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Effects of a Cotinus coggygria Ethyl Acetate Extract on Two Human Normal Cell Lines

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The effect of ethyl acetate extract from *Cotinus coggygria* (sumac) leaves on the viability of two normal human cell lines (BJ and MCF-10A) is tested. Both cell lines are known to express fibroblast activation protein α (FAP) – a serine protease involved in tumorigenesis and tumor progression. The extract is shown to contain one or more components that inhibit FAP. Using the Neutral Red Uptake Test, it is found that in the line of activated human fibroblasts (BJ), the treatment leads to inhibition of cell proliferation. Conversely, the extract has no adverse effect on MCF-10A cells (mammary gland epithelium cells) at low concentrations even increasing the cells proliferation by 13 %. It is concluded that the tested extract contains a potent inhibitor of FAP, which may be useful as an anti-cancer agent, but should be used with caution due to its dual effect on normal epithelial cells.

Key words: Cotinus coggygria, Fibroblast activation protein α, MCF-10A cells, BJ cells

Introduc tion

Secondary metabolism in plants leads to the production of a large number of natural products with historically proven potential for therapeutic use in a number of diseases. *Cotinus coggygria* (sumac) belongs to the family *Anacardiaceae*. Extracts of its roots, stems, leaves and flowers are widely applied in folk and modern medicine because of their antiseptic, anti-inflammatory and hepatoprotective properties [5]. Antioxidant and antitumor activities of those extracts are also documented [1, 3]. Our preliminary studies showed that the ethyl acetate extract of sumac 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. FAP is a trans-membrane serine peptidase, belonging to the S9 family of post-proline specific proteases. It is over-expressed in the stromal fibroblasts of about 90 % of carcinomas and many sarcomas [2]. The enzyme is generally considered as a suitable target for the development of novel anti-cancer therapies [7]. However, there are several malignancies in which FAP acts as a tumor suppressor [8]. For example, the

lack of FAP expression in the membranes of human malignant melanoma cells and non-small cell lung cancer cells is considered to be a part of the tumor phenotype [12, 13]. To elucidate the FAP's dual role in cancer, more studies with the enzyme inhibitors are needed.

The aim of the present study is to assess the effect(s) of ethyl acetate extract of *Cotinus coggygria* leaves as a powerful FAP inhibitor on the cell viability and proliferative activity of two types of human cultured cells, known to express the enzyme – MCF-10A and BJ.

Materials and Methods

Ethyl acetate extract of Cotinus coggygria leaves. Crude ethanol extract of *C. coggygria* leaves was purchased from Vemo 99 Ltd (Sofia, Bulgaria). Five grams of the powdered crude extract were suspended in 20 ml dist. water and 6N hydrochloric acid was added drop wise until pH 3.0. The mixture was extracted twice with ethyl acetate. The organic phase was filtered, washed with brine and dried for several hours over sodium sulfate. Then, the ethyl acetate was partially evaporated *in vacuo* and diisopropyl ether was added in drops. A dark yellow solid fraction was formed, which was filtered and dried.
FAP inhibition by the C. coggygria extract. The inhibitory properties of the sumac leaves extract (1 to 10 μg/ml) were tested on recombinant human FAP (R&D Systems through Biomedica, Bulgaria) in phosphate buffered saline (PBS) with the addition of 1mM EDTA and 80 μM fluorescent FAP 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 emmition every 3 min. The program EnzFilter V2 was used for data processing.
Cell culturing. For the experiments, two human cell lines were used: MCF-10A (immortalized normal epithelial cells of mammary gland) and BJ (activated normal skin fibroblasts). 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 (EGF), insulin and cholera toxin were added in concentrations according to the cell bank instructions. Cells were plated at a density of 2×10^3 in 100 μl culture medium in each well of 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 in culture medium. A concentration range from 0.75 to 100 μg/ml of the extract was applied for 48 h. The neutral red uptake assay was used for the estimation of the cells viability/proliferative activity, exactly as previously described (9). After treatment with Neutral Red medium for 3 h, washing and application of the ethanol/acetic acid solution (NR Desorb), 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

Our previous experiments showed that extraction of crude plant components with ethyl acetate in acid medium leads to fractions containing polyphenols, chlorogenic acids and flavonoids (glycosylated or not) [10, 11]. Although the composition of the present extract from *C. coggycria* leaves is not determined yet, the mode of extraction may lead to the reasonable assumption, that it contains similar components. Our present experiments prove that one or more of those components are powerful inhibitors of human recombinant FAP with IC_{50}=3.7 μg/ml. As the specific inhibition of FAP would be essential for the development of novel anti-cancer strategies [7], the elucidation of FAP inhibitor(s) structure will be an important objective for our future studies.
According to the data, presented in Human Protein Atlas about the cell distribution of FAP (https://www.proteinatlas.org/ENSG00000078098-FAP/cell), it is expressed in BJ cells as this is the case of the activated fibroblasts all together. Recent studies also show that the enzyme is present in the membranes of MCF-10A cells [4]. While the role of the enzyme in activated fibroblasts is associated with an increase in their proliferative activity, its role in mammary epithelial cells is not known yet. One of the assumptions about the presence of FAP in cells of epithelial origin is that the cells are in the process of preparing for epithelial-to-mesenchymal transition [4].

Our results show that even small amounts of the *C. coggygria* extract induce a decrease of BJ cells viability, most probably due to the inhibition of FAP. The extract IC_{50} on BJ cells was estimated to be 52 μg/ml (**Fig. 1**). This result corresponds to the established importance of the enzyme for the division and migration of activated fibroblasts.

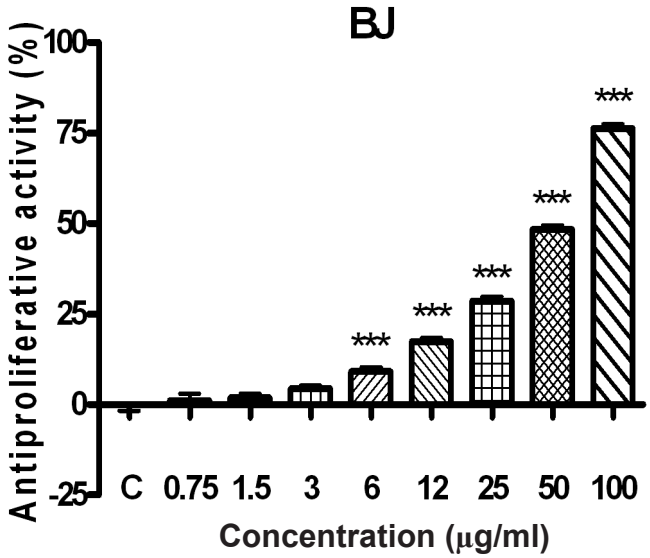

Fig. 1. Effect of *C. coggygria* ethyl acetate extract on the viability/proliferation of BJ cells. Each result represents a mean from three experiments. The anti-proliferative effect of not-treated cells is accepted to be zero.

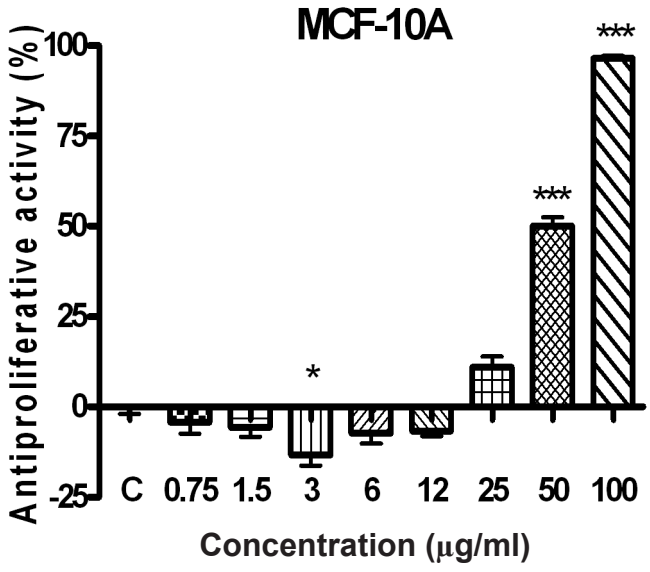

Fig. 2. Effect of *C. coggygria* ethyl acetate extract on the viability/proliferation of MCF-10A cells. Each result represents a mean from three experiments. The anti-proliferative effect of not-treated cells is accepted to be zero.

An interesting result was obtained with MCF-10A cells (**Fig. 2**). Up to concentrations of 12 μg/ml, the *C. coggygria* ethyl acetate extract did not have a negative effect on the cells viability. Conversely, at concentration 3.0 μg/ml a statistically significant increase in cells proliferation activity, estimated to 13 %, was detected. However, concentrations higher than 20 μg/ml decreased the cells proliferation rate with IC_{50}= 52 μg/ml which is equal to that of BJ cells. According to these results, the importance of FAP activity for epithelial mammary gland cells is more complicated. The enzyme may be involved in the mechanisms of cells proliferation control. These results support our hypothesis, expressed in previous studies, that the absence of FAP is a part of the tumor phenotype of human mammary gland epithelial cells [6].

Conclusions

The results, presented here show that ethyl acetate extract of *Cotinus coggygria* leaves possesses one or more FAP inhibitor(s) of yet unidentified structure which may prove to be suited for use both in biomedical research and for the development of novel anti-cancer therapeutic strategies. On the other hand, FAP inhibitors should be considered as convenient tools for suppression of the expansion of tumor fibroblasts, but keeping in mind their dual effect on cells of epithelial origin.

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References

1. **Artun, F. T., A. Karagoz, G. Ozcan, G. Melikoglu, S. Anil, S. Kultur, N. Sutlupinar N.** In vitro anticancer and cytotoxic activities of some plant extracts on HeLa and Vero cell lines. – *JBUON,* **21(3)**, 2016, 720-725.
2. **Gorrell, M. D., J. E. Park.** Fibroblast activation protein α. In: *Handbook of proteolytic enzymes* (Eds. N. D. Rawlings, G. S. Salvesen), Elsevier Ltd, 2013, 3395-3400.
3. **Gospodinova, Z., N. Bózsity, M. Nikolova, M. Krasteva, I. Zupkó.** Antiproliferative properties against human breast, cervical and ovarian cancer cell lines, and antioksidant capacity of leaf aqueous ethanolic extract from *Cotinus coggygria* Scop. – *Acta Medica Bulgarica*, **XLIV**, 2017, 20-25.
4. **Kahounová, Z., D. Kurfürstová, J. Bouchal, G. Kharaishvili, J. Navrátil, J. Remšík,** Š. Šimečková**, V.** Študent**, A. Kozubík, K. Souček K.** The fibroblast surface markers FAP, antifibroblast, and FSP are expressed by cells of epithelial origin and may be altered during epithelial-to-mesenchymal transition. – *Cytometry A.*, **93(9)**, 2018, 941-951.
5. **Matić, S., S. Stanić, M. Mihailović, D. Bogojević.** *Cotinus coggygria* Scop.: An overview of its chemical constituents, pharmacological and toxicological potential. – *Saudi Journal of Biological Sciences,* **23**, 2016, 452-461.
6. **Petrova, V. S.** Studies on the marker role of aminopeptidase A in mammary gland carcinoma with the use of novel substrates and inhibitors. *Author's abstract of PhD thesis*, "Marin Drinov" Publishing house, 2019, Sofia, Bulgaria. [in Bulgarian]
7. **Puré, E., R. B. S. Blomberg.** Pro-tumorigenic roles of fibroblast activation protein in cancer: back to the basics. – *Oncogene*, **37(32)**, 2018, 4343-4357.
8. **Ramirez-Montagut, T., N. E. Blachere, E. V. Sviderskaya, D. C. Bennett, W. J. Rettig, P.Garin-Chesa, A. N. Houghton.** FAP alpha, a surface peptidase expressed during wound healing, is a tumor suppressor. – *Oncogene*, **23**, 2004, 5435-5446.
9. **Repetto, G., A. del Peso, J. L Zurita.** Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. – *Nature Protocols*, **3**, 2008, 1125-1131.
10. **Vasileva, A., M. Dimitrova, I. Iliev, D. Tasheva, I. Ivanov.** Inhibitory effect of plant extracts on postproline enzyme activity in human breast cancer cells. – *Acta Morphol. Anthropol.,* **26**(1-2), 2019, 56-62.
11. **Vasileva. A. M., I. A. Iliev, V. S. Lozanov, M. B. Dimitrova, V. I. Mitev, I. P. Ivanov.** *In vitro* study on the antitumor activity of *Tanacetum vulgare* L. extracts. – *Bulgarian Chemical Communications,* **51**(2), 2019, 249-255.
12. **Wesley, U. V., A. P. Albino, S. Tiwari, A. N. Houghton.** A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. – *J. Exp. Med.*, **190**, 1999, 311-322.
13. **Wesley, U. V., S. Tiwari, A. N. Houghton.** Role for dipeptidyl peptidase IV in tumor suppression of human non-small cell lung carcinoma cells. – *Int. J. Cancer*, **109**, 2004, 855-866.