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PHYTOCHEMICAL INVESTIGATION AND CITOTOXIC ACTIVITY OF *LOTUS CORNICULATUS*

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Abstract

In the present work we reported the phytochemical investigation of *n*-buthanol and chloroform extracts of *Lotus corniculatus* L. a plant belonging to Fabaceae family. Our study reported the isolation of benzoic acid, transilin, isosalicin, soyasaponin I, dehydrosoyasaponin I, medicarpin-3-*O*-*β*-*D*-glucopyranoside, pharbitoside A and *p*-coumaric acid. The structures of all compounds were elucidated on the basis of NMR and MS analysis. The antiproliferative activity of the extracts was evaluated using three continuous murine and human culture cell lines J774A1, HEK-293, and WEHI-164. The *n*-BuOH showed moderate activity cytotoxic.

Key words: Lotus corniculatus, saponins, citotoxic activity

Introduction

Fabaceae family is one of the largest families, with 720-750 genera and 18,000 species [1]. This family has been traditionally divided into three subfamilies, the Caesalpinioideae, Mimosoideae, and Papilionoideae includes herbs, shrubs, trees and vines distributed throughout the world, especially the tropical rain forest. The genus *Lotus*, belonging to the subfamily Papilionoideae is mainly distributed around the Mediterranean region comprising Areas of Europe, Africa, Western Asia and Western North America. Only in Algeria there are about fifteen species [2]. *Lotus corniculatus* also known as "Birdsfoot trefoil" is a perennial dehiscent species, herbaceous member of the pea family (Fabaceae). It can be distinguished from all other members of the pea family by its five leaflets and head-like umbels of bright yellow flowers [1]. *Lotus corniculatus* is a legume species adapted to different climatic and soil conditions. Shows a high resistance to cold, drought and even the excess moisture[3]. Previous phytochemical studies reported the presence of phitoalexins, poliphenols, proanthocyanidins [4], tannins, flavonoids, oleanolic acid and saponins [5]. Different biological activities are reported, in particular the flowers are antispasmodic, cardiotonic and sedative while the whole plant is used externally for a local skin inflammation [6]; the root is carminative, febrifuge, restorative [7]. In our ongoing research for new bioactive compounds from mediterranean medicinal plants [8-10] the aerial parts of *Lotus corniculatus* has been studied.

Material and Methods

Plant Material

Lotus corniculatus L. (Fabaceae) aerial parts, were collected in March 2011 from El-Djbel el ouahch near Constantine City, Algeria. The plant was identified by Pr. M. Bouhroum.

Chemicals

All reagents for cell culture were from Hy-Clone (Euroclone, Paignton Devon, U.K.); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] and 6-mercaptopurine (6-MP) were from Sigma Chemicals (Milan, Italy). *n*-Hexane, *n*-Butanol chloroform, methanol, hydrochloric acid and glacial acetic acid were purchased from VWR (Milano-Italy).

Extraction and isolation

Air dried aerial parts of *Lotus corniculatus* L. (500 g) were macerated four times with 70% MeOH solution by replacing the solution every day with fresh solvent. The hydro-alcoholic solutions were concentrated under reduced pressure to dryness and the residue was dissolved in water (200 mL) and kept in cold overnight. After filtration, the aqueous solution was successively extracted with $CHCl_3$, EtOAc and *n*-BuOH for three times for each solvent, then the $CHCl_3$ (1.7 g), EtOAc (2.5 g) and *n*-BuOH (24.6 g) extracts were concentrated to dryness. Part of *n*-BuOH extract (13 g) was fractionated by a Silica gel 60 (Merck 200-400 mesh) column chromatography eluted with a gradient of CH_2Cl_2/ MeOH with increasing polarity. Fractions of 50 ml were collected, analyzed by TLC and pooled into eight fractions (A–H). Fraction A (322 mg) was purified by RP-HPLC using MeOH–H_2O (2:3) to give benzoic acid (5.0 mg, t_R=18 min). Fraction C (700 mg) was purified by RP-HPLC using MeOH–H_2O (45:55) to give isosalicin (4 mg, t_R =8 min) and transilin (1.1mg, t_R =30 min). Fraction D (800 mg) was purified by RP-HPLC using MeOH–H_2O (1:1) to give soyasaponin I (2.7mg, t_R =32min), dehydrosoyasaponin I (1.7mg, t_R =37 min) and pharbitoside A (2.7mg, t_R =32min). The chloroform extract (1,6 g) was subjected to column chromatography using silica gel and eluted with $CHCl_3$ followed by increasing concentrations of MeOH (from 1% to 100%). Fractions of 50 mL were collected, analyzed by TLC (silica gel plates, in $CHCl_3$ and mixtures $CHCl_3$–MeOH) and grouped into five fractions (A–E). Fraction B (20mg) was pure *p*-coumaric acid. Fraction F (172 mg) was purified by RP-HPLC using MeOH–H_2O (55:45) to give medicarpin-3-*O*-*β*-*D*-glucopyranoside (3mg, t_R =14 min). The purity of each compound was determined by HPLC, and NMR.

Experimental procedures

Column chromatography was performed over silica gel (63-200 µm, Merck, Darmstadt, Germany); TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by $Ce(SO_4)_2$/H_2SO_4 (Sigma-Aldrich, Milano, Italy) solution. HPLC separations were conducted on a Shimadzu LC-20AT Prominence system equipped with a Shimadzu RID-10A refractive index detector, and with a Waters µ-Bondapak C18 column (Waters, Milford, MA). NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K [11, 12]. The NMR data were processed on a Silicon Graphic

Indigo2 Workstation using UXNMR software. HRESIMS spectra were acquired in the positive ion mode on a Q-TOF premier spectrometer equipped with a nanoelectrospray ion source (Waters-Milford, MA, USA).

Antiproliferative assay

J774.A1, murine monocyte/macrophage, WEHI-164, murine fibrosarcoma, and HEK-293, human epithelial kidney cells were grown as reported previously [13]. J774.Al, WEHI-164, and HEK-293 (3.4×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37°C in 5% CO_2 and 95% air for 2 h. Thereafter, the medium was replaced with 50 µL of fresh medium and a 75 µL aliquot of 1:4 serial dilution of each test compound was added and then the cells incubated for 72 h. In some experiments, serial dilutions of 6-MP were added. The cell viability was assessed through an MTT conversion assay [14, 15]. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP [13] was calculated as: % dead cells=100-(OD treated/OD control) x 100. Table 1 shows the results obtained expressed as an IC_{50} value (µM), the concentration that inhibited cell growth by 50% as compared to the control.

Results and Discussion

The phytochemical investigation of *n*-BuOH and chloroform extracts of *Lotus corniculatus* aerial parts was conducted using for the extracts fractionation a Silica gel column chromatography followed on HPLC. Chromatographic and spectroscopic analyses of fractions indicated the presence of eight known compounds, in particular benzoic acid and *p*-coumaric acid [16], a phenolic glucoside isosalicin [17], a flavonoid glycoside transilin [18]; three saponins soyasaponin I, dehydrosoyasaponin I [19], pharbitoside A [20] ; a pterocarpan glycoside medicarpin-3-*O*-*β*-*D*-glucopyranoside [20]. Isosalicin was isolated for the first time in *Lotus corniculatus*; soyasaponin I, dehydrosoyasaponin I, pharbitoside A, medicarpin-3-*O*-*β*-*D*-glucopyranoside and transilin were isolated for the first time in *Lotus* genus.

The antiproliferative activity of extracts and isolated compounds was evaluated against the J774.A1, WEHI-164, and HEK-293 cell lines.

The n-BuOH extract was the only active showing an IC50 of 27±2, 55±2.5 and >100 µg/mL against J774.A1, WEHI-164, and HEK-293 cell lines, respectively. Phenolic derivatives were almost inactive, while data obtained (IC_{50} values) for saponins: soyasaponin I, dehydrosoyasaponin I and pharbitoside A are reported in Table 1. As can be seen from the results, pharbitoside A , was the most active constituents. Generally, cytotoxic effects of compounds saponins were dependent on the number of sugar units: the ones having less sugar moieties were more intense in activity as compared with those having more sugar moieties. A possible explanation is that the number of the sugar moieties determines the hydrophilic properties of a compound; the hydrophilic compounds are less able to pass through the cell membrane of mammalian cells, which is reflected in a lower cytotoxicity.

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Table 1. *In vitro* Antiproliferative Activity.

Compounds	cell line (IC_{50} μM)		
	J774.A1[b]	**HEK-293**[c]	**WEHI-164**[d]
dehydrosoyasaponin I	29.4± 0.6	36.1 ± 0.4	28.0± 0.3
soyasaponin I	56.2 ± 0.8	67.0± 0.2	>100
pharbitoside A	97.0 ± 1.2	>100	>100
6-MP[f]	0.003 ± 0.5	0.007 ± 0.4	0.015 ± 0.6

[a] The IC_{50} value is the concentration of compound that affords 50% reduction in cell growth (after a 3-days incubation).
[b]J774.A1 = murine monocyte/macrophage cell lines.
[c]HEK-293 = human epithelial kidney cell lines.
[d]WEHI-164 = murine fibrosarcoma cell lines.
[e]nd = not determined. [f]6-MP = 6-mercaptopurine.