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Effects of *Cotinus coggygria* Extract on the Transcriptional Levels Of Histone Deacetylase Genes In Breast Cancer Cells *In Vitro*

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ABSTRACT

Despite the significant progress in cancer treatment in recent years, the disease remains a leading cause of death worldwide. Histone deacetylases are a family of enzymes recognized as key regulators of the process of histone acetylation, a main epigenetic event involved in the control of gene transcription. High expression levels of histone deacetylases are distinctive for cancer cells and a searching for new more effective and less toxic histone deacetylase inhibitors with natural origin is a nowadays intensively studied research direction in cancer prevention and therapy. *Cotinus coggygria* is a medicinal plant possessing numerous valuable biological properties. The antitumor potential of *C. coggygria* extracts is poorly studied and the available data concerning the anticancer capacity of the Bulgarian herb are limited only to a previous research of the authors, which detected reduction in viability of breast cancer cell line MCF7 after treatment with aqueous ethanolic leaf extract. The objective of the present study was to evaluate the ability of *C. coggygria* extract to modify the process of histone acetylation by transcription analysis of nine genes coding for histone deacetylases after treatment of MCF7 cells for different time periods through quantitative Reverse Transcription - Polymerase Chain Reaction. The obtained results showed statistically significant reduction in the relative amount of *HDAC5* and *HDAC7* mRNA transcripts at 48 hour after treatment and a tendency of *HDAC3* expression inhibition at 72 hour. Future investigations will be directed to assessment of the extract effect on the expression of genes coding histone acetyltransferases in MCF7 cells.

Keywords: *Cotinus coggygria*, MCF7, HDACs, gene expression

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INTRODUCTION

Nowadays, products with plant origin have become attractive candidates for cancer prevention and therapy because of their higher safety and tolerability in comparison to cytotoxic synthetic agents, and capability to reduce adverse side effects of chemo- and radiation therapy when applied in combination. The combined usage of some plant extracts together with conventional oncotherapeutics may result in synergistic effect and reduction of the doses of applied chemotherapy drugs [1]. Four classes of plant-derived antineoplastic agents are already used in clinical practice: the vinca alkaloids – vinblastine, vincristine, vindesine; the taxanes – paclitaxel and docetaxel; the epipodophyllotoxins – etoposide, teniposide and the camptothecin derivates – camptothecin and irinotecan [2]. A great number of medicinal plants are still being screened for evaluation of their anticancer potential in *in vitro* and *in vivo* model systems.

Epigenetic alterations such as DNA methylation, histone modifications and miRNA-mediated gene silencing play key role in cancer development and progression. Due to the fact that epigenetic modifications are reversible and occur in early stages of carcinogenesis a contemporary direction in oncology is focused on cancer prevention and treatment by phytochemicals-based modulation of epigenetic effects. Studies reported that some plant extracts (*Limoniastrum guyonianum, Thymus serpyllum*) and active compounds from plant sources such as curcumin, sulforaphane, resveratrol, genistein, quercetin, apigenin, epigalocatechin-3-gallate and others could alter the expression of particular tumor suppressor genes and proto-oncogenes by influence on epigenetic events in different cancer types [3-5]. The epigenetic mechanisms of action and targets of plant compounds vary among different cancer types.

The acetylation is a major post-translational modification of histone proteins associated with carcinogenetic process. It represents a transfer of acetyl groups from acetyl coenzyme A on the epsilon - amino group of lysine residues in the amino-terminal tail of the proteins. The regulation of the levels of histone acetylation is a dynamic process under the control of two classes of enzymes: histone acetyltransferases (HATs) – catalyze the process of histone acetylation and histone deacetylases (HDACs), which catalyze the deacetylation and remove acetyl groups from histones. Histone acetylation leads to an open chromatin structure and enables transcription activation and deacetylation is responsible for chromatin condensation and transcriptional repression. At present, 25 HATs and 18 HDACs have been identified. According to their size, homology, subcellular expression and number of enzymatic domains HDACs are divided into four classes [6]: class I HDACs includes HDACs 1, 2, 3, and 8; class II - HDACs 4, 5, 6, 7, 9, and

10; class III HDACs encompasses seven proteins named sirtuins (Sirt) 1–7, which are structurally unrelated to the other classes and class IV consists of HDAC 11.

In last decades numerous investigations are directed to finding novel histone deacetylase inhibitors as potential antineoplastic agents able to restore the normal expression pattern of silenced regulatory genes in cancer cells [7,8]. Promising histone deacetylase inhibitors (HDACi) are the Hyaluronic Acid Butyric ester (HA-But) for treatment of different primary and metastatic tumors [9], Trichostatin A and Valproic acid against breast cancer [10,11], suberoylanilide hydroxamic acid (SAHA) - for T-cell lymphoma [12]. However, available bioactive phytochemicals with lower toxicity and higher efficiency than synthetic inhibitors are in the focus of studies for their potential to modify epigenetic processes for cancer prevention and therapy.

Cotinus coggygria Scop. (Anacardiaceae), also known as Eurasian smoke tree, is a medicinal plant routinely used in traditional folk medicine of various countries. It possesses a wide spectrum of valuable biological activities such as antibacterial, anti-inflammatory, antihaemorragic [13], antioxidant [14] and others. The anticancer properties of the herb extracts are not studied enough. Previous study of the authors revealed cytotoxic activity of Bulgarian *C. coggygria* leaf aqueous ethanolic extract towards breast cancer cell line MCF7 and less toxicity to non-cancerous cell line MCF10A [15].

The present investigation aims to assess the ability of the abovementioned aqueous ethanolic leaf extract of *C. coggygria* from Bulgarian plant population to influence the epigenetic processes in breast cancer cells MCF7 by altering the expression of nine *HDAC* genes.

MATERIALS AND METHODS

Plant extract

Cotinus coggygria aqueous ethanolic extract from plant dry leaves was produced and provided by Vemo 99 Ltd. (Sofia, Bulgaria). It contains (in percent of dry matter): 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.vemo-vsv.com/products/herbal-extracts/cotinus-coggygria/).

Cell culturing

Human breast adenocarcinoma cell line MCF7 was supplied by the American Type Culture Collection - ATCC (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, at 37ºC in a humidified atmosphere containing 5% CO_2. When reached 80-90% confluency the cells were detached with 0.05% trypsin/EDTA-solution. The experiments were carried out during the exponential phase of cell growth.

Extract treatment

Cells were seeded into 100 mm plates (1×10^6 per plate) in a final volume of 10 ml and were incubated in complete cell culture medium. After 24 h cells were treated with *C. coggygria* extract at a final concentration of 40.6 µg/ml for 3, 24, 48 and 72 hours. The applied concentration had been determined to be the IC_{50} (inhibitory concentration 50%) of the *C. coggygria* extract on MCF7 cells in our previous study. Untreated cells were used as controls for each time period (0-72 h). Extract treated and control cells were collected by using Trypsin/EDTA-solution, centrifuged at 150×g for 5 min and then the pellets were washed with ice cold PBS and centrifuged again for 5 min at 4°C, 250×g. Cell pellets were frozen and stored at -40ºC till the time of RNA extraction.

Total RNA extraction

Total RNA was isolated using GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's recommendations. The extracted total RNA was treated with RNase-free DNase I (RNase-free DNase set, Qiagen) to eliminate any potential genomic DNA contamination and then was purified again with GeneJET RNA Purification Kit. The concentration and purity of RNA were determined spectrophotometrically (BioSpec-nano Spectrophotometer - Shimadzu Biotech) and only isolates with absorption ratio 260/280 nm > 1.8 were subjected to subsequent analysis. The integrity and quality of extracted RNA were determined on agarose gel electrophoresis.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR).

One microgram total RNA from each sample was used for the first-strand complementary DNA (cDNA) synthesis by First Strand cDNA Synthesis Kit (Thermo Scientific) with Random Hexamers according to the manufacturer's protocol. Reactions not containing the reverse transcriptase or RNA template were used as Negative controls (NC) and Zero controls (0C), respectively.

The synthesized cDNA was used as a template for detection of the relative mRNA expression levels of nine genes coding HDACs (*HDAC1*, *HDAC2*, *HDAC3*, *HDAC4*, *HDAC5*, *HDAC6*, *HDAC7*, *HDAC8* and *Sirt1*) by qRT-PCR analysis. Housekeeping gene β-actin was used as an endogenous control to normalize the expression of these genes. The primers for the abovementioned genes were synthesized by Microsynth AG (Switzerland) and primer sets

sequences [16,17,18] are listed in Table 1. Quantitative RT-PCR reaction mix was performed in 10 µl volume containing: 1x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 0.3 µM gene specific primers, DNase-RNaseFree water and 50 ng cDNA on PikoRealTM Real-Time PCR System (Thermo Fisher Scientific Inc.). Identical cycling conditions were applied, including: initial denaturation at 95°C for 15 min, followed by 45 cycles at 95°C - 15 sec, 60°C - 30 sec, 72°C - 45 sec. After amplification melting curve analysis was carried out in temperature step of 0.3°C in order to determine the melting temperature of the products. Negative (NC) and no template controls (NTC) were included in each experiment. Data were analyzed using PikoReal Software version 2.1 (Thermo Fisher Scientific Baltics UAB). Pfaffl equation [19] was used to quantify the mRNA relative expression fold changes of the genes of interest relative to the endogenous control. For each gene standard curve for evaluation of the product amplification efficiency according to the template concentration and number of cycles was generated using cDNA dilution series in step 1:10. In order to reduce the influence of *in vitro* cultivation with time, the expression levels of mRNA of each gene in *C. coggygria* treated cells at 3, 24, 48 and 72h was compared to the expression of the same gene in control untreated cells at the same time point, taken as 1.

Table 1: Sequences of primers used in qRT-PCR analysis

Gene name	Forward primer	Reverse primer
HDAC1	5′-ACCGGGCAACGTTACGAAT-3′	5′-CTATCAAAGGACACGCCAAGTG-3′
HDAC2	5′-TCATTGGAAAATTGACAGCATAGT-3′	5′-CATGGTGATGGTGTTGAAGAAG-3′
HDAC3	5′-TTGAGTTCTGCTCGCGTTACA-3′	5′-CCCAGTTAATGGCAATATCACAGAT-3′
HDAC4	5′-AATCTGAACCACTGCATTTCCA-3′	5′-GGTGGTTATAGGAGGTCGACACT-3′
HDAC5	5′-TTGGAGACGTGGAGTACCTTACAG-3′	5′-GACTAGGACCACATCAGGTGAGAAC-3′
HDAC6	5′-TGGCTATTGCATGTTCAACCA-3′	5′-GTCGAAGGTGAACTGTGTTCCT-3′
HDAC7	5′-CTGCATTGGAGGAATGAAGCT-3′	5′-CTGGCACAGCGGATGTTTG-3′
HDAC8	5′-TCCCGAGTATGTCAGTATATATGA-3′	5′-GCTTCAATCAAAGAATGCACCAT-3′
Sirt1	5'-TGGCAAAGGAGCAGATTAGTAGG-3'	5'- CTGCCACAAGAACTAGAGGATAAGA-3'
β actin	5'-GGACTTCGAGCAAGAGATGG-3'	5'-AGCACTGTGTTGGCGTACAG-3'

Each gene name should be exactly against the corresponding primer sequence. We are sending Table 1 as an attachment with font size 10pt and new table size

Each qRT-PCR reaction was performed in at least three replicates in different PCR runs with cDNA templates from two independent treatment experiments (one or two reverse transcriptions per experiment). The results are presented as mean±SEM (standard error of the mean). Statistical differences were evaluated using t-test and values of $p<0.05$ were accepted as statistically significant.

RESULTS AND DISCUSSION

Epigenetic alterations are recognized as a main feature of human cancers. Key genes involved in tumor suppression and control of cell proliferation (*p16, p19, p21, p27* and others) are silenced through HDAC-mediated epigenetic mechanisms [20]. HDACs are among the most promising targets for cancer therapy and HDAC inhibitors regulating the activity of HDACs are in development as antineoplastic drugs [21,22]. Normal cells, contrariwise, demonstrate relative resistance to the effects of HDACi [23]. Among plant-derived HDACi are allyl derivatives from garlic [24], sulforaphane, quercetin and others. Molecular modelling studies with other plant active compounds - biotin, vitamin E metabolites and α-lipoic acid, also suggest HDACi potential [25].

In the present study a screening for presence of effect of medicinal plant *C. coggygria* extract on gene expression of nine representatives of HDAC family (*HDAC1-8* and *Sirt1*) in MCF7 breast cancer cells was performed after treatment for 3, 24, 48 and 72 hours with IC_{50} concentration of the extract (40.6 µg/ml). In each time point untreated cancer cells were used as a control in order to reduce the impact of cell cultivation with time. The results obtained by qRT-PCR analysis showed statistically significant decrease in the relative expression levels of *HDAC5* and *HDAC7* mRNAs after cancer cells treatment with the extract for 48h in comparison to the untreated cells. A tendency of reduction in the relative amount of *HDAC3* mRNA transcripts was also observed at 72h (Figure 1).

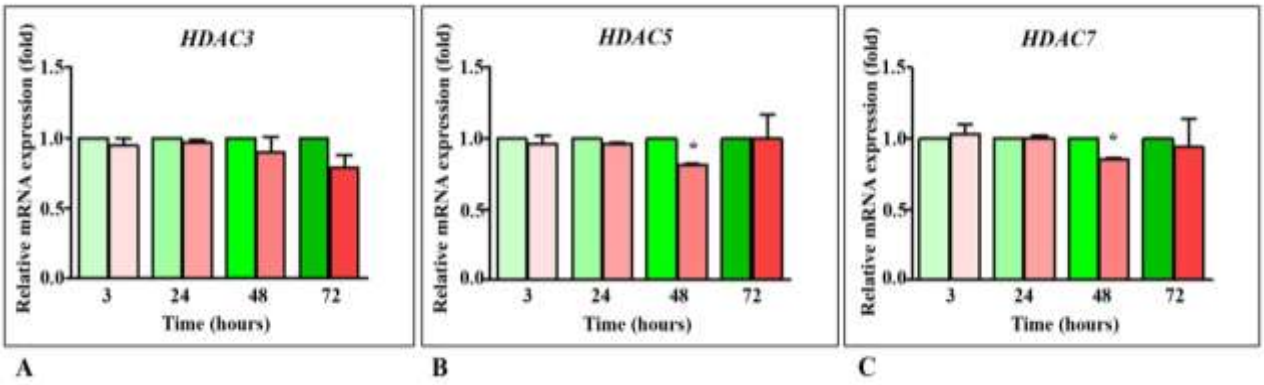

Figure 1: Relative mRNA expression levels of the *HDAC3* (A), *HDAC5* (B), *HDAC7* (C) genes at 3h, 24h, 48h, 72h after treatment of MCF7 cells with *C. coggygria* extract – green columns refer to untreated controls and red columns – to treated cells. Error bars represent standard error of the mean (SEM); * p < 0.05.

The studies regarding the antitumor properties of *C. coggygria* extracts are limited to a few publications, which reported cytotoxic and antiproliferative effect on some cancer cell lines [26-28]. The available data on the Bulgarian plant concerning its anticancer potential are restricted only to our previous study, finding that the extract significantly decreased the viability of MCF7 breast

cancer cells and inhibited in considerably lower degree the viability of MCF10A non-cancerous cells [15]. Currently, to our knowledge, there are no investigations on the effect of *C. coggygria* extracts on the expression levels of genes involved in epigenetic process of histone acetylation in cancer cells. The results obtained in the present study showing influence of the extract treatment on the expression status of some *HDACs* genes in cancer cells are the first in this respect and substantiate further investigations in this direction.

CONCLUSION

The here presented results indicated that leaf aqueous ethanolic extract from the medicinal plant *Cotinus coggygria* with Bulgarian origin possesses the ability to reduce the expression of *HDAC5* and *HDAC7*. A trend in decrease of *HDAC3* transcriptional levels was also observed. The obtained data might contribute to the revealing of some mechanisms of the anticancer action of this plant extract as well as to deepening the research on its pharmacological potential. Further investigations will be directed towards more detailed assessment of the extract effect on epigenetic processes in cancer cells by studying the expression profiles of genes coding for histone acetyltransferases as well as to expand the research at protein level.

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