

POLYGONUM BISTORTA L. HERBA ET FLORES. POLYPHENOLS PROFILE, ANTIOXIDANT PROPERTIES AND CYTOTOXIC EFFECT ON MURINE FIBROBLAST CELL LINE NIH3T3

LUCIA PÎRVU¹, FAWZIA SHA'AT^{2,3*}, LUMINIȚA CLAUDIA MICLEA², TUDOR SAVOPOL², GEORGETA NEAGU¹, DENISA IOANA UDEANU⁴, MIHAELA GEORGETA MOISESCU²

¹National Institute for Chemical - Pharmaceutical Research and Development (INCDCF), 112 Vitan Road, 031299, Bucharest, Romania

²"Carol Davila" University of Medicine and Pharmacy, Faculty of Medicine, Biophysics and Cellular Biotechnology Department, 8 Eroii Sanitari Boulevard, 050474, Bucharest, Romania

³Politehnica University of Bucharest, Faculty of Applied Chemistry and Material Science, Analytical Chemistry and Environmental Engineering Department, 1-7 Gheorghe Polizu Street, 011061, Bucharest, Romania

⁴"Carol Davila" University of Medicine and Pharmacy, Faculty of Pharmacy, Department of Clinical Laboratory and Food Safety, 6 Traian Vuia Street, 020956, Bucharest, Romania

*corresponding author: fawzya.shaat@gmail.com

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Abstract

This work aimed to study the polyphenols content, the antioxidant activity and the potential cytotoxic effects on fibroblast murine cells NIH3T3 of an ethanolic extract (70%, v/v) obtained from the aerial part (*herba et flores*) of *Polygonum bistorta* L. (*Polygonaceae* family). The tested extract was assessed for its total phenols content: 1.15 mg gallic acid equivalents (GAE) per 1 mL extract and showed numerous flavonoids compounds such as quercetin, kaempferol and luteolin derivatives, as well as caffeic and chlorogenic phenolic acids. The chemiluminescence (CL) tests indicated a high antioxidant capacity ($IC_{50} = 0.57 \mu\text{g GAE/mL}$), superior to gallic acid ($IC_{50} = 0.85 \mu\text{g/mL}$) and rutin ($IC_{50} = 2.54 \mu\text{g/mL}$), used as reference compounds. The cytotoxicity test demonstrated that, at concentrations over $25 \mu\text{g GAE/mL}$ extract, the ethanolic extracts from the aerial part of *Polygonum bistorta* plant species have a significant toxicity on mouse embryonic fibroblast NIH3T3 cells.

Rezumat

Scopul studiului a constat în evaluarea compoziției chimice în polifenoli, a potențialului antioxidant și a efectului asupra celulelor fibroblastice murine de șoarece (NIH3T3) a unui extract etanolic (70%, v/v) obținut din partea aeriană (*herba et flores*) de *Polygonum bistorta* L. (fam. *Polygonaceae*). Extractul a prezentat o concentrație în polifenoli totali de 1,15 mg echivalent acid galic (GAE) per 1 mL extract și un conținut bogat în flavonoide, derivați ai quercetinei, kaempferolului și luteolului, precum și acizi fenolici, acid cafeic și acid clorogenic. Testele de chemiluminescență (CL) au indicat un potențial antioxidant foarte puternic pentru extractul testat ($IC_{50} = 0,57 \mu\text{g GAE/mL}$) superior compușilor de referință, acid galic ($IC_{50} = 0,85 \mu\text{g/mL}$) și rutin ($IC_{50} = 2,54 \mu\text{g/mL}$). Testul de citotoxicitate a demonstrat faptul că la concentrații peste $25 \mu\text{g GAE/mL}$, extractele etanolice din partea aeriană de *Polygonum bistorta* prezintă o toxicitate semnificativă asupra celulelor murine fibroblastice tip NIH3T3.

Keywords: *Polygonum bistorta*, polyphenols, antioxidant, cytotoxicity, fibroblast cells, NIH3T3

Introduction

Polygonum bistorta L. (*Polygonaceae* family), commonly known as "bistort", is a valuable phyto-medicine, being used as a root vegetable in Asian regions due to its high content in alimentary starch-like polysaccharides. The root part (*Bistortae rhizoma*) is mostly known for its strong astringent properties, very useful in managing bleeding processes [12-14] and microbial infections [2, 15].

The aerial part has been shown to contain similar amounts of tannin type compounds (15 - 20% total content of which 5 - 10% in leaves pieces) as well as numerous phenolic acids such as ferullic, syringic, p-benzoic, hydroxycoumaric, melilotic, sinapic, vanillic

and ellagic acids [4]. The fresh material has catechinic and gallic acid derivatives and volatile oil compounds (from 0.004% to 0.010%) [2].

Comparative studies on taxons of *Polygonum* L. genus indicated quercetin, kaempferol, (iso)rhamnetin, luteolin, quercetin-3-methyl ether and taxifolin as being the specific free flavonoids aglycones present in *P. bistorta* species [10].

Therefore, the main therapeutic benefits of *P. bistorta* plant derived products are due to their anti-infectious [2, 15] and haemostatic [13] properties, generally attributed to tannins and flavonoids. Studies on methanolic and cold/hot water extracts from the aerial part of bistort showed their effectiveness against

microbial strains: *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Salmonella typhi* [2]. Also, the ethanolic extracts have been reported as very effective against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptococcus pneumonia* strains, the antimicrobial activity being attributed to gallic acid present in the herbal part [5, 15]. Concerning the haemostatic properties, some methylated flavonols [13] have been suggested to be involved.

Given the high interest in both, antimicrobial and anti-haemorrhagic properties of *Polygonum* genus, the present work aimed at studying the polyphenols profile and the antioxidant activity, as well as the cytotoxic effect on NIH3T3 cells in culture (monitoring the dynamic response profile of living cells via the xCELLigence real-time cell analyser and the cellular changes including cell number - cell index, CI) of an ethanolic extract (70%, v/v) from the aerial part of *Polygonum bistorta* species.

Materials and Methods

Plant material: The fresh aerial part (*herba et flores*) of *Polygonum bistorta* L. was collected in the summer time (June) from Romanian Carpathians (1400 m altitude). Taxonomic identification has been performed by the botanist's team of the National Institute for Chemical - Pharmaceutical Research and Development (INCDCP), Bucharest, Romania. A voucher specimen (PBam 2015) is deposited in INCDCP Plant Material Storing Room. The fresh material was shade dried, then grounded to medium-size plant powder.

Instruments: The instruments used for plant derived products analysis were: UV/Vis spectrophotometer (Hélios γ , Thermo Electron Corporation, U.K.) for chemical quantitative measurements; Linomat5 TLC visualizer (CAMAG, Muttenz, Switzerland) for chemical qualitative analyses; Luminometer (Turner BioSystems, USA) for the antioxidant activity assessments; xCELLigence® DP Real-Time Cell Analysis (xC-RTCA) (ACEA Biosciences, USA) for the *in vitro* cell proliferation tests.

Reagents and solutions for plant material characterisation: Chemicals (aluminium chloride, sodium carbonate, sodium acetate), reagents (Folin-Ciocalteu, Natural Product-NP/PEG), solvents (ethyl acetate, methanol, ethanol, chloroform, formic acid and glacial acetic acid) as well as the reference compounds (ref.) (rutin (min. 95%), hyperoside (> 97%), apigenin (> 97%), cosmosiin (97%), vitexin (96%), kaempferol (95%), caffeic acid (99%), chlorogenic acid (> 95%), rosmarinic acid (99%), gallic acid (95%) and protocatechuic acid (> 97%))

were purchased from Fluka and Sigma-Aldrich Co (Germany).

Preparation of plant extracts: Fifty grams of the powdered plant material were twice extracted with 500 mL of 70% (v/v) ethanol, 30 minutes each extraction, at reflux temperature. The combined extracts were paper filtered resulting 750 mL ethanolic extract (code E21). Fifty mL of E21 were further hydrolysed in 4 N HCl for 30 minutes, filtered and concentrated at the residue. The residue was further solved into 50 mL distilled water and manually extracted with ethyl acetate (three times, with 100 mL each extraction). The three ethyl acetate fractions were mixed and concentrated to a *sicc* product. The *sicc* product was dissolved into 96 % (v/v) ethanol to a final volume of 50 mL, obtaining the final hydrolysed sample (code H21).

Quantitative estimation of total phenols content in extracts: Total phenols content was measured using Folin-Ciocalteu reagent, the standard method of the Romanian Pharmacopoeia (FRX) [18]. The results were expressed as mg gallic acid (GAE) equivalents/ 1 mL sample ($r^2 = 0.989$).

Quantitative estimation of total flavones content in extracts: Total flavones were also measured according to the standard method of Romanian Pharmacopoeia (FRX) [18]. The results were expressed as mg rutin (R) equivalents/1 mL sample ($r^2 = 0.999$).

Qualitative analysis of polyphenols in extracts: Studies were performed by (HP)TLC method according to Wagner H. and Bladt S. [17], and Reikh E. and Schibli A. [16]. Polyphenols content was appraised by using two solvent systems: ethyl acetate:formic acid:glacial acetic acid:water 100:11:11:26 - system A and chloroform:glacial acetic acid:methanol:water 30:32:12:8 - system B, as described in a previous work [9].

Antioxidant activity assay: Studies were performed on the E21 extract in six dilution series: non-diluted sample ($\times 1$) and ($\times 2$), ($\times 5$), ($\times 10$), ($\times 50$) ($\times 100$) dilutions. Antioxidant activity tests were performed by chemiluminescence (CL) using luminol - H_2O_2 system [6]. 50 μ L aliquots of each sample in triplicates were mixed with 200 μ L 10^{-3} M luminol/DMSO, 700 μ L 0.2 M TRIS-HCl (pH 8.6) and 50 μ L 10^{-3} M H_2O_2 . As reference, the same mixture as above prepared, in which 50 μ L (70%, v/v) ethanol were used instead of the test sample aliquot. CL intensity (arbitrary units - a.u.) was measured every 5 seconds during one minute. The values at exactly 5 seconds were used for the antioxidant activity (AA%, formula bellow) and CL-IC₅₀ estimation (defined as the test sample concentration inhibiting 50% of the reactive oxygen species generation); CL-IC₅₀ estimation was done by comparison with R and GAE as reference compounds.

$$AA\% = \frac{\text{CL reaction intensity of the reference sample (a.u.)} - \text{CL reaction intensity of the tested sample (a.u.)}{\text{CL reaction intensity of the reference sample (a.u.)}} \times 100$$

Cell cytotoxicity (CC) evaluation: Studies were performed on NIH3T3 mouse embryonic fibroblast cells (CRL-1658™) purchased from ATCC® (USA). Cells were cultured at 37°C, 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal bovine serum, 1% penicillin/streptomycin/neomycin mix and 1% L-glutamine (Sigma-Aldrich, Germany). Cells were seeded in 16 wells E-Plates with gold electrodes on the bottom which allowed measuring the impedance variation in real time. At 24 hours after seeding, different concentrations of *P. bistorta* extract (E21) were added (final concentrations: 1, 5, 10, 25, 50 and 100 µg/mL equivalent GAE). Two different controls were used: one in which no solvent was added (Control) and one in which the final ethanol concentration was equal to the highest one in E21 samples (ethanol 6.08%).

The cytotoxic effect of ethanol itself was evaluated in separate experiments, reproducing the same

concentrations as in the experiments with the test samples/E21 dilutions (Figure 5).

The output of dedicated software (RTCA v2.0.130) of xC-RTCA is the so called Cell Index (CI) which is proportional to the number of cells adherent to gold electrodes [1, 3, 11]. CI was measured every 30 minutes during 4 days. We defined as cellular cytotoxicity the half value (CC-IC₅₀) the extract concentration inhibited 50% of CI values measured at 48 hours after bistorta addition. GraphPad Prism 7.0.0.159 was used to represent CC-IC₅₀. Values of $p \leq 0.05$ were considered significant.

Results and Discussion

(HT)PLC analysis

Figure 1 presents qualitative (HP)TLC aspects, polyphenols content of E21 and H21 samples respectively.

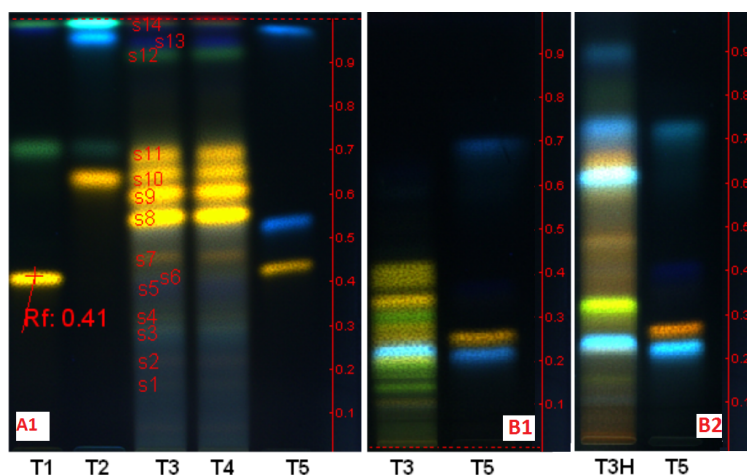


Figure 1.

(HP)TLC studies on the extracts from *Polygonum bistorta herba et flores*

A1: Track T1, rutin, vitexin, protocatechuic acid and apigenin (ref.); Track T2, hyperoside, cosmosiin, rosmarinic acid, kaempferol; Tracks T3 - T4, *P. bistorta herba et flores* E21; Track T5, rutin, gallic and caffeic acid (ref.); **B1:** Track T3, *P. bistorta herba et flores* E21; Track T5, rutin, chlorogenic, gallic and caffeic acid (ref.); **B2:** Track T3H, *P. bistorta herba et flores* hydrolysate sample (H21); Track T5, chlorogenic acid, rutin, gallic acid and caffeic acid (ref.).

System A setting study (the general method for polyphenols assessment) on E21 samples (chromatogram A, Tracks T3 - T4) indicated the existence of four major quercetin derivates (orange fluorescent spots s8, s9, s10, s11), among which: hyperoside/s10 ($R_F \approx 0.65$), iso-quercitrin/s11 ($R_F \approx 0.69$) and two other quercetin glycosides (s8 ($R_F \approx 0.57$) and s9 ($R_F \approx 0.62$)). The latter two probably present free hydroxyl groups at 3rd position which accounts for the observed maximum absorption of E21 sample at the wavelength 433 nm (total flavones content assay). Orange fluorescent spot s7 ($R_F \approx 0.45$) indicated traces of rutin, while the two green fluorescent spots s12 ($R_F \approx 0.94$) and s14

($R_F \approx 0.98$) were attributed to kaempferol derivative and the free aglycone; the blue-indigo spot s13 with $R_F \approx 0.94$ was attributed to gallic acid.

As previously demonstrated very useful for polyphenols free aglycones assessment [9], the system B setting (typically used for saponins' assessment) on E21 sample (chromatogram B1, T3 track) indicated the occurrence of several intense green fluorescent spots, the most probable overlapped by quercetin monoglycosides, further attributed (TLC and UV-VIS supplementary studies) to kaempferol glycosides. Furthermore, studies on the hydrolyzed sample H21 (chromatogram B2, T3H track) indicated luteolin glycoside (yellow fl. spot at $R_F \approx 0.33$) presence,

also overlapped by quercetin derivatives, and caffeic acid (blue fl. zone at $R_F \approx 0.74$) resulted from chlorogenic acid (blue fl. zone at $R_F \approx 0.24$) hydrolysis, as well as high amounts of quercetin (orange zone up to $R_F \approx 0.68$) and kaempferol (dark green zone at $R_F \approx 0.82$) free aglycones also conforming to kaempferol derivatives presence in E21 sample.

Concerning quantitative aspects, the analyses performed indicated 1.15 mg total phenols content expressed as gallic acid (GAE) equivalents and, respectively,

1.26 mg total flavones content expressed as rutin (R) equivalents/1 mL extract, meaning 1.72 g GAE and, respectively, 1.89 g R/100 g plant powder (*P. bistorta herba et flores*).

Antioxidant activity

Figure 2 presents the chemiluminescence/CL assay results (a.u. at each 5 seconds with a total of 60 s) on the E21 sample, the six dilution series (x1 - x2 - x5 - x10 - x50 - x100) respectively, and the comparison with the control sample (M).

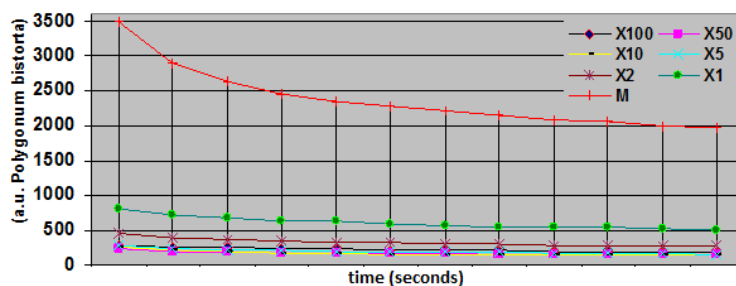


Figure 2.

CL assay on the E21 test sample (six dilution series) compared to control (M)

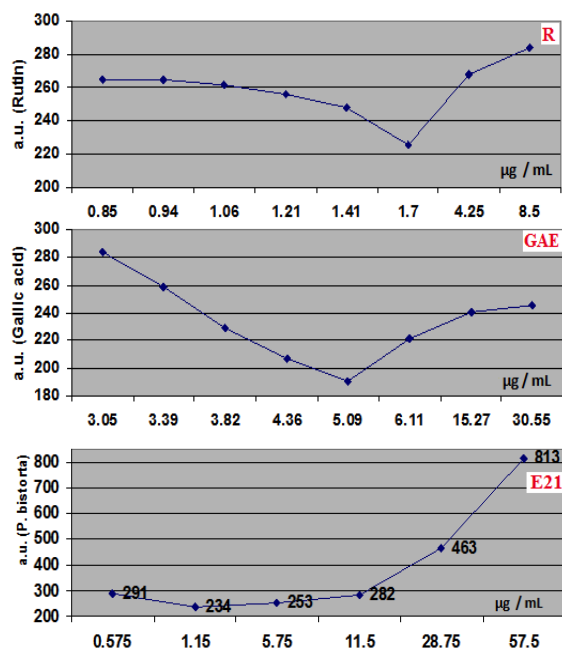


Figure 3.

IC_{50} assay on E21 compared to rutin (R) and gallic acid (GAE)

Rutin [R] and gallic acid [GAE] reference products (10^{-3} M prepared in 70%, v/v ethanol solvent), eight dilution series (x1, x2, x5, x6, x7, x8, x9, x10, x50, x100) respectively, were used as control samples

As results from the study, the ethanolic extract (E21) from *P. bistorta* plant material presents a very high antioxidant activity ($IC_{50} = 0.57 \mu\text{g GAE/mL}$), thus exceeding the antioxidant potency of gallic acid reference product ($IC_{50} = 0.85 \mu\text{g GAE/mL}$), one of the most powerful antioxidants found in plants.

Cell cytotoxicity

Figure 4 illustrates the effect of E21 extract on mouse fibroblastic cells (NIH3T3); the resulted RTCA diagram shows the mean cell index measurements per concentration level of plant extract in time. Thus, no cytotoxic effect of E21 test sample on the NIH3T3 cells at concentration of 1 and, 5 and 10 $\mu\text{g GAE/mL}$ sample, versus a decline in number of cells starting from a concentration of 25 $\mu\text{g GAE/mL}$ sample has been noticed. Approximately 4 h after adding the E21 sample, a slight increase in the number of cells at 25, 50 and 100 $\mu\text{g GAE/mL}$ has noticed, and after other several hours an inhibition of proliferation was also observed.

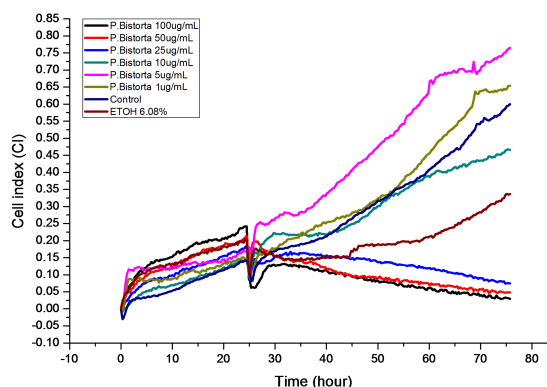


Figure 4.

Real-time monitoring of the effect of E21 on the NIH3T3 cell line

Similarly, Figure 5 illustrates the effect of the sample solvent (70%, v/v ethanol - ETOH) on the NIH3T3 cells. Studies on the six ETOH dilution

series (meaning 6.08 - 3.04 - 1.52 - 0.6 - 0.3 - 0.06 ethanol content, % - v/v, corresponding to 100, 50, 25, 10, 5 and 1 µg GAE/mL E21 dilution series), indicated no cytotoxic activity or other effects of ETOH on the murine cell line.

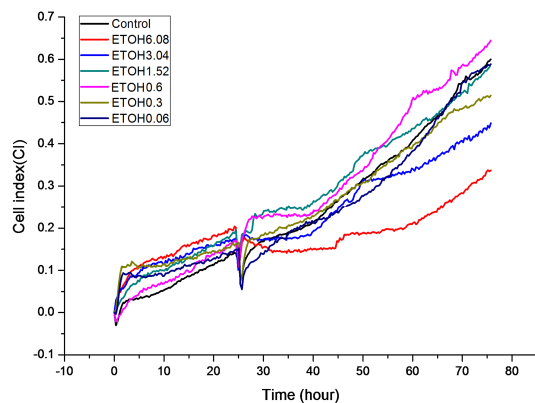


Figure 5.

Real-time monitoring of the effect of ethanol solvent (dilution series, % - v/v) on the NIH3T3 cell line

Figure 6 shows the calculation of IC_{50} value for the test sample E21. As the working principle, a real-time plot of the *xCELLigence* experiment was graphed by the RTCA system, a dose-response curve was plotted and the IC_{50} value was calculated. Briefly, the NIH3T3 cells were exposed to various concentrations (1 - 100 µg/mL) of plant extract for 48 h (6 replicate wells per concentration). The viability inhibition on NIH3T3 cells was measured compared to the control sample (ethanolic solvent at 6.08 - 3.04 - 1.52 - 0.6 - 0.3 - 0.06 % - v/v) and the IC_{50} value was determined from the fitted curve.

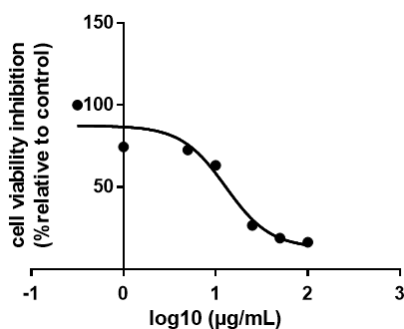


Figure 6.

Dose-response curve and calculated IC_{50} for effects on cell viability of NIH3T3 cells treated with E21 test extract

Therefore, the IC_{50} value, representing the plant extract concentration causing 50% growth inhibition, was appreciated at 12.88 µg GAE/mL sample, thus concluding a *high cytotoxic** activity of the E21 plant extract against murine fibroblast cells.

*According to the U.S. National Cancer Institute plant screening program, plant derived products

showing $IC_{50} < 20$ µg/mL are considered as high cytotoxic; IC_{50} measuring from 20 - 100 µg/mL indicate moderate cytotoxicity; IC_{50} measuring 201 - 500 µg/mL suggest weak cytotoxic activity, while $IC_{50} > 500$ µg/mL no cytotoxic activity [7, 8].

Conclusions

Qualitative (HP)TLC analysis on the (70%, v/v) ethanolic extract (E21) from *Polygonum bistorta herba et flores* plant material indicated the predominance of flavonoid compounds (quercetin, kaempferol and luteolin derivatives), and also several phenyl carboxylic acid derivatives (caffeic and chlorogenic acids respectively).

Even if with very high antioxidant activity ($IC_{50} = 0.57$ µg GAE/mL extract), *in vitro* studies on NIH3T3 cells exposed to ethanolic extract (dilution series) from *Polygonum bistorta herba et flores* plant material indicated a significant decrease in cell adhesion, spreading and proliferation over 25 µg total phenols (GAE)/mL sample ($IC_{50} = 12.88$ µg GAE/mL). Thus, our results are the first to show the cytotoxic effect of *Polygonum bistorta* ethanolic extract in real time on a well-established, suitable transfection host cell type; the solvent sample, 70% (v/v) ethanol respectively, has no effect on NIH3T3 cells viability. However, further mechanistic studies are required to determine the precise mode of action of *Polygonum bistorta* plant derived products and separate compounds.

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