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CICHORIUM INTYBUS L. FROM BULGARIA INHIBITS VIABILITY OF HUMAN BREAST CANCER CELLS *IN VITRO*

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Summary: Medicinal plants are considered as a promising source of biologically active compounds with valuable properties for cancer prevention and treatment. *Cichorium intybus* L. has wide applications in traditional folk medicine and a broad variety of biological activities. To our knowledge, no data are hitherto available in regard to the antitumor properties of the Bulgarian herb. The objective of the present study was to investigate the *in vitro* inhibitory effect of the crude aqueous ethanolic extract of Bulgarian *Cichorium intybus* L. on the viability of human breast cancer cell line MCF7 and normal mammary epithelial cell line MCF10A. The assessment of the antitumor potential was based on MTT cell viability assay in the concentration range of 30-600 μg/ml and examination of cell morphology alterations by light microscopy after extract exposure. The obtained results indicated that the extract possessed a selective inhibitory effect as seen by the predominant decrease in cancer cell viability compared to normal cells (17% at the highest concentration for MCF7 cells and 80% for MCF10A). The 50% inhibitory concentration of the cancer cell line was determined to be 429 μg/ml. A slight time-dependent decrease in tumor cells viability was found. Changes in cancer cells morphology were observed after treatment with the highest extract concentrations, whereas for normal cells, no alterations in cell shape and size were detected.

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Keywords: Antitumor activity; Cell viability; *Cichorium intybus* L.; MCF7; MCF10A.

Abbreviations: DMEM – Dulbecco's Modified Eagle Medium; NEAA – Non-Essential Amino Acids; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS – fetal bovine serum; IC_{50} – half maximal inhibitory concentration.

INTRODUCTION

Plants possess unlimited potential to provide novel agents for prevention and therapy of various diseases regardless of the great importance of the synthetic chemistry for drug discovery. About 10 000 to 15 000 of the worldwide plant species have documented medicinal application and 150-200 are already used in western medicine (McChesney et al., 2007). Plant-derived antineoplastic agents

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like vinblastine, vincristine, paclitaxel, etoposide, topotecan, irinotecan and others are successfully applied at present in the oncology practice for treatment of some cancer types. New agents, flavopiridol and combretastin A4 phosphate, are in clinical development in view of their selective anticancer activity (Cragg and Newman, 2005). However, the number of plants and secondary metabolites, which are chemically characterized and thoroughly examined in a broad panel of models, remains deficient.

Phytochemical properties of plants from different populations of one species could vary considerably in different habitats, depending on environmental factors like soil type, temperature, rainfall quantity, altitude. Bulgarian flora is characterized by vast biodiversity of over 700 medicinal plants species, containing high percentage of active ingredients, which represent 20% of the flora in the country (Evstatieva et al., 2007).

Cichorium intybus L., commonly known as chicory, blue daisy and others, is a woody, perennial herbaceous plant from Asteraceae family, which in Bulgaria occurs from 0 to 1500 m asl. It has natural distribution in Mediterranean region, Middle Asia and North Africa. At present, the plant is cultivated in Europe and North America for food, animal forage, as a coffee substituent and additive, source of inulin, for ethanol production and others.

Phytochemical studies show that a large number of active compounds are present in *C. intybus*, including the flavonoids kaempferol, quercetin, lutedin and epigenins (Heimler et al., 2009), sesquiterpene lactones, vitamins, coumarins (Varotto et al., 2000). The chemical composition also includes alkaloids, carbohydrates (inulin), tannins, triterpenoids, minerals and fatty acids.

The plant has a significant medical importance in Eurasia and parts of Africa. Currently, only one herbal medicinal product containing chicory as a single ingredient is registered in the European Union, representing herbal tea of roots, used against lack of appetite and digestive disorders (European Medicines Agency, 2013). In folk medicine, the plant juice is used against uterine cancer (Judžentienė and Būdienė, 2008). The flowers of chicory are applied as a tonic stimulant and for treatment of gallstones, sinus problems, gastroenteritis, cuts and bruises. Roots and leaves of the plant are used also as diuretic, cardio protective, anti-rheumatic and anti-diabetic agents (Pushparaj et. al., 2007). Antibacterial (Nandagopal and Kumari, 2007), antimalarial (Bischoff et al., 2004), anti-inflammatory (Cavin et al., 2005), antioxidant (Nikolova et al., 2011) and antiallergic activity (Kim et al., 1999) have also been detected.

In regard to the antitumor properties of the plant, data report antiproliferative and cytotoxic activity of n-hexane extract from overground parts of chicory on Jurkat cells (lymphoblastic leukemia) (Saleem et al., 2014). Nawab et al. (2011) found that the aqueous extract of seeds from *C. intybus* exhibited a moderate inhibitory effect on the viability of human breast cancer (T47D), prostate cancer (PC-3) and colon cancer (RKO) cell lines. It was also established that the crude ethanolic extract from roots of the herb significantly inhibited Ehrlich tumor carcinoma in mice (Hazra et al., 2002). Lee et al. (2000) reported that 1β-hydroxyeudesmanolide - magnolialide, isolated from roots of *C. intybus*, inhibited growth of tumor

cell lines and induced differentiation of leukemia HL-60 and U-937 cells to monocyte or macrophage-like cells. Investigations in experimental animal models indicated that inulin-type fructans, extracted from roots of chicory, possessed anticarcinogenic properties and in human tumor cells inulin-derived fermentation products inhibited cell growth, modulated differentiation and decreased metastasis activity (Pool-Zobel, 2005).

Considering the above-mentioned data, the present study was undertaken to examine the *in vitro* antitumor activity of the crude extract of the Bulgarian medicinal plant *Cichorium intybus* L. on a human breast cancer cell line and to compare the effect of the extract on viability of a non-tumorigenic cell line. To our knowledge, the antitumor potential of this Bulgarian herb has not yet been studied.

MATERIALS AND METHODS

Plant extract

Cichorium intybus L. aqueous ethanolic extract was provided by Vemo 99 Ltd (Bulgaria). The powder from the overground parts of the plant was extracted three times with 50% ethanol. The extract was evaporated to dryness under vacuum at a temperature below 50ºC, dissolved in water and extracted by aqueous butanol three times. The butanol extract was evaporated to dryness and subjected to chromatography on ion-exchange D101 resin. The resin was subjected to ethanol gradient elution from 0% to 100%.

The active substances in the plant extract included coumarins, determined as esculin (from 9.0 to 11.0%); flavonoids, determined as apigenin (from 7.0 to 8.5%); tannic compounds, determined as tanin (from 4.5 to 5.5%) (http://www.vemo-vsv.com/products/herbal-extracts/cichorium-intybus).

Cell lines and culture conditions

Two human cell lines were included in the study: tumor (MCF7) and normal (MCF10A). MCF7 is one of the most frequently used for *in vitro* studies human breast cancer cell lines. It is isolated from pleural effusion of a patient with metastatic breast cancer. Phenotypically it represents luminal epithelium. MCF10A is an immortal non-tumorigenic breast epithelial cell line. It arose spontaneously from the mortal MCF10, which derived from a woman with fibrocystic breast disease and possesses the characteristics of normal breast epithelial cells. The cell lines were supplied by the American Type Culture Collection (ATCC).

MCF7 cells were cultured in DMEM medium supplemented with 10% FBS, 1% sodium pyruvate and 1% MEM NEAA, while for the cultivation of MCF10A cell line we used DMEM medium with 10% FBS, 1% sodium pyruvate, 1% MEM NEAA, 20 ng/ml human epidermal growth factor (hEGF), 10 μg/ml insulin and 0.05 mM hydrocortisone. The cells were incubated at 37ºC in humidified atmosphere with 5% CO_2.

MTT assay

The effect of *Cichorium intybus* L. extract on tumor and normal cells viability was evaluated through the MTT assay. The method is colorimetric and is based on the ability of mitochondrial dehydrogenases of viable cells to reduce yellow tetrazolium salt MTT to blue formazan in quantities proportional to the number of living cells (Mosmann, 1983).

Cells were seeded into 12-well plates (1×10^5 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 (bovine serum albumin) and then cells were treated for 24 h with different final concentrations of the tested extract: 30, 50, 70, 90, 110, 130, 150, 200, 250, 350, 400, 450, 500, 600 μg/ml for MCF7 cell line and 30, 110, 200, 250, 400, 450, 600 μg/ml for MCF10A. 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 in 400 μl/well 10% SDS, 0.01M HCI. The absorbance was measured at 570 nm. The percentage of cell viability after extract exposure to the above-mentioned concentrations was determined using the following formula:

$$\text{Viability (\%)} = (\text{Experiment value}/\text{Control value}) \times 100$$

The MTT assay was also applied for assessment of cell viability alteration with the increase of time after treatment with the IC_{50} concentration for 24, 48 and 72 h.

Morphological observation of MCF7 and MCF10A cells

The morphological observation analysis of tumor and normal cell lines after treatment for 24, 48 and 72 h with the above-mentioned concentrations of *Cichorium intybus* L. extract was synchronized with MTT cell viability assay. Morphological changes were observed under an inverted light microscope.

Statistical analysis

The data are presented as means ± standard deviation (SD) of at least two separate experiments, each performed in triplicate. Statistical differences between control and treated groups were evaluated using one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test. Values of $p<0.05$ were considered statistically significant. The IC_{50} value was calculated using GraphPad Prism 5 software.

RESULTS

The screening for antitumor properties of the crude extract from *Cichorium intybus* L. was performed through MTT cell viability assay on breast cancer cell line MCF7 after treatment for 24 h with concentrations in the range of 30 – 600 μg/ml. The results showed a slight inhibition of tumor cells viability at low and medium concentrations while at the higher concentrations (above 350 μg/ml) viability decreased substantially, reaching a minimum value of 17% at 600 μg/ml (Fig. 1). The IC_{50} value was determined to be 429 μg/ml.

Regarding the viability of MCF10A normal cells after treatment, the herb extract demonstrated a weak stimulating effect on cell viability at almost all applied concentrations without dose dependency (Fig. 1). In concentration ranges between 400 μg/ml and 450 μg/ml, including the IC_{50} value of the cancer cell line, normal cells viability was a little above 100%. Only at the highest concentration (600 μg/ml) *C. intybus* extract decreased normal cells viability to 80%. The comparison of the results obtained by the MTT cell viability assay

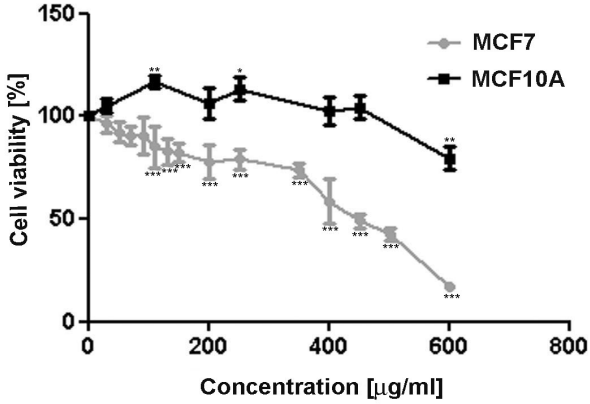

Figure 1. MTT assay of MCF7 and MCF10A cells for 24 h with increasing concentrations of *Cichorium intybus* L. extract. Error bars represent standard deviation. *, ** and *** indicate significant differences from the control group by Dunnett's test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

for tumor and non-tumorigenic cell lines showed a considerable selectivity of the extract inhibitory effect.

Cell morphological alterations after treatment with the extract were studied by light microscopy in parallel with the MTT assay. For the tumor cells, a trend of rounding and shrinkage, a reduction in the number of adherent viable cells and an increase in the number of floating dead cells were observed at a concentration of 400 μg/ml and above (Fig. 2). These

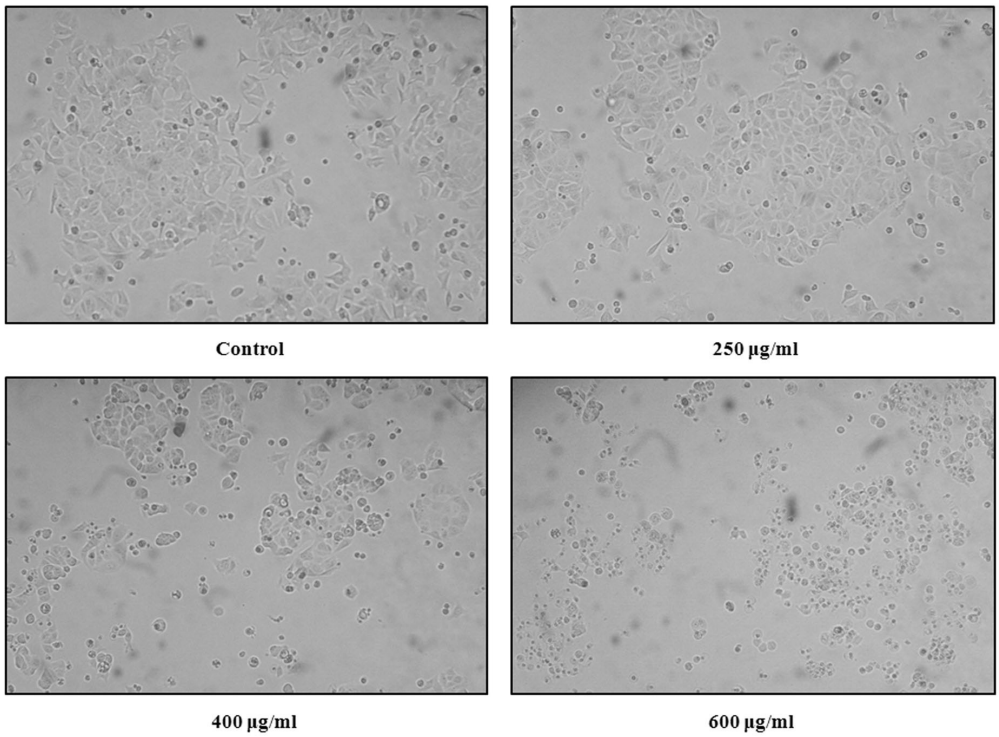

Figure 2. Morphological changes of MCF7 cells after treatment for 24 h with 250, 400 and 600 μg/ml of *Cichorium intybus* L. extract compared to untreated control.

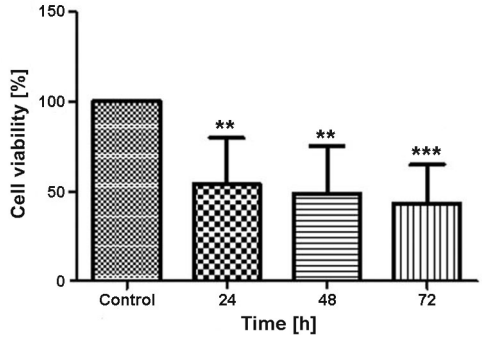

Figure 3. MTT assay of MCF7 cells for 24, 48 and 72 h with IC_{50} of *Cichorium intybus* L. extract. Error bars represent standard deviation. ** and *** indicate significant differences from the control group by Dunnett's test (** $p<0.01$, *** $p<0.001$).

results corresponded to the data from the MTT assay. Light microscopy analysis did not detect changes in the morphology of treated normal cells in comparison to untreated cells at any of the applied concentrations. Only a slight reduction of the cell monolayer density was observed (data not shown).

The subsequent experiments involving exposure of MCF7 cells on extract effect at the IC_{50} concentration for 24, 48 and 72 h demonstrated a slight time-dependent decrease in the tumor cells viability with increasing duration of treatment. Cell viability was reduced weakly to 49% and 43% at 48 h and 72 h, respectively (Fig. 3).

DISCUSSION

Herbal medicinal products are part of the alternative cancer therapies, which are expected to reduce tumor burden in cancer patients for whom no efficient standard anticancer therapy is available. The combined use of alternative medicines with conventional therapeutic drugs may reduce the negative side effects caused by chemotherapy (Goldstein, 2003), improve the effectiveness of oncotherapeutics and increase the strength of the immune system against cancer.

Cichorium intybus L. is a widely used in folk medicine plant with a vast pharmacological potential due to its valuable biological activities. To our knowledge, the present study reports the first data on the antitumor effect of the Bulgarian *C. intybus*. The investigations were carried out on breast adenocarcinoma cell line MCF7 and non-tumorigenic breast epithelial cell line MCF10A using the MTT cell viability assay at a wide range of extract concentrations. The analysis registered a slight influence of *C. intybus* extract activity on MCF7 cells at low and medium concentrations and a considerable increase of the inhibitory effect at the higher doses. The established inhibitory effect did not show a stable dose dependency. The IC_{50} value of the herb extract was calculated to be 429 μg/ml. A weak time-dependent reduction of tumor cells viability was detected.

Studies concerning normal mammary epithelial cell viability demonstrated a minor inhibitory effect only at the highest dose thus indicating marked selectivity of the extract activity.

Recent *in vitro* investigations of Saleem et al. (2014) with n-hexane extract of chicory from Pakistan population detected that after treatment of lymphoblastic leukemia cell line with doses from 10 to 100 μg/ml, the extract reduced significantly the number of viable cells to 50.3%. A study on the effect of an aqueous extract from seeds of Indian *C. intybus* on the viability of human cancer cell lines T47D, RKO and PC-3 showed a modest decrease after 24 h treatment with concentrations 1 - 10% (inhibition in ranges 2-21% for T47D; 6-26% for RKO; 2-30% for PC-3) (Nawab et al., 2011). The IC_{50} value detected in our study takes an intermediate position when compared to the limited available literature data, regarding *in vitro* antitumor effect of crude *C. intybus* extracts. The different values for the inhibitory concentration 50% among publications can be explained by the variations in sensitivity of different cell lines as well as by the distinctions in chemical composition of plants from different geographical regions.

Considering the results presented here, it can be concluded that the crude aqueous ethanolic extract of the medicinal plant *Cichorium intybus* L. from Bulgaria possesses antitumor properties as indicated by its *in vitro* inhibitory effect on breast cancer cell viability. The extract exhibits high selectivity as assumed by the considerably lower inhibitory effect on the viability of the non-tumorigenic breast epithelial cell line.

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