

Anticancer potential of selected *Fallopia* Adans species

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Abstract. The aim of the present study was to determine the anticancer potential of three species belonging to the *Fallopia* genus (Polygonaceae): *Fallopia convolvulus* (*F. convolvulus*), *Fallopia dumetorum* (*F. dumetorum*) and *Fallopia aubertii* (*F. aubertii*). For this purpose, crude extracts were obtained and characterized for their phenolic and flavonoid total content and examined for their anticancer activity on three tumor cell lines: breast cancer (MCF7), colon carcinoma (Caco-2) and cervical cancer (HeLa) cells. The cytotoxic potential of the three species was assessed by MTT assay, cell cycle analysis and by the evaluation of mitochondrial membrane potential (MMP). The acute toxicity of the extracts was evaluated using one *in vitro* cell model (Vero cells, an African Green monkey kidney cell line) and two invertebrate *in vivo* models (*Daphnia magna* and *Artemia salina*). The highest total phenolic and flavonoid content was found in the *F. aubertii* flower extracts. The cytotoxic effects of the extracts from *F. aubertii* and *F. convolvulus* on all three cell lines were examined at concentrations ranging from 3 to 300 µg/ml. G2/M cell cycle arrest was induced by all the extracts, and a significant increase in the subG1 cell population was observed. The hydroethanolic extract from the flowers of *F. aubertii* induced cell apoptosis more rapidly than the other extracts. The MMP indicates the involvement

of the mitochondria in the induction of apoptosis. A positive correlation between the total phenolic content of the extracts and the IC₅₀ values against the HeLa cells was also noted. None of the extracts exhibited significantly toxic effects. Considering the antitumor potential of *F. aubertii* and *F. convolvulus*, these two species may represent a good source of plant extracts with anticancer properties.

Introduction

Cancer is the main cause of mortality and morbidity in Europe following cardiovascular diseases and represents the uncontrolled growth and spread of cells that arises from a change in one single cell (1). Each year, millions of individuals are diagnosed with cancer. The disease accounts for the death of approximately 3.5 million individuals annually worldwide (2). It was estimated that only in Europe, in 2012, 3.45 million new cases of cancer were noted, excluding non-melanoma skin cancer, and 1.75 million deaths occurred from cancer (3).

Throughout history, plant extracts and their purified active components have been the backbone of cancer chemotherapeutics (4). Additionally, structural analogues have been obtained by molecular modifications of the natural compounds and have reinforced the anticancer arsenal (5). It is estimated that over 70% of anticancer compounds are either natural products, or natural product-derived substances (6). The rich diversity of the chemical structures provided by natural resources offers valuable templates for exploring novel molecular scaffolds and is the most significant source of new drug developments (7).

Over the past two decades, flavonoid-rich plant extracts and isolated flavonoids have shown anticancer potential (8). Apigenin, baicalein, luteolin, nobiletin and tangeretin have been shown to be the most effective flavonoids against carcinomas of the stomach, whereas luteolin has been shown to be a promising candidate for the treatment of skin cancer (9,10). Hesperidin has been shown to inhibit human pancreatic cancer cell growth and its use has been suggested for the prevention of pancreatic cancer (11). A number of targets and a variety of action mechanisms have been proposed to explain the cytotoxic effects of flavonoids. Genistein, daidzein, luteolin and quercetin are able to inhibit DNA topoisomerase activity

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Abbreviations: DM, dry material; DMSO, dimethyl sulfoxide; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; MMP, mitochondrial membrane potential; MTT, 3-(4,5-dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; TFC, total flavonoid content; TPC, total phenolic content

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and are considered as potential agents for future use in cancer therapeutics (12). Quercetin, luteolin and kaempferol are promising antitumor agents that can block the cell cycle (13), induce apoptosis (14), inhibit angiogenesis (15) and modulate the expression of several protein kinases (16). Fisetin acts as a dual inhibitor of the phosphatidylinositol 3-kinase (PI3K)/Akt and mammalian target of rapamycin (mTOR) pathways and has been evaluated for its potential inhibitory role against *in vitro* (17). Myricetin and quercetagenin have been shown to inhibit the activity of PI3Ks (18). Given the high antitumor potential of these compounds, a number of plant extracts rich in flavonoids have been investigated in order to evaluate their anticancer properties (19,20). The low production cost of the plant extracts compared to the pure compounds and the synergistic effects of the natural compounds are the main advantages for using natural extracts (21).

Fallopia Adans is a plant genus which contains approximately 15 species (22-24). The species are widespread over the northern hemisphere (25). With the exception of *Fallopia japonica* (Houtt.) Ronse Decr. (syn. *Polygonum cuspidatum*) and *Fallopia multiflora* (Thunb.) Haraldson, the therapeutic potential of all the other species has not been investigated in detail (26). These plants are invasive and can easily produce biomass and can therefore be introduced in crops (27). *Fallopia convolvulus* (*F. convolvulus*) (L.) Á. Löve and *Fallopia dumetorum* (*F. dumetorum*) (L.) Holub (syn. *Polygonum dumetorum* L.) are native to Europe, and *Fallopia aubertii* (*F. aubertii*) (L. Henry) Holub [syn. *Fallopia baldschuanica* (Regel) Holub] is a subsynchronous species introduced from Central Asia as an ornamental plant (25). Chemotaxonomic studies on the genus *Fallopia* have shown that all species contain flavonoids with a profile relatively uniform for all species and that quercetin glycosides are the major constituents (28,29). The flavonoid fraction of *F. convolvulus* consist of glycosides of quercetin, kaempferol, myricetin, apigenin, luteolin, rhamnetin and isorhamnetin (28,30). Three characteristic flavonoid structures have also been found in this species: falloconvolin A and B and quercetin-3-O-(2-E-esinapoxyl)-glucopyranoside (31). In *F. aubertii*, glycosides of quercetin, kaempferol, apigenin, luteolin, myricetin and several chromones structures have been identified (29,32,33). With the exception of rhamnetin, isorhamnetin and characteristic flavonoids, all other flavonoids have also been found in *F. dumetorum* (28).

The aim of this study was to examine the effects of various plant extracts from three *Fallopia* species, *F. convolvulus*, *F. dumetorum* and *F. aubertii*, on cancer cell lines in order to further determine their usefulness. The correlations between the polyphenol and flavonoid content and the cytotoxic effects of these extracts were also evaluated.

Materials and methods

Materials. Folin-Ciocalteu reagent, methanol p.a., ethanol p.a., potassium acetate (CH₃COOK), quercetin trihydrate, colchicine and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Aluminium chloride hexahydrate (AlCl₃ x 6H₂O), sodium carbonate anhydrous (Na₂CO₃) and gallic acid were purchased from Scharlau Co. (Barcelona, Spain). Cervical cancer (HeLa) and colon cancer (Caco-2) cells were purchased from Cellonex,

Separations (Randburg, South Africa) and breast (MCF7) cancer cells were purchased from Highveld Biological (Johannesburg, South Africa). The Coulter[®] DNA Prep[™] reagents kit was purchased from Beckman Coulter (Fullerton, CA, USA). 3-(4,5-Dimethyl-1,3-thiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from Thermo Scientific (Logan, UT, USA).

Plant material and preparation of the extracts. *F. convolvulus* was harvested from Buftea, Ilfov county (July, 2013), *F. dumetorum* from Zimnicea, Telorman county (June, 2013) and *F. aubertii* from Bucharest (October, 2013) Romania. The identity was established by comparing with herbarium specimens from 'Dimitrie Brandza' Botanical Garden, Bucharest and voucher specimens are available in the herbarium collection of the Department of Botany and Cell Biology, 'Carol Davila' University of Medicine and Pharmacy, Bucharest, Romania. *F. convolvulus* (C) and *F. dumetorum* (D) consisted of stems, leaves, flowers and fruits and, *F. aubertii* consisted of flowers (AF), and stems and leaves (AH). A total of 10 g of each material was grounded (mesh 14) and extracted with 3x100 ml solvent (e, ethanol; ha, ethanol 50%; w, water) under reflux, followed by concentration (rotary evaporator, RVO 004; Ingos, Prague, Czech Republic) and lyophilized at -55°C (CoolSafe ScanVac 55; LaboGene, Lyngø, Denmark). For the cell culture experiments, plant extracts (AF_{ha}, AF_e, AF_w and AH_{ha}), were reconstituted in DMSO at a final concentration of 100 mg/ml and stored at -20°C until use. Serial dilutions were prepared in order to obtain the following concentrations: 3, 30, 100 and 300 µg/ml.

Phytochemical determinations. The total polyphenol content (TPC) was determined according to the Folin-Ciocalteu method described by González *et al* (34) (at λ=750 nm) and the total flavonoid content (TFC) was determined using the method with AlCl₃ as described by Chang *et al* (35) and Bazylo *et al* (at λ=429 nm) (36). All determinations were performed in triplicate and were measured using a UV-VIS spectrophotometer (Halo DB-20-220; Dynamica, Salzburg-Mayrwies, Austria).

The results were calculated using linear calibration curves and are expressed as the means ± SEM of the experiments in milligram gallic acid equivalents (GA equiv.) per gram of dry material (DM) and in milligram quercetin equivalents (Q equiv.) per gram of DM.

Assessment of toxicity

In vitro screening of the extracts for their potential cytotoxicity on cancer cell lines. The human cancer cell lines, MCF7 (breast cancer), Caco-2 (colon carcinoma) and HeLa (cervical cancer), were used for the screening process. All cell lines were grown in DMEM supplemented with 10% fetal bovine serum. Each cell line was seeded in 200 µl aliquots at a cell density of 3x10⁴ cells/ml in 96-well plates and left overnight to attach. For the treatment of each cell line, the medium was replaced with fresh medium containing four concentrations (3, 30, 100 and 300 µg/ml) of extract. The treated cells were incubated at 37°C in a humidified 5% CO₂ incubator for 48 h.

The medium containing the various treatments was removed prior to the addition of MTT solution to the cells and replaced with 200 μ l of medium containing 0.5 mg/ml MTT. The cells were incubated for 3 h. Thereafter, the medium was removed and the blue formazan product was solubilized in DMSO. The absorbance was read at 540 nm using a BioTek® PowerWave XS spectrophotometer (BioTek, Winooski, VT, USA).

Optical density (OD) data were analyzed using Excel and the relative cell viability was determined using quadruplicate readings. Untreated cells were considered to have 100% cell viability. Cell viabilities in other test wells were calculated relative to the untreated controls and expressed as a percentage.

Due to the positive correlation between the concentration used and the biological effects, HeLa cells were used for the determination of the IC₅₀ value of the cytotoxicity of the *Fallopia* extracts. HeLa cells were seeded in the same manner as described above for the initial screening protocol. The cells were treated with various concentrations of plant extract (12.5-500 μ g/ml) and exposed to the extract for 48 h. In the same manner as described above for the initial screening protocol, MTT was used to determine cell viability following incubation.

Evaluation of toxicity using a normal cell model in vitro. Extracts exhibiting cytotoxicity were tested against a normal cell line. Vero cells (an African green monkey kidney cell line) were used and seeded at a density of 1×10^5 cells/ml. The determination of the cytotoxicity was performed according to the protocol described above.

Assessment of acute toxicity. The assessment of acute toxicity was determined using two different assays as follows:

i) *Artemia salina* toxicity assay. Brine shrimp (*Artemia salina* L.) lethality assay was performed using the procedures described in the study by Meyer *et al* (37) with some modifications. Brine shrimp cysts were obtained from a local aquarium (Bucharest, Romania) and incubated in artificial sea water (40 g/l salinity) for 24 h in a growth chamber (Sanyo MLR-351H; Sanyo, San Diego, CA, USA) at $25 \pm 1^\circ\text{C}$, under continuous aeration, using a 16-h photoperiod and 8 h of darkness. The newly hatched nauplii were separated from the shells, transferred to fresh sea water with a micropipette and incubated for 24 h. Assays were performed in Petri dishes (d=30 mm). Each dish contained 20 larvae in a final volume of 2,000 μ l. The plant extract concentrations were in the range of 1,000-3,000 μ g/ml (1,000, 1,500, 2,000, 2,500 and 3,000 μ g/ml) and the final DMSO concentration was 1% (v/v). A solution of 1% DMSO in artificial seawater was used as a negative control and colchicine in the range of 0.5-10 μ g/ml as a positive control. The concentrations were selected after no lethality was registered during a preliminary test using plant extracts at concentrations of 1-1,000 μ g/ml. Each sample was performed in duplicate and each test was run twice. Due to the absence of specific information about the stability of the plant extracts in the presence of light, the bioassay was performed in the dark. After 24 h, the number of surviving organisms was counted and recorded. Larvae were considered dead only if they did not move their appendages for 10 sec during observation.

ii) *Daphnia magna* toxicity assay. *Daphnia magna* Straus were maintained parthenogenetically at 'Carol Davila' University (Department of Pharmaceutical Botany and Cell

Biology) from 2012. Prior to the assay, the daphnids were selected according to their size and kept in fresh water under continuous aeration. The bioassay was performed according to the method described in the study by Fan *et al* (38) with some modifications (39). Ten daphnids were inserted in 10 ml graduated test tubes, and the plant extracts were added in synthetic media in order to obtain solutions of 1,000, 1,500, 2,000, 2,500 and 3,000 μ g extract/ml. The final test solutions were 1% DMSO concentration/10 ml final volume. Synthetic medium with 1% DMSO was used as a negative control and colchicine as a positive control. The daphnids were kept under the same conditions as those described above for the *Artemia salina* assay and the number of surviving daphnids was counted after 24 h. Each sample was performed in duplicate and each test was run twice. The daphnids were considered dead only if they did not move their appendages for 30 sec during observations.

Cell cycle analysis. HeLa cells were seeded at 5×10^4 cells/ml in 10 ml aliquots in 10 cm culture dishes and treated with IC₅₀ values of AF_{ha}, AF_e and C_{ha}. The cells were incubated for 16 and 32 h. After the appropriate incubation period, the HeLa cells were trypsinized for 10 min, re-suspended in phosphate-buffered saline (PBS) and transferred to polypropylene tubes. The Coulter DNA Prep reagents kit was used for DNA cell cycle analysis, as per the manufacturer's instructions. Briefly, 100 μ l lysis reagent were added to each tube and incubated for 5 min at room temperature. Thereafter, 500 μ l PI (50 μ g/ml) were added and the tubes were incubated for 15 min at 37°C . Flow cytometric analysis was performed directly following incubation. A Beckman Coulter Cytomics FC500 was used for all flow cytometric analysis. FlowJo_V10 was used for analysis.

Analysis of mitochondrial membrane potential (MMP). A total of 300 μ l aliquots of trypsinized cells used for cell cycle analysis was removed from its respective culture dish and placed in a separate polypropylene tube for the analysis of MMP. The cells were centrifuged at 500 x g for 5 min at room temperature and washed with PBS to remove the trypsin. Thereafter, a lipophilic cation dye, JC-1, was added to a final concentration of 2 μ g/ml. JC-1 was used to determine a change in the MMP. Cells were incubated for 10 min at room temperature in the dark. The cells were washed using 500 μ l PBS and centrifuged at 500 x g for 5 min. The wash step was repeated three times prior to flow cytometric analysis.

Statistical analysis. Data are presented as the means \pm standard deviation (SD) from at least three independent experiments. Statistical significance was established by the Student's t-test at the level of $p < 0.05$. The statistical significance of the differences between means was assessed by ANOVA with Tukey's post-hoc tests. P-values < 0.05 were considered to indicate statistically significant differences.

The lethality percentage (L%) was plotted against the logarithm of concentrations and the lethality, concentration curves were drawn using the least squares fit method and the lethal concentrations that kill 50% of organisms (LC₅₀) were determined using these curves. The upper and lower limits of the 95% confidence interval (CI 95%) and the correlation coefficient (r^2) were also calculated.

Table I. Yield extraction, TFC and TPC for the *Fallopia* extracts.

No.	Extract	Yield of crude extract (%)	TFC (mg Q equiv./g DM)	TPC (mg GAE equiv./g DM)
1	<i>F. convolvulus</i> (hydroethanolic 50% - C _{ha})	18.31	33.43±0.3510	209.24±2.7899
2	<i>F. dumetorum</i> (hydroethanolic 50% - D _{ha})	10.21	22.73±0.3405	77.44±0.8382
3	<i>F. aubertii herba</i> (hydroethanolic 50% - AH _{ha})	13.65	30.02±0.3214	162.33±4.8745
4	<i>F. aubertii flores</i> (hydroethanolic 50% - AF _{ha})	18.28	29.57±0.8453	252.96±6.4306
5	<i>F. aubertii flores</i> (aqueous - AF _w)	23.05	23.43±0.3831	154.85±4.8467
6	<i>F. aubertii flores</i> (ethanol 96% - AF _e)	12.82	48.33±0.7122	207.04±1.6670

Values are the means ± standard deviation of triplicate analyses. GAE, gallic acid; TFC, total flavonoid content; Q, quercetin; TPC, total phenolic content; DM, dry plant material. Superscript letters e, ha and w indicate the solvent used i.e., 96% ethanol, 50% ethanol or w, water, respectively.

Cell viability data and the IC₅₀ values were calculated from the concentration-response data using a mathematical Hill function. All calculations were performed using GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results and Discussion

The present study focused on the cytotoxic effects of some extracts of *F. convolvulus*, *F. dumetorum* and *F. aubertii* on human cancer cell lines (MCF7, Caco-2 and HeLa) in correlation with their content in flavonoids and phenolic compounds. Additionally, the toxicity of the extracts was assessed by alternative toxicity bioassays using an *in vitro* model with confluent African green monkey kidney (Vero) cells and two *in vivo* invertebrate models, *Artemia salina* and *Daphnia magna* bioassays.

Extraction yield. Several steps such as milling, grinding, homogenization and extraction are required in order to obtain pharmacological active extracts from plant material (40). Extraction efficiency is affected by all these factors in different ways. Under the same conditions (e.g., particle size, temperature, extraction time, solvent:plant material ratio), the solvent and plant material composition are the most important parameters (41). In this study, we obtained six extracts from three plant species of the genus *Fallopia*. As *F. convolvulus* and *F. dumetorum* have a high TFC and TPC (42,43), we prepared only the hydroethanolic 50% extract (C_{ha} and D_{ha}). From *F. aubertii*, four extracts were obtained: one from stems and leaves extracted with ethanol 50% (AH_{ha}) and three extracts from flowers with water (AF_w), ethanol 50% (AF_{ha}) and ethanol 96% (AF_e). The extraction yields are presented in Table I. The extraction yields ranged from 10.2 to 23.5%. AF_w exhibited the

highest yield compared to all other extracts, possibly due to the mucilage and other compounds soluble in water. The lowest extraction yield was obtained with ethanol 96%. The results are in agreement with the extraction yields obtained for other medicinal plants rich in flavonoids (44).

Determination of TPC and TFC. Polyphenols are widespread compounds in plant species. Recent studies have reported a positive correlation between TPC/TFC and anticancer properties and have also shown the various mechanisms of action of these compounds in both in *in vitro* and *in vivo* models of cancer (45,46). The TPC of the six different extracts was determined from a linear gallic acid (GAE) standard curve ($y=0.1053x+0.0320$, $r^2=0.9993$) and the TFC was determined from a linear quercetin standard curve ($y=0.0765x-0.0084$, $r^2=0.9997$). The TPC and TFC of the tested extracts are presented in Table I. The TPC in the tested extracts ranged from 77.44 to 252.96 mg GA equiv./g DM. AF_{ha} showed the highest TPC among all the extracts. The decreasing order of the TPC in the extracts was: AF_{ha}>C_{ha}>AF_e>AH_{ha}>AF_w>D_{ha}. All results were statistically significant (ANOVA, $p<0.0001$). However, the Tukey post-hoc ($p<0.05$) test revealed no differences between the TPC of C_{ha} and AF_e. In addition, no significant difference was observed in the TPC between AH_{ha} and AF_w ($p>0.05$).

The TFC in the six extracts ranged from 22.73 to 48.33 mg Q equiv./g DM. The highest TFC was exhibited by AF_e. Ethanol 96% was the best solvent for the extraction of flavonoids for the *F. aubertii* flowers. The result is statistically significant ($p<0.05$) by comparison with the extraction with water and ethanol 50% of the same plant material. The decreasing order of the TFC in the extracts was: AF_e>C_{ha}>AH_{ha}>AF_{ha}>AF_w>D_{ha}. All the results were statistically significant (ANOVA, $p<0.0001$). According to the Tukey range test ($p<0.05$), the TFC value was statistically similar for

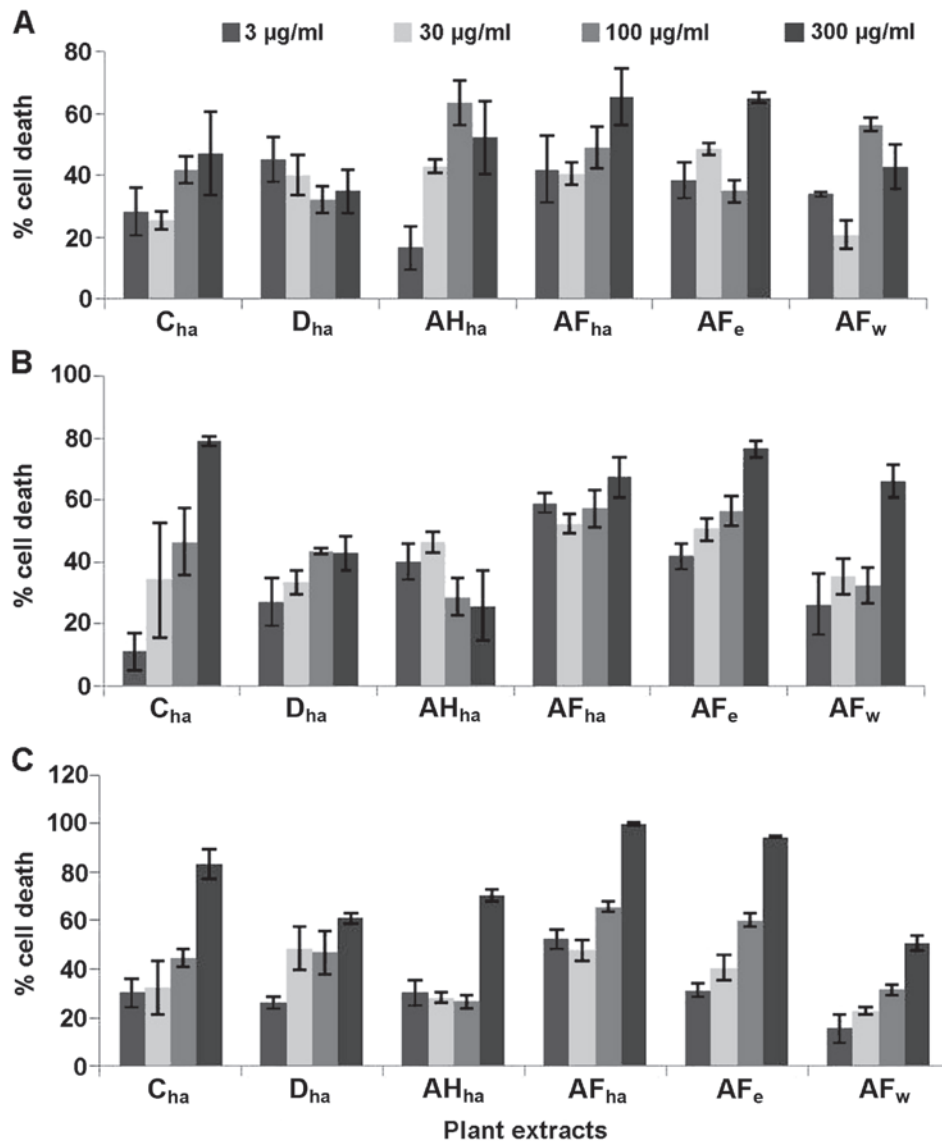


Figure 1. Initial screening of *Fallopia* extracts against a (A) cervical cancer cell line (MCF7), (B) colon carcinoma cell line (Caco-2) and (C) cervical cancer cell line (HeLa) at the concentrations indicated.

the herba and flores hydroalcoholic extracts of *F. aubertii*. Both the TPC and TFC were the lowest in D_{ha}. Among the three species, *F. aubertii* exhibited the highest TPC and TFC.

Assessment of cytotoxicity in vitro

Initial screening of the extracts for their cytotoxic potential. Six *Fallopia* extracts were screened at 4 different concentrations, namely 3, 30, 100 and 300 µg/ml against the HeLa, Caco-2 and MCF7 cells (Fig. 1) for the determination of their cytotoxic potential. Based on these results, dose-response analysis was performed on the extracts AF_{ha}, AF_e, C_{ha} and D_{ha}.

Dose-response analysis and IC₅₀ determination. The cytotoxic effect of the four extracts was determined against the HeLa cells by MTT assay and the IC₅₀ values were determined. From these results, the concentration of the extracts to be used for further experiments was fixed at 125 µg/ml for C_{ha} and AF_e and at 100 µg/ml for AF_{ha} (Table II). The IC₅₀ value of D_{ha} was considered too high to pursue its cytotoxic potential. Cytotoxic evaluation was also performed using confluent African green

monkey kidney (Vero) cells as a control cell line. All four extracts proved to be non-toxic to the Vero cells (data not shown).

Cell cycle analysis. DNA cell cycle analysis was performed using the HeLa cells after 16 and 32 h of exposure to 3 cytotoxic plant extracts. After 16 h of exposure (Fig. 2), a significant increase in the G2/M population was evident for the AF_{ha} and C_{ha} extracts. After 16 h, more than half the cell population treated with AF_e experienced cell death (subG1). After 32 h of extract exposure (Fig. 3), a significant increase in the subG1 cell population was evident with all extracts.

Figs. 2 and 3 show cell cycle analysis used to determine which phase of the cell cycle cells arrest in. It is evident in Fig. 2 that after 16 h of exposure to AF_{ha} and C_{ha}, the cells experienced G2/M phase arrest as there was a significant increase in 4N DNA. After 16 h of treatment with AF_e, there was a marked increase in the subG1 peak, indicating apoptotic cells. This peak indicates the presence of fragmented DNA,

Table II. IC₅₀ of cytotoxicity to HeLa cells and dose-response curve parameters.

Extract	IC ₅₀ ($\mu\text{g/ml}$)	IC 95% of IC ₅₀ ($\mu\text{g/ml}$)	Goodness of fit (r ²)
<i>F. aubertii</i> flores (hydroethanolic 50% - AF _{ha})	106.0±5.94	96.0-138.2	0.9593
<i>F. aubertii</i> flores (ethanol 96% - AF _e)	124.7±8.91	ND	0.9453
<i>F. convolvulus</i> (hydroethanolic 50% - C _{ha})	122.9±6.98	112.9-142.0	0.9751
<i>F. dumetorum</i> (hydroethanolic 50% - D _{ha})	ND	ND	0.5157

ND, not determined. Superscript letters e, ha and w indicate the solvent used i.e., 96% ethanol, 50% ethanol or w, water, respectively.

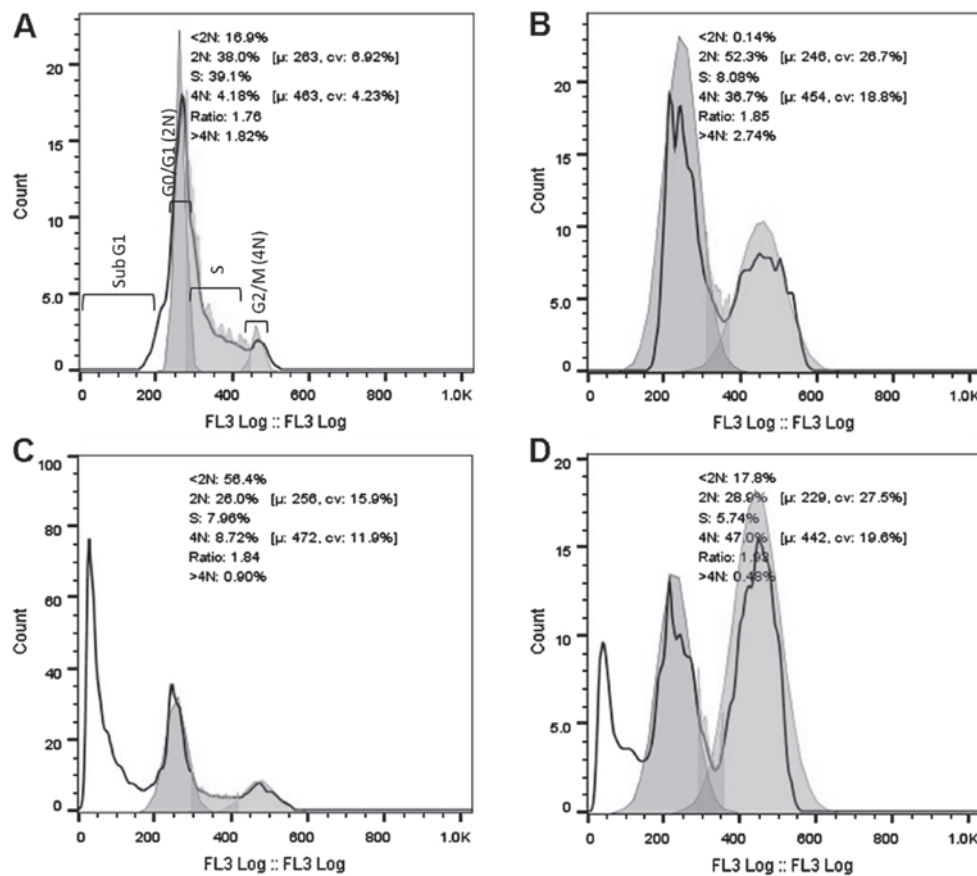


Figure 2. Histograms representing DNA cell cycle analysis after 16 h of treatment of the cervical cancer cells (HeLa cells). HeLa cells were treated with (B) 100 $\mu\text{g/ml}$ AF_{ha}, (C) 125 $\mu\text{g/ml}$ AF_e and (D) 125 $\mu\text{g/ml}$ C_{ha}. (A) Represents untreated control cell population. Cell cycle analysis was performed on a Beckman Coulter Cytomics FC500 flow cytometer following propidium iodide (PI) staining of DNA. FlowJo V10 was used for analysis of results. Ten thousand events were recorded for each sample.

a biochemical hallmark of apoptosis. After 32 h of treatment with the plant extracts, a marked increase in the subG1 cell population was evident, suggesting that the cells were apoptotic.

The mechanism of this G2/M arrest cannot be deduced from propidium iodide (PI) cell cycle analysis and more than one possibility exists. Cdc25B and Cdc25C are phosphatases that regulate the progression of the cell cycle from the G2 phase

through to the M phase. They do so by their activity on Cdc2/cyclin A and Cdc2/cyclin B complexes (47). Active Cdc2 complexed to cyclin B1 is required for the progression from the G2 to the M phase. When DNA damage occurs, Cdc25C is deactivated by a cascade process and this results in the phosphorylation and hence, the inactivity of Cdc2/cyclin B and thus arrest of the cell cycle in the G2 phase. G2/M arrest can also occur by problems in the formation of the mitotic spindle and

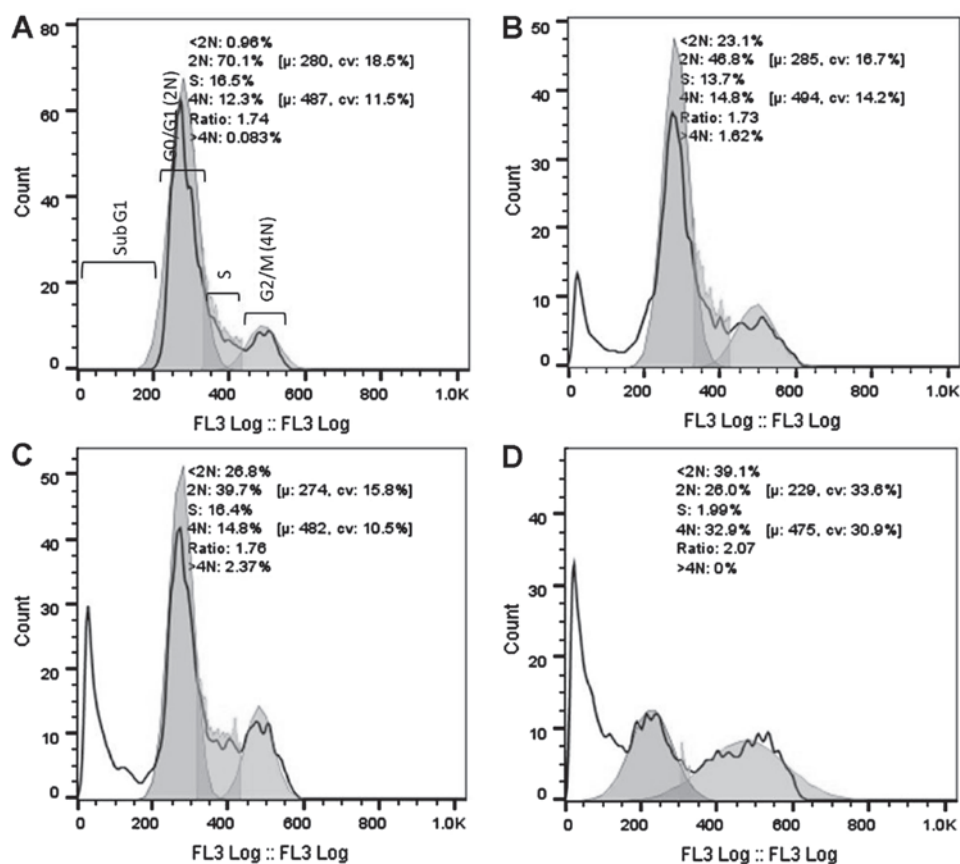


Figure 3. Histograms representing DNA cell cycle analysis after 32 h of treatment of cervical cancer cells (HeLa cells). HeLa cells were treated with (B) 100 $\mu\text{g/ml}$ AF_{ha} , (C) 125 $\mu\text{g/ml}$ AF_e and (D) 125 $\mu\text{g/ml}$ C_{ha} . (A) Represents untreated control cell population. Cell cycle analysis was done on a Beckman Coulter Cytomics FC500 flow cytometer following propidium iodide (PI) staining of DNA. FlowJo V10 was used for analysis of results. Ten thousand events were recorded for each sample.

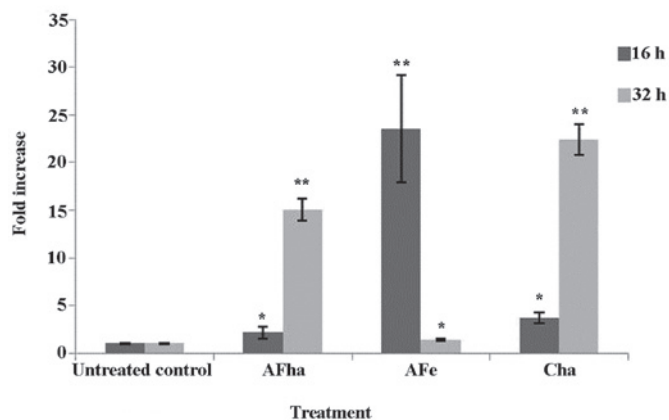


Figure 4. Fold increase in mean green fluorescence intensity indicating the depolarization of the mitochondria after 16 and 32 h of treatment of cervical cancer cells (HeLa cells). Analysis was performed using a Beckman Coulter Cytomics FC500 flow cytometer following JC-1 staining. Ten thousand events were recorded for each sample. Error bars indicate the SD of triplicate values of a single experiment. Significance was determined using the Student's t-test, * $p < 0.05$ and ** $p < 0.005$.

this results in mitotic catastrophe (47). Further studies on the mechanisms of G2/M arrest need to be performed by evaluating the state and levels of Cdc2 and cyclin B proteins, as well as Cdc25C phosphatase. The effects of the plant extracts on tubulin polymerization also need to be determined.

After 16 h of treatment with AF_e , the HeLa cells experienced a significant increase in cell death, as indicated by the large subG1 peak. It is thought that cell cycle arrest may have occurred earlier than 16 h and thus was not seen. In order to determine whether cells experience cell cycle arrest, the analysis of the DNA state can be performed at an earlier time interval.

Evaluation of MMP. To determine the onset of the intrinsic pathway of apoptosis, the MMP was evaluated using the lipophilic cationic dye, JC-1. This dye reversibly changes the colour from green to orange as the membrane potential of the mitochondria increases. Thus, an increase in the mean green fluorescence intensity (MFI) would indicate the depolarization of the mitochondrial membranes and hence the involvement of the mitochondria in the induction of apoptosis.

An increase in the MFI in the green channel was evident after 16 h, but more evidently at 32 h of exposure to the AF_{ha} and C_{ha} extracts (Fig. 4).

Cytotoxic stimuli may induce the permeabilization of cellular membranes and result in the depolarization of the mitochondrial membrane. A method to determine changes in the MMP is by using JC-1. JC-1 forms aggregates when present in high concentrations and the aggregates fluoresce orange (48). If J-aggregates do not form and the dye exists as monomers due to depolarization of the mitochondrial membrane, an increase in green fluorescence will be evident.

Table III. Acute toxicity of the extracts to AS and on DM.

No.	Extract	LC ₅₀ ($\mu\text{g/ml}$)		CI 95% of LC ₅₀ ($\mu\text{g/ml}$)		Goodness of fit (r ²)	
		AS	DM	AS	DM	AS	DM
1.	<i>F. convolvulus</i> (hydroethanolic 50% - C _{ha})	ND	ND	ND	ND	0.8524	ND
2.	<i>F. dumetorum</i> (hydroethanolic 50% - D _{ha})	2689.09	4073.80	2664.09-2714.96	ND	0.9969	0.8769
3.	<i>F. aubertii herba</i> (hydroethanolic 50% - AH _{ha})	2576.36	2884.03	2503.18-2888.15	ND	0.9865	0.8996
4.	<i>F. aubertii flores</i> (hydroethanolic 50% - AF _{ha})	2374.70	2398.83	2222.08-2522.71	2344.22-2454.71	0.9471	0.9847
5.	<i>F. aubertii flores</i> (aqueous - AF _w)	2239.55	3019.95	2186.19-2293.30	ND	0.9913	0.9399
6.	<i>F. aubertii flores</i> (ethanol 96% - AF _e)	1872.16	2951.20	1802.54-1939.97	ND	0.9889	0.9266
7.	Colchicine	1.45	4.74	1.24-1.69	4.50-4.98	0.9483	0.9880

ND, not determined (the value could not be calculated); AS, *Artemia salina*; DM, *Daphnia magna*.

Fig. 4 shows a significant fold increase in green MFI after 16 h of exposure to AF_e and after 32 h of exposure to AF_{ha} and C_{ha}. This suggests that the mitochondria are depolarized due to exposure to the extracts and that the intrinsic pathway of apoptosis is activated.

Once the MMP decreases, proteins that are normally found between the inner and outer membrane of the mitochondria are then released and promote the activation of the apoptotic cascades (49).

Assessment of acute toxicity. The assessment of toxicity using alternative methods (e.g., *Artemia salina* and *Daphnia magna* bioassays) is widely used due to the many advantages as being inexpensive, time saving and having a high degree of correlation with the acute toxicity (LC₅₀) registered in pharmacotoxicology studies on rodents (mice and rats) mammalian models (50-52).

None of the tested extracts were toxic to both the *Artemia salina* and *Daphnia magna* invertebrates. The extracts were first tested in the range of 10 to 1000 $\mu\text{g/ml}$ [10, 50, 100, 250, 500, 750 and 1,000 $\mu\text{g/ml}$, and no toxicity was observed (L% <0.05) at all tested concentrations]. In order to assess the toxicity at higher concentrations, another experiment was carried out at concentrations between 1,000 and 3,000 $\mu\text{g/ml}$. LC₅₀ were calculated only at 24 h of exposure due to the lack of information concerning the stability of the extracts and as the extracts tend to precipitate in aqueous DMSO solutions in the second day of the experiments. The brine shrimp lethality test revealed toxic effects only at high concentrations of the extracts from *F. dumetorum* and *F. aubertii*. The LC₅₀ exhibited by the five extracts ranged from 1872.16 to 2689.09 $\mu\text{g/ml}$ (Table III). Although the LC₅₀ could be calculated, we consider that the

extracts did not present any toxic risk at all. Their toxicity to *A. salina* was far below the limit of 1,000 $\mu\text{g/ml}$ mentioned by Meyer *et al* (37). A positive correlation between the concentration and lethality was observed for all six extracts (r²>0.85). With the exception of C_{Et50}, no significant differences were observed (p<0.05). In comparison with the positive control, all LC₅₀ values are at least 1,000-fold higher, thus the toxicity is significantly lower or non-existent. LC₅₀ induced by *F. convolvulus* extract could not be calculated because of a lethality <35% exhibited at the maximum concentration.

The absence of toxicity observed in the brine shrimp lethality test was supported by results of the *D. magna* bioassay. The LC₅₀ exhibited by the extracts on the daphnids ranged from 2398.83 to 4073.80 $\mu\text{g/ml}$ (Table III). A positive correlation between the concentration and lethality (r²>0.85) and no statistical differences were observed for the determinations performed with the *F. dumetorum* and *F. aubertii* extracts (p<0.05). *F. convolvulus* exhibited no toxicity at all on *Daphnia magna*, the L% induced by the extract at 3,000 $\mu\text{g/ml}$ being <5%. All results were significantly higher than the positive control (4.74 $\mu\text{g/ml}$) and the toxicity threshold reported by Guilhermino *et al* (50) for toxic substances.

In conclusion, of the three species of *Fallopia* investigated in this study, none was significantly toxic to invertebrate models or to the normal cell model. The highest cytotoxicity to the cancer cells was observed with extracts from the *F. convolvulus* and *F. aubertii* flowers. There was a positive correlation between TPC of the extracts and the IC₅₀ values against HeLa cervical cancer cells, with *F. aubertii* flower hydroethanolic extract (AF_{ha}) having the highest TPC content and the lowest IC₅₀. This extract also induced apoptosis at a much earlier time point than the two extracts with the second and third highest

TPC values, namely *F. convolvulus* hydroethanolic extract (C_{ha}) and *F. aubertii* flower ethanolic extract (AF_e), respectively.

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