DOI:10.1021/jf901395t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Pro-Oxidative and Pro-Apoptotic Action of Defatted Seeds of *Oenothera paradoxa* on Human Skin Melanoma Cells

Edyta Jaszewska, Anita Kośmider, Anna K. Kiss, and Marek Naruszewicz*

Department of Pharmacognosy and Molecular Basis of Phytotherapy, Faculty of Pharmacy, Medical University of Warsaw, Poland

Three extracts of defatted seeds of *Oenothera paradoxa* Hudziok, aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract, differing by their total content of phenolic compounds and by their contents of individual polyphenols, were investigated in this study. The extracts exerted cytotoxic action on HTB-140 human skin melanoma cells. After 24 h of incubation, IC_{50} values of 169.7 ± 5.9 μg/mL, 72.4 ± 3.8 μg/mL, and 155.3 ± 6.3 μg/mL were obtained for HTB-140 cells with the aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract at the tested concentrations (5−200 μg/mL), respectively, while IC_{50} for normal fibroblast cells NHDFs was not attained. Moreover, for HTB-140 cells, LD_{50} (concentration at which 50% of cells were dead) of 89.2 ± 4.3 μg/mL and 181.4 ± 6.5 μg/mL were obtained with 60% ethanolic extract and 30% isopropanolic extract, respectively. In melanoma cells, all three extracts caused a concentration-dependent increase of ROS production, GSH, and ATP lowering, and appearance of phosphatidylserine on the external surface of cellular membranes where it was bound to annexin V-FITC; furthermore, apoptosis without activation of caspase-3 took place. The most effective was 60% ethanolic extract, which had the greatest total content of phenolic compounds and the greatest content of pentagalloylglucose (PGG).

KEYWORDS: *Oenothera paradoxa*; polyphenols; pentagalloylglucose; cytotoxicity; reactive oxygen species; apoptosis; HTB-140 cells

INTRODUCTION

Oenothera sp. (Oenotheraceae) are native to Central and South America but are also cultivated in Europe for the production of seeds. Oil from seeds of *Oenothera biennis* L. and *Oenothera paradoxa* Hudziok is the main source of γ-linolenic acid (GLA). Evening primrose oil (EPO) is used for the treatment of atopic eczema, premenstrual syndrome, multiple sclerosis, hypercholesterolemia, rheumatoid arthritis, and diabetic nephropathy (*1*).

In the past few years, there has been a growing interest in evening primrose because of its polyphenolics content. It is well known that *Oenothera* herba contains flavonoids, phenolic acids, and hydrolyzable tannins (ellagitannins and gallotannins) (*2*, *3*). Defatted seeds are rich in hydrolyzable and condensed tannins (procyanidins), but only gallic acid, (+)-catechin, (−)-epicatechin, and a tetrameric procyanidin gallate were isolated as pure compounds from *O. biennis* seeds (*4*−*6*). In our previous study, we have additionally identified (−)-epicatechin, (−)-epicatechin gallate, ellagic acid, caffeic acid, quercetin, penta-*O*-galloylo-β-D-glucose, and procyanidin B3 (*7*). The quantity of polyphenolics in evening primrose seed extracts vary from 240 mg/g to 750 mg/g and is high when compared with that of black currant seed extracts (22−180 mg/g) and sesame seed extracts (14−65 mg/g), and similar to that of borage seed extract (~410 mg/g), grape seed extract (~790 mg/g), or green tea extract (~450 mg/g) (*5*, *7*, *9*).

*Corresponding author. Tel/Fax: +48 22 572 09 85. E-mail: marek.naruszewicz@wum.edu.pl.

A number of studies have been conducted to demonstrate the antioxidant and iron(II) chelation activity of *Oenothera* (*O. biennis* and *O. paradoxa*) defatted seed extracts (*4*−*6*, *8*, *9*). A phenolic fraction purified form of defatted seeds of *Oenothera biennis* promoted selective apoptosis of human and mouse bone marrow-derived cell lines. Analyses of the fraction have revealed that it contains gallic acid, which displayed selective cytotoxicity against a variety of tumor cells with a higher activity than against normal cells (*10*). Other studies have demonstrated that the defatted seed extract of *O. biennis* could induce apoptosis in Ehrlich ascites tumor cells, by increasing the activity of superoxide dismutase and intracellular peroxide levels, which resulted in cytochrome *c* release. However, no activation of caspase-3 was observed (*11*−*13*).

Melanoma is one of the most aggressive skin cancers, difficult to treat, with a high metastatic potential (*14*). Compounds with the phenolic structure, such as polyphenols of witch hazel bark (*15*) and procyanidins of pine bark (*16*), may inhibit proliferation of melanoma cells. Some of these compounds, such as polyphenols of grapes, including resveratrol (*17*) and piceatannol (*18*), polyphenols of black tea, including theaflavins and thearubigins (*19*), and procyanidins of apples (*20*), may also induce melanoma cell apoptosis. In melanoma treatment, the combination of survival inhibitors with pro-apoptotic factors (*21*) is most indicated. Polyphenols have been evidenced to be able to potentiate the action of cytostatic agents, i.e., of decarbazine in the case of (−)-epigallocatechin-3-gallate (EGCG) (*22*) and of cisplatin in the case of apigenin and quercetin (*23*).

Published on Web 08/20/2009

Table 1. Polyphenol Content in the Tested Extracts (mg/g) ± SD

extract	total polyphenols	gallic acid	(+)-catechin	ellagic acid	pentagalloyl glucose
aqueous	468.8 ± 9.8	3.70 ± 0.13	23.43 ± 0.82	1.48 ± 0.02	12.05 ± 0.53
60% ethanolic	745.5 ± 8.8	2.18 ± 0.08	30.41 ± 1.48	1.15 ± 0.05	16.75 ± 0.64
30% isopropanolic	592.0 ± 2.1	13.03 ± 0.13	37.43 ± 0.08	0.99 ± 0.04	9.44 ± 0.07

The objective of this study was to investigate the pro-oxidative and pro-apoptotic actions of the aqueous, 60% ethanolic and 30% isopropanolic extracts obtained from defatted seeds of *Oenothera paradoxa* on human skin melanoma cells of the HTB-140 line. Additionally, the main phenolic compounds, i.e., gallic acid, (+)-catechin, ellagic acid, and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG), were measured in the extracts.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM) and ViaLight Plus Assay Kit were purchased from Lonza Walkersville Inc. (USA). Trypsin-ethylenediaminetetraacetic acid (EDTA), penicillin, streptomycin, and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technologies (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), the glutathione assay kit, and the caspase-3 assay kit were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The cytotoxicity detection kit and Triton X-100 were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Calcein-AM and propidine iodide (PI) were purchased from MoBiTec GmbH (Göttingen, Germany). The protein assay kit was purchased from Pierce Biotechnology (Rockford, USA). Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (USA). Phosphate buffered saline (PBS) was purchased from Biomed (Lublin, Poland). All other materials necessary for cellular cultures were purchased from Greiner Bio One GmbH (Frickenhausen, Germany).

Ellagic acid and gallic acid were purchased from ChromaDex (Santa Ana, CA), and (+)-catechin was purchased from Carl Roth (Karlsruhe, Germany). Pentagalloyl glucose was isolated as described previously (*7*). All substances used were of >95% purity. The Folin−Ciocalteu reagent was purchased from POCh (Gliwice, Poland). All solvents were of HPLC grade.

Plant Material. Three freeze-dried polyphenolic extracts of defatted seeds of *Oenothera paradoxa*, aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract, containing ca. 47%, 75%, and 59% of phenolic compounds, respectively, were obtained from Agropharm S.A. (Tuszyn, Poland). The seeds were obtained from crops cultivated according to the GACP guidelines (EMEA/HMPWP/31/99 Rev. Three of 2002).

Total Phenolic Content. The total phenolic content was determined with the Folin−Ciocalteu reagent using gallic acid as the standard. Accurately weighed 0.02 g of the extract was dissolved in 100 mL of water, then 0.5 mL of the extract was mixed with 0.5 mL of the reagent, and 10 mL of the 10% Na_2CO_3 solution, and then filled up with water to 50 mL. The mixture was incubated in the dark for 30 min. Absorbance was measured spectrophotometrically at 765 nm on a 160A UV−vis spectrophotometer (Shimadzu Corp., Kyoto, Japan).

High-Performance Liquid Chromatography (HPLC) Analysis. Phenolic compounds of the extracts were analyzed by HPLC at room temperature on the LC-10AT equipment consisting of two high-pressure mixing pumps and a diode array detector (SPD-M10A) (Shimadzu Corp., Kyoto, Japan). Separation was performed on a Luna C-18, 25 × 4.6 mm, 5 μm column (Phenomenex, Torrance, CA). The eluent was (A) 2.5% CH_3COOH and (B) CH_3CN + 2.5% CH_3COOH (80:20). A gradient solvent system was used: 7−20% B (45′); 20−40% B (70′); 40−100% B (75′); 100% B (80′). The flow rate was 1 mL/min, and the injection volume was 20 μL. UV spectra were recorded in the range of 200−400 nm; chromatograms were acquired at 254 nm for ellagic acid and 280 nm for other polyphenols.

Accurately weighed samples were dissolved in water to a concentration of 10 mg/mL. Samples and standards were dissolved in MeOH + 2.5% CH_3COOH (1:1). Quantitative determination was carried out using calibration curves from standards solution in the concentration range of 5−125 μg/mL ($y = 61424x + 36311$; $R^2 = 1$; $Rt = 5.3$) for gallic acid, 31.25−500 μg/mL ($y = 142121x - 35276$; $R^2 = 0.999$; $Rt = 17.9$) for (+)-catechin, 3.125−31.25 μg/mL ($y = 128728x - 11878$; $R^2 = 0.996$; $Rt = 49.3$) for ellagic acid, and 15.625−250 μg/mL ($y = 28845x - 51203$; $R^2 = 0.9999$; $Rt = 55.2$) for pentagalloylglucose. The quantities of analyzed polyphenols are presented in **Table 1**.

Cell Culture and Treatment. Human melanoma cells, line Hs 294T (HTB-140), were purchased from American type Culture Collection (Rockville, USA). Human skin fibroblast cells (NHDFs) were purchased from Lonza Walkersville Inc. (USA). HTB-140 and NHDFs cells (from the third to sixth passage) were seeded on 24-well plates (1.5×10^4 cells per well) or 12-well plates (3.5×10^4 cells per well) and incubated for 3−4 days in the DMEM medium with 10% FBS, penicillin and streptomycin, in 5% CO_2 and at 37 °C. Then their medium was changed to serum-free DMEM.

Freeze-dried extracts of defatted seeds of *Oenothera paradoxa* were dissolved in water and sterilized by filtration. Thus, prepared working solutions were diluted in the cellular medium or in PBS to concentrations of 5−200 μg/mL.

Mitochondrial Function Assessment. The function of mitochondria of both cell types was assessed with the use of MTT, which is converted in live cells under the effect of mitochondrial dehydrogenase into insoluble formazan. HTB-140 and NHDFs cultivated on 24-well plates were incubated with the extracts for 24 and 72 h and then with the MTT solution (MTT was dissolved in the RPMI-1640 medium, 0.5 mg/mL) for 1 or 2 h (depends on the cell line: HTB-140 or NHDFs). The converted dye was then solubilized with the use of acidic isopropanol (0.04 M HCl in absolute isopropanol), and absorbance was measured at 570 nm with background subtraction at 650 nm, with the use of the 160A UV−vis spectrophotometer (Shimadzu Corp., Kyoto, Japan). Cell viability was calculated as a percent versus the control (cells incubated in serum-free DMEM without extracts).

Cellular Membrane Integrity Assessment. To assess cellular membrane integrity at 24 and 72 h of incubation of HTB-140 cells and NHDFs on 24-well cells with the extracts, the activity of lactate dehydrogenase (LDH) released from cytosol of damaged cells to the supernatant was measured using the protocol of the cytotoxicity detection kit test described by the manufacturer (Roche Diagnostics, Germany). Absorbance values measured at 490 nm, using the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA) were translated into cytotoxicity rate, using the following formula:

$$\text{LDH release [\%]} = \frac{\text{LDH of sample} - \text{LDH of control}}{\text{LDH of TX100 control} - \text{LDH of control}} \times 100$$

where controls were cells in serum-free DMEM without extracts, and TX100 controls were cells incubated in serum-free DMEM with 10% Triton X-100 (100% LDH release).

Morphology. Changes in morphology of cells of both lines were assessed with the use of the Eclipse TS100F (Nikon, USA) microscope fitted with the Nikon Digital Sight DS-U2 camera, using the NIS-Elements Nikon BR 2.30 software, in visual light and, in the case of HTB-140 cells, also in fluorescence after their prior dyeing with calcein-AM and propidine iodide (as per the protocol of MoBiTec, Germany).

ATP Level Measurement. Measurement of the intracellular level of ATP was performed with the use of the luciferase enzyme, using the protocol for the ViaLight Plus Assay Kit test described by the manufacturer (Lonza Walkersville Inc., USA). HTB-140 cells were incubated on 24-well plates in serum-free DMEM with various concentrations of tested extracts (5−200 μg/mL) for 24 h, and then the cells were rinsed with PBS, tripsinized, and counted in the Bürker's chamber. Luminescence was measured with the use of the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA). The result was presented as the percentage of cells in relation to the control (cells incubated in serum-free DMEM without extracts).

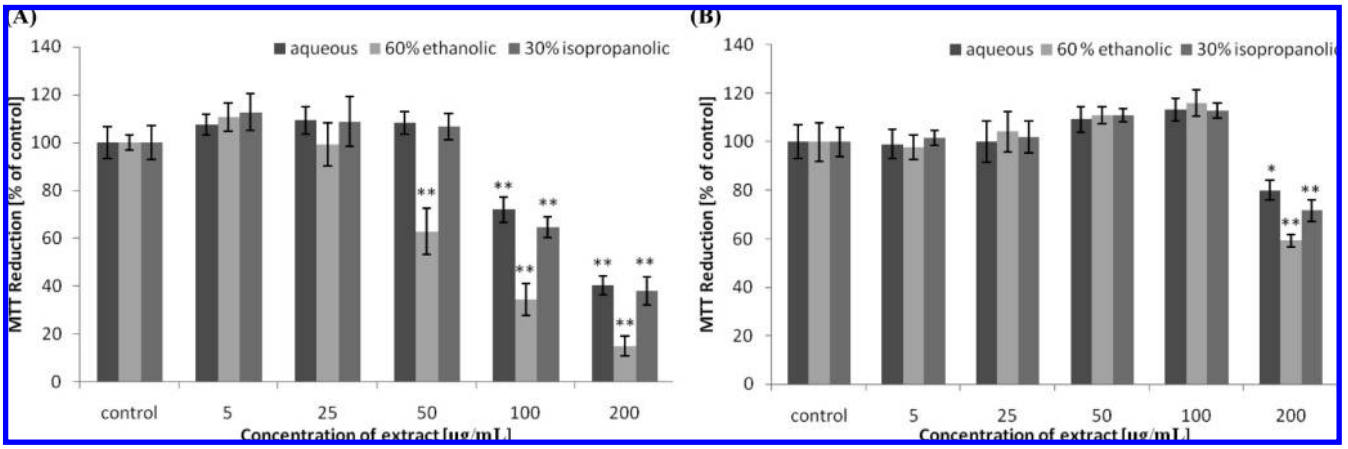

Figure 1. MTT assay results from HTB-140 cells (**A**) and NHDFs (**B**) treated with the tested extracts. Cells were incubated with the indicated concentrations of extracts for 24 h followed by the MTT assay. Data are expressed as means ± SD from four independent experiments performed in triplicate. Statistically significant differences: *, $p < 0.05$; **, $p < 0.001$ refer to the control (untreated cells).

Reactive Oxygen Species (ROS) Production Measurement. Nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), which easily penetrates cells undergoing conversion into strongly fluorescent DCF in the presence of reactive oxygen species (ROS), was used for the measurement of the intracellular ROS level. HTB-140 cells cultivated in 12-well plates in serum-free DMEM were rinsed with PBS and incubated for 1 h in PBS with the tested extracts added at various concentrations (5−200 μg/mL), and then rerinsed with PBS and treated with 250 μM $DCFH_2$-DA. Fluorescence measurement was started immediately after $DCFH_2$-DA was added, using the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA) at an excitation wavelength of 485 nm and emission wavelength of 530 nm, with readings every 5 min.

GSH Level Measurement. The GSH level was assessed by DTNB, using the protocol for the glutathione assay kit (Sigma-Aldrich Chemie, Germany) test. HTB-140 cells were incubated on 24-well plates in serum-free DMEM with the tested extracts at various concentrations (5−200 μg/mL) for 4 h, and then the cells were rinsed with PBS and tripsinized, where 4 wells of the culture plate constituted one sample (about 4×10^5 cells). Protein Assay Kit was used to measure the quantity of protein, using the manufacturer's protocol (Pierce Biotechnology, USA). Absorbance was measured at 412 nm, using the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA); the standard was GSH. The results were expressed in relation to the quantity of protein in the sample.

Staining with Annexin V-FITC/PI. To detect apoptosis/necrosis, HTB-140 cells were incubated on 12-well plates in serum-free DMEM with the tested extracts at various concentrations (5−200 μg/mL) for 6 h. The cells were rinsed with PBS and tripsinized. Then the protocol for the Annexin V-FITC apoptosis detection kit test described by the manufacturer (BD Biosciences, USA) was followed. The samples were analyzed by flow cytometry, using FACSCalibur and the CellQuest software (BD Biosciences, USA). The results were presented as rates of early apoptotic (An^+/Pi^-) and late apoptotic (An^+/Pi^+) cells.

Caspase-3 Activity Measurement. Caspase activity was measured with the use of the caspase-3 assay kit, using the protocol described by the manufacturer (Sigma-Aldrich Chemie, Germany). HTB-140 cells were incubated on 24-well plates in serum-free DMEM with the tested extracts at various concentrations (5−200 μg/mL) for 16 h. Then the cells were rinsed with PBS and tripsinized, where 4 wells of the culture plate constituted one sample (about 4×10^5 cells). Absorbance was measured at 405 nm with the use of the BioTek Synergy 4 microplate reader (BioTek Instruments Inc., USA); the data were translated with the use of the standard curve of *p*-nitroaniline (p-NA) release. Caspase activity was related to the number of μmols of released p-NA/min/mL.

Statistical Analysis. There were $2-4 \times 10^5$ cells per each sample (cell number was evaluated in the Bürker's chamber). The data were presented as means ± SD, compared with the use of one-way analysis of variance (ANOVA) with Tukey's posthoc test, using the STATISTICA software (version 8.0, StatSoft, Poland), with statistical significance determination at two levels: $p < 0.05$ and $p < 0.001$.

RESULTS

Cytotoxic Action on HTB-140 Cells and NHDFs. As shown by the conducted MTT test, the viability of both cellular lines is closely dependent on the type and concentration of the administered extract. At 24 h of incubation of HTB-140 cells, IC_{50} values (50% growth inhibition concentration) of 169.7 ± 5.9 μg/mL, 72.4 ± 3.8 μg/mL, and 155.3 ± 6.3 μg/mL were obtained for the aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract at the tested concentrations (5−200 μg/mL), respectively, while IC_{50} was not attained for the NHDF cells. (**Figure 1**) At 72 h of incubation, IC_{50} for the HTB-140 cells was 69.6 ± 5.8 μg/mL; 17.1 ± 2.6 μg/mL and 34.8 ± 4.3 μg/mL for the aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract, respectively, while for NHDFs, the IC_{50} values were significantly higher and were 81.7 ± 6.9 μg/mL; 43.3 ± 4.8 μg/mL and 62.7 ± 6.4 μg/mL, respectively.

The tested extracts caused, in both cell types, an extract type-dependent and tested concentration-dependent increase in the release of lactate dehydrogenase (LDH) into the culture medium, and the HTB-140 cells proved to be significantly more sensitive to the action of all three extracts than the NHDF cells. At 24 h of incubation, the LD_{50} (concentration at which 50% of cells were dead) values of 89.2 ± 4.3 μg/mL and 181.4 ± 6.5 μg/mL, respectively, were obtained for 60% ethanolic extract and 30% isopropanolic extract at the tested concentrations (5−200 μg/mL). (**Figure 2**) The LD_{50} value was obtained neither at 24 nor at 72 h of incubation for NHDFs cells. The greatest release of LDH by HTB-140 cells at 24 h of incubation was observed at higher concentrations of the tested extracts (100−200 μg/mL), while at 72 h, the significant values of LDH release were observed with lower concentrations (25−50 μg/mL). Cytotoxicity of the tested extracts against NHDFs was similar at both incubation times.

In both tests (MTT and LDH release), the 60% ethanolic extract displayed a significantly more potent action than the aqueous extract ($p < 0.05$).

In the light microscope, significant changes in morphology of HTB-140 cells were observed already under the influence of 25 μg/mL aqueous extract, while fibroblast morphology did not change. All three extracts at concentrations of 50 μg/mL and higher caused a concentration-dependent and incubation time-dependent change in morphology of the cells of both lines. In fluorescence, after prior staining of HTB-140 cells with calcein-AM and propidine iodide, a progressive and strictly concentration-dependent increase in the number of PI (+) cells was observed. (**Figure 3**)

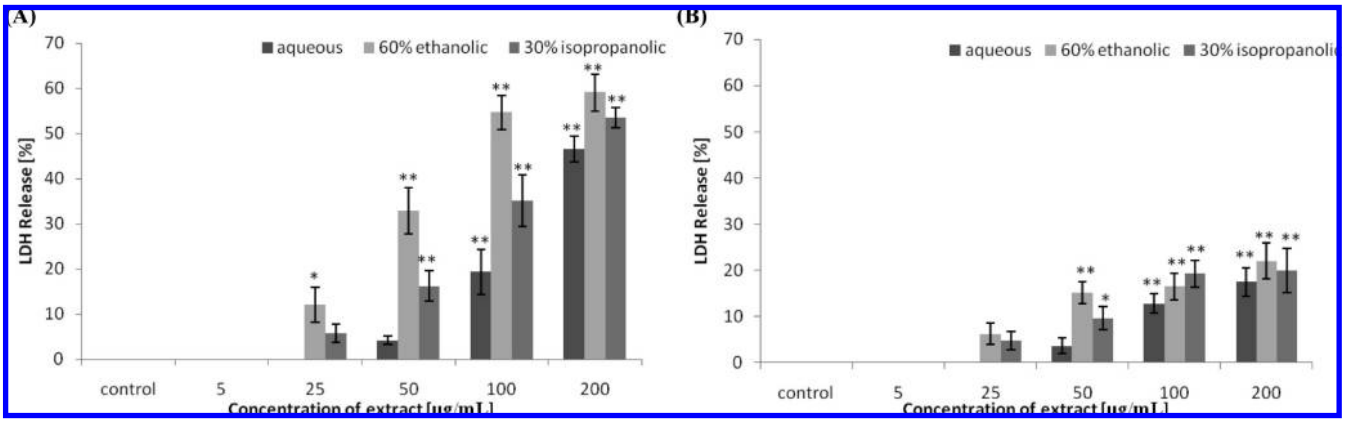

Figure 2. LDH release assay from HTB-140 cells (**A**) and NHDFs (**B**) after 24 h incubation with the indicated concentrations of extracts. Results are presented as means ± SD from four independent experiments performed in triplicate. Statistically significant differences: *, $p < 0.05$; **, $p < 0.001$ refer to the control (untreated cells).

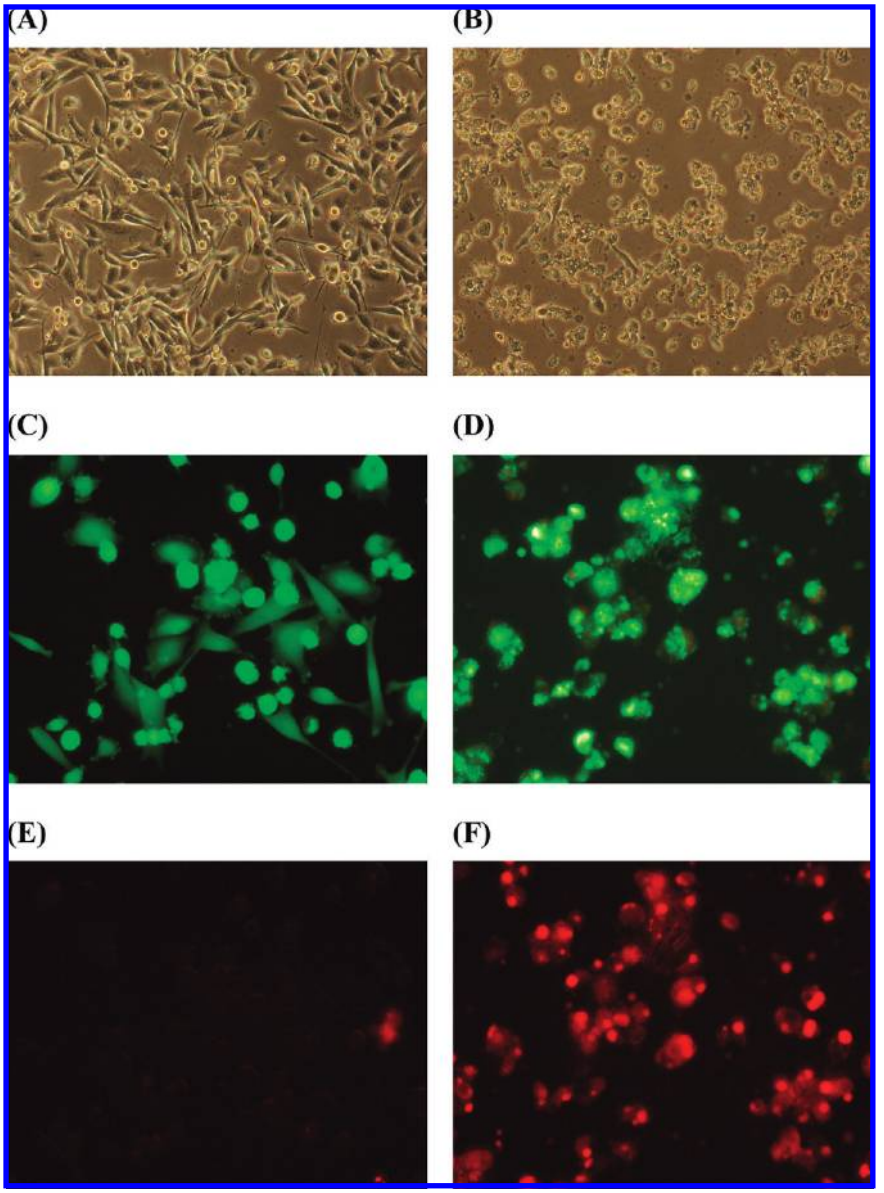

Figure 3. Morphologic changes of HTB-140 cells treated with 60% ethanolic extract at a concentration of 200 µg/mL for 24 h observed in the light microscope (100×): untreated cells (**A**) and cells incubated with the extract (**B**). In fluorescence (200×) after calcein-AM staining: untreated cells (**C**) and cells incubated with the extract (**D**). In fluorescence (200×) after PI staining: untreated cells (**E**) and cells incubated with the extract (**F**). The calcein in viable cells emits green fluorescence, while PI emits red fluorescence only in dead cells.

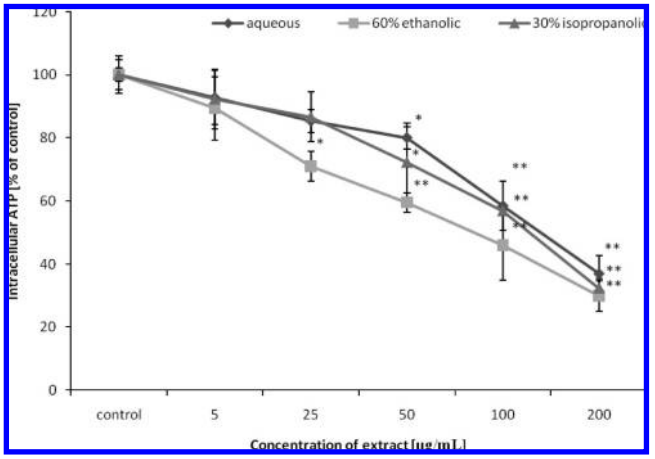

Figure 4. Changes in ATP levels of HTB-140 cells after 24 h treatment with the tested extracts. Data are presented as the means ± SD from three independent experiments performed in triplicate. Statistically significant differences: *, $p < 0.05$; **, $p < 0.001$ refer to the control (untreated cells).

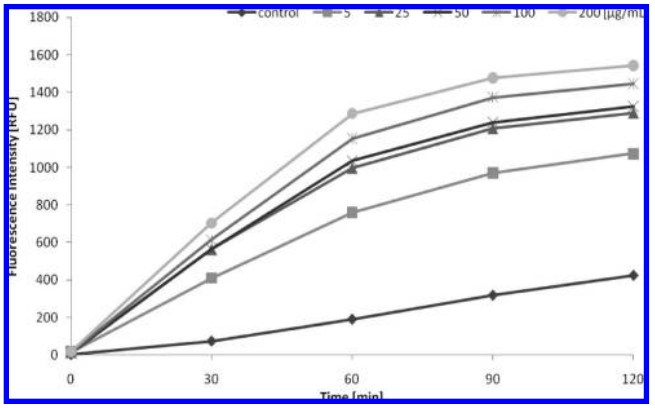

Figure 5. ROS formation in HTB-140 cells treated with 60% ethanolic extract. Results are presented as means of four independent experiments performed in triplicate. Level of significance after 120 min reading for each concentration was $p < 0.001$ as compared to that of the control (untreated cells).

Effect on ATP Level in HTB-140 Melanoma Cells. At 24 h of incubation of HTB-140 cells with the tested extracts, the intracellular ATP level was significantly lowered with concentrations of 25−200 μg/mL for 60% ethanolic extract and 50−200 μg/mL for the aqueous extract and 30% isopropanolic extract, attaining the lowest value of 29.9 ± 4.8% (versus the control) for 60% ethanolic extract at a concentration of 200 μg/mL. The aqueous extract and 30% isopropanolic extract at a concentration of 200 μg/mL lowered the ATP level to 37.0 ± 5.8% and 32.4 ± 3.0%, respectively (in relation to the control). The ATP reduction was strictly concentration-dependent. (**Figure 4**)

Effect on ROS Production in HTB-140 Melanoma Cells. The reduction in quantity of ATP synthesized in the cell could be associated with damage to the respiratory chain and overproduction of free radicals. To check the quantities of ROS produced in HTB-140 cells after their incubation for 1 h with the tested extracts, DCF fluorescence was measured. Throughout 2 h of result reading, a progressive concentration-dependent increase in ROS production was obtained, expressed as an increase in DCF fluorescence. (**Figure 5**) At 2 h after $DCFH_2$-DA was added to the cells, 60% ethanolic extract and 30% isopropanolic extract at all the tested concentrations and the aqueous extract at 25−200 μg/mL

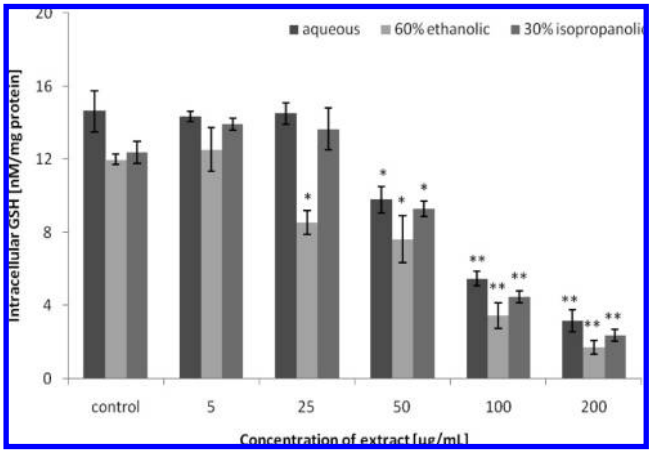

Figure 6. Changes in GSH levels of HTB-140 cells after 4 h treatment with the tested extracts. Data are expressed as means ± SD from two independent experiments performed in triplicate. Statistically significant differences: *, $p < 0.05$; **, $p < 0.001$ refer to the control (untreated cells).

caused a statistically significant increase in ROS production, which was about 3.6- 3.1-, and 3.0-fold, respectively (in relation to the control) for the 200 μg/mL concentration.

Effect on the GSH Level in HTB-140 Melanoma Cells. Overproduction of free radicals may cause GSH level lowering in HTB-140 cells. To verify this hypothesis, after 4 h of cell incubation in serum-free DMEM with the tested extracts (5−200 μg/mL), the intracellular GSH level was measured with the use of DTNB. Sixty percent ethanolic extract at 25−200 μg/mL significantly lowered the GSH level, which attained about 14% of the control value for 200 μg/mL. The aqueous extract and the 30% isopropanolic extract at 50−200 μg/mL significantly reduced the GSH level, and at 200 μg/mL, this level attained approximately 22% and 19% of the control value (**Figure 6**).

Increase in the Number of Cells in Early (An^+/Pi^-) and Late (An^+/Pi^+) Apoptosis. Reduction in GSH (*24*) and ATP (*25*) levels makes the cells enter the pathway of apoptotic death. To check the effect of the tested extracts on the quantity of HTB-140 cells in apoptosis at 6 h of incubation, cell staining with annexin V labeled with FITC and propidine iodide was performed (**Figure 7**). Sixty percent ethanolic extract as well as the aqueous and 30% isopropanolic extracts increased in a concentration-dependent fashion the number of cells in early and late apoptosis, while the number of early apoptotic cells was always higher than that of late apoptotic cells. The total quantity of cells stained with annexin (An^+) was 75%, 86%, and 77% for the aqueous extract, 60% ethanolic extract, and 30% isopropanolic extract at 200 μg/mL, respectively.

Effect on Activity of Caspase-3 in HTB-140 Melanoma Cells. Appearance of phosphatidylserine on the external surface of cellular membranes where it was bound to annexin V, giving an increased number of annexin-positive (An^+) cells, is a sign that the cells entered apoptosis. To assess the mechanism of further changes, caspase-3 activity was tested with Ac-DEVD-pNA as a substrate. Despite the 16 h period of incubation of HTB-140 cells with the tested extracts, no significant differences were observed between the control (cells incubated in serum-free DMEM, without extracts) and the tested samples.

DISCUSSION

In this study, cytotoxic action of standardized extracts of defatted seeds of *Oenothera paradoxa* against HTB-140 melanoma cells was evidenced on the basis of IC_{50} for all extracts and LD_{50} for the 60% ethanolic extract and 30% isopropanolic

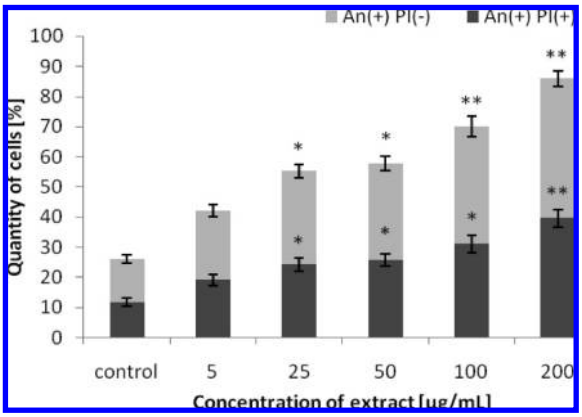

Figure 7. Number of HTB-140 cells in early (An^+/Pi^-) and late (An^+/Pi^+) apoptosis evaluated using Annexin V-FITC/PI staining followed by flow cytometric analysis. Cells were treated with 60% ethanolic extract for 6 h. Data are presented as the means ± SD from two independent experiments performed in triplicate. Statistically significant differences: *, $p < 0.05$; **, $p < 0.001$ refer to the control (untreated cells).

extract already at 24 h of incubation. At the same time, only a minimum effect on viability of physiologically normal cells was found. The potency of the antiproliferative action of extracts of defatted seeds of *Oenothera paradoxa* on HTB-140 cells was as follows: aqueous extract < 30% isopropanolic extract < 60% ethanolic extract, which may be related to their total contents of phenolic compounds as well as to the differences in content of individual polyphenols. A significant amount of (+)-catechin was detected in all three extracts, but it had only a weak pro-oxidative action (*26*, *27*). Potent pro-oxidative properties inducing apoptosis of cancer cells are displayed by gallic acid (*28*), the greatest quantity of which was found in 30% isopropanolic extract, and pentagalloylglucose (PGG) (*29*), the greatest quantity of which was found in the most effective 60% ethanolic extract. The most potent action of this extract may be caused by its greatest total content of phenolic compounds and by more potent pro-oxidative properties and higher cytotoxicity against cancer cells displayed by PGG as compared to gallic acid (*30*).

Many plant substances with proven antioxidant action also display pro-oxidative action on cancer cells, inducing their apoptosis (*31*, *32*). This study has shown that extracts of defatted seeds of *Oenothera paradoxa* cause an increase in ROS production in human skin melanoma cells. ROS are constantly produced in mitochondria, in the respiratory chain, from where these molecules are released mainly into the mitochondrial matrix, and the main target of their action are mitochondrial DNA (mtDNA) and membrane proteins as well as unsaturated fatty acid building membrane lipids. The effect of changes in mtDNA is impairment of synthesis of the respiratory chain proteins coded by it, which interferes with the chain function and leads to the generation of additional quantities of reactive oxygen species, consequentially causing cell death (*33*, *34*). Therefore, the oxidoreduction potential of cells remains under strict control of antioxidant enzymes and low-weight molecules such as reduced glutathione (GSH), the level of which (the GSH/GSSG ratio) is an index of ROS production in mitochondria (*35*, *36*). In this study, a significant reduction of the intracellular GSH level in human skin melanoma cells under the influence of the tested extracts was shown. A significant reduction of the GSH level in the presence of a large amount of ROS may cause, already after a few hours of stimulus occurrence, inactivation of mitochondrial complexes I−IV (*37*, *38*) and reduced ATP synthesis, which was evidenced in our studies. Chronic oxidative stress may cause breaking of the internal mitochondrial membrane and release of the content of the mitochondrial intermembrane space into the cytosol (*39*). Cytochrome *c* translocation from the mitochondrion to cytosol causes the formation of an apoptosome. The consequence of apoptosome formation is the activation of effector caspases-3, -6, and -7 (*40*). However, no caspase-3 activity was found in our study, despite evidence by cytometric analysis that melanoma cells entered the pathway of apoptotic death. The lack of caspase-3 activity might be connected with the reduced expression of the Apaf-1 effector protein in melanoma cells, which precludes the formation of the apoptosome (*41*, *42*). Therefore, for comparison, the action of the tested extracts on caspase-3 activity in HepG2 hepatic cancer cells was examined, with the same result (data not shown). It is possible that AIF, whose translocation from the mitochondrion to the nucleus initiates degradation of nuclear structure by a noncaspase-dependent route (*43*), is responsible for the apoptosis of melanoma cells caused by extracts of defatted seeds of *Oenothera paradoxa*. Studies on defatted seeds of *Oenothera biennis* evening primrose evidenced that ethanolic extract increased the production of oxygen free radicals in Ehrlich ascites tumor cells (*11*), causing apoptosis through AIF translocation from the mitochondrion to the nucleus (*12*). A similar mechanism may exist in the case of extracts obtained from *Oenothera paradoxa*. Changes typical for apoptosis take place in the cells, and phosphatidylserine, whose presence was detected by the cytometric method with the use of annexin V labeled with FITC, appears on the external surface of cellular membranes (*44*).

In conclusion, extracts of defatted seeds of *Oenothera paradoxa* exert a cytotoxic action against HTB-140 human skin melanoma cells. They disturb the oxidoreductive balance of melanoma cells through an increase of ROS production and related lowering of the GSH level. GSH lowering results most probably in damaging of the respiratory chain and a reduction in production of mitochondrial ATP, and this makes the cells enter the pathway of apoptotic death, while the apoptotic pathway is not associated with activation of caspase-3. The 60% ethanolic extract proved to be the most effective; at the same time, it contained the greatest total amount of phenolic compounds and the greatest quantity of pentagalloylglucose.

ACKNOWLEDGMENT

We thank Dr. Maria Król for help with cytometric tests, Agnieszka Filipek, MSc, for support in operating the microplate reader, and Roman Rudecki, Eng., for laboratory help with the assay of total content of phenolic compounds.

LITERATURE CITED

(1) Newall, C. A.; Anderson, L. A.; Phillipson, J. D. *Herbal Medicines, a Guide for Health-Care Professionals*; The Pharmaceutical Press: London, UK, 1996; pp 110−113.

(2) Hatano, T.; Yasuhara, T.; Matsuda, M.; Yazaki, K.; Yoshida, T.; Okuda, T. Oenothein B, a dimeric hydrolizable tannin of cyclic structure. *Chem. Pharm. Bull.* **1989**, *37*, 2269–2271.

(3) Zinsmeister, H. D.; Bartl, S. The phenolic compounds of *Oenothera*. *Phytochemistry* **1971**, *10*, 3129–3132.

(4) Wettasinghe, M.; Shahidi, F.; Amarowicz, R. Identification and quantification of low molecular weight phenolic antioxidants in seeds of evening primrose (*Oenothera biennis* L.). *J. Agric. Food Chem.* **2002**, *50*, 1267–1271.

(5) Wettasinghe, M.; Shahidi, F. Iron (II) chelation activity of extracts of borage and evening primrose meals. *Food Res. Int.* **2002**, *35*, 65–71.

(6) Lu, F.; Foo, L. Y. *Phenolic Antioxidant Components of Evening Primrose. Nutrition, Lipids, Health, and Disease*; Niki, E., Packer, L., Eds.;AOCS Press: Champaign, IL, 1995; pp 86−95.
(7) Kiss, A. K.; Derwińska, M.; Dawidowska, A.; Naruszewicz, M. Novel biological properties of *Oenothera paradoxa* defatted seed extracts. Effects on the metallopeptidases activity. *J. Agric. Food Chem.* **2008**, *56*, 7845–52.
(8) Bałasińska, B.; Troszyńska, A. Total antioxidative activity of evening primrose (*Oenothera paradoxa*) cake extract measured *in vitro* by liposome model and murine L1210 cells. *J. Agric. Food Chem.* **1998**, *46*, 3558–3563.
(9) Peschel, W.; Dieckmann, W.; Sonnenschein, M.; Peschel, A. High antioxidant potential of pressing residues from evening primrose in comparison to other oilseed cakes and plant antioxidant. *Ind. Crops Prod.* **2007**, *25*, 44–54.
(10) Pellegrina, C. D.; Padovani, G.; Mainente, F.; Zoccatelli, G.; Bissoli, G.; Mosconi, S.; Veneri, G.; Peruffo, A.; Andrihetto, G.; Rizzi, C.; Chignola, R. Anti-tumor potential of a gallic acid-containing phenolic fraction from *Oenothera biennis*. *Cancer Lett.* **2005**, *226*, 17–25.
(11) Arimura, T.; Kojima-Yuasa, A.; Watanabe, S.; Suzuki, M.; Kennedy, D. O.; Matsui-Yuasa, I. Role of intracellular reactive oxygen species and mitochondrial dysfunction in evening primrose extract-induced apoptosis in Ehrlich ascites tumor cells. *Chem.-Biol. Interact.* **2003**, *145*, 337–347.
(12) Arimura, T.; Kojima-Yuasa, A.; Suzuki, M.; Kennedy, D. O.; Matsui-Yuasa, I. Caspase-independent apoptosis induced by evening primrose extract in Ehrlich ascites tumor cells. *Cancer Lett.* **2003**, *201*, 9–16.
(13) Arimura, T.; Kojima-Yuasa, A.; Kennedy, D. O.; Matsui-Yuasa, I. Reactive oxygen species-independent G1 arrest induced by evening primrose extract in Ehrlich ascites tumor cells. *Cancer Lett.* **2004**, *207*, 19–25.
(14) Hoang, M. T.; Eichenfield, L. F. The rising incidence of melanoma in children and adolescents. *Dermatol. Nurs.* **2000**, *121*, 192–193.
(15) Touriño, S.; Lizárraga, D.; Carreras, A.; Lorenzo, S.; Ugartondo, V.; Mitjans, M.; Vinardell, M. P.; Juliá, L.; Cascante, M.; Torres, J. L. Highly galloylated tannin fractions from witch hazal (*Hamamelis virginiana*) bark: electron transfer capacity, *in vitro* antioxidant activity, and effects on skin-related cells. *Chem. Res. Toxicol.* **2008**, *21*, 696–704.
(16) Touriño, S.; Selga, A.; Jiménez, A.; Juliá, L.; Lozano, C.; Lizárraga, D.; Cascante, M.; Torres, J. L. Procyanidin fractions from pine (*Pinus pinaster*) bark: radical scavenging power in solution, antioxidant activity in emulsion, and antiproliferative effect in melanoma cells. *J. Agric. Food Chem.* **2005**, *53*, 4728–4735.
(17) Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C. Grape polyphenol resveratrol and the related molecule 4-hydroxystilbene induce growth inhibition, apoptosis, S-phase arrest, and upregulation of cyclins A, E, and B1 in human SK-Mel-28 melanoma cells. *J. Agric. Food Chem.* **2003**, *51*, 4576–4584.
(18) Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C. The grape and wine polyphenol piceatannol is a potent inducer of apoptosis in human SK-Mel-28 melanoma cells. *Eur. J. Nutr.* **2004**, *43*, 275–284.
(19) Halder, B.; Bhattacharya, U.; Mukhopadhyay, S.; Giri, A. K. Molecular mechanism of black tea polyphenols induced apoptosis in human skin cancer cells: involvement of Bax translocation and mitochondria mediated death cascade. *Carcinogenesis* **2008**, *29*, 129–138.
(20) Miura, T.; Chiba, M.; Kasai, K.; Nozaka, H.; Nakamura, T.; Shoji, T.; Kanda, T.; Ohtake, Y.; Sato, T. Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. *Carcinogenesis* **2008**, *29*, 585–593.
(21) Eberle, J.; Kurbanov, B. M.; Hossini, A. M.; Trefzer, U.; Fecker, L. F. Overcoming apoptosis deficiency of melanoma-Hope for new therapeutic approaches. *Drug Resist. Updates* **2007**, *10*, 218–234.
(22) Liu, J. D.; Chen, S. H.; Lin, C. L.; Tsai, S. H.; Liang, Y. C. Inhibition of melanoma growth and metastasis by combination with (−)-epigallocatechin-3-gallate and decarbazine in mice. *J. Cell Biochem.* **2001**, *83*, 631–642.
(23) Caltagirone, S.; Rossi, C.; Poggi, A.; Ranelletti, F. O.; Natali, P. G.; Brunetti, M.; Aiello, F. B.; Piantelli, M. Flavonoids apigenin and quercetin inhibit melanoma growth and metastatic potential. *Int. J. Cancer.* **2000**, *87*, 595–600.
(24) Naoi, M.; Maruyama, W.; Yi, H.; Yamaoka, Y.; Shamoto-Nagai, M.; Akao, Y.; Gerlach, M.; Tanaka, M.; Riederer, P. Neuromelanin selectively induces apoptosis in dopaminergic SH-SY5Y cells by deglutathionylation in mitochondria: involvement of the protein and melanin component. *J. Neurochem.* **2008**, *105*, 2489–2500.
(25) Obajimi, O.; Melera, P. W. The depletion of cellular ATP by AG2034 mediates cell death or cytostasis in a hypoxanthine-dependent manner in human prostate cancer cells. *Cancer Chemother. Pharmacol.* **2008**, *62*, 215–226.
(26) Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* **1998**, *46*, 1887–1892.
(27) Labuda, J.; Bucková, M.; Heilerová, L.; Silhár, S.; Stepánek, I. Evaluation of the redox properties and anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor. *Anal. Bioanal. Chem.* **2003**, *376*, 168–173.
(28) Sakaguchi, N.; Inoue, M.; Ogihara, Y. Reactive oxygen species and intracellular Ca^{2+}, common signals for apoptosis induced by gallic acid. *Biochem. Pharmacol.* **1998**, *55*, 1973–1981.
(29) Hu, H.; Lee, H. J.; Jiang, C.; Zhang, J.; Wang, L.; Zhao, Y.; Xiang, Q.; Lee, E. O.; Kim, S. H.; Lü, J. Penta-1,2,3,4,6-O-galloyl-beta-D-glucose induces p53 and inhibits STAT3 in prostate cancer cells in vitro and suppresses prostate xenograft tumor growth *in vivo*. *Mol. Cancer Ther.* **2008**, *7*, 2681–2691.
(30) Abdelwahed, A.; Bouhlel, I.; Skandrani, I.; Valenti, K.; Kadri, M.; Guiraud, P.; Steiman, R.; Mariotte, A. M.; Ghedira, K.; Laporte, F.; Dijoux-Franca, M. G.; Chekir-Ghedira, L. Study of antimutagenic and antioxidant activities of gallic acid and 1,2,3,4,6-pentagalloylglucose from *Pistacia lentiscus*. Confirmation by microarray expression profiling. *Chem.-Biol. Interact.* **2007**, *165*, 1–13.
(31) Nakazato, T.; Ito, K.; Ikeda, Y.; Kizaki, M. Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. *Clin. Cancer Res.* **2005**, *11*, 6040–6049.
(32) Robaszkiewicz, A.; Balcerczyk, A.; Bartosz, G. Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell Biol. Int.* **2007**, *31*, 1245–1250.
(33) Fleury, C.; Mignotte, B.; Vayssière, J. L. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* **2002**, *84*, 131–141.
(34) Szewczyk, A.; Wojtczak, L. Mitochondria as a pharmacological target. *Pharmacol. Rev.* **2002**, *54*, 101–127.
(35) Wojtczak, L.; Sleshenkov, V. S. Protection by pantothenic acid against apoptosis and cell damage by oxygen free radicals − the role of glutathione. *BioFactors* **2003**, *17*, 61–73.
(36) Schafer, F. Q.; Buettner, G. R. Redox environment of the cell as viewed through the redox state of the glutatione disulfide/glutathione couple. *Free Radical Biol. Med.* **2001**, *30*, 1191–1212.
(37) Hsu, M.; Srinivas, B.; Kumar, J.; Subramanian, R.; Andersen, J. Glutathione depletion resulting in selective mitochondrial complex I inhibition in dopaminergic cells is via an NO-mediated pathway not involving peroxynitrite: implications for Parkinson's disease. *J. Neurochem.* **2005**, *92*, 1091–1103.
(38) Seyfried, J.; Soldner, F.; Schulz, J. B.; Klockgether, T.; Kovar, K. A.; Wüllner, U. Differential effects of L-buthionine sulfoximine and ethacrynic acid on glutathione levels and mitochondrial function in PC12 cells. *Neurosci. Lett.* **1999**, *264*, 1–4.
(39) Czarna, M.; Jarmuszkiewicz, W. Role of mitochondria in reactive oxygen species generation and removal; relevance to signaling and programmed cell death. *Postepy Biochem.* **2006**, *52*, 145–156.
(40) Grądzka, I. Mechanisms and regulation of the programmed cell death. *Postepy Biochem.* **2006**, *52*, 157–165.
(41) Mustika, R.; Budiyanto, A.; Nishigori, C.; Ichihashi, M.; Ueda, M. Decreased expression of Apaf-1 with progression of melanoma. *Pigment Cell Res.* **2005**, *18*, 59–62.

(42) Dai, D. L.; Martinka, M.; Bush, J. A.; Li, G. Reduced Apaf-1 expression in human cutaneous melanomas. *Br. J. Cancer*. **2004**, *91*, 1089–1095.
(43) Candé, C.; Cohen, I.; Daugas, E.; Ravagnan, L.; Larochette, N.; Zamzami, N.; Kroemer, G. Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. *Biochimie* **2002**, *84*, 215–222.
(44) Cregan, S. P.; Fortin, A.; MacLaurin, J. G.; Callaghan, S. M.; Cecconi, F.; Yu, S. W.; Dawson, T. M.; Dawson, V. L.; Park, D. S.; Kroemer, G.; Slack, R. S. Apoptosis inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol*. **2002**, *158*, 507–517.

Received April 28, 2009. Revised manuscript received June 30, 2009. Accepted August 2, 2009. The research study was cofinanced by the European Social Fund and by the State Budget of Poland under the Integrated Regional Development Operational Program.