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Original paper

Tamarix gallica extract induces apoptosis via an intrinsic mechanism

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Abstract

Tamarix gallica (T. gallica) is a source of phenolic compounds which show biological properties, such as antimicrobial, anti-inflammatory, antioxidant, hepatoprotective, chemopreventive, anti-microbial, etc. These pharmacodynamic properties, availability and strength, are determined by the source region. The present study evaluated some biological effects at cellular and molecular level of the *T.gallica* extract on a tumoral cell line (HEp-2 cell line).

Treated or untreated cells were evaluated using microscopy, CellTiter 96® Aqueous One Solution Cell Proliferation Assay and Annexin V-FITC / Propidium Iodide Assay to quantify cell cytotoxicity and cell death. In order to characterize the apoptotic events that appeared after *T. gallica* treatment, the Carboxyfluorescein Multi-Caspase Activity Kit, Bio-Plex Pro™ RBM Apoptosis Multiplex Assay and Proteome Profiler Array – Human Apoptosis Array Kit were used.

We established an IC50 value of *T. gallica* extract at a concentration of 0.107%. *T. gallica* extract treatment induced morphological changes with the features of apoptosis. The analysis of multi-caspase activity revealed an increased caspase activity in treated cells compared to untreated cells. The pro-apoptotic proteins (caspase 3 and Cytochrome c) levels were increased and anti-apoptotic, Bcl xL and IAP family members (Survivin, cIAP 1 and cIAP 2), were decreased in *T. gallica* treated cells. The Bio-Plex Pro RBM test confirmed that the mechanism leading to the *T. gallica* induced apoptosis is based on the commitment, onset and induction of intrinsic apoptosis.

Our study demonstrates that the *T. gallica* extract had cytotoxic activity on Hep2 cells and a concentration of 0.1% proved pro-apoptotic activity by an intrinsic mechanism of action.

Keywords Cytotoxicity, alcoholic extract, *Tamarix gallica*, apoptosis.

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Introduction

Medicinal plants synthesize a number of pharmacologically active substances, representing a valuable ecological alternative for classic treatments. These botanicals have a relatively safe and widely accepted status for consumers, due to the reduced risk or absence of side effects and various biological activities, including immunomodulatory, antimicrobial, antiviral and anti-tumoral effect (MIHAILA & al [1]). According to their complex composition, medicinal plant extracts act concomitantly and synergistically through different mechanisms (cell wall degradation, inhibition of microbial biofilm formation, damage of cell membrane and/or membrane proteins, cytoplasmic coagulation, inhibition of glycolysis and glucose transport etc.) (BURT [2]).

Tamarix gallica may have chemopreventive potential against liver carcinogenesis through multiple actions, including restoration of cellular antioxidant enzymes, detoxifying enzymes, ODC activity and DNA synthesis (SEHRAWAT & al [3]). The extracts of *T. gallica* have long been used in the treatment of liver disorders, traditionally being considered a natural hepatostimulant. It is one of the constituents of the Liv.52® formula along with *Capparis spinosa, Cichorium Intybus, Mandur bhasma, Solanum nigrum, Terminalia arjuna, Cassia occidentalis, Achillea millefolium*. The acidic fraction of *T.gallica* extract has an inhibitory effect on the growth of calcium oxalate crystals *in vitro* and thus could inhibit the formation of nephrolithiasis (BENSATAL & al [4]). The extract obtained by infusing the flowers of *T. gallica* harbored an antioxidant activity higher than the infusion obtained from the leaves (KSOURI & al [5]).

Regarding the active compounds identified in *T. gallica* extract, there are studies that show the differences in extract composition, corresponding to the plant organ, the origin of the plant and the extraction method used. The compounds were identified based on their retention times and spectral characteristics of their peaks vs. standards. The flower phenolic fingerprint obtained through RP-HPLC contained seven phenolic acids (gallic, sinapic, chlorogenic, syringic, vanillic, p-coumaric, and trans-cinnamic acids) and six flavonoids((+)-catechin, isoquercetin, quercetin, apigenin, amentoflavone, and flavone). The leaves analysis revealed 12 phenolic compounds – gallic, sinapic, chlorogenic, syringic, vanillic, rosmarinic, p-coumaric, ferulic, and trans-cinnamic acids, as well as two flavonoids (quercetin and amentoflavone) (KSOURI & al [5]). Quercetin, rhamnetin, rhamnazin, tamarixetin, kaempferol, quercetin 3-*O*-β-D-glucuronide, quercetin 3-*O*-β-D-glucuronide methyl ester, kaempferol 3-*O*-β-D-glucuronide, kaempferol 3-*O*-β-D-glucuronide methyl ester are fractions and sub fractions of *T. galica* (aerial parts) ethanol extract, as showed by Ben Hmidene A. and colleagues (2017) using chromatography on silica gel column (BEN HMIDENE & al [6]). All these components act as external factors in order to obtain the specific effects at cellular and tissue level.

When an uncharacterized substance is being investigated for its biological effects, *in-vitro* studies are needed in order to investigate the mechanisms of action and these often begin with experiments aimed to show the cellular response. Induced apoptosis and growth arrest during cell cycle are the first to be investigated when a decrease in proliferation appears in cells treated with the tested substance. In our study we evaluated the cytotoxic effects of *T. gallica* alcoholic extract on HEp-2 cell line and investigated apoptosis and its mechanism of action.

Methods

Plant sampling and preparation for extract: The extract of *T. gallica* was prepared according to Ionescu et al, (2014) (IONESCU & al [7]). Briefly, the leaves and flowers were rinsed with water, dried, macerated in 70% ethanol and filtrated. The stock solution of the extract was obtained after ethanol evaporation under vacuum and stored at 4ºC until analysis.

Cell lines and primary cell culture: The eukaryotic cell culture used was HEp-2 cell line (ATCC® CCL-23™). The cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (Sigma, USA) supplemented with 10% heat-inactivated foetal bovine serum (Sigma, USA) at 37ºC, 5% CO2, in a humid atmosphere.

Cytotoxic effect: HEp-2 cells were seeded in 96-well plate at a density of $5x10^3$ cells/well. After 24 hours, the cells were treated with binary serial dilutions of *T. gallica* extract starting with 0.5% up to 0.00393%. Morphological alteration of cells after the treatment with the extracts at different dilutions was verified at 24 h using an inverted microscope, ZEIS Axio Observer. Cytotoxic effect was evaluated using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega Corporation, USA) according to manufacturer protocol. Briefly, 20 µl of CellTiter 96® AQueous One Solution Reagent were added into each well containing the treated and untreated cells in 100µl of culture medium. The plate was further incubated at 37°C for 1 hour and the absorbance at 490 nm was measured using a 96-well plate spectrophotometer reader Tristar²S LB942 Multimode Reader, Berthold Technologies, USA.

Detection of apoptosis by flow cytometry using the Anexin V-FITC / Propidium Iodide (PI) kit

HEp-2 cells were seeded in 24-well plate at a density of $7.5x10^4$ cells/well. 24 hours after treatment with 0.1% and 0.5% dilutions of the *T. gallica* extract stock, the cells were harvested by trypsinization, washed in PBS and resuspended in 200 µL of pre-diluted binding buffer, adjusting the density at 2×10^5 cells/mL. Subsequently, the cell suspension was labeled with 5 µL Annexin V: FITC, incubated for 10 minutes in the dark, at room temperature followed by washing with pre-diluted binding buffer and stained with 10 µL PI solution from kit for detecting dead cells (BOTEZATU & al [8]).

Carboxifluorescein multi-caspase activity kit

HEp-2 cells were seeded at a density of $3x10^5$ cells/well in 6-well plates. Treatments with 0.1% of the

stock solution of *T. gallica* extract and 0.04 mg/mL quercetin were done. Cells were harvested at 24 hours, counted and divided into two aliquots at a concentration of 10^5 cells/100 µL and processed according to manufacturer recommendations (Carboxyfluorescein Multi-Caspase Activity Kit, Enzo Life Sciences, Inc., USA). An aliquot was treated with 2.5 µL FAM-VAD-FMK/100 µL cell suspension, shaken and incubated for one hour in dark, at 37°C. The cells were washed twice with washing fluid, and 100 µL of each cell suspension was duplicated in a black microtiter plate. The fluorescence intensity at 490 nm excitation, 520 nm emission was quantified at the spectrophotometer. The results are presented as a log of the ratio between the integrated values of the treated vs the untreated cells.

Bio-plex Pro RBM Apoptosis Assay

HEp-2 cells were treated for 24 hours with 0.1% alcohol extract of *T.gallica* and 0.04mg/ml quercetin. The treated and untreated cells (control) were harvested by trypsinization and apoptotic pathway activation was evaluated using Bio-Plex Pro™ RBM Apoptosis Multiplex Assay (Bio-Rad Laboratories, Inc., USA) according to manufacturers' protocol. Briefly, harvested cells were suspended in lysis buffer, homogenized by sonication, centrifuged and the protein concentration of the supernatant was quantified using Bradford kit. The samples were brought to the final concentrations of 500 µg/mL by diluting with lysis buffer and incubated for 1 hour, at room temperature with the microspheres coated with antibodies specific to Active caspase-3, Bcl-xL/Bak dimer, Mcl-1/Bak dimer, Survivin. After 3 washes to remove unbound protein, the biotinylated detection antibody was added to create a sandwich complex. The final detection complex was formed by the addition of streptavidin-phycoerythrin (SA-PE). Quantification was performed using Bio-Plex 200, an instrument based on xMAP technology, with two lasers and associated optics for measuring different molecules bound to the surface of the microspheres. Data analysis was performed with Bio-Plex Manager software.

Proteome Profiler Array – Human Apoptosis Array Kit

The expression profiles of apoptotic proteins were analysed using the Proteome Profiler Array – Human Apoptosis Array Kit (R&D Systems, Inc., USA) according to the manufacturers' instructions. At 24 hours after treatment with 0.1% *T. gallica* extract, the treated and untreated cells were harvested, washed with phosphate buffered saline (PBS), lysed in lysis buffer for 30 minutes, and centrifuged at 14000xg, 5 minutes. The protein concentration of supernatant was quantified using Bradford kit. The nitrocellulose membranes, containing 35 different antibodies spotted in duplicate, were incubated with 250 µl of cell lysate (300 µg total protein/array), overnight at 2-8°C on a rocking platform shaker. After membranes washing for 3 times, the samples were incubated with detection antibodies for one hour, and thereafter with Streptavidin-HRP complex for 30 minutes. The membranes were finally incubated with 1 mL of Chemi Reagent Mix for 1 minute and exposed in the chemiluminescence module of the CHEMIDOC device and the chemiluminescent signal was recorded. The pixel density was analyzed using ImageJ 1.52a image analysis software. A matrix was created to analyse the pixel density in each spot of the array. The average value of the signals (pixel density) of the pairs of duplicate spots was determined for each protein from the array. The background signal was subtracted from each spot. In order to determine the relative variations in the concentrations of the proteins, we compared the signals corresponding to the different arrays between different samples.

Results and Discussions

Cytotoxic effect

For cytotoxic effect evaluated on HEp2 cells, we used the concentrations as described above and obtained the half maximal inhibitory concentration (IC50). IC50 value in Hep2 cells was 0.107% (Fig. 1). So in our further *in vitro* investigation we used 0.1% concentration as the effective dose.

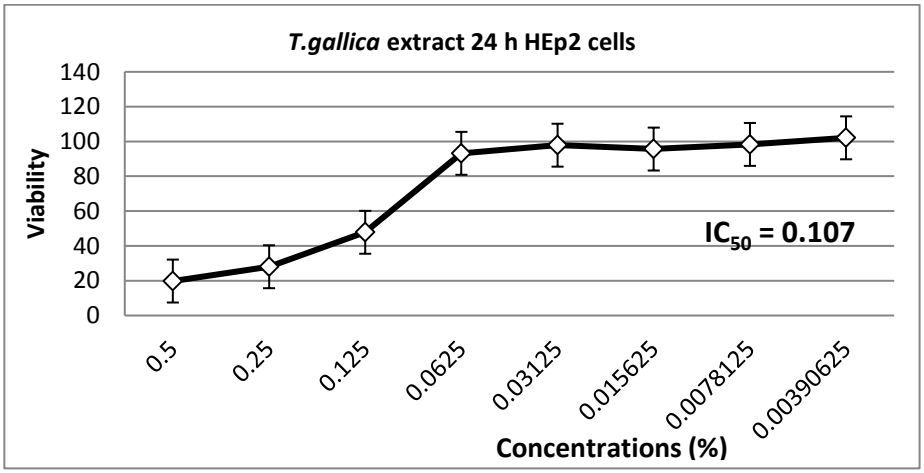

Figure 1. Effect of *T. gallica* extract on HEp2 cell viability. Cells were cultured in the presence of the indicated concentrations of *T. gallica* for 24 h, and cell viability was determined by a colorimetric assay. The results of a representative experiment are shown. Each point represents the mean of triplicate determinations.

In order to characterize some biological effects of *T. gallica*, *the* morphological alterations of cells were verified using an inverted microscope. We observed that when treated with the highest concentrations (0.5%) of the extract the cells were fixed on the flask wall. At slightly smaller concentrations, we observed some structural changes like cellular lysis, cytoplasmic inclusions, appearance of lipid droplets-like, generally associated with cell death induction. The images are representative for intracytoplasmic inclusions are presented in Figure 2.

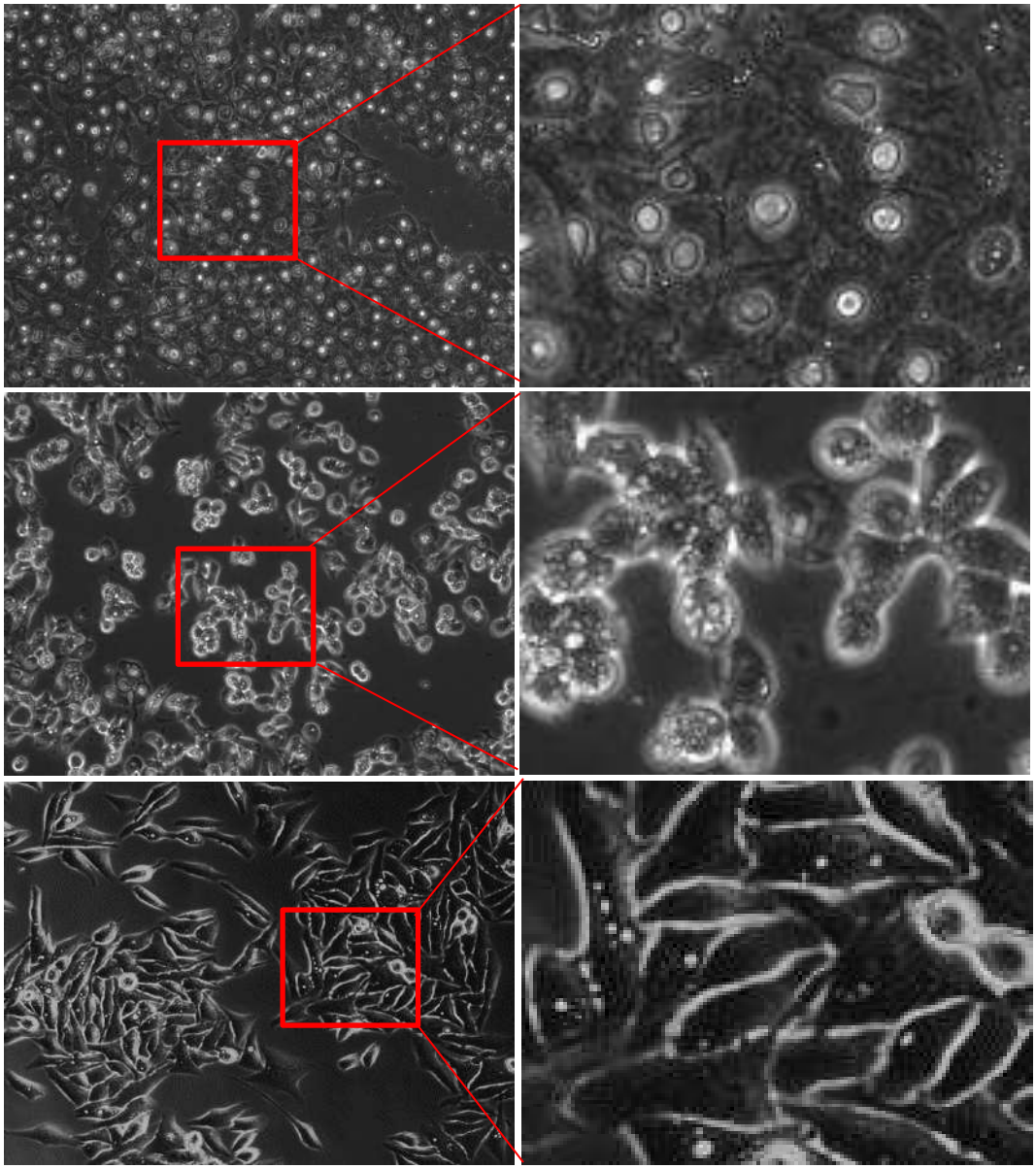

Figure 2. Effect of *T. gallica* extract on Hep2 cell morphology. Cells were cultured in the presence of the *T.gallica* extract in a concentration of a) 0.5%, b) 0.1%; c) 0.05%. Phase contrast, 200x.

Detection with Annexin V-FITC/PI

Further, to study whether the reduced viability was due to cell apoptosis triggered by *T. gallica* extract treatment, Hep2 cells were analyzed by flow cytometry after labelling with annexin-V-FITC and propidium iodide, which allowed discrimination between intact and apoptotic cells. At 0.1% and 0.5% of the stock solution an increase in apoptosis and necrosis was observed. Thereby, when treated with 0.1% *T. gallica* 76.11% were viable cells, 2.2% and 5.24% were cells in early respectively late apoptosis, 16.37% were necrotic cells. When treated with 0.5% *T. gallica* there was a decrease of viable cells (67.83%), still over 50%. The apoptotic number of cells increase, 3.63% were early apoptotic and 7.17% late apoptotic cells and also the necrotic cells were 21.37% (Fig. 3).

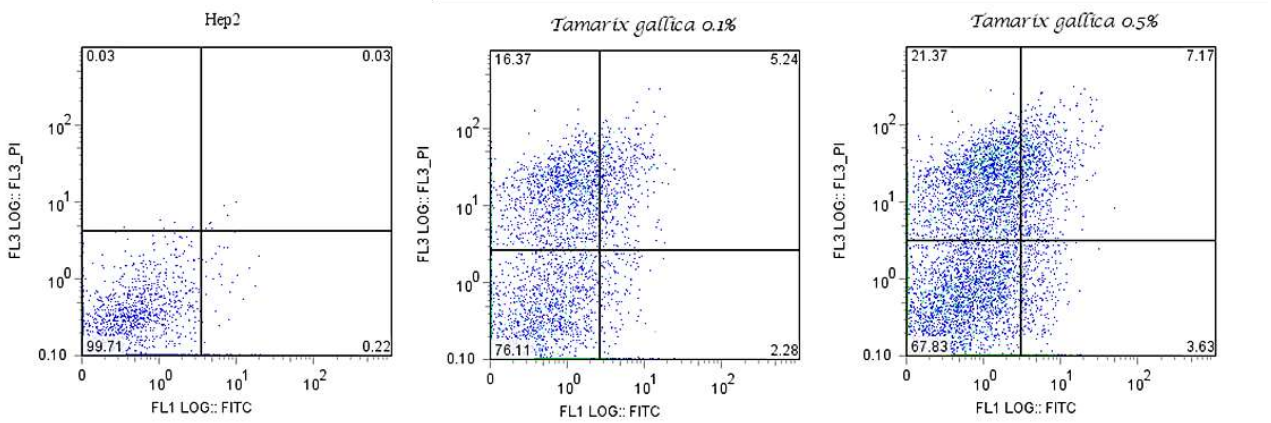

Figure 3. The apoptotic effect induced by *T. gallica* treatment on Hep2 cells, at 24 h post treatment. The method discriminates between intact and apoptotic/necrotic cells. The results of a representative experiment are shown.

Carboxifluorescein multi-caspase activity kit

In order to detect a total caspase activity we used a carboxyfluorescein multi-caspase activity kit. In our experiments the treatments on HEp2 cells with alcoholic extract induced the activation of global caspase activity (Fig.4). We also observed that the effect on the overall caspase activity of the treatment with 0.1% *T. gallica* extract is similar to the effect induced by the treatment with 0.04 mg/ml quercetin. We used quercetin as a standard flavonoid, considering that tamarixetin is a monomethoxi-flavone that is methylated quercetin in the O-4 'position, a metabolite of quercetin, and can also be isolated from *T. gallica* extract (Fig. 4). Quercetin, a plant-derived flavonoid, has been known to induce apoptosis and cell cycle arrest, but the mechanism of activity has not been elucidated. In different studies these effects appeared via modification of Foxo3a signalling (NGUYEN & al [9]) or by the growth arrest and DNA damage-inducible gene 45 (gadd45) induced expression (YOSHIDA & al [10]), and also by the modulation of the EGF-mediated signalling pathway (LEE & al [11]). If tamarixetin, or whole *T. gallica* extract, use the same pathways it will be established in further studies.

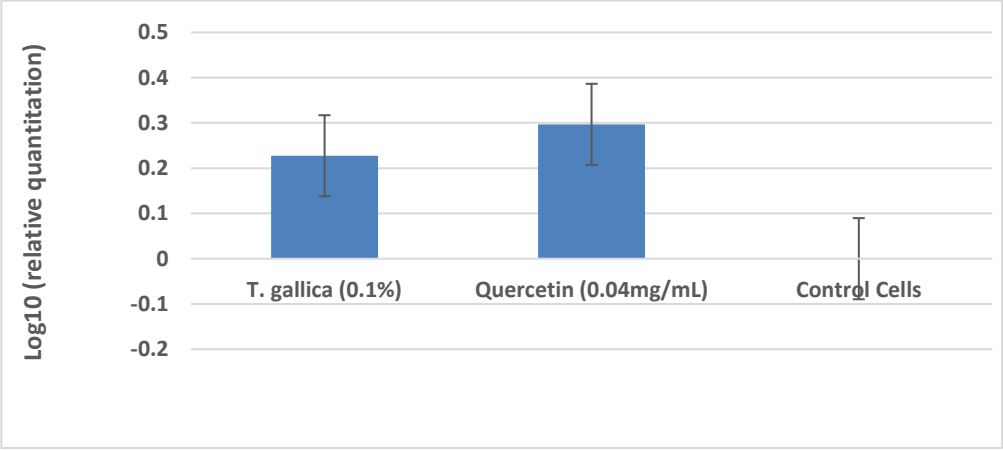

Figure 4. Multi-caspase activity in cells treated with *T. gallica* and standardized Quercetin. Results are expressed as fold change from control (untreated HEp2 cells).

Proteome Profiler Array – Human Apoptosis Array Kit

After we established that the effect observed by microscopy analysis was partially due to induced apoptosis by *T. gallica* extract, we wanted to know what pathway for the apoptosis induction was activated by this extract. Based on the signal initiation, apoptosis occurs mainly through two pathways: 1) the extrinsic pathway initiated by cell death receptors from the cell membrane, and 2) the intrinsic or mitochondrial pathway controlled by the BCL-2 family of proteins and is completed by permeabilization of the mitochondrial membrane and release of apoptogenic effectors (eg Cytochrome c). Both pathways lead to the activation of the caspase cascade (KALKAVAN & al [12]). In the cells treated with *T. gallica* extract compared to the untreated cells, the expression of BAD and BAX is increased and the expression of BCL-XL was decreased,

supporting the idea that apoptosis is activated by treatment, and this process is initiated by the intrinsic pathway (Fig. 5). Moreover, the initiation of apoptosis following treatment with *T. gallica* extract by the intrinsic rather than the extrinsic pathway is sustained by low expression of TRAIL R1/DR4, TRAIL R2/DR5, FADD, Fas/TNFSF6, members of TNF superfamily that mediates initiation by the extrinsic pathway of apoptosis.

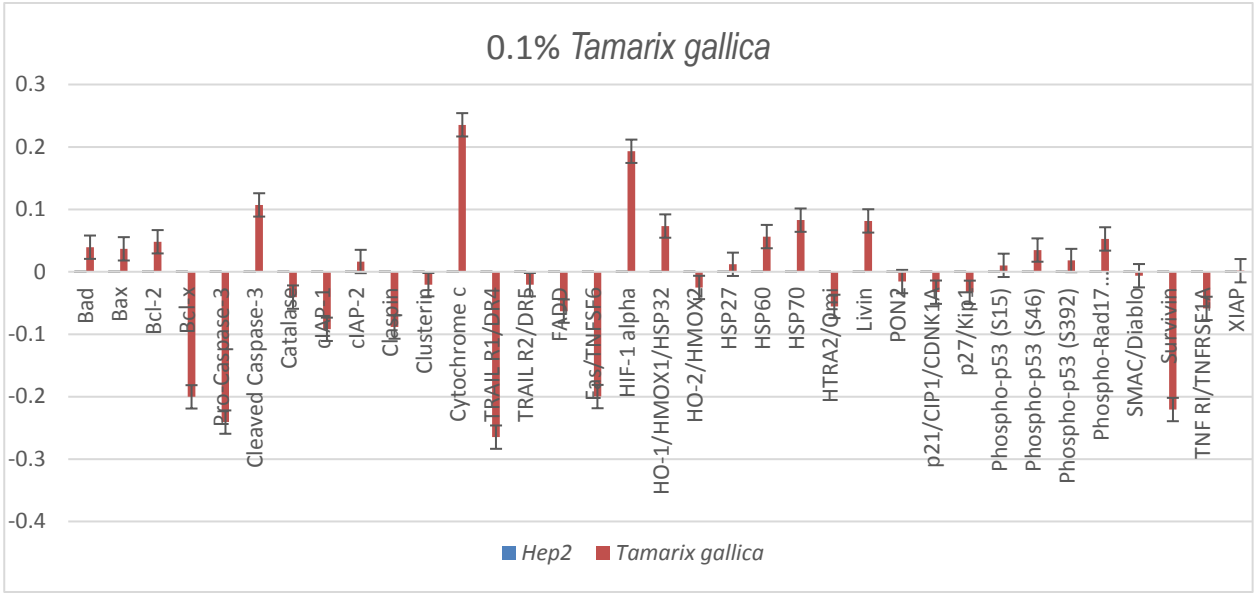

Figure 5. Proteome Profiler Array- expression profiles of apoptosis-associated proteins; The results are expressed as fold change from control (untreated HEp2 cells) after the pixel density was analysed. Cells were harvested at 24 h after treatments. For this experiment only the 0.1% concentration was considered. Proteins analysed: Bad, Bax, Bcl-2, Bcl-x, Pro-Caspase-3, Cleaved Caspase-3, Catalase, cIAP-1, cIAP-2, Claspin, Clusterin, Cytochrome c, TRAIL R1 / DR4, TRAIL R2 / DR5, FADD, Fas / TNFSF6, HIF-1 alpha, HO-1 / HMOX1 / HSP32, HO-2 / HMOX2, HSP27, HSP60, HSP70, HTRA2 / Omi, Livin, PON2, p21 / CIP1 / CDNK1A, p27 / Kip1, Phospho -p53 (S15), Phospho-p53 (S46), Phospho-p53 (S392), Phospho-Rad17 (S635), SMAC / Diablo, Survivin, TNF RI / TNFRSF1A, XIAP.

In our experiments, we observed an increase in cleaved caspase 3 (activated), Cytochrome c and HIF-1 alpha, which indicate apoptosis activation under stress conditions.

In addition, treatment with alcoholic *T. gallica* extract induced increased expression of phosphorylated p53 proteins. The p53 protein is considered the guardian of the cell being involved in the control of the cell cycle, inducing blockages at the G1 and G2 levels and in the control of apoptosis in response to abnormal proliferative signals, cellular stress and alterations at the DNA level (BENCHIMOL [13]). The activity of p53 is regulated by numerous post-translational modifications: phosphorylation, acetylation, methylation, ubiquitination, which may occur at the level of the amino acids to which the enzymes are linked, specific to each of the modifications listed above (kinases, acetyl-transferases, methyl-transferases, etc.). In the healthy cell the p53 protein has a relatively short lifespan and is degraded by binding to enzymes such as MDM2, PirH2, COP-1 and CHIP (ubiquitin). Following cellular stress, p53 undergoes phosphorylation at multiple residues, modifying its biochemical activity to act as a transcription factor (MACLAINE & al [14]). Among the dozens of phospho-acceptor sites in our results we obtained an increased expression for Phospho-p53 (S15), Phospho-p53 (S46), Phospho-p53 (S392). All 3 have been reported in the literature as being involved in the process by which Phospho-p53 acts as a pro-apoptotic factor. After phosphorylation, Phospho-p53 can interact either as a transcription factor in the nucleus, but also has an extranuclear function to bind directly, physically with BCL-2 and BCL-XL in mitochondria, and its translocation into mitochondria precedes the release of Cytochrome c and reduction of mitochondrial membrane potential by blocking these two anti-apoptotic proteins (PARK & al [15]) (ZETTERSTROM & al [16])

The expression of Survivin, a member of the IAP (Apoptosis Inhibitors) family, known to have a dual function as inhibitor of apoptosis and regulator of cell division, was very low. According to Garg et al, (2016), with these two separate functions, it is possible for Survivin expression to act as a control point in inducing programmed cell death in cells where aberrant changes in cell division occur (GARG & al [17]). On the other hand, Survivin down-regulation are mediated through a complex containing XIAP (X-linked inhibitor of apoptosis) (ARORA & al [18]), a protein that directly suppress apoptotic cell death pathways by the unique interactions with caspases -3, -7 and -9 through binding to their catalytically active sites, ubiquitinate, exerting thereafter an anti-caspase activity and inhibiting thereby their proteolytic activity (GALBAN & al [19]). Overall, Survivin inhibition associated with unmodified

expression of XIAP and decreased expression of Pro-Caspase-3 but increased expression of their active form (cleaved Caspase-3) sustain the pro apoptotic activity of *T. gallica* extract.

Heat shock proteins HSP27, HSP60, HSP70 are involved in protecting the cell against stressors and act as activators of apoptosis by inhibiting antiapoptotic pathways (eg AKT) (IKWEGBUE & al [20]). Their expression is also increased in the cells treated with *T. gallica*.

So, the tested extract increased the level of pro-apoptotic proteins together with the increase in the level of heat shock proteins (Hsp27, 60 and 70), HIF-1a, Livin, phosphorylated p53, and Cytochrome c and decreased level of anti-apoptotic proteins (Fig. 5).

Bio-plex Pro RBM Apoptosis Assay

The intrinsic pathway activation leading to apoptosis was confirmed using the Bio-Plex Pro RBM test, that quantify some proteins involved in the commitment, onset and induction of apoptosis by the intrinsic pathway.

It was demonstrated that the effect of the interactions between BCL-2 protein family members is the trigger of apoptosis by pore formation (BAK, BAX homo-oligomerization) in the mitochondrial membrane and release of pro-apoptogenic factors from the mitochondrial intermembrane space into the cytosol. If the caspase cascade is activated these events end in cell death through apoptosis. On the other hand, BCL-XL, MCL-1 respectively BCL-2 exert their anti-apoptotic function by forming dimers with BAK, BAX. This sequestration inhibit their function of pore formation in the mitochondrial membrane. In their turn BCL-2 and BCL-XL could also be sequestrated by the pro-apoptotic proteins BAD, BID, BIK, BIM, BMF, HRK, NOXA, PUMA, etc. (TAIT & al [21]).

Using the Bio-plex Pro RBM Apoptosis Assay we observed not only the expression of pro or anti apoptotic proteins but also the dimmer formation. So, an increase in the Bcl-xl/Bak dimers associated with the decrease of the Mcl-1/Bak dimers was observed and as we presented above, in healthy cells Bak is controlled by Mcl-1 and Bcl-xL and the release from these complexes is associated with apoptosis induction (Fig. 6). The results of this assay correlated with Proteome Profiler Array sustain that the expression of BCL-XL is decreased (Fig. 5) because is sequestrated by BAK (Fig. 6). So, it is not the BCL-XL pathway that leads to apoptosis but rather the BCL-2 pathway taking account of the increased expression of BCL 2 (Fig. 5). Also, the MCL1/BAK dimers expression is inhibited and this could also lead to apoptosis induced by *T.gallica* extract treatments. On the other hand, taking into account the increased levels of active caspase-3 and the inhibition of Survivin (Fig. 5), we consider that after treatment with 0.1% *T. gallica* extract, the intrinsic pathway of apoptosis is activated in cells harvested at 24 hours.

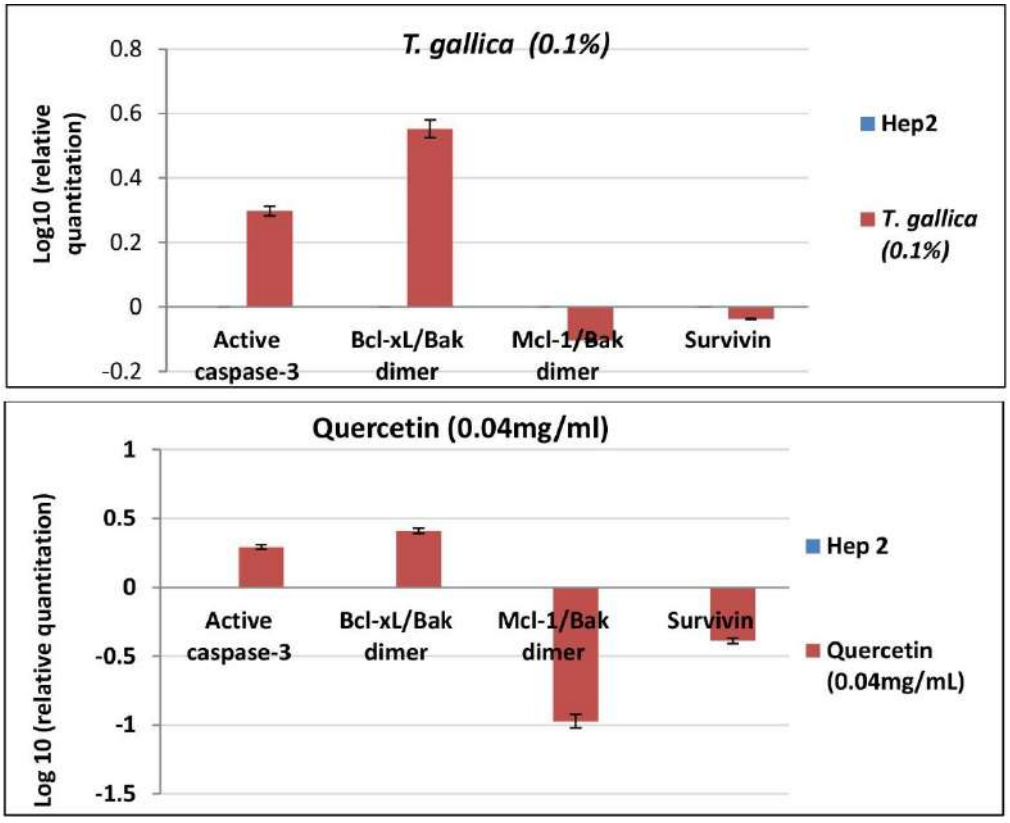

Figure 6. Quantification of protein expression: Active caspase 3, BCL-XL/BAK dimer, MCL1/BAK dimer and Survivin. Cells treated with *T. gallica* and standardized Quercetin at mentioned concentrations. Results are expressed as fold change from control (untreated HEp2 cells).

Conclusions

Our study demonstrated that the *T. gallica* extract had cytotoxic activity on Hep2 cells and a concentration of 0.1% proved pro-apoptotic activity by an intrinsic mechanism of action. The *in vivo* studies and further clinical trials are necessary to confirm, the scientific results obtained *in-vitro*, and the safety and efficacy of the *T. gallica* use in human.

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