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Evaluation of anticancer and antimicrobial activities of the *Polygonum maritimum* ethanol extract

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Abstract: *Polygonum maritimum* is a traditional herbal remedy that produces abundant flavonoid secondary metabolites. The ethanol extract of *P. maritimum* aerial parts (*POM*) was chemically characterized and tested for antimicrobial properties and cytotoxicity. Results of LC-MS/MS analysis showed high contents of gallic acid, epigallocatechin gallate and catechin, and significant amounts of quercetin-3-O-galactoside and quercetin-3-O-glucoside. Evaluation of the antifungal properties revealed that *POM* induced notable growth inhibition of *Alternaria alternata* (34.3%), *Penicillium* spp. (30.6%), *Fusarium semitectum* (20.2%) and *Aspergillus* spp. (19.6%). Evaluation of cytotoxicity against human hepatoma HepG2 cells included monitoring the effects of both *POM* alone and its combination with cytostatic doxorubicin (Dox). Cell viability, apoptosis and cell cycle distribution and the expression of antioxidant enzymes (superoxide-dismutases SOD1 and SOD2 and catalase) were determined. A dose-dependent decrease in cell viability was detected, but a remarkably stronger effect was obtained when *POM* and Dox were applied in combination as compared to individual treatments. IC_{50} values were determined to be 393 μg/mL (*POM*) and 2.24 μg/mL (Dox) in combination, but 1153 μg/mL (*POM*) and 12.56 μg/mL (Dox) in a single treatment. The value of the Loewe index, determined for IC_{50}, was notably lower than 1 (LI=0.51), clearly indicating synergism of *POM* and Dox. Additionally, *POM* and *POM* +Dox induced early/late apoptosis and G2/M cell cycle arrest. Furthermore, *POM* increased, while Dox decreased the expression levels of SODs and catalase. The obtained results encourage further examination of the potential use of *POM* in modern phytotherapy.

Key words: *P. maritimum*; antifungal effect; synergism with doxorubicin; flow cytometry; antioxidant enzymes

Abbreviations and acronyms: *Polygonum maritimum* extract *(POM)*; Doxorubicin (Dox); superoxide dismutase 1 (SOD1); superoxide dismutase 2 (SOD2) ; catalase (CAT); liquid chromatography tandem-mass spectrometry (LC-MS/MS); fetal bovine serum (FBS); phosphate buffered saline (PBS); dimethyl sulfoxide (DMSO); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); fluorescence-activated cell sorting (FACS); Loewe index (LI); 7-aminoactinomycin D (7-AAD); nuclear factor erythroid-2 related factor 2 (Nrf2); 25% inhibitory concentration (IC_{25}); 50% inhibitory concentration (IC_{50}); 75% inhibitory concentration (IC_{75})

INTRODUCTION

The genus *Polygonum* belongs to the family Polygonaceae, one of the dominant families in the flora of most countries, especially in the Northern Hemisphere [1]. Being native to Europe, Asia and Africa, *Polygonum* herbs have been used as traditional medicines in many different cultures for treating dysentery, articular pain, inflammation and diabetes [2-4]. As reported in recent publications, plants belonging to *Polygonum* genus are known to produce a large number of biologically important secondary metabolites, such as flavonoids, anthraquinones, stilbenoids, glycolipids and terpenes [1, 5-7]. These secondary metabolites are largely

viewed as potential sources of novel anticancer and antiallergic agents, antiinflammatory drugs, antibiotics, insecticides and herbicides. The pharmacological effects of *Polygonum* spp. include antioxidant, antibacterial, antifungal, neuroprotective, antidiabetic and antiinflammatory activities [1,5-8]. Furthermore, the species *Polygonum maritimum*, commonly known as sea knotgrass, has been described for its antioxidant activities [1]. It contains quercetin, quercitrin and catechin, known for their numerous bioactivities [9].

Considering the existence of biodegrading and storage-contaminating fungi in foods, as well as fungal toxicity and potential pathogenicity, there is a constant need for their growth control. Since synthetic substances targeting plant fungal pathogens can produce toxic residues, the preservative activity of herbs and spices has received notable attention [10]. In addition, the resistance of fungi to conventional antimycotics further stimulates the search for natural inhibitors [11].

The use of cytostatics in treating different cancers is burdened with numerous problems, including many side effects and the frequent development of drug resistance in tumor cells [12,13].

Taking into account the abovementioned facts, the aims of this study were to examine the cytotoxic and antifungal properties of the ethanolic extract of *P. maritimum* (*POM*). Its cytotoxicity was assessed in human hepatocarcinoma HepG2 cells and included an evaluation of its effect on antioxidant enzyme expression and its potential to modulate the cell's response to the common cytostatic doxorubicin (Dox). To the best of our knowledge, none of this has been previously reported. Furthermore, the antifungal effect was examined against *Penicillium* spp., *Alternaria alternata*, *Aspergillus* spp., *Fusarium semitectum* and *Fusarium oxysporum* by monitoring growth inhibition and mycelial decolorization.

MATERIALS AND METHODS

Chemicals and reagents

Reference standards of the phenolic compounds and quinic acid were obtained from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from Chromadex (Santa Ana, USA). HPLC gradient grade methanol was purchased from J. T. Baker (Deventer, The Netherlands), and p.a. formic acid from Merck (Darmstadt, Germany). William's medium, fetal bovine serum (FBS), penicillin-streptomycin mixtures, phosphate buffered saline (PBS), trypsin from porcine pancreas, dimethyl sulfoxide (DMSO), protease inhibitor cocktails, Triton® X-100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Steinheim, Germany). Reagents for apoptosis and cell cycle assays were obtained from Invitrogen Life Technologies™ (FITC-AnexinV, Binding puffers 2x, Rnase A Pure Link™, Massachusetts, USA), and 7-aminoactinomycin D was provided by Pharmingen™ (New Jersey, USA). The Folin-Ciocalteu reagent was obtained from Fisher Scientific (Leicestershire, UK). Doxorubicin (Cas. No. 25316-40-9) was provided by Actavis, S.C. Sindan-Pharma S.R.L. (Buchurest, Romania). Primary and secondary antibodies were purchased from Abcam (Cambridge, UK). All the other chemicals and reagents were obtained from local companies and were molecular biology grade.

Plant material and extract preparation

Aerial parts of *POM* were collected along the Adriatic coast, in Ulcinj, Montenegro. The voucher specimen was deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia (BUNS Herbarium) under the reference number 2-1670. The dried plant material (5-15g) was extracted with 80% ethanol (1g per 10 mL) and filtered. The obtained extract was evaporated to dryness in a vacuum evaporator (<45°C). Raw extract was suspended in water and purified by liquid-liquid extraction with petroleum ether. The petroleum ether layer was washed with methanol and the methanol extract was pooled with the aqueous layer. Subsequently, the extract was evaporated and dissolved in DMSO to a final concentration of 100 mg/mL.

Identification of compounds

The phytochemical profile of *P. maritimum* was evaluated by measuring the total phenolic and total flavonoid contents [14] and determined by quantitative LC-MS/MS analysis [15].

In vitro antibacterial activity

The microdilution method was used to assess the antibacterial activity of *POM* (concentration range of 0.039-5 mg/mL) against seven bacterial strains, including Gram-negative bacteria: *Escherichia coli* (ATCC 8739), *Shigella flexneri* (ATCC 9199), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella enteritidis* (ATCC 13076), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19111), and *Enterococcus faecalis* (ATCC 29212).

In vitro antifungal activity

The antifungal activity of the plant extract was determined by the agar plate method [16]. The fungi *Penicillium* spp., *Alternaria alternata*, *Aspergillus* spp., *Fusarium semitectum* and *Fusarium oxysporum* were obtained from the Institute of Medicinal Plants Research "Dr. Josif Pančić", Pančevo, Serbia. Micromycetes were maintained on potato dextrose agar (PDA). One hundred μL of the extract with a particular concentration were brushed with a glass sterile stick in the PDA substrate. The *POM* was tested at a concentration of 1 mg/mL against *Penicillium* spp., *Fusarium oxysporum* and *Alternaria alternata*, and at a concentration of 5 mg/mL against *Fusarium semitectum* and *Aspergillus* spp. Seven-day-old agar disks (5 mm in diameter) bearing the desired fungus growth were transferred to the center of Petri plates with PDA. The fungus cultures were incubated at 25±2°C for 7 days. The experiment was set for up to four replicates and repeated three times. The percentage of mycelial growth inhibition was calculated according to the following formula:

$$\%I=100\times(dc\times dt)/dc$$

where I – mycelial growth inhibition in %, dc – mycelial growth in control in mm, dt – mycelial growth with *POM* in mm.

Cell culture

Human hepatocellular carcinoma HepG2 cells (ATCC HB-8065, USA) were grown in William's medium containing 15% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine. The cells were maintained in an incubator at 37°C with 5.0% CO_2 in a humidified atmosphere. The cells were subcultured at 90% confluence, twice each week, using 0.1% trypsin. Cell viability was determined by the trypan blue dye exclusion method. Cells in the logarithmic growth phase were used in all experiments.

Cytotoxicity assay

The cytotoxic effects of the plant extract and doxorubicin, both as single compounds and in mixture, were assessed by a modified MTT assay [17]. Briefly, HepG2 cells were seeded into 96-well plates at a density $2x10^4$ cells/well and incubated overnight at 5% CO_2, 37°C. The cells were exposed to a series of two-fold dilutions of test substances and their combinations to obtain concentrations ranging from 4-0.125 mg/mL of *POM*, and from 22.8-0.712 μg/mL of Dox. After incubation for 24 h, the medium containing test substances was displaced with MTT (final concentration 0.5 mg/mL) and incubated for additional 3 h. At the end of the incubation with MTT, the medium was removed, and the formazan crystals were dissolved in DMSO. The optical density was measured at 570 nm using a microplate reading spectrophotometer (Multiskan FC, Thermo Scientific, Shanghai, China). Three independent experiments were conducted, and external wells were excluded.

Analysis of apoptosis by flow cytometry

HepG2 cells ($1x10^6$ cells/plate) were cultured with plant extract, *POM* (0.5 and 1 mg/mL) with and without Dox (2.8 and 5.7 μg/mL) for 24 h. After the treatment, the cells were harvested, washed with PBS, stained with Annexin V_FITC and 7-AAD, and analyzed by fluorescence activated cell sorting (FACS) (Calibur Becton Dickinson, Heidelberg, Germany) flow cytometer and Cell Quest computer software, according to the manufacturer's protocol.

Cell cycle analysis

HepG2 cells were treated as described above. After the treatment and incubation for 24h, cells were harvested and fixed with ice-cold 70% ethanol at -20°C for 30 min. Subsequently, the cells were resuspended in PBS containing propidium iodide and RNase A and incubated for 30 min at room temperature. Distribution of the cells was measured by FACS analysis.

Western blot analysis

After treatment with the test compounds, the cell lysates were prepared in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail solutions. The protein concentration was determined according to the Lowry method [18] at 750 nm, using bovine serum albumin (BSA) as a standard. Protein expression of CuZn superoxide dismutase (SOD1), Mn superoxide dismutase (SOD2) and catalase (CAT) were determined by Western blot analysis. Cell lysates (20 μg of samples per lane for SOD1 and SOD2 and 50 μg per lane for CAT) were poured on 12% SDS-polyacrylamide gels and transferred overnight to 0.45 mm polyvinylidene difluoride (PVDF) membranes after electrophoresis. The membranes were blocked in 5% dry non-fat milk in Tris buffered saline containing 0.1% Tween 20 (TBST) for 1.5 h. Anti-SOD1, -SOD2 and -CAT rabbit polyclonal antibodies (1:2000) were used as primary antibodies. Anti-β actin mouse monoclonal antibody (1:1000) was used as a loading control. Horseradish peroxidase-conjugated secondary goat anti-rabbit polyclonal antibodies (for SOD1, SOD2 and CAT, 1:5000) or rabbit anti-mouse polyclonal antibody (for beta-actin, 1:5000) were used as a secondary antibody. The immunoreactive bands were visualized using the enhanced chemiluminescence detection kit (ECLplus, Amersham, Bucks, UK) on a ChemiDoc-It2 detection system (UVP, Jena, Germany). The intensity of signals was evaluated by the ImageJ program. The amount of protein in each lane was normalized to β-actin.

Statistical analysis

To analyze the type of pharmacokinetic interactions of Dox and the extract in combination, the Loewe additivity model (LI) was used and data from the MTT assay were subjected to statistical analysis with the mixLow package in R-studio [19]. Data from apoptosis, cell cycle and Western blotting were analyzed by analysis of variance (ANOVA) using GraphPad Prism software. The level of statistical significance was defined as $p<0.05$. Microsoft Excel software was used to perform the statistical analyses for antifungal experiments.

RESULTS

Identification of compounds

The obtained results showed that the *P. maritimum* extract of the aerial parts has a high total phenolic content (483 mg eqv. gallic acid/1g dry extract) but a lower total flavonoid content (1.4 mg eqv. quercetin/1g dry extract). The dominant compounds of the *POM* extract could be presented in following order: gallic acid > epigallocatechin gallate > catechin > quercetin-3-O-galactoside > quercetin-3-O-glucoside (Table 1).

Table 1. Results of phenols quantification in the *P. maritimum* herbal extract.

Compound	***P. maritimum* L. Content [mg/g DW]***
Quinic acid	0.773 ± 0.077
Gallic acid	**5.541±0.499**
Protocatechuic acid	0.081±0.007
2,5-dihydroxybenzoic acid	0.002±0.000
5-*O*-caffeoylquinic acid	0.029±0.001
Epigallocatechin gallate	**3.734±1.120**
p-hydroxybenzoic acid	0.026±0.002
Caffeic acid	0.014±0.001
Vanillic acid	0.039±0.012
Syringic acid	0.114±0.023
Coumaric acid	0.026±0.002
Ferulic acid	0.023±0.002
Sinapic acid	0.012±0.001
Catechin	**2.628±0.263**
Epicatechin	0.056±0.006
Vitexin	ND
Luteolin-7-*O*-glucoside	0.001±0.000
Quercetin-3-O-galactoside	**0.967±0.058**
Rutin	ND
Quercetin-3-*O*-glucoside	**0.482±0.014**
Apigenin-7-*O*-glucoside	ND
Myricetin	0.167±0.050
Quercetin-3-*O*-L-rhamnoside	0.074±0.004
Kaempferol-3-*O*-glucoside	0.113±0.005
Quercetin	0.194±0.058
Naringenin	0.007±0.001
Luteolin	0.001±0.000
Apigenin	ND
Kaempferol	0.008±0.001
Esculetin	0.004±0.000

*given in mg per dry extract (DW)±relative standard deviation of repeatability; nd – not detected – peak not observed, concentration is lower than the limit of detection

In vitro antimicrobial activity

Antibacterial screening performed by the microdilution assay against a panel of seven pathogenic strains showed no antibacterial activity (in the given range of concentrations; less than 5 mg/mL). Antifungal activity was evaluated by comparing the growth of mycelia in the presence of *POM* and in the control sample (untreated). Apart from *Fusarium oxysporum*, *POM* inhibited the growth of all other tested strains. The obtained results showed a notable growth inhibitory effect on *Alternaria alternata*, *Penicillium* spp., *Fusarium semitectum* and *Aspergillus* spp., with inhibitions determined to be 34.3%, 30.6%, 20.24% and 19.6%, respectively (Table 2). Moreover, depigmentation and morphological changes of the *Aspergillus* spp. mycelium were additional indications of *POM* toxicity.

Cytotoxicity assay

The cytotoxic effect of the herbal extract, Dox, and their combinations, evaluated on HepG2 cells, is presented in dose-response curves (Fig.1). The IC_{25}, IC_{50} and IC_{75} values were determined from these curves (Table 3). In order to quantify the mode of interaction between the tested substances, the Loewe index (LI) was calculated, with LI<1, LI=1, LI>1 indicating synergistic, additive and antagonistic effects, respectively (Fig. 2).

Table 2. Inhibition of mycelium growth after treatment with *POM*.

Fungi	Growth inhibition [%]
Alternaria alternata	34.3
Penicillium spp.	30.6
Fusarium semitectum	20.24
Aspergillus spp.	19.6
Fusarium oxysporum	nd*

POM was tested at concentrations of 1 mg/mL against *Penicillium* spp., *Fusarium oxysporum* and *Alternaria alternata* and 5 mg/mL against *Fusarium semitectum* and *Aspergillus* spp.

*nd – not detected – inhibition of growth is not detected

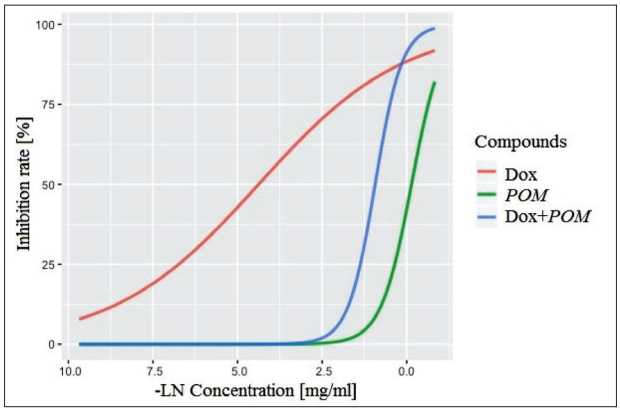

Fig. 1. Inhibition of HepG2 cell viability after treatment with *POM*, Dox and their combination. The tested concentrations of *POM*, Dox and their combination are presented as values of natural logarithms (LN values). *POM* was tested in a concentration range from 4-0.125 mg/mL (that corresponded to the range from 1.386 to -2.079 presented in LN values). Dox was tested in a concentration range from 0.0228-0.0007125 mg/mL (that corresponded to the range from -3.780 to -7.246 presented in LN values).

Although antagonism was obtained when the binary mixture constituents were applied in low concentrations (for the IC_{25} value LI was 1.56), with the increase in their concentrations clear synergism was obtained

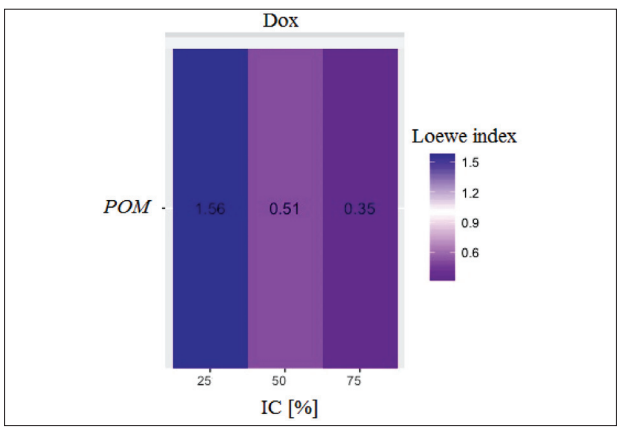

Fig. 2. Loewe Index values calculated at the IC_{25}, IC_{50} and IC_{75} level. Loewe index (LI), interaction index; LI<1 indicates synergism; LI=1 indicates an additive effect; LI>1 indicates antagonism.

Table 3. The cytotoxicity of *POM* and Dox, either alone or in a binary-drug combination on HepG2 cells.

	IC_{25} of *POM*	IC_{25} of Dox	IC_{50} of *POM*	IC_{50} of Dox	IC_{75} of *POM*	IC_{75} of Dox
P. maritimum	702		1153		1880	
Dox		1.30		12.56		138
P. maritimum+Dox	253	1.44	393	2.24	608	3.47

* Values are presented as IC_{25} IC_{50} and IC_{75}, in μg/mL, from three independent experiments by the MTT assay. IC_{25}-$_{75}$-the concentration of sample required to inhibit cell survival by 25%, 50% and 75%, respectively.

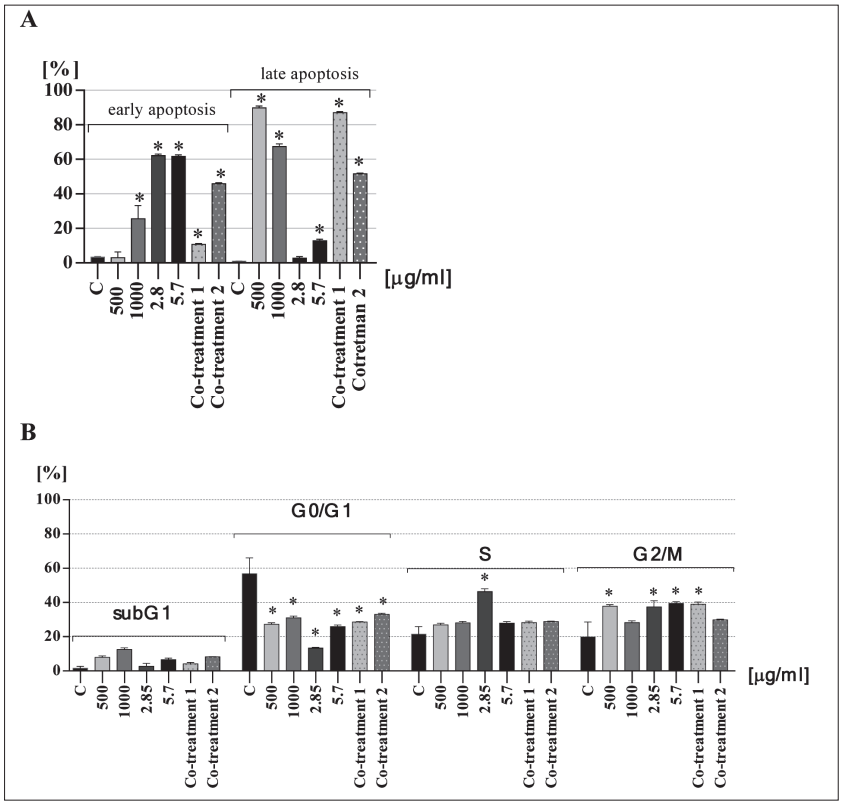

Fig. 3. Analysis of apoptosis (**A**) and cell cycle arrest (**B**) in HepG2 cells treated with *POM*, Dox and their combination. Co-treatment=simultaneously applied *POM* and Dox (Co-treatment 1=500 μg/mL *POM* and 2.28 μg/mL Dox; Co-treatment 2=1000 μg/mL *POM* and 5.7 μg/mL Dox. Results are expressed as the difference (%) in comparison with the untreated control (C); *p≤0.05.

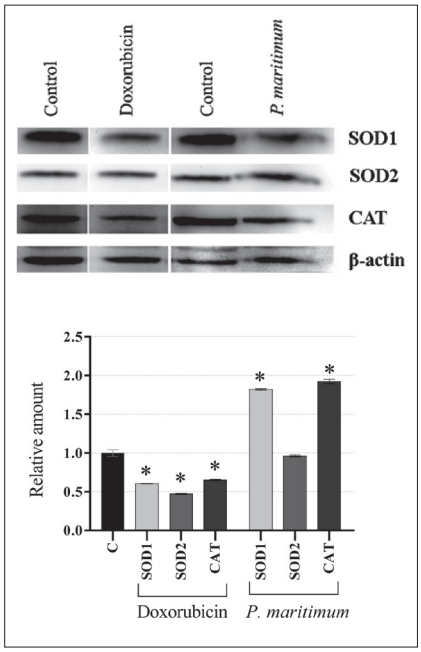

Fig. 4. The effect of *POM* and Dox on the induction of the antioxidative defense enzymes. **A** – Blots were normalized to β-actin as the internal control. **B** – Histograms of densitometric analyses of SOD1, SOD2 and CAT levels following treatment of HepG2 cells with *POM* or Dox (*POM* 0.5 mg/mL, Dox 1.4 μg/mL); C – untreated control * p≤0.05.

(for the IC_{50} and IC_{75} values, LIs were determined to be 0.51 and even 0.35, respectively). For example, 50% lethality was obtained with strikingly lower doses in the cotreatment (393 μg/mL of *POM* and 2.24 μg/mL of Dox), compared to the treatment with *POM* (1153 μg/mL) or Dox alone (12.56 μg/mL).

Assessment of apoptosis and cell cycle

To determine whether the cytotoxicity of *POM* and its combination with Dox is related to apoptosis induction and if it is mediated by cell cycle arrest, flow cytometry was applied. The *POM* extract (500 μg/mL), alone and in combination with Dox (2.8 μg/mL), increased the percentage of cells in late apoptosis (89.85% and 87.11%, respectively; Fig. 3A). Nonetheless, the combination of lower concentrations of *POM* and Dox increased the number of early apoptotic cells. Furthermore, a statistically significant increase in early and late apoptotic cells was observed after the cell treatment with 1000 μg/mL of *POM* alone (25.51% and 67.5%, respectively), and in combination with Dox (45.88% and 51.6%, respectively). As expected, Dox increased the number of apoptotic cells as follows: when applied in a lower dose (2.8 μg/mL) only cells in early phase (62.15%) were observed, but when applied in a higher dose (5.7 μg/mL), both early and late phases (61.65% and 12.75%, respectively) were observed. The cell cycle phase distribution after the treatment of cells with *POM*, Dox and their combinations is presented in Fig. 3B. Lower concentrations of the extract, Dox, and their combination induced cell cycle arrest in G2/M phase, causing a significant decrease in cell number in the G0/G1 phase. Higher concentrations and their combination did not significantly affect the cell cycle.

Western blot analysis

To examine the effect of *POM* on the antioxidant enzymes SOD1, SOD2 and CAT, we employed Western blotting. HepG2 cells were treated either with the

extract (500 μg/mL) or with Dox (1.4 μg/mL). The obtained results showed that Dox decreased the expression of CAT, SOD1 and SOD2, while *POM* extract significantly increased CAT and SOD1 expression but had no influence on SOD2 (Fig. 4).

DISCUSSION

Many reports have highlighted the potential benefits of using *Polygonum* species in phytomedicine, revealing the close correlation between their phenolic composition and the pharmacological effects they exhibit, including antioxidant, anticancer and antimicrobial properties. In the course of the examination of the biological activities of *POM*, its antimicrobial potential was examined first. While no antibacterial activity was found, a notable antifungal effect against food-contaminating micromycetes was observed. Absence of an antibacterial potential is contrary to the results of El-Haci et al. [1], who determined an antibacterial activity of *P. maritimum* extract against some human pathogens. This discrepancy could be attributed to the difference in the process of extraction [20]. On the other hand, the obtained antifungal effect is consistent with literature data showing that extracts of various *Polygonum* species, such as *P. hydropiper*, *P. aviculare*, *P. punctatum* and *P. acuminatum* produced fungicidal effects [5,21-24]. In addition, the antimicrobial activity of gallic acid and catechin, being dominant in *POM*, was also shown [25].

The cytotoxicity of *POM* was monitored in human hepatoma HepG2 cells, which have been widely used as an *in vitro* model for assessing drug effectiveness and toxicity [26]. Since chemosensitizing phytochemicals can be combined with conventional chemotherapeutic agents to improve cancer treatment and clinical outcomes, we also monitored the effect of its combinations with doxorubicin (Dox). The benefits of this approach include a reduction in the doses of applied chemotherapeutic drugs, which is important in view of the severe toxicity of conventional cytostatics. This can decrease the toxicity without compromising treatment efficacy through the induction of more diverse mechanisms of action [27]. Dox-induced toxicity is mediated by reactive oxygen species (ROS) production and consequently increases lipid peroxidation [28]. Furthermore, the medical application of Dox is limited due to its side effects, mainly hepatotoxicity and cardiotoxicity, but also due to frequent development of drug resistance in tumor cells [29]. Taking into account that the active principals of *POM*, including the most dominant flavan-3-ols (catechin and epigallocatechin gallate), flavon-3-ols (quercetin and myricetin derivatives) and free gallic acid, possessing an antioxidative potential [25,30], *POM* could protect against toxicity mediated by Dox-induced ROS, and certainly affect the expression of antioxidative enzymes. In line with this are the results indicating that extracts of *P. glabrum* and *P. orientale* provided hepatoprotective activity in normal hepatocytes *in vivo* [31,32].

The low cytotoxic effect of *POM* is in agreement with literature data showing that plant species from the Polygonaceae family have an antiproliferative effect on hepatocarcinoma cells, including Bel-7402 and Hepa 1-6 [33]. Concerning the effect of combinations (*POM*+Dox), both antagonistic and synergistic interactions were obtained. This could be attributed to the dual effect of antioxidants, which induce a prooxidative effect at higher concentrations [34]. In light of this, *POM* at lower tested concentration induced an antioxidant effect and consequently reduced the ROS-mediated toxicity of Dox. On the other hand, with increasing *POM* concentration, its effect became prooxidative and enhanced Dox toxicity, inducing a remarkable synergistic effect.

In order to analyze the mode of obtained cytotoxicity, we monitored the effect on apoptosis and cell cycle arrest. Our results indicate that *POM* alone and combined with Dox significantly induced early and late apoptosis and G2/M cell cycle arrest. This is consistent with recent studies showing that various extracts of *Polygonum* species and their active compounds also induced a proapoptotic effect and arrested HepG2 cells in the G2/M phase [35,36]. In addition, literature data confirmed that the cytotoxic activity of gallic acid and catechin, as dominant constituents of *POM*, could also be linked with the proapoptotic effect and G2/M cell cycle arrest [3,25,27,30]. The authors emphasized their ability to induce cell cycle arrest in malignant cells, which contributes to cancer cell proliferation inhibition while leaving normal cells unscathed. Furthermore, gallic acid-based indanone derivatives have been shown to exhibit substantial anticancer activity against HepG2 cells [25].

Medications based on plants, including *Polygonum* extracts, and conventional cytostatic drugs can influence the metabolism of toxic substances by modulating both nonspecific and specific detoxifying enzymes and by disturbing the balance between apoptosis, the regulation of cell death and proliferation [37,38]. Herein we monitored the impact of *POM* and Dox on the modulation of nonspecific detoxifying/antioxidant enzymes such as SOD1, SOD2 and CAT. The results indicated that *POM* enhanced, whilst Dox suppressed SOD1 and CAT activities in HepG2 cells. Some studies reported higher expression of SOD enzymes in hepatocytes after treatment with *P. orientale* and *P. multiflorum* extracts, which is in accordance with our findings [32,39]. The main constituents of *POM*, gallic acid and catechin, exert their ability to elevate the hepatic levels of CAT, and therefore may provide protection against the deleterious effects of lipid peroxides and hydrogen peroxide [40]. Moreover, gallic acid and epigallocatechin gallate affect the level of antioxidant enzymes by stimulating Nrf2 protein expression through the Keap1-Nrf2-ARE signaling pathway in HepG2 cells [41]. It is well known that activation of the Nrf2-ARE cellular defense system is an important mechanism for protecting cells against oxidative and xenobiotic stresses [26,41,42].

Considering all the obtained results, the *POM*-induced dual protective and cytotoxic effects depended mainly on the applied dose. A similar observation was described by another research group that showed that plant extracts had a protective effect on hepatocytes *in vivo* as a result of enhanced antioxidant enzyme activities and the cytotoxic effect obtained through increased apoptosis and alterations of the cell cycle [43].

In conclusion, the *Polygonum maritimum* extract could be a significant natural source of biologically active compounds that possess antifungal and cytotoxic properties. It is important to emphasize its ability to potentiate the chemotherapeutic activity of the conventional cytostatic doxorubicin. However, further investigation is needed to elucidate the mechanism of action of *POM* and to examine its application both as a fungicide and adjuvant in chemotherapy.

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Author contributions: MJ participated in all the experiments, wrote the draft and performed the literature search; TSR contributed to the flow cytometric analysis; ES prepared the extract, performed the chemical analysis and reviewed the manuscript; SB performed the statistical analysis and prepared the figures; BN supervised the research project in some parts and formed the final version of manuscript; NJ contributed to the Western blots, TS contributed to the antifungal experiments; JKV critically reviewed the manuscript; DMĆ provided the concept of the study and supervised all phases of the research. All authors read and approved the final manuscript.

Conflicts of interest disclosure: The authors have no conflicts of interest to declare.

REFERENCES

1. El-Haci IA, Bekkara FA, Mazari W, Hassani F, Didi MA. Screening of biological activities of *Polygonum maritimum L.* from Algerian coast. Asian Pac J Trop Biomed. 2013;3(8):611-6.
2. Tunon H, Olavsdotter C, Bohlin L. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. J Ethnopharmacol. 1995;48(2):61-76.
3. Wang KJ, Zhang YJ, Yang CR. Antioxidant phenolic compounds from rhizomes of *Polygonum paleaceum*. J Ethnopharmacol. 2005;96(3):483-7.
4. Ravipati AS, Zhang L, Koyyalamudi SR, Jeong SC, Reddy N, Bartlett J, Smith PT, Shanmugam K, Münch G, Wu MJ, Satyanarayanan M. Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content. BMC Complement Altern Med. 2012;12(1):173.
5. Dong X, Fu J, Yin X, Li X, Wang B, Cao S, Zhang J, Zhang H, Zhao Y, Ni J. Pharmacological and other Bioactivities of the Genus *Polygonum*-A Review. Trop J Pharm Res. 2014;13(10):1749-59.
6. Nkuété AH, Migliolo L, Wabo HK, Tane P, Franco OL. Evaluation of Multiple Functions of *Polygonum* Genus Compounds. European J Med Plants. 2015;6(1):1-16.
7. Rodrigues MJ, Custódio L, Lopes A, Oliveira M, Neng NR, Nogueira JM, Martins A, Rauter AP, Varela J, Barreira L. Unlocking the in vitro anti-inflammatory and anti-diabetic potential of *Polygonum maritimum* L. Pharm Biol. 2017;55(1):1348-57.
8. Rodrigues MJ, Slusarczyk S, Pecio Ł, Matkowski A, Salmas RE, Durdagi S, Pereira C, Varela J, Barreira L, Custódio L. *In vitro* and *in silico* approaches to appraise *Polygonum maritimum L.* as a source of innovative products with anti-ageing potential. Ind Crops Prod. 2018;111:391-9.
9. Alía M, Mateos R, Ramos S, Lecumberri E, Bravo L, Goya L. Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). Eur J Nutr. 2006;45(1):19-28.
10. Özcan M, Boyraz N. Antifungal properties of some herb decoctions. Eur. Food Res. Technol. 2000;212(1):86-8.

11. Brent KJ, Hollomon DW. Fungicide resistance in crop pathogens: How can it be managed? 2nd rev. ed. Brussels: Fungicide Resistance Action Committee; 2007. 50 p.
12. Ullah MF. Cancer multidrug resistance (MDR): a major impediment to effective chemotherapy. Asian Pac J Cancer Prev. 2008;9(1):1-6.
13. Shields M. Chemotherapeutics. In: Pharmacognosy: Fundamentals, Applications and Strategies.McCreath SB, Delgoda R, editors. London: Academic Press; 2017. 738 p.
14. Beara IN, Lesjak MM, Jovin ED, Balog KJ, Anačkov GT, Orčić DZ, Mimica-Dukić NM. Plantain (*Plantago* L.) species as novel sources of flavonoid antioxidants. J Agric Food Chem. 2009;57(19):9268-73.
15. Orčić D, Francišković M, Bekvalac K, Svirčev E, Beara I, Lesjak M, Mimica-Dukić N. Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. Food Chem. 2014;143:48-53.
16. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal. 2016;6(2):71-9.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75.
19. Boik JC, Narasimhan B. An R package for assessing drug synergism/antagonism. J Stat Softw. 2010;34(6):1-8.
20. Mohapatra DP, Thakur V, Brar SK. Antibacterial efficacy of raw and processed honey. Biotechnol Res Int. 2011;2011:1-6.
21. Ayaz M, Junaid M, Ullah F, Sadiq A, Ovais M, Ahmad W, Zeb A. Chemical profiling, antimicrobial and insecticidal evaluations of *Polygonum hydropiper* L. BMC Complement Altern Med. 2016;16(1):502.
22. Waqas M, Shad AA, Wasila H, Khan EA, Bashir O, Rashid MU, Ahmad I. Antimicrobial activity of Polygonium hydropiper leaves against plant and human pathogenic fungal and bacterial strains. Pure Appl Biol. 2016;5(4):856-61.
23. Salama HM, Marraiki N. Antimicrobial activity and phytochemical analyses of *Polygonum aviculare* L. (Polygonaceae), naturally growing in Egypt. Saudi J Biol Sci. 2010;17(1):57-63.
24. Derita MG, Leiva ML, Zacchino SA. Influence of plant part, season of collection and content of the main active constituent, on the antifungal properties of *Polygonum acuminatum* Kunth. J Ethnopharmacol. 2009;124(3):377-83.
25. Badhani B, Sharma N, Kakkar R. Gallic acid: a versatile antioxidant with promising therapeutic and industrial applications. Rsc Advances. 2015;5(35):27540-57.
26. Yeh CT, Yen GC. Involvement of p38 MAPK and Nrf2 in phenolic acid-induced P-form phenol sulfotransferase expression in human hepatoma HepG 2 cells. Carcinogenesis. 2005;27(5):1008-17.
27. Pinmai K, Chunlaratthanabhorn S, Ngamkitidechakul C, Soonthornchareon N, Hahnvajanawong C. Synergistic growth inhibitory effects of *Phyllanthus emblica* and *Terminalia bellerica* extracts with conventional cytotoxic agents: doxorubicin and cisplatin against human hepatocellular carcinoma and lung cancer cells. World J Gastroenterol. 2008;14(10):1491-7.
28. Ichihara S, Yamada Y, Kawai Y, Osawa T, Furuhashi K, Duan Z, Ichihara G. Roles of oxidative stress and Akt signaling in doxorubicin cardiotoxicity. Biochem Biophys Res Commun. 2007;359(1):27-33.
29. Eid SY, El-Readi MZ, Wink M. Synergism of three-drug combinations of sanguinarine and other plant secondary metabolites with digitonin and doxorubicin in multi-drug resistant cancer cells. Phytomedicine. 2012;19(14):1288-97
30. Ow YY, Stupans I. Gallic acid and gallic acid derivatives: effects on drug metabolizing enzymes. Curr Drug Metab. 2003;4(3):241-8.
31. Raja S, Ramya I. A comprehensive review on *Polygonum glabrum*. Int J Phytomedicine. 2017; 8(4):457-67.
32. Chiu YJ, Chou SC, Chiu CS, Kao CP, Wu KC, Chen CJ, Tsai JC, Peng WH. Hepatoprotective effect of the ethanol extract of *Polygonum orientale* on carbon tetrachloride-induced acute liver injury in mice. J Food Drug Anal. 2017;26(1):369-379.
33. Hu B, An HM, Shen KP, Song HY, Deng S. *Polygonum cuspidatum* extract induces anoikis in hepatocarcinoma cells associated with generation of reactive oxygen species and down regulation of focal adhesion kinase. Evid Based Complement Alternat Med. 2012; 2012: 607675.
34. Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 2011;82(4):513-23.
35. Shieh DE, Chen YY, Yen MH, Chiang LC, Lin CC. Emodin-induced apoptosis through p53-dependent pathway in human hepatoma cells. Life Sci. 2004;74(18):2279-90.
36. Lee BH, Huang YY, Wu SC. Hepatoprotective activity of fresh *Polygonum multiflorum* against HepG2 hepatocarcinoma cell proliferation. J Food Drug Anal. 2011;19(1):26-32.
37. Wang YY, Li J, Wu ZR, Zhang B, Yang HB, Wang Q, Cai YC, Liu GX, Li WH, Tang Y. Insights into the molecular mechanisms of *Polygonum multiflorum* Thunb-induced liver injury: a computational systems toxicology approach. Acta Pharmacologica Sinica. 2017; 38(5):719-32.
38. Altay A, Kartal Dİ, Sadi G, Güray T, Yaprak AE. Modulation of mRNA expression and activities of xenobiotic metabolizing enzymes, CYP1A1, CYP1A2, CYP2E1, GPx and GSTP1 by the Salicornia freitagii extract in HT-29 human colon cancer cells. Arch Biol Sci. 2017;69(3):439-48.
39. Lin L, Ni B, Lin H, Zhang M, Li X, Yin X, Qu C, Ni J. Traditional usages, botany, phytochemistry, pharmacology and toxicology of *Polygonum multiflorum* Thunb.: a review. J Ethnopharmacol. 2015;15(159):158-83.
40. Yoo KM, Lee CH, Lee H, Moon B, Lee CY. Relative antioxidant and cytoprotective activities of common herbs. Food Chem. 2008;106(3):929-36.
41. Han X, Shen T, Lou H. Dietary polyphenols and their biological significance. Int J Mol Sci. 2007;8(9):950-88.
42. Silva IC, Polaquini CR, Regasini LO, Ferreira H, Pavan FR. Evaluation of cytotoxic, apoptotic, mutagenic, and chemopreventive activities of semi-synthetic esters of gallic acid. Food Chem Toxicol. 2017;105:300-7.
43. Wang YY, Li J, Wu ZR, Zhang B, Yang HB, Wang Q, Cai YC, Liu GX, Li WH, Tang Y. Insights into the molecular mechanisms of *Polygonum multiflorum* Thunb-induced liver injury: a computational systems toxicology approach. Acta Pharmacol Sin. 2017;38(5):719-32.