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Research article

Cytotoxic effects of *Anagallis arvensis* and *Anagallis foemina* in neuronal and colonic adenocarcinoma cell lines

Víctor López [a,*], Rita Yolanda Cavero [b], Maria Isabel Calvo [c]

[a] Department of Pharmacy, Faculty of Health Sciences, San Jorge University, Autovía A-23 Zaragoza-Huesca km. 299, 50830 Villanueva de Gállego (Zaragoza), Spain
[b] Department of Plant Biology, School of Sciences, University of Navarra, Irunlarrea sn, 31008 Pamplona, Spain
[c] Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, Irunlarrea sn, 31008 Pamplona, Spain

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ABSTRACT

Anagallis arvensis L. and *Anagallis foemina* Mill. (Primulaceae) have been used in Navarra (Spain) as wound healing remedies within a context of traditional medicine. The species have previously demonstrated antimicrobial and COX-inhibiting properties. Cytotoxic effects of the plants have never been established though they are popularly known to be toxic at high doses and/or long term oral administration. Cytotoxicity was evaluated in PC12 and DHD/K12PROb cells using spectrophotometric methods such as the MTT and LDH assays. Both plants reduced cell survival and induced cell damage (LDH release) in a dose-dependent manner, PC12 cells being more sensitive to the extracts than DHD/K12PROb cells. Methanol extracts were significantly more cytotoxic and doses over 80 μg/ml reduced cell survival above 50%. Results suggest that these plants may be responsible for the toxic effects that have been described in traditional medicine.

1. Introduction

Anagallis arvensis L. and *Anagallis foemina* Mill. (Primulaceae) are known as “the scarlet pimpernel” and “the blue pimpernel” respectively due to its red and blue flowers. However, the scarlet pimpernel (*A. arvensis*) also has a variety with blue flowers (*A. arvensis* L. var. *caerulea* Gouan), which is very similar to the blue pimpernel (*A. foemina*).[1] The taxonomic difference between the species is that the corolla lobe margins in *A. arvensis* is fringed with a large number (35–70) of 3-celled glands where the top cell is enlarged, whereas *A. foemina* has a smaller number of 3 or 4-celled glands (less than 30) with all cells of equal size.[2]

Both species are used in Navarra (Iberian Peninsula, Spain) regarding wound healing properties in human and veterinary ethnomedicine.[3–5] Aerial parts of both species are used to prepare an ointment for the treatment of external infections such as and wounds or infected spots; in some cases, an infusion is prepared with the plants to threat internal or systemic infections though they are popularly known to be toxic at high doses or long term consumption.[6]

In this sense cytotoxicity was evaluated in two cell lines: PC12, which is used as a model of dopaminergic cells in neurosciences, and DHD/K12PROb considered a model of colon adenocarcinoma cells.

2. Material and methods

2.1. Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich. The LDH cytotoxicity detection kit was purchased from Roche (Indianapolis, USA). Culture media, penicillin-streptomycin, fetal bovine serum (FBS), horse serum (HS), sodium pyruvate and Dulbecco’s phosphate buffered saline (PBS) were obtained from Gibco (Barcelona, Spain).

2.2. Plant material

Aerial parts of *A. arvensis* and *A. foemina* were collected in Navarra in spring time 2008 and authenticated by Silvia Akerreta and Rita Yolanda Cavero (Department of Plant Biology, University of Navarra). Voucher specimens have been deposited in the PAMP Herbarium of the University of Navarra: *A. arvensis* (PAMP 18927) and *A. foemina* (PAMP 18718).

2.3. Preparation of extracts

Lyophilized methanol and aqueous extracts prepared for a previous study[7] and kept at −40 °C were used for *in vitro* cytotoxicity assays. Extracts were reconstituted in PBS and filtered before use.

* Corresponding author. Tel.: +34 976 060 100; fax: +34 976 077 584.
E-mail address: ilopez@usj.es (V. López).

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2.4. Assessment of cell viability

2.4.1. Cell culture

Rat pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection. Cells were grown in DMEM supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, penicillin (10 U/ml), streptomycin (10 μg/ml) and 0.2 mM sodium pyruvate. DHD/K12PROb cells were obtained from a colon adenocarcinoma induced in syngenic BD-XI rats and maintained in a mixture of DMEM and Ham's F-10 supplemented with 10% fetal bovine serum and 0.01% gentamicin. Cultures were incubated in the presence of 5% CO_2 at 37 °C and 100% relative humidified atmosphere.

2.4.2. Cell survival by the MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a yellow tetrazolium salt that is converted into a purple compound (formazan) in viable cells by mitochondrial enzymes. This property is used as an index of cell survival.[8] PC12 cells were seeded in 96-multiwell plates at 2×10^4 cells/well. After 48 h, the medium was replaced by low serum medium (1% heat-inactivated horse serum), the plant extracts were added to the wells at different concentrations and incubated for 24 h. Then, the medium was removed and an MTT-1% DMEM solution (0.3 mg/ml MTT final concentration) was added for 1 h incubation at 37 °C. The MTT was removed and formazan crystals were dissolved in DMSO. Absorbance was measured at 550 nm in a microplate reader (Bio-Tek, USA). DHD/K12PROb cells were also seeded at 2×10^4 cells/well. After 24 h, cells were treated with plant extracts and incubated for 24 h. Cell survival was measured as above but using DMEM-Ham's F-10 supplemented with 10% fetal bovine serum and 0.01% gentamicin as culture medium.

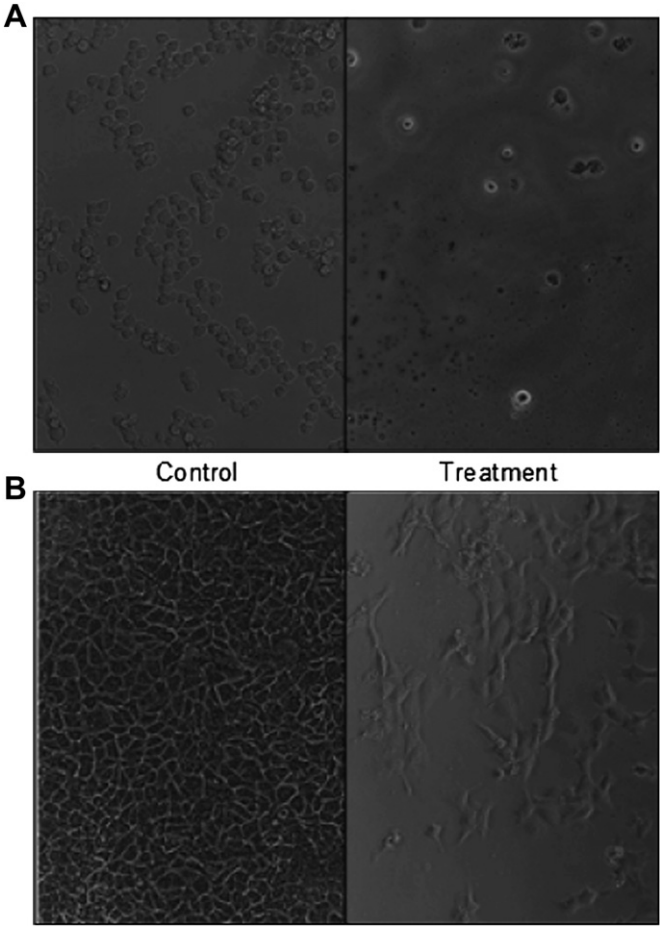

Fig. 1. Changes in morphology and growth of PC12 (A) and DHD/K12PROb (B) cells after treatment with 60 μg/ml of methanolic extract obtained from *Anagallis arvensis*.

2.4.3. Cell death by the LDH assay

Cytotoxicity of extracts was also studied by the determination of lactate dehydrogenase (LDH) into the incubation medium using a commercial kit from Roche. LDH is a cytosolic enzyme released into the medium when the integrity of the cell membrane deteriorates suffering from necrotic cell death.[9] Cells were seeded in 96-multiwell plates at 2×10^3 cells/well. After 48 h incubation, cells were exposed to different concentrations of plant extracts with renewed 1% DMEM for 24 h. DHD/K12PROb were seeded at the same density but treated after 24 h incubation. The assay was carried out following the instructions of the manufacturer. Background interferences were deducted by calculating the LDH activity of the medium. Spontaneous release of LDH was also calculated by measuring LDH activity of untreated cells. Total intracellular LDH was measured in cell lysates obtained by treatment with the manufacturer lysis solution.

2.5. Statistical analysis

Data are expressed as means ± S.D. of three independent experiments. One-way ANOVA followed by Dunnett's multiple comparison tests was used to compare control and treatments.

3. Results

Cellular damage induced by methanol and aqueous extracts after 24 h treatment was observed under a phase contrast

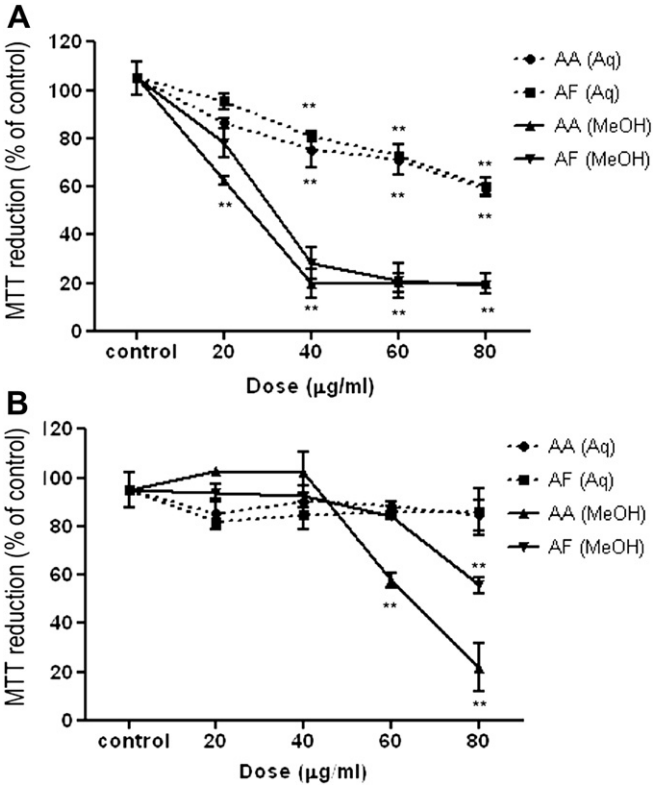

Fig. 2. Cell viability by the MTT assay in PC12 (A) and DHD/K12PROb (B) cells. $^{**}p < 0.01$ versus control. AA: *Anagallis arvensis*; AF: *Anagallis foemina*; MeOH: methanolic extract; Aq: aqueous extract. Triton X-100 (5%) was used as control substance to induce cell death.

microscope (Fig. 1) and quantified by the MTT and LDH assays (Figs. 2 and 3). Both spectrophotometric methods (MTT and LDH) produce complementary data as the MTT assay consist of measuring cell survival whereas the LDH method evaluates cellular damage trough lactate dehydrogenase leakage.

Changes in cell morphology were observed. Most of them lost their polygonal shape becoming smaller and circular and loosing their ability to adhere to the plate surface.

Fig. 2 shows a significant reduction on PC12 and DHD/K12PROb cell survival. The viability of PC12 cells measured by the MTT was greatly reduced in a dose-dependent manner when cells treated with increasing doses (20–80 μg/ml) of methanol and aqueous extracts of *A. arvensis* and *A. foemina*. DHD/K12PROb cells were sensitive to methanol extracts of both species, but not to the aqueous extracts. According to other studies regarding cytotoxicity of medicinal plants, we could state that the cytotoxic events in this case are moderate.

Data of LDH assay (Fig. 3) confirmed what was observed by the MTT method, but in this case in terms of released LDH into the incubation medium. In general, methanol extracts were much more cytotoxic than aqueous and PC12 cells, considered as neuronal-like cells, were more sensitive to the plants than colonic adenocarcinoma cells (DHD/K12PROb). However, significant differences within plant species were not observed.

4. Discussion

A. arvensis and *A. foemina* are traditionally used in the province of Navarra (Iberian Peninsula) for dermatological purposes related to external infections. A previous study by the authors revealed that both species exerted antimicrobial and anti-inflammatory properties as well as presence of saponins and flavonoids in the samples.[7] In certain situations, the plants can be administered orally as infusions to treat internal infections; however, the plants are reported as toxic species during long term oral consumption. Other studies concluded that *A. arvensis* possesses antioxidant,[10] antifungal,[11] molluscicidal,[12] and poisoning[13,14] effects but we demonstrate for the first time the toxic effects of the plants using *in vitro* cell systems.

The extracts were tested in two different cell lines: PC12 cells, commonly used in neurosciences due to its phenotypic characteristics with sympathetic neurons[15] and DHD/K12PROb cells, used as model for antitumor agents against colon adenocarcinoma.[16] The traditional knowledge indicated that those plants can be very toxic and some studies put forward the idea that *A. arvensis* works as a poison in animals.[13] Saponins, that have been previously detected in the extracts[7,17,18] might be involved in the cytotoxic phenomenon as some of them are known to interact with cellular membranes increasing permeability and producing cell damage.[19]

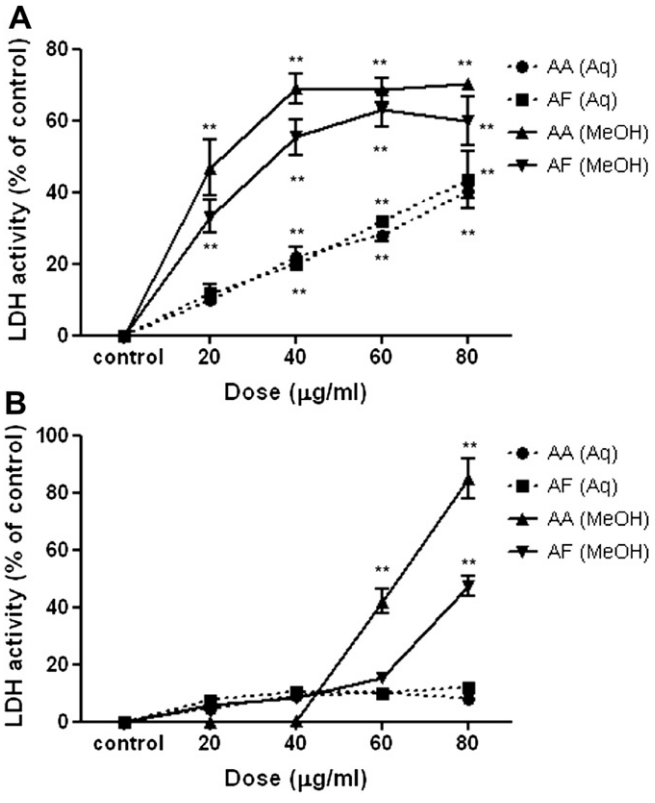

Fig. 3. Cytotoxicity of plant extracts by the LDH assay in PC12 (A) and DHD/K12PROb (B) cells. $^{**}p < 0.01$ versus control. AA: *Anagallis arvensis*; AF: *Anagallis foemina*. MeOH: methanolic extract; Aq: aqueous extract. Triton X-100 (5%) was used as control substance to induce cell death.

5. Conclusions

Results indicate that these plants may be responsible for the toxic effects that have traditionally been described. In general, methanol extracts were much more cytotoxic than aqueous and PC12 cells, considered as neuronal-like cells, were more sensitive to the plants than colonic adenocarcinoma cells. Further studies with the extracts may be of interest to identify new leads and compounds with cytotoxic properties.

Conflicts of interest

All authors have none to declare.

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