymes, hormones, phenols (SUZUKI *et al.*, 2008; KARADENIZ *et al.*, 2011; ORŠOLIĆ, 2013). Due to its complex composition, RJ has demonstrated various pharmacological activities: antitumor, anti-inflammatory, antioxidant, antibiotic, immunomodulatory, hypotensive, antihypercholesterolemic, hypoglycemic, anti-allergic, antiaging, hepatoprotective, and many other (IZUTA *et al.*, 2009; PAVEL *et al.*, 2011; SUGIYAMA *et al.*, 2012; SUPABPHOL and SUPABPHOL, 2013; MOFID *et al.*, 2016).

According to our knowledge, no data regarding the effect of royal jelly originating from Serbia on ROS concentration, energy status, relative expression of apoptosis and biotransformation genes in cancer cell lines is available in the literature. The aim of this study was to characterize the biological activity of RJ in normal human fibroblast (MRC-5) and colorectal cancer (HCT-116 and SW-480) cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) and Phosphate Buffered Saline (PBS) were obtained from GIPCO, Invitrogen, USA. Fetal Bovine Serum (FBS) and Trypsin EDTA was obtained from PAA (The Cell Culture Company, Pasching, Austria). Dimethyl Sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Nitro Blue tetrazolium (NBT), Ethidium bromide (EB), PMS (N-methylphenazonium methyl sulfate), INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride), NAD (nicotinamide adenine dinucleotide) and chloroform were purchased from SERVA, Germany. Acridine Orange (AO) was obtained from Acros organics, USA. ATP assay kit and Triton X-100 were

purchased from Sigma Aldrich, USA. TRIzol and molecular biology grade water were obtained from Ambion, USA. High-Capacity cDNA Reverse Transcription Kit Applied Biosystems were obtained from Thermo Fisher Scientific, USA. SensiFAST SYBR Lo-ROX kit was from Bioline, UK. Primers used for relative expression of genes were from Eurofins Genomics, Ebersberg. All other used solvents and chemicals were of analytical grade.

Royal jelly preparation

Native RJ sample (originated from *Apis mellifera* L.) was produced by bee culture located in Velika Plana, Podunavlje District of Central Serbia, and collected in the spring of 2017. Fresh RJ was stored at -20 °C until used. Prior to the analysis, RJ was defrosted and dissolved in PBS, and then diluted with the culture medium. The stock solution (1 mg/ml) was sterilized by filtropur S 0.2 for sterile filtration and diluted by medium to various working concentrations.

Cell preparation and culturing

Normal lung fibroblasts MRC-5 and colorectal cancer cell lines HCT-116 and SW-480 (obtained from American Type Culture Collection) were maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in humidified atmosphere with 5% CO₂ at 37 °C, grown in 75 cm² cell culture flask until cell confluence at 70 to 80%. After a few passages, cells were seeded for different assays.

Cell viability assay (MTT assay)

Cytotoxic effects of RJ were determined by MTT assay (MOSSMAN, 1983) and previously described in detail (ĆURČIĆ *et al.*, 2012). MRC-5, HCT-116 and SW-480 cells were treated with various concentrations of RJ (1, 10, 50, 100, 250 and 500 µg/ml) for 24 and 72 h of initial treatment.

Fluorescence microscope analysis of cell death

For the analysis of cell death, microscopic fluorescent assay Acridine orange/ethidium bromide (AO/EB) double staining was used (BASKIĆ *et al.*, 2006) and previously described in detail (ĆURČIĆ *et al.*, 2012). MRC-5, HCT-116 and SW-480 cells were seeded in a 96-well plate (10⁴ cells per well) and after 24 h were treated with 100 µl of RJ (250 µg/ml) for 24 and 72 h.

Determination of Superoxide Anion Radical (NBT assay)

The concentration of superoxide anion radical (O₂^{•-}) was determined by spectrophotometric method (AUCLAIR and VOISIN, 1985), previously described in detail (ŽIŽIĆ *et al.*, 2013). Cells were seeded in 96-well plate (10⁴ cells per well) and treated with RJ (10, 100 and 250 µg/ml) for 24 h.

Evaluation of energy status parameters

Determination of lactate dehydrogenase concentration (LDH assay)

The glycolysis intensity and cell damage were detected by spectrophotometric method - LDH assay (CHAN *et al.*, 2013). Lactate dehydrogenase (LDH) is stable soluble enzyme present in almost all living cells. The principle of this colorimetric assay is based on activity

of LDH, as glycolytic oxidoreductase, which catalyzes conversion of lactate to pyruvate and produces nicotinamide adenine dinucleotide (NADH). Diaphorase (PMS) is enzyme, which uses NADH to catalyze reduction of tetrazolium salt (INT) into colored formazan. The consumption of NADH is proportional to LDH activity and can be spectrophotometrically measured. Red formazan absorbs light at 490 nm. Concentration of LDH was observed both in medium and in lysate.

MRC-5, HCT-116 and SW-480 cells were seeded in 6-well plate at a density of 1×10^5 cells per well in 1 ml media. Cells were treated with 1 ml of RJ (100 and 250 $\mu\text{g}/\text{ml}$), for 24 and 48 h. The untreated cells served as a control. Medium without cells was transferred from 6-well plate and centrifuged for 15 min at 1200 rpm at 4 °C. After that, 400 μl of 9% Triton X-100 was added to lyse the cells in the lysis wells and mixed thoroughly using a pipette to make sure all cell membranes have degraded. Lysate was then centrifuged for 5 min at 1200 rpm at 4 °C. The supernatants (50 μl) of both media and lysates were harvested and transferred to a 96-well plate. Prior to the assay, 2x LDH assay buffer (INT, PMS, NAD, lactic acid, pH 8.0) was made and 100 μl of assay buffer was added to each of the assay wells. The assay plate was incubated for 30 min at room temperature in the dark. Absorbance was measured at 490 nm on Microplate Reader (ELISA RT-2100C).

Measurement of adenosine triphosphate concentration (ATP assay)

Measurement of intracellular ATP level was performed using colorimetric assay kit (Abcam, USA). Method is based on glycerol phosphorylation resulting in red colored reaction (CHEN *et al.*, 2011). MRC-5, HCT-116 and SW-480 cells were seeded in T-25 cm^2 cell culture flask (10^6 cells per flask) and after 24 h of incubation were treated with 5 ml of RJ (100 and 250 $\mu\text{g}/\text{ml}$) for 24 and 48 h. The untreated cells served as a control. After incubation with treatment, supernatant was rejected, and cells were lysed in 200 μl of lysis buffer and mixed thoroughly using a pipette to make sure all cell membranes have degraded. Lysate was centrifuged for 2 min at 13000 rpm at 4 °C. 50 μl of supernatant was added to a new 96-well plate and followed by the addition of 50 μl of the Reaction Mix to each well with samples (work was performed on ice). After the incubation period in dark for 30 min at room temperature, the absorbance was measured at 570 nm using Microplate Reader (ELISA RT-2100C). Concentration of ATP ($\mu\text{mol}/\mu\text{g}$) was calculated as:

$$\text{ATP concentration} = \left(\frac{T_s}{S_v} \right) \times D$$

T_s = amount of ATP in sample well calculated from standard curve generated at the same time

S_v = sample volume added in the sample wells

D = sample dilution factor

Apoptotic and biotransformation genes expression

Isolation of RNA from cultured cells

RNA was isolated from normal fibroblast and colorectal cancer cells using protocol of CHOMCZYNSKI and SACCHI (1987). T-25 cm^2 cell culture flasks were used to seed cells, and at confluency over 90% of cells were treated with RJ at concentration 100 $\mu\text{g}/\text{ml}$, while the untreated cells served as control. After 24 h of treatment, cells were trypsinized and centrifuged at 1200 rpm for 10 min. Cells were resuspended in TRIzol at concentration of 10^6 cells/ml of TRIzol. 200 μl of chloroform was added to each sample, and after 15 seconds of mixing, samples were left for incubation 2-3 min at room temperature. After centrifugation of samples for 15 min/12000 rpm at 4 °C three visible phases were observed. The supernatant (containing RNA) was transferred to a new microtube, 500 μl of isopropanol was added and

RNA precipitated for 10 min at room temperature. Samples were centrifuged 10 min/12000 rpm at 4 °C. Supernatant was removed and remaining residue washed with 1 ml of 80% ethanol. After centrifuge (5 min/7500 rpm/4 °C) supernatant was removed and ethanol was left for air drying 2-3 min. RNA residue was resuspended in 20 µl molecular biology grade water, and then incubated at 55 °C at thermoblock for 2-3 min. RNA concentration was measured in every sample using biophotometer (Eppendorf BioPhotometer Plus). Indicator of RNA purity was considered relation between absorbance in the range of 1.8 up to 2.0. Samples were aliquoted and preserved at -80 °C until further use.

Reverse transcription (RT-PCR)

Translation of mRNA into a complementary DNA (cDNA) was performed using reverse transcriptase enzyme (BUSTIN, 2000). Commercially available kit High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was applied, consisting of 2 µl 10x Buffer RT, 2 µl dNTP (concentration 5 mM), 2 µl oligo-dT primers, 11 µl molecular biology grade water, 1 µl Reverse Transcriptase (*Sensiscript Reverse Transcriptase*). 2 µl of RNA sample (concentration 1 µg/µl) was added to Master Mix. Eppendorf Mastercycler-PCR was programed and adapted according to manufacturer's instructions. The resulting cDNA was stored at -80 °C until further use.

Quantitative polymerase chain reaction (qPCR)

Resulting cDNA was used to evaluate relative gene expression (ZHAI *et al.*, 2005), applying commercially available kit (sensiFAST SYBR LO-Rox kit, Bioline). Kit components were vortexed before use and reaction mixture was made for each target gene separately adding forward/reverse primer mix (0.5 µl Forward primer and 0.5 µl Reverse primer, concentration 10 µM) 10 µl qPCR Master Mix. Reaction mixture was poured into a PCR assay plate and 2 µl of cDNA sample was added and 7 µl water molecular biology grade. Each qPCR cycle started with polymerase activation step (95 °C/2 min), which was followed by 40 cycles repeating, each of them consisting of three steps: 1) denaturation of DNA at 95 °C/5 sec; 2) primer hybridization (annealing) at 60 °C/10 sec; 3) extension at 72 °C/20 sec. After amplification was done, the result evaluation was made using Applied Biosystems 7500/7500 Real-Time PCR software. β-actin gene was selected as internal control gene for the experiment (SCHIMITTGEN and LIVAK, 2008). Calculation of relative gene expression in examined cells was performed by using following formula:

$$2^{-\Delta\Delta Ct} = \Delta Ct_1 - \Delta Ct_2$$

ΔCt_1 = difference between CT values of examined gene in sample and CT values of β-actin in the sample;

ΔCt_2 = difference between CT values of examined gene in control cells and CT values of β-actin in control cells.

Forward and Reverse primer sequences used in experiments were:

Genes	Forward sequence	Reverse sequence
<i>β-actin</i>	5'-AAGCAGGAGTATGACGAGTCCG-3'	5'-GCCTTCATACATCTCAAGTTGG-3'
<i>BAX</i>	5'-GGACGAACTGGACAGTAACATGG-3'	5'-GCAAAGTAGAAAAGGGCGACAAC-3'
<i>BCL-2</i>	5'-CTACGAGTGGGATGCGGGAGATG-3'	5'-GGTTCAGGTAAGTCAATCCACAG--3'
<i>CYP1A1</i>	5'-GCCACAGCCCAGATAGCAA-3'	5'-GGTCTGGCCAGGTCTAGGCA-3'
<i>GSTP1</i>	5'-TCAAAGCCTCTGCCTATAC-3'	5'-AGGTGACGCAGGATGGTATT-3'
<i>MRP-2</i>	5'-ATACCAATCCAAGCCTCTAC-3'	5'-GAATTGTCAACCTGTAAGAG-3'

Statistical analysis

The data are expressed as mean \pm standard error (SE). Statistical significance was determined using the Student's t-test or the one-way ANOVA test for multiple comparisons. A p value < 0.05 was considered significant. The magnitude of correlation between variables was done using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver.17, 2008). The IC₅₀ values were calculated from the dose curves by a computer program (CalcuSyn).

The results for NBT, LDH and ATP assays were presented in relation to the number of viable cells on the basis data given in MTT test.

RESULTS

Cytotoxic effects

Evaluation of RJ effects on MRC-5, HCT-116 and SW-480 cell viability was determined by MTT assay. Based on the results, RJ showed no significant cytotoxic effect on all tested cell lines. Increased cell viability is evident after 24 h of treatment, and proliferative effect induced by RJ in lower concentrations in all three tested cell lines (Fig. 1). Higher RJ concentrations induced decrease in cell viability in all tested cell lines, but in cancer cells their recovery is noticeable. Slight cytotoxic effect of RJ was evident in tested cancer cell lines at 250 $\mu\text{g/ml}$ concentration. Cancer cells exerted slightly higher sensitivity to the treatment than normal fibroblast cells.

The effect of RJ was expressed by IC₅₀ values (inhibitory dose that inhibit cell growth by 50%), used as a parameter for cytotoxicity, was higher than 500 $\mu\text{g/ml}$. Considering weak antiproliferative effects, IC₅₀ values are higher than the highest applied RJ concentration and could not be calculated. Per these criteria, RJ showed no cytotoxic effect on investigated cell lines.

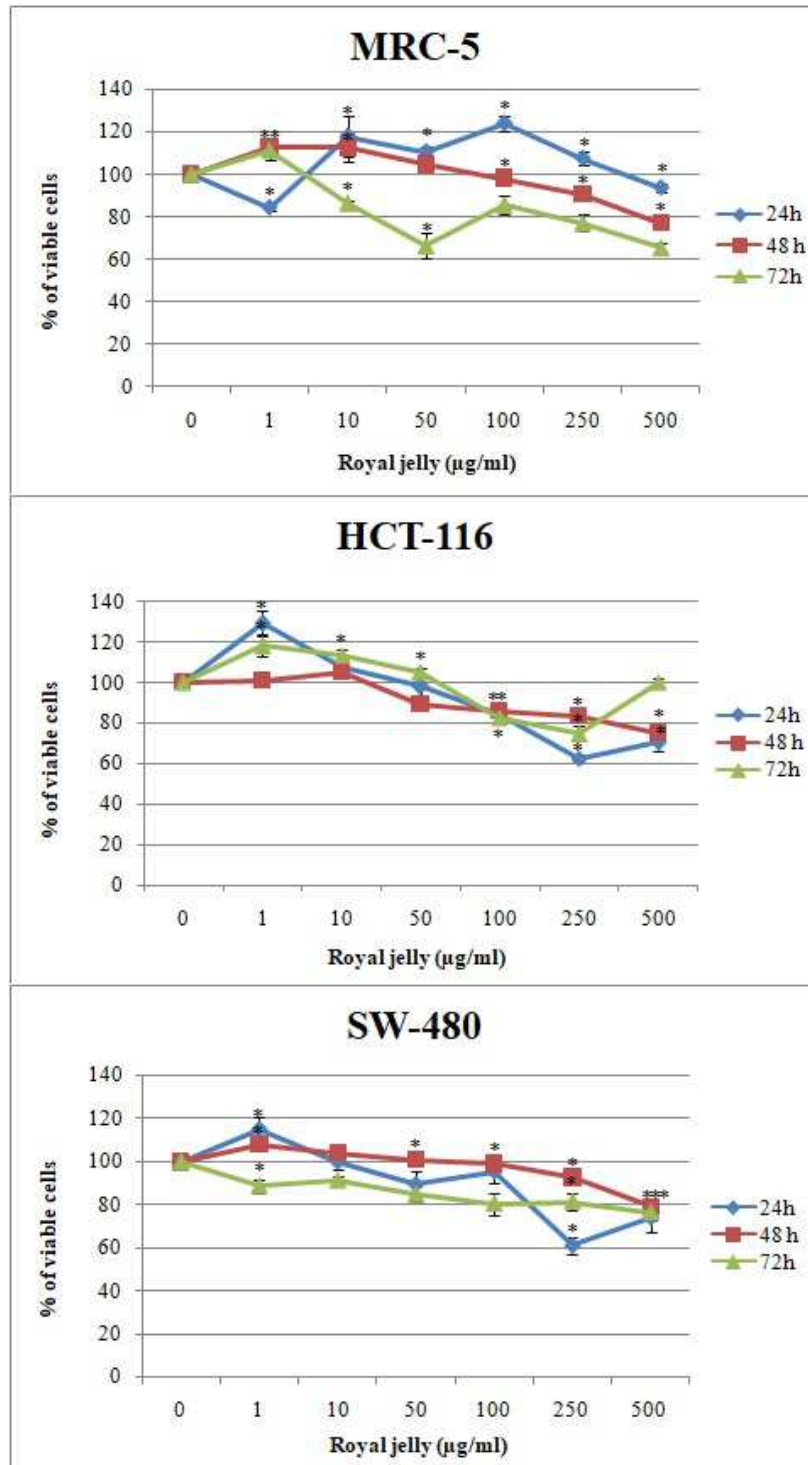


Figure 1. Effects of RJ on MRC-5, HCT-116 and SW-480 cell viability. Results are presented as means \pm SE of 3 independent experiments. *statistically significant difference ($p < 0.05$) compared to control.

Fluorescence microscope analysis of cell death

In order to determine whether the inhibition of cell proliferation by RJ was due to the induction of apoptosis, AO/EB staining was performed. Cells were treated with RJ (250 $\mu\text{g/ml}$, concentration that induced slight cytotoxic effect in all tested cell lines) for 24 and 72 h, while untreated cells served as control. Table 1 presents the percentage of viable cells, cells

in early apoptosis, late apoptosis and necrosis MRC-5, HCT-116 and SW-480 cells after examined treatment. The RJ treatment did not cause significant visible morphological changes in investigated cell lines. RJ treatment mainly induced slight apoptosis (predominantly early apoptosis), while late apoptotic cells were absent, and low percentages of necrotic cells were observed. Generally, percentage of apoptosis (mainly early apoptosis) and necrosis was very low after applied treatment. Apoptosis occurred in control cells 72 h after RJ treatment was due to proliferation, outgrowing of cells and lack of nutrients, thus cell death.

Table 1. Effect of RJ on apoptosis in MRC-5, HCT-116 and SW-480 cells, stained with AO/EB.

24 h	Viable cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Necrosis cells (%)
MRC-5				
Control	100.00±0.01	/	/	/
Treatment	98.55±0.14 *	1.46±0.14 *	/	/
HCT-116				
Control	99.06±0.29	0.94±0.29 *	/	/
Treatment	97.70±0.17 *	2.02±0.19 *	/	0.29±0.02 *
SW-480				
Control	98.65±0.24 *	0.90±0.06 *	0.30±0.02 *	/
Treatment	97.47±0.10 *	2.25±0.12 *	/	0.28±0.02 *
72 h				
MRC-5				
Control	98.43±0.16 *	1.15±0.30 *	/	0.43±0.14 *
Treatment	97.64±0.22 *	2.06±0.23 *	/	0.30±0.01 *
HCT-116				
Control	96.67±1.14 *	3.05±1.14 *	/	0.29±0.02 *
Treatment	96.57±0.94 *	2.88±0.63 *	/	0.56±0.31 *
SW-480				
Control	97.97±0.02 *	1.59±0.13 *	/	0.44±0.15 *
Treatment	97.54±0.32 *	1.89±0.06 *	/	0.57±0.26 *

Results are presented as means ± SE of 3 independent experiments.

*statistically significant difference ($p < 0.05$) compared to control

Concentration of Superoxide Anion Radical (O_2^-)

Assessment of RJ effects on O_2^- concentration in MRC-5, HCT-116 and SW-480 cells was determined by NBT assay and results are presented in Figure 2. Concentration of O_2^- in MRC-5 cells was lower compared to control cells, 24 h of treatment, but increase of O_2^- production was detected after 48 and 72 h of treatment. RJ treatment generally increased concentration of O_2^- in tested cancer lines. Dose dependent increase in O_2^- production in HCT-116 cell line was observed after 24 and 48 h of treatment, while the highest statistically significant increase of O_2^- concentration was noticeable 72 h after treatment. SW-480 cell line expressed slight increase in O_2^- concentration at RJ concentration of 10 $\mu\text{g/ml}$, compared to control, while the highest increase was expressed at concentration 250 $\mu\text{g/ml}$. Cancer cell line exhibited more evident sensitivity to the treatment regarding changes in O_2^- concentrations compared to normal fibroblast cells.

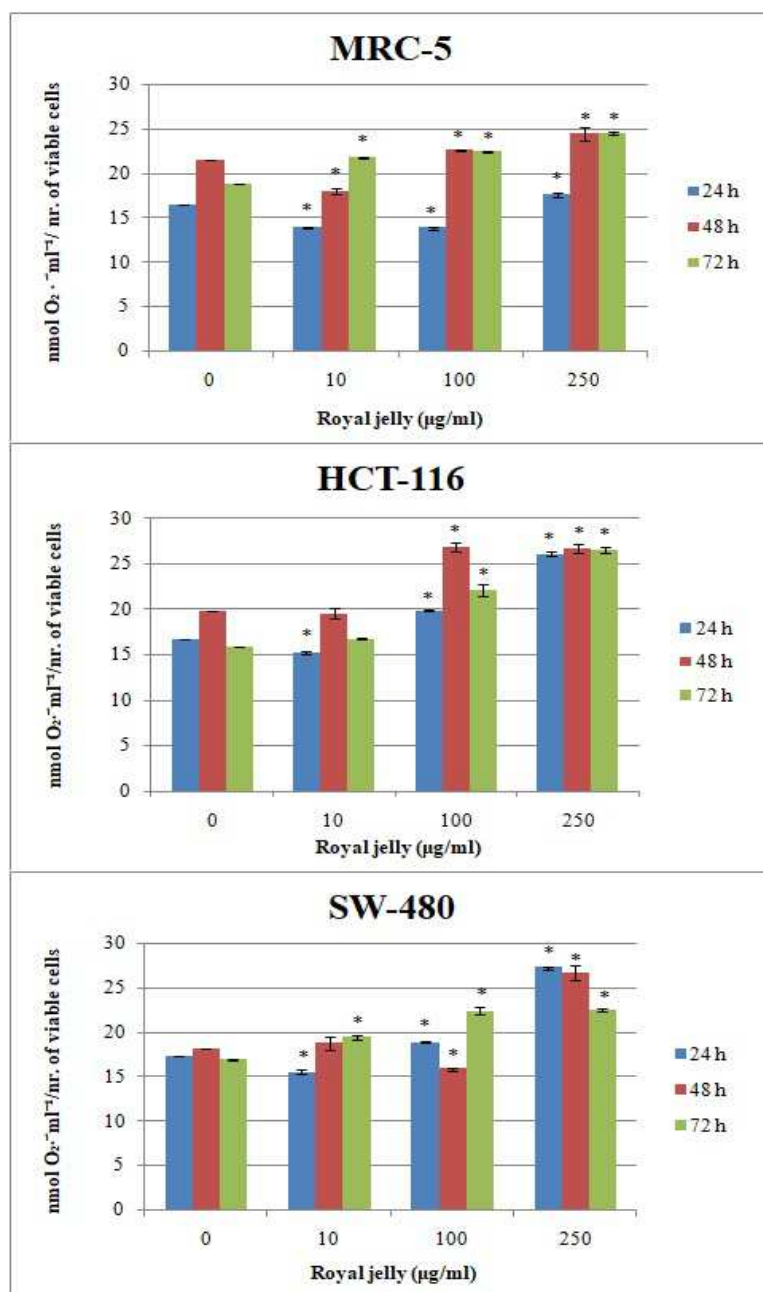


Figure 2. Effects of RJ on O_2^- concentration (nmol/ml in relation to number of viable cells) in MRC-5, HCT-116 and SW-480 cell lines. Results are presented as means \pm SE of 3 independent experiments. *statistically significant difference ($p < 0.05$) compared to control.

Evaluation of energy status parameters

Lactate dehydrogenase concentration

The basal LDH concentration in medium as well in lysate was similar both in normal fibroblast and cancer cells (Figs. 3 and 4) indicating equal rate of glycolysis.

RJ decreased LDH level in medium of MRC-5 cells after both 24 and 48 h, while this parameter changed in cancer cells. Namely, LDH concentration slightly increased in HCT-116 cells in dose-dependent manner after 24 and 48 h of treatment, which suggests to the eventual necrosis of cells. In SW-480 cells increased LDH level was expressed at RJ concentration 250 $\mu\text{g/ml}$ after 24 h. Our results showed weaker changes in LDH concentration in medium after 48 h of treatment (Fig. 3).

LDH concentration in lysate of MRC-5 cells remained similar to the basal concentration. In cancer cells, increase of LDH level was significant and more pronounced after 24 h of treatment (Fig. 4). Cell selectivity of RJ treatment was prominent, apparently, HCT-116 cells exerted higher sensitivity to treatment than MRC-5 and SW-480 cells. Significant increase of this parameter in lysate of cancer cells, compared to normal fibroblast cells, indicate higher glycolysis intensity.

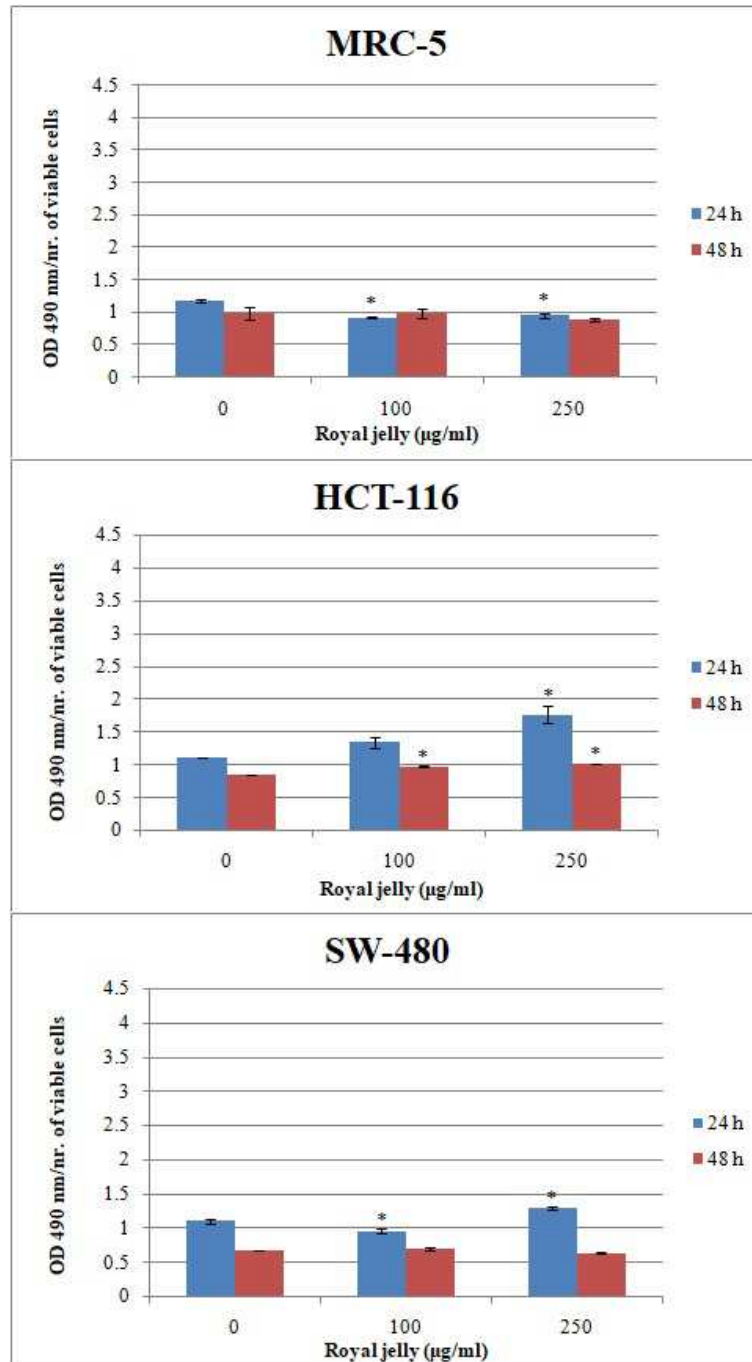


Figure 3. Effects of RJ on LDH concentration (OD 490 nm in relation to number of viable cells) in medium of MRC-5, HCT-116 and SW-480 cell lines.

Results are expressed as means \pm SE for 3 independent determinations. *statistically significant difference ($p < 0.05$) compared to control.

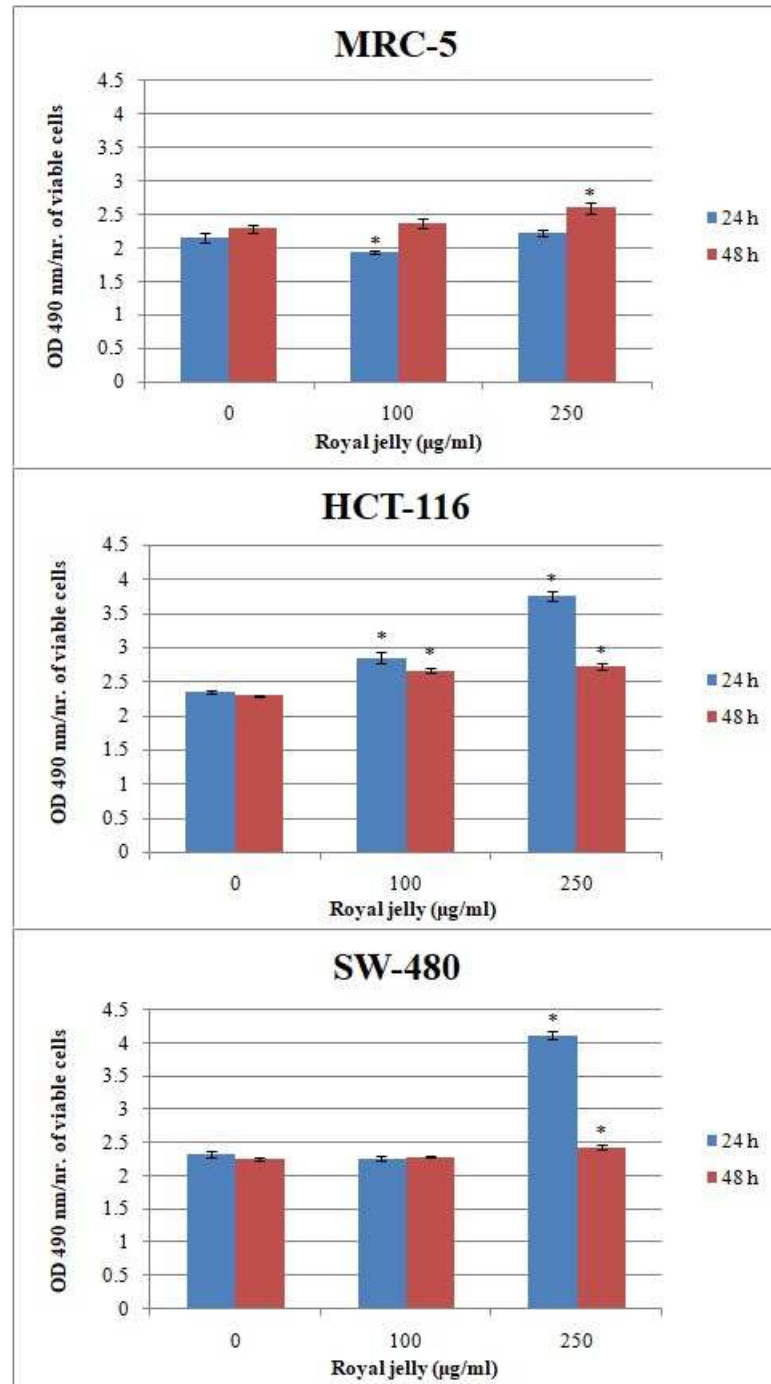


Figure 4. Effects of RJ on LDH concentration (OD 490 nm in relation to number of viable cells) in lysate of MRC-5, HCT-116 and SW-480 cell lines.

Results are expressed as means \pm SE for 3 independent determinations. *statistically significant difference ($p < 0.05$) compared to control.

The adenosine triphosphate concentration

ATP concentration was measured in normal fibroblast MRC-5 and HCT-116 and SW-480 cancer cell lines by performing ATP colorimetric assay. In MRC-5 cells increase of this parameter was obvious after 24 h of treatment at concentration of 100 $\mu\text{g/ml}$ of RJ, while at higher concentration the ATP level was decreased (Fig. 5). RJ induced decrease of ATP levels in MRC-5 cells at both applied concentrations (100 and 250 $\mu\text{g/ml}$) after 48 h of treatment. After both treatment incubation periods, ATP concentration was significantly increased in

cancer cell lines, indicating cell selective effect of RJ treatment. After 24 h of treatment, increase of ATP concentrations in cancer cells was prominent in dose-dependent manner, compared to the control. Increase of ATP level cells in dose-dependent manner was evident in HCT-116 after 48 h of treatment, while in SW-480 cells lower RJ concentration (100 $\mu\text{g/ml}$) induced increase in ATP, while the higher RJ concentration (250 $\mu\text{g/ml}$) induced decrease of ATP level (Fig. 5).

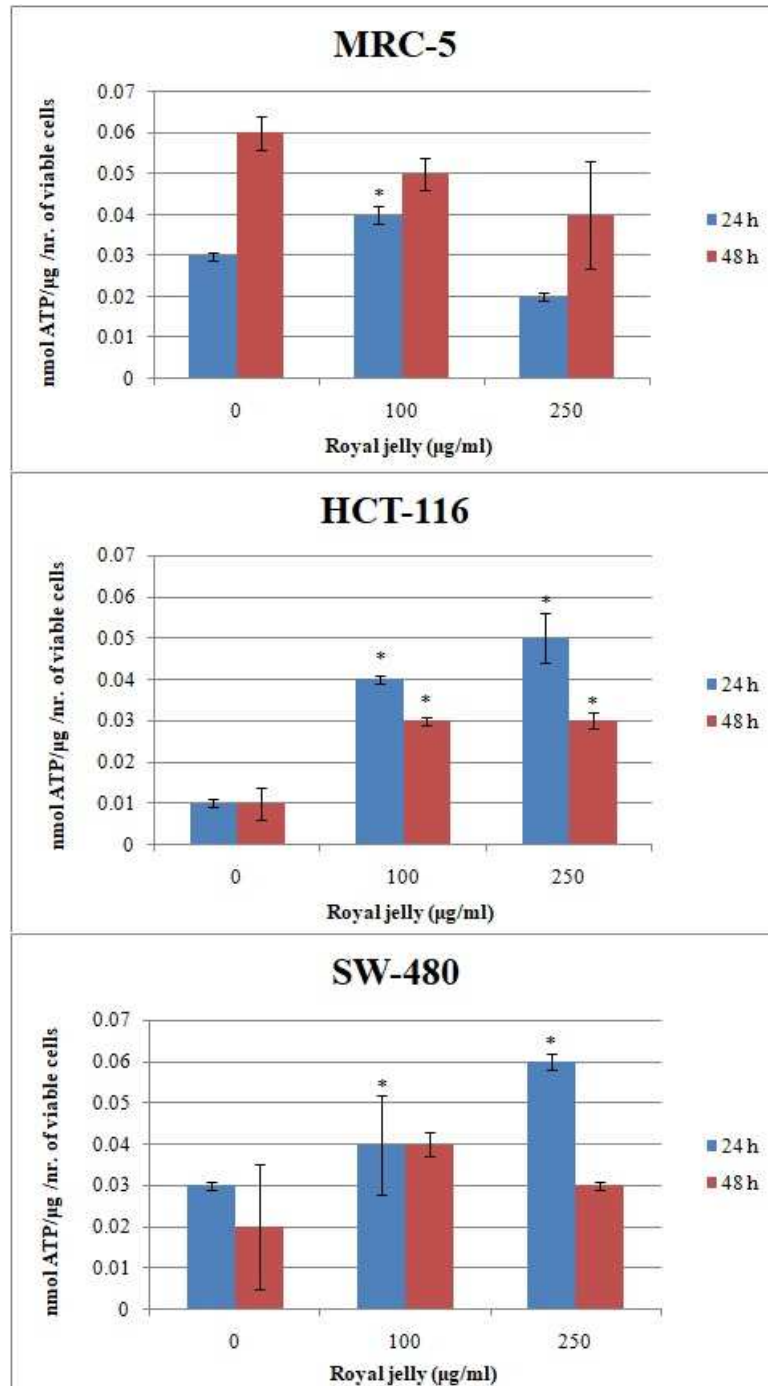


Figure 5. Effects of RJ on ATP concentration (nmol/ μg in relation to number of viable cells) in MRC-5, HCT-116 and SW-480 cell lines. Results are expressed as means \pm SE for 2 independent determinations. *statistically significant difference ($p < 0.05$) compared to control.

Relative gene expression

The expression of genes whose protein products were included in apoptosis (*BAX*, *BCL-2*) and biotransformation of xenobiotics (*CYP1A1*, *GSTP1*, *MRP-2*) were monitored by qPCR method. The relative expression of target genes was calculated in relation of β -actin as housekeeping gene (endogenous control). Results presented as relative expression of target genes in treated cells in relation of control (untreated) cells (1) showed changes in genes expression in all tested cell lines after RJ treatment (Fig. 6). RJ treatment affected expression of apoptotic genes in all tested cell lines by increasing *BAX* and *BCL-2*. RJ showed cell selective effect in this case, namely, more pronounced, higher expression was observed in normal fibroblast than in cancer cells. Inhibition of *CYP1A1* gene expression is evident in normal as well as in cancer cells. RJ increased expression of *GSTP1* gene in MRC-5 and SW-480, while the opposite effect was observed in HCT-116 cells. The increase of *MRP-2* gene was evident in all tested cell lines after RJ treatment.

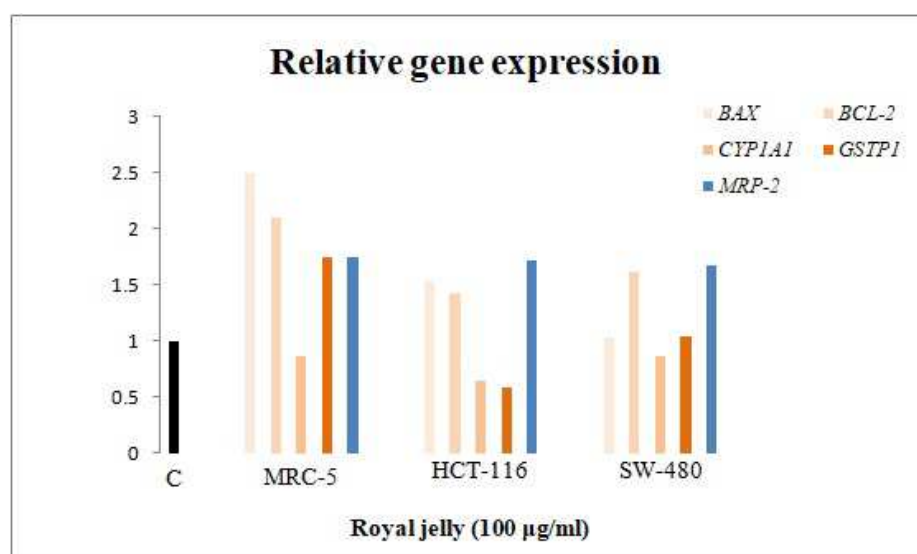


Figure 6. Effects of RJ on relative expression of *BAX*, *BCL-2*, *CYP1A1*, *GSTP1*, *MRP-2* genes in MRC-5, HCT-116 and SW-480 cell lines.

DISCUSSION

The effects of RJ on cell proliferation have been previously reported, but there are no studies on HCT-116 and SW-480 cancer cells. In recent studies performed by FILIPIČ *et al.* (2015) the relatively low antiproliferative activity of RJ on CaCo2 colorectal adenocarcinoma cells, was noticed. Antiproliferative effect of RJ was suggested to be due to its protein fraction (major royal jelly proteins - MRJP), which prevented proliferation of MCF-7 human breast cancer cells induced by bisphenol A and decreased HeLa cells population by 50% (RAMADAN and AL-GHAMDI, 2012; FILIPIČ *et al.*, 2015). On the contrary, lipid estrogenic component present in RJ increased proliferation of MCF-7 cells (PAVEL *et al.*, 2011). RJ protein fraction also showed stimulative effect on human monocytes (FILIPIČ *et al.*, 2015) and rat hepatocytes, suggesting that MRJP in RJ probably functions as growth factor (TORRES-ACOSTA *et al.*, 2015). According to our results, RJ applied in low concentrations stimulated cell proliferation of tested cell lines, while concentration 250 μ g/ml exhibited weak cytotoxic effect in cancer cell lines. Cell selective effect of RJ treatment can be noticed since cancer cells show slightly more sensitive response of cancer cells to the treatment than normal fibroblast cells. In order to determine type of cell death at this concentration, we performed

AO/EB staining and concluded that cytotoxicity was due apoptosis. Investigated treatment did not induce necrosis. Also, level of LDH in cell culture medium, as indicator of necrosis, was relatively low compared to control, in tested cell lines, 24 and 48 h after RJ treatment, indicating that RJ did not induce necrosis.

Phenolic compounds are shown to induce pro-oxidant effect in cancer cells causing increased ROS production (ĆURČIĆ *et al.*, 2014). Antioxidative properties of RJ are well known and attributed to polyphenols, flavonoids (flavonoles, flavanones, flavones) and cinnamic acid derivatives present in RJ (RAMADAN and AL-GHAMDI, 2012). In our study, pro-oxidant effect occurred in cancer cells could be due to polyphenolics present in RJ. These compounds affect cancer cells targeting different signaling pathways such as cell proliferation and modulation of redox status (ZHOU *et al.*, 2016). Changes in mitochondrial membrane permeability caused by increased levels of ROS are resulting in cytochrome c release and inducing apoptosis pathway (ĆURČIĆ *et al.*, 2014). Our study showed elevated ROS production in cancer cells comparing to normal fibroblast MRC-5 and control values, especially concentration of 250 µg/ml. This may suggest that slight cytotoxic effects in cancer cells could be result of oxidative stress caused by RJ treatment.

Apoptosis is process highly regulated by members of BCL-2 protein family, categorized into anti-apoptotic and pro-apoptotic proteins (BAIG *et al.*, 2016). We assessed expression of anti-apoptotic *BCL-2* and pro-apoptotic *BAX* genes after exposure to RJ. Treatment induced notable higher expression of both genes in MRC-5 cells than in cancer cells. The treatment induced changes in expression of both genes in HCT-116 cells, with higher obtained values of pro-apoptotic *BAX* gene expression, which is consistent with stronger cytotoxicity of RJ treatment in this cell line. Expression of anti-apoptotic *BCL-2* gene was higher in SW-480 cells compared to pro-apoptotic *BAX*, which is in correlation with unchanged values of cell viability at RJ concentration of 100 µg/ml.

Certain metabolites, especially fatty acids and nucleic acids are prerequisite in tumor cell for growth and proliferation under low oxygen levels. Overexpression of transcription factor like *HIF-1α* in hypoxic conditions, which regulates expression of LDH, leads to increased levels of LDH in cancer. However, it was observed that estrogen can also influence the expression of LDH (MIAO *et al.*, 2013). It was reported previously that RJ exhibits estrogenic activity *in vitro*, through binding to estrogen receptor β (ER β). This signaling pathway resulted in transcription modulation via estrogen response element (ERE) in DNA, as well as proliferation of tumor cells (SUZUKI *et al.*, 2008). Our study showed that basal LDH concentration in lysate was similar in normal fibroblast and cancer cells, indicating equal rate of glycolysis. Cell selective effect of RJ was noted, by increasing levels of LDH in cancer cell lysate, while this parameter remained similar in normal fibroblast cells. Elevated LDH content in cancer cell lysate indicates hypoxic conditions and increased rate of glycolysis. Increased LDH concentration leads to higher activity of this enzyme and higher production of ATP, which is in accordance to our results. Elevated energy metabolism insures greater ATP production, needed for cell proliferation, as well as for apoptosis, energy-dependent cascade of molecular events (ELMORE, 2007). ATP as source of energy is needed for many metabolic processes in the cell, including biotransformation of xenobiotics (CROOM, 2012).

Our study revealed that RJ affected expression of biotransformation genes in normal fibroblast and cancer cells. Important enzyme included in metabolism of xenobiotics and oxygenation of organic substrates by reducing of molecular oxygen is cytochrome P450 CYP1A1, one of three members of CYP1 family (TAMASI *et al.*, 2011; HRYCAY and BANDIERA, 2015). ROS can be generated as byproduct of molecular oxygen reduction process catalyzed by CYP enzymes as a consequence of control loss of this process (HRYCAY and BANDIERA, 2015). Expression of *CYP1A1* is often altered in cancer, resulting in maintenance of drug resistance (TAMASI *et al.*, 2011). Therefore, by suppressing the expression of

CYP1A1, drug resistance in cancer could be reduced. According to our results, RJ treatment suppressed *CYP1A1* expression in cancer cells compared to control values.

Another important enzyme with vital role in detoxification and metabolism of xenobiotics, including anticancer agents is glutathione S-transferase P1 (GSTP1), protein product of *GSTP1* gene. GSTP1 catalyze addition of glutathione (GSH) to xenobiotics or metabolites of oxidative stress, thus neutralizing them. Trans-membrane MRPs are removing glutathione conjugates from the cell (NISSAR *et al.*, 2017). We showed in our study that RJ treatment suppressed *CYP1A* and *GSTP1* in HCT-116 cells. This ability of RJ to suppress expression of genes or activity of their protein products with important role in metabolism of cytostatics, could be very perspective in effectiveness of anticancer drugs applied in combination with RJ. Increased *GSTP1* and *MRP-2* expression is expected in normal fibroblasts and other healthy cells, due their role in detoxification. This mechanism protects normal cells from xenobiotics, including any exogenous substances (PAJAUD *et al.*, 2012).

MRP-2 protein is the major trans-membrane efflux transporter, member of MRP subfamily of ABC transporters, with role in ATP-dependent excretion of glutathione conjugates and other endogenous and xenobiotic compounds. According to previous reports, *MRP-2* is expressed in cancer tissues, such as colorectal cancer, thus multidrug resistance of cancer cells depends on *MRP-2* expression level (SODANI *et al.*, 2012). In our study, increased expression of *MRP-2* as a result of RJ treatment can be observed in all tested cell lines. RJ treatment, although non-toxic at applied concentration (100 µg/ml), was obviously recognized as xenobiotic by tested cells and thus possibly excreted from cells. Overexpression of *MRP-2* requires ATP, which explains increasing of ATP concentration in treated cells.

CONCLUSION

Royal jelly originating from Serbia stimulates cells proliferation of all examined cell lines; however, at higher applied concentrations cytotoxic effects were evident in cancer cell lines. These cytotoxic effects were caused mainly by apoptosis, without necrotic effects, which was confirmed by LDH concentration in cell culture medium. The parameters of energy status were clearly raised to the higher level, suggesting that RJ increased glycolysis rate in cancer cells and amount of ATP probably needed for apoptosis and *MRP-2* activity. Slight cytotoxic effect in cancer cells, induced by the treatment, could be due to oxidative stress provoked probably by polyphenols present in RJ. Our data indicate that the expression of apoptosis and biotransformation genes was changed in cells by treatment. Biotransformation of xenobiotics by CYPs and GSTP1 enzymes plays immense role in anticancer therapy, thus suppressing of these genes by RJ treatment could be very desirable. Namely, potential application of RJ with appropriate cytostatics could provide better anticancer effect. Although the significant cell selective effects of RJ were observed, further studies are necessary to clarify molecular mechanisms of RJ activity, investigate ability of RJ to affect specific components in different signal pathways or estimate eventual specificities in royal jelly content originating from Serbia. Royal jelly originating from Serbia could be considered as potential source of abundant bioactive substances used in anticancer therapy of certain cancer types.

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