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Cytotoxic effect of *Plantago* spp. on cancer cell lines

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Abstract

Methanolic extracts from seven *Plantago* species used in traditional medicine for the treatment of cancer, were evaluated for cytotoxic activity against three human cancer cell lines recommended by the National Cancer Institute (NCI, USA). The results showed that *Plantago* species exhibited cytotoxic activity, showing a certain degree of selectivity against the tested cells in culture.

Since the flavonoids are able to strongly inhibit the proliferation of human cancer cell lines, we have identified luteolin-7-*O*-β-glucoside as major flavonoid present in most of the *Plantago* species. Also, we have evaluated this compound and its aglycon, luteolin, for their cytotoxic and DNA topoisomerase I poisons activities. These results could justify the traditional use of the *Plantago* species and topoisomerase-mediated DNA damage might be a possible mechanism by which flavonoids of *Plantago* exert their cytotoxicity potential.

Keywords: Plantago; Cytotoxicity; Topoisomerases; Luteolin-7-*O*-β-glucoside; Tumour cell lines

1. Introduction

Plantago species play a minor role in several areas of the world where they are indigenous. Hartwell, in his monumental work on the use of plants by humans against cancer (Duke, 1985; Hartwell, 1982) published the use of *Plantago cordata*, *Plantago coronopus*, *Plantago griesebachii*, *Plantago lagopus*, *Plantago lanceolata*, *Plantago macrostachys*, *Plantago major*, *Plantago media*, *Plantago mexicana*, *Plantago minor*, *Plantago ovata*, *Plantago psyllium*, *Plantago rocae*, *Plantago rugelii*, *Plantago sericea*, *Plantago tomentosa*, and *Plantago ureades*. A recent review of plants used ethnomedically against cancer presents the results of a query of the NAPRALERT database and has reported the use of *Plantago asiatica* on Easter Island for internal cancers, *Plantago hirtella* in Mexico against cancerous diseases and *Plantago paralias* in Argentina as an antitumour agent (Graham et al., 2000). Furthermore, a review of traditional uses of *Plantago major* (Samuelsen, 2000) shows that it is used in Canary Islands, Chile, Venezuela and Panama in the treatment of tumours.

Flavonoids constitute one of the most characteristic classes of compounds in Plantago. Kawashty et al. (1994), studied the flavonoid profile of 18 species of *Plantago* and they indicated that luteolin-7-*O*-β-glucoside was the major component in most *Plantago* species. Various studies are in line with the hypothesis that flavonoids prevent or inhibit cancer development (Hertog et al., 1993; Hertog, 1996).

The precise mechanism responsible for the antitumour effect of flavonoids is not still thoroughly understood. It is clear that flavonoids affect various metabolic pathways such as activation of glycolytic enzymes or protein synthesis (Lee et al., 1994; Middleton and Kandaswami, 1993). However, their cytotoxic effect cannot be clearly assigned to a specific cellular target yet. Some studies, associate this cytotoxic action with DNA scission, moreover, some flavonoids have been shown to induce topoisomerase I- and II-mediated DNA cleavage complex (Boege et al., 1996; Martín-Cordero et al., 2000; López-Lázaro et al., 2000, 2002). Thus, topoisomerase-mediated DNA damage seems to be a candidate mechanism, by which some flavonoids may exert their cytotoxic potential.

As part of our continuing work in the search for biologically active compounds, we have assessed methanolic extracts from leaves of seven species of *Plantago* genus: *Plantago afra* Linn. (syn. *Plantago psyllium* L.), *Plantago bellardii* All., *Plantago coronopus* L., *Plantago lagopus* L., *Plantago lanceolata* L., *Plantago major* L., and *Plantago serraria* L. (Family—Plantaginaceae), against three human

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cell lines, using etoposide as positive control. The tumoural cellular lines were renal adenocarcinoma (TK-10), breast adenocarcinoma (MCF-7), and melanoma (UACC-62), recommended by the National Cancer Institute (NCI, USA).

Here, we have isolated the flavone luteolin-7-*O*-β-glucoside from *Plantago lagopus* and shown its ability to stabilise the covalent topoisomerase I–DNA complex in vitro, besides its aglycon, luteolin, obtained by hydrolysis.

2. Methodology

2.1. Plant material

Plantago afra, *Plantago bellardii*, *Plantago coronopus*, *Plantago lagopus*, *Plantago lanceolata*, and *Plantago serraria* were collected around Badajoz (Spain) and *Plantago major* in Galdar, Gran Canaria (Spain) during April 2000. They were authenticated by Dr. Abelardo Aparicio, Department of Botany, Pharmacy Faculty, University of Seville, Seville. Voucher specimens are kept in the Herbarium of the Seville University.

2.2. Extraction procedure and isolation

Fifty grams of each air-dried and powdered leaves were extracted by Soxhlet for 48 h with MeOH and filtered. Extracts were taken to dryness at 40 °C under vacuum, obtaining a residue. To remove chlorophylls and waxes, these methanolic extracts were washed with *n*-hexanic fractions, using sonication bath at controlled temperature and times, till all non-polar compounds were removed. The washed methanolic extracts were object of our study. The recovery of each extract was: *Plantago afra* (18% w/w), *Plantago bellardii* (16%), *Plantago coronopus* (21.3%), *Plantago lagopus* (16.5%), *Plantago lanceolata* (29%), *Plantago major* (18%), and *Plantago serraria* (24%).

4.5 g of the MeOH extract from *Plantago lagopus* was chromatographed on Silica gel column using gradient elution with mixtures of EtOAc:MeOH:H_2O at different proportions, increasing the polarity progressively. A flavonoid glycoside was crystallised from the intermediate fractions and purified in MeOH and its aglycon was obtained by acid hydrolysis according to standard procedures (Mabry et al., 1970).

2.3. Enzymes and nucleic acids

Purified enzyme, supercoiled pRYG DNA and the positive control etoposide were purchased from TopoGen, Inc. (Columbus, OH, USA). Proteinase K was from Sigma Chemical Co. Stock solutions of these compounds were dissolved in dimethylsulfoxide (DMSO) at 40 mM and were diluted in water containing 2.5% DMSO before use.

2.4. Assay for cytotoxic activity: human cell lines

The following three human cancer cell lines were used in these experiments: the human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines. They were kindly provided by Dr. G. Cragg, Department of NCI, Maryland, USA. The human tumour cytotoxicities were determined following protocols established by NCI (Monks et al., 1991). TK-10, MCF-7, UACC-62 cell lines were cultured in RPMI 1640 medium (Bio Whittaker) containing 20% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

All cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week. According to their growth profiles, the optimal plating densities of each cell line was determined (15×10^3, 5×10^3 and 100×10^3 cells/well for TK-10, MCF-7 and UACC-62, respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analysed by the SRB assay.

2.5. Testing procedure and data processing

The sulphorhodamine B (SRB) assay was used in this study to assess growth inhibition. This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. For the assay, cells were detached with 0.1% trypsin-EDTA (Sigma Chemical Co.) to make single-cell suspensions, and viable cells were counted using a Coulter counter and diluted with medium to give final densities of 15×10^4, 5×10^4 and 100×10^4 cells/ml for TK-10, MCF-7 and UACC-62, respectively. One hundred microlitres per well of these cell suspensions was seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h, the cells were treated with the serial concentrations of extracts and compounds. They were initially dissolved in an amount of 100% DMSO (1 mg/ml for extracts and 40 mM for compounds) and further diluted in medium to produce five concentrations. One hundred microlitres per well of each concentration was added to the plates to obtain final concentration of 250, 25, 2.5, 0.25 and 0.025 μg/ml for the extracts and 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M for the flavonoids and for the positive control. The DMSO concentration for the tested dilutions was not greater than 0.25% (v/v), the same as in solvent control wells. The final volume in each well was 200 μl. The plates were incubated for 48 h.

2.6. Sulphorhodamine B method

After incubating for 48 h, adherent cell cultures were fixed in situ by adding 50 μl of cold 50% (w/v) trichloroacetic

acid (TCA) and incubating for 60 min at 4 °C. The supernatant was then discarded and the plates washed five times with deionised water and dried. One hundred microlitres of SRB solution (0.4% w/v in 1% acetic acid) was added to each microtiter well and the culture was incubated for 30 min at room temperature. Unbound SRB was removed by washing five times with 1% acetic acid. Then, the plates were air-dried. Bound stain is solubilised with Tris buffer and the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 492 nm. At the end, GI_{50} values (concentrations required to inhibit cell growth by 50%), TGI (concentration resulting in total growth inhibition) and LC_{50} (concentration causing 50% of net cell killing) were calculated according to the previously described protocols (Monks et al., 1991). Two or three experiments were carried out for each extract or compound. The data are given as the mean of two or three different assays ±S.E.M.

2.7. DNA cleavage reactions with topoisomerase I

Cleavage topo I buffer contained 10 mM Tris–HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM Spermidine and 5% glycerol. The cleavage reaction (20 μl) contained water, cleavage buffer, the tested drugs dissolved in 2 μl DMSO/H_2O (2.5%), supercoiled DNA (0.25 μg in 1 μl of buffer), and 2.5 μl (5 U) of topoisomerase I storage buffer were mixed in this order in ice/water. Reactions were carry out by incubation at 37 °C for 30 min, terminated by the addition of 2 μl SDS 10% and 1 μl proteinase K 20 μg/ml and followed by an additional 30 min incubation at 37 °C. Subsequently, the samples were extracted with chloroform:isoamyl alcohol, and 2 μl bromophenol blue. Samples were loaded on 1% agarose gels and electrophoresed at 3 V/cm for 6 h in Tris–Acetate–EDTA buffer with ethidium bromide to a final concentration of 0.5 μg/ml. The gels were washed in a large amount of water. For the quantitative determination of topo I activity, videoimpression was densitometrically measured using a PCBAS software. After integration of the bands, nicked open circle (OC) DNA forms were expressed as a percentage of total DNA.

3. Results

3.1. Isolation and identification of luteolin-O-β-glucoside and luteolin

The identification of luteolin-7-*O*-β-glucoside isolated from the methanolic extract of the leaves from *Plantago lagopus* and its aglycon, obtained by acid hydrolysis, were based on the comparison of spectral data (UV, MS, ^{1}H NMR, ^{13}C NMR) with data registered in the literature (Mabry et al., 1970; Velazquez Fiz et al., 2000).

Table 1
Extracts concentration (μg/ml) required to inhibit cell growth by 50% (GI_{50}), to produce total growth inhibition (TGI) and to cause 50% of net cell killing (LC_{50})

Methanolic extracts	Inhibition parameters	TK-10	MCF-7	UACC-62
Plantago afra	GI_{50}	>250	76.07 ± 19.25	53.83 ± 16.08
	TGI	>250	>250	238.09 ± 117.22
	LC_{50}	>250	>250	>250
Plantago bellardii	GI_{50}	86.32 ± 21.46	42.01 ± 2.71	34.77 ± 4.15
	TGI	>250	87.49 ± 9.13	84.21 ± 7.92
	LC_{50}	>250	>250	>250
Plantago coronopus	GI_{50}	>250	32.57 ± 11.90	40.98 ± 5.80
	TGI	>250	74.30 ± 14.10	89.94 ± 13.31
	LC_{50}	>250	169.42 ± 6.97	198.63 ± 34.3
Plantago lagopus	GI_{50}	>250	114.45 ± 20.24	66.07 ± 8.76
	TGI	>250	>250	244.56 ± 78.95
	LC_{50}	>250	>250	>250
Plantago lanceolata	GI_{50}	>250	47.17 ± 6.80	50.58 ± 11.15
	TGI	>250	99.97 ± 1.50	>250
	LC_{50}	>250	212.95 ± 22.19	>250
Plantago major	GI_{50}	>250	46.5 ± 7.1	46.5 ± 8.2
	TGI	>250	97.5 ± 1.8	112.5 ± 2.1
	LC_{50}	>250	207 ± 18.20	247 ± 12.3
Plantago serraria	GI_{50}	>250	55.12 ± 11.33	48.94 ± 8.17
	TGI	>250	120.88 ± 16.21	118.29 ± 28.27
	LC_{50}	>250	274.45 ± 12.44	>250

The range of doses assayed was 0.025–250 μg/ml. Results are mean ± S.E.M. ($n = 3$).

Table 2
GI_{50} values (μM) of flavonoids and positive control tested against the cell lines TK-10, MCF-7 and UACC-62

Tested compounds	Inhibition parameters	TK-10	MCF-7	UACC-62
Luteolin-7-*O*-β-glucoside	GI_{50}	62.1 ± 12.60	40.8 ± 3.0	20.9 ± 3.3
	TGI	>100	>100	52.8 ± 10.7
	LC_{50}	>100	>100	>100
Luteolin	GI_{50}	30.6 ± 6.95	74.8 ± 6.5	10.14 ± 1.9
	TGI	>100	>100	>100
	LC_{50}	>100	>100	>100
Etoposide	GI_{50}	9.95 ± 0.08	0.87 ± 0.21	1.13 ± 0.21
	TGI	52.4 ± 0.4	>100	13.3 ± 2.4
	LC_{50}	>100	>100	>100

Results expressed as mean ± S.E.M. ($n = 2$).

3.2. Cytotoxic activity of Plantago methanolic extracts and flavonoids on tumoural cell lines

The results depicted in Tables 1 and 2 summarise the cytotoxic effects of the seven methanolic extracts and the two flavonoids on TK-10, MCF-7 and UACC-62 cell lines. The antineoplastic agent, etoposide, was taken as positive control. The seven extracts showed cytotoxic activity on the breast adenocarcinoma (MCF-7) and melanoma (UACC-62) tumoural cell lines in a concentration-dependent manner at the recommended NCI (USA) doses. However, neither methanolic extract, with the exception of *Plantago bellardii* (GI_{50} = 86 μg/ml), showed any cytotoxic activity against renal adenocarcinoma (TK-10) cells. The methanolic extracts from *Plantago coronopus* and *Plantago bellardii* were the most cytotoxic on MCF-7 and UACC-62 cell lines (GI_{50} = 32 and 34 μg/ml, respectively). The growth of MCF-7 cells was totally inhibited by extracts from *Plantago bellardii*, *Plantago coronopus*, *Plantago lanceolata*, *Plantago major* and *Plantago serraria* (TGI = 87, 74, 99, 97 and 120 μg/ml, respectively) and *Plantago afra*, *Plantago bellardii*, *Plantago coronopus*, *Plantago lagopus*, *Plantago major* and *Plantago serraria* methanolic extracts demonstrated total growth inhibition on UACC-62 cell line (TGI = 238, 84, 89, 244, 112 and 118 μg/ml, respectively). On the other hand, none of the tested extracts showed total growth inhibition on TK-10 cells. Furthermore, *Plantago coronopus*, *Plantago lanceolata*, *Plantago major* and *Plantago serraria* extracts on MCF-7 cell line produced a 50% of net killing (LC_{50}) at the doses 169, 212, 207 and 274 μg/ml, respectively, and on UACC-62 cell line, *Plantago coronopus* and *Plantago major* extracts showed LC_{50} at the doses 198 and 247 μg/ml.

Luteolin-7-*O*-β-glucoside isolated from the methanolic extract of *Plantago lagopus* and its aglycon luteolin were found to possess cytotoxic activity on the three cell lines at the tested doses. Luteolin-7-*O*-β-glucoside was the most active on breast adenocarcinoma cell line (MCF-7), with GI_{50} value of 40 μg/ml; while luteolin was the most active in the melanoma cell line (UACC-62) (GI_{50} = 10 μg/ml).

3.3. Stabilisation of cleavage complex

The gel presented in Fig. 1 shows the stabilisation effects of the luteolin-7-*O*-β-glucoside and luteolin on cleavage complex formation at concentrations of 50 and 100 μM. It is observed that the two flavones in the presence of topoisomerase induces formation of OC in a similar fashion to camptothecin tested at 100 μM, used as positive control in the topo I assay. For the lower dose assayed, 50 μM, luteolin-7-*O*-β-glucoside induces 8.56% OC and its aglycon, 8.58%. At the highest dose, 100 μM, luteolin-7-*O*-β-glucoside induces 9.02% OC and its aglycon, 8.65%. Camptothecin at 100 μM induces 14.54% OC formation (Fig. 2).

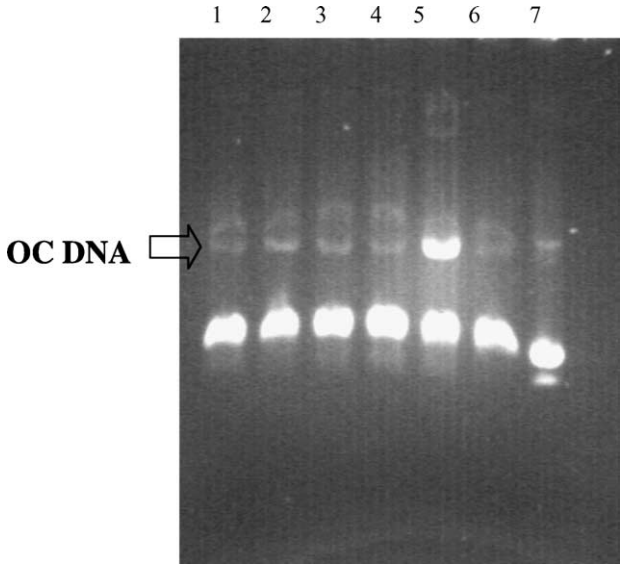

Fig. 1. DNA topoisomerase-I-mediated DNA cleavage: (1) topo I + supercoiled DNA + 50 μM luteolin-7-*O*-β-glucoside; (2) topo I + supercoiled DNA + 100 μM luteolin-7-*O*-β-glucoside; (3) topo I + supercoiled DNA + 50 μM luteolin; (4) topo I + supercoiled DNA + 100 μM luteolin; (5) topo I + supercoiled DNA + 100 μM camptothecin; (6) topo I + supercoiled DNA; (7) supercoiled DNA (OC DNA: open circular DNA).

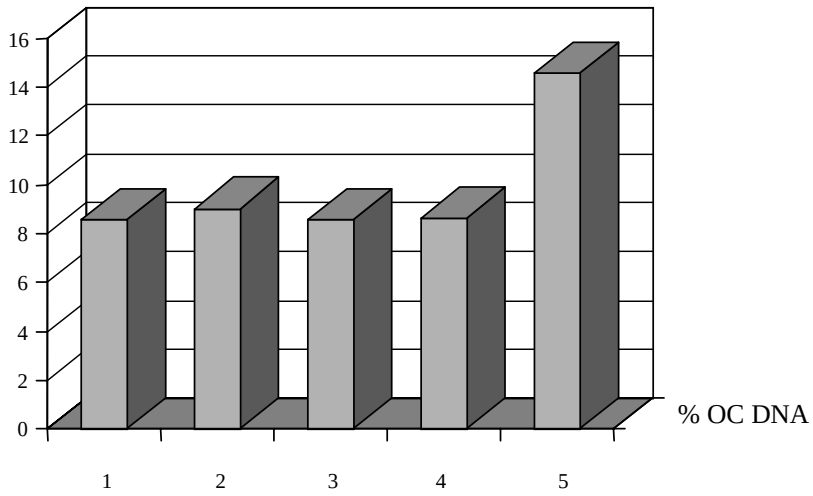

Fig. 2. Quantitative comparison of topoisomerase-I-mediated cleavage induced by (1) 50 μM luteolin-7-glucoside, (2) 100 μM luteolin-7-glucoside, (3) 50 μM luteolin, (4) 100 μM luteolin, (5) 100 μM camptothecin.

4. Discussion and conclusions

The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and nowadays are used to treat human tumours. The ethnopharmacological knowledge is helpful to lead the search for plants with potential cytotoxic activity.

Different species of *Plantago* have been reported to be utilised as remedies against cancer, however, there is not any scientific validation. One goal of this evaluation is to understand better its use. Our results demonstrate that *Plantago* extracts have growth inhibitory and cytotoxic effects on breast adenocarcinoma and melanoma cell lines recommended by NCI. These preliminary results could be justified by the cytotoxic activity of the flavone, luteolin-7-*O*-β-glucoside, the major flavonoid in *Plantago* species. Similar results have been established by Pettit et al. (1996), who isolated luteolin as an active component of *Terminalia arjuna* in cancer cell lines, which justifies the underlying use of these species in traditional cancer treatment.

Luteolin has been shown to inhibit a series of human cancer cell lines (renal A-549, ovary SK-OV-3, melanoma SK-MEL-2, XF-498, HCT15, gastric HGC-27) (Matsukawa et al., 1993; Ryu et al., 1994) breast MCF-7 (Le Bail et al., 1998) and human leukaemia cells (Post and Varma, 1992). Our results suggest that luteolin-7-*O*-β-glucoside could be an interesting anti-cancer molecule, more active in the breast adenocarcinoma cell than luteolin.

The precise mechanism responsible for the cytotoxic activity of luteolin-7-*O*-β-glucoside, the major flavonoid found in all species of *Plantago*, is not thoroughly understood. The topoisomerase-mediated DNA damage seems to be a candidate mechanism, by which some flavonoids may exert their cytotoxic potential (Martín-Cordero et al., 2000; López-Lázaro et al., 2000, 2002). In this work, luteolin-7-*O*-β-glucoside acted as a potent DNA topoisomerase I poisons as well as its aglycon luteolin, this last result agree with Chowdhury et al. (2002). This is the first report of luteolin-7-*O*-β-glucoside acting as a topoisomerase I poison. Thus, there is now preliminary scientific validation for the use of some of these medicinal plants for anticancer.

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