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Antitumor activity of Bulgarian herb *Tribulus terrestris* L. on human breast cancer cells

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ABSTRACT

Medicinal plants have been intensively studied as a source of antitumor compounds. Due to the beneficial climate conditions Bulgarian herbs have high pharmacological potential. Currently, the antitumor effect of the Bulgarian medicinal plant *Tribulus terrestris* L. on human cancer cell lines is not studied. The main active compounds of the plant are the steroid saponins.

The present study aims to analyze the effect on cell viability and apoptotic activity of total extract and saponin fraction of Bulgarian *Tribulus terrestris* L. on human breast cancer (MCF7) and normal (MCF10A) cell lines. Antitumor effect was established by MTT cell viability assay and assessment of apoptotic potential was done through analysis of genomic integrity (DNA fragmentation assay) and analysis of morphological cell changes (Fluorescence microscopy). The results showed that total extract of the herb has a marked dose-dependent inhibitory effect on viability of MCF7 cells (half maximal inhibitory concentration is 15 µg/ml). Cell viability of MCF10A was moderately decreased without visible dose-dependent effect. The saponin fraction has increased inhibitory effect on breast cancer cells compared to total extract. Morphological changes and DNA fragmentation were observed as markers for early and late apoptosis predominantly in tumor cells after treatment. Apoptotic processes were intensified with the increase of treatment duration.

The obtained results are the first showing selective antitumor activity of Bulgarian *Tribulus terrestris* L. on human cancer cells *in vitro*. Apoptotic processes are involved in the antitumor mechanisms induced by the herb. This results give directions for future investigations concerning detailed assessment of its pharmacological potential.

Key words: *Tribulus terrestris* L., saponins, breast cancer cell line, antitumor activity, apoptosis

Introduction

The complex nature of tumorigenesis is the main reason for absence of effective and universal treatment. At present, together with the application of conventional approaches in oncological practice, antitumor activity of medicinal plants is intensively studied. The final purpose is the development of therapeutic agents, which selectively and locally to inhibit the growth of tumors without damaging the normal tissues. In this regard, antitumor therapy is aiming at finding of active

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substances, which inhibit growth and/or repress proliferation of cancer cells only. One basic approach is the study of antitumor effect of biologically active compounds in total extracts and purified fractions of plants, known and used for therapy in traditional medicine. Currently, by the use of highly sensitive methods and technologies it became known that some active herb compounds possess selective anticancer effect (Taraphdar et al., 2001). However, the exact mechanisms underlying this effect are not clear at this time.

Bulgarian flora includes a variety of plant species (about 3600), and many of them (about 650) are classified as medicinal. Currently, pharmacological and curative action is studied for a limited number of medicinal plants. Often the extensive experience of traditional medicine has served as a basis for an in-depth analysis of the active compounds. Bulgarian herbs contain high percentage of biologically active substances as a result of favorable and diverse climate and soil conditions. They are rich in alkaloids, flavonoids, glycosides, saponins, polysaccharides, polyphenols, tannins and others. There is evidence for some of the active herb substances for induction of apoptosis in different types of cancer cells (Wang G et al., 2006).

Tribulus terrestris L. is an annual herbaceous plant from *Zygophyllaceae* family. It thrives in sandy and dry soil. In Bulgaria it is widespread in the Southern part of the country and the plants from the Southeastern areas are considered to have the richest composition. *T. terrestris* is distributed in dry climate regions of the world - Europe (Mediterranean region), Asia and America. It is used in traditional medicine of India, China, Turkey. In Bulgaria the herb is applied for general energizing, revitalizing and improving of general condition and status of both healthy and seriously diseased people. Known is the usage of herbal *T. terrestris* extracts also in treatment of urological infections, prostatic hypertrophy, edema, boils and some diseases of the cardiovascular system. Extracts from *T. terrestris* have immunostimulatory and antimicrobial effect (Sengul et al., 2009). According to the traditional Chinese medicine the plant is a strong aphrodisiac and may stimulate men sexual behavior, strengthen the heart muscle and reduce the level of cholesterin (Antonio et al., 2000). Plant fruits are applied to treat eye deseases, abdominal diseases and vitiligo (Cai et al., 2001).

At present, some of the main active compounds of the plant are identified and purified (Cai et al., 2001; Ganzera et al., 2001; Bedir et al., 2002; De Combarieu et al., 2003), including steroidal furostanol and spirostanol saponins - protodioscin, terrestrinins A and B, gitogenin, hecogenin, diosgenin and others. Pharmacological value of the herb is determined by the amount of the active compounds, which vary considerably depending on the area of plant growing and the used part. According to Kostova and Dinchev (2005), the contents of furostanol saponins in Bulgarian herb extract can reach 45%, while in other regions is only 15-20%. The amount of the furostanol saponin protodioscin is highest in the Bulgarian and Moldavian plants.

It is considered that the medicinal qualities of *T. terrestris* are due exactly to the high contents of saponins. They are glycosides with complex structure and likely serve as reserve compounds. Saponins are highly active surface substances. It was found that they possess hemolytic activity, help for reduction of cholesterin contents (Chu et al., 2003) and for the resorption of the fats and carbohydrates in the body. Based on high contents and biological activity of saponins in *T. terrestris*, herbal mixtures and drugs were developed in some countries (Cai et al., 2001; De Combarieu et al., 2003), which are used in the complex therapy of atherosclerosis of general, cerebral and coronary vessels ("Tribosponin") and for treatment of sexual dysfunction („Tribestan"). Similar herbal mixtures are applied also in China and India.

Limited are the data for antitumor activity of *T. terrestris.* There are restricted number of publications regarding *in vitro* studies on the effect of different steroid spirostanol and furostanol saponin components of *T. terrestris* extracts on human malignant melanoma, oral epidermoid carcinoma, breast and ovary carcinoma (Bedir et al., 2002 ; Hu & Yao, 2003; Sun et al., 2003). Many of the studies in these publications are based on *T. terrestris* from Chinese population. Data for antitumor activity of the Bulgarian plant are extremely limited. There is evidence for antiproliferative effect of *T. terrestris* saponins on mouse carcinoma lines (Ivanova et al., 2009). In addition, Neychev et al. (2007) found that saponin fraction from *T. terrestris* is less toxic to normal human fibroblasts.

The molecular mechanisms underlying the anticancer effect are associated with initiation of apoptosis or suppression of cancer cell proliferation. Apoptosis is a process of programmed cell death, which involves a variety of biochemical processes and irreversible changes in cell morphology, incompatible with the normal cell function. Morphological characteristics of apoptosis include plasma membrane destruction, cell compression, chromatin condensation, genomic DNA fragmentation. It was found that other saponins, structurally similar to diosgenin, present in *T. terrestris* extracts, may block cell cycle, suppress

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proliferation and induce apoptosis in human sarcoma cell lines (Trouillas et al., 2005).

In this study we investigated the antitumor effect of total extract and saponin fraction of Bulgarian *T. terrestris* through a comparative analysis of viability (MTT assay) of normal (MCF10A) and breast cancer cell line (MCF7) after treatment. Assessment of the apoptotic potential of total extract and saponin *T. terrestris* fraction was also done through analysis of genomic integrity (DNA fragmentation assay) and analysis of morphological cell changes (Fluorescence microscopy).

Materials and Methods

Preparation and purification of Tribulus terrestris extracts and fractions

Tribulus terrestris (TT) total extract and saponin fraction were prepared according to the methodology of Cai et al. (2001). The powder from the plant leaves was extracted three times with 70% ethanol. The extract was evaporated to dryness under vacuum at temperature below 50ºC, dissolved in water and extracted by aqueous butanol three times. The butanol extract was evaporated to dryness and was subjected to chromatography on D101 resin. The resin was subjected to ethanol gradient elution from 0% to 100%. Saponins containing fractions were evaporated to dryness and subjected to chromatography on silica gel using mixture of $CHCl_3$-MeOH-H_2O (50:10:1, v/v/v). The contents of saponins in the fraction selected for the experiments was determined to be more than 99% by photometric analysis conducted according to Gyulemetova et al. (1982). The final fraction was standardized on base of protodioscin contents by RP-HPLC, using commercially available protodioscin (ChromaDex, Inc., Santa Ana, CA) as an external standard according to Ganzera et al. (2001).

Cell lines and cell culturing

Breast cancer (MCF7) and normal (MCF10A) human cell lines were included in the study. MCF7 is isolated from pleural effusion of a patient with metastatic breast cancer. MCF10A cell line is an immortal non-tumorigenic breast epithelial cell line derived from a woman with fibrocystic breast disease. MCF10A cells possess the characteristics of normal breast epithelial cells and are not tumorigenic in immunosuppressed mice. The cells were supplied by the American Type Culture Collection (ATCC).

The MCF7 cells were cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% sodium pyruvate, while MCF10A cell line – in DMEM medium, with 5% FBS, 1% sodium pyruvate, 20 ng/ml human epidermal growth factor (hEGF), 10 µg/ml insulin and 0.05 mM hydrocortisone. The cells were incubated at 37ºC in a 5% CO_2. All procedures were performed under strict sterile conditions. Fabric sterile consumables and chemicals were used and the work surface was treated with 70% isopropanol.

MTT assay

Cell viability was determined through MTT assay. Indicative for antitumor activity is the IC_{50} value (half maximal inhibitory concentration) – the concentration of active compound needed to reduce the cell viability to 50%.

Cells were plated into 12-well tissue culture plates ($1x10^5$ per well) in a final volume of 1 ml and were incubated for 24 h in complete cell culture medium. For the next 24 h cells were starved in serum-free medium, supplemented with 0.1% BSA. Then they were treated with different concentrations of the tested substance (from 0.06 to 100 µg TT/ml medium) for 24 h using cultivating medium as a solvent. Wells with serum-free medium were used as negative controls. During the last 3 h of the incubation an aliquot of 100 µl MTT per well was added (a stock solution of 5 mg/ml MTT was used). After incubation, the medium was removed and the formazan complex was solubilized with 400 µl/well 10% SDS in 0.01M HCI. The absorbance was subsequently measured at 570 nm. The experiment was repeated three times and each concentration had three repeats.

DNA fragmentation analysis

$1x10^5$ cells per well were plated into 6-well culture plates. After treatment with TT total extract and saponin fraction for 24 h, 48 h and 72 h the cells were detached with 0.05% trypsin-0.53 mM EDTA, incubated for 15 min at 37ºC and collected. After centrifugation for 5 min at 125G the supernatant was discarded and 200 µl PBS pH 7.2 (50 mM KH_2PO_4, 150 mM NaCl) was added to the cell pellet. DNA was isolated as described by the manufacturers' protocol using DNeasy Blood & Tissue Kit (Qiagen). An aliquot of 2.5 µg genomic DNA was analysed on 1.5% agarose gel in 1xTBE, at 80 V and 50 mA for 1 h. The samples were visualized under UV after gel staining with ethidium bromide.

Fluorescence microscopy

Cancer cell line MCF7 was treated with total extract and saponins for 24 h, 48 h and 72 h. Treated MCF7 cells and untreated MCF7 controls were detached with 0.05% trypsin-

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0.53 mM EDTA, incubated for 15 min at 37ºC and harvested. After centrifugation for 5 min at 125G the resulting cell pellet was washed twice with PBS and stained with propidium iodide (PI) and anexin (Annexin-V-FLUOS Staining Kit, Roche). After dark incubation for 10 min at room temperature, the cells were analyzed under fluorescence microscope (Olympus BX-41).

The cells in early apoptosis were stained in green; those in late apoptosis had a red stained nuclear area and green stained membrane; the necrotic cells were stained in red; the cells which did not undergo apoptosis remained unstained. Two fluorescence microscopy filters were used: FITC (560-600nm) for cells in apoptosis and Texas Red (595-605 nm) for cells in necrosis.

Results

Cell viability analysis

The range of concentrations was selected according to previous data for an effect of saponin fraction from Bulgarian *T. terrestris* on viability of normal human fibroblasts (Neychev et al., 2007).

Untreated tumor and normal cells were used as controls and their viability was accepted as 100%. The results showed that extract from *T. terrestris* had a marked dose-dependent effect on viability of MCF7 cell line. In the range of lower concentrations (up to 2 µg TT/ml medium) the viability of tumor cells is comparable to this in the control and with the increase of concentration a steady decrease in cancer cells viability was observed (Figure 1). Fifty percents reduction of tumor cell viability was observed at a concentration of 15 µg TT/ml medium.

In respect to the normal cell line, at the lower concentrations (up to 8 µg TT/ml) a moderate reduction in cell viability (up to 84%) without dose-dependent effect was found. In the area of the inhibitory concentration (15 µg TT/ml medium) the normal cells viability significantly decreased, reaching a lowest value of about 70%. With the increase of concentration (over 20 µg TT/ml), however, a tendency of increased viability of MCF10A cells was registered. At a concentration of 90 µg TT/ml a strong selective effect on tumor cells viability was observed, where cancer cells viability is lowest (34%), at a maximal normal cells viability (88%).

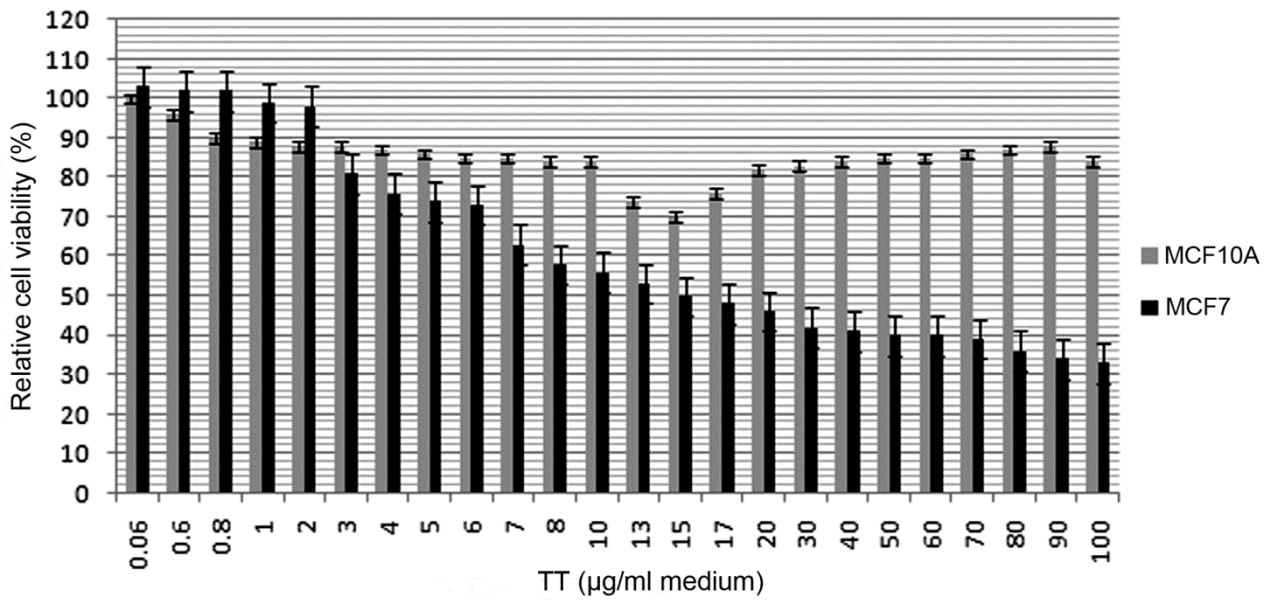

Figure 1. *MTT assay of normal MCF10A and cancer MCF7 cell lines treated for 24 h with increasing range of concentrations of total extract from Tribulus terrestris.*

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MTT assay of saponin fraction was conducted at the inhibitory concentration IC_{50} determined for the total extract. The results showed a slight decrease in tumor cells viability under treatment of saponin fraction in comparison with the total extract (43% towards 50%) (Figure 2). Upon treatment of normal cells both with total extract and saponin fraction, viability remained in the range of 70%.

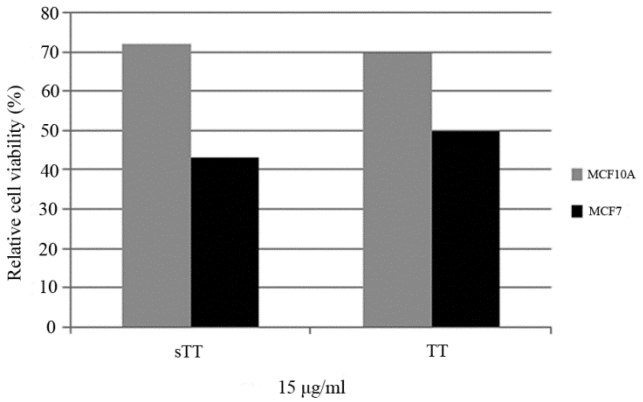

Figure 2. *MTT assay of normal MCF10A and breast cancer MCF7 lines treated for 24h with inhibitory concentration (15 µg/ml medium) of total extract (TT) and saponin fraction (sTT) from Tribulus terrestris.*

Analysis of apoptotic potential

To assess the apoptotic potential of total extract and saponin fraction of *Tribulus terrestris* the following approaches were applied: 1) analysis of genomic integrity (DNA fragmentation analysis) and 2) analysis of morphological changes in treated cells (fluorescence microscopy).

DNA fragmentation analysis

Cells from MCF10A and MCF7 lines were treated with 15 µg/ml of total extract and saponin fraction for 24 h, 48 h and 72 h (Figure 3).

The results showed presence of DNA fragmentation both in tumor and normal cells after 24 h treatment with total extract and saponin fraction. The detected level of fragmentation, however, was higher in tumor cells. Saponin fraction did not show a stronger effect on genomic integrity of tumor and normal cells when compared to the total extract. The results showed a slight increase in the level of DNA fragmentation in cancer cells with extention of duration treatment (48 h and 72 h) both with the total extract and the saponin fraction.

Fluorescence microscopy

Fluorescence microscopy analysis included treatment of tumor cells with total extract and saponin fraction, respectively, at a concentration of 15 µg/ml for 24 h, 48 h and 72 h, staining of treated cells with Annexin V and Propidium iodide and observation under fluorescent microscope. The results revealed presence of cellular morphological changes related to apoptotic processes in breast cancer cells, after treatment both with the total extract and the saponin fraction (data not shown). The observed morphological abnormalities were associated both with early and late apoptosis. A proportional increase in the number of tumor cells in apoptosis with the increase in time of treatment was detected. This association was found both under total extract and saponin fraction treatment (data not shown).

Discussion

The first antitumor drugs from plants with an application in cancer chemotherapy were developed five decades ago. A great achievement in this respect is the elaboration of drugs such as: vinblastine and vincristine (*Catharanthus roseus*), paclitaxel (*Taxus brevifolia*), silvestrol (*Aglaia foveolata*), eliptinium (*Bleekeria vitensis*) (Cragg & Newman, 2005), chrysin (*Passiflora incarnate*), artemisinin (*Artemisia annua*) (Newman & Cragg, 2007) and others. A large number of the medicinal products nowadays are of natural origin (Newman & Cragg, 2007). In this respect, medicinal plants are actively studied as a potential source of new active components with high antitumor activity.

The high steroid saponins levels in *T. terrestris* and the data on their cytotoxic activity towards a number of tumor cell lines presumes a high antitumor potential of this medicinal plant (Hu & Yao, 2001). So far, most thoroughly studied is the cytotoxic effect of the Chinese *T. terrestris* (Bedir & Khan, 2000; Bedir et al., 2002; Hu & Yao, 2003; Sun et al., 2003, 2004; Wang et al., 2009). The data on the antiproliferative activity of the Bulgarian herb are limited to the publications of Neychev et al. (2007) and Ivanova et al. (2009). Until now, there is no evidence of an antitumor effect of Bulgarian medicinal plant *T. terrestris* on human cancer cell lines. The data in the present study are the first in this respect and contribute to the assessment of the viability of breast cancer and normal cells treated with total extract and saponin fraction of *T. terrestris* from Bulgarian plant population.

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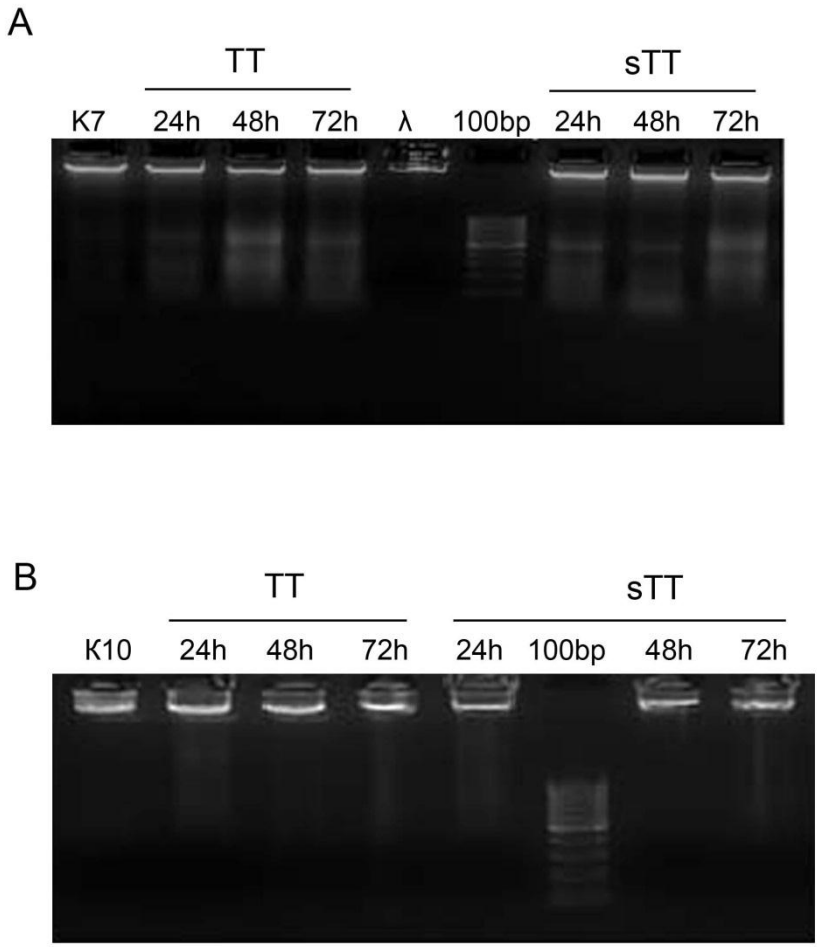

Figure 3. *DNA fragmentation analysis in 1.5% agarose gel electrophoresis:* ***A*** *- DNA from MCF7 cells treated with TT and sTT fraction (15µg/ml) for 24h, 48h, 72h. K7 – DNA from untreated tumor cells;* ***B*** *- MCF10A cells treated with TT and sTT fraction (15µg/ml) for 24h, 48h, 72h. K10 – DNA from untreated control cells.*

The obtained results outline some of the possible mechanisms underlying the antitumor effect.

The results of the performed MTT analysis showed that the total extract of *T. terrestris* has a pronounced dose-dependent inhibitory effect on viability of human breast cancer cells. With regard to the normal cell line, lower concentrations of *T. terrestris* moderately decreased cell viability without visible dose-dependent effect. With the increase of concentration, however, an increase in normal cells viability was found. The saponin fraction had stronger inhibitory effect on viability of breast cancer cells compared to the total extract. Similar to our results are the results of Bedir et al. (2002), Sun et al. (2003, 2004) and Su et al. (2009). The authors found decreased viability of several tumor cell lines including HL-60, Bcap-37, BEL-7402, SK-MEL, KB, BT-549 and SK-OV, treated with saponins and glucosides of the Chinese *T. terrestris.* The detected IC_{50} values vary widely between publications. The reason for this high variability is probably due to the different chemical nature and composition of active saponins in *T. terrestris* from different geographical areas, and also to differences in the extent and mechanism of their action. It is known that the Bulgarian herb is rich in active furostanol saponins, which are in smaller amount or are missing in the plants from Asia region (Dinchev et al., 2008). Of importance for the differences in cytotoxic activity is also the type of treated tumor cells. In this connection Hu & Yao (2001) found that from the 60 analyzed cell lines most sensitive to methyl

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protogracillin are the cell lines from leukemia, colon cancer and prostate cancer, while ovarian cancer and renal cancer cell lines are the least sensitive. In this analysis (Hu & Yao, 2001) MCF7 cell line is described as moderately sensitive in respect to tests with steroid saponins. Here we found that IC_{50} of total extract from Bulgarian *T. terrestris* is 15 μg/ml. Considering the data of Hu et al. for a moderate sensitivity of MCF7 and our MTT assay results, it can be assumed that the Bulgarian herb could have even stronger antitumor effect on cancer cell lines with higher sensitivity to steroid saponins.

Molecular mechanisms of action of biologically active substances derived from plants are associated with cytotoxicity, cytostaticity and/or with apoptosis. Apoptosis (programmed cell death) is initiated in response to damages in hereditary material and represents a series of genetically controlled events, resulting in elimination of damaged cells. The process is associated with activation of cellular endonucleases, which digest cellular DNA to well-differentiated fragments that can be visualized through gel electrophoresis (Arden & Betenbaugh, 2004). Our results showed induction of apoptosis after treatment with total *T. terrestris* extract or saponin fraction both in breast cancer and normal cells. However, the level of fragmentation in tumor cells was higher. This observation is in accordance with the data from the MTT assay, which showed lower viability of tumor cell line in comparison to the normal. By increasing the duration of treatment both with total extract and saponin fraction, DNA fragmentation level in tumor cells slightly increased. This is consistent with the observations that DNA fragmentation occurs in the later stages of the apoptosis (Johnson et al., 2000). Similar data for induction of apoptosis are also obtained in the analysis of the effect of Chinese herb *T. terrestris* on different types of cancer cell lines (Hu & Yao, 2003; Sun et al., 2003, 2004).

Nuclear alterations are the most common characteristics of the apoptotic process. Noticeable morphological changes in the nucleus are also chromatin condensation and nuclear fragmentation in later stages. The changes in the nucleus are clearly visible under light microscope with the use of DNA intercalating dyes. The conducted fluorescence microscopic analysis confirmed presence of morphological changes in treated with total extract and saponin fraction of *T. terrestris* breast cancer cells, which are associated with relocalization of the membrane receptors as an indication of early apoptosis. These changes were dependent on the duration of the treatment and their number increased with the increase in the period of exposure. The presence of fragmented nuclear and plasma membranes as indication of late apoptosis in breast cancer cells was also visible. Similar observations were found upon treatment of HL-60, HepG2 and K562 cell lines with the saponins dioscin (Wang Y et al., 2006) and methyl protodioscin (Wang G et al., 2006).

In conclusion, total extract of the Bulgarian *T. terrestris* has a marked dose-dependent inhibitory effect on viability of breast cancer cells. Saponin fraction has increased inhibitory effect compared to the total extract. In the mechanisms of antitumor activity of *T. terrestris* apoptotic processes are involved. Morphological changes and DNA fragmentation were observed as markers for early and late apoptosis in tumor cells after treatment. The obtained results are the first showing an antitumor activity of the Bulgarian medicinal plant *T. terrestris* in human cancer cells and give directions for future investigations concerning detailed assessment of the pharmacological potential of the herb.

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References

Antonio J, Uelmen J, Rodriguez R, Earnest C. 2000. The effects of *Tribulus terrestris* on body composition and exercise performance in resistance-trained males. Int. J. Sport Nutr. Exerc. Metab., 10(2): 208-215.

Arden N, Betenbaugh MJ. 2004. Life and death in mammalian cell culture: strategies for apoptosis inhibition. Trends Biotechnol., 22(4): 174-180.

Bedir E, Khan IA. 2000. New steroidal glycosides from the fruits of Tribulus Terrestris. J. Nat. Prod., 63(12): 1699-1701.

Bedir E, Khan IA, Walker LA. 2002. Biologically active steroidal glycosides from *Tribulus terrestris*. Pharmazie, 57(7): 491-493.

Cai L, Wu Y, Zhang J, Pei F, Xu Y, Xie S, Xu D. 2001. Steroidal saponins from *Tribulus terrestris*. Planta Med., 67(2): 196-198.

Chu S, Qu W, Pang X, Sunand B, Huang X. 2003. Effect of saponin from *Tribulus terrestris* on hyperlipidemia. Zhong Yao Cai, 26: 341-344.

Cragg GM, Newman DJ. 2005. Plants as source of anticancer agents. J. Ethnopharmacol., 100: 72-79.

De Combarieu E, Fuzzati N, Lovati M, Mercalli E. 2003. Furostanol saponins from *Tribulus terrestris*. Fitoterapia, 74(6): 583-591.

Dinchev D, Janda B, Evstatieva L, Oleszek W, Aslani MR, Kostova I. 2008. Distribution of steroidal saponins in *Tribulus terrestris* from different geographical regions. Phytochemistry, 69(1): 176-186.

Ivanova A, Serly J, Dinchev D, Ocsovszki I, Kostova I, Molnar J. 2009. Screening of some saponins and phenolic components of

RESEARCH ARTICLE

Tribulus terrestris and *Smilax excelsa* as MDR modulators. In Vivo, 23(4): 545-550.

Ganzera M, Bedir E, Khan IA. 2001. Determination of steroidal saponins in *Tribulus terrestris* by reversed-phase high-performance liquid chromatography and evaporative light scattering detection. J. Pharm. Sci., 90(11): 1752-1758.

Gyulemetova R, Tomova M, Simova M, Pangarova T, Peeva S. 1982. Determination of furostanol saponins in the preparation tribestan. Pharmazie, 37: 296.

Hu K, Yao X. 2001. Methyl protogracillin (NSC-698792): the spectrum of cytotoxicity against 60 human cancer cell lines in the National Cancer Institute's anticancer drug screen panel. Anticancer Drugs, 12(6): 541-547.

Hu K, Yao X. 2003. The cytotoxicity of methyl protodioscin against human cancer lines in vitro. Cancer Invest., 21(3): 389-393.

Johnson VL, Ko SC, Holmstrom TH, Eriksson JE, Chow SC. 2000. Effector caspases are dispensable for the early nuclear morphological changes during chemical-induced apoptosis. J. Cell Sci., 113(Pt 17): 2941-2953.

Kostova I, Dinchev D. 2005. Saponins in *Tribulus terrestris* - chemistry and bioactivity. Phytochemistry Reviews, 4: 111-137.

Neychev VK, Nicolova E, Zhelev N, Mitev VI. 2007. Soponins from *Tribulus terrestris* L. are less toxic for normal human fibroblasts than for many cancer lines: Influence on apoptosis and proliferation. Experimental Biology and Medicine, 232: 126-133.

Newman DJ, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 years. J. Nat. Prod., 70(3): 461-77.

Sengul M, Yildiz H, Gungor N, Cetin B, Eser Z, Ercisli S. 2009. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. Pak. J. Pharm. Sci., 22(1): 102-106.

Su L, Chen G, Feng SG, Wang W, Li ZF, Chen H, Liu YX, Pei YH. 2009. Steroidal saponins from *Tribulus terrestris*. Steroids, 74(4-5): 399-403.

Sun B, Qu W, Baiet Z. 2003. The inhibitory effect of saponins from *Tribulus terrestris* on Bcap-37 breast cancer line *in vitro*. Zhong Yao Cai, 26: 104-106.

Sun B, Qu WJ, Zhang XL. 2004. Ivestigation on inhibitory and apoptosis-inducing effects of saponins from *Tribulus terrestris* on hepatoma cell line BEL-7402. Zhongguo Zhong Yao Za Zhi, 29(7): 681-684.

Taraphdar AK, Madhumita R, Bhattacharya RK. 2001. Natural products as inducers of apoptosis: implication for cancer therapy and prevention. Current Science, 80(11): 1387-1396.

Trouillas P, Corbière C, Liagre B, Duroux JL, Beneytout JL. 2005. Structure-function relationship for saponin effects on cell cycle arrest and apoptosis in the human 1547 osteosarcoma cells: a molecular modelling approach of natural molecules structurally close to diosgenin. Bioorg. Med. Chem., 13(4): 1141-1149.

Wang Y, Cheung YH, Yang Z, Chiu JF, Che CM, He QY. 2006. Proteomic approach to study the cytotoxicity of dioscin (saponin). Proteomics, 6: 2422-2432.

Wang G, Chen H, Huang M, Wang N, Zhang J, Zhang Y, Bai G, Fong WF, Yang M, Yao X. 2006. Methyl protodioscin induces G2/M cell cycle arrest and apoptosis in HepG2 liver cancer cells. Cancer Lett., 241(1): 102-109.

Wang J, Zu X, Jiang Y. 2009. Five furostanol saponins from fruits of *Tribulus terrestris* and their cytotoxic activities. Nat. Prod. Res., 23(15): 1436-1444.