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Hypericum perforatum L. EXTRACTS EXERT CYTOTOXIC EFFECTS AND SHOW DIFFERENT MIRNA SIGNATURES IN PC-3 AND DU 145 PROSTATE CANCER CELLS

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Phytochemicals and bioactive substances derived from a wide range of plant extracts have been reported to exert various anticancer effects. Prostate cancer is one of the leading causes of cancer-related deaths within the male population. Prostate cancer-specific miRNA signatures were associated with cancer formation and progression, with various subtypes, and response to therapy. MicroRNA levels of expression were shown to change after the treatment of various compounds and substances extracted from natural products. Natural herbal compounds were shown to induce variations in miRNA expression levels in cancer cells.

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The aims of this study were to investigate the cytotoxic effects of methanol, ethyl-acetate, and hexane extracts obtained from branch-body part and flowers of *Hypericum perforatum* L. against humane PC-3 and DU 145 and to test potential miRNA-128/133b/155/193a/206/21/335 signature changes and differences between the two prostate cancer cell lines.

Cytotoxic activity of *H. perforatum* extracts, their effects on cell cycle distribution, and miRNA expression levels were examined in humane PC-3 and DU 145 prostate cancer cells by MTT cell survival assay, flow cytometry, and quantitative real-time PCR.

Hexane extract of flowers showed the strongest intensity of cytotoxic activity against PC-3 and DU 145 cells. The highest increase in the percentage of PC-3 cells in the subG1 phase was observed in cell samples treated with hexane extract of flowers and branch-body part. Significant differences in miRNA-128/133b/155/193a/206/21/335 levels were observed between PC-3 and DU 145 cell lines, especially in samples treated with flower extracts compared with the branch-body part.

Conclusions: Investigated extracts have significant anticancer potential not only from the aspects of cytotoxicity and cell cycle effects but also from the aspect of lowering oncogenic or increasing tumor-suppressive miRNAs. The best effect might be the increase of tumor-suppressive miR-128 (accompanied by miR-193a) induced by the hexane extract of the flowers, which also exerted the highest cytotoxic activity. Hexane extract of flowers may be the candidate for further investigation for improving the efficiency of standard therapies for PCa. A miRNA signature might be cell-type specific after the treatment with *H. perforatum* extracts.

Key words: Hypericum perforatum L.; PC-3; DU 145; Cytotoxic activity; MicroRNA

INTRODUCTION

Prostate cancer is one of the most frequent male malignancies, second by diagnosis rates, and the fifth leading cause of cancer-related deaths (CUZICK *et al.*, 2014; TAITT, 2018). Besides standard therapeutic approaches, such as endocrine therapy, chemotherapy, and radiotherapy, cancer cell apoptosis might be triggered by phytochemicals and bioactive substances derived from a wide variety of plant extracts. Natural herbal compounds have been associated with miRNA expression levels change in different cancer cells (TUNCA *et al.*, 2012; HONG *et al.*, 2015). MicroRNA molecules are small non-coding RNAs shown to govern entangled mechanisms underlying cancer development and progression. Latest research has shown that different miRNA profiles are associated with prostate cancer (PCa) pathology and response to therapy (LUU *et al.*, 2017; TAN *et al.*, 2018). For example, miR-135a has the ability to induce apoptosis in prostate cancer cells (XU *et al.*, 2019). One of the most prominent oncogenic miRNAs in cancer is miR-21 (PAN *et al.*, 2010). MicroRNA-21 silence mRNA transcribed from the programmed cell death 4 (*PDCD4*) gene and activates components of the TGF-β pathway, thus promoting tumor growth, migration, and invasion of cancer cells (SHI *et al.*, 2010). On the contrary, miR-21 inhibition releases *PDCD4* translation and thus inhibition of

PCa cell growth *in vitro* and *in vivo* (FOLINI *et al.*, 2010). MicroRNA-128 was described as a tumor suppressor, significantly underexpressed in PCa tissue compared with matched non-transformed control samples. Furthermore, experiments with miR-128 replacement sensitized DU 145 and LNCaP prostate cancer cells to cisplatin and reduced invasive potential (SUN *et al.*, 2015). MicroRNA-133b is characterized as a tumor suppressor in prostate cancer. Transfection with miR-133b induces apoptosis in PC-3 cells influences on crucial apoptotic signaling pathways and was associated with prostate cancer progression (PATRON *et al.*, 2012). Lower expression levels of miR-133b were detected in PC-3 and DU 145 cell lines (TAO *et al.*, 2012). Interestingly, induced miR-133b overexpression with miRNA mimics in LNCaP cells elevated proliferation rates and induced cell-cycle progression, and lowered apoptosis. MicroRNA-133b in LNCaP cells that are less aggressive than PC-3 in the terms of tumorigenic features, might expedite tumorigenesis, while in PC-3 cells miR-133b acts as a tumor suppressor (LI *et al.*, 2014), indicating that heterogeneity of miRNAs expression pattern is the term which should be included while investigating various tumor subtypes (PETROVIC *et al.*, 2017). CAI *et al*. (2015) have shown overexpression of miR-155 in PC-3, LNCaP, DU 145, and 22RV1 PCa cell lines and PCa tissue, proving its oncogenic role in prostate cancer. MicroRNA-193-5p was marked as a tumor suppressor in PCa, because higher levels resulted in inhibition of PC-3 and DU 145 cell proliferation (LIU *et al.*, 2017). Downregulation of miR-206 was detected in prostate cancer tissue, as well as in DU-145 and PC-3 prostate cancer cell lines, compared with non-transformed tissue and cells, while overexpression of miR-206 inhibited pro-tumorigenic features in PCa tissue and cells (WANG *et al.*, 2018). MicroRNA-335 acts as a tumor suppressor in prostate tissue and cells. Lower expression was detected in PCA cell lines PC-3, DU 145, LNCaP, and in prostate tissue compared with benign prostate hyperplasia and non-transformed tissue. Lower expression of miR-335 is associated with a higher Gleason's score, as well (PNG *et al.*, 2011).

Hypericum perforatum L. (St. John's wort) represents an herbaceous plant that grows in open, aggravated temperate climate regions. It has been used in ancient history for the treatment of various illnesses and conditions such as anxiety and depression (ZIRAK *et al.*, 2019). *H. perforatum* has also been used for its anti-inflammatory and antibacterial properties (HUANG *et al.*, 2011; OKMEN and BALPINAR, 2016). *H. perforatum* has the potential to reduce malignant growth in various types of cancer (MENEGAZZI *et al.*, 2021). This is a verdant plant with various representations and amounts of bioactive molecules such as hyperforin, hypericin, hyperoside, quercetin, etc. The amount and abundance of active substances and secondary metabolites depend on the geography and season of plant harvesting (MENEGAZZI *et al.*, 2021). In our previous study, we have shown that *H. perforatum* extracts of various plant parts and obtained by different extraction solvents contain different compounds such as quercetin, pseudohypericin, adhyperforin, hypericin, and biapigenin (MATIĆ *et al.*, 2021).

In the present study, we aimed to investigate cytotoxic effects of six extracts: methanol, ethyl-acetate, and hexane extracts obtained from branch-body parts (stem, branches, and leaves), and from the flowers of *H. perforatum* against humane PC-3 and DU 145 prostate cancer cell lines. The second aim was to investigate if extracts with cytotoxic activity change miRNA-128/133b/155/193-5p/206/21/335 expression levels in prostate cancer cells. The third goal was

to investigate potential differences in miRNA-128/133b/155/193-5p/206/21/335 signature between PC-3 and DU 145 cell line samples treated with extracts of *H. perforatum*.

MATERIALS AND METHODS

Preparation and characterization of Hypericum perforatum L. extracts

Hypericum perforatum L. plants were harvested at Alacam (Samsun, Turkey), during the first half of August 2018. The detailed procedure related to the preparation of plant extracts is described by MATIĆ *et al.* (2021). Flowers were separated from blossoms before extraction. The branch-body parts (stem, branches, and leaves) as well as flowers were washed, dried, and then powdered. One gram of flower/branch-body powder samples was mixed with 10 mL of hexane, methanol, and ethyl-acetate in an ultrasonic bath at 42°C for 60 min, and then filtered through No. 1 Whatman filter paper. In the next step, all extracts were concentrated in a vacuum with a rotavapor at 40°C which was repeated three times, dried and placed in the dark at 20°C temperature. Dried extracts were dissolved in DMSO at a stock concentration of 25 mg/mL, and stored at +4°C. The chemical characterization of *H. perforatum* extracts was performed by LC-MS analysis and ^{1}H-NMR, as reported previously (MATIĆ *et al.*, 2021). The most abundant compounds: quercetin, I3, II8-Biapigenin, pseudohypericin, and adhyperforin were found only in hexane extracts of flowers and branch-body parts; hyperforin was found in hexane extracts of flowers and branch-body parts, ethyl-acetate of branch-body, and methanol extract of flowers; hypericin was found in methanol and hexane extracts of flowers and branch-body (MATIĆ *et al.*, 2021).

Statistical analysis was performed in GraphPad Prism version 9.0.0 for Windows, Graph-Pad Software, San Diego, California USA. P values less than 0.05 were considered statistically significant, and p values between 0.1 and 0.05 were considered a statistical trend. For the comparison of groups, Mann-Whitney U-test was used to compare the sum of ranks.

Determination of cytotoxic activity

Cytotoxic effects of the six *H. perforatum* extracts were examined against two human prostate cancer cell lines: prostate grade IV adenocarcinoma PC-3 and prostate carcinoma DU 145.

Cancer cell lines were maintained in RPMI-1640 nutrient medium enriched with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, and a solution of penicillin-streptomycin (100 U/mL of penicillin and 0.1 mg/mL of streptomycin), as described elsewhere (MATIĆ *et al.*, 2021). PC-3 (5000 cells per well) and DU 145 (5000 cells per well) were placed in 96-well microtiter plates, and the next day the cells were treated with five different concentrations of the extracts (ranging from 3.125 µg/mL to 50 µg/mL, or from 6.25 µg/mL to 100 µg/mL or from 12.5 µg/mL to 200 or 400 µg/mL, depending on the intensity of the cytotoxic activity of the particular extract against specific cell line). The nutrient medium was added to control cells. The cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay after 72 h incubation period according to the standard protocol

firstly described by Mosmann (1983), and which was modified by Ohno and Abe (OHNO and ABE, 1991). In brief, after 72 h treatment, 10 µL of MTT solution (5 mg/mL in phosphate buffered saline, PBS) was added to the wells with cells and to the corresponding blank wells. After 4 h of incubation, the 100 µL of solution of 10% sodium dodecyl sulfate was added to each well. The absorbance was measured the next day at 570 nm using Thermo Scientific Multiskan EX plate reader. Each of the three independent experiments was performed in triplicate. The IC_{50} concentration was defined as the concentration of the extract that inhibited cell survival by 50%, compared with the control cell sample. The chemotherapy drug cisplatin was used as a positive control. All reagents used in the cell culture experiments were products of Sigma Aldrich. Thermo Scientific™ Biolite™ 96-well microplates were used in this research.

Cell cycle analysis

The effects of the extracts on cell cycle phase distribution of PC-3 and DU 145 cells were analyzed by flow cytometry. PC-3 and DU 145 cells (250000 cells per well) were incubated with IC_{50} and $2IC_{50}$ concentrations of the extracts 2-6 for 24 h. After 24 h incubation, the cells were collected by trypsinization, washed with PBS, and fixed in 70% ethanol on ice, according to standard procedure (ORMEROD, 2000). Cell samples were stored at -20°C for at least one week before analysis. On the day of analysis, the cells were washed, resuspended in PBS with RNase A (applied at a concentration of 200 µg/mL), and incubated for 30 min at 37°C. Subsequently, the propidium iodide solution (final concentration of 40 µg/mL) was added to the cells and incubated for 10 min before analysis. Percentages of prostate cancer cells within specific phases of the cell cycle were determined using a FACSCalibur flow cytometer (BD Biosciences). The analyses of acquired data (10000 events collected for each gated cell sample) were performed using BD CellQuest Pro™ software. All reagents for cell cycle analysis were products of Sigma Aldrich. Thermo Scientific™ Biolite™ 6-well plates were used for these experiments.

RNA extraction, reverse transcription and RT-qPCR

PC-3 and DU 145 cells were seeded into 75 cm^2 cell culture flasks ($4x10^6$ cells). The next day, the nutrient medium was removed, and a fresh medium containing IC_{50} concentrations of the extracts was added to the adherent cells. The applied concentrations were subtoxic IC_{20} concentrations, determined by MTT assay after 72 h incubation of cells with the extracts. To control sample, a fresh nutrient medium was added. The applied concentrations were determined after 72 h incubation with the extracts by MTT assay and were subtoxic. After 24 h incubation, the cells were collected by trypsinization, washed with PBS, and the cell samples were frozen at -80°C.

Total RNA was extracted from PC-3 and DU 145 cell samples with TRI Reagent (Sigma Aldrich), 200 µL chloroform per 1 mL of TRI Reagent, 1 mL of isopropanol, and 1 mL of 75% ethanol -diethylpyrocarbonate (DEPC) water. RNA samples were quantified on Biospec nano (Shimadzu Japan). All samples had 260/280 nm ratios between 1.9 and 2.1 at. Ten nanograms of total RNA was used with TaqMan MicroRNA Reverse Transcription Kit (Applied

Biosystems, Foster City, California, USA), and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) and Taqman Universal master mix, No UNG amperase for the reaction of real-time quantitative PCR (qRT-PCR). We used TaqMan miR-21 (ID: 000397); miR-155 (ID:002623), miR-133b (ID:002247), miR-206 (ID:000510), and Qiagen-miScript assays for miR-128 (Hs_miR-128-2-5p, MIMAT0031095), miR-193a (Hs_miR-193a-5p_1, MIMAT0004614), miR-335 (Hs_miR-335_1, MIMAT0000765), small nuclear RNA RNU6B (ID:001093) and SNORD48 (MS00007511, miScript, Qiagen) were used as endogenous controls. Real-Time PCR 7500 (Applied Biosystems, Foster City, California, USA) system and on Rotor Gene Q instrument (Qiagen, GmbH, Manheim, Germany) as follows: 10 min at 95◦C, and 40 cycles of 15 sec at 95 ◦C and 60 sec at 60 ◦C. Expression levels of miRNAs were presented in relative units, as relative quantity (RQ). Control (non-treated) samples were used as calibrator (1 x RQ), and analyzed with ddCt method on 7500 System SDS Software (Applied Biosystems, Foster City, California, USA), where RQ sample = $2^{-(\text{dCt sample - dCt calibrator})}$; $\text{dCt} = \text{Ct}_{\text{miR-128/133b/155/193a/206/21/335}} - \text{Ct}_{\text{RNU6B/SNORD48}}$.

RESULTS

Cytotoxic effects of Hypericum perforatum L. extracts

The extracts obtained from branch-body parts (1-3) and flower parts (4-6) of the plant *H. perforatum* exerted concentration-dependent cytotoxic activities against human prostate adenocarcinoma PC-3 and prostate carcinoma DU 145 cell lines (Table 1).

Table 1. Concentrations of Hypericum perforatum L. extracts which induced 50% decrease in prostate cancer cell survival

	PC-3	DU 145
	IC_{50} [µg/mL] average ± SD; for cisplatin [µM]	
Extract 1	>200	381.31 ± 7.35
Extract 2	47.04 ± 4.63	58.24 ± 0.03
Extract 3	16.25 ± 0.58	21.10 ± 0.60
Extract 4	46.43 ± 0.32	62.75 ± 1.26
Extract 5	27.57 ± 2.04	31.57 ± 3.38
Extract 6	11.06 ± 0.87	11.70 ± 0.80
Cisplatin	12.29 ± 1.60	5.05 ± 0.65

Extracts are as follows:1-methanol extract of branch-body part, 2-ethyl-acetate extract of branch-body part, 3-hexane extract of branch-body part, 4-methanol extract of flowers, 5-ethyl-acetate extract of flowers, 6-hexane extract of flowers. The results are presented as the average ± standard deviation of three independent experiments performed in triplicate. MTT assay was used the for determination of cell survival after 72 h treatment.

Among tested extracts, hexane extract of flowers (6) showed the strongest cytotoxic effect on both tested prostate cancer cell lines with IC_{50} values of 11.06 µg/mL for PC-3 cells and 11.70 µg/mL for DU 145 cells. Hexane extract of the branch-body part (3) also showed strong cytotoxic activity against PC-3 cells (IC_{50} value of 16.25 µg/mL) and DU 145 cells (IC_{50}

value of 21.10 μg/mL). Ethyl-acetate extract of flowers (5) exhibited pronounced cytotoxicity on tested prostate cancer cell lines (IC_{50} values of 27.57 μg/mL for PC-3 cells and 31.57 μg/mL for DU 145 cells). Ethyl-acetate extract of the branch-body part (2) and methanol extract of flowers (4) demonstrated moderate cytotoxic activities against PC-3 and DU 145 prostate cancer cells (IC_{50} values of 47.04 μg/mL and 46.43 μg/mL for PC-3 cells; IC_{50} values of 58.24 μg/mL and 62.75 μg/mL for DU 145 cells). Methanol extract of the branch-body part (1) showed very low cytotoxicity on both tested prostate cancer cell lines with IC_{50} values higher than 200 μg/mL. At tested concentrations up to 200 μg/mL this extract was inactive against these cell lines.

Effects of Hypericum perforatum L. extracts on cell cycle phase distribution

To explore the mechanisms of cytotoxic activities of five *H. perforatum* extracts (2-6), their effects on cell cycle phase distribution of PC-3 and DU 145 cells treated for 24 h with IC_{50} and $2IC_{50}$ concentrations of the extracts were analyzed. Each of the five tested *H. perforatum* extracts showed a concentration-dependent increase in the percentage of PC-3 cells in the subG1 phase of the cell cycle after 24 h incubation, as presented in Figure 1. When extracts were applied at IC_{50} concentrations, the highest increase in the percentage of PC-3 cells within the subG1 phase was observed in cell samples exposed to hexane extract of the branch-body part (3) and hexane extract of flowers (6) (6.82% and 7.31% respectively) in comparison with this percentage in the control cell sample (0.62%). Furthermore, when extracts were applied at $2IC_{50}$ concentrations, the hexane extract of flowers (6) and ethyl-acetate extract of the branch-body part (2) caused the highest increase in the percentage of PC-3 cells in subG1 cell cycle phase (16.28% and 21.6%).

Ethyl-acetate extract of the branch-body part (2) and ethyl-acetate extract of flowers (5) applied at IC_{50} concentrations induced an increase in the percentage of PC-3 cells in G1 phase of the cell cycle in comparison with control PC-3 cells. The treatment of PC-3 cells with other three tested extracts (3, 4, and 6) caused low increase in the percentage of PC-3 cells in G1 phase. This effect was not observed when PC-3 cells were incubated with $2IC_{50}$ concentrations of the extracts.

All five examined *H. perforatum* extracts demonstrated concentration-dependent increase in the percentage of DU 145 cells within subG1 cell cycle phase after 24 h treatment when compared with this percentage in control untreated DU 145 cells (Figure 2). Ethyl-acetate extract of flowers (5) applied at both tested concentrations caused the highest increase in the percentage of cells in the subG1 phase of the cell cycle (6.53% for IC_{50} concentration and 16.60% for $2IC_{50}$ concentration). Hexane extract of the branch-body part (3), methanol extract of flowers (4), and hexane extract of flowers (6) applied at IC_{50} and $2IC_{50}$ concentrations triggered the increase in the percentage of DU 145 cells within the G1 phase of the cell cycle in comparison with this percentage in control cells. Ethyl-acetate extract of flowers (5) applied at IC_{50} concentration also caused the increase in the percentage of cells in the G1 phase.

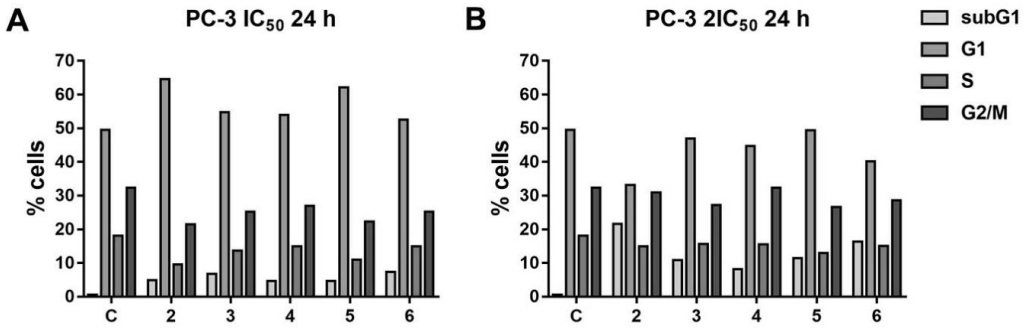

Figure 1. Changes in the cell cycle phase distribution of PC-3 cells treated with *Hypericum perforatum* extracts applied at concentrations corresponding to IC_{50} (A) and $2IC_{50}$ (B) after 24 h. C–control, extracts are as follows: 2-ethyl-acetate extract of branch-body part, 3-hexane extract of branch-body part, 4-methanol extract of flowers, 5-ethyl-acetate extract of flowers, 6-hexane extract of flowers. Results of one representative experiment are shown.

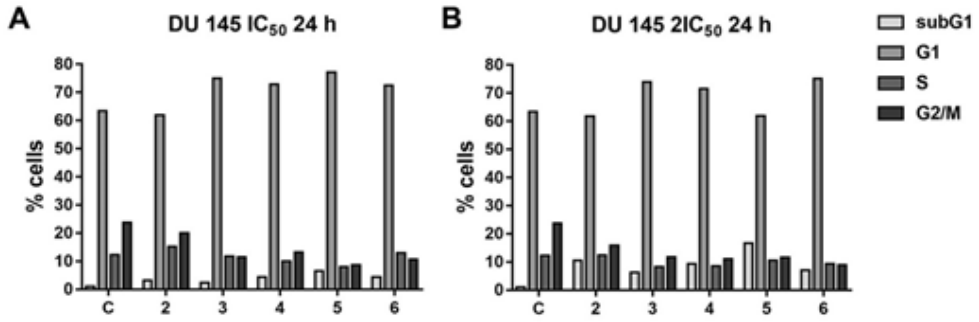

Figure 2. Changes in the cell cycle phase distribution of DU 145 cells treated with *Hypericum perforatum* extracts applied at concentrations corresponding to IC_{50} (A) and $2IC_{50}$ (B) after 24 h. C – control, extracts are as follows: 2-ethyl-acetate extract of branch-body part, 3-hexane extract of branch-body part, 4-methanol extract of flowers, 5-ethyl-acetate extract of flowers, 6-hexane extract of flowers. Results of one representative experiment are shown.

MicroRNA relative expression

Out of six investigated extracts, five have shown good cytotoxic activity on PC-3 and DU 145 cell lines. MicroRNA expression level changes were evaluated in six samples (five treated with extracts and one non-treated control) from both cell lines. We have observed the potential influence of 3 variables on miRNA expression levels: part of the plant; type of extraction; type of cell line. Changes in treated samples compared with non-treated control sample were also described.

MicroRNA levels of expression in prostate adenocarcinoma PC-3 cell line

In PC-3 cells each plant body part in samples from all investigated types of extraction increased levels of miR-21/128/133b/155/193a/206/335 compared with the control cell sample. The highest increase of miR-128 levels was detected after the treatment with hexane extract of flowers (6) (67.2 times higher than control), followed by ethyl-acetate extract of flowers (5) (22.3 times higher than control). In the case of miR-128 in PC-3 cells, the influence of the plant part (flowers in particular) was more eminent than the influence of the extraction type (Figure 3). Also, it is evident that miR-128 was higher in cells treated with hexane extracts compared with ethyl acetate extracts of both branch-body part and flowers. MicroRNA-128 was the microRNA with the highest-level changes, accompanied by miR-193a, both increased, and both with tumor suppressor characteristics.

MicroRNA levels of expression in DU 145 cell line

Prostate cancer cells treated with methanol extract of flowers (4) had the highest levels of tumor-suppressive miR-133b, compared with these levels in cells treated with other extracts, and control cells (Figure 3). Cells treated with hexane extract of the branch-body part (3) and cells traded with ethyl-acetate extract of flowers (5) showed the highest levels of miR-206. Hexane extract of the branch-body part (3), methanol extract of flowers (4), ethyl-acetate extract of the branch-body part, and ethyl-acetate extract of flowers (5) showed similarly increased levels of miR-206 among each other in treated PC-3 cells (Figure 4). Levels of miR-21 were just slightly increased in cells treated with hexane extract of the branch-body part (3), while cells treated with methanol extract of flowers (4) had the most increased levels of miR-21. Cells treated with ethyl-acetate extract of flowers (5) showed the smallest increase of miR-21, and miR-155, but slightly higher than in the control cell sample (Figures 3, 4).

In DU 145 cells treated with ethyl-acetate and hexane extract of the branch-body part, we detected higher levels of miR-133b/155/21 compared with the control, while miR-206 levels were lowered (Figure 3, 4). Ethyl acetate extract of the branch-body part (2) and ethyl-acetate extracts of flowers (5) increased miR-335 levels. Ethyl-acetate extracts of flowers (5) increased miR-128 levels, while all investigated extracts combined with different plant parts increased miR-193a levels. Ethyl-acetate extracts of the branch-body part (2) showed the highest increase in miR-128 expression in DU 145. The same extract slightly increased levels of miR-133b. In DU 145 samples treated with methanol extract of flowers, miR-133b and miR-206 were lower than the control, while miR-21 and miR-155 were higher. DU 145 cells treated with ethyl-acetate

extract of flowers (5) had lower levels of miR-133b and miR-206, but slightly higher levels of miR-21 and miR-155. DU 145 cells treated with hexane extract of flowers (6) had lower miR-133b/206 levels and higher miR-21/155 levels (Figure 3, 4).

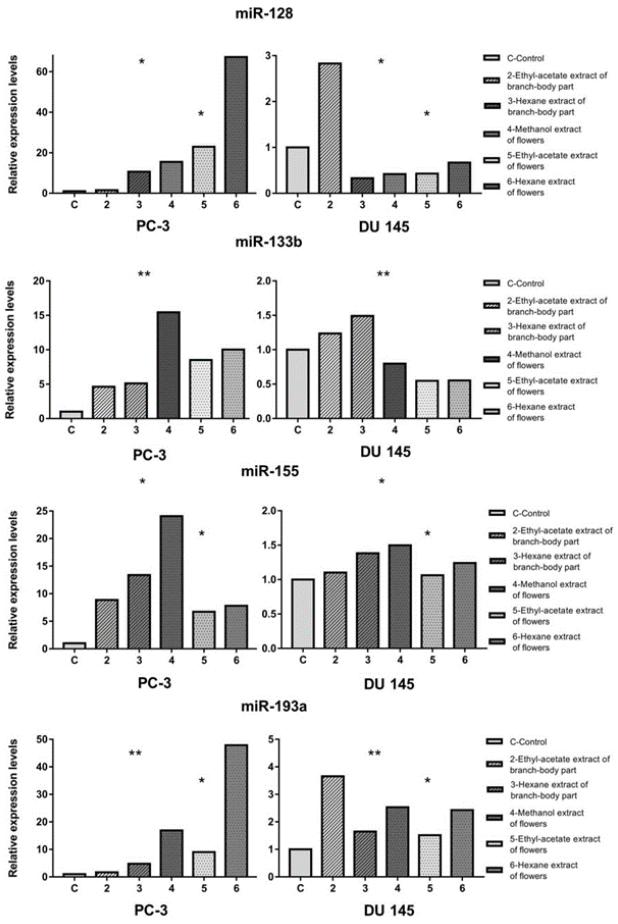

Figure 3. Relative expression of miR-128/133b/155/193a molecules in PC-3 and DU 145 cell lines treated with 2-ethyl-acetate extract of branch-body part, 3-hexane extract of branch-body part, 4-methanol extract of flowers, 5-ethyl-acetate extract of flowers, 6-hexane extract of flowers, and C-control. Significant differences among PC-3 and DU 145 samples are presented with an asterisk (*) for p less than 0.05, and ** for p less than 0.01. Each miRNA is represented in a different shade, while the extracts are presented in different shades and patterns.

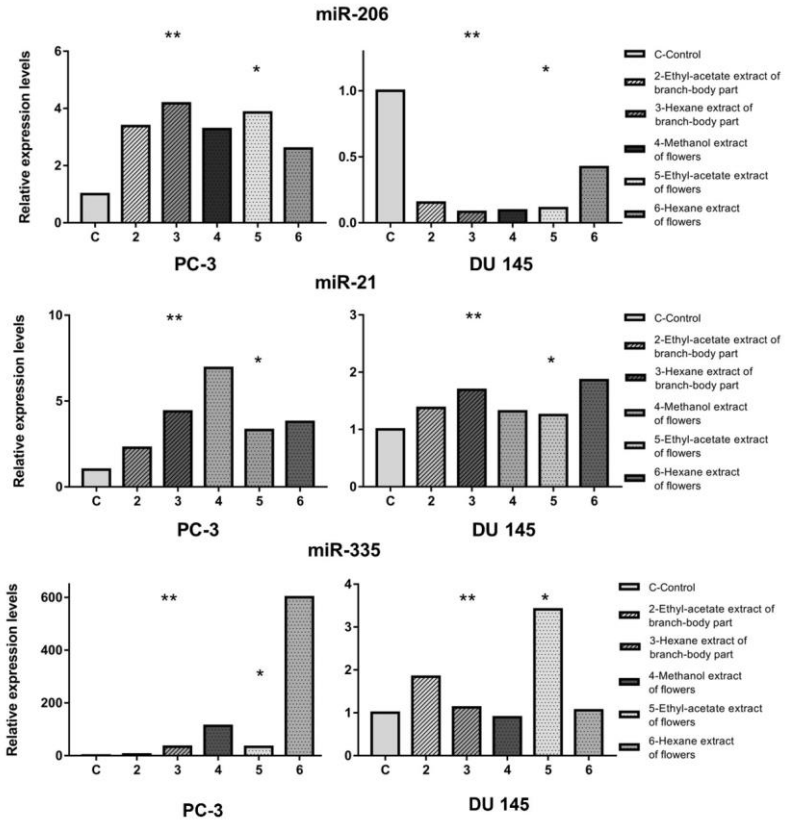

Figure 4. Relative expression of miR-206/21/335 molecules in PC-3 and DU 145 cell lines treated with 2-ethyl-acetate extract of branch-body part, 3-hexane extract of branch-body part, 4-methanol extract of flowers, 5-ethyl-acetate extract of flowers, 6-hexane extract of flowers, and C-control. Significant differences among PC-3 and DU 145 samples are presented with an asterisk (*) for p less than 0.05, and ** for p less than 0.01. Each miRNA is represented in a different shade, while the extracts are presented in different shades and patterns.

Differences in miRNA expression levels between cell lines, plant part extracts, and type of extraction

PC-3 cells showed significantly higher levels of miR-133b/155/193a/206/335 (Mann-Whitney U test, $p = 0.009$), and miR-128 (Mann-Whitney U test, $p = 0.016$, Table 2) compared with those levels in DU 145 cell samples.

Table 2. Differences between PC-3 and DU-145 and plant parts

MicroRNA relative expression (median)					
Part of the plant	PC-3	$p^{PC\ 3\ bbp/f}$	DU 145	$p^{DU\ 145\ bbp/f}$	$p^{PC\text{-}3/DU\ 145}$ $p^{bbp\ PC\text{-}3/DU\ 145}$ $p^{f\ PC\text{-}3/DU\ 145}$
miR-128					
	15.455		0.429		*0.016*
Branch-body part	4.572	*0.083*	0.429	*> 0.999*	*0.439**
Flowers	22.943		1.250		*0.050***
miR-133b					
	8.504		0.798		*0.009*
Branch-body part	4.867	*0.083*	1.363	*0.083*	*0.121*
Flowers	10.032		0.552		*0.121*
miR-155					
	8.865		1.241		*0.009*
Branch-body part	11.126	*0.564*	1.243	*> 0.999*	*0.121*
Flowers	37.809		1.241		*0.050*
miR-193a					
	9.000		0.611		*0.009*
Branch-body part	3.236	*0.083*	0.703	*0.248*	*0.121*
Flowers	16.912		0.483		*0.050*
miR-206					
	3.382		0.116		*0.009*
Branch-body part	3.778	*0.248*	0.118	*0.564*	*0.121*
Flowers	3.279		0.111		*0.050*
miR-21					
	3.791		1.376		*0.009*
Branch-body part	3.334	*0.564*	1.534	*0.564*	*0.121*
Flowers	3.791		1.315		*0.050*
miR-335					
	33.591		1.125		*0.009*
Branch-body part	19.109	*0.248*	1.483	*0.564*	*0.121*
Flowers	112.986		1.057		*0.050*

$p^{PC\text{-}3/DU\ 145}$ value represents the differences between all PC-3 and DU 145 cell samples; $p^{PC\ 3\ bbp/f}$ value represents the differences between PC-3 cells treated with bbp-branch-body part extracts and PC-3 cells treated with f-flower extracts; $p^{DU\ 145\ bbp/f}$ value representing the differences between DU 145 cells treated with bbp-branch-body part extracts and DU 145 cells treated with f-flower extracts; $p^{bbp\ PC\text{-}3/DU\ 145}$ *p-value representing the differences between PC-3 and DU 145 cells treated with bbp extracts and $p^{f\ PC\text{-}3/DU\ 145}$ ** treated with flower extracts. P values are in italic format. Significant p values are less than 0.05, and p values less than 0.01, and higher than 0.05 were considered as a statistical trend (bold font format). Median values of groups are presented in non-italic, non-bold font format.

Additionally, statistically significant higher expression of miR-128 was detected in PC-3 cells compared with expression level in DU 145 cells after the flower extract treatments (Mann-Whitney U test, p = 0.050, Table 2), while the difference between cell types was not detected after the branch-body part extract treatments. Furthermore, we have observed a statistical trend toward higher miR-128 expression levels in PC-3 cells incubated with flower extracts compared with branch-body part extract treated samples. (Mann-Whitney U test, p = 0.083, Table 2).

Table 3. Differences between PC-3 and DU-145 prostate cancer cells and the type of extraction

MicroRNA relative expression (median)					
Extraction type	PC-3	$p^{PC\text{-}3\ h/e}$	DU 145	$p^{DU\ 145\ h/e}$	$\frac{p^{h\ PC\text{-}3/DU\ 145}}{p^{e\ PC\text{-}3/DU\ 145}}$
miR-128					
Hexane	12.214	*0.439*	1.120	*> 0.999*	*0.439*
Ethyl-acetate	38.906		0.171		*0.121*
miR-133b					
Hexane	7.567	*0.439*	1.021	*0.437*	*0.121*
Ethyl-acetate	6.567		0.892		*0.121*
miR-155					
Hexane	10.596	*0.439*	1.312	*0.121*	*0.121*
Ethyl-acetate	7.789		1.082		*0.121*
miR-193a					
Hexane	26.313	*0.439*	0.373	*0.439*	*0.121*
Ethyl-acetate	5.341		0.639		*0.121*
miR-206					
Hexane	3.387	*> 0.999*	0.252	*> 0.999*	*0.121*
Ethyl-acetate	3.620		0.132		*0.121*
miR-21					
Hexane	4.092	*0.121*	1.776	*0.121*	*0.121*
Ethyl-acetate	2.794		1.314		*0.121*
miR-335					
Hexane	18.992	*0.121*	2.626	*0.564*	*0.121*
Ethyl-acetate	317.041		1.091		*0.121*

$p^{PC\text{-}3\ h/e}$ value represents the differences between treatment with hexane and ethyl-acetate extracts in PC-3 cell line; **$p^{DU\ 145\ h/e}$** value represents the differences between treatment with hexane and ethyl-acetate extracts in DU 145 cell line; **$p^{h\ PC\text{-}3/DU\ 145}$** value represents the differences between PC-3 and DU 145 treated with hexane extracts; **$p^{e\ PC\text{-}3/DU\ 145}$** value represents the differences between PC-3 and DU 145 cell treated with ethyl-acetate extracts. P values are in italic format. Median values of groups

In PC-3 cell line, miR-133b levels were higher in cells treated with flower extracts compared with cells treated with branch-body part extracts (Mann-Whitney U test, p = 0.083, Table 2). In DU 145 cells the situation was quite opposite- treatment with branch-body part extracts followed a trend towards higher miR-133b levels compared with treatment with flower extracts (Mann-Whitney U test, p = 0.083, Table 2).

Levels of miR-155 were significantly higher in PC-3 cells exposed to extracts of flowers compared with DU 145 exposed to extracts of flowers (Mann-Whitney U test, p = 0.050, Table 2). Treatment with extracts of flowers of PC-3 cells showed a trend towards higher levels of miR-193a compared with treatment with branch-body part extracts (Mann-Whitney U test, p = 0.050, Table 2). In addition, PC-3 cells treated with flower extracts had significantly higher levels of miR-193a than flower extracts of DU 145, and the same statistically significant effect was observed for miR-206, miR-21, and miR-335 levels (Mann-Whitney U test, p = 0.050). MicroRNA levels did not show significant differences between PC-3 and DU 145 cells treated with hexane and ethyl-acetate extracts, or between these two cell lines (Table 3).

DISCUSSION

During the past decade, miRNA molecules were examined in the combination with medicinal herbs as promising indicators of malignant potential and response to therapy in different pathological conditions, including various malignancies. MicroRNAs with tumor-suppressive role should be restored, and increased, while oncogenic are expected to be lowered in order to reduce malignant tumor growth and propagation. Also, it has been proposed that miRNA molecules might be utilized for the modulation of resistance to anticancer therapy via silencing transcripts of drug-resistance-related genes which are associated with cell death (apoptosis) and differentiation (SI *et al*., 2019).

The five extracts obtained from branch-body parts and flowers of wild plant populations of *H. perforatum* from Samsun in Turkey showed cytotoxic effects on two prostate cancer cell lines- PC-3 and DU 145. Hexane extract of flowers (6) and hexane extract of the branch-body part (3) demonstrated the best cytotoxic activity against tested prostate cancer cells. Prostate grade IV adenocarcinoma PC-3 and prostate carcinoma DU 145 cell lines had equal sensitivity to the strong cytotoxic activity of hexane extract of flowers (6). PC-3 cells were more sensitive to the cytotoxic effects of hexane extract of the branch-body part (3) when compared with the sensitivity of DU 145 cells. Both cell lines exerted similar sensitivity to the cytotoxicity of ethyl-acetate extract of flowers (5) which exerted pronounced cytotoxic activity. The prostate cancer cells had lower, but similar sensitivity to the cytotoxicity of ethyl-acetate extract of the branch-body part of the plant (2). PC-3 cells had similar sensitivity to the cytotoxic effects of the ethyl-acetate extract of the branch-body part (2) and methanol extract of flowers (4), and this cell line was more sensitive to extract 4 when compared with DU 145 cells.

The results of this research are in agreement with our previously published study that reported pronounced cytotoxic activities of hexane extract of flowers (6), hexane extract of the branch-body part (3), and ethyl-acetate extract of flowers (5) obtained from *H. perforatum*, collected at Samsun in Turkey against human cervical adenocarcinoma HeLa cells, chronic

myelogenous leukemia K562 cells, and lung carcinoma A549 cells (MATIĆ *et al.*, 2021). The hexane extract of flowers (6) had similar high intensity of cytotoxic activity against prostate cancer cell lines examined in this study and previously examined leukemia K562 cell line (MATIĆ *et al.*, 2021). This extract showed approximately two times higher cytotoxic activity against PC-3 and DU 145 prostate cancer cells when compared with its reported activity against lung carcinoma A549 cells (MATIĆ *et al.*, 2021). Furthermore, one of the *H. perforatum* extracts with good cytotoxicity-hexane extract of the branch-body part (3) exerted similar high intensity of cytotoxic activity against prostate adenocarcinoma PC-3 cells and leukemia K562 cells (MATIĆ *et al.*, 2021). Ethyl-acetate extract of flowers (5) showed lower intensity of cytotoxic activity against prostate cancer cell lines when compared with its reported activity against HeLa, K562, and A549 cancer cell lines (MATIĆ *et al.*, 2021). When considering the cytotoxicity of ethyl-acetate extract of the branch-body part (2) and methanol extract of flowers (4), these extracts showed higher cytotoxic effects against HeLa and K562 cell lines in comparison with effects against PC-3 and DU 145 cells. However, DU 145 cells and A549 lung carcinoma cells had similar sensitivity to the cytotoxicity of ethyl-acetate extract of the branch-body part (2), while PC-3 cells were to some extent more sensitive. In contrast to these results, the methanol extract of flowers (4) had higher intensity of cytotoxic activity against prostate cancer cell lines in comparison with activity against lung carcinoma A549 cells (MATIĆ *et al.*, 2021).

Examination of the effects of five *Hypericum perforatum* L. extracts (2-6) on cell cycle distribution revealed an increase in the percentage of PC-3 and DU 145 prostate cancer cells within the subG1 phase of the cell cycle after 24 h treatment with IC_{50} and $2IC_{50}$ concentrations of all tested extracts. The observed effect in prostate cancer cell lines confirms the ability of extracts to induce cell death in treated cells as has been reported in our previous study (MATIĆ *et al.*, 2021). It has been shown that all five investigated extracts (2-6) showed the ability to induce apoptosis in HeLa cells through the activation of effector caspase-3 (MATIĆ *et al.*, 2021). These results are in line with other studies reporting proapoptotic effects of various *H. perforatum* extracts against different cancer cell lines such as breast adenocarcinoma MCF7, human myelogenous leukemia K562, and colorectal adenocarcinoma HT-29 (VALLETTA *et al.*, 2018; YOU *et al.*, 2018; ARASH A. YEGANI *et al.*, 2020).

Differences in the qualitative and quantitative phytochemical composition of six *Hypericum perforatum* L. extracts extracted from plant branch-body parts and flowers using three different solvents caused differences in intensities of cytotoxic activities of extracts against prostate cancer cell lines and other previously tested cancer cell lines (MATIĆ *et al.*, 2021). The main bioactive phytochemicals identified in the hexane extract of flowers (6) and hexane extract of the branch-body part (3) which exerted the strongest cytotoxicity against PC-3 and DU 145 cells were quercetin, hypericin, pseudohypericin, hyperforin, adhyperforin, and biapigenin (MATIĆ *et al.*, 2021). Diverse extraction procedures of different parts of the plant *Hypericum perforatum* L. in addition to plant habitat and seasonal variations in the content of secondary metabolites may lead to differences in the reported intensities of cytotoxic activity of distinct *H. perforatum* extracts. Results of from MARTARELLI *et al*. (2004) study demonstrated the cytotoxic effect of *H. perforatum* methanol extract containing hyperforin and hypericin against PC-3 cells

with IC_{50} value of 420 µg/mL. While our methanol extract of the branch-body part (1) exerted very weak cytotoxic properties against prostate cancer cells only at concentrations higher than 200 µg/mL, the methanol extract of flowers (4) showed approximately ten times higher intensity of cytotoxic activity against PC-3 cells when comparing the intensity of activity of extract reported by MARTARELLI *et al*. (2004).

Restoration of tumor-suppressive miR-128 was shown to have an inhibitory effect on prostate cancer cell invasion, and improved sensitivity to cisplatin (SUN *et al.*, 2015). So, restoration of miR-128 by plant extracts might be useful to improve response to therapy. In the PC-3 cell line, we have observed that the hexane extract of flowers (6) may be quite promising in the elevation of miR-128 expression levels. In DU 145 cells treatment with ethyl-acetate extract of the branch-body part (2) of *H. perforatum* might be the right choice to elevate miR-128 in order to prevent prostate cancer progression.

MicroRNA-21 is a well-known oncomiRNA elevated in every known solid cancer (PAN *et al.*, 2010). In our experiment, we have shown that two tumor suppressor miRNAs were elevated in cells treated with extract, compared with control. But, interestingly, miRNA-21 and miR-155 as well-known oncomiRNAs showed higher levels compared with control. The mechanisms affecting miR-21-mediated signaling pathways in PCa are still unclear. MicroRNA-21 was shown to be low-expressed in metastatic PCa (SONG *et al.*, 2018). Also, it is matter of question does miR-21 have oncogenic and malignant potential, or the potential to increase the proliferation of non-androgen-dependent cells. Because, as one of the AR-responsive miRNAs, higher miR-21 levels are usually associated with androgen-dependent prostate cancer proliferation, while the PC-3 cell line model does not express androgen receptor (RIBAS *et al.*, 2009), but that is also a matter of contradictory reports because both PC-3 and DU 145 contain mRNA transcribed from AR (ALIMIRAH *et al.*, 2006), but the response to androgen is questionable. So, increased expression of AR-responsive miR-21 in PC-3 cells treated with *Hypericum perforatum* L. extracts could be logical with the absence of androgen factor. In the case of miR-155, HESSVIK *et al*. (2013) investigated if there were any differences in the miRNA expression between PC-3 and the non-cancerous prostate cell line RWPE-1 cells. They showed that 20 miRNAs were identified selectively in PC-3 cells, whereas 26 miRNAs, including miR-155, were observed only in RWPE-1 cells (HESSVIK *et al.*, 2013). This supports that *H. perforatum* L. induces RWPE-1 phenotype, instead of PC-3, with respect to differential miRNA profiles. Moreover, according to a study performed by JI *et al*. (2014) arsenic trioxide (As_2O_3) attenuated angiogenic ability through miR-155-mediated inhibition of transforming growth factor beta (TGF-β)/SMAD signal pathway in PC-3 *in vitro*. As As_2O_3 in this example, *H. perforatum* extracts improved the expression of miR-155 in our study. Therefore, miR-155-mediated tumor suppressive effect of *H. perforatum* may make it a potential chemopreventive agent for prostate cancer, like As_2O_3. Another possible explanation for the increased expression of miR-21 and miR-155 may be that *H. perforatum-dependent* upregulation of miR-133b and miR-206 causes an increase in miR-155 and miR-21 expressions in accordance with ceRNA hypothesis (ERGUN and OZTUZCU, 2015). According to ceRNA hypothesis, there are many genes targeted by miR-133b, miR-206, miR-155, and miR-21 combination and these genes having

miRNA target sites on them for these miRNAs can show sponge activity in different strengths for each of them. In our case, common targets of four concerned miRNAs may be downregulated by increased miR-133b and miR-206 expressions, the sponge activities of these common target genes on miR-155 and miR-21 may decrease and this may eventually cause an increase in the expression levels of miR-155 and miR-21. Furthermore, miRNA expressions are generally regulated together with a gene especially when it is located in 3'UTR region. For example, miR-21 is located in 3'UTR of TMEM49 gene (RIBAS and LUPOLD, 2010). We can discuss the effects of the extracts through TMEM49 and other genes regulated together. Up to now, the relationship between TMEM49 and prostate cancer has become a mystery. In invasive breast and kidney cancer metastasis TMEM49 was characterized as tumor-suppressive (MANN *et al.*, 2008). This information supports our finding that miR-21, a miRNA docking on TMEM49, expression is increased in prostate cancer cells treated with *H. perforatum* extracts.

In PC-3 cells, miR-133b has the ability to induce proapoptotic pathways, and overexpression of miR-206 reduces tumor promotion and propagation, which indicates that examined extracts might have antitumorigenic effects (CAI *et al.*, 2015; WANG *et al.*, 2018) that involve miR-206 expression changes.

It has been shown that miR-193a has anti-oncogenic properties, and inhibits cell growth in of PC-3 and DU 145 cell cells (LIU *et al.*, 2017). Methanol and hexane extracts of flowers increase miR-193a levels in PC-3, while all extracts especially ethyl-acetate of branch-body part and methanol/hexane extracts of flowers in DU 145 cells, indicating that *H. perforatum* may have great potential to increase this tumor suppressor, regardless the type of extraction and plant part. Considering that miR-335 acts as a tumor-suppressive feature in PCa (PNG *et al.*, 2011), hexane extracts of flowers in PC-3, and ethyl-acetatete extracts of flowers may have the best anti-tumor effect.

PC-3 is a high-grade (IV) PCa cell line with high metastatic potential, while DU 145 shows moderate metastatic potential (RAVENNA *et al.*, 2014). Significant differences in all investigated miRNA expression levels between the two investigated prostate cell lines suggest that future additional treatment approaches might be specific to tumor subtype, tumor grade, and stage (VERMA *et al.*, 2019). VERMA *et al.* (2019) have listed miRNAs that significantly differed between the PC-3 and DU 145 cell lines. None of our investigated miRNAs was on that list, except the miR-128 precursor, not mature (VERMA *et al.*, 2019). To summarize, PC-3 and DU 145 cell lines treated with *Hypericum perforatum* L. extracts showed different miRNA signatures.

CONCLUSIONS

Hexane extracts of *Hypericum perforatum* L. flowers and branch-body parts exerted the most prominent cytotoxic effects against prostate cancer PC-3 and DU 145 cell lines. Each of the five examined extracts caused an increase in the percentage of PC-3 and DU 145 cells within the subG1 phase of the cell cycle. Ethyl-acetate extract of flowers might be the most efficient from the aspect of miRNA level changes, because it might have the best ability to increase protective miR-133b and miR-206 levels, and just slightly and non-significantly increase the level of the

two oncomiRNAs in PC-3 cell line, but not in DU 145. Hexane extract of flowers has the highest potential to increase miR-128, and miR-193a levels, the miRNAs with a tumor-suppressive role in PCa.

Considering the fact that miRNA molecules are one of the major regulators of gene expression, especially during cancer pathogenesis, it is very important among others, to investigate the influence of phytochemicals with anticancer effects, and it is also very important to bear in mind the heterogeneity in their behavior in various tumor subtypes, so miRNA might not be universal biomarkers or therapeutic targets, but might be very useful in specific tumor subtypes, as we here have seen those differences between two humane prostate cancer cell lines. A miRNA signature can be utilized for the prediction of response to various therapeutic strategies. Also, several miRNAs at the same time might be considered to be targeted in combination with other treatment modalities.

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Hypericum perforatum L. EKSTRAKTI POKAZUJU CITOTOKSIČNI EFEKAT I RAZLIČIT MIKRO RNK POTPIS U PC-3 I DU 145 ĆELIJAMA RAKA PROSTATE

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Izvod

Rak prostate je jedan od vodećih uzroka smrti u muškoj populaciji. Specifični potpisi mikro RNK su povezani sa nastankom i progresijom raka prostate i odgovorom na terapiju. Pokazano je da prirodna jedinjenja biljaka dovode do promena u nivoima mikro RNK u ćelijama različitih tumora. U ovoj studiji ispitana je citotoksična aktivnost ekstrakata *Hipericum perforatum* L., MTT testom preživljavanja ćelija, kao i njihovi efekti na distribuciju PC-3 i DU 145 ćelija u određenim fazama ćelijskog ciklusa protočnom citometrijom. Takođe, ispitani su i nivoi ekspresije mikro RNK reakcijom RT-qPCR. Heksanski ekstrakt cvetova je pokazao najjači intenzitet citotoksične aktivnosti prema ćelijama PC-3 i DU 145. Najveći porast procenta PC-3 ćelija u subG1 fazi zabeležen je u uzorcima ćelija tretiranim heksanskim ekstraktom cvetova i nadzemnog dela biljke bez cvetova. Uočene su značajne razlike u nivoima ekspresije mikro RNK-128/133b/155/193a/206/21/335 između PC-3 i DU 145 ćelijskih linija, posebno u uzorcima tretiranim ekstraktima cvetova u poređenju sa ekstraktima nadzemnog dela biljke bez cvetova. Ispitivani ekstrakti imaju značajan antitumorski potencijal, ne samo zbog citotoksičnosti i efekata na ćelijski ciklus, već i zbog mogućnosti snižavanja nivoa onkogenih i povećanja nivoa tumor-supresornih mikro RNK. Najznačajniji efekat bi mogao biti povećanje tumor-supresorne miR-128 (zajedno sa miR-193a) nakon tretmana heksanskim ekstraktom cvetova, koji je takođe ispoljio i najvišu citotoksičnu aktivnost. Heksanski ekstrakt cvetova bi mogao biti kandidat za poboljšanje efikasnosti standardnih terapija. Potpis mikro RNK može biti specifičan za tip ćelije/maligniteta nakon tretmana ekstraktima *Hipericum perforatum* L, što bi se moglo koristiti za podelu pacijenata u specifične grupe za određenu kombinaciju terapije.

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