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Cotinus coggygria Non-Volatile Fraction Affects the Survival of Human Cultured Cells

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Ethyl acetate extract from *Cotinus coggygria* (smoke tree) leaves contains non-volatile components, some of which are potent inhibitors of prolyl oligopeptidase (POP) and fibroblast activation protein α (FAP). Those enzymes are known to participate in tumorigenesis and tumor growth. Effects of the above extract on several human cultured cells, originating from the most common and aggressive cancers were examined using the Neutral Red Uptake Test. The IC_{50} values were determined and selectivity indices (SI) versus non-tumorigenic cell lines MCF-10A and BJ were calculated. According to the results, *C. coggygria* extract has a highly selective effect on HeLa cells and can be considered as a potential therapeutic agent in cervical carcinoma. Additionally, it is shown that the simultaneous suppression of POP and FAP has a pronounced impact on the cell proliferation of both tumor and normal human cells at concentrations > 12 μg/ml, which proves the enzymes' role in the control of cell proliferation.

Key words: Cotinus coggygria, prolyl oligopeptidase, fibroblast activation protein α, human cultured cells, cell survival

Introduction

Cotinus coggygria (smoke tree) is a flowering plant from the family *Anacardiaceae*, also known as smoke bush, Venetian sumach, or dyer's sumach. Extracts from both aerial and underground parts of the plant are used as antiseptic, anti-inflammatory and hepatoprotective agents [6]. Alcoholic extracts contain considerable amount of polyphenols, fusetin and other ingredients with a pronounced antitumor activity (see e.g [2]). Our previous study showed that the ethyl acetate extract of smoke tree leaves inhibits fibroblast activation protein α (FAP, EC 3.4.21.B28) – a protease involved in the development of a large number of solid tumors [3]. On the other hand, FAP has similar substrates to another serine protease – prolyl oligopeptidase (POP). Thus, a possibility exists that POP can also

be suppressed by the above extract. POP (EC 3.4.21.26) is a cytosolic peptidase of S9 family, hydrolysing peptide bonds at the C-terminus of proline from short peptides (up to 30 amino acids). In actively proliferating cells, the enzyme localizes in the nucleus, where, by a yet unknown mechanism, it is involved in the stimulation of cell division and cell differentiation [7]. Increased levels of POP have been found in a variety of solid tumors [5]. It is believed that the administration of POP inhibitors may result in a suppression of cell proliferation and restriction of tumor growth [4].

The aim of the present study is to assess the effect(s) of ethyl acetate extract of *Cotinus coggygria* leaves on the activity of POP as well as on the cell viability and proliferative activity of human tumor and non-tumorigenic cultured cells.

Materials and Methods

Ethyl acetate extract of Cotinus coggygria leaves. This extract was obtained exactly as described previously [3]. In brief, crude ethanol extract of *C. coggygria* leaves (Vemo 99 Ltd, Sofia, Bulgaria) was suspended in dist. water and acidified to pH 3.0 with 6N hydrochloric acid. The mixture was extracted with ethyl acetate, filtered, washed with brine and dried over sodium sulfate. Then, diisopropyl ether was added in drops and the formed dark yellow solid was filtered and dried.

POP inhibition by the C. coggygria extract. POP inhibition properties of the above extract (1 to 10 μg/ml) were tested on recombinant human POP (R&D Systems through Biomedica, Bulgaria) in phosphate buffered saline (PBS, pH 7.4) with the addition of 1 mM EDTA, 5 mM dithiotreitol (DTT) and 80 μM fluorogenic substrate Z-glycyl-prolyl-methylcoumaryl amide (Z-Gly-Pro-MCA, Bachem, Switzerland) at 37ºC. Enzyme assays were carried out in 96-well plates on Varioscan Fluorescence spectrofluorimeter at 360 nm excitation and 460 nm emission every 3 min. The program EnzFilter V2 was used for data processing.

Cell culturing and treatment. For the experiments, the following human cell lines were used: **MCF-10A** (immortalized normal epithelial cells of mammary gland), **BJ** (activated normal skin fibroblasts), **MCF-7** (luminal type A breast carcinoma), **MD-MBA-231** (triple negative breast carcinoma), **A549** (lung alveolar adenocarcinoma), **HeLa** (cervical cancer), **HT-29** (colorectal adenocarcinoma), **H1299** (non-small cell lung carcinoma cells), **HepG2** (hepatocellular carcinoma) and **PC3** (prostate adenocarcinoma). They were cultured in Dulbecco's Modified Eagle's medium – high glucose (DMEM 4,5 g/l glucose), supplied with 10 % fetal bovine serum and antibiotics in usual concentrations in a humidified atmosphere with 5 % CO_2 at 37.5ºC. In the case of MCF-10A cells, epidermal growth factor, insulin and cholera toxin were added in concentrations corresponding to the cell bank instructions. Cells were plated at a density of 2×10^3 in 100 μl culture medium in 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with *C. coggygria* extract. The extract was dissolved in DMSO and diluted with culture medium. A concentration range from 0.75 to 100μg/ml was applied for 48 h. After treatment with Neutral Red for 3 h, washing and application of the ethanol/acetic acid solution (NR Desorb) (after [10]), the absorption was measured on ELISA microplate reader (TECAN, SunriseTM, Grödig/ Salzburg, Austria) at a wavelength of 540 nm. GraphPad Prizm5 software was used for the processing of the results. All experiments were performed in triplicate.

Results and Discussion

In a recent paper, we showed that ethyl acetate extract of *C. coggygria* leaves possesses one or more components inhibiting FAP with an IC_{50} value of 3.7 μg/ml [3]. However, FAP and POP are both serine-type post-proline specific endopeptidases with similar substrate specificity. Thus, it is reasonable to suggest that the extract can inhibit POP, too. Our present study shows an inhibition of human recombinant POP by IC_{50}= 0.12μg/ml, i.e. the extract is 30 times more effective inhibitor of POP than of FAP.

POP is widely expressed in human organs and tissues with the highest levels in brain, testis and kidney [8]**.** Physiological functions of the enzyme include processing of neuropeptides, signal transduction, control of protein secretion, etc. It is shown to be involved in cell division and differentiation and in the nervous system – in learning and memory (reviewed in [1]). POP expression is elevated in many types of carcinomas, suggesting it may promote cancer development and growth [5]. Furthermore, it was shown that treatment with the inhibitor J94 (inhibits both POP and FAP) suppressed growth of human colon cancer xenograft tumors in mice by >90% [4]. Recent studies of Perez et al. [9] using the POP inhibitor Y-29794 demonstrate a lowered survival of *in vivo* tumor growth of triple-negative breast cancer, implanted in mice. Thus, POP inhibitors are recognized as potential therapeutics in cancer. That is why we decided to test the impact of *C. coggygria* ethyl acetate extract on the survival/proliferation of human tumor cells of different origin.

In the Atlas ARCHS4 of tissue expression of a number of genes, POP's expression (https://maayanlab.cloud/archs4/gene/PREPL#tissueexpression) and this one of the FAP (https://maayanlab.cloud/archs4/gene/FAP#tissueexpressionexpressions) in the most commonly used cell lines are presented in provisional units from zero to fourteen. According to this database, POP is expressed in all the cells, studied here, in large quantities from 9 to 12. It has the lowest amount in H1299 (9.5) and highest in HeLa cells (11.8). Effects *of C. coggygria* ethyl acetate extract on the proliferative activity of human tumor cells are illustrated in **Fig. 1** and **Fig. 2**. Low extract concentrations (up to 12 μg/ml) showed a minimal or no effect. In HT-29, HepG2 and PC3 cells at concentrations about 1.5 – 3.0 μg/ml a statistically significant pro-proliferative activity was observed (**Fig. 2**). It should be noted, that cells of none or reverse effect have a zero or close to zero FAP expression. The most powerful anti-proliferative activity was observed in HeLa cells which expresses FAP by 5.9 provisional units. In view of the above findings, it might be suggested that the proliferative activity of tumor cells depends on both enzymes but most of all, on the tissue origin. Results for the non-tumorigenic MCF-10A and BJ cells were given in our previous paper [3]. In the present study, we repeated the experiment to obtain very close results for the two cell lines. In Table 1, IC_{50} and SI values for the cells are presented. Since IC_{50} for the two "normal" cell lines are very close, SI indices calculated versus MCF-10A and BJ are almost equal. From these results, it becomes clear that SI is very promising only for HeLa cells (SI > 11). A moderately good selectivity was obtained also for HepG2 cells (SI around 2.5).

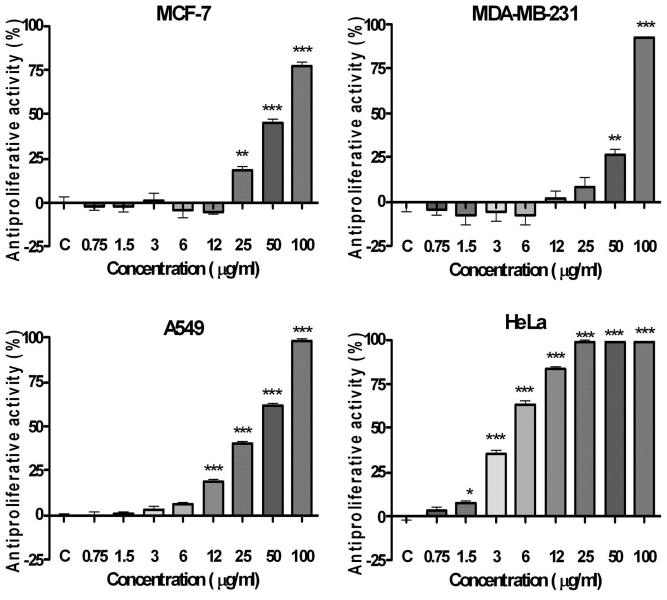

Fig. 1. Effect of C. coggygria ethyl acetate extract on the proliferative activity of the cells MCF-7, MDA-MB-231, A549 and HeLa. The effect in not-treated control cells was accepted to be zero (C). Each value is a mean of three independent experiments.

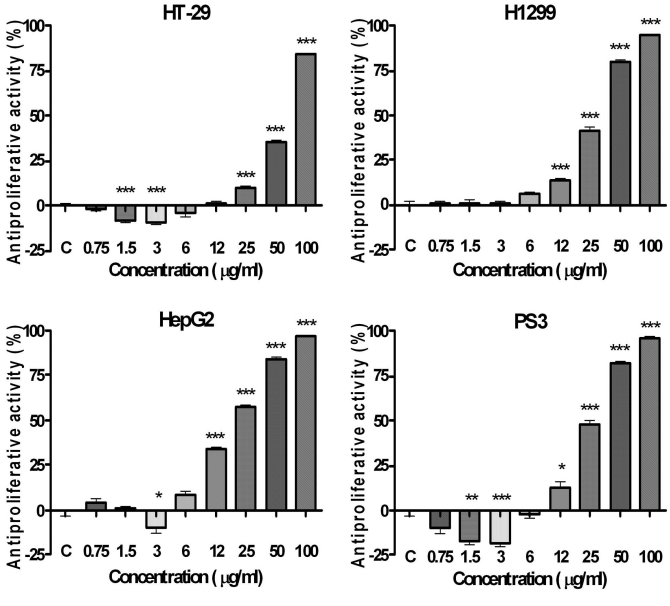

Fig. 2. Effect of C. coggygria ethyl acetate extract on the proliferative activity of the cells HT-29, H1299, HepG2 and PC3. The effect in not-treated control cells was accepted to be zero (C). Each value is a mean of three independent experiments.

Table 1. IC_{50} and SI values of different cultured cells after application of *C. coggygria* ethyl acetate extract.

Cell line	IC_{50} [μg/ml] mean ± SD	SI versus MCF-10A	SI versus BJ
MCF-10A	50,79 ± 4,645	1,00	1,05
BJ	53,34 ± 4,64	0,95	1,00
MCF-7	57,32 ± 4,702	0,89	0,93
MDA-MB-231	67,63 ± 3,67	0,75	0,79
A549	36,25 ± 3,47	1,40	1,47
HeLa	4,53 ± 0,36	11,2	11,78
HT-29	65,24 ± 2,92	0,78	0,82
H1299	30,32 ± 2,48	1,68	1,76
HepG2	21,03 ± 1,71	2,42	2,54
PC3	26,64 ± 3,5	1,91	2,00

Conclusions

According to the results presented here, *C. coggygria* extract has a highly selective effect on HeLa cells and can be considered as a potential therapeutic agent in cervical carcinoma. Additionally, it is shown that the simultaneous suppression of POP and FAP has a pronounced impact on the cell proliferation of both tumor and normal human cells at concentrations > 12 μg/ml, which proves the enzymes' role in the control of cell proliferation.

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