Acta Poloniae Pharmaceutica – Drug Research, Vol. 74 No. 2 pp. 688–698, 2017 ISSN 0001-6837
Polish Pharmaceutical Society

ANTIOXIDANT AND CANCER CHEMOPREVENTIVE ACTIVITIES OF CISTUS AND POMEGRANATE POLYPHENOLS

HELENA MOREIRA[1], ALEKSANDRA ŚLĘZAK[1]*, ANNA SZYJKA[1], JAN OSZMIAŃSKI[2] and KAZIMIERZ GĄSIOROWSKI[1]

[1]Department of Basic Medical Sciences, Wroclaw Medical University, Borowska 211, 50-556 Wrocław, Poland
[2]Department of Fruit, Vegetable and Cereals Technology, The Faculty of Food Sciences, Wrocław University of Environmental and Life Sciences, Chełmońskiego 37/41, 51-630 Wrocław, Poland

Abstract: Polyphenol rich extracts obtained from cistus herb (*Cistus incanus* L.) and pomegranate peel (*Punica granatum* L.) exhibited significant antioxidant activity in V79 cell culture (Chinese hamster lung fibroblasts) - cistus extract reduced intracellular content of reactive oxygen species (ROS) by 30-40% and pomegranate extract by 29-36%. In human breast (MCF-7) and colon (LOVO) cancer cell lines cistus and pomegranate extracts decreased cancer cell growth both in drug-sensitive cells by 15-30% and in drug resistant (doxorubicin-resistant; DX) sublines by 5-20%. However, the extracts did not influence on cell growth of normal hamster fibroblast cultures (V79). The extracts induced apoptosis in the tested cancer cell lines. Significantly higher proapoptotic impact of the extracts was observed in drug-sensitive than in drug-resistant sublines. The results suggest potential usefulness of the tested polyphenol rich extracts in people exposed to oxidative stress. Their potential use as adjuvant therapy of human cancers needs further studies.

Keywords: *Cistus incanus* L., *Punica granatum* L., polyphenols, antioxidant, anticancer

Wide range of phytochemicals exhibit antioxidant and anticancer properties and total biological effect of plant extracts is usually attributed to the additive and synergistic effects of the complex mixture of phytochemicals present in plants (1). The aim of the present study wass to evaluate an impact of polyphenolic rich extracts obtained from *Cistus incanus* L. herb and from *Punica granatum* L. peel on oxidative stress in normal hamster lung fibroblasts (V79) and on proliferation of several human cancer cell lines.

Cistus incanus L. is the Mediterranean plant found in the regions of Southern Europe and North Africa. In traditional medicine, cistus herbal teas have been used for the treatment of diarrhea and as an antispasmodic or anti-inflammatory agents. Cistus extracts have also been known for their antimicrobial, antimycotic, antiviral and antibacterial activities (2, 3). Recently, antioxidant capacities of cistus extracts have been described (4). However, anticancer activity of cistus extract was only scanty described. It was shown that aqueous extracts of cistus was active in suppressing the growth of human epithelial prostate cells (5) and revealed cytotoxicity of cistus extracts against KB, P-388 and NSCLC N6 cell line (6).

Punica granatum L. (pomegranate) is an original native of Persia, later naturalized in the Mediterranean region. Nowadays, pomegranate fruits are widely consumed in many countries in form of juice, wines, or crude fruit (7). Literature data describe its therapeutic potential such as antiatherogenic, antiparasitic, antimicrobial, anti-inflammatory, antioxidant and cancer preventing effects (7). Putative mechanisms of anticancer activity of pomegranate includes antiproliferative, antioxidant, anti-invasive, anti-metastatic, antigenotoxic effects, as well as induction of apoptosis through the modulation of Bcl-2 proteins, upregulation of p21 and p27 protein, and downregulation of cyclin-cdk network (8, 9). Anticancerogenic activity of whole pomegranate fruits was shown to be superior to those observed with their isolated active compounds (10).

* Corresponding author: e-mail: ola.sle@interia.pl

EXPERIMENTAL

Chemicals

Trolox ((R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DCF-DA (2,7-dichlorofluorescin diacetate), TCA (trichloroacetic acid), SRB (sulforhodamine B, sodium salt) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell proliferation kit II (XTT) was purchased from ROCHE (Mannheim, Germany). FITC Annexin V Apoptosis Detecting Kit was obtained from BD PHARMIGEN Biosciences (Franklin Lakes, NJ, USA). Cell culture media: EMEM (Eagle's Minimum Essential Medium), DMEM F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) and DMEM (Dulbecco's Modified Eagle Medium), HBSS (Hank's Balanced Salt Solution), FBS (fetal bovine serum), ultraglutamine 1 and gentamicin sulfate were purchased from Lonza (Verviers, Belgium). TrypLE™ Express was obtained from GIBCO (Waltham, MA, USA). All other chemicals were from POCH S.A. (Gliwice, Poland).

Plant material

Raw material for preparation of *Punica granatum* L. peel the fruit delivered from Israel and herb *Cistus incanus* L. from Albania.

Samples were directly frozen in liquid nitrogen and freeze-dried (24 h; Alpha 1-4 LSC, Christ, Germany). The homogeneous powders were obtained by crushing the dried tissues using a closed laboratory mill to avoid hydration. Powders were kept in a refrigerator (-80^{o}C) until extract preparation.

Extraction procedure and the content of polyphenols

The extraction procedure of polyphenols was described previously (11). Polyphenols were isolated from *Punica granatum* L. peels by extraction with 50% methanol, the ratio of this solvent to dry materials being 3 : 1 (v/v). The extract after methanol evaporation was adsorbed on Purolite AP 400 resin (U.K.) for further purification. The polyphenols were then eluted out with 80% ethanol, concentrated, and freeze-dried.

Extracts from herb of *Cistus incanus* L. was prepared according to its own method (12), wherein the extract ethyl acetate was selectively precipitated from the group of catechin and procyanidin eliminating other polyphenols.

The content of polyphenols in individual preparations was determined by means of UPLC/DAD and the UPLC/ESI/MS method of analyses described earlier (13).

Just before addition to cell cultures, the extracts solutions were freshly prepared by dissolving in HBSS at concentration of 1 mg/mL (stock solution).

Cell lines

V79 (Chinese hamster pulmonary fibroblasts), LOVO (human colon adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cell lines were purchased from ATCC (American Type Culture Collection, Washington, DC, USA). The doxorubicin resistant cell sublines: LOVO/DX and MCF-7/DX were derived from the original drug-sensitive LOVO and MCF-7 cell lines by 3-months cultivation in the presence of low concentration of doxorubicin.

Cells were cultured in recommended culture media: EMEM (V79), DMEM F12 (LOVO) and DMEM (MCF-7), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25 μg/mL of gentamicin, in 75 cm^2 plastic flasks at 37^{o}C in CO_2-incubator and were subcultured twice a week using TrypLE™ Express solution.

Methods

Detection of intracellular ROS with the DCF-DA assay

The DCF-DA assay was carried out according to the protocol described in the literature 1(14). Briefly, V79 cells were removed from the culture flask using TrypLE™ Express solution and washed with HBSS, spun down and pelleted. Cells were then suspended (1×10^6 cells/mL) in fresh culture medium containing DCF-DA (20 μM) in plastic Falcon tubes (1 mL/sample). The tested extracts were immediately added to the samples (the final concentrations range: 0.1-1 μg/mL), and Trolox, the standard antioxidant (a peroxyl radical scavenger), was used as a positive control. Samples were incubated for 1 h at 37^{o}C, afterwards they were placed on ice and immediately analyzed with CyFlow® SPACE flow cytometer (Sysmex-Partec, Görlitz, Germany). The laser excitation 488 nm (50 mW) and the filter 536/40 (BP) were used for measurements of cell associated DCF fluorescence. The results were analyzed using FCS Express 4 flow software (De Novo Software, Glendale, CA, USA).

Cells viability assay (XTT assay)

Cell viability assay was carried out with the Cell proliferation kit II (XTT), according to manufacturer's instructions. In short, V79 cells were

seeded into 96-well plate at a density of 2×10^4 cell at 100 µL of culture medium and incubated overnight at 37°C in CO_2-incubator. Then, cells were suspended in a fresh medium and the extracts solutions were added to culture wells to the final concentrations 1-100 µg/mL. After 24-h incubation, 50 µL of XTT solution was added to each well and cells were incubated for 4 h at 37°C. Finally, the absorbance at 475 nm were measured in cell samples with Wallac1420 VICTOR2 microplate reader (Perkin Elmer, Waltham, MA, USA).

SRB assay

Growth inhibitory effect of the tested extracts were measured using the sulforhodamine B (SRB) assay, as previously described (15). LOVO, LOVO/DX, MCF-7 and MCF-7/DX cells grown in 48-well plates at a density of 4×10^5 cell per well were incubated for 24 h at 37°C in CO_2-incubator. The extracts solutions were added to the second plate to the required final concentrations (1-75 µg/mL). Following addition of the extracts, cell cultures were incubated for additional 48 h at 37°C in CO_2-incubator, then, cells on plastic culture wells were fixed with cold TCA (final concentration 10%, w/v) for 1 h at 4°C, washed with tap water and air-dried at room temperature. A mildly acidic SRB solution (0.4% dye solution in 1% acetic acid) was added to wells for 30 min at 25°C, and unbound stain was removed by rinsing with aqueous solution of 1% (v/v) acetic acid. Afterwards, culture plates were allowed to dry at room temperature. The SRB bound to the cellular proteins was dissolved in 10 mM Trizma-base solution (pH 10.5) for 10 min shaking vigorously and the absorbance of the SRB solution was estimated at 540 nm in a Victor 2 microplate reader (Perkin-Elmer, MA, USA).

Apoptosis detection assay

Apoptosis was detected in cancer cell cultures with flow cytometry after staining of cells with fluorochrome mixture: annexin V-FITC and PI, using the FITC Annexin V Apoptosis Detection Kit I. LOVO, LOVO/DX, MCF-7 and MCF-7/DX cells (4×10^5) were incubated for 24 h in 24-well culture plate in the presence of the extracts (37°C, 5% CO_2). Then, cells were detached with TrypLE™ Express, washed with cold PBS and resuspended in 100 µL of ice-cold 1X binding buffer, stained with 5 µL of Annexin V-FITC and 5 µL of PI for 10 min in the dark at room temperature. Samples were analyzed with the flow cytometer CyFlow® SPACE (Sysmex-Partec, Görlitz, Germany) applying laser excitation 488 nm (50 mW), filters: 536/40 (BP) for Annexin V-FITC and 675/20 (BP) for PI. The results were analyzed with FCS express 4 flow software.

Statistical analysis

Statistical significance of the results was calculated with paired Student's *t* test.

RESULTS

Polyphenolic composition of cistus and pomegranate extracts' preparations as determined by means of UPLC/DAD and the UPLC/ESI/MS is given in Table 1 (cistus) and in Table 2 (pomegranate).

The major polyphenolic constituents of the cistus extract are myricetin glycosides (61.81 g/100 g of the extract dry mass), including myricetin 3-rhamnoside representing 47.3% of the extract mass (Table 1). Other polyphenols present in the extract (although in markedly lower content) are quercetin glycosides (9.28 g/100 g), gallocatechins (6.27 g/100 g), epicatechins (3.07 g/100 g), kaempferol glycosides (1.97 g/100 g) and bis-HHDP-glucose (1.22 g/100 g). Gallic acid, punicalagin, cornussin B, prodelphinidin and pendunculagin are present in very low quantity (< 1 g/100 g).

Pomegranate extract consists of the highest body of HHDP-gallagyl-hexoside (punicalagin) (58.88 g/100 g) together with punicalagin isomer (2.40 g/100 g) and punicalagin α/A (2.04 g/100 g). The extract is also rich in ellagic acid glycosides (7.68 g/100 g) and ellagic acid derivatives (5.34 g/100 g) and ellagitannin (1.96 g/100 g). Other constituents: kaempferol glycosides, granatin A and B, pendunculagin II, gallocatechin are present in small quantities (< 1 g/100 g).

Evaluation of ROS scavenging activities

Effects of cistus and pomegranate extracts on ROS level inV79 cells, measured with the DCF-DA assay, are shown in Figure 1.

The tested concentration range (0.1–1.0 µg/mL) of both extracts strongly decreased ROS level in V79 cells, by 30-40% (cistus) and by 29-36% (pomegranate). The observed effects were statistically significant and comparable to the effect of Trolox (10 µM), a water-soluble analog of vitamin E. It is essential to note that flavonoids present in plant extracts solution do not directly interact with the DCF-DA (16).

Effect of the extracts on V79 cells viability

The influence of 24-h incubation with the tested extract on viability of the V79 cell cultures, is given in Figure 2.

Table 1. Retention times, UV/Vis spectra characteristics of phenolic compounds of cistus water extract.

Compound	Rt [min]	α_{max} [nm]	MS-	MS/MS-	Content g/100g
Galloyl glucose	2.01	280	331		
Gallic acid	2.22	280	169		0.68
Punicalin isomer	2.32	370	781	601/301	
Punicalin isomer	2.48	370	781	601/301	
Gallocatechin trimer	2.54	280	913	305	0.12
Gallocatechin dimer	2.73	280	609	423/305	0.51
Gallocatechin-(4α-8)-catechin	3.08	280	593	289	0.08
Digalloyl glucose	3.19	280	483	169	0.04
Gallocatechin	3.30	280	305		3.66
Gallocatechin dimer	3.45	280	609	423/305	0.10
Gallocatechin-(4α-8)-catechin	3.55	280	593	289	0.32
Gallocatechin dimer	3.64	280	609	423/305	0.23
Gallocatechin trimer	3.70	280	913	305	0.27
Gallocatechin dimer	3.84	280	609	423/305	0.51
Punicalagin isomer	3.89	370	1083	781/301	0.15
Cornusiin B	4.00	370	1085	451/301	0.08
bis-HHDP-glucose	4.29	280	783	301	1.04
bis-HHDP-glucose	4.36	280	783	301	0.18
Prodelphinidin dimer	4.53	280	593	305	0.46
Punicalagin isomer	4.61	370	1083	781/601/301	0.32
Cornusiin B	4.72	370	1085	783/451/301	0.15
HHDP-digalloyl-glucoside	4.75	370	785	301	0.44
(-)Epicatechin	4.89	280	289		3.07
Galloyl-HHDP-glucoside	4.99	280	633	301	0.06
Punicalagin gallate	5.05	370	1251	1083/ 781/601/ 301	0.00
Galloylprodelphinidin trimer	5.18	280	1085	591/305/167	0.28
Gallocatechin dimer	5.36	280	609	423/305	0.47
HHDP-hex	5.37	370	481	301	
Galloyl-prodelphinidin trimmer	5.45	280	1065	897/593/305/168	0.32
Digalloyl-HDDP-glucoside(pedunculagin II)	5.55	370	785	483/301	0.57
Myricetin-3-O-galactoside	6.55	350	479	316	5.72
Myricetin-3-O-glucoside	6.65	350	479	316	0.70
Myricetin-O-xyloside2	7.15	350	449	316	7.10
Ellagic acid rutinoside	7.27	360	609	463/301	
Myricitrin Myricetin 3-rhamnoside	7.32	350	463	316	47.30
Quercetin-3-O-galactoside	7.44	350	463	301	2.04
Quercetin-3-O-glucoside	7.56	350	463	301	0.43
Myricetin-pentoside	7.73	350	449	316	
Quercetin-pentoside	8.02	350	433	301	1.94
Kaempferoldimethyletherhexoside	8.22	350	475	285	0.59
Kaempferol -3-O-glucoside	8.30	350	447	285	0.61
Quercetin-3-O-rhamnoside	8.38	350	447	301	4.73
Myricetin -rutinoside	9.26	350	625	479/316	0.85
Myricetin -rhamno-glucoside	9.70	350	625	479/316	0.14
Quercetin rhamno-glucoside	9.99	350	609	463/301	0.14
Kaempferol -O-rutinoside	10.65	350	593	447/285	0.59
Kaempferol rhamno-glucoside	11.02	350	593	285	0.18

The results display that viability of the V79 cells was not significantly changed by the extracts. Depending on the concentration of the extracts, cell cultures viability (reflected by the activity of mitochondrial enzymes in the XTT assay) varied from 92% to 112% (cistus) and from 95% to 105% (pomegranate).

Human cancer cells growth inhibition by the tested extracts

Impact of the extracts on human breast (MCF-7) and colon (LOVO) cancer cell lines was determined using the SRB assay, separately in cell lines on average sensitive to cytostatic drugs (LOVO/WT and MCF-7/WT) and in sublines resistant to doxorubicin (LOVO/DX and MCF-7/DX). Cells were exposed to cistus and pomegranate extracts for 48 h. Results of the SRB test are given in Figure 3 (cistus) and in Figure 4 (pomegranate).

As shown in Figure 3, cistus extract exhibited the most significant growth inhibitory activity towards LOVO/WT (decrease by 16-27%) and MCF-7/DX cells (decrease by 15-28%). The effects were statistically significant, although not concentration dependent. No significant changes of the

Table 2. Retention times, UV/Vis spectra characteristics of phenolic compounds of pomegranate water extract.

Compound	Rt [min]	α_{max} [nm]	MS-	MS/MS-	Content g/100g
Galloyl-glucose	1.66	261/376	331	271/169	0.18
Punicalin α/A	1.89	257/377	781	601, 721	2.04
HHDP-galloyl-hexoside (punicalagin)	2.09	257/377	1083	611/331/146	0.80
Punicalagin isomer	2.18	257/377	1083	781/622/301	2.40
Ellagitannin	2.43	255/376	933	631/450/301	1.11
Di(HHDP-galloylglucose)-pentose	2.60	255/376	1415	1397, 783	0.19
(+)-Gallocatechin	2.68	280	305	219, 261	0.05
HHDP-gallagyl-hexoside (punicalagin)	2.90	257/377	1083	781/301	20.29
Valoneic acid bilactone	3.00	254/373	469	425, 301	0.99
Ellagic acid derivative	3.20	254/377	1085	907/783/301	0.63
HHDP-gallagyl-hexoside (punicalagin)	3.74	257/377	1083	781/301	37.79
Ellagic acid derivative	3.98	257/377	1085	907/783/301	0.93
Granatin A	4.26	257/365	799	301	0.19
Ellagitannin	4.38	257/377	783	481/301	0.11
Galloyl-bis-HHDP-hexoside(casuarinin)	4.44	257/377	935	659/301	0.15
Digalloyl-gallagyl-hexoside	4.56	257/377	1085	933/301	0.50
Ellagitannin	4.72	257/367	783	481/301	0.17
Ellagitannin	4.95	257/367	783	481/301	0.31
Ellagitannin	5.08	257/367	783	481/301	0.26
Ellagic acid hexoside	5.24	272/361	463	301	5.64
Digalloyl-HDDP-glucoside (pedunculagin II)	5.58	285/367	785	483/301	0.14
Granatin B	5.98	257/367	951	933/765/301	0.17
Galloyl-HHDP-DHHDP-hex (granatin B)	6.04	257/367	951	907/635/301	0.10
Ellagic acid derivative	6.23	257/367	433	352/301	0.26
Ellagic acid derivative	6.41	257/367	433	352/302	1.63
Ellagic acid rhamnoside	6.47	252/361	447	352/301	1.88
Ellagic acid derivative	6.65	252/361	301	275/217/169	1.89
Ellagic acid-(p-coumaroyl)hexoside	6.87	263/313	609	463/445/301	0.16
Isorhamnetin-glucuronide	7.18	350	491	352/146	0.49
Kaempferol- rutinoside	7.64	355	593	285, 547	0.44
Kaempferol-hexoside	7.92	350	447	285	0.22

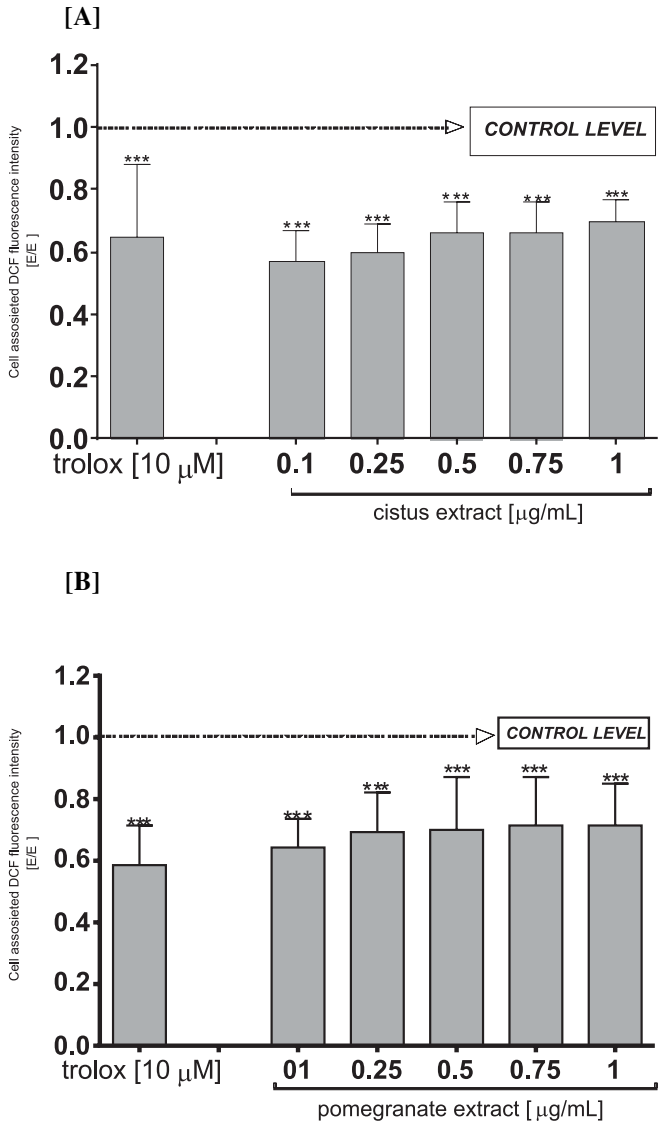

Figure 1. Effect of cistus [**A**] and pomegranate [**B**] extracts on intracellular ROS level in V79 cell line (DCF-DA test). The results obtained in the presence of the extracts (E) were compared to the relevant control (E_0), i.e., V79 cells exposed to the vehicle (H_2O). Trolox, the standard antioxidative analog of vitamin E, was used as a positive control. The values are expressed as the mean ± SD, n = 4 (*** $p \leq 0.0001$)

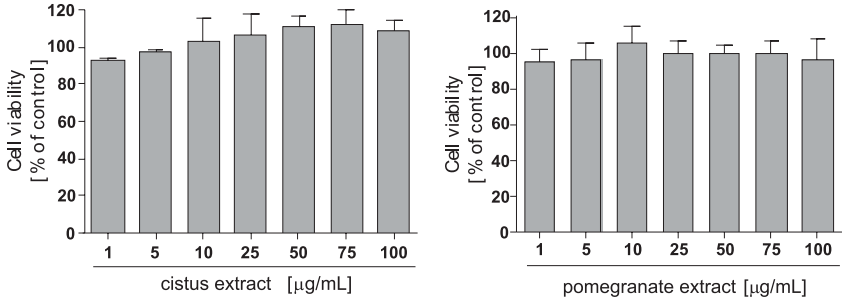

Figure 2. Cell viability (XTT) assay of normal hamster fibroblasts, the V79 cells, after treatment with cistus and pomegranate extracts. The results are presented as a percentage of the control sample (cells incubated without the extracts). The data are expressed as the mean ± SD, n = 3

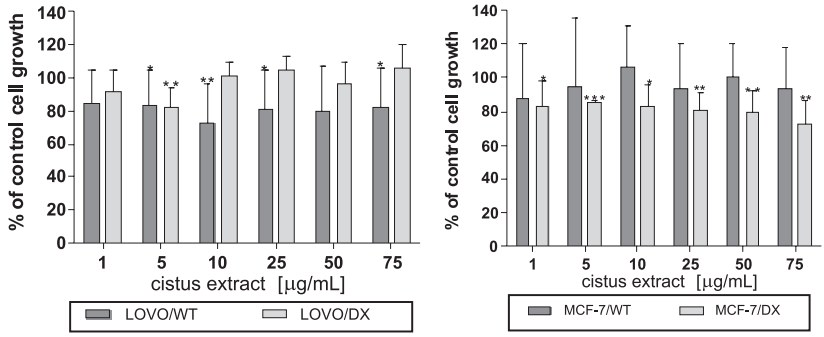

Figure 3. Impact of cistus extract on growth inhibition of human cancer cells (the SRB assay). The results are presented as a percentage of the control cell growth (cell cultures carried out without the tested extracts). Data are expressed as the mean ± SD, n = 6 (* $p < 0.05$; ** $p \leq 0.001$; *** $p \leq 0.0001$)

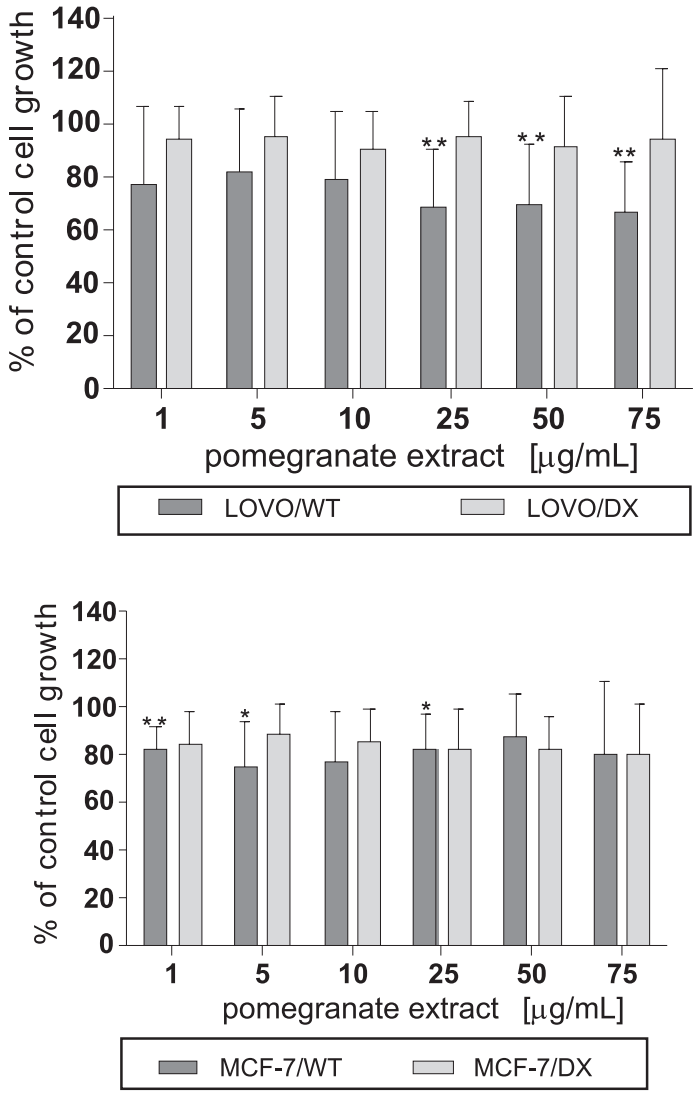

Figure 4. Impact of pomegranate extract on growth inhibition of human cancer cells (the SRB assay). The results are presented as a percentage of the control cell growth (cell cultures carried out without the tested extracts). Data are expressed as the mean ± SD, n = 6, (* $p < 0.05$; ** $p \leq 0.001$)

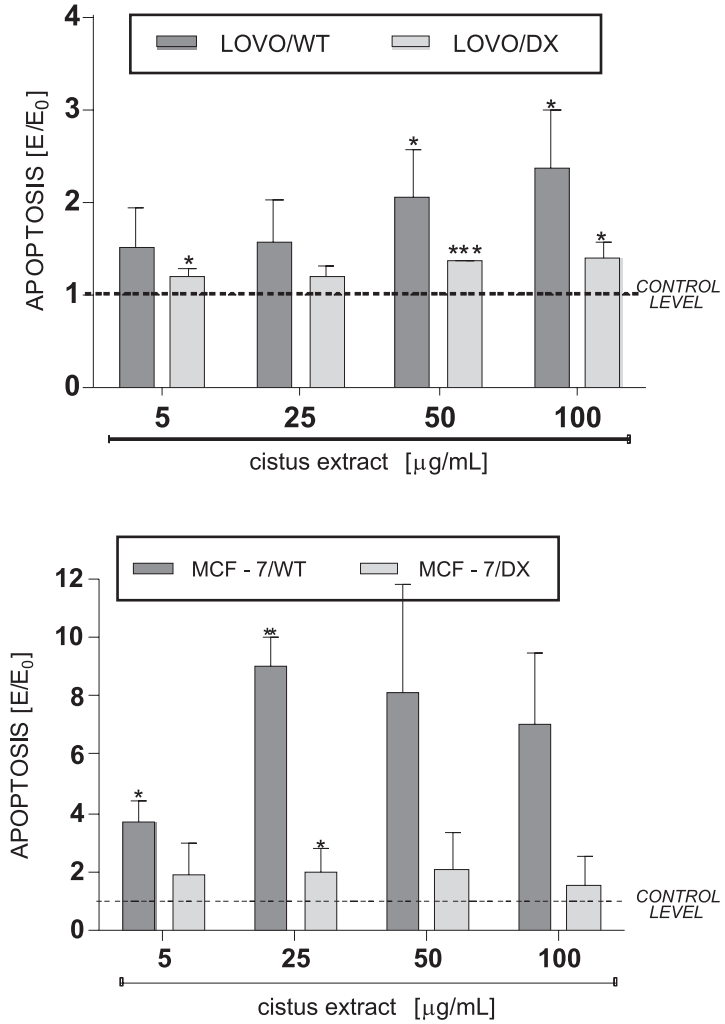

Figure 5. Effect of cistus extract on frequency of apoptosis in cancer cell cultures. The result obtained in the presence of cistus extract (E) were compared to the relevant control culture (E_0) (without the tested extract). The data are expressed as the mean ± SD, n = 3, (* $p < 0.05$, ** $p \leq 0.001$, *** $p \leq 0.0001$)

LOVO/DX and MCF-7/WT cells growth was found in the presence of the tested cistus extract.

Pomegranate extract inhibited cell growth in all tested cancer cell cultures (Fig. 4), by 18-34% (LOVO/WT), 5-10% (LOVO/DX), 13-25% (MCF-7/WT) and 11-20% (MCF-7/DX), especially at higher pomegranate extract concentrations (25-75 μg/mL).

Induction of apoptosis by the extracts in human cancer cells

Pro-apoptotic activities of cistus and pomegranate extracts were carried out in cultures of doxorubicin-sensitive and -resistant colon (LOVO/WT, LOVO/DX) and breast (MCF-7/WT, MCF-7/DX) cancer cells after 24-h incubation with the extracts.

Impact of cistus extract on apoptotic cell frequency in tested cancer cell cultures is shown in Figure 5.

As could be seen in Figure 5, cistus extract (100 μg/mL) caused 2.35-fold increase in apoptotic cells frequency in LOVO/WT cell culture and 1.38 increase in LOVO/DX cell culture, when compared to control culture (without the tested extracts), and the effects were proportional to concentration of the extract. In MCF-7/WT cell culture, cistus extract exhibited the strongest influence on frequency of apoptotic cells causing 9-fold increase (25 μg/mL). In MCF-7/DX cell cultures 2-fold increase of apoptotic cells frequency was noted.

The influence of pomegranate extract on frequency of apoptosis in human cancer cell cultures is given in Figure 6.

Pomegranate extract caused 2.5-fold increase of apoptotic cell frequency in LOVO/WT cell culture (at 50 and 100 µg/mL) and 1.46-fold increase in LOVO/DX cell culture (at 25 and 50 µg/mL) compared to the control cultures (without the extract). Pro-apoptotic effect was markedly stronger in MCF-7/WT than in MCF-7/DX cell cultures (7.84-fold *versus* 2.48-fold increase, respectively).

DISCUSSION AND CONCLUSION

Extracts of *Cistus incanus* exert important antioxidant capacity that could be attributed to their polyphenolic composition, especially to the concentration of flavonol compounds, including myricetins, quercetins, catechins, kaempferol and gallic acid (4).

In the present study the polyphenol rich extract from *cistus incanus* contains mostly myricetins, gallocatechins, quercetins, kaempferols, wherein myricetin 3-rhamnoside is the main constituent, representing 47.3% of the extract weight. The polyphenols present in the pomegranate extract include punicalagins, punicalin α/A, ellagitannins, ellagic acids, kaempferols, granatin B and gallocatechin, however, particularly rich in punicalagins is HHDP-gallagyl-hexoside, which constitutes 58.08% of the extract weight. Literature data show that antioxidant effect of whole pomegranate homogenates correlated with the content of the hydrolyzable tannins predominantly to punicalagin content (17). Punicalagin was reported as compound responsible for 50% of the pomegranate juice's antioxidative potential (7, 10). Antioxidant activities and phenolic content of juices

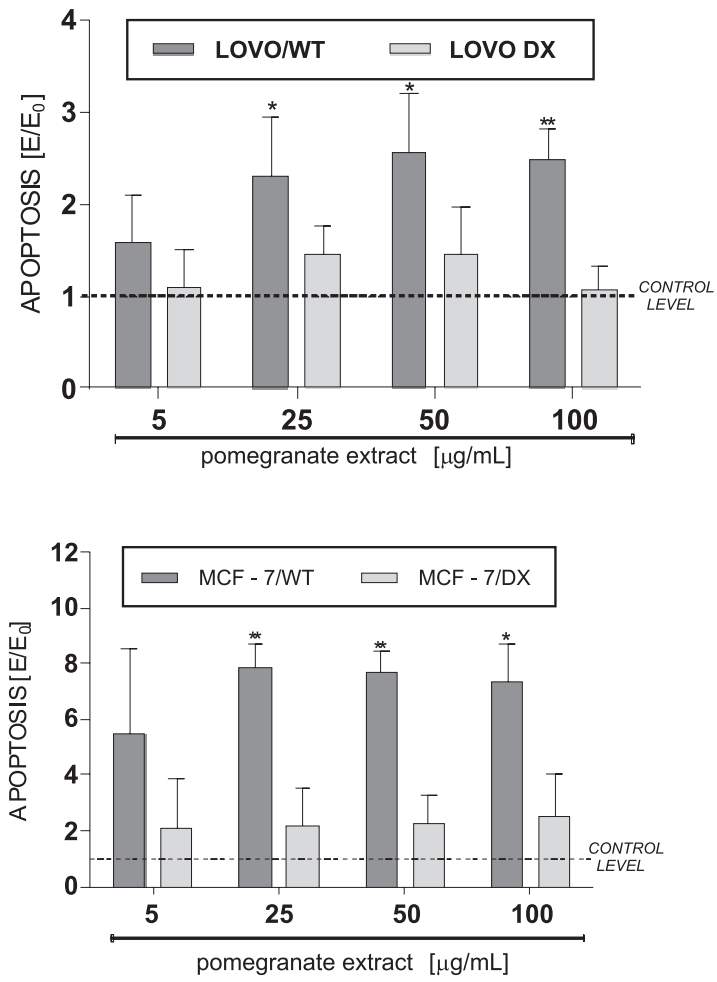

Figure 6. Effect of pomegranate extract on frequency of apoptosis in cancer cell cultures. The result obtained in the presence of pomegranate extract (E) were compared to the relevant control culture (E_0), (without the tested extract). Results are expressed as the mean ± SD, n = 3 (* $p<0.05$, ** $p \leq 0.001$)

from whole pomegranate fruit were significantly higher than juices from pomegranate arils (18). Both the tested extracts exhibited significant free radical scavenging activity decreasing intracellular ROS in V79 cells by 30-40% (cistus) and by 29-36% (pomegranate). The strength of antioxidative activities were comparable to the activity of the standard antioxidant - vitamin E (α-tocopherol, Trolox).

The antioxidant properties of the plant polyphenols are linked to their anticarcinogenic activities. It is well documented that carcinogenesis is associated with increased level of ROS due to downregulation of ROS scavengers and/or antioxidant enzymes (19-21). Oxidative stress induces mutations which could lead to increase in cancer cells survival and proliferation potential, the inactivation of tumor suppressor genes as p53 and the activation of pro-survival factors such as NF-κB and AP-1 (10). However, plant polyphenols, in addition to their direct antioxidant action, are thought to influence on intracellular signaling pathways that alter regulation of proteins involving in cancer cells survival, such as transcription factors: signal transducer and activator of transcription (STAT) or NF-κB (22). As was documented in the literature, anticancer activity of plant polyphenols involves various mechanisms which finally lead to inhibition of cell growth, decrease of protein kinase activities, induction of apoptosis, inhibition of MMP secretion, attenuation of tumor cell invasion, inhibition of adhesion/spreading of cells and anti-angiogenic properties (23).

Cistus incanus and *Punica granatum* plants are believed to have beneficial anticancer effects due to their high polyphenolic content. In the present paper, the tested extracts were evaluated for their growth-inhibitory and pro-apoptotic effects in breast and colon cancer cell cultures. Cistus extract was mostly effective in drug-sensitive colon cancer cells (LOVO/WT) and doxorubicin-resistant breast cancer cells (MCF-7/DX). Pomegranate extract displayed growth-inhibitory effects against all tested cancer cell lines, i.e., sensitive- and doxorubicin-resistant breast and colon cancer cells. Importantly, no growth inhibitory activity was found in normal V79 cells for both extracts. Induction of cancer cells apoptosis was significantly higher in cultures of drug-sensitive cancer cells (LOVO/WT and MCF-7/WT) compared to doxorubicin-resistant cancer cells (LOVO/DX and MCF-7/DX). This is consistent with literature data, that showed that polyphenolic compounds were stronger inducers of apoptosis in drug-sensitive than in drug-resistant adenocarcinoma cell cultures (38) and also in leukemia cell cultures (24). The cell type-depending difference were also observed in propoptotic activity of cistus and pomegranate extracts. In MCF-7/WT cells culture, cistus extract caused 9-fold increase in apoptotic cells and pomegranate extract 7-fold increase when compared to relative control cultures (without the tested extracts), whereas in LOVO/WT cells 2-fold and 2.5-fold increase, respectively, was noted. Significant part of pro-apoptotic activity of the tested extracts could be linked to the presence of major phenolic compound: myricetin (cistus) and punicalagin (pomegranate). It was documented in the literature, that myricetin increased the level of Bax and promoted release of apoptosis inducing factor and cytochrome C from the mitochondria in colon cancer cell lines (25). Apoptosis-promoting activity of myricetin, could be a result of their suppression of anti-apoptotic signaling pathways, i.e., inhibition of extracellular mitogen-activated protein kinase (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K) as was shown in pancreatic (26), lung (27) and cervical (28) cancers. Punicalagin was shown to induce apoptosis through the activation of the caspase-9/caspase-3 cascade and inhibition of poly(ADP-ribose) polymerase (PARP) in glioma cells (29). In ovarian cancer, punicalagin inhibited β-catenin signaling which caused G1/S phase transition arrest and evolved pro-apoptotic pathways (30). However it should be pointed out, that pro-apoptotic activity and inhibition of cancer cell growth by the complex pomegranate extracts were significantly higher than that of single polyphenolic compounds isolated from this fruit (11).

In conclusion, cistus and pomegranate polyphenol rich extracts revealed strong antioxidant activities due to suppression of intracellular ROS generation and should be used by people exposed to oxidative stress. In addition, both extract significantly decreased cancer cell *in vitro* proliferation and induced apoptosis. These results suggest that both extracts should be further studied for their potential use as adjuvants to the standard therapy in the treatment of human cancers.

Acknowledgment

This study was supported by the Wroclaw Medical University Grant no. ST.D130.16.009.

REFERENCES

1. Liu R.H.: Am. J. Clin. Nutr. 78, 517S (2003).
2. Wittpahl G., Kölling-Speer I., Basche S., Herrmann E., Hannig M. et al.: Planta Med. 81, 1727 (2015).

3. Rebensburg S., Helfer M., Schneider M., Koppensteiner H., Eberle J. et al.: Sci. Rep. 6, 20394 (2016).
4. Gori A., Ferrini F., Marzano M.C., Tattini M., Centritto M. et al.: Int. J. Mol. Sci. 17, 1 (2016).
5. Vitali F., Pennisi G., Attaguile G., Savoca F., Tita B.: Nat. Prod. Res. 25, 188 (2011).
6. Chinou C. Demetzos C. Harvala C. Roussakis J.F.V.: Planta Med. 60, 34 (1994).
7. Tyagi S., Singh A., Bhardwaj P., Sahu S., Yadav A.P., Kori M.: Acad. J. Plant Sci. 5, 45 (2012).
8. Syed D.N., Afaq F., Mukhtar H.: Semin. Cancer Biol. 17, 377 (2007).
9. Turrini E., Ferruzzi L., Fimognari C.: Oxid. Med. Cell. Longev. 2015, 938475 (2015).
10. Seeram N.P., Adams L.S., Henning S.M., Niu Y., Zhang Y. et al.: J. Nutr. Biochem. 16, 360 (2005).
11. Gąsiorowski K., Szyba K., Brokos B., Kołaczyńska B., Jankowiak-Włodarczyk M. et al.: Cancer Lett. 119, 37 (1997).
12. Oszmiański J., Bourzeix M.: Pol. J. Food Nutr. Sci. 4(45), 91 (1995).
13. Kolniak-Ostek J., Oszmiański J.: Int. J. Mass Spectrom. 392, 154 (2015).
14. Eruslanov E., Kusmartsev S.: Methods Mol. Biol. 594, 57 (2010).
15. Vichai V., Kirtikara K.: Nat. Protoc. 1, 1112 (2006).
16. Matsuo M., Sasaki N., Saga K., Kaneko T.: Biol. Pharm. Bull. 28, 253 (2005).
17. Tzulker R., Glazer I., Bar-Ilan I., Holland D., Aviram M. et al.: J. Agric. Food Chem. 55, 9559 (2007).
18. Akhavan H., Barzegar M., Weidlich H., Zimmermann B.F.: J. Chem. 2015, 11 (2015).
19. Valko M., Rhodes C.J., Moncol J., Izakovic M., Mazur M.: Chem. Biol. Interact. 160, 1 (2006).
20. Ziech D., Franco R., Georgakilas A.G., Georgakila S., Malamou-Mitsi V. et al.: Chem. Biol. Interact. 188, 334 (2010).
21. Dreher D., Junod A.F.: Eur. J. Cancer 32A, 30 (1996).
22. Greenwell M., Rahman P.K.S.M.: Int. J. Pharm. Sci. Res. 6, 4103 (2015).
23. Kanadaswami C., Lee L.T., Lee P.P.H., Hwang J.J., Ke F.C. et al.: In Vivo (Brooklyn) 19, 895 (2005).
24. Tolomeo M., Grimaudo S., Di Cristina A., Roberti M., Pizzirani D. et al.: Int. J. Biochem. Cell Biol. 37, 1709 (2005).
25. Koosha S., Alshawsh M.A., Yeng L.C., Seyedan A., Mohamed Z.: Int. J. Med. Sci. 13, 374 (2016).
26. Phillips P.A., Sangwan V., Borja-Cacho D., Dudeja V., Vickers S.M. et al.: Cancer Lett. 308, 181 (2011).
27. Shih Y.-W., Wu P.-F., Lee Y.-C., Shi M.-D., Chiang T.-A.: J. Agric. Food Chem. 57, 3490 (2009).
28. Li H.G., Chen J.X., Xiong J.H., Zhu J.W.: J. BUON. 21, 182 (2016).
29. Wang S., Huang M., Li J., Lai F., Lee H. et al.: Acta Pharmacol. Sin. 34, 1411 (2013).
30. Tang J., Min J., Li B., Hong S., Liu C. et al.: Int. J. Gynecol. Cancer 26, 1557 (2016).