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Human DNA topoisomerase inhibitors from *Potentilla argentea* and their cytotoxic effect against MCF-7

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Two polyphenolics, kaempferol 3-*O*-β-D-(6″-*E*-*p*-coumaroyl)-glucopyranoside (tiliroside) (**1**) and methyl brevifolincarboxylate (**2**) isolated from aerial parts of *Potentilla argentea* L. (Rosaceae) were evaluated for their cytotoxicities against human breast carcionoma cell line (MCF-7) and their DNA-binding ability. The DNA-binding ability of these compounds was studied by means of the human DNA topoisomerase I and II inhibition assay and ethidium displacement assay using calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$. Compound **2** was much more active and showed a higher level of cytotoxic potency than compound **1**, with IC_{50} values of 1.11 ± 2 μM and 21.60 ± 2 μM, respectively. In DNA topoisomerase I and II inhibition *in vitro* assays both investigated compounds **1** and **2** were more effective against topoisomerase II than I. The results of DNA binding studies reveal that methyl brevifolincarboxylate had a greater DNA binding affinity that tiliroside, which correlates with its greater potency as a topoisomerase I/II inhibitor.

1. Introduction

Numerous naturally occurring polyphenolic compounds are possessing anti-inflammatory, antioxidant, anti-allergic, antiviral, antibacterial activities in addition to affecting certain aspects of mammalian metabolism. Various species of the *Potentilla* genus are known for their different therapeutic properties and are used as astringents in the treatment of diarrhoea, dysentery and sore throats. Recent studies have also shown that some *Potentilla* extracts exhibit antitumour activity (Syiem et al. 2003). There is increasing interest in evaluating certain polyphenolics as antineoplastic agents.

Flavonoids and other polyphenolic compounds can inhibit the DNA-maintenance enzymes topoisomerases. These regulate the supercoiling of chromosomal DNA, and play a pivotal role in the replication, transcription, recombination, segregation, condensation and repair of chromosomes (Wang 2002; Constantinou et al. 1995; Webb and Ebeler 2004). Topoisomerases are classified as type I and type II on the basis of distinct differences in enzyme sequence, subunit composition and function. Type II enzymes are dimeric, transiently break both strands of duplex DNA (using a catalytic tyrosine residue in each subunit), and pass an intact DNA duplex through this transient double-stranded break in an ATP-dependent manner. Type I topoisomerases, by contrast, are monomeric, require no energy source, and relax superhelical DNA by cleaving a single strand of duplex DNA (Berger 1998). Because of their central role in DNA replication, transcription and repair, topoisomerases enzymes are important in both current and future strategies for cancer chemotherapy, especially since overexpression of these proteins has been demonstrated in many human tumour types, including breast cancer. While topoisomerase I is the specific target for only a limited group of drugs acting as poisons of the enzyme, such as camptothecin and derivatives, topoisomerase II is the primary target of poisoning by an increasing number of cytotoxic drugs of diverse nature currently available for the clinical treatment of human cancers.

Previous studies have shown that tiliroside exhibits cytotoxic activity against two of the nine tested human laeukemic cell lines, viz. CCRF-CEM and NAMALWA, but is inactive against most human cell lines (Dimas et al. 2000; Nshimo et al. 1993). Esteves-Souza et al. (2002) have studied the effect of tiliroside on murine Ehrlich carcinoma and human K562 leukaemia cultured cells. The compound showed concentration dependent activity on human leukaemia cells. In a recent work, Rao et al. (2007) reported that tiliroside also possesses significant cytotoxic potency against different human cell lines: Jurkat (lymphocytic), PC-3 (prostate), HepG2 (hepatoma), Colon 205 (colonic), and normal cells PBMCs (peripheral blood mononuclear cells). To our knowledge, little is known about the biological activity of methyl brevifolincarboxylate. Recently, Iizuka et al. (2006, 2007) confirmed that the compound is a potent inhibitor of platelet aggregation comparable to adenosine despite differences in the inhibitory mechanism and inhibited NE-induced vasocontractions. This was in part attributable to the involvement of $[Ca^{2+}]_I$ through receptor-operated Ca^{2+} channels.

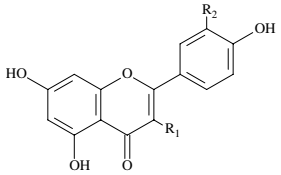
The present study examined the DNA-binding ability of kaempferol 3-*O*-β-D-(6″-*E*-*p*-coumaroyl)-glucopyranoside

Table 1: Viability of MCF-7 cells treated for 24 h with different concentrations of 1 and 2

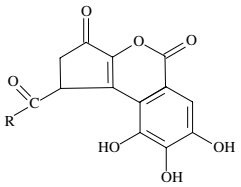
Compd.	Concentration (μM)					IC_{50} (μM)	Statistical data (model y = ax + b)		
	1	10	50	100	150		a	b	R^2
1	39%	49%	63%	72%	88%	21.60 ± 2	0.0030	0.4351	0.9647
2	46%	77%	92%	97%	96%	1.11 ± 2	0.1031	0.4896	0.9665

[a] Percent of non-viable (apoptotic and necrotic) MCF-7 mammalian tumour cells

(tiliroside) and methyl brevifolincarboxylate and their cytotoxicity against cultured breast cancer MCF-7 cells. The DNA-binding ability of these compounds was studied employing the topoisomerase I/II inhibition assay and the ethidium displacement assay using calf thymus DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$.



1: R_1 = coumaroyl-glu R_2 = H



2: R = O-methyl

2. Investigations, results and discussion

In order to compare the cytotoxicity of compounds **1** and **2**, cell viability of breast cancer MCF-7 cells was measured using acridine orange. Compound **2** proved to be more potent than compound **1**, with IC_{50} values of 1.11 ± 2 μM and 21.60 ± 2 μM, respectively (Table 1). To analyse whether the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in the presence of compounds **1** and **2** (Table 2). All of the tested compounds showed concentration dependent activity, yet with different potencies. The concentrations of **1** and **2** needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) in MCF-7 was found to be 29 ± 2 μM, and 16 ± 2 μM, respectively.

These compounds induced both apoptosis and necrosis in breast cancer MCF-7 cells. Interestingly, the apoptotic cell death was mainly observed for **1** (44–46%) and for **2** (43–60%) in a concentration range of 10 to 100 μM, as is shown in Fig. 1A and B. These results also show that cell death is predominantly necrotic for both compounds at a concentration of 150 μM (72% and 50%, respectively).

To test whether cytotoxic properties were related to DNA-binding and topoisomerase I/II inhibition, compounds **1** and **2** were evaluated in a cell-free system. The binding affinities of compounds **1** and **2**, to calf thymus DNA, and synthetic polymers poly(dA-dT)$_2$ and poly(dG-dC)$_2$, were compared using the ethidium displacement assay (Debart et al. 1989). The concentration of test compound in the study needed to reduce fluorescence by 50% was measured in each case. The association constants were determined using these values, and the data are presented in Table 3. Compound **1** was shown to have very moderate binding affinities for DNA in ethidium bromide displacement assays when compared with compound **2** (Table 3). The homopolymer DNA-binding data reported in Table 3 characterise the affinity of compounds **1** and **2** for a more limited set of DNA-binding sites and can give an indication of base-sequence specificity for DNA-binding molecules. The binding constant obtained here for the binding of compounds **1** and **2** to poly(dC-dG)$_2$ polymer are almost the same as the association constant for the binding of compounds **1** and **2** to poly(dA-dT)$_2$.

A number of flavonoids inhibit the catalytic activity of isolated topoisomerases. The ability of compounds **1** and **2** to inhibit topoisomerase I and II activity was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing ligand concentration using agarose gel electrophoresis. As Fig. 2 indicates, the super helical plasmid (lane 1) was relaxed by topoisomerases (lane 2). Figure 2A demonstrates that compound **1** has little impact on the ability of topoisomerase I to transform supercoiled DNA into several topoisomer forms of relaxed DNA at a concentration 100 μM. Compound **2** induces topological changes at a concentration of 5 μM, but even at a concentration of 200 μM, it does not inhibit topoisomerase I completely. Figure 2B shows that both investigated compounds are more effective against topoisomerase II, although compound **2** is more active than compound **1** in this case as well. As can be seen here, the inhibition activity of compound **1** starts at a concentration 50 μM whereas that of compound **2** starts at 10 μM. Complete inhibition of DNA cleavage (positive controls) was obtained using camptothecin (Cpt) and etoposide (Et), drugs which are potent inhibitors of topoisomerase I and topoisomerase II, respectively.

The results of DNA binding studies reveal that compound **2** has a greater DNA binding affinity than compound **1**, which correlates with its greater potency as a topoisomerase I/II

Table 2: Cytotoxic effects of compounds 1 and 2 on the cultured breast cancer MCF-7 cells measured by inhibition of [^{3}H]thymidine incorporation into DNA

Concentration (μM)	Compound **1** (% control)	Compound **2** (% control)
10	89 ± 2	87 ± 2
20	68 ± 2	47 ± 2
30	52 ± 2	39 ± 2
40	44 ± 2	31 ± 2
50	35 ± 2	26 ± 2

Table 3: DNA binding of compounds 1 and 2

Ligand	Calf thymus DNA[a] ($K_{app} \times 10^5\ M^{-1}$)	poly(dA-dT)$_2$[a] ($K_{app} \times 10^5\ M^{-1}$)	poly(dG-dC)$_2$[a] ($K_{app} \times 10^5\ M^{-1}$)
1	0.6	0.7	0.5
2	2.6	2.9	2.4

[a] The error for compounds **1** and **2** is ± $0.2 \times 10^5\ M^{-1}$

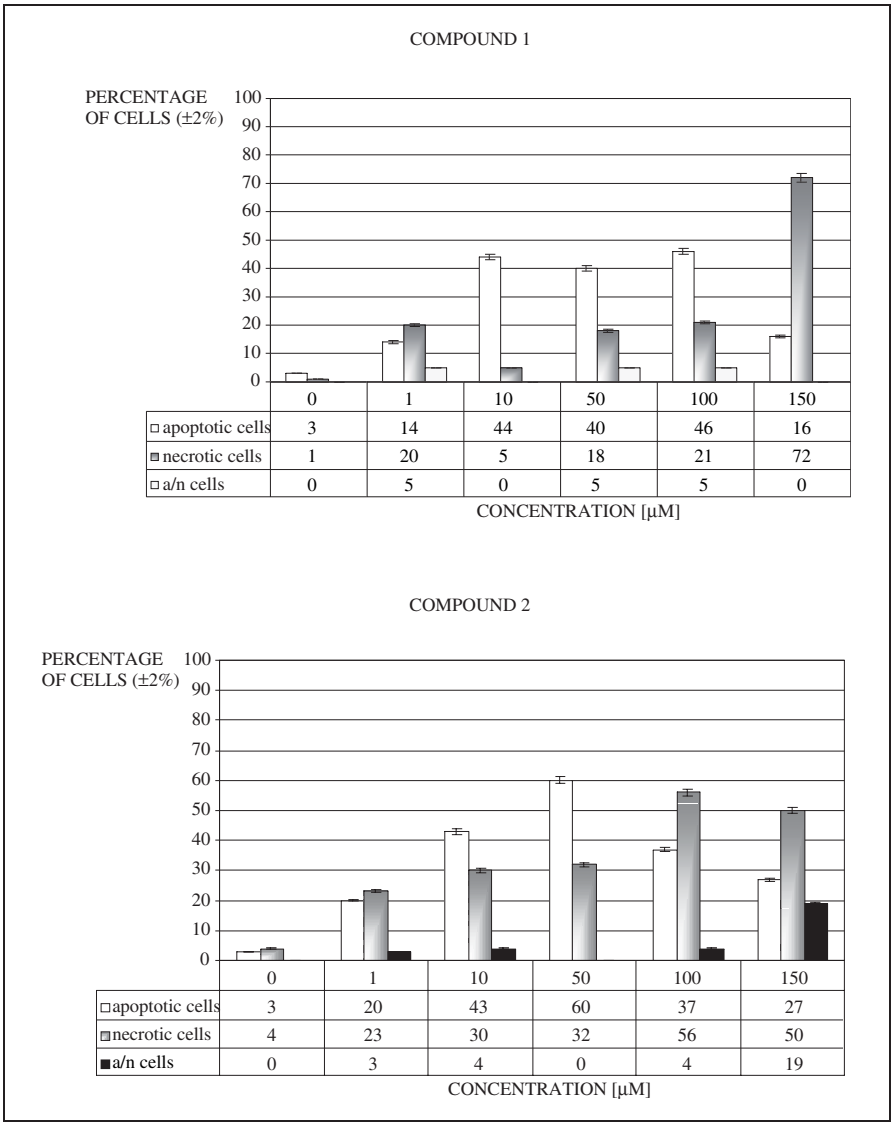


Fig. 1:
The percentage of apoptotic, necrotic and apoptotic/necrotic (a/n) MCF-7 cells after drugs treatment.
100% = {apoptotic(%) + necrotic(%) + a/n(%)}.
Control = 0 μM

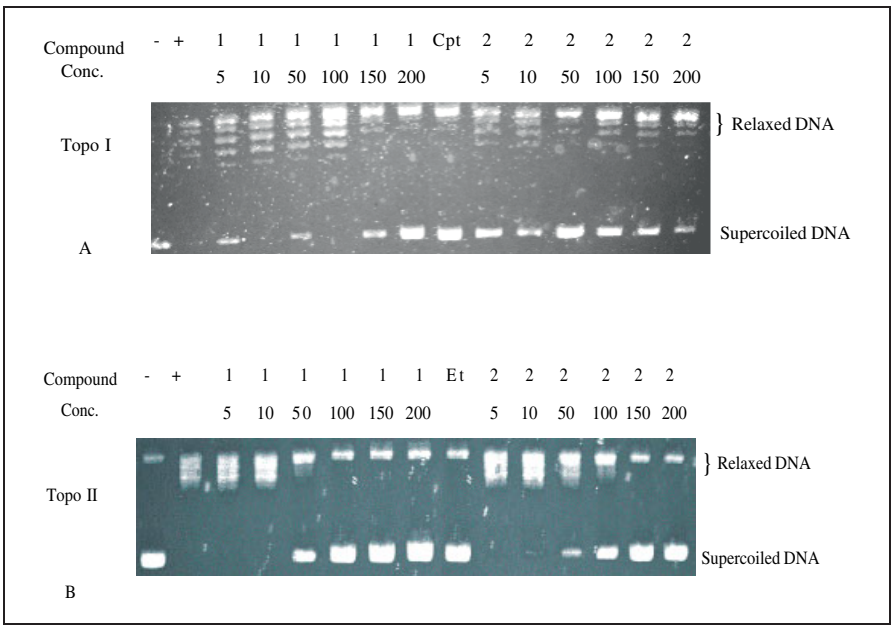


Fig. 2:
Effect of tiliroside (**1**), methyl brevifolincarboxylate (**2**) and camptothecin (Cpt)/etoposide (Et) respectively, on the relaxation of supercoiled plasmid DNA by topoisomerase I (Topo I – A) or II (Topo II – B)

inhibitor. It is possible that compounds **1** and **2** induce conformational DNA changes and hinder the formation of enzyme-DNA cleavable complexes. This suggests that DNA-binding may be also implicated in the cytotoxicity of compounds **1** and **2**, possibly by inhibiting interactions between topoisomerases and their DNA targets.

3. Experimental

3.1. Plant material, extraction, isolation

The air-dried and powdered aerial parts of *Potentilla argentea* (2.0 kg) were extracted with petrol and $CHCl_3$ in a Soxhlet apparatus. Plant material, purified in this way, was successively extracted with MeOH. The MeOH extract concentrated under vacuum was dissolved in H_2O and further successively partitioned between Et_2O, EtOAc and n-BuOH, affording 12.9, 38.0 and 28.0 g of each dried fraction, respectively. The Et_2O extract was chromatographed on a polyamide column eluting with MeOH–H_2O with increasing polarity. Fractions eluted with MeOH–H_2O (5.5 : 4.5, v/v) to yield a pure methyl brevifolincarboxylate (**2**, 106 mg) and eluted with MeOH–H_2O (7 : 3, v/v) to afford kaempferol 3-*O*-β-D-(6″-*E*-*p*-coumaroyl)-glucopyranoside, tiliroside (**1**, 245 mg). The purity of both compounds was confirmed based on the following: by the appearance of a single spot in TLC [silica gel G plates, Merck, Germany; EtOAc/MeOH/H20 (18 : 1 : 1)] and by HPLC analysis (Waters HPLC system (Milford, MA, USA) 600E pump, 600 Controller, 996 DAD detector; column Symmetry C18, (5 mm; 3.9 × 150 mm); mobile phase: A: water + 0.02% TFA, B: methanol + 0.02% TFA. Profile: 0–5 min 75% A; 10 min 70% A; 16 min 55% A; 18 min 55% A; 25 min 20% A; 30 min 20% A; 40 min 75% A.; a flow of 0.5 ml/min PDA detection at 275 nm.; compound **1** and **2** ($t_R = 21.67$ min, $t_R = 30.55$ min, respectively). Further identification of both compounds was carried out on the basis of spectroscopic experiments (UV; ^1H and ^{13}C NMR, DEPT; 2D-NMR; MS and confirmed by comparison with those reported in literature. In addition, the structure of **1** was investigated in the solid-state ^{13}C NMR technique (Wolniak et al. 2006).

3.2. Inhibition of human DNA topoisomerases

3.2.1. Topoisomerase I relaxation assay

Native pBR322 plasmid DNA (0.20 μg) was incubated with 10 unit human topoisomerase I (Amersham Biosciences) in a standard reaction buffer (50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 M NaCl, 1 mM dithiothreitol) in the absence or presence of varying concentrations of tested compounds and camptothecin (2 μM) , in a final volume of 10 μl. The mixture was incubated at 37 °C for 30 min and the reaction was terminated by adding 2 μl of 10% SDS. The reaction mixture was subjected to electrophoresis (3 h, 90 V) through a 1.0% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained with ethidium bromide solution (0.5 μg/ml) for 30 min. The DNA was visualised using a 312 nm wavelength transilluminator and photographed (Canon PowerShot G6, 7.1 mln megapixels).

3.2.2. Topoisomerase II relaxation assay

Native pBR322 plasmid DNA (0.20 μg) was incubated with 10 unit human topoisomerase II (Amersham Biosciences) in a standard reaction buffer (10 mM Tris-HCl (pH 7.9), 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, and 15 μg/ml bovine serum albumin) in the absence or presence of varying concentrations of tested compounds and etoposide (10 μM), in a final volume of 10 μl. The mixture was incubated at 37 °C for 30 min and the reaction was terminated by adding 2 μl of 10% SDS. The reaction mixture was subjected to electrophoresis (3 h, 90 V) through a 1.0% agarose gel in TBE buffer (90 mM Tris-borate and 2 mM EDTA). The gels were stained with ethidium bromide solution (0.5 μg/ml) for 30 min. The DNA was visualised using a 312 nm wavelength transilluminator and photographed (Canon PowerShot G6, 7.1 mln megapixels).

3.3. Cytotoxicity assay

3.3.1. MCF-7 cells

The antiproliferative activities of the drugs were assayed *in vitro* against the breast (MCF-7) cancer cell line (purchased from the American Type Culture Collection, Rockville, MD). Stock cultures of cells were maintained in continuously exponential growth by weekly passage in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% FBS (Sigma), 50 μg/ml streptomycin, 100 U/ml penicillin at 37 °C in a humid atmosphere containing 5% CO_2. Cells were cultivated in Costar flasks and subconfluent detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate-buffered saline. The study was carried out using cells from passages 3–7, grown as a monolayer in six-well plates (Nunc) (5×10^5 cells per well) and preincubated 24 h without phenol red.

3.3.2. Determination of apoptotic index and cell viability

The compounds were dissolved in DMSO and used at concentrations of 1, 10, 50, 100 and 150 μM. Microscopic observations of cell monolayers were made using a Nikon optiphot microscope. Wright-Giemsa staining was performed using the Fisher Leuko Stat Kit. MCF-7 cells were mixed with a dye mixture (10 μM acridine orange and 10 μM ethidium bromide, prepared in phosphate-buffered saline) after 24 h of treatment. At the end of each experimental time point, all the media were removed and the cells were harvested by incubation with 0.05% trypsin and 0.02% EDTA for 1 min and washed with the medium. Then, 250 μl of cell suspension was mixed with 10 μl of the dye mix and 200 cells per sample were examined by fluorescence microscopy, according to following criteria:

- viable cells with normal nuclei (a fine reticular pattern stained green in the nucleus and red-orange granules in the cytoplasm);
- viable cells with apoptotic nuclei (green chromatin which is highly condensed or fragmented and uniformly stained by acridine orange);
- nonviable cells with normal nuclei (bright orange chromatin with organised structure);
- nonviable cells with apoptotic nuclei (bright orange chromatin which is highly condensed or fragmented).

Table 1 and Fig. 1 show the antitumour activity of the investigated compounds as a percentage of non-viable (apoptotic, necrotic and apoptotic/necrotic) cells.

3.3.3. Statistical analysis

In this experiment, the mean values for three independent and duplicated assays (n = 3) were calculated along with their standard deviations. The results were subjected to least squares analysis, accepting a coefficient of determination in the range $0.9600 < R^2 < 1$. The IC_{50} data are presented in Table 1.

3.4. Ethidium displacement assay

Fluorescence was measured using a Hitachi F-2500 spectrophotometer (Tokyo, Japan) at room temperature. The DNA-ethidium complex was excited at 546 nm and the fluorescence was measured at 595 nm. The maximum fluorescence was measured after adding 75 mM NaCl buffer solution, containing 25 μl of DNA solution ($A_{260} = 2$) to 2 ml of ethidium bromide (5.0×10^{-6} M) in 10 mM Tris-HCl (pH 7.4). Aliquots of a 10 mM stock of the tested compound solution were then added to the DNA-ethidium solution, and the fluorescence was measured after each addition until it had been reduced by 50%. Theoretical curves were fitted to the fluorescence intensity data points using nonlinear least-squares computer routines. The apparent binding constant was calculated from $K_{EtBr}[EtBr] = K_{app}[drug]$, where [drug] = the concentration of the test compound at a 50% reduction of fluorescence and K_{EtBr} is known (Debart et al. 1989). Compounds **1** and **2** and their complexes with DNA showed neither optical absorption nor fluorescence at 595 nm and did not interfere with the fluorescence of an unbound ethidium bromide.

3.5. DNA synthesis assay

To examine the effects of the studied compounds on cell proliferation, MCF-7 cells were seeded in 6-well plates and grown as described above. Cell cultures were incubated with varying concentrations of compounds **1** and **2** and 0.5 μCi of [^{3}H]thymidine for 24 h at 37 °C. The cells were then harvested by trypsinization and washed (with cold phosphate-buffered saline) with centrifugation for 10 min at 1500 g several times (4–5) until the dpm in the washes were similar to the reagent control. Radioactivity was determined by liquid scintillation counting. [^{3}H]thymidine uptake was expressed as dpm/well.

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