Screening for Free Radical Scavenging and Cell Aggregation Inhibitory Activities by Secondary Metabolites from Turkish *Verbascum* species

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Free radical scavenging and cell aggregation inhibitory activities of 36 secondary metabolites isolated from the methanolic extracts of *Verbascum cilicicum* Boiss., *V. lasianthum* Boiss. ex Bentham, *V. pterocalycinum* var. *mutense* Hub.-Mor., and *V. salviifolium* Boiss. (Scrophulariaceae) were investigated. The isolated compounds, 6-*O*-vaniloyl ajugol (**1**), ilwensisaponin A (**2**), ilwensisaponin C (**3**), verbascoside (**4**), β-hydroxyacteoside (**5**), martynoside (**6**), poliumoside (**7**), forsythoside B (**8**), angoroside A (**9**), dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**10**), dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**11**), apigenin 7-*O*-β-glucopyranoside (**12**), luteolin 7-*O*-β-glucopyranoside (**13**), luteolin 3′-*O*-β-glucopyranoside (**14**) and chrysoeriol 7-*O*-β-glucopyranoside (**15**), exhibited a dose-dependent inhibition of bioautographic and spectrophotometric DPPH activities. Verbascoside (**4**) was the most active (IC_{50} 4.0 μg/ml) comparing it to vitamin C (IC_{50} 4.4 μg/ml) to inhibit phorbol 12-myristate 13-acetate (PMA)-induced peroxide-catalyzed oxidation of 2′,7′-dichlorofluorescein (DCFH) by reactive oxygen species (ROS) within human promyelocytic HL-60 cells. Ilwensisaponin A (**2**) (MIC 6.9 μg/ml) showed moderate *in vitro* activity on lymphocyte-associated antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1)-mediated aggregation using the HL-60 cell line [positive control was cytochalasin B (MIC 2.3 μg/ml)]. None of the other compounds showed free radical scavenging and cell aggregation inhibitory activities.

Key words: Verbascum sp., Free Radical Scavenging Activity, Cell Aggregation Inhibitory Activity

Introduction

Verbascum species (Scrophulariaceae), commonly known as "mullein", are reported to have expectorant, mucolytic and demulcent properties and are used to treat respiratory problems such as bronchitis, dry coughs, tuberculosis and asthma in traditional Turkish medicine. The species are also used to treat haemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhoea, and have inhibitory activities against the murine lymphocytic leukaemia and influenza viruses A2 and B (Baytop, 1999; Turker and Camper, 2002).

Our previous studies have resulted in the isolation of 1 monoterpene and 21 iridoid glycosides, 2 oleanane-type triterpenoid saponins, 6 phenylethanoid, 2 neolignan and 4 flavonoid glycosides from *V. cilicicum* Boiss. (Tatli *et al.*, 2003), *V. lasianthum* Boiss. ex Bentham (Akdemir *et al.*, 2004a, b), *V. pterocalycinum* var. *mutense* Hub.-Mor. (Tatli *et al.*, 2004), and *V. salviifolium* Boiss. (Akdemir *et al.*, 2003, 2004c, 2005).

Reactive oxygen species (ROS) have been implicated in lipid peroxidation, inflammation, heart diseases, atherosclerosis, cataract, cognitive dysfunction, AIDS, cancer and aging. This has been of world-wide interest in endogenous and exogenous antioxidants. Plants have proven to be rich sources of radical scavengers. Therefore, it is important to look for effective radical scavengers by using various screening methods (Takamatsu *et al.*, 2003a, b). Cell adhesion process also plays roles in pathological conditions such as chronic inflammation and cancer metastasis. Cell adhesion molecules (CAMs) are important in the regulation of the immune response and inflammation (Takamatsu *et al.*, 2004).

In these contexts, natural compounds are receiving increasing attention. As a part of our ongoing research on *Verbascum* species growing in Turkey,

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we here report the possible biological activities of secondary metabolites from four Turkish *Verbascum* species.

Material and Methods

Plant material

Verbascum cilicicum Boiss. (Scrophulariaceae) was collected from Adana, between Pozantı and Ulukışla, Alihoca village, in July 2000. *V. lasianthum* Boiss. ex Bentham was collected in Urla, in August 1999. *V. pterocalycinum* var. *mutense* Hub.-Mor. was collected from İçel, between Mut and Karaman, 930–1100 m, in July 2000. *V. salviifolium* Boiss. was collected from Burdur, Yesilova, Southwest of Burdur Lake, 880 m, in June 2002 (Huber-Morath, 1978). Voucher specimens were deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (H. Duman and I. I. Tatli HUEF 00183, Z. S. Akdemir HUEF 99130, H. Duman and I. I. Tatli HUEF 00184, I. I. Tatli HUEF 02003, respectively).

Extraction and isolation

The extraction procedures of *V. cilicicum*, *V. lasianthum*, *V. pterocalycinum* var. *mutense* and *V. salviifolium*, the isolation and the structure elucidation of active compounds were given in detail in our previous papers (Akdemir *et al*., 2003, 2004a, b, c; Tatli *et al*., 2004).

Pharmacological studies

Reduction of DPPH radical by bioautographic assay

Methanolic solutions (0.1%) of compounds **1**–**36** were chromatographed on a silica gel TLC plate using $CHCl_3/CH_3OH/H_2O$ (61:32:7). After drying, TLC plates were sprayed with a 0.2% DPPH (1,1-diphenyl-2-picrylhydrazyl, Sigma) solution in CH_3OH. Compounds **1**–**15**, showing a yellow-on-purple spot, were regarded as antioxidants (Takao *et al*., 1994).

Reduction of DPPH radical by spectrophotometric assay

The radical scavenging activity of the potent isolated compounds **1**–**15** was examined with the DPPH radical by a spectrophotometric method. (±)-α-Tocopherol (vitamin E, Sigma), ascorbic acid (vitamin C, Sigma) and 3-*t*-butyl-4-hydroxyanisole (3-BHA, Sigma) were used as control. CH_3OH solutions of compounds **1**–**15** at various concentrations (10, 25, 50, 100, 200 μM) were added to 1.5×10^{-5} M DPPH in CH_3OH. The absorbance of the remaining DPPH was measured in 1-cm cuvettes with a Shimadzu UV-160A spectrophotometer at 520 nm, after 30 min incubation at room temperature. Decreasing of the DPPH solution absorbance indicated an increase of the DPPH radical scavenging activity. This activity is given as % DPPH radical scavenging that is calculated using the equation: [(control absorbance – sample absorbance)/control absorbance] $\times$ 100. A DPPH solution without sample solution was used as control (Hatano *et al*., 1989).

Microplate assay for the detection of oxidative products with DCFH-DA

This method is based on a fluorimetric assay described by Rosenkranz *et al.* (1992). Myelomonocytic HL-60 cells (1×10^6 cells/ml, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C in 5% CO_2/95% air. 125 μl of the cell suspension were added to a well of a 96-well plate. After treatment with a different concentration of the test materials for 30 min, cells were stimulated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Furthermore, cells were incubated for 15 min after the addition of 5 μg/ml 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyze DCFH-DA to DCFH. Reactive oxygen species generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA-treated control incubations with and without the test materials. Levels of DCF were measured using a CytoFluor 2350 fluorescense measurement system (Millipore) with excitation at 485 nm (bandwidth 20 nm) and emission at 530 nm (bandwidth 25 nm) (Rosenkranz *et al*., 1992).

Cell aggregation assay

HL-60 cells were suspended at a density of 1×10^6 cells/ml. 150 μl of the cell suspension were added to a well of a 96-well plate. After incubation with sample for 10 min, PMA (10 ng/ml, final concentration) was added. The mixture was placed in a CO_2 incubator and aggregation of the cells was observed 16 h after the PMA addition. The cell aggregation inhibitor cytochalasin B, which is an

exoenzyme from *Clostridium botulinum*, anti-LFA-1 (lymphocyte-associated antigen-1), and anti-ICAM-1 (intercellular adhesion molecule-1) monoclonal antibodies (Santa Cruz Biotech, Inc., Santa Cruz, CA) were used as positive controls (Katagiri *et al*., 1999).

XTT assay for cytotoxicity

After sample exposure on cells for 18 h, XTT assay was performed using the methods described by Scudiero *et al.* (1988). Briefly, 25 μl of XTT-PMS solution (1 mg/ml XTT solution supplement by 25 μM of PMS) were added to HL-60 cells (2×10^4 cells in 225 μl medium) in each well on the microplates. After incubating for 4 h at 37 °C, absorbance at 450 nm was measured by a microplate reader (reference absorbance at 650 nm) (Musza *et al*., 1994; Scudiero *et al*., 1988).

Results and Discussion

In a primary screening, 36 pure secondary metabolites from the *Verbascum* species examined and 15 compounds (**1**–**15**) showed antioxidant activities in TLC autographic assays with the DPPH radical. In the spectrophotometric assay, antioxidants **1**–**15** reacted with the stable free radical DPPH, resulting in the production of colourless 1,1-diphenyl-2-picrylhydrazyl, and showed a dose-dependent reduction of DPPH, corresponding to the intensity of quenching of the DPPH radical (Hatano *et al*., 1989). The results are given in Table I.

So far many *in vitro* assay systems for the evaluation of antioxidants were reported, but it is important how to feedback the results of an *in vitro* assay into an evaluation *in vivo*. Fluorescent technology made it possible to evaluate antioxidants in live cells using specific probes such as DCFH-DA. A cell-based method using a fluorescent technology is useful to directly examine the ability of natural products to penetrate cell membranes and inhibit ROS-catalyzed oxidation in living human cells (Bass *et al*., 1983). Therefore, the pure natural products **1**–**15** were also examined in the DCFH-DA cellular-based assay. The inhibition effects of the compounds on ROS-catalyzed oxidation of DCFH in HL-60 cells are shown in Table II.

Among the compounds **1**–**15**, verbascoside (**4**) showed a potent antioxidant effect (IC_{50} 4.0 μg/ml). On the other hand, ilwensisaponin A (**2**; IC_{50}

Table I. Free radical scavenging effects of natural compounds **1**–**15** on DPPH radical.

Compound	Source	10 μM[a]	25 μM	50 μM	100 μM	200 μM
6-*O*-Vaniloyl ajugol (**1**)	*Verbascum lasianthum*	35.4[b]	46.0	67.4	58.9	71.8
Ilwensisaponin A (**2**)	*Verbascum pterocalycinum* var. *mutense*	39.8	64.8	29.9	77.7	77.5
Ilwensisaponin C (**3**)	*Verbascum pterocalycinum* var. *mutense*	35.4	29.9	25.8	15.5	17.1
Verbascoside (**4**)	*Verbascum lasianthum* *Verbascum pterocalycinum* var. *mutense* *Verbascum salviifolium*	IA[c]	23.1	41.0	89.9	73.9
β-Hydroxyacteoside (**5**)	*Verbascum salviifolium*	IA	11.8	51.7	46.8	65.0
Martynoside (**6**)	*Verbascum salviifolium*	10.8	9.2	45.8	71.1	76.2
Poliumoside (**7**)	*Verbascum salviifolium*	13.9	32.8	46.3	68.8	73.7
Forsythoside B (**8**)	*Verbascum salviifolium*	IA	13.2	IA	52.1	81.4
Angoroside A (**9**)	*Verbascum salviifolium*	IA	27.7	28.7	46.2	38.8
Dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**10**)	*Verbascum salviifolium*	3.9	7.7	18.2	19.9	62.9
Dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**11**)	*Verbascum salviifolium*	3.9	7.7	18.2	19.9	62.9
Apigenin 7-*O*-β-glucopyranoside (**12**)	*Verbascum salviifolium*	78.4	80.4	85.3	95.0	91.7
Luteolin 7-*O*-β-glucopyranoside (**13**)	*Verbascum salviifolium*	94.4	88.6	69.4	89.3	75.0
Luteolin 3′-*O*-β-glucopyranoside (**14**)	*Verbascum salviifolium*	73.4	70.1	79.9	83.1	91.1
Chrysoeriol 7-*O*-β-glucopyranoside (**15**)	*Verbascum salviifolium*	62.7	68.0	72.0	78.3	90.0
3-BHA[d]		67.3	51.8	56.8	69.3	83.6
Vitamin C[d]		87.0	79.8	85.7	94.4	IA
Vitamin E[d]		81.0	89.9	81.9	93.7	98.3

[a] Values are means of three independent determinations. [b] % DPPH radical scavenging. [c] IA, inactive. [d] Used as a standard.

Table II. Effects of natural products **1**–**15** on reactive oxygen species and HL-60 cell- aggregation and proliferation.

Compound	Antioxidant (DCFH) IC_{50} [μg/ml][a]	A. Cell-aggregation MIC[b] [μg/ml]	B. Cell-proliferation-XTT IC_{50} [μg/ml][a]	Specific index (B/A)
6-*O*-Vaniloyl ajugol (**1**)	40.0 ± 1.2	IA	IA	–
Ilwensisaponin A (**2**)	8.5 ± 0.6	6.9	4.0 ± 0.7	0.58
Ilwensisaponin C (**3**)	45.0 ± 1.3	62.5	33.0 ± 1.1	0.53
Verbascoside (**4**)	4.0 ± 0.4	62.5	> 62.5	1
β-Hydroxyacteoside (**5**)	44.4 ± 1.5	IA	IA	–
Martynoside (**6**)	30.0 ± 2.2	IA	IA	–
Poliumoside (**7**)	20.0 ± 1.0	IA	IA	–
Forsythoside B (**8**)	16.4 ± 0.9	IA	IA	–
Angoroside A (**9**)	17.3 ± 0.7	IA	IA	–
Dehydrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**10**)	IA[c]	IA	IA	–
Dehydrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**11**)	IA	IA	IA	–
Apigenin 7-*O*-β-glucopyranoside (**12**)	41.8 ± 1.7	IA	IA	–
Luteolin 7-*O*-β-glucopyranoside (**13**)	17.0 ± 1.1	IA	IA	–
Luteolin 3′-*O*-β-glucopyranoside (**14**)	22.0 ± 0.7	IA	IA	–
Chrysoeriol 7-*O*-β-glucopyranoside (**15**)	34.2 ± 1.3	IA	IA	–
Vitamin C[d]	4.4 ± 0.8			
Cytochalasin B[d]		2.3	43.0 ± 2.2	18.7

[a] Values are means of three independent determinations ± SE. [b] MIC, minimum inhibitory concentration. [c] IA, inactive. [d] Used as a standard.

8.5 μg/ml) showed a moderate antioxidant activity, while the other compounds displayed weak activity, except for compounds **10** and **11** which were inactive.

In order to determine the effects of isolated compounds on immunological and inflammatory reactions, they were also evaluated for their *in vitro* effects on LFA-1/ICAM-1-mediated cell aggregation using the HL-60 cell line. In a primary screening the cell aggregation and XTT method were reasonable assays for the selection of cell adhesion inhibitors (Takamatsu *et al*., 2004). Therefore, the isolated compounds **1**–**15** were evaluated as having inhibitory activity for LFA-1/ICAM-1-mediated cell aggregation of HL-60 cells (Table II). Ilwensisaponin A (**2**) inhibited the cell aggregation (MIC 6.9 μg/ml) as compared to cytochalasin B (MIC 2.3 μg/ml). Ilwensisaponin C (**3**; MIC 62.5 μg/ml) and verbascoside (**4**; MIC 62.5 μg/ml) were weakly active in the primary cell aggregation assay, while ilwensisaponin A (**2**) was >10-fold more cytotoxic (IC_{50} 4.0 μg/ml) than cytochalasin B (IC_{50} 43.0 μg/ml) as determined by the XTT assay. Furthermore, none of the other compounds were active in the cell aggregation assay.

Conclusion

Several phenolic and terpenic compounds have been reported to be good antioxidants in some *in vitro* antioxidative assay models (Chander *et al*., 1992; Harborne, 1994; Hostettmann and Marston, 1995; Jimenez and Riguera, 1994; Zhao *et al*., 2005). However, there is no report concerning the ROS-catalyzed oxidation of isolated compounds in living human cells.

According to the structure-activity relationship, martynoside (**6**), forsythoside B (**8**), angoroside A (**9**) and poliumoside (**7**) showed weak activity as compared to verbascoside (**4**). Thus, antioxidative activity might be mainly related to the number of aromatic hydroxy groups, the structure of the acyl moiety and the number of sugar moieties. However, modification of the sugar chain or replacement of hydroxy groups by methoxy groups in the structure might of minor importance under this experiment condition. Besides He *et al*. (2000) found that poliumoside (**7**) had shown significant inhibitory effects on free radical-induced hemolysis of red blood cells and free radical scavenging activities *in vitro* due to the presence of the rhamnosyl at C-6″. However, poliumoside (**7**) has a weak activity to penetrate cell membranes and inhibit ROS-catalyzed oxidation in living human cells in the present study (He *et al*., 2000).

The antioxidant activities of flavonoids decreased in the following order: luteolin 7-*O*-*β*-glucopyranoside (**13**) > luteolin 3′-*O*-*β*-glucopyranoside (**14**) > chrysoeriol 7-*O*-*β*-glucopyranoside (**15**) > apigenin 7-*O*-*β*-glucopyranoside (**12**). The C2–C3 double bond of the C ring appears to increase the scavenger activity because it confers stability to the phenoxy radicals produced, while the 4-oxo group increases the free radical scavenger activity by delocalizing electrons from the B ring. The presence of both 7- and 5- and additionally 3-hydroxy groups in rings A and C is required for maximum radical scavenging potential. Besides, some of the activities such as antitumour and cytotoxic effects of phenolic compounds are mainly dependent on the *ortho*-dihydroxy aromatic systems in their structures. Methylations of at least one of the *ortho*-dihydroxy groups abolishes the activity (Takamatsu *et al*., 2003a). In the present study, the finding is that luteolin 7-*O*-*β*-glucopyranoside (**13**) with *ortho*-dihydroxy groups elicited stronger activity than chrysoeriol 7-*O*-*β*-glucopyranoside (**15**) and apigenin 7-*O*-*β*-glucopyranoside (**12**). Moreover, in the same manner, it was also found to be more active than luteolin 3′-*O*-*β*-glucopyranoside (**14**) due to the presence of a glucopyranosyl unit at C-3′ position.

Additionally, it was displayed that vaniloyl ajugol showed moderate activity, because of its phenolic system, although few reports appeared in the literature on the antioxidant activity of iridoid glycosides (Chander *et al*., 1992).

The antioxidant activity of triterpenic saponins was found to be mainly dependent on their olefinic structures and the number of sugars, depending on the type, their linkage to each other, and location within the molecule. In addition monodesmosidic saponins in which the sugar units are attached to the aglycone at the C-3 position are more potent in this antioxidant system. On the other hand, methylation of at least one of the hydroxy groups may abolish the activity as evidenced by the observation that methoxylated derivatives like ilwensisaponin C may lost their activity (Hostettmann and Marston, 1995).

Potent antioxidants were identified by using the solution-based chemical assay along with the cellular-based assay, and we were able to compare the results in both systems (Tables I, II). Most of the compounds, except for compounds **10** and **11**, not only act as antioxidants in solution-based antioxidant assays but can also be taken up by living cells maintaining their activity. Compounds **10** and **11** were shown to be active in the solution-based chemical assay, but had no significant activity inside cells. This suggests that these compounds do not enter the cells due to poor cellular uptake or reduced medium solubility, or perhaps lack the capacity to quench DCF fluorescence inside the cell (Takamatsu *et al*., 2003b).

ROS including hydroperoxide and lipid peroxides are thought not only to injure cells but also to induce the expression of intercellular adhesion molecule-1. Therefore, the antioxidant effects produced by compounds **1**–**9**, **12**–**15** could partially contribute to the cell aggregation inhibitory effect. As results of our screening programme, ilwensisaponin A (**2**), ilwensisaponin C (**3**) and verbascoside (**4**) inhibited cell aggregation, while none of the other compounds were specific inhibitors of LFA-1/ICAM-1-mediated cell aggregation. But the results were reproducible and the dose-response behaviour is characteristic for the cell aggregation assay (Takamatsu *et al*., 2004).

In conclusion, the structure-activity relationship studies of oleanan-type triterpenoid saponins suggests that the magnitude of the cell aggregation activity has often been linked with anti-inflammatory and immunostimulant activities of the saponins. The availability of a longer sugar chain and the number of hydroxy groups lead to an increase of activity. Monodesmosidic saponins are more active than the bidesmosides in killing cancer cells (Hostettmann and Marston, 1995). On the other hand, phenylethanoid glycosides, especially verbascoside (**4**), are considered to show a biphasic effect on cancer cells, that is cytotoxic activity. Furthermore, this effect depends on the type of cells. In the case of HeLa cells, the cytotoxic activity at low concentration could not be so strong (Inoue *et al*., 1998).

Finally, it is intriguing to note that the cytotoxic and antioxidant activity of Turkish *Verbascum* species is contributed to, mainly, saponins **2**, **3** and the phenylethanoid glycoside **4**. In order to correlate the obtained data with other effects in the field of cytotoxicity and radical scavenging activity of effective compounds, further examinations in different cellular and enzymatic assays should be evaluated. Examination of *Verbascum* species with regard to their biological activity and chemical contents support the traditional use of *Verbascum* species and may give a new insight into the usage of *Verbascum* in Turkey.

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