# 1090A:Oncotherapeutic and gastro-entero-hepatoprotective potential of bioflavonoids: the example of fustin, isolated from the heartwood of Eurasian smoketree (*Cotinus coggygria* Scop.), Anacardiaceae

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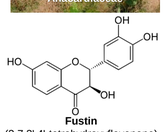
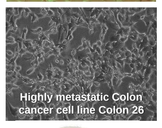
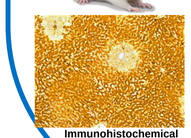
## Overview

The research on biologically active compounds and identification of new molecular targets are relevant to the development of drug therapy. From all approved antitumor drugs from 1946 to 2019, 79% are with natural origin, natural product derivatives, or structures inspired by natural products. However, considering the vast variety of plant metabolites, only small percentage of bioflavonoids have been fully analyzed for their possible potential and future application in oncotherapy. For this reason, in-depth research into more effective and safer plant anticancer substances is of particular importance. The medicinal plant *Cotinus coggygria* has a high content of polyphenols, including dihydroflavonol **fustin**, which is poorly studied in preclinical experiments. Our *aim* is to explore the pharmacological activity of fustin in experimental models with human tumour cell lines and *Wistar* rats.


**Fustin**
(3,7,3',4'-tetrahydroxyflavanone)
PubChem CID: 5317435

Immunohistochemical staining for NF-κB in rat liver

Fustin was isolated and purified from *C. coggygria* heartwood by RP-HPLC and NMR-spectroscopy.

The *in vitro* anticancer potential was assessed on human cell lines from breast cancer, colon cancer, malignant skin melanoma and squamous cell skin carcinoma. Gene expression analysis of the ability of fustin to affect mechanisms of apoptosis, migration and adhesion, and block the cell cycle was performed by qRT-PCR.

The *in vivo* study of anti-inflammatory and organoprotective effects of fustin was conducted by biochemical analyses, histopathological evaluation and immunohistochemical tests on 4 rat models of:

- carrageenan-induced acute paw inflammation (n=30);
- indomethacin-induced gastric ulceration (n=30);
- trinitrobenzene sulfonate-induced colitis (n=40);
- paracetamol-induced hepatotoxicity (n=48).

Proapoptotic and anti-migratory activity of fustin on breast cancer and colon cancer cells was found. Its anticancer properties are associated with alterations in transcriptional levels of nine genes involved in apoptosis, autophagy and cell cycle control. Fustin suppresses the first phase of acute inflammation, similar to NSAIDs. It exerted a gastro-protective action, reduced the expression of NF-κB and ameliorated some histopathological parameters of acute colitis and hepatotoxicity.

## Introduction

In recent decades cancer treatment is evolving, but nevertheless it is still not completely effective, precise and health-sparing. Some of the many challenges, often life-threatening, that patients encounter are low cellular permeability, dose-related toxicity, congenital and/or acquired chemotherapy resistance and various types of treatment-related adverse events (trAEs). For these reasons, the search for an appropriate and effective treatment approach is particularly imperative. It is well known that plant bioactive molecules have the potential to overcome or reduce some of the negative side effects observed after the administration of conventional anticancer drugs.

***Cotinus coggygria*** Scop., a deciduous shrub native to southern Europe, central Asia and China, has been used for therapeutic purposes since ancient times. In traditional medicine, it is used for the treatment of skin and mucosal lesions, gingivitis, pharyngitis, stomatitis, and gastritis, as well as an antipyretic remedy, against cardiac and urinary diseases and diabetes. It has a high content of polyphenols, including fustin, which is poorly studied in preclinical experiments. Our **aim** is to explore the *in vitro* and *in vivo* pharmacological activity of fustin in experimental models with human tumour cell lines and Wistar rats.

## Methods

❶***Isolation and purification of fustin***: Air-dried *C. coggygria* heartwood milled to 1 kg fine powder, extracted 3 times with 10 L $CH_2Cl_2/CH_3OH$ 1:1 for 24 h; After evaporation, 76 g crude extract fractionated by CC on Merck silica gel column (750 × 45 mm) with particle size 0.063-0.200 mm; $CH_2Cl_2/CH_3OH$ gradient solvent system; CC screened by TLC on aluminium plates precoated with Merck silica gel 60 F254 (0.25 mm thickness), fractions with similar Rf values combined; Fustin-rich fractions, detected by NMR-spectroscopy eluted with $CH_2Cl_2/CH_3OH$ at 90/10 to 80/20; Pure fustin isolated by RP-semi-preparative-HPLC on an Agilent Technologies 1100 Series with Zorbax Eclipse XDB C18 column (150 × 9.4 mm, i.d. 5 µm) at 254 nm for detection, and $H_2O/CH_3CN$ system: 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 purified up to 98% on RP-HPLC using the following program: 0-20 min, 25-40% $CH_3CN$ (Rt= 5.1 min); NMR-spectroscopy for structure elucidation on a Bruker Avance III 500 (500 MHz for $^1H$; 125 MHz for $^{13}C$), in $CD_3OD$ as solvent. Chemical shifts (δ) were expressed in ppm and coupling constants (J) in hertz (Hz).

❷***Assessment of in vitro anticancer potential of fustin*** on 5 tumor cell lines: human breast cancer (MCF7, MDA-MB-231), murine colon cancer (Colon 26), human malignant skin melanoma (A375) and human squamous cell skin carcinoma (A431), as well as human non-tumorigenic breast epithelial cell line (MCF10A) and normal human dermal cell line (BJ).

*Cells cultivation:* Cell lines obtained from ATCC (Manassas, Virginia, USA); cultured in DMEM+10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% $CO_2$; subcultured twice a week at a split ratio of 1:4.

*MTT tests:* Cell proliferation was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay; Cells seeded in 96-well plates, density of $5\times10^3$ cells per well, after overnight incubation treated with 5 - 135 µg/ml fustinin a new medium for 72 h; Untreated cells were negative control. During the last 4 h of incubation 20 µl MTT 5 mg/ml per well added; medium then removed, formazan complex dissolved in 10% SDS, 0.01M HCl; absorbance measured at 570 nm on microplate reader (Thermo Scientific Multiscan Spectrum). Cell proliferation (%) was calculated using the formula:

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

*Fluorescence microscopy analysis of cell death*: Cells seeded in 6-well plates, density of $1 \times 10^5$ cells/mL; after 24 h treated with 35 and 75 µg/mL fustin for 72 h. Treated cells and untreated negative controls detached with 0.05 % trypsin-0.53 mM EDTA, harvested, centrifugated for 5 min at 200×g, washed twice with PBS, stained with Annexin V and propidium iodide (PI) according to Annexin-V-FLUOS Staining Kit (Roche). After incubation at dark for 15 min, cells analyzed under a fluorescence microscope (Olympus BX-41), as two fluorescence microscopy filters were used: FITC (560-600nm) for apoptosis and Texas Red (595-605 nm) for necrosis.

*Fluorescence imaging of mitochondrial morphology*: BioTracker™ 488 Green Mitochondria Dye (Sigma-Aldrich) applied; $5 \times 10^4$ cells/mL seeded in 6-well plates, incubated for 24 h, treated with 35, 55, and 75 µg/mL fustin for 72 h, than incubated in medium + 100nM Mitochondria dye for 15 minutes at 37°C; observed under a fluorescence microscope (Olympus BX-41). Images analyzed by Image J software version 1.54d (NIH, Bethesda, MD, USA).

*Wound healing assay*: Cells seeded at density of $4\times10^5$ cells in 6-well plates; cultivated for 48 h to reach confluence 90 - 95%; cell monolayer wounded by 200 µl plastic pipette tip, cell debris removed via washing with PBS; then incubated with fresh medium + 1% FBS and 35, 55, and 75 µg/mL fustin for 24 and 72 h; untreated cells were negative controls. The migrating cells in the wound area monitored and photographed under an inverted microscope (Leica DMI3000 B, Leica Microsystems GmbH, Wetzlar, Germany) at 0, 24, and 72 h. Cell-free zone was measured by Image J software version 1.54d, percentage of wound closure rate estimated using the formula:

*% wound closure = (wound area at 0 h — wound are at 24 or 72)/wound area at 0 h*100.*

*Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)*: Cells treated with 35, 55, and 75 µg/mL fustin for 72 h; untreated cells were negative controls; then collected by trypsinization, centrifuged at 200×g for 5 min, washed with ice cold PBS, centrifuged again at 250×g for 5 min at 4°C. Total RNA isolated using GeneJET RNA Purification Kit (Thermo Scientific Inc.); concentration and purity of extracted RNA determined by BioSpec-nano Spectrophotometer (Shimadzu Biotech). 1 µg total RNA from each sample subjected to first-strand complementary DNA (cDNA) synthesis using First Strand cDNA Synthesis Kit (Thermo Scientific Inc.). Relative expression levels of thirty four genes associated with the programmed cell death, cell cycle control and cell proliferation, metastatic and adhesion abilities of cancer cells, and epigenetic events were studied by qRT-PCR analysis. The housekeeping gene β-actin used as endogenous control to normalize genes expression. The quantitative RT-PCR analysis was performed on PikoRealTM Real-Time PCR System (Thermo Fisher Scientific Inc.) using 1x Luna® Universal qPCR Master Mix (New England Biolabs Inc.). For amplification, initial denaturation at 95°C for 15 min applied, followed by 45 cycles at 95°C – 15 s, 60°C – 30 s, 72°C – 45 s. Gene expression data were analyzed using PikoReal Software version 2.1 (Thermo Fisher Scientific Baltics UAB).

*Functional and pathway enrichment analysis*: Functional analysis of the identified differentially expressed genes (DEGs) (DAXX, BAX, BECN1, ATM, CDKN1A, MYC, and SIRT1) was performed with g:Profiler v:e111_eg58_p18_30541362 (https://biit.cs.ut.ee/gprofiler/gost) web server. The corresponding results from the databases of Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, miRTarBase, CORUM and Human Protein Atlas were characterized from a g:SCS significance threshold of 0.05. The analysis of the identified DEGs, converted to ID Entrez using GO Molecular function (GO:MF), GO Biological Process (GO:BP), GO Cellular Component (GO:CC), KEGG pathways, Reactome Pathways, etc.

❸***In vivo study of anti-inflammatory and organoprotective effects of fustin*** in four Wistar rat models:

*Experimental animals:* All procedures in animal treatment and experimentation carried out in accordance with EU directive 2010/63/EU, and approved by the Bulgarian Food Safety Agency (Protocol № 23/April 15th, 2021, Permission № 305/June 28th, 2021). Experiments performed on 148 male *Wistar* rats, housed in plastic cages at a room temperature 21±1° C and on a 12/12 light/dark cycle with free access to food and water.

*Induction of hind paw edema*: Rats pretreated by orogastric tube for one week: control group received distilled water (10 ml/kg, containing 50 µl Tween 80 as vehicle); F10 and F20 animals received 10 ml/kg suspensions of fustin in two doses: 10 mg/kg and 20 mg/kg + vehicle. On day 7, 1 mg of λ-carrageenan (Cat. № 22049 of Sigma-Aldrich), dissolved in 100 µl of saline administered subcutaneously in rat left hind paw. Using a digital plethysmometer LE7500 (Panlab, Barcelona), paw volume (ml) measured 0, 30, 60, 120, 180, 240, and 300 min after the injection. Paw edema (ml) was calculated using the formula: Paw edema (ml) = (Vs-$V_0$), where Vs: paw volume measured at time interval after carrageenan injection; $V_0$: initial paw volume.

*Induction of gastric ulcer*: Rats pretreated daily for 1 week by an orogastric tube; Fustin as suspension in vehicle (50 µl Tween 80 per 10 mL distilled water), F10 group received fustin (10 mg/kg) as 10 mL/kg suspension. Control and Indo rats received the vehicle (10 ml/kg). On the 7th day of the experiment, 1 h after the pretreatment with fustin, groups Indo and F10 received 100 mg/kg indomethacin as a 10 ml/kg suspension given by orogastric tube; Control rats: 10 ml/kg of the vehicle Tween 80. Rats anaesthetized with diethyl ether 4 hours after the treatment. Blood collected from the sublingual veins for biochemical analysis. After decapitation, stomachs were removed, opened along the great curvature, washed and inspected macroscopically.

*Induction of colitis:* Combination of ketamine/xylazine applied intraperitoneally as anesthesia. TNBS at a dose of 10 mg dissolved in 0.25 ml 50% ethanol administered by a soft cannula in colon at a depth of 8 cm from the anus. In the control animals, physiological saline was applied intrarectally. Oral treatment by orogastric cannula initiated 24 h after colitis induction and lasted for 8 days. Fustin, 10 and 20 mg/kg, prepared as a suspension in distilled water and Tween 80 (volume of 10 ml/kg) administered to group TNBS+F10/F20. Groups Control and TNBS received the vehicle (volume of 10 ml/kg). On the 10th day of the experiment, 24 h after the last fustin treatment, the animals were sacrificed under diethyl ether anesthesia.

*Induction of acute liver damage*: From day 1 to day 9, rats were treated once daily by direct stomach intubation as follows: Control and PCM rats received 50 µl Tween 80 in 10 ml distilled water as vehicle (10 ml/kg). F5+PCM and F10+PCM animals received 10 ml/kg suspension of fustin at doses of 5 mg/kg and 10 ml/kg + vehicle. On the 7th day, 1 hour after the oral treatment, groups PCM, F5+PCM and F10+PCM injected intraperitoneally with PCM (1.0 g/kg) as suspension with Tween 80 (volume 4 ml/kg). Control rats received 4 ml/kg vehicle. On day 9, 48 hours after PCM administration, rats anaesthetized with diethyl ether 2 hours after the last fustin treatment

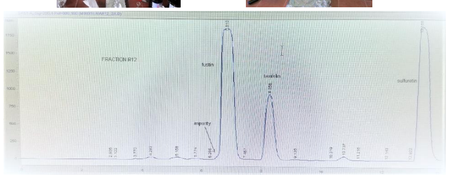
*Histopathological evaluation*: Tissue samples of stomach, colon and liver fixed in 10% neutral-buffered $CH_2O$ solution, stained with hematoxylin and eosin; observed with light microscopy. Deep erosions, hemorrhages, epithelial necrosis, inflammatory cells infiltration, destruction of colon epithelial cells and glands, colon edema, liver steatosis, ballooning degeneration, portal inflammation, zonal necrosis, confluent necrosis and apoptosis evaluated by the following scale: 0 – no; 1 – low; 2 – moderate; 3 – high.

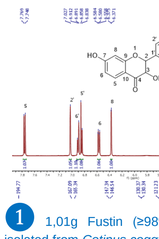
*Immunohistochemical tests of NF-kB expression*: Tissue sections from rat stomach, colon and liver, 4 µm thick, placed on silanized slides. NF-κB expression determined by rabbit anti NF-kB-p100 polyclonal antibody (E-AB-32222; Elabscience, USA), diluted 1:200, following protocol of EnVision FLEX. Immunohistochemical evaluation determined semi-quantitatively in 50 cells of each sample using the following score for cytosol staining: 1 – no, 2 – weak, 3 – moderate, 4 – strong. The average intensity of the immune reaction given as: number of cells of each type x corresponding coefficient (1, 2, 3 or 4) x total number of cells$^{-1}$.

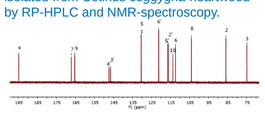
*Biochemical assays*: Blood samples centrifuged at 2000 rpm for 10 min and serum collected. 1 g Stomach/Colon/Liver tissue mixed with 10 ml 50 mM Tris/HCL buffer (pH=7.4) andwas homogenized for 3-4 min at 3500 rpm; homogenate centrifuged at 4°C and 3000 rpm for 15 min; supernatant collected. Using colorimetric kit (Biomaxima, Poland), alanine aminotransferase (ALT) levels determined. MDA and SOD colorimetric assays on multi-mode microplate reader (Synergy 2, BioTek Instruments, USA): Superoxide Dismutase (SOD) Activity Kit: Product №19160, Sigma-Aldrich; Malondialdehyde (MDA) Kit: Product № MAK085, Sigma-Aldrich.

*Statistical analysis:* GraphPad Prism 7.00 statistical software (San Diego, California): one-way ANOVA, followed by Dunnett's multiple comparisons test, and Student's t-test to compare two independent groups. Results were presented as mean ± SEM. A value of $p < 0.05$ indicated a statistical significance.

## Results



❶ 1,01g Fustin (≥98% Purity) was isolated from *Cotinus coggygria* heartwood by RP-HPLC and NMR-spectroscopy.

| Atom Nr | $\delta_H$, J (Hz) | $\delta_C$ |
|---|---|---|
| 1 | - | - |
| 2 | 4.98 d (12.0) | 85.9 |
| 3 | 4.52 d (12.0) | 74.8 |
| 4 | - | 194.8 |
| 5 | 7.75 d (8.4) | 130.3 |
| 6 | 6.57 dd (8.8, 1.6) | 112.4 |
| 7 | - | 167.1 |
| 8 | 6.37 d (1.6) | 104.0 |
| 9 | - | 165.3 |
| 10 | - | 113.7 |
| 1' | - | 130.4 |
| 2' | 7.03 d (1.6) | 116.2 |
| 3' | - | 146.5 |
| 4' | - | 147.3 |
| 5' | 6.85 d (8.0) | 116.4 |
| 6' | 6.90 dd (8.0, 1.6) | 121.2 |

NMR Spectrometer: Bruker Avance III 500 400 MHz for $^1H$, 100 MHz for $^{13}C$
Solvent: $CD_3OD$

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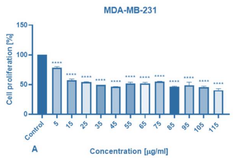
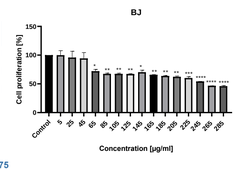
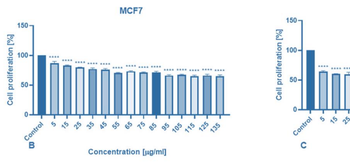
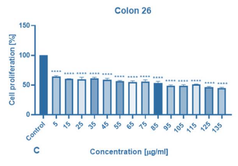
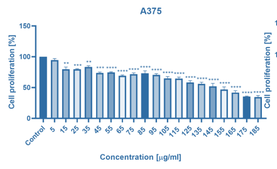


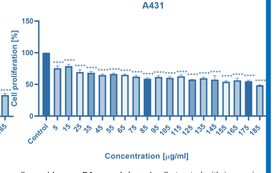

| Cell lines | | $IC_{50}$ [µg/ml] | Selectivity Index (SI) |
|---|---|---|---|
| MDA-MB-231 | triple negative breast cancer | 56.02 | 4.97 |
| MCF7 | luminal A breast cancer | 187.80 | 1.48 |
| Colon 26 | murine colon adenocarcinoma | 78.07 | 3.57 |
| A375 | malignant skin melanoma | 149.10 | 1.87 |
| A431 | epidermoid carcinoma | 151.20 | 1.84 |
| BJ | normal dermal cells | 278.60 | |



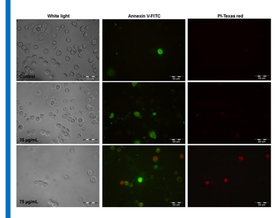



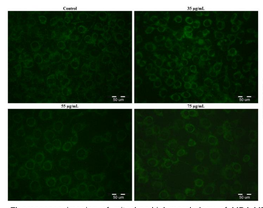



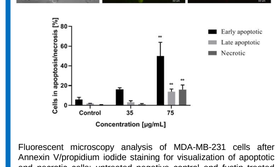

MTT cell proliferation assay of human MDA-MB-231 (A) and MCF7 (B) breast cancer cells, and murine Colon 26 adenocarcinoma cells (C) treated for 72 h with increasing concentrations of fustin. Error bars represent standard error of the mean (SEM). **** indicates significant differences from the control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

MTT cell proliferation assay of human A375 and A431 skin cancer cells, and human BJ normal dermal cells treated with increasing concentrations of fustin for 72 h. 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, ***p<0.001, ****p<0.0001).

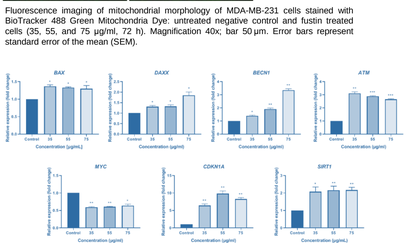



Fluorescence imaging of mitochondrial morphology of MDA-MB-231 cells stained with BioTracker 488 Green Mitochondria Dye: untreated negative control and fustin treated cells (35, 55, and 75 µg/ml, 72 h). Magnification 40x; bar 50 µm. Error bars represent standard error of the mean (SEM).




Fluorescent microscopy analysis of MDA-MB-231 cells after Annexin V/propidium iodide staining for visualization of apoptotic and necrotic cells: untreated negative control and fustin treated cells (35 and 75 µg/ml, 72 h). Magnification 40x; bar 50 µm.




Relative expression levels of BAX, DAXX, BECN1, ATM, MYC, CDKN1A, and SIRT1 genes at 72 h after treatment of MDA-MB-231 cells with fustin in concentrations of 35, 55, and 75 µg/mL – black columns refer to untreated controls. Error bars represent standard error of the mean (SEM). *, ** and *** indicate significant difference from the control group (*p < 0.05, **p < 0.01, ***p < 0.001).

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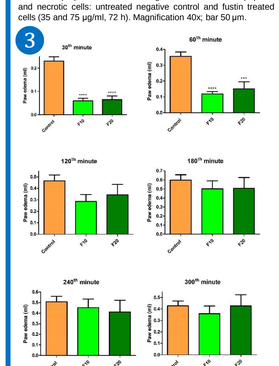
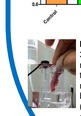
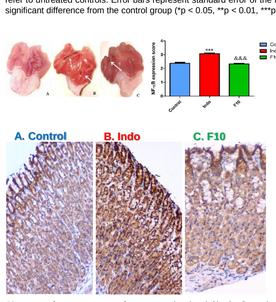


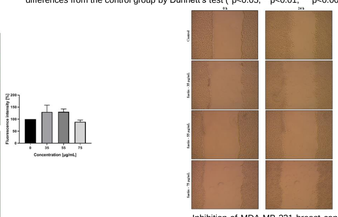



**Left hind paw edema voliume** (ml) on the 30th, 60th, 120th, 180th, 240th and 300th minute after λ-carrageenan injection in rats, treated for 7 days with fustin at doses of 10 mg kg-1 (F10) and 20 mg kg-1 (F20); Error bars represent standard error of the mean (SEM). *** and **** indicate significant differences from the control group by Dunnett's test (***p<0.001, ****p<0.0001)

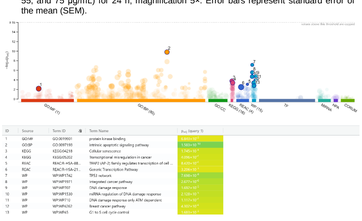



**Macroscopic appearance** of rat stomachs (up left): A. Control group (normal appearance); B. Group Indo (many long linear ulcerations); C. Group F10 (decreased number or no ulcers); **Immunohistochemical score** (up right) and **staining** (down) for NF-κB gastric mucosa expression in rat model of indomethacin-induced gastric ulceration with 7-days fustin pretreatment (n=30): Scale: 1 – lacking, 2 – weak, 3 – moderate, 4 – strong; Significant differences from the Control/Indo group by Dunnett's test: ***p<0.001 vs. Control; &&&p<0.001 vs. Indo; magnification x 200

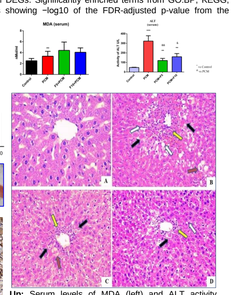
**Superoxide dismutase (SOD)** mean inhibition activity in colon tissue homogenate (up left) and **Immunohistochemical score** (up right); **Macroscopic appearance** of rat colon (center) and **staining** (down) for NF-κB colon expression in rat model of TNBS-induced colitis with 10-days fustin treatment (n=30); Scale: 1 – lacking, 2 – weak, 3 – moderate, 4 – strong; Significant differences from the Control/TNBS group by Dunnett's test: **p < 0.01 vs. control; ***p < 0.001 vs. control; ###p < 0.001 vs. group TNBS; magnification x 200




Inhibition of MDA-MB-231 breast cancer cell migration after treatment with fustin (35, 55, and 75 µg/mL) for 24 h; magnification 5×. Error bars represent standard error of the mean (SEM).



g:Profiler enrichment analysis of DEGs. Significantly enriched terms from GO:BP, KEGG, REAC and MIRNA with Y-axis showing −log10 of the FDR-adjusted p-value from the enrichment test.




**Up:** Serum levels of MDA (left) and ALT activity (right) in a rat model of paracetamol (PCM)-induced hepatotoxicity with 7-days fustin pretreatment (n=48); **p<0.01 vs. Control; ***p<0.001 vs. Control; &&p<0.01 vs. PCM, &p<0.05 vs. PCM; **Down: Liver** of (A) control: preserved architectonics; (B) PCM: necrosis (black arrow), steatosis (white), ballooning degeneration (red) and inflammatory infiltration (yellow); (C) F5+PCM: less ballooning degeneration, reduced apoptosis, steatosis, and confluent necrosis; (D) F10+PCM: moderate ballooning degeneration, less inflammation, confluent necrosis covers a smaller area of parenchyma; magnification x 200

## Conclusions

The obtained results indicate that fustin is a perspective candidate for further detailed analyses of its oncotherapeutic potential. Our future studies will be focused on the examination of *in vivo* anticancer potential of fustin in animal model systems.

## Acknowledgements

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