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COTINUS COGGYGRIA SCOP. LEAF EXTRACT EXERTS HIGH BUT NOT DOSE- AND TIME-DEPENDENT IN VITRO CYTOTOXIC ACTIVITY ON HUMAN BREAST CANCER CELLS

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Summary: Plants are among the major sources of biologically active substances possessing remarkable therapeutic values for treatment of various diseases, including cancer. Bulgarian flora includes about 770 medicinal plants species, 200 of which are sources for herbal drugs with application in medicine, cosmetics and food industry. Cotinus coggygria Scop. is a medicinal plant widely used in traditional phytotherapy with a variety of valuable biological properties. Currently, the plant is an object of intensive studies for assessment of its pharmacological capacity against different diseases. The objective of the present research was to evaluate the antitumor cytotoxic potential of *Cotinus coggygria* leaf aqueous ethanolic extract against human breast cancer cell line MCF7. The investigation of cancer cell viability inhibitory effect was performed by the MTT assay after treatment for 24 h with the extract at concentrations ranging from 5 μ g/ml to 400 μ g/ml. Light microscopy observations were performed to explore changes in cell morphology upon treatment. The obtained results revealed significant though not dosedependent cytotoxic activity of the extract with a detected value for the half maximal inhibitory concentration (IC₅₀) of 40.6 μ g/ml. The kinetics of the antitumor activity was followed after extract exposure for 24, 48 and 72 h with the IC_{50} concentration. The inhibitory effect decreased with time showing no linear time-dependency. In the present study, we report novel data on the in vitro antitumor cytotoxic activity of the Bulgarian herb Cotinus coggygria delegating it high antineoplastic potential.

Keywords: Breast cancer; Cotinus coggygria Scop.; cytotoxic activity; MCF7; medicinal plant.

Abbreviations: FBS – fetal bovine serum; IC_{50} – half maximal inhibitory concentration; MTT -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SEM - standard error of themean.

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INTRODUCTION

Cancer is a major medico-social problem with a complex heterogeneous nature. Breast cancer is the most frequent cancer type in women with 1.7 million

new cancer cases (25.1% of all cancer types) for 2012 and 6.3 million women who had been diagnosed with the disease in the previous five years (Ferlay et al.,

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2013). It is also the most common cause of cancer death among women (522 000 cases representing 14.7% of all cases of lethality). Despite the advances, achieved in the chemotherapeutic practice over the last decades, the conventional cancer therapy is still facing substantial obstacles. The search for prospective candidates for development of new pharmaceutical oncotherapeutics with cytotoxic activity among the medicinal plants, used in traditional phytotherapy due to their beneficial properties, is a widespread tendency all over the world.

Cotinus coggygria Scop. (Anacardiaceae), also known with local names "smradlika" or "tetra", is a medicinal plant commonly used in Bulgaria with a wide application not only in folk herbal medicine but also as a health supplement, in perfumery, cosmetic and medicinal products. The species is а flowering, deciduous shrub or a small tree, native from southern Europe to central China and introduced to North America. C. coggygria possesses a broad range of biological and pharmacological properties such as antimicrobial, antifungal (Matić et al., 2011), antiviral (Jing et al., 2012), immunomodulatory (Bilen et al., 2013), anti-inflammatory (Marčetić et al., 2013), antioxidant (Gospodinova at al., 2017), hepatoprotective (Pavlov et al., 2013a), wound-healing (Demirci et al., 2003), gastroprotective (Pavlov et al., 2013b), antipyretic (Huang, 1999) and others. External uses of the herb are mainly reported and although some authors consider it poisonous due to a large amount of gallotannins (Landzhev, 2010), a lot of internal applications have been described against paradontosis, gastric, duodenal ulcer and many other illnesses

(Ivanova et al., 2005).

Though reported, the antitumor properties of the medicinal plant have been limitedly studied. The anticancer cytotoxic potential of C. coggygria extracts has been an object of in vitro studies concerning plant populations from Serbia and Italy (Savikin et al., 2009; Pollio et al., 2016). No data are so far available on the antitumor cytotoxic activity of the Bulgarian herb. In regard to this, the aim of the present investigation was to assess the cell viability inhibitory effect of Bulgarian Cotinus coggygria aqueous ethanolic leaves extract applied at different concentrations and time periods on MCF7 breast cancer cells.

MATERIALS AND METHODS

Plant extract

Leaf aqueous ethanolic extract from *Cotinus coggygria* was produced and provided by Vemo99 Ltd. (Sofia, Bulgaria). The extract contained (in percent of dry matter): total polyphenols, determined as catechin (from 27.0 to 32.0%); flavonoids, determined as apigenin (not less than 15.0%); flavonoids, determined as quercetin (not less than 2.0%) (http://www.vemovsv.com/products/herbal-extracts/cotinus-coggygria/).

Cell line and maintenance

Human breast adenocarcinoma cell line MCF7 was supplied by the American Type Culture Collection - ATCC (Manassas, Virginia, USA). Cells were cultivated 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 maintained at 37°C in a humidified atmosphere containing 5% CO_2 and were kept free from fungal, bacterial and mycoplasma contamination. When reached 80-90% confluence, the cells were detached with 0.05% trypsin/EDTA-solution. Cells were passaged 2 times per week in a split ratio 1:3. The experiments were carried out during the exponential phase of cell growth.

MTT cell cytotoxicity assay

The cytotoxic potential of the C. coggygria extract on breast cancer cell line MCF7 was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide] assay (Mosmann, 1983). Cells were seeded into 12-well tissue culture plates in complete cell culture medium at a concentration of 1×10^5 per well and after 24 h incubation, cells were starved for the next 24 h in serum-free medium supplemented with 0.1% BSA (bovine serum albumin). Subsequently cells were treated with the plant extract applied at different concentrations (5, 10, 20, 30, 50, 90, 130, 150, 250, 300, 400 µg/ml) for 24 h using cultivating medium as a solvent. Untreated cell samples with serum-free medium were used as negative controls. During the last 3 h of the incubation MTT reagent (0.5 mg/ml final concentration) was added. After incubation, the medium was removed and the formazan complex was dissolved in 10% SDS, 0.01M HCI. The absorbance was subsequently measured at 570 nm using microplate reader. The percentage of cytotoxicity was determined by the following equation:

Inhibition of cell viability (%) = 100 – (Absorbance of examined sample / Absorbance of control) × 100 The IC_{50} value was calculated by means of GraphPad Prism 5 software.

The MTT assay was performed with the IC₅₀ concentration for 24, 48 and 72 h to assess the dynamics of cell cytotoxicity alterations during the treatment period.

Microscopic observation of cell morphology

Observation analysis under inverted light microscope was performed parallel with the MTT cell cytotoxicity assay in order to examine the effect of *C. coggygria* extract on the morphology of MCF7 cells.

Statistical analysis

The data are presented as means \pm standard error of the mean (SEM) of at least two independent 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. Results are considered statistically significant at values of p < 0.05.

RESULTS

Cell cytotoxicity analysis

The inhibitory effect of *C. coggygria* leaf aqueous ethanolic extract on cell viability of human breast cancer cell line MCF7 was evaluated by the MTT assay. The obtained results demonstrated a strong though not dose-dependent cytotoxic effect of *C. coggygria* extract (Fig. 1, Table 1). In the range of the lower concentrations (5 μ g/ml and 10 μ g/ml), a weak inhibition of tumor cell viability was observed (10.65% and 10.56%, respectively). With the increase of the extract concentration (from

20 µg/ml to 130 µg/ml), the inhibitory effect became more intense reaching a maximum value of 77.06%. However, at the higher concentrations (from 150 to 400 µg/ml) tumor cell viability inhibition showed a tendency of a gradual diminution (from 76.64% to 57.99%). Statistically significant differences (p<0.001) between treated and untreated tumor cells were established for all studied concentrations from 20 µg/ml to 400 µg/ml.

An indicator for the antitumor activity of tested substances is the value of the IC_{50} parameter, i.e. the concentration needed to reduce cell viability to 50%. The IC_{50} value for the *C. coggygria* extract on MCF7 cells was calculated to be 40.6 µg/ml.

Morphology observation of *C. coggygria* treated MCF7 cells performed alongside with the MTT analysis revealed comparable results (Fig. 2). Rounding, shrinking and monolayer detachment of tumor cells subjected to the extract activity were visible and the tendency followed that established by the MTT assay. Untreated control cells remained normal in shape and monolayer adherent.

Antitumor activity kinetics

Tumor cell viability inhibition with time was subsequently studied by the MTT assay after treatment of MCF7 cells with *C. coggygria* extract applied at a concentration corresponding to the detected IC₅₀ (40.6 µg/ml) for a period of 24, 48 and 72 h (Fig. 3). The results showed an overall reduction of the inhibitory activity over time decreasing to 19.82% at the 48 h followed by a slight increase to 35.97% at the 72 h. Statistically significant differences between treated cells and untreated controls were established.



Figure 1. MTT analysis of cell viability inhibition on MCF7 tumor cells treated with *Cotinus coggygria* extract with increasing concentrations for 24 h.

Morphology observation analysis of MCF7 cells after treatment for 24, 48 and 72 h with 40.6 μ g/ml *C. coggygria* extract confirmed the data obtained from the MTT assay (Fig. 4).

Table 1. Statistical analysis of MTTcytotoxicity assay results.

| Concentration [µg/ml] | Inhibition of cell viability $[\% \pm SEM]$ |
|--------------------------|---|
| 5 | 10.65 ± 3.32 |
| 10 | 10.56 ± 4.69 |
| 20 | $23.51 \pm 5.49^{***}$ |
| 30 | $43.69 \pm 5.38^{***}$ |
| 50 | $64.29 \pm 3.73^{***}$ |
| 90 | $75.71 \pm 1.39^{***}$ |
| 130 | $77.06 \pm 0.93^{***}$ |
| 150 | $76.64 \pm 1.00^{***}$ |
| 250 | $64.86 \pm 0.71^{***}$ |
| 300 | $61.96 \pm 1.92^{***}$ |
| 400 | $57.99 \pm 1.67^{***}$ |

Data are presented as means \pm SEM. *** Indicates significant differences from the control group with p<0.001.



130 µg/ml

400 μg/ml

Figure 2. Changes in morphology of MCF7 cells after treatment with *C. coggygria* extract at concentrations of 50 μ g/ml, 130 μ g/ml and 400 μ g/ml for 24 h.



Figure 3. MTT analysis of MCF7 tumor cells treated with 40.6 μ g/ml *C*. *coggygria* extract for 24, 48 and 72 h. Error bars represent SEM. ** (p<0.01) and *** (p<0.001) indicate significant differences from the control group.

DISCUSSION

The here-performed study outlined a strong cytotoxic activity of Bulgarian C. coggygria leaf aqueous ethanolic extract on the human breast cancer cell line MCF7 with IC_{50} value of 40.6 μ g/ml. The observed cell viability inhibitory effect of the extract was highly selective to tumor cells as our previously published results demonstrated a minor influence and less toxicity to the human normal mammary epithelial cell line MCF10A (Gospodinova et al., 2014). Additionally, our recently reported data (Gospodinova at al., 2017) have shown significant selective antiproliferative activity of the same C. coggygria extract



Figure 4. Morphological changes of MCF7 cells after treatment with 40.6 µg/ml *C. coggygria* extract for 24, 48 and 72 h.

on human breast, ovarian and cervical cancer cell lines. These results attribute high antitumor potential of the Bulgarian herb.

The available data concerning the anticancer cytotoxic activity of C. coggygria extracts are restricted to only two in vitro studies. Pollio et al. (2016) established that methanol extract from aerial part of C. coggygria from Italian plant population decreased cell viability of MCF7 breast cancer and A549 lung cancer cell lines. Another study found a considerable cytotoxic effect of methanol extracts of leaves and flowers of the plant from Serbia on human cervical cancer cell line HeLa (IC $_{50}$ values are 9.01 µg/ml and 29.4 µg/ml, respectively) and colon cancer cell line LS174 (65.4 μ g/ml and 41.3 μ g/ ml) (Savikin et al., 2009). The here-found IC_{50} for the Bulgarian herb, in regard to cell viability inhibitory effect on MCF7 cells, was higher when compared to the study of the extract of *C. coggygria* from Serbian plant population on HeLa cells but in comparison to LS174 cells showed a lower IC₅₀ value.

The detected in the present study *C*. coggygria antitumor cytotoxicity was neither dose- nor time-dependent and seemed to weaken with the increase of the applied extract concentrations (150 µg/ml and above) or treatment period. In contrast, the study of Pollio et al. (2016) displayed dose-dependency on MCF7 cells after treatment at concentrations 0.05%, 0.1%, and 0.15% (v/v) and the effect was found to be irreversible only at the highest used concentration. Such a phenomenon for lack of a steady dose-dependency together with a decrease in antitumor activities at the higher doses is not unusual and has been observed for various plant extracts and chemotherapeutic agents. Examples in this respect are some antimetabolite chemotherapeutic drugs (among which folate analogs, purine analogs, pyrimidine analogs, substituted ureas), whose action is specific according to the phase of the cell cycle, being highest at the S-phase. Their cytotoxic activity shows a nonlinear dose response and after reaching a certain dose, no more cells are killed despite increasing doses (Malhotra and Perry, 2003). One possible explanation for the inhibition reduction of the studied C. coggygria extract could be the saturation with the increase of the dose and time that may lead to depletion of the specific targets of plant active substances. Epigenetic adaptive mechanisms surmounting the cytotoxic activity of the herb could also be projected.

In the present study, we report novel data on the *in vitro* anticancer cytotoxic activity of the Bulgarian *C. coggygria*, which give the grounds for future elaboration of the molecular targets and mechanisms underlying the antitumor effect of the plant extract.

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REFERENCES

- Bilen S, S Yılmaz, AM Bilen, 2013. Influence of Tetra (*Cotinus coggygria*) extract against *Vibrio Anguillarum* infection in Koi Carp, *Cyprinus carpio* with reference to haematological and immunological changes. *Turk J Fish Aquat Sci*, 13: 517–522.
- Demirci B, F Demirci, KHC Başer, 2003. Composition of the essential oil of *Cotinus coggygria* Scop. from Turkey. *Flavour Frag J*, 18: 43–44.
- Ferlay J, I Soerjomataram, M Ervik, R Dikshit, S Eser, C Mathers, M Rebelo, DM Parkin, D Forman, F Bray, 2013.
 GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet].
 Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr.
- Gospodinova Z, G Antov, R Christova, M Krasteva, 2014. Extracts from *Tanacetum vulgare* L. and *Cotinus coggygria* Scop. exert a lower *in vitro* inhibitory effect on normal mammary epithelial cell viability than on breast cancer. *Anticancer Research*, Abstracts of the Ninth International Conference of Anticancer Research, 34: 5928.
- Gospodinova Z, N Bózsity, M Nikolova, M Krasteva, I Zupkó, 2017. Antiproliferative properties against human breast, cervical and ovarian cancer cell lines, and antioxidant capacity of leaf aqueous ethanolic extract from *Cotinus coggygria* Scop. *Acta Medica Bulgarica*, 44(2): 20–25.
- Huang KC, 1999. The pharmacology of Chinese herbs. 2nd Edition. CRC Press Inc., Boca Raton, FL.

- Ivanova D, D Gerova, T Chervenkov, T Yankova, 2005. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *J Ethnopharmacol*, 96: 145–150.
- Jing B, Z Ma, J Feng, H Liang, C Li, X Zhang, 2012. Evaluation of the antiviral activity of extracts from plants grown in the qinling region of China against infection by Tobacco mosaic virus (TMV). *J Phytopathol*, 160: 181–186.
- Landzhev I, 2010. In: Encyclopedia of medicinal plants in Bulgaria. Trud Publ., Sofia, 372–374 (In Bulg.).
- Malhotra V, MC Perry, 2003. Classical chemotherapy: mechanisms, toxicities and the therapeutic window. *Cancer Biol Ther*, 2(4 Suppl 1): S2–S4.
- Marčetić M, D Božić, M Milenković, N Malešević, S Radulović, N Kovačević, 2013. Antimicrobial, antioxidant and anti-inflammatory activity of young shoots of the smoke tree, *Cotinus coggygria* Scop. *Phytother Res*, 27: 1658–1663.
- Matić S, S Stanić, S Solujić, T Milošević, N Niciforović, 2011. Biological properties of the *Cotinus coggygria* methanol extract. *Period Biol*, 113: 87–92.
- Mosmann T, 1983. Rapid colorimetric

assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, 65: 55–63.

- Pavlov D, M Eftimov, M Tzaneva, D Ivanova, M Nashar, I Kobakova, S Valcheva-Kuzmanova, 2013b. Gastroprotective effect of ethanol infusion from *Cotinus coggygria* wood in rats. *Scripta Scientifica Medica*, 45: 7–12.
- Pavlov D, M Nashar, M Eftimov, K Kalchev, S Valcheva-Kuzmanova, M Tzaneva, D Ivanova, 2013a.
 Subchronic toxicity study of aqueous infusion from *Cotinus coggygria* leaves in Wistar rats. *Compt rend Acad bulg Sci*, 66: 749–756.
- Pollio A, A Zarrelli, V Romanucci, A Di Mauro, F Barra, G Pinto, E Crescenzi, E Roscetto, G Palumbo, 2016.
 Polyphenolic profile and targeted bioactivity of methanolic extracts from Mediterranean ethnomedicinal plants on human cancer cell lines. *Molecules*, 21(4): 395.
- Savikin K, G Zdunic, T Jankovic, T Stanojkovic, Z Juranic, N Menkovic, 2009. *In vitro* cytotoxic and antioxidative activity of *Cornus mas* and *Cotinus coggygria*. *Nat Prod Res*, 23(18): 1731–1739.