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Original Research Article

Complex assessment of *in vitro* anti-melanoma action of medicinal plant *Cotinus coggygria* Scop

Georgi G Antov[1], Mariyana S Georgieva[1], Irina I Boycheva[1], Svetla J Todinova[2], Avgustina K Danailova[2], Milena T Nikolova[3], Natalia A Krasteva[2], Zlatina I Gospodinova[1]*

[1]Institute of Plant Physiology and Genetics, [2]Institute of Biophysics and Biomedical Engineering, [3]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria

For correspondence: **Email: zlatina.go@abv.bg; **Tel:** +359 (2) 979 2617*

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Abstract

Purpose: *To elucidate the anticancer mechanism and molecular targets of the aqueous fraction from Cotinus coggygria leaf extract in A375 malignant melanoma cells.*
Methods: *Aqueous ethanol extract from the leaves of C. coggygria was fractionated into the aqueous and chloroform fractions by solvent–solvent partition. A375 cells were treated with the aqueous fraction at various concentrations and incubated for 72 h. The effect of the aqueous fraction on programmed cell death by staining with Annexin V/Propidium iodide ias well as on cell viability and adhesion through labeling with fluorescein diacetate were determined under fluorescence microscopy. In addition, the clonogenicity assessment, comet assay evaluation of genotoxic properties, characterization of thermodynamic behavior of cells by differential scanning calorimetry, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on the expression of genes involved in processes of apoptosis and cell cycle control were determined.*
Results: *The number of viable adherent cells was reduced by 38.00 and 85.06 % at 20 and 80 µg/mL, respectively compared to untreated cells. In addition, a significant, dose-dependent reduction in the clonogenicity of melanoma cells was observed, culminating in an almost complete inhibition of cancer cells colony-forming ability at 40 and 60 µg/mL of the fraction. Treatment with the fraction increased comets of Classes 3 and 4 to 35 %, while comets of Class 5, which corresponds to the highest level of DNA fragmentation, increased to 2.25 %. Also, the gene damage index (GDI) was highest (1.2) at the concentration of 40 µg/mL. Furthermore, the expression levels of BAX, CASP8 and CDKN1A were upregulated and MYC expression decreased after treatment with the aqueous fraction.*
Conclusion: *Cotinus coggygria possesses significant in vitro anti-melanoma potential. Future studies will be focused on the in vivo assessment of its therapeutic qualities in experimental animals.*

Keywords: *Cotinus coggygria Scop, A375 melanoma cell line, Apoptosis, Colony formation inhibition, Genotoxic effects, Cell growth inhibition*

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INTRODUCTION

Skin cancer, such as non-melanoma and melanoma, represents the most common malignancy in Caucasians worldwide. Malignant melanoma is the most severe and lethal form of skin cancer. The incidence of cutaneous melanoma has increased globally in the last decade as there were approximately 280, 000 new cases and 60, 000 deaths in 2018 [1]. The

disease is highly metastatic and resistant to conventional chemotherapy. In recent years, research has focused on the isolation of novel plant-derived anti-neoplastic agents with higher efficiency and without side effects.

Cotinus coggygria Scop. is an important medicinal plant species with a significant therapeutic potential and it is widely distributed from Southern Europe to Central China. The herb is used in traditional folk medicine predominantly against different skin disorders and has been intensively studied in recent years due to its various biological qualities [2]. Few studies have focused on the anti-cancer properties of *C. coggygria* in the past but in recent times, the plant has generated increasing interest among researchers due to its anti-cancer properties [3]. Previously, the ethanol extract of the leaves of Bulgarian *C. coggygria* has been shown to possess considerable anti-proliferative activity against human breast, ovarian and cervical cancer cell lines [4,5]. In addition, the crude extract and its chloroform and aqueous fractions have been shown to exhibit significant selective cell growth inhibitory properties against A375 melanoma as well as the A431 non-melanoma squamous cell carcinoma cells [6]. Furthermore, the strongest anti-proliferative effect was produced by aqueous fraction of the extract against A375 melanoma cells.

The present research aims to elucidate the molecular targets and mechanism of action of the aqueous fraction of *C. coggygria* leaf extract on A375 human skin melanoma cells.

EXPERIMENTAL

Plant extract and fractionation

The aqueous ethanol extract of the leaves of *Cotinus coggygria* was produced and provided by Vemo 99 Ltd. (Sofia, Bulgaria). Fractionation of the crude extract was performed by solvent–solvent partition with distilled water and chloroform (1:1 v/v) and both fractions were then evaporated to dryness using a rotary evaporator. The aqueous (polar) fraction of *C. coggygria* crude leaf extract was used for subsequent analyses.

Cell line and culturing conditions

Human skin malignant melanoma cell line A375 (ATCC, Manassas, Virginia, USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) in a humidified atmosphere and appropriate experimental conditions (37 °C and 5 % CO_2).

Fluorescein diacetate staining

Melanoma cells (1.25×10^4 cells/well) were seeded on round coverslips placed in 24-well plates, incubated overnight for attachment, and treated with the aqueous fraction of *C. coggygria* extract at concentrations of 20, 40, 60, and 80 µg/mL and incubated for 72 h. The treated cells were subsequently stained for 2 min with 0.001 % fluorescein diacetate (FDA) dissolved in acetone and washed with 1X phosphate-buffered saline (PBS).

The cells were then observed under an Axiovert 25 fluorescence microscope (Carl Zeiss, Germany) equipped with a digital camera and micrograph taken at 10X magnification. Subsequently, the number of attached viable cells was quantified from the micrographs using the ImageJ software.

Annexin V/propidium iodide staining

For assessment of the pro-apoptotic properties of the aqueous fraction, melanoma cell line, A375, was seeded in a 6-well plate (2×10^5 cells/well) and after 24 h, the cells were treated with an aqueous fraction of *C. coggygria* extract at concentrations 40 and 80 µg/mL for 72 h. Treated A375 cells and untreated negative controls were detached with 0.05 % trypsin- 0.53 mM EDTA, harvested and centrifuged for 5 min at 200 ×g, washed twice with PBS and stained with Annexin V and Propidium iodide (PI) (Annexin-V-FLUOS Staining Kit, Roche). After incubating in the dark for 15 min at room temperature, the cells were analyzed under a fluorescence microscope (Olympus BX-41). Apoptotic cells were differentiated from necrotic cells using two separate filters as follows: FITC (560 – 600 nm) for cells in apoptosis and Texas Red (595 - 605 nm) for cells in necrosis.

Colony-forming assay

Cells were seeded at a density of 1×10^3 cells per well in 6-well plates, allowed to adhere overnight and thereafter treated with an aqueous fraction of *C. coggygria* extract at concentrations of 20, 40 and 60 µg/mL. Untreated cells were used in the analysis as negative control. After 7 days, cells were fixed and stained with 2 % methylene blue in 50 % ethanol for 20 min for colony visualization. Colonies containing more than 50 cells were scored under the microscope. Colony-forming ability (percent clonogenicity) was calculated as a percent of the number of

colonies formed after melanoma treatment compared to the control untreated cells.

Alkaline comet assay

Melanoma A375 cells were cultured in 12-well plates at a density of 2.5×10^4 cells/well for 24 h. Subsequently, cells were treated with an aqueous fraction of *C. coggygria* extract at concentrations of 20, 40, and 60 µg/mL for 72 h. Untreated cells in a complete culture medium served as a negative control. The cells were collected by trypsinization, washed with PBS, centrifuged (250×g, 4 °C for 5 min), and re-suspended in ice-cold PBS. The genotoxicity of the aqueous fraction of *C. coggygria* extract was determined by an alkaline comet assay with minor modification [5,7]. A 50 µL cell suspension and 50 µL low-melting-point (LMP) agarose (0.5 %) were homogenized and transferred to slides previously coated with normal agarose (1 %). The slides were cooled at 4 °C for 10 min and were placed in freshly prepared alkaline lysis solution (2.5 M NaCl, 100 mM EDTA-Na_2, and 10 mM Tris (pH > 10), 5 mL of DMSO, and 0.5 mL of Triton X-100). The slides were then transferred to an electrophoresis chamber containing an alkaline buffer (300 mM NaOH, 1 mM EDTA, pH >13.0) and incubated for 15 min at 4 °C for DNA denaturation. Electrophoresis was performed at 23 V and 300 mA (1 V/cm) for 20 min. After electrophoresis, the slides were neutralized in 0.4 M Tris-HCl (pH 7.5) for 15 min, dehydrated twice with 90 and 96 % ethanol for 5 min each, and air dried.

The slides were then stained with 40 µL DAPI (4 g/L) and scoring of Comets was performed using a fluorescence microscope Olympus BX 41 (Hamburg, Germany). The images were analyzed using the CometScore System version 1.5s software. At least fifty randomly selected cells per slide were scored and recorded for each experiment. The experiments were repeated three times and the mean values were calculated. Comets were categorized according to the percentage of DNA in the 'comet' tail [8] as follows: class 1 – no damage (< 5 %); class 2 – low damage (5.1 – 17 %); class 3 – moderate damage (17.1 – 35 %); class 4 – high damage (35.1 – 60 %); class 5 – extremely high damage (over 60 % of DNA material in the comet tail). The genetic damage index (GDI) was calculated using Eq 1.

$$GDI = \{1(n1) + 2(n2) + 3(n3) + 4(n4)\}/N(n0\text{-}n4) \quad \ldots.(1)$$

Where: n0, is the number of cells with class 1; *n*1 = the number of cells with class 2; *n*2 = the number of cells with class 3; *n*3 = the number of cells with class 4; *n*4 = the number of cells with class 5.

Differential scanning calorimetry (DSC)

Malignant melanoma cells (3×10^7 cells), treated for 72 h with 60, 90 and 150 µg/mL aqueous fraction of *C. coggygria* extract, and untreated cells, serving as negative control, were detached by trypsinization, washed with PBS, centrifuged and re-suspended in 800 µL PBS. Differential scanning colorimetry (DSC) was performed on a DASM4 micro-calorimetric system (Biopribor, Pushchino) in a temperature range from 30 – 120 °C at a scanning rate of 1 °C/min. An instrumental (buffer/buffer) baseline was subtracted from the excess heat capacity (CP^{ex}) curves and a linear baseline fit was applied to each calorimetric curve and normalized to the DNA concentration. The thermograms were deconvoluted mathematically using theoretical Gaussian fitting and were analyzed using the Origin software package.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For qRT-PCR analysis, A375 cells in a complete culture medium were treated with *C. coggygria* aqueous fraction at concentrations of 40 and 80 µg/mL for 72 h. Untreated cells incubated for the same period were used as negative controls. The cells were subsequently collected using trypsinization, centrifuged and washed with ice-cold PBS and further centrifuged (250×g for 5 min at 4 °C). Total RNA was isolated using GeneJET RNA Purification Kit (Thermo Scientific) according to the manufacturer's protocol. The concentration and purity of extracted RNA were determined using a BioSpec-nano Spectrophotometer (Shimadzu Biotech). Next, 1 µg total RNA from each sample was reverse transcribed to complementary DNA (cDNA) using First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's recommendations. The relative expression levels of fifteen genes (Table 1) involved in the processes of programmed cell death, cell cycle control, and cell proliferation were studied using qRT-PCR. The housekeeping gene, β-actin, was used as an endogenous control to normalize gene expression. The real-time PCR (RT-PCR) analysis was performed with a PikoRealTM Real-Time PCR System (Thermo Fisher Scientific Inc.) using 1x HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). For amplification, initial denaturation at 95 °C for 15 min, followed by 45 cycles of denaturation (95 °C for 15 sec), annealing (60 °C for 30 sec), and elongation (72

°C for 45 sec) was applied. Gene expression data were analyzed using PikoReal Software version 2.1 (Thermo Fisher Scientific Baltics UAB). Each qRT-PCR reaction was performed in at least five replicates in different PCR runs with cDNA templates from two independent biological experiments. The results are expressed as mean ± SEM.

Data analyses

Statistical differences between untreated control and treated groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test and t-test. A significance level of $p < 0.05$ was employed to denote statistical significance.

RESULTS

A375 cell morphology and adhesion

The morphological characteristics as well as the parallel evaluation of the number of viable adherent cells after treatment with varying concentrations of *C. coggygria* were analyzed by fluorescence microscopy.

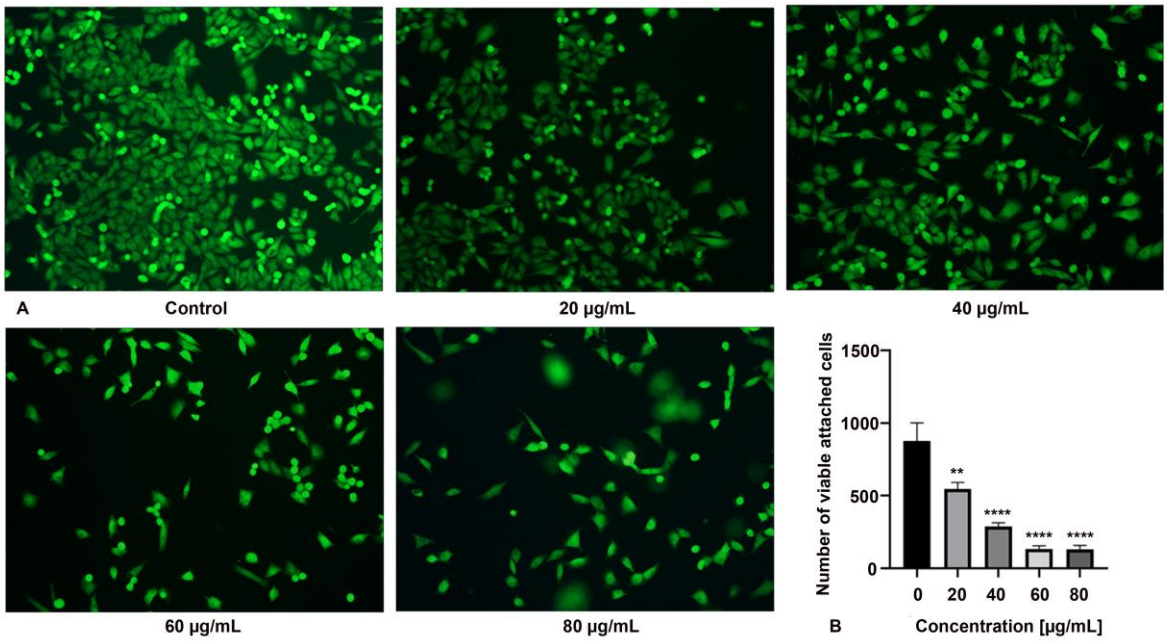

Figure 1: Fluorescent microscopy analysis after labeling with fluorescein diacetate of A375 cells treated with *C. coggygria* aqueous fraction (20, 40, 60 and 80 µg/ml) for 72 h: **A** - Fluorescent micrographs; magnification 10×; **B** - Quantitative evaluation of viable adherent A375 cells. Error bars represent standard error of the mean (SEM). ** $p < 0.01$, **** $p < 0.0001$ vs. control

Table 1: Oligonucleotide sequences of primers used in qRT-PCR analysis

Gene	Forward primer sequence (5′-3')	Reverse primer sequence (5′-3')
p53	GCACTGGTGTTTTGTTGTGG	GTGGTTTCAAGGCCAGATGT
BAX	ATGGACGGTCCGGGGAGCAGC	CCCCAGTTGAAGTTGCCGTCAG
Bcl-2	GGTGCCACCTGTGGTCCACCTG	CTTCACTTGTGGCCCAGATAGG
CASP3	ATGGAGAACACTGAAAACTCA	TTAGTGATAAAAATAGAGTTC
CASP8	AAGCAAACCTCGGGGATACT	GGGGCTTGATCTCAAAATGA
CASP9	CCAGAGATTCGCAAACCAGAGG	GAGCACCGACATCACCAAATCC
Daxx	TCTACAACTTTGGCTGTCACCTC	GTCTCTTCTGTCTCTCGCCCT
BIRC5	AGAACTGGCCCTTCTTGGAGG	CTTTTTATGTTCCTCTATGGGGTC
LKB1	AGGGATGCTTGAGTACGAACC	GTCCTCCAAGTACGGCACC
ATM	TTGGCAAGCTGGAACTCTTT	TGAGCAACTGACTGGCAAAC
CHEK2	GCGCCTGAAGTTCTTGTTTC	CGTAAAACGTGCCTTTGGAT
MYC	TCAAGAGGCGAACACACAAC	TTTCCGCAACAAGTCCTCTT
KRAS	CACGGTCATCCAGTGTTGTC	CACCACCCCAAAATCTCAAC
CDKN1A	TAGCAGCGGAACAAGGAG	AAACGGGAACCAGGACAC
p16INK4a	TGCCCAACGCACCGAATAGTTA	GTGCAGCACCACCAGCGTGTCC
β-actin	CAGAGCAAGAGAGGCATCC	GTTGAAGGTCTCAAACATGATC

The results showed a statistically significant reduction in the number of viable adherent cells compared to the control containing untreated A375 cells (Figure 1). The number of attached cells was reduced by about 38 % even at the lowest applied concentration of 20 µg/mL. Furthermore, the decrease in the number of adherent cells reached 85.06 % at a concentration of 80 µg/mL of the aqueous fraction.

Induction of apoptosis

The results of Annexin V/Propidium iodide staining of malignant melanoma cells treated with varying concentrations of *C. coggygria* aqueous fraction are presented in Figure 2. The results revealed altered A375 cell morphology associated with different phases of apoptosis. In the analysis, A375 cells that did not undergo apoptosis remained unstained, cells in early apoptosis were stained green, and those in late apoptosis had a red-stained nuclear area and green-stained membrane while the necrotic cells were stained in red. Furthermore, a dose-dependent induction of early and late apoptotic events as well as necrotic events were observed.

A375 clonogenicity reduction

Clonogenic analysis, which allows an assessment of the capability of a single cell to proliferate and form a colony or a clone after treatment with specific agents for a long-term period, was performed. Clonogenicity of A375 melanoma cells was determined after treatment for 7 days with concentrations of 20, 40 and 60 µg/mL of the aqueous fraction of *C. coggygria* leaf extract. Significant dose-dependent anti-clonogenic effect was found in *C. coggygria*-treated cells at all the concentrations tested while well-developed colonies were observed in untreated control cells (Figure 3 A). Even at the lowest applied concentration of the fraction (20 µg/mL), a significant reduction in the clonogenicity of melanoma cells to 20.61 % was seen, while exposure to 40 and 60 µg/mL resulted in almost complete inhibition of cancer cells colony-forming ability (Figure 3 B).

The genotoxic capacity of *C. coggygria* aqueous fraction

The genotoxic effect of aqueous fraction of *C. coggygria* extract was determined through alkaline comet assay performed 72 h after treatment with three concentrations of 20, 40 and 60 µg/mL and was compared to negative untreated control groups. The findings showed that the aqueous fraction of *C. coggygria* extract significantly increased the percent of DNA in the tail when compared to the negative control group ($p < 0.001$). The results show that the fraction possesses genotoxic characteristics at concentrations higher than 20 µg/mL ($p < 0.001$; Figure 4).

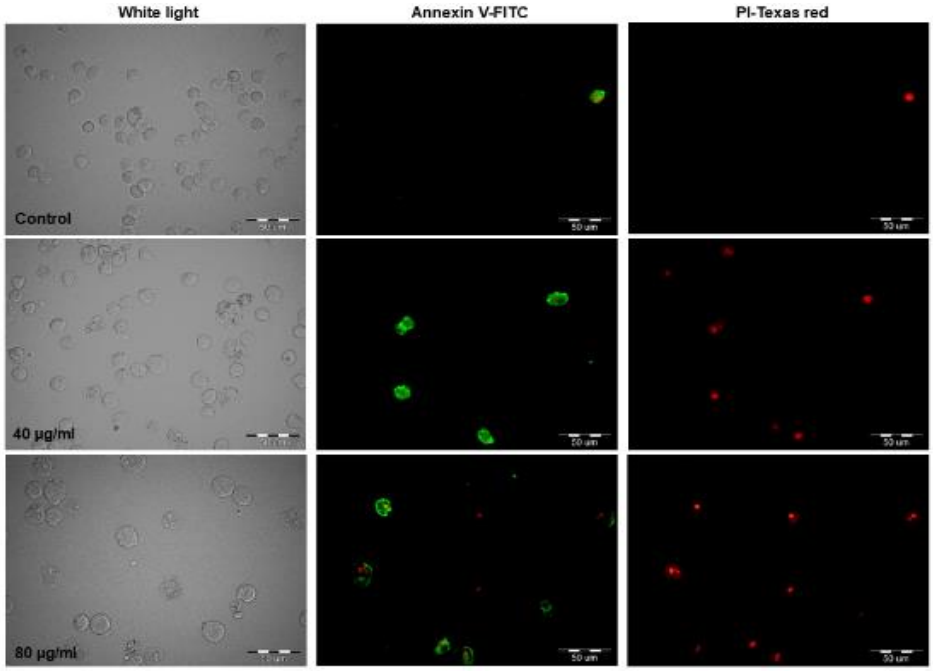

Figure 2: Fluorescent microscopy analysis of A375 cells after Annexin V/propidium iodide staining for visualization of apoptotic and necrotic cells: untreated negative control and *C. coggygria* aqueous fraction treated cells (40 and 80 µg/mL, 72 h). Magnification 40x; bar 50 µm

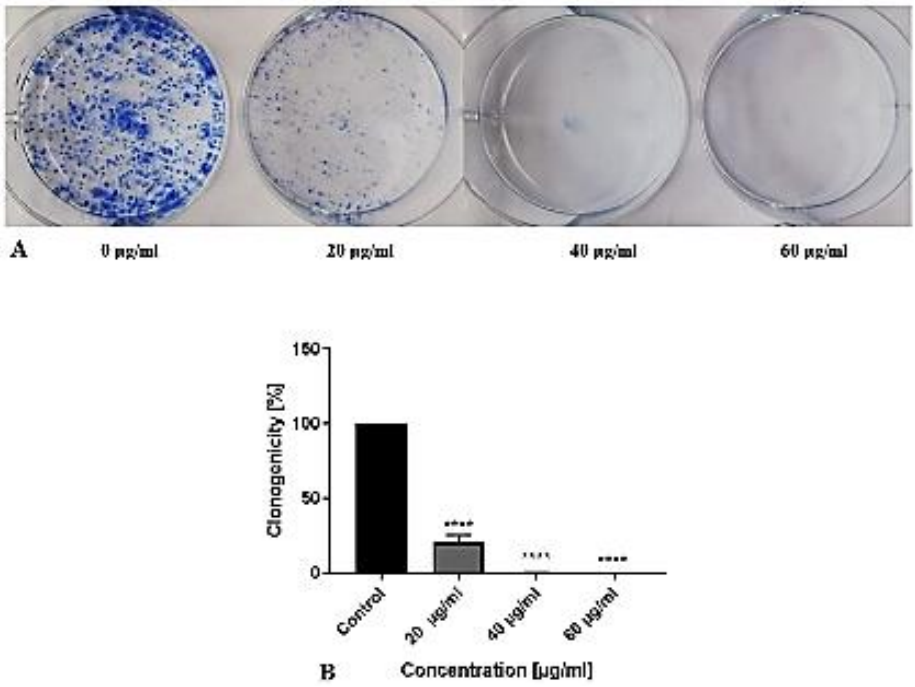

Figure 3: (**A)** Effect of aqueous fraction of *C. coggygria* extract on clonogenicity of A375 cells after treatment for 7 days with 20, 40 and 60 µg/mL when compared to untreated control; (**B)** Quantitative evaluation of A375 cells clonogenicity. Error bars represent standard error of the mean (SEM). **** p <0.0001 vs. control

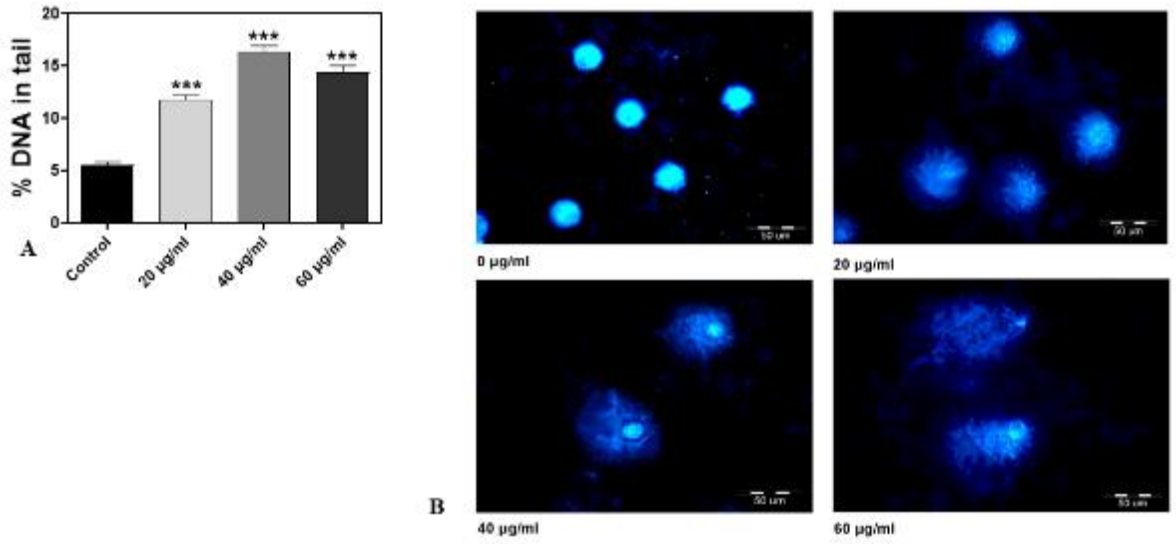

Figure 4: (A) The effect of aqueous fraction of *C. coggygria* extract on % DNA in the tail of melanoma cells A375. Results are presented as mean ± SEM from at least three separate experiments. ***p < 0.001 vs. control; **(B)** Representative A375 comet nucleoids treated without or with aqueous fraction of *C. coggygria* extract to reveal DNA strand breaks

Furthermore, the values of the percentage of DNA in the comet tail were used to classify the analyzed nucleoids (Figure 5) according to the level of DNA damage. Almost all of the nucleoids (more than 90 %) from the control group formed comets belonging to Class 1 and Class 2 with scanty migration of DNA during electrophoresis (Figure 5A). Only a small portion of the nucleoids in the control corresponded to Class 3 (approximately 6 %) and Class 4 (2.6 %) based on the percentage of DNA in the comet tail (Figure 5A). Exposure to the aqueous fraction of *C. coggygria* extract resulted in a significant increase in DNA damage evident in the redistribution of comet classes (Figure 5 B-D). At 20 µg/mL concentration, there was a decrease in the population of the Class 1 and also an increase in Class 2 comets. In addition, there was an increase in the appearance of Class 3, Class 4 and Class 5 comets, signifying increased

fragmentation of DNA. The largest scale changes were observed in the nucleoids of the melanoma treated with 40 µg/mL and 60 µg/mL of the fraction. The number of comets of Classes 3 and 4 increased to ~ 35 %, while comets of Class 5, which corresponds to the highest level of DNA fragmentation, increased to 2.25 %.

Table 2: Genetic damage index (GDI) in A375 melanoma cells exposed to aqueous fraction of *C. coggygria* extract

Genetic damage index (GDI)	
Control	0.42
20 µg/mL	0.87
40 µg/mL	1.2
60 µg/mL	1.05

In addition, the genetic damage index (GDI), which is based on the quantitative distribution of the comet classes, was calculated and is presented in Table 2. The data show that the treatment of the melanoma cells with the plant fraction resulted in a statistically significant increase in DNA migration during electrophoresis and in an increase in the GDI indicating moderate fragmentation of the genome. The GDI was highest at the concentration of 40 µg/mL.

Thermodynamic properties of A375 cells

Differential scanning calorimetry evaluation of the effect of *C. coggygria* aqueous fraction on the thermodynamic cell characteristics was determined after treatment for 72 h with fraction concentrations of 60, 90 and 150 µg/mL and compared to untreated cells.

The DSC profiles of untreated and treated melanoma cells are presented in Figure 6. Alterations of the thermal transitions in the low- and high-temperature regions of the calorimetric profiles in the treated cells were established. This could be associated with a slight stabilization of cytoplasmic proteins and structural destabilization in the nuclear matrix.

Expression of genes involved in cell proliferation and apoptosis

In order to elucidate the target molecules and mechanisms through which the aqueous fraction of *C. coggygria* extract accomplishes its anticancer effect, the relative expression levels of specific genes with a key role in the processes of apoptosis induction, cell cycle control and cell proliferation (15 genes) were evaluated by qRT-PCR. The expression levels in treated cells at 72 h were compared to the gene expression in untreated cells at the same point in time. The results obtained using qRT-PCR analysis detected statistically significant changes in the relative expression levels of four genes viz: *BAX*, *CASP8*, *MYC* and *CDKN1A* after treatment with the fraction in comparison to the untreated cells (Figure 7).

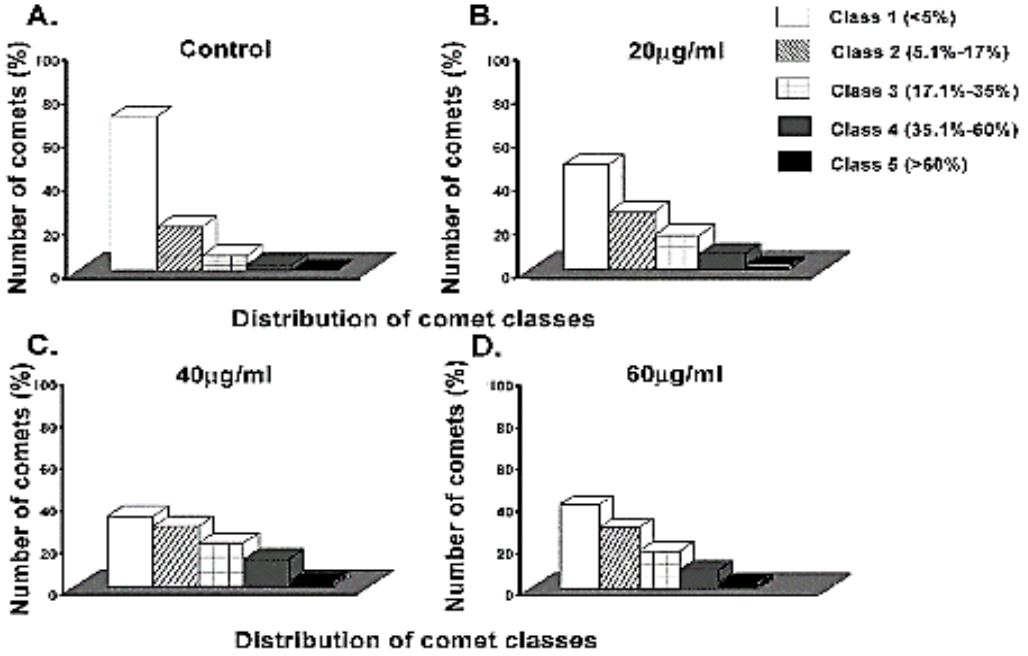

Figure 5: Classification and distribution of nucleoids after treatment with aqueous fraction of *C. coggygria* extract **(A)** without treatment; **(B)** 20 µg/mL; **(C)** 40 µg/mL; **(D)** 60 µg/mL. The graph shows that all three treatments exhibit comets of greater % DNA in the tail compared to control

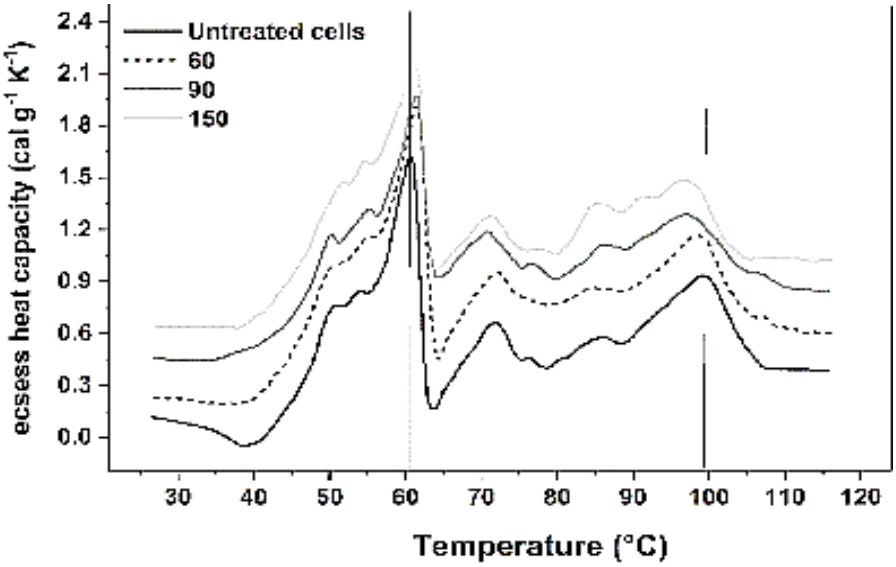

Figure 6: DSC thermogram of control A375 cells (black line) and A375 cells treated with *C. coggygria* aqueous fraction for 72 h with concentrations of 60 µg/mL (dash), 90 µg/mL (dark gray line) and 150 µg/mL (light gray line)

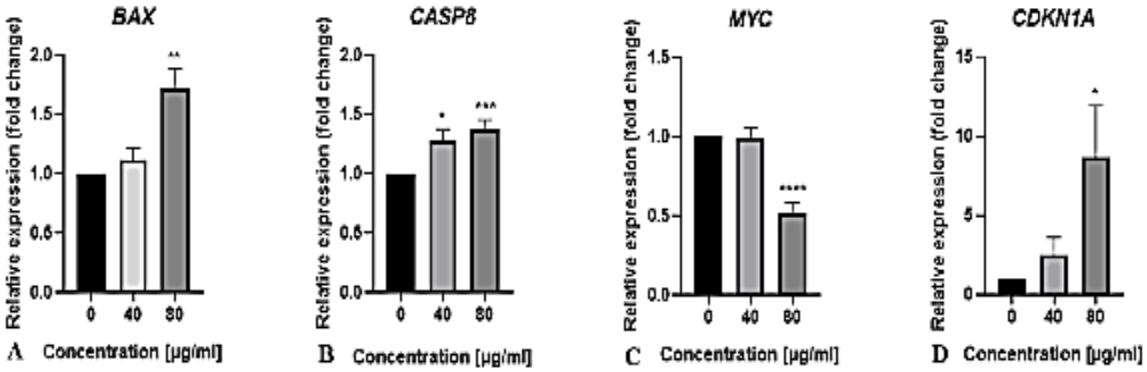

Figure 7: Relative expression levels of *BAX* (A), *CASP8* (B), *MYC* (C) and *CDKN1A* (D) genes at 72 h after treatment of A375 cells with aqueous fraction of *C. coggygria* extract at concentrations of 40 and 80 µg/mL. Black columns refer to untreated controls. Error bars represent standard error of the mean (SEM); β-actin was used as an endogenous control to normalize the expression of the genes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. control group

The fraction increased the number of mRNA transcripts of proapoptotic gene, *BAX,* at a concentration of 80 µg/mL, upregulates *CASP8* in a dose-dependent manner, significantly reduced *MYC* expression and increased more than 8-fold, the relative expression levels of *CDKN1A* gene after treatment at a concentration of 80 µg/mL.

DISCUSSION

Plant-based medicinal products with multi-target properties, effectiveness against various types of oncological diseases, and capability to potentiate chemosensitivity and to produce little or no side effects, are of great interest in oncopharmacology. The antitumor potential of *Cotinus coggygria* on skin cancer model system, which is the most prevalent oncology disease among the European population, has not been explored. Previously, our group reported strong antiproliferative properties of *C. coggygria* against A375 melanoma and A431 nonmelanoma cell lines [6].

In this work, induction of apoptosis and strong inhibition of the clonogenic activity of melanoma cells was confirmed by qRT-PCR analysis, which found alterations in relative expression of genes involved in apoptotic pathways and regulation of cell proliferation. A considerable increase in the relative expression levels of *CDKN1A* (Cyclin Dependent Kinase Inhibitor 1A) gene encoding the p21 protein, which functions as a negative regulator of cell cycle progression at G1, was observed. The qRT-PCR result also showed a statistically significant reduction in the expression of the *MYC* proto-oncogene. *MYC* is involved in the pathogenesis of most types of cancer in humans and its activation is associated with

autonomous proliferation and growth, unrestricted DNA replication, increased protein biogenesis, changes in cellular metabolism and induction of angiogenesis. These observations corroborate our previous data obtained by MTT analysis, which established strong cytostatic activity of *C. coggygria* in A375 cells [6]. This result indicates cell growth inhibition as a leading mechanism of anticancer action in A375 malignant melanoma cells. In addition, fluorescence microscopic analysis, comet assay and qRT-PCR analyses showed pro-apoptotic activity of *C. coggygria.* A statistically significant increase in the transcriptional levels of *BAX* and *CASP8* genes in A375 cells was observed. *BAX* is a proapoptotic gene and mediates cell death while *CASP8* mediates apoptosis by stimulating both death receptors and/or the mitochondrial apoptotic pathway [9].

Presently, no study on the mechanism of action of *C. coggygria* in skin cancer cell lines has been conducted. However, Pollio and co-workers reported that the methanol extract of *C. coggygria* branches and leaves showed considerable cytotoxic and cytostatic effects on A549 lung cancer cell line, MCF7 breast cancer cells, U937 histiocytic lymphoma, and TK6 human B lymphoblastoid cells [10]. In MCF7 cell line, the extract modulates the expression of proteins with a key role in cell cycle control and cell death regulation, increases p53 and p21 expression and decreases the expression of Cdk2. Another study showed that in glioblastoma cells, *C. coggygria* ethanol extract from roots and stems inhibits cell proliferation and induces apoptosis via Akt inhibition resulting in suppression of the PI3K/Akt pathway [11]. A significant influence on the expression of ERK and p-ERK proteins was also reported which leads to cell proliferation inhibition and induction of apoptosis.

This report identifies the specific mechanisms and pathways by which the antitumor properties of the medicinal plant in the specific skin cancer model system are manifested. The effect of the aqueous fraction on other melanoma cell lines as well as on animal models needs to be ascertained in the future to determine the reproducibility of the results.

CONCLUSION

The results of the present research demonstrate that the aqueous fraction from the leaves of the *Cotinus coggygria* possesses significant anti-melanoma potential. The fraction exhibits proapoptotic effects against A375 human malignant melanoma skin cancer cells, significantly decreases the number of viable adherent cells, inhibits cell cycle progression, represses A375 colony formation capacity, induces primary DNA damage in melanoma cells, and influences cytoplasmic proteins and matrix components. Future studies will be directed to *in vivo* assessment of the anti-melanoma properties of the plant on an animal experimental model system.

DECLARATIONS

Acknowledgements

None provided.

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. ZG and GA designed the study; all authors performed experiments; ZG, GA, MG, ST, and AD analyzed the results; ZG wrote the main part of the manuscript, and supervised the study; GA and MG participated in the writing of the manuscript; all authors read the article and approved the final version of the manuscript for publication.

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