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Effects of the Flavonoid Pilloin Isolated from *Marrubium cylleneum* on Mitogen-Induced Lymphocyte Transformation

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Abstract

Flavonoids are known to exhibit a wide range of biological functions. In the present study, the possible immunomodulatory and cytotoxic effects of pilloin, a flavone extracted from the Greek endemic herb *Marrubium cylleneum* were investigated by using the *in vitro* lymphocyte transformation and the cytotoxicity assays, respectively. Apigenin was used as the control flavonoid. Pilloin exerted a cytotoxic action targeted at the transformed lymphoblasts. On the other hand, the glycosylated flavonoid chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside was inactive, providing further evidence that glycosylation may eliminate the effects of aglycones.

Keywords: Lymphocyte, *Marrubium cylleneum*, Lamiaceae, flavonoids, cytotoxicity.

Introduction

Flavonoids, a widely distributed category of plant metabolites, are well known for the diversity of their biological effects (Middleton, 1998; Ielpo et al., 2000). Numerous studies have indicated the effects of flavonoids on T and B lymphocyte function and proliferation (Namgoong et al., 1993; You et al., 1998; Bouic & Lamprecht, 1999). In this study, we examined the *in vitro* effects of the flavone pilloin (luteolin-7,4′-dimethylether) and two other flavonoids, apigenin and chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside, extracted from *Marrubium cylleneum* Boiss. & Heldr., an endemic herb of Southern Greece with ovate to sub-orbicular leaves and yellowish corolla belonging to the Lamiaceae family (Baden, 1991), on mitogen-induced lymphocyte transformation, and conducted a cytotoxicity test using the Trypan blue exclusion method.

Materials and methods

The plant was collected from the mountain Menalon (Peloponnisos), in May 1997. A voucher specimen has been kept in the Herbarium of Patras University, Greece, under the number Skaltsa & Lazari 111. Shade-dried aerial parts of the plant (0.7 kg) were finely ground and extracted at room temperature with dichloromethane and methanol, successively. The extracts were evaporated under reduced pressure to obtain a viscous mass. The methanol residue (3.5 g) was subjected to vacuum liquid chromatography (VLC), performed over silica gel (Merck, Art.7736 – 11 fractions of 400 ml), using CH_2Cl_2-MeOH mixtures of increasing polarity as eluents. Salvigenin, pectolinaringenin, luteolin-7,4′-dimethylether (pilloin) and 6-hydroxyluteolin,7-methylether were obtained from the fraction (226.9 mg) eluted from VLC with CH_2Cl_2-MeOH 85 : 15 by further purification on column chromatography (CC) over Sephadex LH-20, using methanol as eluent and thin-layer chromatography (TLC) (Merck, Kieselgel plates; Art. 5721 with CH_2Cl_2-MeOH-HCOOH 9 : 2 : 1). The fraction eluted from VLC with CH_2Cl_2-MeOH 80 : 20 (467.4 mg) was further purified on CC (Kieselgel, Merck, Art. 9385; 72 fractions; 200 ml), using EtOAc-MeOH mixtures of increasing polarity. From this CC, the fraction eluted with EtOAc yielded chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside, apigenin and genkwanin by further purification on CC (Sephadex LH-20; MeOH; 30 fractions of 30 ml) and TLC (Merck, Kieselgel plates; Art. 5721 with CH_2Cl_2-MeOH-HCOOH 9 : 2 : 1) while the fraction eluted with EtOAc-MeOH 97 : 3 yielded chrysoeriol-7-*O*-β-D-glucopyranoside by further purification on TLC (Merck, Cellulose plates; Art. 5716 with 30% aqueous solution of acetic acid).

The isolated compounds were identified by spectroscopic methods (UV, ^{1}H-NMR, ^{13}C-NMR and 2D NMR). ^{1}H- and

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^{13}C-NMR spectra were taken on a Bruker-AC 200 spectrometer in DMSO-d_6 and $CDCl_3$ solutions and chemical shifts were recorded in δ-values. ^{1}H–^{1}H COSY, HMQC and HMBC were obtained using standard pulse sequence on a Bruker-DRX 400 spectrometer. UV spectra were registered on a Shimadzu UV-160A spectrophotometer, according to standard procedures (Mabry et al., 1970).

Peripheral arterial blood containing 160 IU/ml heparin (Leo, Denmark) from male rabbits, which were kept under controlled temperature and light conditions, was used. Each sample consisted of 0.5 ml of blood and 4.5 ml culture medium (RPMI supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine – Seromed, Germany). Lymphocyte transformation was induced with 5 µg/ml phytohemagglutinin (PHA – Seromed, Germany). One hundred µl of the flavonoid to be tested, dissolved in DMSO at a final concentration of 0.3–0.03 mM were added. Control samples containing DMSO or 50 µg/ml cyclosporin (Sandimmun, Novartis) were also prepared. Following incubation for 48 h at 37 °C, in 5% CO_2/95% air, the samples were centrifuged at 500 ×g for 10 min. Seven ml of 75 mM KCl were added to the precipitate, followed by centrifugation as above. The samples were then centrifuged twice after adding 6 ml of 3 : 1 methanol/glacial acetic acid. Aliquots of the fixed lymphocyte suspension were stained with 5% Giemsa prepared with Sørensens' buffer. Small, deeply basophilic lymphocytes and larger, lighter transformed blasts as well as aberrant cells were counted under a light microscope, at a magnification of ×1000 (Nicolic et al., 1976; Rabatic & Dekaris, 1979; Yao et al., 1994). Aberrant cells were counted as any cell without an intact membrane, thus being considered destroyed before the incubation and cell isolation was complete.

The cytotoxicity of pilloin was evaluated using Trypan blue on lymphocytes separated by a Ficoll density gradient (Sfikakis et al., 1996).

Statistical analysis of the results was performed by ANOVA followed by Scheffé test, $p < 0.05$ being considered as statistically significant.

Results

Apigenin, genkwanin, salvigenin, pectolinaringenin, luteolin-7,4′-dimethylether (pilloin), 6-hydroxyluteolin,7-methylether, chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside and chrysoeriol-7-*O*-β-D-glucopyranoside were isolated from the aerial parts of *M. cylleneum*. Pilloin, apigenin and chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside were evaluated for their effects on lymphocyte transformation.

At a concentration of 0.3 mM, pilloin significantly decreased the transformation of cells ($p < 0.01$) and increased the aberrant cells ($p < 0.05$), while the percentage of inactivated lymphocytes remained unchanged ($p < 0.05$) (Fig. 1), thus indicating a cytotoxic action directed at the activated lymphocytes, but not at the inactivated cells.

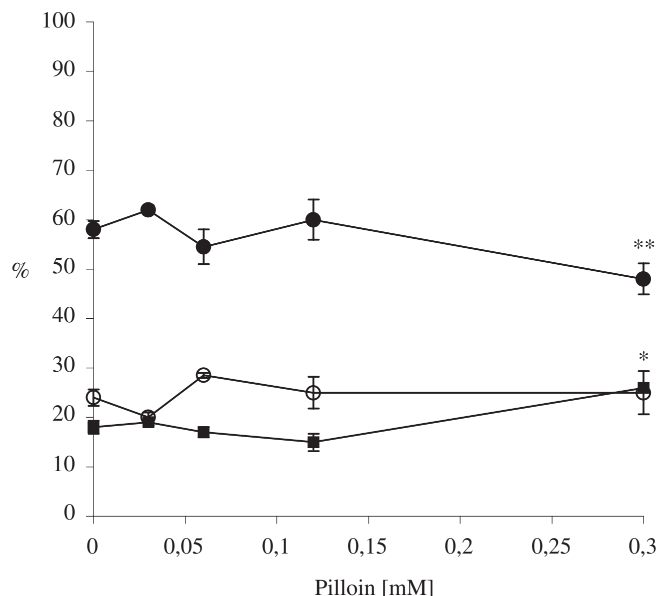

Figure 1. Effect of pilloin on the percentage of inactivated lymphocytes (○), activated blasts (●) and aberrant cells (■), following 48 h incubation in the presence of phytohemagglutinin. Results are expressed as mean ± SEM (n = 5). In the case of control, the mean was calculated from 23 observations. * $p < 0.05$, ** $p < 0.01$ *vs* absence of pilloin.

At 0.3 mM, apigenin (Baden, 1991), which was used as the control flavonoid, showed similar results to pilloin (Fig. 2), while cyclosporin, which was used as the control immunosuppressant, reversed the inactivated lymphocyte/lymphoblast ratio. Chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside, at 0.3 mM, showed no difference with the control sample (Fig. 2). Cell counting of fixed lymphocyte suspension samples showed no statistically significant difference in total lymphocyte count between the control samples and the pilloin samples (data not shown).

Finally, during the first few hours of incubation there was no significant difference in the viability of the cells between the control groups and the samples containing 0.3 mM of pilloin (Table 1), thus indicating that pilloin has no immediate cytotoxic effect. At 24 h, however, pilloin decreased cell viability ($p < 0.05$). Similar results were observed at 48 h, in particular in the samples in which PHA has been added, indicating that the increased lymphocyte transformation due to mitogen presence makes the cells more prone to cytotoxic action.

Discussion

The genus *Marrubium* comprises *ca.* 30 species, indigenous in Europe, the Mediterranean area and Asia (Mabberlay, 1997). Most of the species are annual or rhizomatous perennial herbs with a distinct indumentum of often very complex hairs and verticillasters subtended by floral leaves. A number

of active metabolites, terpenoids and flavonoids including luteolin, apigenin and chrysoeriol derivatives have been extracted from various species of this genus. These include *M. alysson* (Saleh et al., 1981), *M. friwaldskyanum* (Savona et al., 1984), *M. vulgare* (Nawwar et al., 1989), *M. supinum, M. peregrinum* (Tomás-Barberán et al., 1992) and *M. polydon* (Hatam et al., 1995).

The data presented here indicate that the flavone pilloin affects the lymphocyte transformation process by exerting cytotoxic action targeted at the transformed lymphoblasts. This was shown by the decrease of the lymphoblast percentage and the increase of the aberrant cell proportion, while the relative number of untransformed lymphocytes remained unchanged. This was also shown by the cytotoxicity tests, which indicated that lymphoblasts are more vulnerable to this cytotoxic action. Apigenin showed results similar to those of pilloin on the lymphocyte transformation assay. On the other hand, the glycosylated flavonoid showed no effect, a result which correlates with previous findings that glycosylation of various flavonoid aglycones eliminated the immunosuppressive effects of the aglycones alone, although no aberrant cells have been reported in these studies (Namgoong et al., 1993).

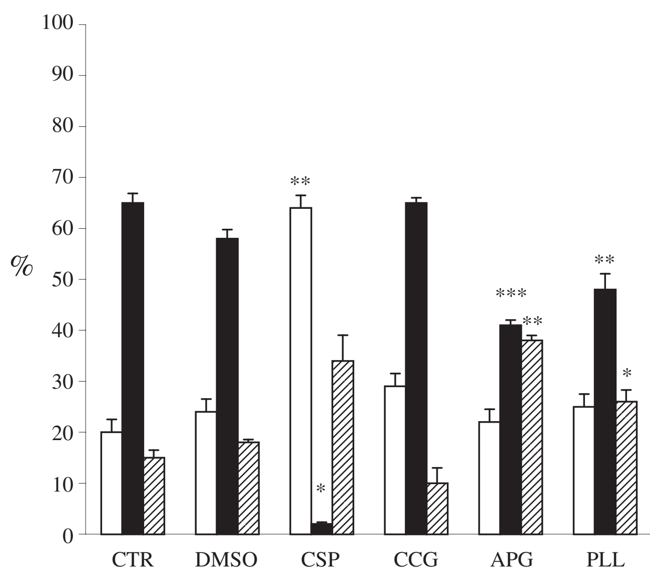

Figure 2. Effect of DMSO, 50 µg/ml cyclosporin (CSP) and 0.3 mM of the flavonoids chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside (CCG), apigenin (APG) and pilloin (PLL) on the percentage of inactivated lymphocytes (open columns), activated transformed lymphocytes (closed columns) and aberrant cells (striped columns), following 48 h incubation in the presence of phytohemagglutinin (PHA). Results are expressed as mean ± SEM from 3–5 observations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* the proportions of cells in the presence of the solvent only (DMSO).

Pilloin is a luteolin-type derivative, which has demonstrated a variety of biologic actions. Flavones and particularly luteolin have been proven to be active inhibitors of protein kinase C and tyrosine kinases (Agullo et al., 1997), enzymes which are known to play an important role in signal transduction and cell transformation. It is through these mechanisms that flavonoids exhibit their diverse immunomodulating properties (Middleton, 1998). Furthermore, flavonoids including luteolin have shown strong inhibitory effects on the growth of certain cell lines including human leukemic CEM-C1 and CEM-C7 cells (Post & Varma, 1992), possibly by the inhibition of the enzymes previously mentioned (Agullo et al., 1997). Moreover, luteolin and other flavonoids have been found to activate in a dose-dependent manner wild-type p53 accumulation and apoptosis in tumour and non-tumour cell lines (Plaumann et al., 1996).

It is possible that the mechanism of cell death induced by pilloin on proliferating transformed lymphocytes involves the inhibition of one or more of the enzyme systems mentioned above. The aberrant cells, which are also present in the control groups, but to a lesser extent, showed nuclear condensation and destruction of cell structure, which may be indicators of apoptotic cell death, although they did not exhibit characteristic membrane blebbing (Jakobsen et al., 1994). Pilloin characteristically did not exhibit cytotoxic action during the first few hours of incubation, thus indirectly indicating the possible role of apoptosis, as opposed to a direct cytolytic mechanism of cell death.

The mechanism of action remains elusive, although it is possible that pilloin may induce effects similar to those of other related flavonoids and that its biological activity may make it medicinally useful.

Table 1. Viability of cultured peripheral blood lymphocytes. Results are presented as % viable cells in each sample (determined by 4% Trypan blue).

	Period of incubation (h)				
	1	2	6	24	48
Control	100 ± 0	99 ± 0.5	94 ± 4.0	70 ± 7.5	39 ± 1.4
PHA 5 µg/ml	100 ± 0	96 ± 2.8	82 ± 4.5	46 ± 6.5	36 ± 1.2
Pilloin 0.3 mM	100 ± 0	98 ± 0.0	92 ± 1.3	57 ± 2.2*	21 ± 2.5*
Pilloin + PHA	99 ± 0.5	94 ± 3.5	82 ± 3.2	34 ± 4.8	11 ± 2.1***

* $p < 0.05$ *vs* control, *** $p < 0.001$ *vs* viability in the presence of phytohemagglutinin (PHA).

Acknowledgements

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