

with 1 N BaCO₃ and filtered. The filtrate was extracted with CHCl₃ (3 × 10 ml). In order to identify the constituents of the sugars, the aqueous layer was evaporated. The residue was purified by silica gel (16 g) column chromatography using CHCl₃-MeOH-H₂O (6:4:1, 350 ml) as an eluant, which resulted in 78.7 mg of rhamnose (R_f 0.32) and 8.6 mg of glucose (R_f 0.56). The optical rotation of the purified sugars were equivalent to those of standard L-rhamnose and D-glucose.

Tumor cell lines and cytotoxicity assay: Human stomach cancer (SNU-1), human lung (A549), leukemia (K-562), human ovarian (SKOV-3), and human melanoma (SKMEL-2) cancer cell lines were supplied from the College of Medicine, Seoul National University. The *in vitro* cytotoxicity assay was performed as described previously (10–12).

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Corosolic Acid Isolated from the Fruit of *Crataegus pinnatifida* var. *psilosa* is a Protein Kinase C Inhibitor as well as a Cytotoxic Agent

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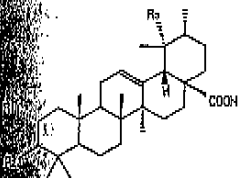
Abstract: Corosolic acid isolated from the fruit of *Crataegus pinnatifida* var. *psilosa* was tested for anticancer activity. Corosolic acid displayed about the same potent cytotoxic activity as ursolic acid against several human cancer cell lines. In addition, the compound displayed antagonistic activity against the phorbol ester-induced morphological modification of K-562 leukemia cells, indicating the suppression of protein kinase C (PKC) activity by the cytotoxic compound. The compound showed PKC inhibition with dose-dependent pattern in an *in vitro* PKC assay.

Abbreviations:

DMEM:	Dulbecco's Modified Essential Medium
DMSO:	Dimethyl sulfoxide
ED ₅₀ :	Effective dose at 50% survival
EtOH:	Ethyl alcohol
FBS:	Fetal bovine serum
PBS:	Phosphate buffered saline
PDBu:	Phorbol 12,13-dibutyrate
PKC:	Protein kinase C
SRB:	Sulforhodamine B

The fruits of *Crataegus pinnatifida* Bunge var. *psilosa* Schneide have been used traditionally as a peptic agent in oriental medicine (1). Three triterpenoid compounds (Fig. 1), ursolic acid (USA, 3β-hydroxy-12-urs-12-en-28-oic acid), corosolic acid (CSA, 2α-hydroxyursolic acid), and euscopic acid (ECA, 2α,3α,19α-trihydroxyurs-12-en-28-oic acid), and two other glucosides were isolated from the fruit of *C. pinnatifida* var. *psilosa* in the previous work (2). Ursolic acid is a well-known terpenoid compound that exists widely in foods, medicinal herbs and other plants (3), but corosolic acid was first isolated from *C. pinnatifida*. The pharmacological activities of these terpenoid compounds were limitedly known except ursolic acid. It has recently been reported that ursolic acid displayed anti-invasive activity on metastatic HT1080 human fibrosarcoma cells (4), the cell cycle arrest of MCF-7 breast cancer cell at G1 phase (5), inhibition of skin tumorigenesis (6), and antiviral activity (7). In this work, the cytotoxicity of corosolic acid purified from the fruits of *C. pinnatifida* var. *psilosa* against various human cancer cell lines and *in vitro* PKC inhibition by corosolic acid were elucidated.

Little is known about the pharmacological activities of corosolic acid as yet, and this is the first report on antitumor activity of corosolic acid. Corosolic acid displayed potent cytotoxic activity



- I : R₁=OH, R₂=β-OH, R₃=H (corosolic acid)
 II : R₁=R₂=H, R₃=β-OH (ursolic acid)
 III : R₁=OH, R₂=α-OH, R₃=OH (euscaptic acid)

Fig. 1. Structure of terpenoids isolated from the fruit of *C. pinnatifida* var. *psilosa*.

similar to ursolic acid against several human cancer cell lines established from various origins. The ED₅₀ of corosolic acid was estimated as 0.4–5.0 μg/ml depending on the cell lines (Table 1). Both compounds were more cytotoxic against HeLa S₃ and SNU-C₄ cells than against other cancer cells indicating that these compounds have strong sensitivity to solid cancer cells. Probably the cytotoxicity against cancer cells was partly due to the inhibition of PKC activity by corosolic acid because corosolic acid displayed an antagonistic effect on the morphological change of K-562 human myelogenous leukemic cells with phorbol esters which has been known as a PKC activator and potent tumor promoter (unpublished data). Also this morphological change was reported to correlate with the initial cancer promoting activity by way of abnormal PKC activation (8). Actually *in vitro* PKC assay showed that corosolic acid inhibited PKC activity with dose-dependent patterns (Table 2) indicating that corosolic acid displayed the cytotoxic activity related to PKC inhibition.

The above results show the growth inhibition with about 0.4–5.0 μg/ml but incomplete inhibition of rat brain PKC activity *in vitro* by concentrations higher than about 20 μg/ml. The discrepancy in effective concentrations is probably due to the low selectivity of corosolic acid on PKC because various amphiphilic triterpenoids were known as relatively ineffective inhibitors of protein kinases including PKC, except for cyclic AMP-dependent protein kinase that was inhibited potently by the triterpenoids (9). This means that corosolic acid may affect other susceptible targets related to the cytotoxicity. According to another report, ursolic acid is known to be relatively non-toxic and effective in protecting against chemically induced liver injury (10). In this respect, it is difficult to clarify the mechanisms for the pharmacological effects of the triterpenoid compounds on cytotoxicity as yet. We will try to clarify whether the cytotoxic activity of corosolic acid and ursolic acid is related directly to the mechanism of PKC inhibition or not. In many cases, it was known that PKC inhibition was related to a reduction of the multi-drug resistance (MDR) activity (11, 12); unfortunately however, corosolic acid could not overcome the MDR of competent cell lines (data not shown). Whether corosolic acid can be used as anticancer agent or not remains to be elucidated conclusively. Even so, the above results indicate that the cytotoxicity of corosolic acid is strongly related with its PKC inhibition.

Table 2 Effect of corosolic acid and ursolic acid on PKC inhibition (numbers represent mean ± standard deviations).

Inhibitor	concentration (μg/ml)	³² P incorporated (cpm)*	% decrease in PKC activity
Control		4820 ± 352	100
Corosolic acid	0.1	4579 ± 153	95
	1	4240 ± 102	88
	10	2985 ± 220	62
	50	1926 ± 82	40
Ursolic acid	50	2070 ± 105	43
Staurosporine (1 nM)		2406 ± 13	50

* All experiments were triplicated.

Materials and Methods

Plant material and purification procedure: Fruits of *Crataegus pinnatifida* var. *psilosa* were collected at Kwangduk-San (Kwangwon-do, Korea) in October 1994 and seeds were removed. A voucher specimen is maintained in the Korea Research Institute of Bioscience and Biotechnology. Dried fruits of *Crataegus pinnatifida* var. *psilosa* (7 kg) were ground and extracted with MeOH (20 liters, 3 ×) followed by evaporation under reduced pressure. The crude extract (2 kg) was dissolved in distilled water (0.5 liter) and extracted with diethyl ether (2 liter, × 5) followed by drying under vacuum. The solvent fraction (100 g) were separated by silica gel column chromatography with a step-gradient of solvent (CH₂Cl₂: MeOH = 60:1 → 1:1). Purification and structure analysis of corosolic acid were conducted as in the previous report (13).

Cell lines and culture media: K-562 (ATCC CCL 243), Hep G₂ (ATCC HB 8065), HeLa S₃ (ATCC CCL 2.2), and A-549 (ATCC CCL 185) cell lines were obtained from the American Tissue Culture Collection (Rockville, Maryland, U.S.A.). SNU-C₄ (KCLB 00004) was obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). Dulbecco's Modified Essential Medium (DMEM) and RPMI-1640 were from Gibco-BRL (Grand Island, NY, U.S.A.). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) were from Hyclone (Logan, UT, U.S.A.).

Biological activities: Cytotoxic activity was evaluated by the same procedure as used in the previous report (14) applying the SRB method (15). For the assay of phorbol ester-antagonistic activity, K-562 cells were diluted to 1 × 10⁵ cells/ml and 100 μl of the cell suspension was dispensed in each well of a 96-well plate. Aliquots of 10 μl of each sample extract (50 mg/ml) were added into the wells and the cells were incubated for 1 h at 37 °C. Phorbol 12,13-dibutyrate (PDBu) was then added to make the final concentration of 0.1 μg/ml. After 30 min, bleb-bearing K-562 cells were counted under a microscope and

Table 1 Effects of corosolic acid on the cytotoxicity against various human cancer cell lines (numbers represent means ± standard deviations).

Sample	Cell Line (ED ₅₀ , μg/ml)*				
	Hep G ₂	A549	SNU-C ₄	HeLa S ₃	K-562
Corosolic acid	4.8 (± 1.2)	5.0 (± 0.8)	0.4 (± 0.1)	1.0 (± 0.3)	4.3 (± 1.5)
Ursolic acid	3.0 (± 0.6)	1.85 (± 0.3)	1.4 (± 0.5)	1.5 (± 0.8)	12.5 (± 4.3)

* All experiments were triplicated.

compared with the positive and the negative controls treated with and without 1 nM staurosporine, respectively. PKC activity was determined as in the previous report (14).

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Analysis of the Essential Oil of *Grindelia discoidea*

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Abstract: The essential oil from aerial parts of *Grindelia discoidea* was analyzed by GC and GC/MS. Forty-six components were identified, representing more than 95% of the oil. The main constituents were (*E,E*)-farnesol (> 9.0%) and (*Z,E*)-farnesol (15.7%).

As a continuation of our project of screening new essential oils from medicinal plants of Argentina (1–4), and because sometimes the aromatic properties of a homeopathic remedy could influence its uses, we have examined the natural variation in *Grindelia discoidea* essential oil. About 60 species belong to genus *Grindelia* Will. (5). *Grindelia discoidea* Hooker Arn. is a resinous shrub (30 cm height) of yellow flowers. It is found in Uruguay and the central area of Argentina (6). Its common name is caá-pé-mini and/or caá-pé-aici and is widely used homeopathically for the treatment of asthma and stomachache (7).

The components of the essential oils from *G. discoidea* populations studied are given in Table 1. Forty-six compounds were identified, representing more than 95% of the oil.

The monoterpene fraction was present in relatively low amounts in almost all the populations, with the exception of 3AG (21.4%). The essential oil of the 3AG population contained (*Z*)- β -ocimene (7.2%) as the most abundant monoterpene. Oxygen-containing monoterpenes constituted the minor part of these oils (< 2.0%). Another distinguishable feature was the presence, in small amount, of *trans*- and *cis*-*p*-mentha-2,8-dien-1-ol and *trans*- and *cis*-*p*-mentha-2,8-dien-2-ol, previously described in the essential oils obtained from the leaves and flowers of *Cymbopogon desiflorus* (11) and *Chenopodium ambrosoides* (12). Bornyl acetate, bornyl and α -pinene were the major compounds of the monoterpene fraction of *Grindelia robusta* and *Grindelia squarrosa* (13, 15).

The sesquiterpene hydrocarbons ranged from 24.3% in the population 8S to 40.4% in 5AG population. This fraction contained mainly γ -cadinene (< 9.4%), valencene (< 2.4%) and β -selinene (< 1.1%). Mina Clavero populations (1) and 2MC had considerable amounts of β -caryophyllene (6.2% and 8.6%, respectively), (*Z,E*)-Farnesol (< 15.7%), (*E,E*)-farnesol (< 9.0%) and globulol (< 6.2%) were the major compounds.

