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In vitro antitumor potential of Bulgarian *Tanacetum vulgare* L. on human breast adenocarcinoma cells

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ABSTRACT

In recent decades the search for new oncotherapeutic agents of natural origin with selective antitumor activity is of significant priority for cancer treatment. The antitumor potential of the medicinal plant Tanacetum vulgare L. *is limitedly studied and no data are so far available concerning the Bulgarian plant. The objective of the present study was to evaluate the in vitro antitumor potential of crude aqueous ethanolic extract of Bulgarian Tanacetum vulgare L. Anticancer effect was investigated by MTT cell viability assay on human breast cancer cell line MCF7. Cell morphological characteristics were examined under light microscope. The analysis showed that plant extract exposure decreased considerably cancer cell viability in dose- and time dependent manner. The 50% inhibitory concentration was determined to be 286.8 µg/ml. Morphological alterations were found in treated tumor cells compared to untreated control cells. The here-presented results are the first demonstrating antitumor properties of the Bulgarian Tanacetum vulgare* L. *and are indicative for further more detailed investigations on the active compounds and molecular mechanisms underlying the antitumor activity.*

Keywords: *Tanacetum vulgare* L.; Breast cancer cell line; Cell viability; Antitumor potential

INTRODUCTION

Cancer is a socially significant disease, a leading cause of mortality worldwide and a reason for 8.2 million deaths in 2012 [1]. The newly diagnosed cases amount to 14.1 million as it is expected new cancer cases per year to reach 19.3 million by 2025. Cancer process includes uncontrollable division of abnormal cells, which possess the ability to invade other tissues and in some cases to metastasize. Conventional therapy covers surgical operation, radiation therapy, hormonal- and chemo-therapy. Tumor cells resistance to chemotherapeutic agents and adverse side effects of radiation- and chemo-therapy are main problems in cancer clinical treatment. Therefore, the elaboration of alternative medicines with natural origin, which selectively eliminate cancer cells without affecting the normal tissues, is of essential importance. In the recent years, medicinal plants are one of the most promising sources for development of novel therapeutics for various diseases, including cancer. Bulgarian flora encompasses considerable biodiversity of about 600 medicinal plant species, which are widely used in traditional phytotherapy [2] and are potentially a source of anticancer compounds.

Tanacetum vulgare L. (Asteraceae), also known as Tansy, is a perennial herbaceous plant. It is native to Europe and Asia and is also introduced to the United States. In Bulgaria the plant grows in all phytogeographical areas from 0 to 2000 m asl. Since ages, the herb has been widely used in traditional medicine for treating rheumatism, fever, gout, epilepsy, digestive disorders, helminthes and others. *Tanacetum vulgare* L. is toxic and its consumption may cause convulsions and even death. The intake of this plant should be done only on medical doctor's prescription. At present, some pharmacopoeias have described the use of Tansy in some medicines for treatment of colds and fever [3]. Plant extracts and isolated compounds possess variety of biological activities including antimicrobial [4], anti-inflammatory [5], immunomodulatory [6], antioxidant [7], antiviral [8] and antitumor [9]. The aqueous extract of the herb exerts vasorelaxing effect *in vitro* [10].

Drug active compounds mainly comprise of flavonoids, essential oils, bitter compounds, alkaloids and tannins. In respect to the chemical constituents, significant

intra-specific variability between some *T. vulgare* populations and subspecies is observed due to plant adaptation to environmental conditions.

In regard to the antitumor properties of the herb, some studies on human cancer cell lines from breast, colon, lung and acute T leukemia indicate *in vitro* cytotoxic activity of *T. vulgare* L. extracts [11, 12]. Extract of *T. vulgare* also inhibited mouse leukemia L1210 cells [13]. Members of a major group of active constituents of essential oils, which are associated with the vast spectrum of herb activities, called STLs (sesquiterpene lactones), are able to induce *in vitro* cytotoxic effect on human lung carcinoma epithelial-like (A549) and Chinese hamster lung fibroblast-like (V79379A) cells [14]. A publication reported that eupatorin, which belongs to another class of second metabolites with profound anticancer properties – flavonoids, and which is found in a number of medicinal plants, including *Tanacetum vulgare*, possesses selective antiproliferative activity against human breast carcinoma cell line MDA-MB-468 but minor effect on normal breast cells [15]. Its cell growth inhibitory effect is attributed to CYP1 family-mediated metabolism.

Based on the above-mentioned data the current study was designed to investigate the *in vitro* antitumor potential of crude extract of *Tanacetum vulgare* L. from Bulgarian plant population. To our knowledge no data is so far available concerning the antitumor activity of the Bulgarian herb. The antitumor effect was determined through analysis of viability of breast adenocarcinoma cell line MCF7 after extract exposure. Morphological changes of treated cancer cells were observed.

MATERIALS AND METHODS

Plant extraction

Tanacetum vulgare L. aqueous ethanolic extract was provided by Vemo 99 Ltd. The powder from the plant overground parts was extracted three times with 50% 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%.

Cell line and cultivation

MCF7 is one of the most frequently used human breast cancer cell lines. It is isolated from pleural effusion of a 69-year old Caucasian woman with metastatic breast cancer. The cell line was supplied by the American Type Culture Collection (ATCC).

Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate and 1% MEM Non-Essential Amino Acids. The cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2. The cell line was kept free from fungal or bacterial contamination.

Trypan blue exclusion assay

Trypan blue exclusion assay was carried out to calculate percentage of MCF7 cell line viability. An aliquot of 50µl cell suspension was mixed with an equal volume of 0.4% trypan blue solution. Viable (unstained) and nonviable (dark blue stained) cells were counted under inverted light microscope. Percentage viability was calculated by the formula:

Viability (%) = (Live cell count/Total cell count) × 100

MTT assay

Cell viability was evaluated through MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) assay. Cells were seeded into 12-well tissue culture plates (1×10^5 per well) in a final volume of 1 ml. After incubation 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. Subsequently cells were treated with different final concentrations of tested extract (200, 250, 300, 350, 400, 500 and 600 µg/ml medium) for 24 h using cultivating medium as a solvent. Cell samples 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 (stock solution of 5 mg/ml MTT). After incubation, the medium was removed and the formazan complex was dissolved with 400 µl/well 10% SDS in 0.01M HCI. The absorbance was subsequently measured at 570 nm using ELISA microplate reader. The MTT experiments were repeated at least twice and each concentration had three repeats.

The percentage of cell viability after extract exposure of the above mentioned concentrations was determined using the following formula:

Viability (%) = (Experiment value/Control value) × 100

MTT analysis was also applied to establishment of cell viability alteration with time after treatment with IC_{50} (half maximal inhibitory concentration) for 24, 48 and 72 h.

Morphological observation of MCF7 cells

MCF7 cells were plated into 12-well plates, cultivated and treated for 24, 48 and 72 h with the same concentrations, as described above. Morphological changes were observed using inverted light microscope. The morphological observation analysis was synchronized with MTT cell viability assay.

Statistical analysis

The data were presented as means ± standard deviation (SD) of at least two separate experiments performed in triplicate. IC_{50} value was calculated using GraphPad Prism 5 software. Statistical differences between control and treated groups were evaluated

using one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test and paired T test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cell viability

Percentage cell line viability was assessed using Trypan blue exclusion test. The results showed that the percentage viability of MCF7 cell line was 94.08 ± 2.41% (SD), which is suitable to perform the following analysis.

The antitumor effect of *Tanacetum vulgate* L. crude extract was examined through MTT cell viability assay. Untreated cells were used as controls. The screening was performed at multiple concentrations ranging from 200 to 600 µg/ml. The results showed that after treatment the cell viability was reduced in dose dependent manner (Fig. 1A, 1B). Viability decreased from 72.43% (at 200 µg/ml) and reached a minimum value of 19.39% (at 600 µg/ml). The IC_{50} value was found to be 286.8 µg/ml. IC_{50} is the concentration that inhibits cell viability to 50%. In comparison, VSPK Sankara Aditya et al. [16] reported 85.4% of MCF7 cell inhibition (i.e. 14.6% cell viability) upon tamoxifen treatment in concentration of 200 µg/ml. Our further experiments involving incubation of tumor cells with *Tanacetum vulgate* L. extract with IC_{50} equivalent concentration for 24, 48 and 72 h revealed time dependent manner of cell viability reduction. It reached 41.07% at the 48th h and 27.39% - at the 72th (Fig. 1C).

The statistical analysis according to GraphPad Prism 5 indicated considerable significant differences between control and treated groups with p-values of less than 0.05.

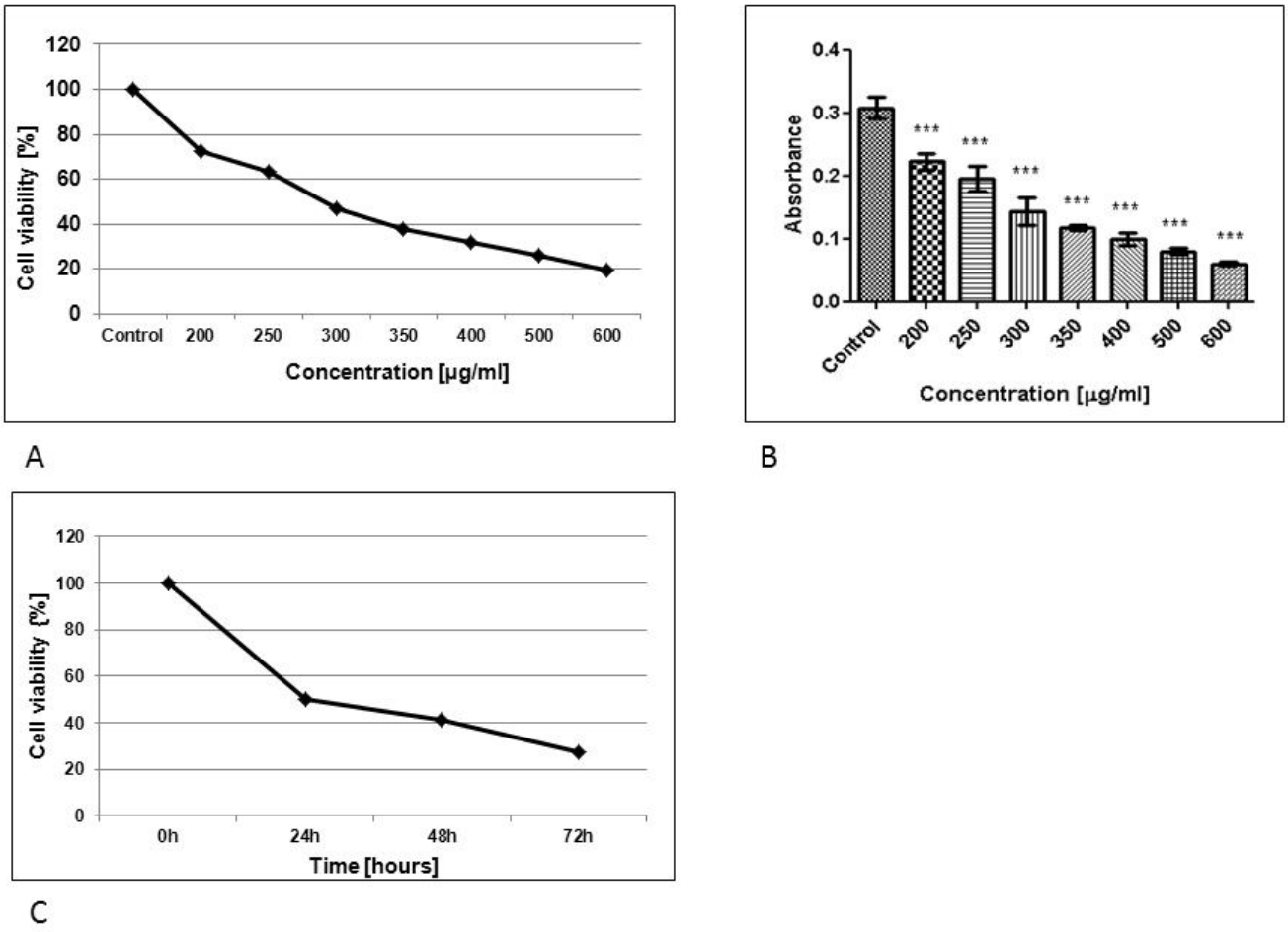

Figure 1. A and B - Effect of *Tanacetum vulgate* L. crude extract on MCF7 cell viability assessed by MTT assay for 24 h with increasing range of concentrations,*** Indicates significant difference from the control group by Dunnett's test ($p < 0.0001$), Error bars represent standard deviation. C - Effect after IC_{50} treatment for 24, 48 and 72 h; $p < 0.05$

Morphological alterations

Morphological changes of treated breast cancer cells were observed under inverted light microscope. Untreated control cells exhibited normal shape, whereas extract treated cells became round and shrank (Fig. 2). Evident reduction in the number of viable cells which were monolayer adherent and increased number of floating dead or dying cells after treatment with concentrations above 250 µg/ml were observed. The number of tumor cells detached from the monolayer arose with the increase in extract concentration.

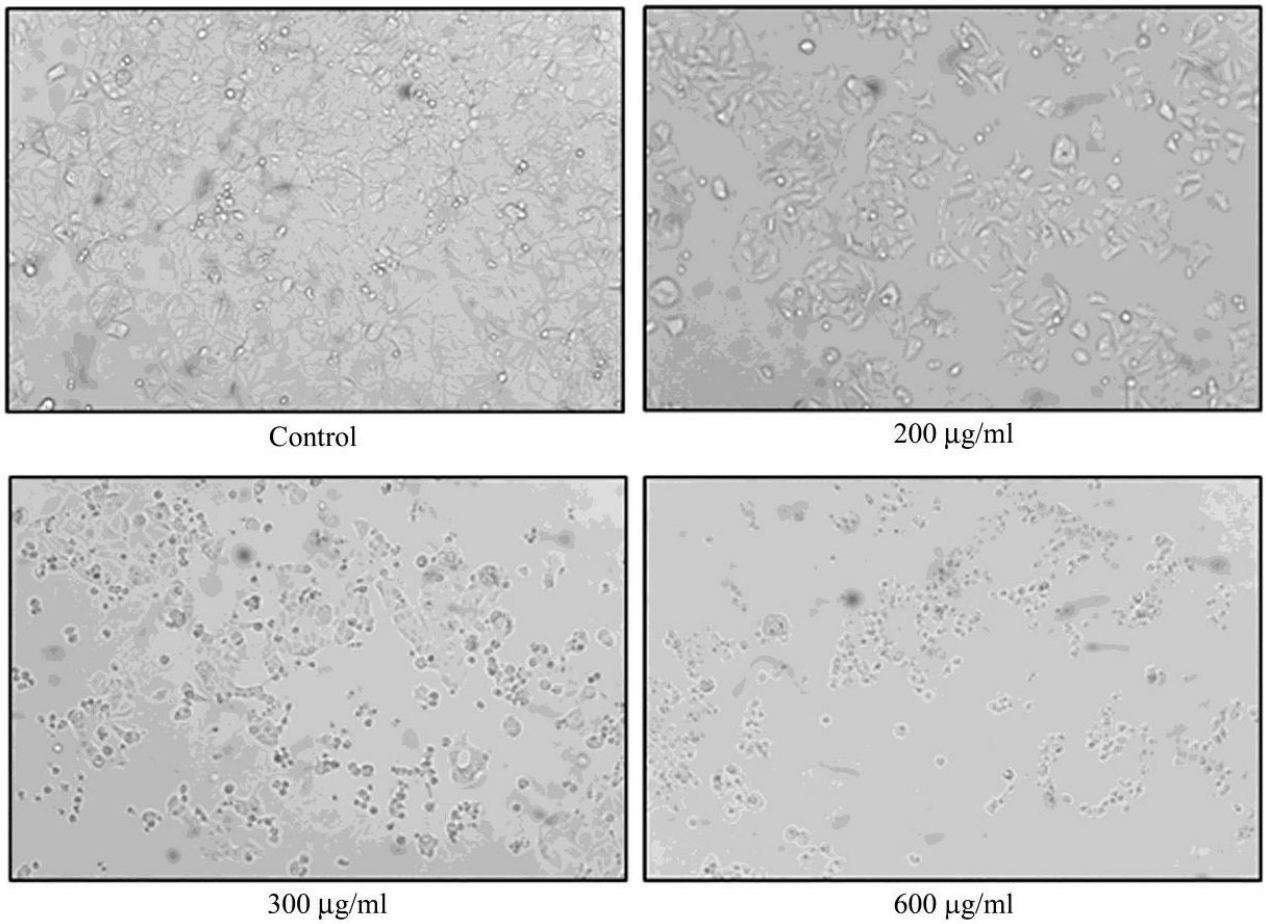

Figure 2. Morphological changes of MCF7 cells after treatment for 24 h with 200 µg/ml, 300 µg/ml, 600 µg/ml of *Tanacetum vulgate* L. extract compared to untreated control.

Similar tendency in morphological alterations in tumor cells was observed after IC_{50} incubation with time (24, 48, 72h).

At present, the available data on the antitumor effect of *T. vulgare* extracts are limited to a few studies. Wegiera et al. [12] reported *in vitro* cytotoxic properties of ethanol extracts from *T. vulgare* on J-45.01 human acute T leukemia cell line. The IC_{50} values the authors found were comparable to the here-found and were in the range of 360 µg/ml (for the herb), 200 µg/ml (inflorescents) and 300 µg/ml (roots). Considerably higher *in vitro* cytotoxicity in colon (WiDr), breast (MDA-MB-231) and lung (NCI-417) cancer cell lines exhibited chloroform extracts of *Tanacetum vulgare* L. aerial parts (IC_{50} from 2.4 to 9.1µg/ml) [11]. Wu et al. [17] investigated the inhibitory activity of an extract from another member of *Tanacetum* genus - *Tanacetum parthenium* (feverfew) against two human breast cancer cell lines (Hs605T and MCF-7) and one human cervical cancer cell line (SiHa). EC_{50} (half-effective concentration) values were 1.5 mg/ml against Hs605T, 2.1 mg/ml against MCF-7, and 0.6 mg/ml against SiHa, which is considerably higher compared to here-found.

The variations in IC_{50} values between publications are probably due both to the different tumor cell lines sensitivity towards the tested agent as well as the intra-genus and intra-species diversity in chemical composition of plants from different geographical areas, due to plant adaptation to specific environmental surroundings.

CONCLUSION

In view of the obtained results it can be concluded that crude aqueous ethanolic extract of Bulgarian *Tanacetum vulgare* L. has a marked dose- and time dependent *in vitro* inhibitory effect on viability of breast cancer cells. The here-reported results are initial indicating for further more detailed investigations. Future studies might be directed to screening for the active compounds to which the antitumor activity could be attributed, as well as the possible mechanisms underlying the antitumor effect, which may include inhibition of proliferation, apoptosis of cancer cells and others.

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