Studies on Constituents with Cytotoxic and Cytostatic Activity of Two Turkish Medicinal Plants *Phlomis armeniaca* and *Scutellaria salviifolia*

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Ten known glycosidic compounds, betulalbuside A (1), 8-hydroxylinaloyl,3-*O*-β-D-glucopyranoside (2) (monoterpen glycosides), ipolamiide (3) (iridoid glycoside), acteoside (verbascoside) (4), leucosceptoside A (5), martynoside (6), forsythoside B (7), phlinoside B (8), phlinoside C (9), and teucrioside (10) (phenylpropanoid glycosides) were isolated from methanolic extracts of *Phlomis armeniaca* and *Scutellaria salviifolia* (Labiatae). Structure elucidations were carried out using ^{1}H-, ^{13}C-NMR and FAB-MS spectra, as well as chemical evidence. The cytotoxic and cytostatic activities of isolated compounds were investigated by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) method. Among the glycosides obtained here, caffeic acid-containing phenylpropanoid (or phenethyl alcohol, or phenylethanoid) glycosides were found to show activity against several kinds of cancer cells. However, they didn't affect the growth and viability of primary-cultured rat hepatocytes. Study of the structure–activity relationship indicated that ortho-dihydroxy aromatic systems of phenylpropanoid glycosides are necessary for their cytotoxic and cytostatic activities.

Key words *Phlomis armeniaca*; *Scutellaria salviifolia*; Labiatae; phenylpropanoid glycoside; cytotoxic activity; cytostatic activity

Labiatae family plants have been used widely as folk medicine in Turkey. Infusions of most of the species are used as the following: stimulant, tonic, antidiarrheaic, hemostatic, for healing and in gastric disorders.[1] In our systematic phytochemical investigations on plants of the family Labiatae, we have previously studied the phenylpropanoid and iridoid glycosides of *Phlomis linearis*,[2-4] *Scutellaria albida* subsp. *colchica*,[5,6] and *Scutellaria orientalis* subsp. *pinnatifida*.[7] As part of the continuing studies on the secondary metabolites (especially phenylpropanoid and iridoid glycosides) of the genus *Phlomis* and *Scutellaria*, in this paper we reported the constituents of *Phlomis armeniaca* WILLD., and *Scutellaria salviifolia* BENTHAM possessing cytotoxic and cytostatic activities, and their structure–activity relationships against several cancer cells.

Phenylpropanoid compounds are glycosides of phenylethyl alcohol esterified by a cinnamic acid molecule (*e.g.*, caffeic, ferulic, or *p*-coumaric acid). Several phenylpropanoid glycosides were found to be active against bacteria and fungi.[8-10] Some of them showed enzyme and hormone inhibitory activity.[11] Especially, acteoside (verbascoside), hydroxyacteoside, suspensaside, forsythiaside, plantamoside, and hellicoside inhibited 5-lipoxygenase, which has an active role in allergic diseases and inflammation specifically, but not competitively.[12,13] Some phenylpropanoids also inhibited cyclic-AMP phosphodiesterase,[14] aldose reductase,[11] and protein kinase C (PKC).[15] PKC is involved in cell proliferation and differentiation and further in the consequent pathologies (tumor proliferation).[11] Some molecules which inhibit PKC activity also show antitumoral activity. The inhibitory effect of acteoside (verbascoside) against PKC was described recently by Herbert *at al.*,[15] who demonstrated that acteoside inhibits PKC by acting directly on the catalytic site of the enzyme.

Acteoside was also found to inhibit the proliferation of P-388 cells[16] and L-1210 cells,[15] which could be partially due to PKC inhibition.

The effects of phenylpropanoid glycosides on the immune system have also been studied, and several of these glycosides, including acteoside, were found to be active as immunosuppressors.[17]

All these results prompted us to examine the cytotoxic activities of isolated phenylpropanoid glycosides.

MATERIALS AND METHODS

Plant Materials *Phlomis armeniaca*, and *Scutellaria salviifolia* were collected from Corum, Hattusas, Turkey, in July 1991, and Ankara, Beynam Forest, Turkey, in July 1992, respectively. Voucher specimens are deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 91-028, 92-045).

General ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GSX-400 and/or JNM A500 FT-NMR. FAB-MS were recorded on a JEOL JMS-HX 110 mass spectrometer. The lobar column used was RQ-2/ODS-Q3 (Fuji gel) for reversed phase medium pressure liquid chromatography (MPLC). TLC was conducted on pre-coated silica gel plates (Merck 60F-254) with $CHCl_3$–MeOH–H_2O (61 : 32 : 7, and 80 : 20 : 2) as a developing solvent. Compounds were detected by UV fluorescence and/or spraying with 1% vanillin–H_2SO_4, followed by heating at 100 °C for 5 min. Column chromatography was carried out on silica gel (Merck, Kieselgel 60), polyamide C-200 (Wako), and Sephadex LH-20 (Pharmacia).

Cells dRLh-84 cell (rat hepatoma), HeLa cell (human epithelial carcinoma), P-388-D1 cell (mouse lymphoid neoplasma), and S-180 (sarcoma) were provided from Japan Cancer Research Resources Bank. Primary-cultured hepatocytes were isolated according to the method of Seglen.[18]

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Chemicals Fetal bovine serum, bovine serum, RPMI 1640, and MEM medium were provided from Irvine Scientific Co. (Santa Ana, CA, U.S.A.). William's E medium, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and antibiotics (penicillin streptomycin) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trypsin was from Merck Co. (Frankfurt, Germany).

Extraction and Isolation Air-dried aerial parts (355 g) of *Phlomis armeniaca* were extracted twice with MeOH at 50 °C. The combined extracts were evaporated under vacuum nearly to dryness. 250 ml H_2O was added and the water-insoluble materials removed by filtration. The filtrate was extracted with petroleum ether (× 4250 ml) and the petroleum ether phase was rejected. The aqueous phase was evaporated to dryness, dissolved in 50 ml water, applied to a polyamide column (200 g), and then eluted with H_2O, followed by increasing concentrations of MeOH to separate the non-phenolic and phenolic fractions. Finally, three main fractions (frs. A—C) were obtained. The fractions eluted with H_2O (fr. A) were chromatographed over silica gel by stepwise elution with $CHCl_3$–MeOH–H_2O (80 : 20 : 2→60 : 40 : 4) and four fractions were obtained (frs. A1—A4). Fr. A2 was subjected to reversed-phase column chromatography (RQ-2/ODS-Q3 Fuji gel, (360 × 24 mm)) eluted with 20% CH_3CN in H_2O to yield compounds **1** and **2** (monoterpene glycosides). From fr. A3, we obtained compound **3** (iridoid glycoside) in a pure form. The fraction eluted with 50% MeOH from a polyamide column (fr. B) was rich in phenylpropanoid glycosides and applied to silica gel column chromatography by stepwise elution with $CHCl_3$–MeOH–H_2O (80 : 20 : 2→60 : 40 : 4) to give fractions B1—B5. Fractions B1—B3 were separately applied to Sephadex LH-20 column chromatography (RQ-2/ODS-Q3 Fuji gel, (360 × 24 mm) and eluted with H_2O to give compounds **4**—**6** in pure form, respectively. Fractions. B4 and B5 were separately chromatographed by using a reversed-phase column, and were eluted with gradiently increasing MeOH in H_2O (30→50%). Compounds **7** and **8** were obtained individually, in pure form, as amorphous powders.

Air-dried aerial parts (360 g) of *Scutellaria salviifolia* were also extracted and purified by the same methods with *Phlomis armeniaca*. The fraction obtained from polyamide column chromatography by elution with 50% MeOH, which was rich in phenylpropanoid glycosides, was chromatographed over silica gel by stepwise elution with $CHCl_3$–MeOH–H_2O (80 : 20 : 2→60 : 40 : 4) to give crude phenylpropanoid mixtures (frs. B1—B5). Fractions B1—B3 were separately applied to Sephadex LH-20 column chromatography and eluted with H_2O to give compounds **4**—**6** in pure form, respectively. Fractions B4 and B5 were separately chromatographed over a reversed-phase column and eluted with gradiently increasing MeOH in H_2O (30→50%) to give compounds **9** and **10** individually, in pure form.

Structure elucidation of the isolated compounds was carried out by using ^{1}H-, ^{13}C-NMR (^{1}H,^{1}H- correlation spectroscopy (COSY), ^{1}H,^{13}C-COSY, heteronuclear multiple-bond correlation spectroscopy (HMBC)) and FAB-MS spectra, as well as chemical evidence. The structures were determined as betulalbuside A[19] (**1**), 8-hydroxylinaloyl-3-*O*-*β*-D-glucopyranoside[20] (**2**), ipolamiide[4] (**3**), acteoside(verbascoside)[21] (**4**), leucosceptoside A[21] (**5**), martynoside[21] (**6**), forsythoside B[22] (**7**), phlinoside B[1] (**8**), phlinoside C[1] (**9**), and teucrioside[23] (**10**).

Cytotoxicity Assay 0.1 ml of cell suspension at the concentration of 3×10^4—1.5×10^5 cells/ml were inoculated into a 96-multi-well plate and cultured for 24 h. After washing the cells with phosphate-buffered saline (PBS), 0.1 ml of medium, containing each of compounds (**1**—**10**) at appropriate concentrations, was added and then incubated for another 48 h. The culture medium was discarded and the remaining compounds were removed by a thorough washing with fresh medium. The surviving cell number was determined by the MTT method.[24]

RESULTS AND DISCUSSION

Separation of biologically active components from MeOH extracts of *Phlomis armeniaca* and *Scutellaria salviifolia* afforded ten glycosidic compounds (**1**—**10**). Their chemical structures are shown in Fig. 1.

We first defined cell-growth inhibitory activity as activity which suppressed the increase in cell number without causing cell death, and cytotoxic activity as activity which decreased cell number with cell death. These activities were determined by microscopic observation and the dye exclusion method. Then we examined the cytostatic and cytotoxic activities of all isolated compounds by the MTT method and found that only the phenylpropanoid glycosides showed such activities.

Acteoside and teucrioside isolated from *Teucrium polium* and *Teucrium chamaedrys* subsp. *syspirense*, were also used in a cytotoxic and cytostatic assay,[25] whereas the amount of phlinoside C was too small to test its activity.

The results of cytotoxic and cytostatic activity tests are shown in Figs. 2 and 3. IC_{50} (50% cytotoxic or cytostatic concentration) values were also given in Tables 1 and 2, respectively.

Acteoside, forsythoside B, phlinoside B, and teucrioside showed cytotoxic activity against dRLh-84 cells (Fig. 2A), while the methoxylated derivatives martynoside and leucosceptoside A didn't have any activity. The cytotoxic activity of teucrioside was strongest, with an IC_{50} of 37.5 μg/ml; acteoside followed it with an IC_{50} of 62 μg/ml, and phlinoside B and forsythoside B were less active than acteoside and teucrioside (Table 1).

The same glycosides showed cytostatic activity against HeLa cells at a low concentration (Fig. 2B). In this case, the IC_{50} values of acteoside, forsythoside B, phlinoside B, and teucrioside were around at 7—10 μg/ml (Table 2). However, teucrioside showed cytotoxic activity with an IC_{50} of 100 μg/ml against HeLa cells (Fig. 2B). The cytotoxicity of other glycosides was not significant at a concentration of 200 μg/ml, because 30—45% of the cells were still alive at this concentration (Fig. 2B). Methoxylated derivatives, martynoside, and leucosceptoside A didn't show any activity.

Acteoside, forsythoside B, and teucrioside were found to be toxic against S-180 cells (Fig. 2C). Acteoside showed

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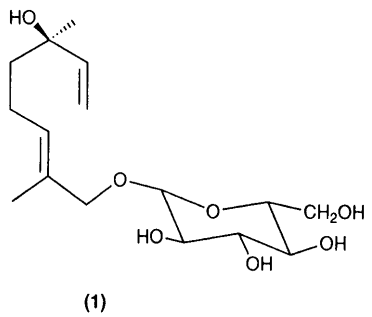
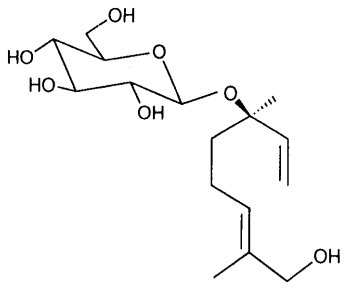
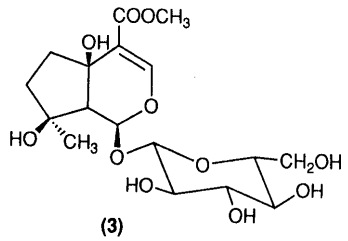


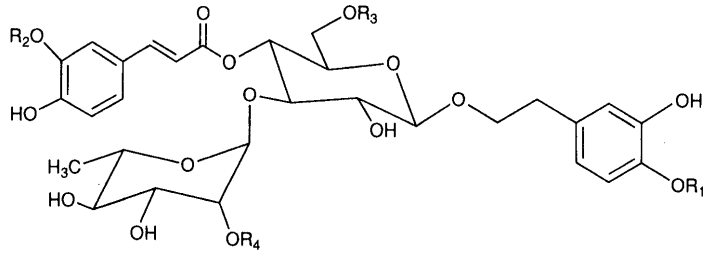
(1)
Betulalbuside A



(2)
8-Hydroxylinaloyl,3-O-β-D-Glucopyranoside



(3)
Ipolamiide



	R_1	R_2	R_3	R_4	Compound
(4)	H	H	H	H	Acteoside
(5)	H	CH_3	H	H	Leucosceptoside A
(6)	CH_3	CH_3	H	H	Martynoside
(7)	H	H	β-Apiose	H	Forsythoside B
(8)	H	H	H	β-Xylose	Phlinoside B
(9)	H	H	H	α-Rhamnose	Phlinoside C
(10)	H	H	H	α-Lyxose	Teucrioside

Fig. 1. Structures of Compounds Isolated and Examined in the Present Study

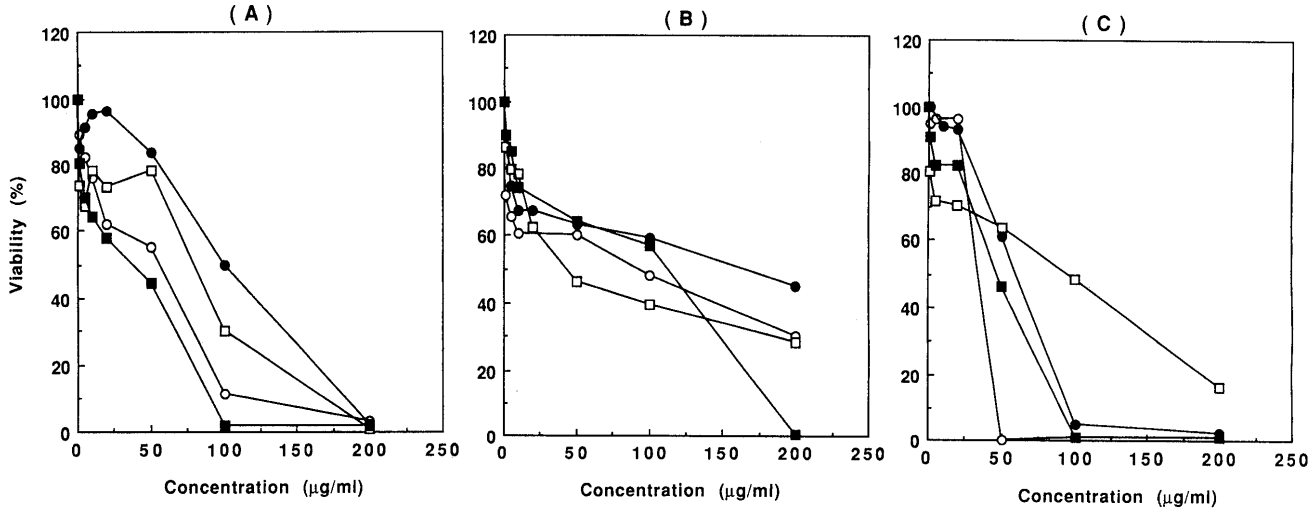


Fig. 2. Effects of Phenylpropanoid Glycosides against Cancer Cells

Cells were incubated with phenylpropanoid glycosides at various concentrations for 48 h. After removing compounds by washing them with fresh medium, the viable cell number was determined by the MTT method. Cell number was adjusted as indicated below in order to obtain the absorbance of around 0.7 at phenylpropanoid concentration (0 μg/ml) after 48 h incubation. (A), dRLh-84 (3×10^4 cells/ml); (B), HeLa (4×10^4 cells/ml); (C), S-180 (3×10^4 cells/ml). ○, acteoside; ●, forsythoside B; □, phlinoside B; ■, teucrioside. Values represent means of 6 wells.

the strongest activity with an IC_{50} of 29.6 μg/ml, and teucrioside and forsythoside B followed it (Table 1). On the other hand, phlinoside B showed cytostatic activity against S-180 cells with IC_{50} of 7.6 μg/ml (Fig. 2C, Table 2) while it showed cytotoxicity with an IC_{50} of 90 μg/ml (Fig. 2C, Table 1). Martynoside and leucosceptoside A didn't show any activity against S-180 cells.

The cytotoxic activities of tested compounds against P-388/D1 cells were not strong and significant (Table 1).

To determine whether phenylpropanoid glycosides show any cytotoxic activity against normal cells, the effects of the compounds against primary-cultured rat hepatocytes were also investigated. As shown in Fig. 3, phenylpropanoid glycosides didn't show any cytotoxic effects against

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Table 1. IC_{50} Values of Cytotoxic Activity Exhibited by Phenylpropanoids

Phenylpropanoids	dRLh-84		HeLa		S-180		P-388/D1		Hepatocyte
	μg/ml	M	μg/ml	M	μg/ml	M	μg/ml	M	μg/ml
Acteoside	61.98	9.93×10^{-5}	>200		29.6	4.70×10^{-5}	221.0	3.50×10^{-4}	Inactive
Leucosceptoside A	Inactive		Inactive		Inactive		Inactive		ND
Martynoside	Inactive		Inactive		Inactive		Inactive		ND
Forsythoside B	108.3	1.40×10^{-4}	>200		56.7	7.50×10^{-5}	121.8	1.60×10^{-4}	Inactive
Phlinoside B	74.2	9.80×10^{-5}	>200		90.4	1.20×10^{-4}	Inactive		Inactive
Teucrioside	37.5	4.96×10^{-5}	108.0	1.40×10^{-4}	47.8	6.30×10^{-5}	123.8	1.64×10^{-4}	Inactive

ND: Not determined.

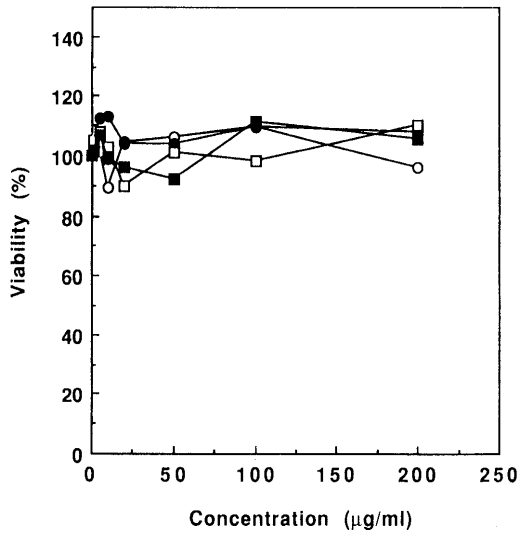

Fig. 3. Effects of Phenylpropanoid Glycosides on Primary-Cultured Rat Hepatocytes

Hepatocytes at the concentration of 1.5×10^5 cells/ml were incubated with phenylpropanoid glycosides at various concentrations for 48 h. After removing the compounds by complete washing in fresh medium completely, the viable cell number was determined by the MTT method. ○, acteoside; ●, forsythoside B; □, phlinoside B; ■, teucrioside. Values represent means of 6 wells.

primary-cultured rat hepatocytes.

In conclusion, phenylpropanoid glycosides are considered to show a biphasic effect on cancer cells, that is, both cytostatic and cytotoxic activities. Furthermore, these effects depend on the types of cells . In the case of HeLa cells, cytostatic activity at a low concentration could be interpreted as the cytotoxic activity not being so strong. However, in the cases of dRLh-84 and S-180 cells, the cytostatic activity couldn't be discriminated from cytotoxic activity, because the cytotoxic activity was strong.

The cytotoxic and cytostatic activities of phenylpropanoid glycosides were found to be mainly dependent on the ortho-dihydroxy aromatic (phenolic) systems in their structures, because methylation of at least one of the phenolic hydroxy groups abolished the activity, as evidenced by the observation that methoxylated derivatives martynoside and leucosceptoside A completely lost their activities.

On the other hand, the results further indicated that glycosylation, depending on the type of sugars and location within the molecule, was also important to the cytotoxic and cytostatic activities of phenylpropanoid glycosides.

When we compared the activity of the disaccharide ester, acteoside, with trisaccharide esters, we found different

Table 2. IC_{50} Values of Cytostatic Activity Exhibited by Phenylpropanoids

Phenylpropanoids	HeLa		S-180	
	μg/ml	M	μg/ml	M
Acteoside	7.8	1.25×10^{-5}	ND	
Forsythoside B	10.5	1.39×10^{-5}	ND	
Phlinoside B	7.7	1.00×10^{-5}	7.6	1.00×10^{-5}
Teucrioside	8.4	1.10×10^{-5}	ND	

ND: Not detected.

effects depending on the cells. Acteoside showed the strongest cytotoxic activity against S-180 cells and trisaccharide esters teucrioside and forsythoside B followed it at almost the same IC_{50} values (Fig. 2C, Table 1). However, the activity of phlinoside B, which contains a xylose molecule as a third sugar at rhamnose C-3,was lower than the others and it had cytostatic activity, too (Fig. 2C, Tables 1, 2). In the case of dRLh-84 cells, trisaccharide ester teucrioside, which contains a lyxose molecule as a third sugar at rhamnose C-3, showed the strongest activity. The activity of phlinoside B was lower. Forsythoside B, which contains an apiose molecule as a third sugar at central glucose C-6, was the least active compound of them all (Fig. 2A, Table 1). Against HeLa cells, the IC_{50} values of disaccharide and trisaccharide esters were found to be similar (Fig. 2B, Table 2), but teucrioside showed cytotoxicity at higher concentrations (Fig. 2B, Table 1).

As a result, the cytotoxic and cell-growth inhibitory activities of tested phenylpropanoid glycosides were found to be mainly dependent on their phenolic structures, the number of free phenolic hydroxy groups, and different sugars, depending on the type and location within the molecule. We have not yet determined whether the cytotoxic activity of phenylpropanoid glycosides shown in this experiment is due to anti-oxidative activity, pro-oxidant activity, or other characteristics of phenylpropanoid glycosides.[11] The mechanism by which phenylpropanoid glycosides show activity is under investigation.

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