Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 4757–4760
Bioorganic & Medicinal Chemistry Letters

Potential antitumor agents: Flavones and their derivatives from *Linaria reflexa* Desf.

Rosa Tundis,[a,*] Brigitte Deguin,[b] Monica R. Loizzo,[a] Marco Bonesi,[a] Giancarlo A. Statti,[a] François Tillequin[b] and Francesco Menichini[a]

[a]*Department of Pharmaceutical Sciences, University of Calabria, I-87030 Rende (CS), Italy*
[b]*Laboratoire de Pharmacognosie de l'Université René Descartes, U.M.R./C.N.R.S. No. 8638, Faculté des Sciences Pharmaceutiques et Biologiques, 4, Avenue de l'Observatoire, F-75006 Paris, France*

Received 23 May 2005; revised 20 July 2005; accepted 22 July 2005
Available online 25 August 2005

Abstract—The antiproliferative activity of several flavonoids isolated from *Linaria reflexa* Desf. (Scrophulariaceae) was evaluated in vitro by the SRB assay against the large cell lung carcinoma cell line COR-L23, hepatocellular carcinoma cell line HepG-2, renal adenocarcinoma cell line ACHN, amelanotic melanoma cell line C32, colorectal adenocarcinoma cell line Caco-2, and normal human fetal lung MRC5. Chemical modifications, that is acetylation, hydrolysis of rutinose unit, and hydrolysis of the terminal rhamnose unit, were performed on pectolinarin. Pectolinarin exhibited strong cytotoxic activity on COR-L23, Caco-2, and C32 cell lines with an IC_{50} of 5.03, 6.18, and 7.17 μM. Similar activities were recorded for the three natural monoacetyl pectolinarin derivatives linariin, isolinariin A, and isolinariin B. In contrast, *per*acetylpectolinarin displayed only marginal activity.

It is well-established that natural products are an excellent source of chemical structures with a wide variety of biological activities, including anticancer property.[1] This has opened up new fields of investigation of potential antitumor compounds, some of which are already widely used in cancer chemotherapy. Cytotoxic screening models provide important preliminary data to select plants with potential anticancer compounds.

Flavonoids are a group of polyphenolic secondary metabolites present in a wide variety of plants.

These compounds have been reported to display a large panel of biochemical properties, including antioxidant activity, inhibition of tyrosine kinases and cAMP phosphodiesterase, and induction of phase II metabolizing enzymes both in vivo and in vitro.[2,3] These biochemical interferences elicited by flavonoids in some cell systems have been associated with their capacity to control cell growth or destroy pathogen organisms, such as fungi and viruses.[4,5] One of the most interesting biological properties of some flavonoids is their cytotoxicity. Several polyalkoxylated flavonoid aglycones have shown interesting cytotoxic and/or antitumor properties, including flavone,[6,7] flavonol,[8] and flavanone.[9]

Nagao et al. reported the cytotoxicity activity of flavonoids and discussed their structure–activity relationships. However, confining the materials to the flavones, what can be deduced from those findings is that the presence of hydroxyl groups at C_5 and C_7 in ring A, and at C_3' and C_4' in ring B, appears to be important for enhanced activity, but the influence of the other substituents at other positions is not clear. In the flavone series, compounds bearing a free 5-hydroxy group were shown to exhibit antitumor activity by a mechanism that involved topoisomerase I inhibition.[10]

Flavonoid glycosides, particularly pectolinarin and linariin, are good chemotaxonomic markers of the Scrophulariaceae family. As part of a screening program that considers the search for *Linaria* species and other natural products with anticancer properties, the aim of the present investigation was to explore the potential cytotoxic effect on human cancer cell lines of flavones from *L. reflexa* and pectolinarin derivatives. The genus *Linaria* (Scrophulariaceae) comprises about 200 species with only about 20 species reported in Italy.[11] It contains several species used in folk medicine as laxative,

Keywords: Linaria reflexa; Flavones; Flavone derivatives; Cytotoxicity.
* Corresponding author. Tel.: +39 984 493169; fax: +39 984 493298; e-mail: rosa.tundis@unical.it

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.07.029

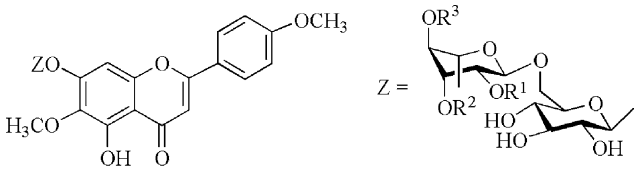
spasmolytic, cholagogic, and anti-inflammatory drugs.[12,13] No previous phytochemical or biological studies on *L. reflexa* Desf. have been reported.

Fresh aerial parts of *L. reflexa* [1 kg, collected (February 2003) in Calabria, Italy, voucher specimen P1001: Herbarium of University of Calabria, CLU] were extracted with methanol three times at room temperature. After evaporation of the solvent under vacuum, the crude extract (80.82 g, 8.08%) was dissolved in methanol; upon cooling, a solid precipitate was obtained by filtration (16 g). The methanolic extract was evaporated to dryness (64 g, 6.4%) and an aliquot (10 g), after extraction with *n*-hexane, was subjected to repeated column chromatography over silica gel (SiO_2) 20–45 μm (CH_2Cl_2/MeOH 95:5, 9:1, 7:3, 1:1), to afford two known iridoid glycosides: antirrhide (15.5 mg) and antirrhinoside (120 mg), pectolinarin (6 mg), and β-sitosterol 3-*O*-β-D-glucopyranoside (7 mg). The solid precipitate was dissolved in H_2O, acidified with HCl (1 N), and extracted with EtOAc, to give a residue and an EtOAc-soluble fraction.

The EtOAc-soluble fraction was subjected to column chromatography over silica gel 20– 45 μm (CH_2Cl_2/MeOH 85:15), to afford linariin (1.97 g) and a fraction of 70 mg.

This fraction was purified by HPLC [JASCO RP-18 250 × 20 mm i.d.; the elution solvents used were A (aqueous 0.01 M phosphoric acid) and B (100% MeOH) with the following gradient: 5% B as initial condition; 50% B for 10 min; 70% B for 5 min; 80% B for 5 min; and finally 100% in B for 5 min] to afford isolinariin A (30 mg) and isolinariin B (35 mg). The residue was crystallized from MeOH to afford pectolinarin (2.50 g; Fig. 1). The structures of the compounds were determined on the basis of the spectral data (UV, IR, MS, ^{1}H NMR, and ^{13}C NMR), identical with those previously described.[9,14–18]

To explore the structure–activity relationships for several flavones' cytotoxicity, pectolinarin (100 mg) was converted into a corresponding *per*acetylated derivative with a mixture of Ac_2O (3 mL) and pyridine (2 mL) at reflux overnight. The solid was washed with water and dried. The product was recrystallized from aqueous ethanol to give 103 mg of colorless crystals (119–121 °C).[19]



Compound	R_1	R_2	R_3
Pectolinarin	H	H	H
Linariin	H	H	Ac
Isolinariin A	Ac	H	H
Isolinariin B	H	Ac	H

Figure 1. Flavones isolated from *L. reflexa*.

The hydrolysis of the rutinose unit of pectolinarin (100 mg; 0.161 mmol) was performed with a solution of H_2SO_4 0.04 N in ethanol 50% overnight at reflux. The reaction mixture was then neutralized with a solution of NaOH and extracted with ethyl acetate. This extract was subjected to column chromatography over silica gel 20–45 μm (CH_2Cl_2/MeOH 85:15), to afford pectolinarigenin (5,7-dihydroxy-6,4′-dimethoxyflavone) (42 mg, 0.134 mmol; 83%).[20,21]

A solution of pectolinarin (150 mg; 0.241 mmol), tartrate (2 g) in water (15 mL) was set at pH 4 by the addition of 0.5 N aqueous HCl and heated at 40 °C. Naringinase (EC 3.2.1.21) (74 mg; Sigma N 1385) was added the reaction mixture was stirred at 40 °C for 2 h. The cloudy mixture was diluted with DMF (5 mL), neutralized with 10% aqueous $NaHCO_3$, and extracted with *n*-butanol. After evaporation of the organic layer, the dried residue was crystallized from MeOH to yield pure pectolinarigenin-7-*O*-β-glucoside (100.9 mg, 0.205 mmol; 85%; 266–268 °C).[22,23]

The protein-staining sulforhodamine B (SRB) assay, developed by the National Cancer Institute for in vitro anticancer screening,[24,25] was used in this study to estimate cell number indirectly by providing a sensitive index of total cellular protein content that is linear to cell density.

Five cancer cell lines, large cell carcinoma COR-L23 (ECACC No.: 92031919), colorectal adenocarcinoma Caco-2 (ATCC No.: HTB-37), amelanotic melanoma C32 (ATCC No.: CRL-1585), hepatocellular carcinoma HepG-2 (ECACC No.: 85011430), renal cell adenocarcinoma ACHN (ATCC No.: CRL-1611), and one normal cell line human fetal lung MRC-5 (ATCC No.: CCL-171), were used in this experiment. The COR-L23, C32, and ACHN cells were cultured in RPMI 1640 medium, while MRC-5, Caco-2, and HepG-2 cells were cultured in DMEM. Both media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. The cell lines were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. The optimal plating density of each cell line was determined over a concentration range of 2×10^4–5×10^4 to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 540 nm and cell number where analyzed by the SRB assay. For the assay, cells were detached with 0.1% trypsin–EDTA to make a single-cell suspension, and viable cells were counted by trypan blue exclusion in a hemocytometer and diluted with medium to give a final concentration of 2–5×10^4 cells/well.

One hundred microliters per well of this cell suspension was seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h, the cells were treated with serial dilutions of pure compounds. Each compound was initially dissolved in an amount of DMSO and diluted further in medium to produce six concentrations. One hundred microliters per well of each concentration was added to the plates in six replicates to obtain final concentrations of 0.25, 0.5, 1, 2.5, 5, 12.5, 25, and

Table 1. Cytotoxic profile of compounds isolated from *L. reflexa* and pectolinarin derivatives against selected human cancer cell lines

Compound	IC_{50} (μM)[a]					
	MRC5	HepG2	Caco-2	COR-L23	ACHN	C32
Pectolinarin	>80.3	14.12 (±3.3)	6.18 (±2.2)	5.03 (±2.3)	17.22 (±3.1)	7.17 (±3.2)
Linariin	>75.3	23.21 (±2.5)	8.01 (±2.7)	5.17 (±2.8)	11.25 (±2.2)	12.60 (±3.3)
Isolinariin A	>75.3	16.36 (±2.8)	8.54 (±3.2)	6.68 (±2.6)	13.95 (±1.3)	11.76 (±2.1)
Isolinariin B	>75.3	10.93 (±1.9)	13.97 (±3.3)	10.37 (±3.3)	16.90 (±3.7)	21.47 (±2.4)
*Per*acetylpectolinarin	>54.5	30.62 (±3.2)	23.41 (±1.8)	28.42 (±3.1)	25.23 (±2.2)	15.56 (±2.1)
Pectolinarigenin	>159.2	NT	NT	4.07 (±2.4)	15.23 (±1.8)	7.02 (±4.1)
Pectolinarigenin-7-*O*-β-glc	>101.6	17.4 (±1.8)	12.7 (±2.8)	6.9 (±2.9)	16.54 (±2.9)	8.9 (±3.8)
Antirrhide	>151.5	>151.5	>151.5	>151.5	>151.5	>151.5
Antirrhinoside	>138.1	>138.1	>138.1	>138.1	>138.1	>138.1

NT: not tested. Vinblastine sulfate salt was used as positive control for MRC5, HepG2, Caco2, COR-L23, and C32 cell lines, while taxol was used for ACHN cell line.

[a] IC_{50} values are means of three experiments, standard deviation is given in parentheses.

50 μg/mL. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of the solvent (DMSO), the same as in the solvent-control wells. The final volume in each well was 200 μL. The plates were incubated for a select exposure time of 48 h. At the end of exposure time, 100 μL of ice-cold 40% trichloroacetic acid (TCA) was added to each well, left at 4 °C for 1 h, and washed five times with distilled water. The TCA-fixed cells were stained for 30 min with 50 μL of 0.4% (w/v) SRB in 1% HOAc. The plates were washed five times with 1% HOAc and air-dried overnight. Vinblastine sulfate salt was used as positive control for MRC5, HepG2, Caco-2, COR-L23, and C32, while taxol was used for ACHN cell line.

On the day of reading the plates, bound dye was solubilized with 100 μL of 10 mM TRIS base (Tris[hydroxymethyl]aminomethane). The absorbance of each well was read on an ELISA reader at 564 nm. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). All values are expressed as means ± standard deviation of the mean (SD). All products are purchased from Sigma, Italy. The inhibitory concentration 50% (IC_{50}) was calculated from a dose–response curve obtained by plotting the percentage of inhibition versus the concentrations with the use of GraphPad Prism 4 software.

Cytotoxicity is commonly used as a target for discovery of novel anticancer compounds and the Sulforhodamine B (SRB) assay, used in this study, is commonly employed. The isolated compounds from *L. reflexa* and their derivatives were screened for cytotoxicity against a panel of cultured cancer cell lines. The results are given in Table 1. All flavonoids showed interesting activity against Caco-2 and COR-L23.

Pectolinarigenin was previously shown to be active against human gastric adenocarcinoma MK-1, human uterus carcinoma HeLa, and murine melanoma B16F10 cells in vitro.[26] It was also active against GLC4, a human small cell lung carcinoma cell line, and against COLO 320, a human colorectal cancer cell line.[8] In our study, pectolinarigenin showed an IC_{50} of 7.02 and 4.07 μM against amelanotic melanoma and large cell carcinoma cell lines, respectively.

Interestingly, the corresponding pectolinarigenin glycosides, which had never been previously tested, also exhibited significant cytotoxic activities against the same cell lines. Indeed, the hydrosoluble flavonoid, pectolinarin, showed the highest cytotoxic activity on COR-L23, Caco-2, and C32 cell lines with IC_{50} of 5.03, 6.18, and 7.17 μM. Similar activities were recorded for the three natural monoacetyl pectolinarin derivatives linariin, isolinariin A, isolinariin B, and pectolinarigenin-7-*O*-β-glucoside. The less sensitive cell line to the *L. reflexa* flavones and their derivatives was the renal adenocarcinoma cell line. The acetylation of all hydroxyl groups decreased the cytotoxic activity against the cancer cell lines used in this study. The iridoids antirrhide and antirrhinoside were inactive.

The inhibitory activity of flavones of *L. reflexa* on the proliferation of cancer cell lines but not on the normal human fetal lung MRC-5 suggests a specific mechanism of action interfering with abnormal proliferation.

Acknowledgments

The authors thank Prof. G. Cesca of the Botany Department of University of Calabria (Italy) for supplying the herb samples used in this study.

References and notes

1. Cragg, G. M.; Newman, D. J. *Expert Opin. Investig. Drugs* **2000**, *9*, 2783.
2. *The Flavonoids. Advances in Research since 1986*; Harborne, J. B., Ed.; Chapman and Hall: London, 1999.
3. Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. *Pharmacol. Rev.* **2000**, *52*, 673.
4. Tahara, S.; Katagiri, Y.; Ingham, J.; Mizutami, J. *Phytochemistry* **1994**, *36*, 1261.
5. Amoros, M.; Simões, C. M. O.; Girre, L.; Sauvager, F.; Cormier, M. *J. Nat. Prod.* **1992**, *55*, 1732.
6. Beutler, J. A.; Cardellina, J. H., II; Lin, C. M.; Hamel, E.; Cragg, G. M.; Boyd, M. R. *Bioorg. Med. Chem. Lett.* **1993**, *03*, 581.
7. Kupchan, S. M.; Sigel, C. W.; Hemingway, R. J.; Knox, J. R.; Udayamurthy, M. S. *Tetrahedron* **1969**, *25*, 1603.
8. Woerdenbag, H. J.; Merfort, I.; Passreiter, C. M.; Schmidt, T. J.; Willuhn, G.; van Uden, W.; Pras, N.;

Kampinga, H. H.; Konings, A. W. T. *Planta Med.* **1994**, *60*, 434.
9. Pouget, C.; Lauthier, F.; Simon, A.; Fagnere, C.; Basly, J. P.; Delage, C.; Chulia, A. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3095.
10. Nagao, T.; Abf, F.; Kinjo, J.; Okabb, H. *Biol. Pharm. Bull.* **2002**, *25*, 875.
11. Pignatti, S., *Flora D'Italia*; Edagricole, Ed.; Bologna: Italy, 1982, pp 542–543.
12. Dobrescu, D.; Cristea, A.; Susanu, M. *Farmacia* **1985**, *33*, 215.
13. Kitagawa, I.; Tani, T.; Akita, K.; Yosioka, I. *Chem. Pharm. Bull.* **1973**, *21*, 1978.
14. Handjieva, N. V.; Ilieva, E. I.; Spassov, S. L.; Popov, S. S. *Tetrahedron* **1993**, *49*, 9261.
15. El-Naggar, L.; Beal, J. L. *J. Nat. Prod.* **1980**, *43*, 649.
16. Boros, C.; Stermitz, F. R. *J. Nat. Prod.* **1990**, *53*, 1056.
17. Lin, C. N.; Arisawa, M.; Shimizu, M.; Morita, N. *Chem. Pharm. Bull.* **1978**, *26*, 2036.
18. Otsuka, H. *J. Nat. Prod.* **1992**, *55*, 1252.
19. Markham, K. R.; Ternai, B.; Stanley, R.; Geiger, H.; Mabry, T. J. *Tetrahedron* **1978**, *34*, 1389.
20. Hase, T.; Ohtani, K.; Kasai, R.; Yamasaki, K.; Picheansoonthon, C. *Phytochemistry* **1995**, *40*, 287.
21. Markham, K. R.; Charj, V. M. In *The Flavonoids: Advances in Research*; Harborne, J. B., Ed.; Chapman and Hall: New York, 1982, pp 19–134.
22. Farag, S. F.; Backheet, E. Y.; El-Emary, N. A.; Niwa, M. *Phytochemistry* **1999**, *50*, 1407.
23. Mahato, S. B.; Sahu, N. P.; Roy, S. K.; Sharma, O. P. *Tetrahedron* **1994**, *50*, 9439.
24. Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, S.; Skehan, P.; Scudiero, D. A.; Boyd, M. R. *J. Nat. Cancer Inst.* **1990**, *82*, 1113.
25. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, I.; Vistica, D.; Waren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Nat. Cancer Inst.* **1990**, *82*, 1107.
26. Zahir, A.; Jossang, A.; Bodo, B.; Proost, J.; Cosson, J. P.; Sévenet, T. *J. Nat. Prod.* **1996**, *59*, 701.