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ORIGINAL ARTICLE

ANTIPROLIFERATIVE ACTIVITY OF NATURAL FLAVONOID FUSTIN ISOLATED FROM THE HEARTWOOD OF *COTINUS COGGYGRIA* SCOP. AGAINST BREAST AND COLON CANCER CELL LINES

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Abstract. Background and objective: *Cotinus coggygria Scop. is a valuable medicinal plant species with pronounced pharmacological potential due to its numerous biological activities. The herb is characterized by a high content of polyphenols among which is fustin. The anticancer activities of fustin, however, are extremely weakly studied. The aim of the present study was to investigate the in vitro antiproliferative potential of fustin isolated from the heartwood of C. coggygria against cell lines originating from two of the most common cancer types – breast (MDA-MB-231 and MCF7), and colon cancer (Colon 26).* ***Materials and methods:*** *Cell growth inhibitory properties of fustin were examined by MTT assay. Subsequently, phase-contrast and fluorescence microscopy analysis as well as colony-forming assay were carried out on the most sensitive to the cytostatic action of the fustin cell line.* ***Results:*** *The obtained results showed that fustin reduced the proliferation of all studied cell lines. The highest cytostatic effect was registered towards breast cancer MDA-MB-231 cells with a half maximal inhibitory concentration (IC_{50}) value of 56.02 μg/ml followed by colon cancer cells with an IC_{50} of 78.07 μg/ml. MCF7 cell proliferation was least affected with a calculated IC_{50} of 187.8 μg/ml. Further investigations on breast cancer MDA-MB-231 cells indicated decreased density of cell monolayer and some morphological alterations, significant attenuation in the number of viable cells, and diminished clonogenic ability of cells after fustin exposure.* ***Conclusion:*** *It could be concluded that fustin isolated from the heartwood of medicinal plant C. coggygria possesses marked antiproliferative properties against breast cancer cell line MDA-MB-231 which will be a subject of our more detailed future investigations.*

Key words: *fustin, C. coggygria Scop., antiproliferative effect, MDA-MB-231, MCF7, Colon 26*

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INTRODUCTION

Cancer is one of the leading causes of mortality worldwide with about 10 million deaths reported in 2020 [1]. Considering the adverse and often life-threatening side effects of cancer treatment, the need for more effective, non-toxic, well-tolerated, and at the same time cost-effective and readily available anticancer agents is imperative.

Medicinal plants play an increasingly important role in the therapy and prevention of a number of diseases, including cancer, both independently or complementary to conventional therapy. *Cotinus coggygria* Scop. (Anacardiaceae), also known as European smoke tree, is a medicinal plant with a wide range of distribution from southern Europe, Central Asia and the Himalayas to northern China. The herb is traditionally used in folk medicine for treatment of skin ailments, paradontosis, gastric and duodenal ulcers, diarrhea, cardiac and renal diseases, diabetes, asthma, cough, liver disease, cancer, and many others. The plant also possesses various proven biological and therapeutic properties, such as antihaemorrhagic, antipyretic, antiseptic, immunomodulatory, antioxidant, anti-inflammatory, antigenotoxic, anticancer, hepatoprotective, antibacterial, antiviral, etc. [2, 3].

C. coggygria is well-distinguished by the presence of wide range of polyphenolic secondary metabolites, including sulfuretin (3′,4′,6-trihydroxyaurone), fisetin (3′,4′,7-trihydroxyflavonol), taxifolin (5,7,3’,4’-flavanon-ol), quercetin (3,3’,4’,5,7-pentahydroxyflavone), butein (trans-2’,3,4,4’-tetrahydroxychalcone), butin (3’,4’,7-trihydroxyflavanone), rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside),liquiritigenin(4’,7-dihydroxyflavanone), isoliquiritigenin (trans-2’,4,4’-trihydroxychalcone), gallic acid (3,4,5-trihydroxybenzoic acid), 4’,5,7-trihydroxyflavanone, eriodictyol (3′,4′,5,7-tetrahydroxyflavanone), fisetinidol-(4α→8)-(+)-catechin, epifisetinidol-(4β→8)-(+)-catechin, epoxide (2,10-oxy-10-methoxysulfuretin), cotinignan A, etc. [4-6]. Among the polyphenolic compounds in the medicinal shrub is fustin (3’,4’,7-trihydroxyflavanol), also known as “dihydrofisetin”. Fustin is a flavanonol subtype of flavonoid.

Flavonoids are a major class of phytochemicals with about 4000 types reported in plants [7] and are intensively studied in recent years due to their chemopreventive and chemotherapeutic properties against different kinds of cancer [8].

Fustin is weakly studied in regard to its anticancer and chemopreventive qualities. There are only a few reports stating that fustin promoted cell death of multiple myeloma cells and suppressed tumor cell growth *in vivo* in BALB/c mice in combination with epigallocatechin-3-O-gallate [9], and possesses protective activity against chromosome aberrations in peripheral human lymphocytes [10].

AIM OF THE STUDY

Taking into account the limited data about the anticancer potential of the natural flavonoid fustin the aim of the present research was to assess the *in vitro* antiproliferative capacity of fustin isolated from the heartwood of *C. coggygria* against cell lines originating from two of the most common cancer types – breast and colon cancer.

MATERIALS AND METHODS

Plant material and extraction

The *C. coggygria* heartwood was collected at Deliblatska Peščara (Deliblato Sand), Vojvodina province, Serbia, in May 2019. Plant material was identified by Prof. Milan Veljic, Faculty of Biology, University of Belgrade, and voucher specimen BEOU 17422 was deposited at the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Belgrade, Serbia. The heartwood was air-dried and milled to a fine powder. Wood powder, 1 kg, was extracted three times with 10 l of methylene chloride/methanol 1:1 for 24 h at room temperature to give 76 g of the crude extract which was subjected to fractionation by Si gel CC.

Isolation and identification of fustin

For column chromatography (CC) Merck silica gel (Si gel) (particle size 0.063-0.200 mm), methanol and methylene chloride were used. Analytical TLC was performed on aluminium plates precoated with Merck silica gel 60 F254 (0.25 mm thickness). The NMR spectra were obtained on a Bruker Avance III 500 (500 MHz for ^{1}H; 125 MHz for ^{13}C), in CD_3OD as solvent. Chemical shifts (d) were expressed in

ppm and coupling constants (J) in hertz (Hz). Semi-preparative reversed phased HPLC was performed on Agilent Technologies 1100 Series HPLC-DAD and Zorbax Eclipse XDB C18 column (150 × 9.4 mm, i.d. 5 µm) was used.

Crude extract was chromatographed on a Si gel CC column (750 × 45 mm), with methylene chloride/methanol (gradient elution – from 97/3 to 60/40). This step was repeated seven times to obtain high amounts of fustin. Column chromatography was monitored by TLC, and the fractions with similar Rf values were combined. Fustin was found in fractions eluted with methylene chloride/methanol approximately 80:20. Pure fustin was isolated from these fractions by reversed phase semi-preparative HPLC using water/acetonitrile system, 254 nm for detection and the following program: 0-20 min, 20-37% CH_3CN; 20-21 min, 37-50% CH_3CN; 21-27 min, 50% CH_3CN; and 27-30 min, 50-100% CH_3CN. Fustin was purified up to 98% on reversed phase semiprep. HPLC using the following program: 0-20 min, 25-40% CH_3CN (Rt = 5.1 min).

Cell lines and culturing conditions

Two human breast cancer cell lines, MDA-MB-231 (triple negative breast cancer subtype – TNBC) and MCF7 (luminal A breast cancer subtype), and a murine colon adenocarcinoma cell line Colon 26 were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

MTT cell proliferation assay

Cell proliferation was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [11]. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and after overnight incubation were treated with increasing concentrations of fustin (5-135 µg/ml) in a new medium for 72 h. As a negative control were used untreated cells cultured in a medium for the same time period.

During the last 4 h of the incubation 20 µl MTT solution with a concentration of 5 mg/ml per well was added and the samples were incubated in dark. At the end of incubation the medium was removed, the formazan complex was dissolved in 10% SDS and 0.01 M HCl, and the absorbance was measured at 570 nm on a microplate reader (Thermo Scientific Multiskan Spectrum). The percentage of cell proliferation was calculated using the following formula:

Cell proliferation (%) = (Absorbance test sample/Absorbance control) × 100

Phase-Contrast Light Microscopy

Phase-contrast observation of the cells was done in order to monitor any alterations in their morphology after treatment with fustin at concentrations of 35, 55, and 75 µg/ml for 72 h. Phase-contrast micrographs were taken at magnifications of 10× with a Leitz microscope equipped with a digital camera.

Fluorescent Microscopy

Fluorescein diacetate (FDA) staining of cells was performed for visualization and a quantitative evaluation of viable metabolic active cells after 72 h treatment with the studied bioflavonoid at concentrations of 35, 55, and 75 µg/ml. At the initial step of the analysis, 1.25×10^4 cells were seeded on round coverslips placed in a 24-well plate, incubated overnight for attachment and treated on the next day with fustin. At the end of the incubation time, cells were observed at a magnification of 10× under fluorescent microscope Axiovert 25 (Carl Zeiss, Germany) equipped with a digital camera after staining for 2 min with 0.001% FDA dissolved in acetone and washed with 1× Phosphate Buffered Saline (PBS). Afterward, the micrographs were analyzed by ImageJ software to count the number of viable attached cells.

Colony-forming assay

For the analysis evaluating cell capability to proliferate and form a colony, cells were seeded at a density of 1×10^3 cells per well in 6-well plates, allowed to attach overnight, and treated with 35 µg/ml fustin. After 5 days, cells were fixed and stained with 2% methylene blue in 50% ethanol for 20 min for colony visualization.

Statistical analysis

Data were presented as means ± standard error of the mean (SEM) of three independent experiments each performed in at least three parallel repeats. The half maximal inhibitory concentration (IC_{50}) values were calculated by GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Statistical differences between control untreated and treated groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test and the results were considered significant if $p < 0.05$.

RESULTS

Antiproliferative potential of C. coggygria isolated fustin on MDA-MB-231, MCF7 and Colon 26 cells

The antiproliferative properties of fustin isolated from the heartwood of *Cotinus coggygria* were studied on human breast cancer cell lines MDA-MB-231 and MCF7, and a murine colon adenocarcinoma cell line Colon 26

after 72 h period of treatment in the range of concentrations from 5 to 135 μg/ml through MTT assay.

The obtained results displayed cytostatic effect of fustin on all tested cell lines (Fig. 1) with the highest cell growth inhibitory activity against the MDA-MB-231 cell line representing triple negative breast cancer subtype with an IC_{50} value of 56.02 μg/ml and maximal registered reduction of proliferation to 40.39% at 115 μg/ml. A considerable cytostatic effect was also found for colon adenocarcinoma cells Colon 26 (IC_{50} = 78.07 μg/ml). The antiproliferative effect was weakest for the MCF7 cell line, the luminal A breast cancer subtype, with a calculated IC_{50} concentration of fustin of 187.8 μg/ml. Statistically significant differences between treated and control groups with p-values of less than 0.0001 were found for all of the tested concentrations of fustin, even at the lowest doses of 5 μg/ml, in the three cancer cell lines (Fig. 1).

MDA-MB-231 cell morphological examination

Phase-contrast micrographs showed that 72 h treatment of MDA-MB-231 cells with fustin at concentrations of 35, 55, and 75 μg/ml reduced significantly the density of cell monolayer when compared to the control cells. As can be seen in Fig. 2, most cells displayed a well-spread polygonal shape with the formation of large lamellipodium. Many cells with an elongated shape with long and thin extensions were also observed as well as cells with a round shape mostly in the samples treated with 35 μg/ml fustin.

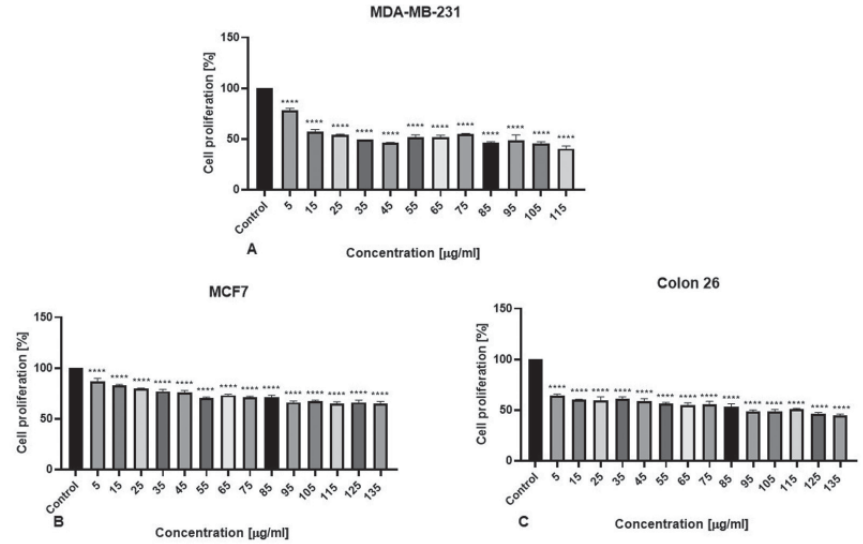

Fig. 1. MTT assay of MDA-MB-231 (A), MCF7 (B) and Colon 26 (C) cells treated for 72 h with increasing concentrations of fustin. Error bars represent standard error of the mean (SEM). **** indicates significant differences from the control group by Dunnett's test (**** $p < 0.0001$)

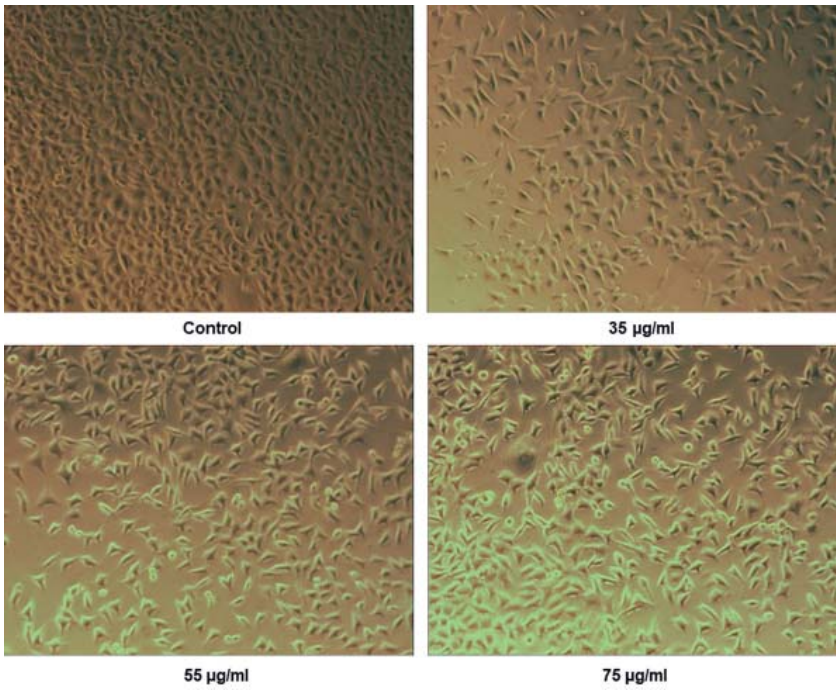

Fig. 2. Phase-contrast micrographs of MDA-MB-231 cells incubated for 72 h with fustin at concentrations of 35, 55, and 75 μg/ml; magnification 10×

Effects of fustin on MDA-MB-231 cell viability

Fluorescein diacetate is an uncharged, non-fluorescent, lipid-soluble dye that crosses biological membranes and can be hydrolyzed by intracellular esterases of the living cells to polar and fluorescent fluorescein. Free fluorescein is retained by intact living cells and its accumulation results in fluorescing cells.

Fluorescence microscopic analysis of cells stained with FDA was carried out to visualize MDA-MB-231 overall cell morphology and to evaluate the number of viable adherent cells 72 h after cell exposure to the fustin in concentrations of 35, 55, and 75 µg/ml.

A quantitative analysis of fluorescent micrographs showed a statistically significant decrease in the number of viable attached cells in comparison to the control containing untreated breast cancer cells which corresponds to the data from the MTT assay (Fig. 3). The registered effect was not dose-dependent and the strongest inhibitory effect on cell viability was observed at the lowest applied concentration of fustin of 35 µg/ml where the percentage of living cells was decreased to 37%, while at the highest applied concentration of 75 µg/ml the survival rate was 61.6%.

Inhibitory effect of fustin on clonogenicity of MDA-MB-231 cells

Clonogenic potential of breast cancer cells MDA-MB-231 was investigated after treatment with a concentration of 35 µg/ml of fustin for a period of 5 days. A considerable anticlonogenic effect in treated cancer cells was observed in comparison to the untreated controls (Fig. 4), which is an indication for inhibitory action of the studied phytochemical on breast cancer cells' reproductive integrity.

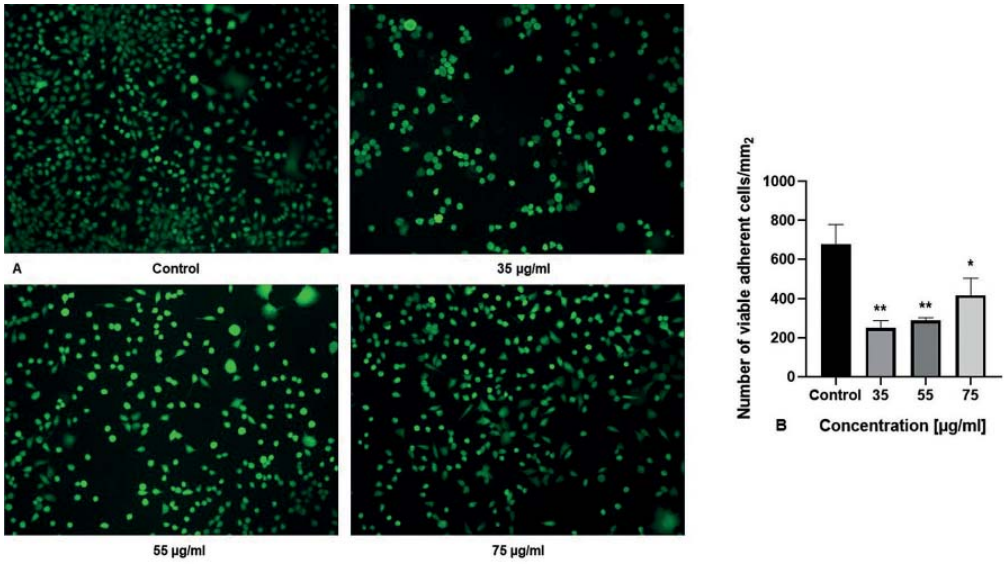

Fig. 3. Fluorescent microscopy analysis of MDA-MB-231 cells stained with fluorescein diacetate after treatment with fustin (35, 55, and 75 µg/ml) for 72 h: A – Fluorescent micrographs; magnification 10×; B – Quantitative evaluation of viable attached MDA-MB-231 cells. Error bars represent standard error of the mean (SEM). * and ** indicate significant differences from the control group by Dunnett's test (* $p < 0.05$, ** $p < 0.01$)

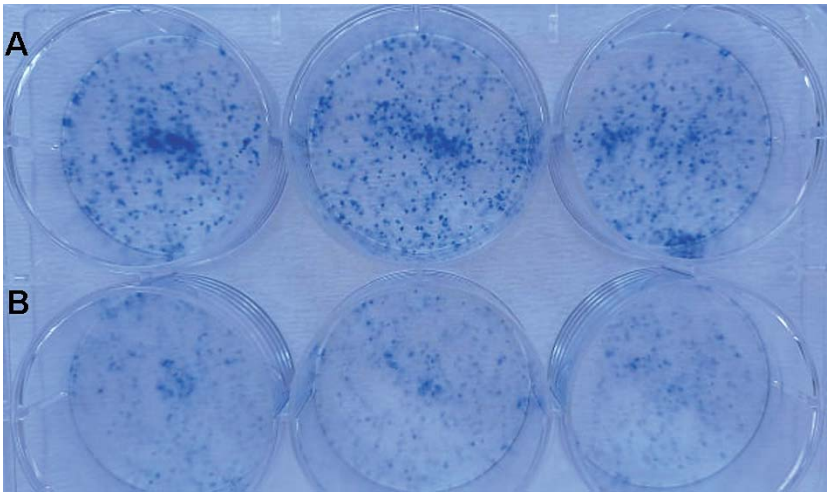

Fig. 4. Effects of fustin isolated from *C. coggygria* on clonogenicity of MDA-MB-231 cells after treatment for 5 days with 35 µg/ml (B) compared to untreated control (A)

DISSCUSSION

In the present research, we studied the antiproliferative potential of the flavonoid fustin isolated from the heartwood of *C. coggygria* in three cancer cell lines – breast (MDA-MB-231 and MCF7), and colon (Colon 26) and found that fustin can significantly inhibit cell growth of all studied cell lines. We chose the above-mentioned cell lines in view of the fact that they originate from two of the most frequent cancer types. According to the World Health Organization (WHO), newly diagnosed cases for 2020 for breast cancer amounted to 2 260 000 and for colon cancer – 1 930 000 [1]. The number of deaths associated with the disease are 684 996 (amounting to 6.9% of all cases) for breast cancer, and 935 173 (9.4%) in regard to colon cancer. Among the studied cell lines, the most sensitive to the fustin action was the MDA-MB-231 breast adenocarcinoma cell line. Therefore, we decided to perform further investigations including microscopic observation and clonogenic assay only of MDA-MB-231 cells after fustin exposure. Fluorescent microscopy observation after staining with FDA of treated MDA-MB-231 cells revealed a pronounced but not dose-dependent decrease in the number of viable attached cells. The results obtained from the clonogenic assay showed a high reduction in the ability of MDA-MB-231 cells to form colonies after treatment with fustin at a concentration of 35 μg/ml. The absence of dose-dependence and reduction of antitumor activity at high doses of application is not unusual for some plant extracts, active compounds, and chemotherapeutic agents [12, 13]. A possible reason for the lack of concentration dependence of the anticancer effects could be the depletion of specific targets of action.

Breast cancer represents a heterogeneous disease with high rates of intra- and intertumoral diversity and variability of response to different treatments. The MDA-MB-231 cell line used in the current research is triple negative breast cancer (TNBC) subtype defined by the absence of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) amplification/overexpression. Even though it accounts for about 15-20% of all breast cancers, it is an aggressive subgroup, associated with poorer prognosis and a higher rate of distant recurrence compared to estrogen receptor-positive subtypes. MCF7 cell line represents the luminal A breast cancer subtype, characterized by ER- and PR-positive and HER2-negative molecular profile. The luminal A subtype is the most common of all breast cancers, accounting for 50-60% of the cases. It is characterized by a low histological grade and a good prognosis with a low relapse rate. TNBC has restricted therapy options when compared to the other types of breast cancer with a growing need for novel naturally-derived treatment approaches with improved efficacy and reduced toxicity.

Flavonoids are polyphenolic compounds with a wide range of pharmacological activities, including anticancer [14]. Among their anticancer effects are induction of programmed cell death, cell cycle arrest, metastasis and invasion inhibition, and modulation of ROS-scavenging enzyme activities [15-17]. The observed in this study *in vitro* anticancer properties of the flavonoid fustin are of great interest in order to evaluate the antineoplastic potential of this still weakly studied flavonoid. To the best of our knowledge, this is the first study reporting an anticancer effect of fustin on breast and colon cancer cell lines. The obtained data, including cytostatic, cell viability inhibiting and anticlonogenic effects, indicate fustin as a perspective candidate for further more detailed analysis of its therapeutic potential against triple negative breast cancer subtype.

In conclusion, the here presented results revealed that the flavonoid fustin isolated from *Cotinus coggygria* heartwood possesses antiproliferative properties against three cancer cell lines: breast (MDA-MB-231 and MCF7), and colon (Colon 26) cancer. The strongest cell growth inhibitory effect was established against the MDA-MB-231 triple negative breast cancer cell line. Fustin caused a significant reduction in the number of viable MDA-MB-231 cells and demonstrated considerable anticlonogenic properties. Further studies will be focused on more detailed characterization of molecular mechanisms and targets of fustin anticancer action.

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Z. Gospodinova and G. Antov have contributed equally to this work.

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