

ANTIPROLIFERATIVE PROPERTIES AGAINST HUMAN BREAST, CERVICAL AND OVARIAN CANCER CELL LINES, AND ANTIOXIDANT CAPACITY OF LEAF AQUEOUS ETHANOLIC EXTRACT FROM *COTINUS COGGYGRIA* SCOP.

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Abstract. *Cotinus coggygia* Scop. leaf aqueous ethanolic extract was examined for its *in vitro* antiproliferative and antioxidant activity. Antiproliferative effect was assessed on four human gynecological cancer cell lines: breast (MCF7, T47D), cervical (HeLa) and ovarian (A2780) and compared to the cell growth inhibitory effect on non-cancerous breast epithelial cell line MCF10A using MTT cell proliferation assay. Radical scavenging assay with DPPH was applied to evaluate antioxidant potential of the extract. The obtained results showed that the herb inhibited cell growth of all of the tested cancer cell lines and the highest was the cytostatic effect on A2780 cells with a half maximal inhibitory concentration (IC_{50}) value of 30.8 $\mu\text{g/ml}$. For the other cell lines the IC_{50} values were in the range of 55–122.7 $\mu\text{g/ml}$. Additionally, the extract exerted considerably weaker reduction in cell proliferation of the non-cancerous cell line MCF10A compared to cancer cells, which indicates for antiproliferative selectivity. *C. coggygia* extract showed high free radical scavenging activity with an IC_{50} value of 11.2 $\mu\text{g/ml}$. The obtained data provide evidence for pharmacological potential of the tested extract and future more detailed studies concerning the molecular mechanisms of the anticancer effect of the herb are needed.

Key words: *cotinus coggygia* Scop., antiproliferative effect, cancer cell lines, antioxidant activity

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INTRODUCTION

Plant kingdom is a source of a wide variety of biologically active compounds with substantial pharmacological potential in the development of novel drugs against different diseases, including cancer. Cancer chemoprevention and treatment with

natural phytochemicals is in the focus of considerable interest in the last decades, to reduce the adverse side effects, to improve the efficacy of chemotherapy and radiation therapy, and also to increase drug accumulation or to reduce cancer cells drug resistance, producing synergistic effects. Furthermore, plant products are less toxic and detrimental than synthetic drugs.

Cotinus coggygria Scop. (syn. *Rhus cotinus* L.), also known as Eurasian smoke tree (local name – tetra, smradlika) is an important commercial medicinal plant routinely used in Balkan folk medicine and one of the fifteen most commonly used herbs in Bulgaria [18]. It is a deciduous, multiple-branching shrub or small tree from the Anacardiaceae family. Plant distribution involves southern Europe, the Mediterranean, the Caucasus, south-eastern and eastern Asia. In Bulgaria it occurs up to about 800 m asl. The plant is widely applied in the folk medicine of many countries due to its antimicrobial, anti-inflammatory, antihemorrhagic [6] and antipyretic properties [11]. The usage of Eurasian smoke tree is predominantly external and though some authors consider it poisonous [26], it has also been applied orally for treatment of throat inflammations, paradontosis, gastric and duodenal ulcer, diarrhea [12], diabetes [14] and others. In Serbian folk medicine decoction of the *C. coggygria* bark has been used against cancer [15]. In Asia the herb is applied against hepatitis and anemia [1]. Syrup from the plant protects liver from chemical damaging, increases bile flow and the immunity of the body [23].

Phytochemical analyses of *C. coggygria* have identified broad range of compounds from various parts of the plant, such as total phenols; flavonoids; tannins; gallic acid [24]; 1,2,3,4,6-Penta-O-galloyl- β -D-glucose [4]; terpenoides; saponins [20] and others.

The antitumor properties of *C. coggygria* extracts are poorly studied and are limited to a few publications. Savikin et al. [22] reported that methanol extracts from leaves and flowers of *C. coggygria* from Serbia reduced significantly cell viability of human cervix carcinoma (HeLa) and colon carcinoma cell line (LS174). The methanol extract from the aerial part of Italian *C. coggygria* affected the cell cycle in four human cancer cells line (A549 – lung adenocarcinoma, MCF7 – breast cancer, U937 – histiocytic lymphoma and TK6 – human B lymphoblastoid cells) and exerted cytotoxic effects against A549 and MCF7 [19]. Antiproliferative effect on HeLa cells was observed after treatment with leaves methanolic extract of *C. coggygria* from Turkey [3]. Diethyl ether-soluble fraction of methanol *C. coggygria* wood extract from Romania inhibited considerably cell growth of ovarian cancer cell line (A2780) but displayed weaker effect on breast (MCF7, MDA-MB-231) and cervical (HeLa) cancer cell lines [2]. Among the active constituents of the plant, antineoplastic properties are ascribed to the gallic acid [13] and the flavonoids myricetin [27], apigenin [21], quercetin [7].

The antioxidant properties of the plant represent a great interest of intensive studies and some authors reported that the high content of polyphenols in *C. cog-*

gygria correlates with high antioxidant potential [9, 12, 17, 20]. According to Ivanova et al. [12] the antioxidant activity of smoke tree leaves infusions is the highest when compared to another twenty studied Bulgarian medicinal plants and was comparable to that of black and green tea and even higher than the worldwide famous teas rooibos, honeybush and mate.

AIM OF THE STUDY

Taking into account the numerous valuable biological activities of *C. coggygria* determining considerable pharmacological potential and its slightly examined antitumor activity, the present study was undertaken to evaluate the antiproliferative properties of aqueous ethanolic leaf extract of the Bulgarian herb against four human cancer cell lines representing three of the most commonly diagnosed cancer types affecting women worldwide (breast, cervical and ovarian) and to compare the effect of the extract on cell growth of a non-cancerous cell line. The *in vitro* antioxidant capacity of the extract by assessment of the free radical scavenging activity was also studied.

MATERIAL AND METHODS

Plant extract

Cotinus coggygria Scop. aqueous ethanolic extract from dry leaves was produced and provided by Vemo 99 Ltd. (Sofia, Bulgaria). The active substances in the extract included (in percent of dry matter): total polyphenols, determined as catechin (from 27.0 to 32.0%); flavonoids, determined as apigenin (15.0%); flavonoids, determined as quercetin (2.0%) (<http://www.vemo-vsv.com/products/herbal-extracts/cotinus-coggygria/>) and others.

Cell lines and cultivation

Human breast cancer cell lines MCF7 and T47D, cervical cancer cell line HeLa and ovarian cancer cell line A2780 were purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK) and were cultivated in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and an antibiotic-antimycotic mixture (Life Technologies, Paisley, Scotland, UK). Non-cancerous breast epithelial cell line MCF10A was supplied by the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and was cultivated in MEM medium with 5% FBS, 1% sodium pyruvate, 1% NEAA, 20 ng/ml human epidermal growth factor (hEGF), 10 μ g/ml insulin and 0.05 mM hydrocortisone (Sigma-Aldrich, Germany). The cells were grown in a humidified atmosphere contain-

ing 5% CO₂ at 37°C. Cell counts were accomplished with a Z1 Coulter Particle Counter (Beckman Coulter Hungary Ltd., Budapest, Hungary).

MTT cell proliferation assay

Cell proliferation was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay [16]. Cell lines were seeded into 96-well microplates (5000 cells/well) in a final volume of 200 µl and attached to the bottom of the well overnight. On the next day, the medium was removed and 200 µl new medium containing the studied extract in final concentrations of 10, 30, 60, 90, 120, 150, 180 µg/ml was added. Wells with untreated cells were used as controls. After 72 h incubation, cell proliferation was determined by the addition of 20 µl of MTT solution (5 mg/ml) for 4 h. The medium was then removed, the formazan complex was solubilized in DMSO and the absorbance was measured at 545 nm with an ELISA reader. Stock solution of the plant extract (at concentration 60 mg/ml) was prepared with DMSO, while the final concentrations (10-180 µg/ml) were prepared in culture medium. In a previous set of experiments we found that concentrations of DMSO up to 0.3% have no statistically significant effect on the cell proliferation as determined under the same conditions.

The data were presented as means ± standard error of the mean (SEM) of two separate experiments each performed in at least five parallel repeats. IC₅₀ values were calculated by means of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Statistical differences between control and treated groups were evaluated using one-way analysis of variance (ANOVA) followed by the Dunnett's post-hoc test. A value of p < 0.05 was considered statistically significant.

DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the studied samples [25]. Different concentrations of *C. coggygia* extract and the synthetic antioxidant butylated hydroxytoluene (BHT) solution (5, 10, 20, 50 and 100 µg/ml in methanol) as well as of gallic acid (1, 2, 5, 10, 20, 50 and 100 µg/ml) were added at an equal volume (2.5 ml) to methanol solution of DPPH (0.3 mM, 1 ml). After 30 min at room temperature, the absorbance (Ab) values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity (\%)} = [1 - (\text{Ab}_{\text{sample}} - \text{Ab}_{\text{blank}}) / \text{Ab}_{\text{control}}] \times 100$$

Methanol (1.0 ml) plus plant extract solution (2.5 ml) was used as a blank, while DPPH solution plus methanol was

used as a control. The measurements of each sample were carried out in triplicate and the data were presented as means ± standard error of the mean (SEM). The IC₅₀ values were calculated by Software Prizm 3.00.

RESULTS

Antiproliferative activity of *C. coggygia* extract on MCF7, T47D, HeLa, A2780 and MCF10A cells

The antiproliferative potential of the extract was assessed on a panel of four human tumor cell lines MCF7, T47D, HeLa, A2780 in comparison with control non-cancerous cell line MCF10A after 72 h treatment period in the range of concentrations from 10 to 180 µg/ml using the MTT assay.

The obtained results showed that the extract decreased significant cell growth of all tested cancer cell lines (Fig. 1) and the detected cytostatic effect was most considerable against ovarian cancer cells A2780 with IC₅₀ value of 30.8 µg/ml. The calculated IC₅₀ concentrations of the extract for MCF7, T47D and HeLa cell lines were 55 µg/ml, 61.1 µg/ml and 122.7 µg/ml, respectively. For the most sensitive cell line – A2780, cell proliferation reached the lowest value of 7.42% at concentration 90 µg/ml. From all of the tested cancer cell lines, the proliferation of cervical cancer cells HeLa was less affected by the extract with reduced cell growth of 31.56% at 180 µg/ml.

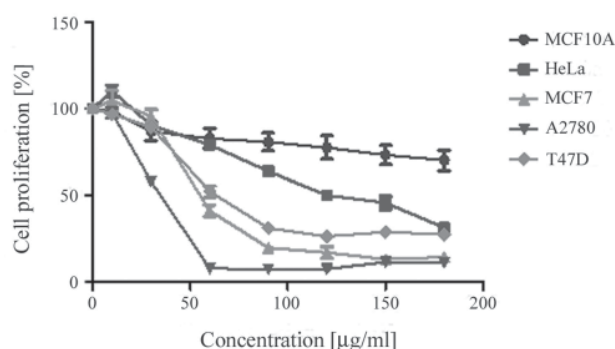


Fig. 1. MTT cell proliferation assay of MCF7, T47D, HeLa, A2780 and MCF10A cells treated with increasing concentrations of *Cottinus coggygia* Scop. extract for 72 h. Error bars represent standard error of the mean (SEM)

In regard to MCF10A cells, a slight dose-dependent reduction in the cell growth was observed after 72 h extract exposure in a considerably lower rate compared to the cancer cells. Cell proliferation decreased from 98.54% at concentration of 10 µg/ml to 70.28% at the highest concentration of 180 µg/ml (IC₅₀ > 180 µg/ml).

Statistical analysis was performed indicating considerable significant differences between treated groups and control with p-values of less than 0.05 (Table 1).

Antioxidant activity of *C. coggygia* extract

The methanolic solution of *C. coggygia* extract, synthetic antioxidant BHT as well as gallic acid as a compound of the plant extract were evaluated for free radical scavenging activity using DPPH assay. Antioxidant activity presented as half maximal inhibitory concentration was found to be 11.2, 12.6 and 1.4 µg/ml for *C. coggygia* extract, BHT and gallic acid, respectively. The methanolic solution of gallic acid showed the strongest antioxidant activity (Fig. 2).

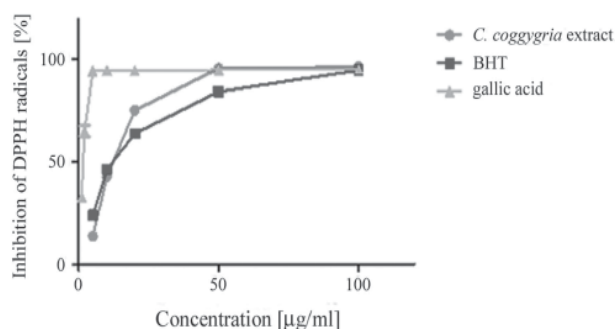


Fig. 2. Free radical scavenging activity of the *Cotinus coggygia* Scop. extract using DPPH. Error bars represent standard error of the mean (SEM)

Table 1. Statistical analysis of MTT cell proliferation assay results (mean ± SEM)

Concentration [µg/ml]	Cell proliferation [% ± SEM]				
	MCF7	T47D	HeLa	A2780	MCF10A
10	104.84 ± 5.87	96.98 ± 2.28	110.29 ± 3.31	97.70 ± 3.05	98.54 ± 0.71
30	95.57 ± 4.15	89.36 ± 2.87	90.46 ± 1.83	58.06 ± 2.32	86.88 ± 5.34
60	41.27 ± 3.40	52.49 ± 3.15	79.14 ± 2.71	8.10 ± 1.36	82.83 ± 5.68
90	19.96 ± 2.22	31.34 ± 1.77	63.89 ± 2.06	7.42 ± 0.71	80.86 ± 5.22
120	17.25 ± 3.27	26.60 ± 1.75	50.22 ± 2.27	7.68 ± 0.78	77.59 ± 6.70
150	13.28 ± 1.28	29.07 ± 2.13	45.89 ± 3.80	11.69 ± 1.31	73.33 ± 5.48
180	14.61 ± 2.33	27.73 ± 2.05	31.56 ± 2.40	11.31 ± 0.80	70.28 ± 5.85

*, ** and *** indicate significant differences from the control group by Dunnett's test (* p < 0.05, ** p < 0.01, *** p < 0.001).

DISCUSSION

Nowadays, over 60% of the applied anticancer agents originate from natural sources such as plants, marine flora and fauna, and microorganisms [5], among which plants species are the most used due to their wide availability, great variety and numerous valuable properties.

As a species with a wide range of pharmacological activities *C. coggygia* is of great scientific interest and finds application in cosmetic and medical products. The antitumor properties of the herb extracts are slightly investigated. In respect to the Bulgarian plant the available data concerning its anticancer properties are restricted only to a previous study of the authors [10], which detected cytotoxic activity of the extract on human breast cancer cell line MCF7 (IC₅₀ value 40.6 µg/ml) and less toxicity to non-cancerous cell line MCF10A.

In vitro studies on human cervical cancer cell line HeLa and colon cancer cell line LS174 demonstrated that methanol extracts of leaves and flowers of *C. coggygia* exhibited significant cytotoxic activity with IC₅₀ values of 19.01 µg/ml and 29.4 µg/ml, respectively for HeLa cells, and 65.4 µg/ml and 41.3 µg/ml, respectively against LS174 cells [22]. Another research concerning HeLa treatment with methanolic plant leaves extract indicated inhibitory effect on cancer cell growth (IC₅₀ concentration of 293 µg/ml) and considerably weaker antiproliferative properties against normal Vero cell line [3]. Cell viability of lung cancer (A549) and breast cancer (MCF7) cell lines was decreased by methanol extract from aerial part of *C. coggygia* and in regard to MCF7 cells reduction was in a dose-dependent manner after treatment at concentrations 0.05%, 0.1%, and 0.15% v/v. The cytotoxic effect in MCF7 was found to be reversible at first two concentrations but irreversible at the high-

est dose. A dose-dependent effect of the extract was also observed on the cell cycle distribution of breast cancer cell line with significant increase in the percentage of cells in G1 phase. *C. coggygia* extract blocked cell cycle of A549, MCF7, U937 (histiocytic lymphoma) and TK6 (human B lymphoblastoid) cells after 24 h exposure at concentration 0.15% v/v [19]. IC₅₀ concentration for cervical cancer cells HeLa calculated in our study (122.7 µg/ml) takes an intermediate position when compared to the values reported in another studies and is the highest with respect to IC₅₀ concentrations detected in the present research for breast and ovarian cancer cell lines. The above-mentioned values indicate that among the four here tested cancer cell lines, HeLa cells are the most resistant to the cell growth inhibitory effect of the herb aqueous ethanolic extract, while A2780 ovarian cancer cell line is the most sensitive with the lowest value of IC₅₀. From all four tested cancer cell lines dose-dependency in the antiproliferative effect of the extract was observed on HeLa cells, while for A2780, MCF7 and T47D cells it was not dose-dependent for all concentration ranges. A slight reduction of the inhibitory effect at the highest applied concentrations was observed when compared to the middle-range concentrations, which is not unusual for some plants extracts.

In order to evaluate the utility of a substance as a potential pharmacological agent, the prevalence of therapeutic effect than toxicity is of significant importance. Our results, revealing weak effect of the herb extract on the cell proliferation of the non-cancerous cell line MCF10A compared to the cancer cells, are indicative for considerable selectivity in the antiproliferative effect. In regard to the cytotoxic properties towards non-cancerous cell lines a publication reported that ethanol and water extracts of the herb decreased cell viability of human gingival fibroblasts (HGF-1) and keratinocytes (HaCaT) in concentration of 5 mg/ml, which is much higher than the doses applied in the present study [8].

The antioxidant activity of the *C. coggygia* extracts and its second metabolites is of great research interest and is elaborately studied. The here obtained results on the antioxidant activity of the Bulgarian plant extract are consistent with previous data concerning other *C. coggygia* extracts [9, 12], which showed marked antioxidant properties of the herb. The found IC₅₀ value of 11.2 µg/ml for *C. coggygia* extract was even lower in comparison to the IC₅₀ concentration of the synthetic antioxidant BHT (12.6 µg/ml), but higher when compared to the gallic acid (1.4 µg/ml).

In conclusion, the here presented results revealed that leaf aqueous ethanolic extract from Bulgarian medicinal plant *Cotinus coggygia* Scop. possesses antiproliferative properties against four human cancer cell lines and the strongest inhibitory effect is detected against A2780 ovarian cancer cells. The proliferation of the non-cancerous cell line MCF10A was affected to a considerably lower degree which is indicative for high selectivity. The extract also exhibited considerable free-radical scavenging activity. Further investigations will be directed to clarification of the molecular mechanisms underlying the anticancer activity of the extract.

Acknowledgements

The authors are grateful to Vemo 99 Ltd. for providing the extract of *Cotinus coggygia* Scop. This work was supported by the grant №BG051PO001-3.3.06-0025, financed by the European Social Fund and Operational Programme Human Resources Development (2007–2013) and co-financed by Bulgarian Ministry of Education and Science.

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