

Influence of *Chelidonium majus* Water Extract onto Cancer Cells and Platelets

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Abstract

The plant *C. majus* (Celandine) is a known source of biologically active compounds that are used in traditional medicine for preventing or curing cancer diseases. The growing interest in this plant is due to discovery of a number of plant-derived cytostatics and anti-blastic drugs. The goal of our study was to discover the influence of a water extract from these plants on cancer cells and to characterize the obtained extract using different analytical methods.

Water extract of leaves of *C. majus* were obtained and lyophilized on TelStar LyoQuest (Spain). Composition of the extract was characterized using HPLC Agilent 1100, SDS-PAGE electrophoresis and MLADI-TOF Mass-spectrometry. ADP-induced platelet aggregation in the presence of obtained mixture was studied by aggregometry using Solar2110 (Belorussia). Effects of the extracts on proliferation of cancer cells was estimated using MTT-test.

Investigation of *C. majus* extract demonstrated presence of chelerythrine and precursors of berberine alkaloids in it.

Addition of *C. majus* extract to platelet rich plasma induced moderate platelet activation (up to 10%). This aggregation was reversible. Further ADP-induced activation of platelets pre-incubated with the extract was diminished on 30%. The extract of *C. majus* leaves also was shown to possess anti-proliferative agent that was demonstrated on MCF-7 cancer cells. The surviving of MCF-7 coulter cells was decreased to 40% in the presence of extract.

In conclusion, direct action of *C. majus* extract components on platelet aggregation and cancer cell proliferation was observed. Further fractionation for the determination of functionally active components of *C. majus* extract is promising for biotechnology and medicine.

Keywords: *C. majus*; Extract; Cancer cells; Platelets

Introduction

Wild plant *C. majus* (Celandine) is a known source of biologically active compounds that are used in traditional medicine for preventing or curing of cancer diseases. The compounds of natural plant origin are being studied as modern agents for antitumor therapy. The growing interest for these compounds is explained by the discovery and detailed study of a number of plant-derived cytostatics and the successful application of such anti-blastic drugs as colchamine, vinblastine, vincristine, podophilin, and others [1-3].

Alkaloids should be named among the compounds of plant origin. This is a group of biologically active heterocyclic nitrogen-containing substances. Extract of celandine (*C. majus* L.) contains about 35 alkaloids [1,4] with protopinic, protoberberin and benzophenanthridine structure. Among alkaloids contained in the extracts of *C. majus* are chelidonin, sanguinarine, berberin, narcissine and coptisin.

The biological activity of many compounds of *C. majus* leaves were demonstrated in the recent years. In particular, it has been established that the chelidonin interacts with tubulin causing the termination of the mitosis phase [5] chelidonine has several pharmacological effects [6,7]. Heliterrin, sanguinarine, berberine and coptisin affect the processes of respiration in mitochondria, berberine affects platelet activation [8], berberine is a reversible inhibitor of acetylcholinesterase [9] and pure berberin is the trombin inhibitor and inhibitor of platelets aggregation [10]. It was steadied in case of cardiovascular and thrombotic deceases [11], but FDA declined it in clinical trials, because of risk cytotoxicity [12]. Also, it demonstrates effect onto arachidonic acid metabolism [13] and the thromboxane B2 (TXB2) and 6-keto-prostaglandin F1 alpha (6-keto-PGF1 alpha) [14], caused to platelet

aggregation and makes effect onto endothelial cells. It was found, that berberine is NF-k-B activator, inhibitor of N-acetyltransferase, cyclooxygenase-2 and topoisomerase and and is an inducer of apoptosis [15,16]. Chelerythrine is a protein kinase C inhibitor [17], protopine inhibits voltage- and receptor-operated calcium channels [18], and sanguinarine is a potent inhibitor of nuclear factor (NF-kB) involved in the activation of tumor necrosis factor gene expression [19]. Due to the presence of iminogroups, sanguinarine and helitrin can inhibit the activity of enzymes that have nucleophilic groups in the active center [20]. Some alkaloids of *C. majus* act as inhibitors of protein kinase, blocking the activity of the transcription factor NF-kB, which is a component of signaling pathways involved in induction of cell proliferation or apoptosis [21]. Authors pay attention to the ability of individual alkaloids of celandine to interact with DNA by penetrating their planar molecular structures in DNA strands [2].

Development of alkaloid-based drugs with antitumor activity is an important issue of biotechnology [22-24]. The mechanisms of alkaloids suppression of the growth of tumors is not well understood,

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in particular, the mechanisms of action of the celandine alkaloids, which are involved in induction of death of tumor cells, remain poorly understood and needs to be clarified. It is unclear, which of the alkaloids of *C. majus* is the most effective pro-apoptotic agent and whether there are other types of cell death that can be induced under the influence of alkaloids of *C. majus*.

That is why the present study deals with effects of water lyophilised extracts of *C. majus* leaves and flowers on isolated human platelets and adenocarcinoma cells of mammal gland, MCF-7.

Materials and Methods

Chemicals

In this study we used: acetonitrile, ammonium sulfate HPLC, acrylamide and bisacrylamide, Glycine and TRIS, SDS, HCl HPLC-grade, cultural medium DMEM, fetal bovine serum, heparin sodium salt, Ficoll Histopaque, Pencillin-streptomycin solution, Fetal bovine serum, (Sigma Aldrich, USA), MTT (Abcam, USA) and trypan blue dye (Thermo Fisher Scientific, USA), W.B.C. diluting fluid (Qualigen, India). Tris and sodium chloride HPLC grade (Fluka, Monte Carlo), ADP chemistry pure (Merck, Germany).

Chelidonium majus extract

Water extracts of leaves of medicinal plant was obtained from dried plants (leaves and flowers, obtained in the period of flowering, drying at +20°C in the shadow). For preparation extract with volume 200 ml 4 g of milled and dried plant were taken. It was washed by bubbling up water and infused 30 minutes under closed lid. Then it was filtered through paper filter and concentrated using lyophilization on TelStar LyoQuest (Spain). Firstly, extract was frozen in the flask using liquid nitrogen, than flask was connected to TelStar LyoQuest with heating and vacuum during 24 hours. Lyophilized brown colored powder was removed to 1.5 ml plastic flasks. For characterization with HPLC powder was dissolved in deionized distilled water in concentration of 3 mg per ml.

Platelet rich plasma

Platelet rich plasma samples were obtained from blood of healthy donors. Volunteers signed informed consent prior to blood sampling according to the Helsinki declaration. Platelet rich plasma (PRP) was prepared from human citrated blood by centrifugation at 1000 rpm during 20 min.

MCF-7 cell line

MCF-7 breast cancer cell lines (Sigma collection obtained) [25,26]. Cells were cultured in a medium with 4500 mg/l glucose (DMEM, Dubecco's Modified Eagle Medium, Sigma, USA) and supplemented with 10% fetal bovine serum (FBS, Sigma, USA) for 3 h at 37°C, 5% CO₂ in CO₂ incubator and grown to confluence at 37°C in 5% CO₂ in a humidified atmosphere in a CO₂ incubator (MMM, Medcenter Entrichungen GmbH MM-Group). The cells were trypsinized [500 µl of 0.025% Trypsin in PBS/0.5 mM EDTA solution (Sigma, USA)] for 2 min and passaged in 96-well Assay Plate, Corning Incorporation, COSTAR) in complete aseptic conditions.

Lymphocyte preparation from human blood

Lymphocytes were obtained from human venous blood using Ficoll gradient method, as described [27,28]. Number of living cells were controlled with trypan blue strain under microscope. Cells were cultured in DMEM culture as described [29,30]. Cultivation of human

lymphocytes was carried out at the same condition as MCF-7 and by the same protocol.

HPLC-chromatography

Chromatographic system Agilent 1100 was used for analysis of extract with column Dupont Instrument (250 mm long and 4.7 mm over) with Zorbax Silicogel (20 mkm) with phenyl inoculation in pressure of 140 bar and flow 1.5 ml per minute. There was used buffer gradient decreasing 0.15 M TRIS HCl pH 6.5, 0.13 M NaCl and incising 50% Acetonitrill contained buffer with 0.1% trifluoroacetic acid in 70 minutes long.

SDS-PAGE

Dried *C. majus* extract was dissolved in sample buffer containing 4% SDS, 0.5% β-mercaptoethanol, 5% glycerol and blue dye bromophenol. Then it was analyzed by SDS-PAGE 15% gel according to Laemmli [31].

Xanthoproteic Test

Xanthoproteic Test was used for characterization of protein content of the extract. One volume of extract was mixed with an equal volume of nitric. Mixture was heated and then cooled, then it was slowly mixed with 40% sodium hydroxide until the mixture becomes alkaline and a color change is noted. Yellow or orange color indicates the presence of an aromatic amino acid.

Steady-state absorption spectra

The differential absorbance spectra were recorded in the 200-700 nm range using spectrophotometer Optizen-POP (Optizen, Korea).

MALDI-TOF analysis

MALDI-TOF analysis of *C. majus* extract was performed using a Voyager-DE (Applied Biosystems, USA). H⁺-matrix ionization of polypeptides with sinapine acid (Sigma-Aldrich) was used. Results were analyzed by Data Explorer 4.0.0.0 (Applied Biosystems) [32].

Cell line experiments

MCF-7 cells were incubated in DMEM medium supplemented with 10% FBS, 40 mg/ml gentamicin at standard conditions (at 37°C in 5% CO₂) during two days. *C. majus* extract was dissolved in the cell's medium DMEM, used for cells treatment. Dissolved extract was dose titrated by binary dissolution from 3 mg/ml to 0.012 mg/ml in final concentration in the cell's medium DMEM with serum.

Cell viability was measured by MTT-colorymetric test. The cell proliferation was expressed as percentage of the viable cell number of the control (non-treated cells). MCF-7 cell surviving was analyzed using trypan blue treatment [33]. It was added with 0.4% w/v freshly filtered trypan blue dissolved in an identical medium to cells. Thereafter, MCF-7 cells are counted on a microscope in Haryev chamber, and the percentage viability is calculated by dividing the number of viable (unstained) cells by the number of all counted cells (stained and unstained).

Platelet aggregation study

CaCl₂ (0.1 mM) and 10-20 µL of *C. majus* extract were added to 200 µL of PRP and incubated in the aggregometer tube at constant spinning during 5 min. The extent of aggregation was monitored by SOLAR aggregometer (Belorussia). Then aggregation was started by addition of 12.5 µM ADP and also monitored as described above.

Results

C. majus extract characterization

Dried extract of *C. majus* was characterized by HPLC, MALDI-TOF analysis, SDS-PAGE and spectra adsorption study. All these method allowed as directly or indirectly estimate the content of water extract that we aimed to study.

Absorption study demonstrated maximal absorption in UV diapason, with main peak of 210 nm Figure 1. Thus we could suggest that main components of studied extracts are alkaloids that have

maximal adsorption meanings in this region of spectra [34].

SDS-PAGE and Xanthoproteic Test showed no proteins in the mixture even after concentrating by ten times so we can conclude that any possible biological effects of extracts can be caused by protein content.

HPLC fractionation of extract of *C. majus* demonstrated the presence of several major fractions. In particular, we detected small high molecular weight fraction and two or three low-molecular weight fractions that we assumed as alkaloids (Figure 2).

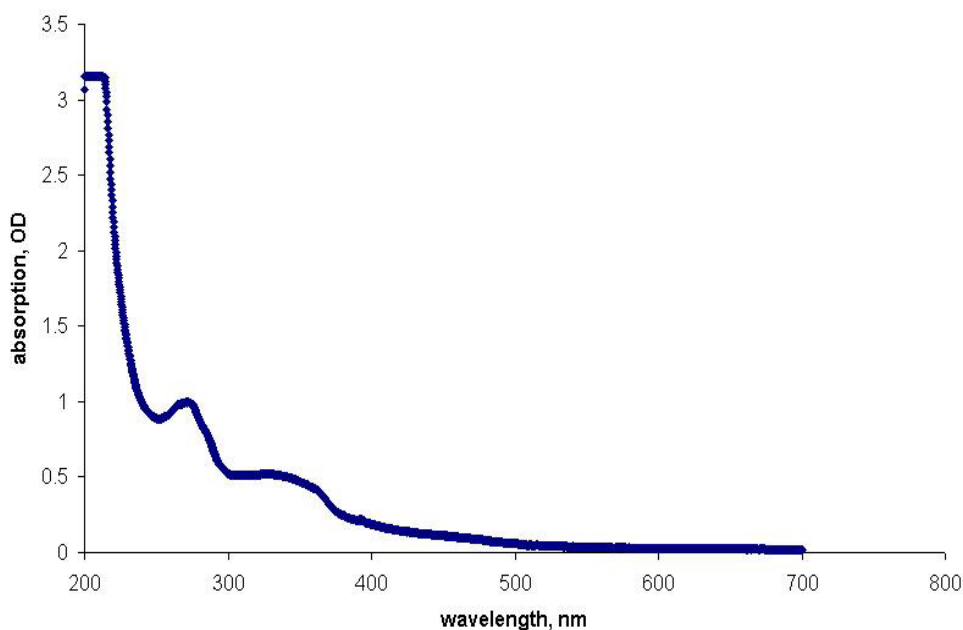


Figure 1: Spectra of adsorption of *Chelidonium majus* extract with maximal absorption at 210 nm.

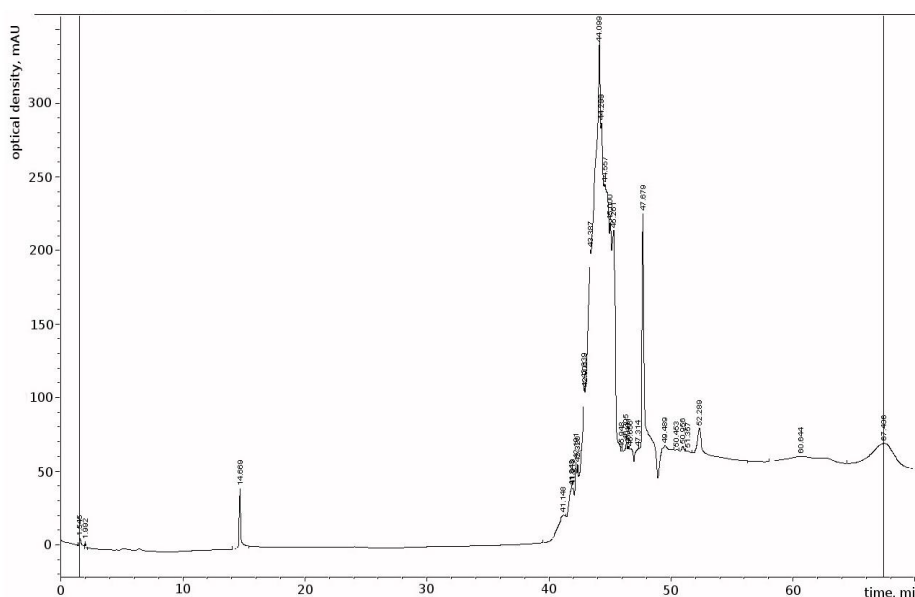


Figure 2: HPLC chromatogram of *Chelidonium majus* extract performed on chromatographic system Agilent 1100, column Dupont Instrument (250 mm long and 4.7 mm over) with Zorbax Silicogel (20 μ m) with phenyl inoculation in pressure of 140 bar and flow 1.5 ml per minute. There was used buffer gradient decreasing 0.15M TRIS HCl pH 6.5, 0.13M NaCl and incising 50% Acetonitrill contained buffer with 0.1% trifluoroacetic acid in 70 minutes long.

To prove this suggestion we performed MALDI-TOF analysis that allowed us to identify two major components of extract with molecular weight 338 Da and 320 Da (Figure 3). First peak most probably belongs to alkaloids from Berberine family, that has molecular weight 338 Da. Possible, it were jatrorrhizine, N-methylstylopine, tetracosane, N-methyltetrahydrocoptisine, 13-hydroxyberberraline, columbamine, dehydrocorytenchine. All this alkaloids are precursoric methabolites of berberine, coptisine and jatrorrhizine. The second represents chelerythrine that is major alkaloid component of species of the tribe Chelidoniaeae of the Papaveraceae family and has molecular weight of 320 Da.

Possible, it were 9,10-didemethylchelerythrine or 7,8-didemethylchelerythrine. Both alkaloids were previously found in plants from the family of Papaveraceae and could be found in *C. majus* extract [34,35].

Thus, we obtained the extract of *C. majus* that was characterized and lyophilized for further studies. It contained mostly alkaloids precursors of berberine and chelerythrine that were identified using MALDI-TOF mass-spectrometry [36-39].

Action of *C. majus* extract on platelet aggregation

In further studies we examined the effect of *C. majus* extract on platelet aggregation. We aimed to investigate its effects on resting or ADP-activated platelets by aggregometry.

It was demonstrated that *C. majus* extract taken in final concentration 0.1 mg/ml and 0.06 mg/ml induced platelet activation that leads to moderate aggregation (rate of aggregation 10%) (Figure 4).

When platelets that were pre-incubated with *C. majus* extract during 1 min were activated by ADP the decreasing of the rate of aggregation was observed (Figure 5). The rate of aggregation of platelets pre-incubated with 0.1 mg/ml or 0.06 mg/ml of extract decreased on 30% and 40% respectively (Figure 5). It can be explained by initial activation of platelets by the extract that take some part of cells out of the aggregation process induced by ADP.

Thus, we suggested that this effect must be caused by chelerythrine or berberine precursors and berberine-like alkaloids content of the extract. This is an opposite effect, that shown for pure berberine [10-14]. We thought, that we got precursors of main alkaloids not because of degradation them during extraction, but because of harvesting young flowering plants, where main alkaloids has not been synthesized yet. In accordance with farmacognosia recommendation, often were used root of this plant, harvesting in autumn. It has different alkaloids composition. So, anticancer and platelets activation of extract, obtained from flowering plant was study firstly. And in comparison with another pure anticancer alkaloids, derived from different plants, our extract demonstrated lower activity, because of presents ballast compounds with a high molecular weight, and lower concentration of active compounds [40].

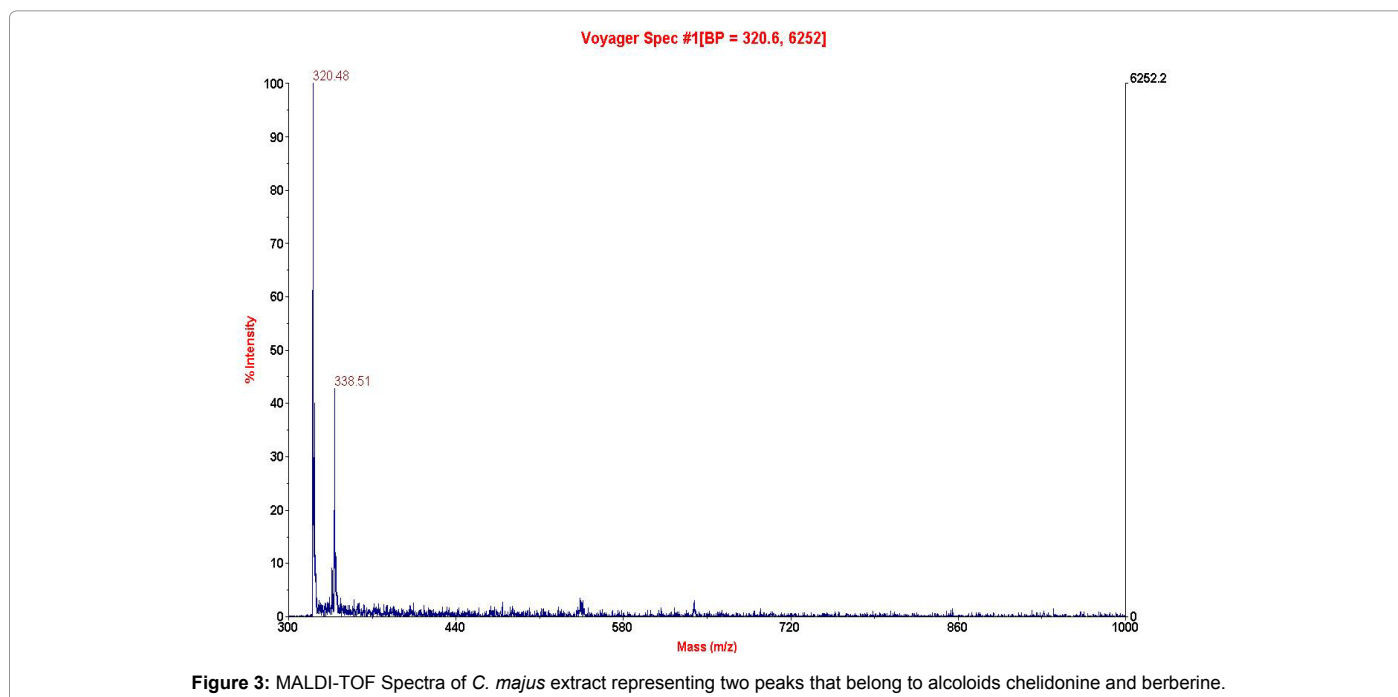
Antiproliferative action of *Chelidonium majus* extract

Also *C. majus* extract taken in concentration of 3 mg/ml and 1.5 mg/ml demonstrated cytopathogenic effect on MCF-7 cells (Figures 6 and 7). Addition of extract decreased the surviving of MCF-7 coulter cells determined using MTT-test to 40% and showed total mortality in test with trypan blue treatment.

The dose-dependent cytopatogenic effect of the studied extract is clearly demonstrated in Figure 6. Viability of cells determined with MTT-test was decreased significantly in the presence of *C. majus* extract. In particular it decreased in 50% in the presence of 0.375 mg/ml of extract.

In comparison with positive control on human lymphocytes showed present of cytotoxic effect with concentrations of extract upper 2 mg/ml, as it described in Figure 7. An optimal dose that inhibits MCF-7 cells and has low toxicity was between 1 and 2 mg/ml, as it was shown for dose 1.5 mg/ml.

Also, it was shown an influence of *Chelidonium majus* extract onto cell membrane permeability. Analysis of the number of dead cells using trypan blue demonstrated that the dose of 0.375 mg/ml possessed



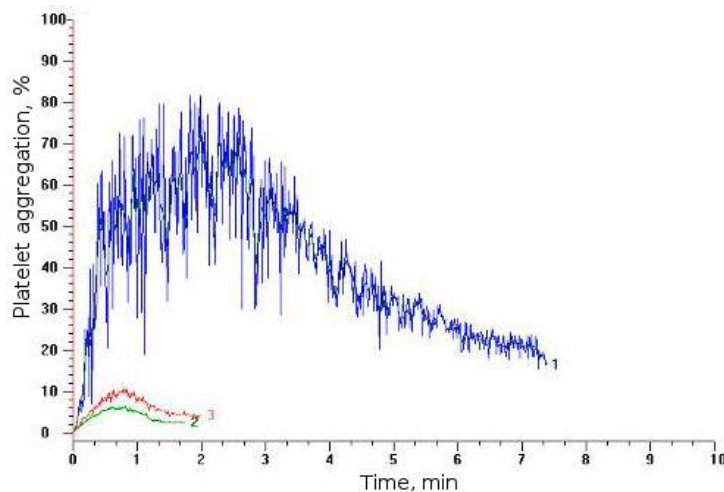


Figure 4: Aggregation of platelets in PRP induced by *Chelidonium majus* extract (3 mg/ml) without ADP (2-10 mkl, 3-20 mkl) and control (ADP-induced aggregation of platelets-1).

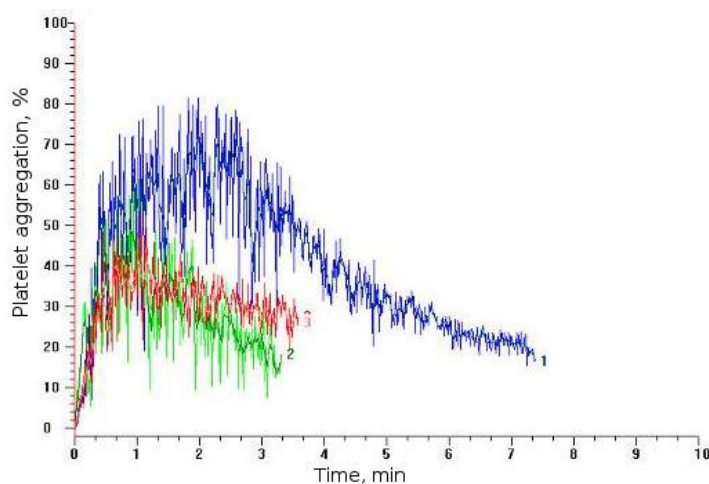


Figure 5: ADP-induced aggregation of platelets in PRP after 5 min incubation with *Chelidonium majus* extract (2-0.1 mg/ml; 3-0.06 mg/ml) and control (incubated with equivalent volume of buffer-1).

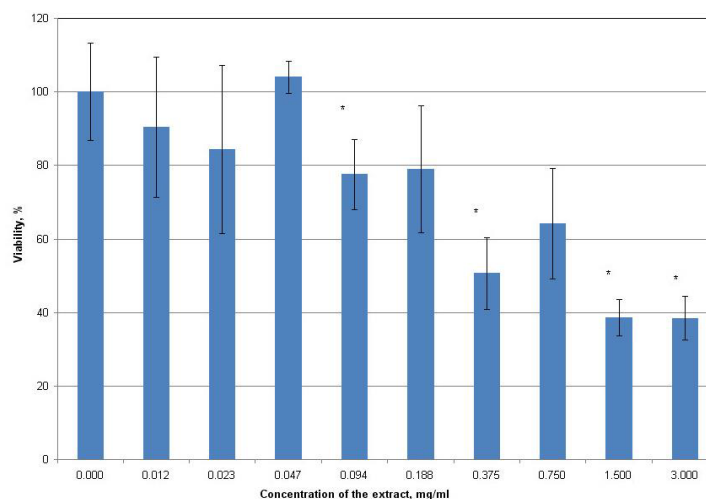


Figure 6: MTT test of surviving MCF-7 cells under treatment with *Chelidonium majus* extract. *results were significant for $p < 0.05$.

effective antiproliferative action and the maximal dose decreased the level of living cells almost to the baseline (Figure 8).

Data of optical microscopy presented in Figure 9 demonstrates the cell layers in the presence of *C. majus* extract in comparison to control plates. Decreasing of cell number was detected and the staining with trypan blue indicated that most of cells in experimental sample were dead (Figure 9).

Conclusion

Spectra adsorption, HPLC and mass spectra demonstrated that dried extract of *C. majus* consist of alkaloids with fluorescence at 220-480 nm and maximum adsorption at 210 nm. MALDI-TOF analysis identified in the extract alkaloids from berberine family and chelerythrine. In our studies the moderate platelet-activating effect of

the studied extract we showed too. We assumed that this effect was caused mainly by berberin precursors.

Chelerythrine that was identified in the extract was shown to possess anti-proliferative activity towards MCF-7 cancer cells. It was shown that under concentration of extract in medium 1.5-3 mg/ml all cells died and MTT test showed residual activity in the rank of 40%.

As to control lymphocytes a cytotoxic effect of the extract was observed only at concentration 3 mg/ml that was twice higher compared to the optimal inhibition dose shown for MCF-7 cells (1.5 mg/ml).

Further studies of the biological effects of plant extracts containing precursors of berberine and chelerythrine can be promising for the development of antithrombotic and anticancer drugs.

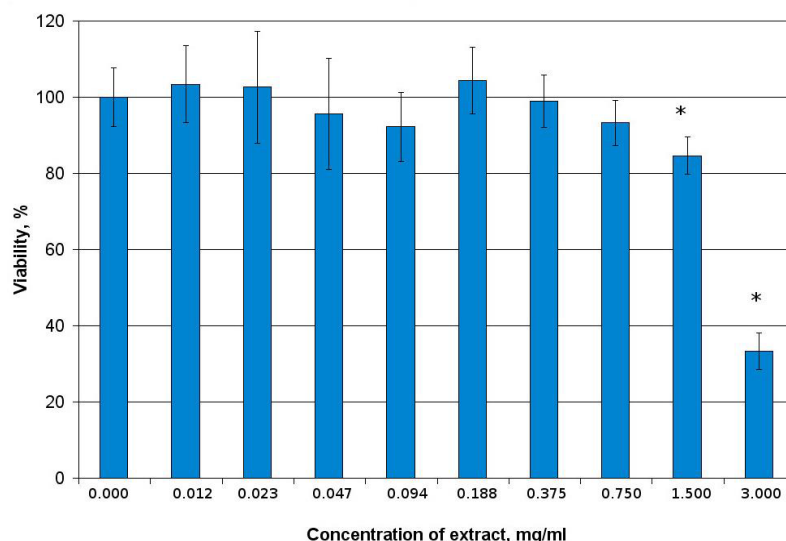


Figure 7: MTT test of surviving lymphocytes under treatment with *Chelidonium majus* extract. All results were significant for $p < 0.05$.

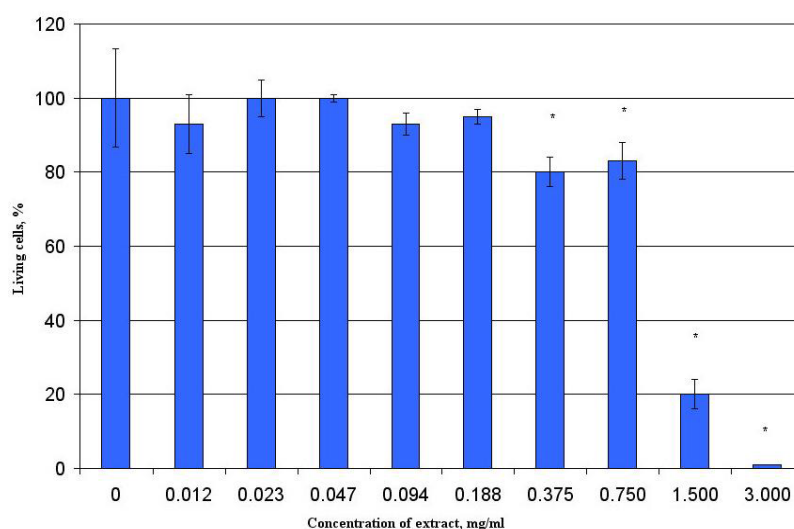


Figure 8: Total mortality in test with trypan blue treatment of MCF-7 cells under treatment with *Chelidonium majus* extract. *results were significant for $p < 0.05$.

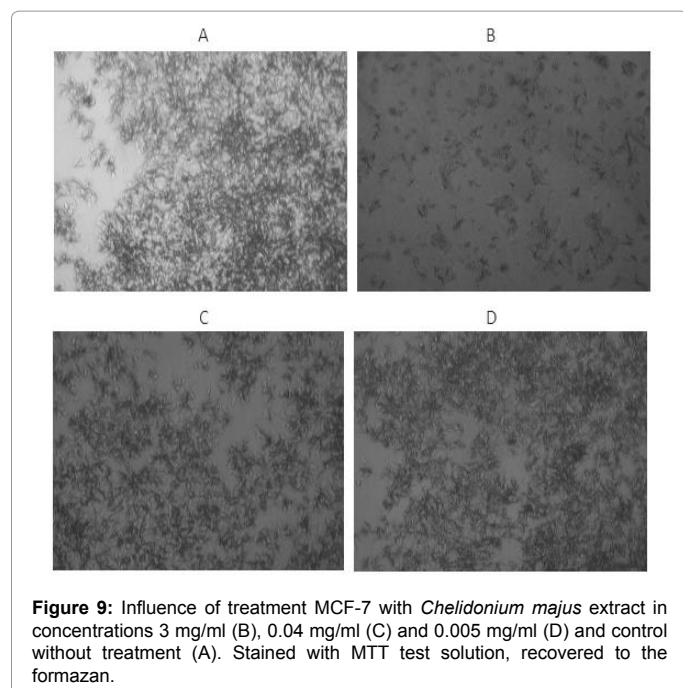


Figure 9: Influence of treatment MCF-7 with *Chelidonium majus* extract in concentrations 3 mg/ml (B), 0.04 mg/ml (C) and 0.005 mg/ml (D) and control without treatment (A). Stained with MTT test solution, recovered to the formazan.

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