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Antineopastic Natural Products and the Analogues IV.

Aurapten, the cytotoxic coumarin from *Poncirus trifoliata* against L1210 cell.

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Abstract □ A cytotoxic coumarin against L1210 cell was isolated from the unripe fruit of *Poncirus trifoliata* (ED_{50}=10.2 μg/ml). Its structure was identified as aurapten, 7-geranyloxycoumarin. Hydrolysis of the substance gave umbelliferone and geraniol. Only geraniol showed the cytotoxic activity (ED_{50}= 6.5 μg/ml) while umbelliferone and its methyl or allyl derivatives were not active.

Keyword □ *Poncirus trifoliata*, Rutaceae, Aurapten, Cytotoxic, L1210, Geraniol.

In continuing search for potential antineoplastic agents from the Korean traditional medicines, it was found that six crude drugs among thirty-eight ones screened showed a considerable cytotoxicity against L1210 cells[1]. Among the six ones, the cytotoxic principle of "Whanggum", the dried roots of *Scutellaria baicalensis*, was isolated and identified as skullcapflavon II[2]. The cytotoxic activity of skullcapflavon II against L1210 cell was confirmed by total synthesis[3]. Another interesting one, "Jisil", the dried, unripe fruit of *Poncirus trifoliata*, has been used as a cough remedy and contains essential oils and coumarin derivatives[4,5,6], but the cytotoxic principle against L1210 cell remains so far unsolved.

EXPERIMENTAL METHODS

Melting points were measured by capillary method and uncorrected. UV spectra were obtained with Pye Unicam PU8800 spectrophotometer and IR spectra were taken on a Perkin Elmer Model 783 Infrared Spectrophotometer. NMR spectra were recorded on a Varian FT80A spectrometer in $CDCl_3$ using TMS as internal standard. Silica gel 60 (70-230 mesh ASTM, Merck) and precoated silica gel 60 TLC plates (Merck) were used as adsorbants for column and thin layer chromatography, respectively.

Biological

The culture of L1210 cells has been maintained in Fisher's medium (GIBCO Laboratories, Grand Island, New York) fortified with horse serum. The value of ED_{50} which is the concentration of a test compound to inhibit the growth of L1210 by 50% was determined following the procedures described by Thayer *et al*[7] with minor modifications. The initial concentration of L1210 cells in Fisher's medium was adjusted to 5×10^4 per milliliter and the growth ratio, Y, for each dose of test substance was calculated following the equation,

$$\frac{T-Co}{C-Co} \times 100 = Y(\%)$$

where T=mean cell count for each dose after 48 hours incubation; C=mean cell count for control after 48 hours incubation; Co=mean cell count at the start of incubation. The ED_{50} values were obtained graphically by plotting Y values against doses of test substances semi-logarithmically.

Extraction and isolation of the active substance

The dried Jisil (2 kg) was ground and extracted with methanol (6 l). The methanol extract was evaporated in vacuum. The residue (300 g) was suspended in water (500 ml) and extracted with petroleum ether (2 l), ethyl ether (2 l) and ethyl acetate (2 l), in sequence. The petroleum ether fraction was refractionated over a silica gel column (400×30 mm dia.) with n-hexane/acetone (9:1). The subfraction 5, which was fluorescent under 365 nm and yellow brown in iodine vapor, was crystallized three times from n-hexane. White needles (450 mg), mp 71°C. UV λ_{max}^{MeOH} nm (logε): 323 (4.22), IR ν_{max}^{KBr} cm^{-1}: 1505, 1610 (benzene), 1720 (C=O). NMR ($CDCl_3$)δ ppm: 1.61, 1.67 (2×3H, each s, 2 × C7′—CH_3), 1.77(3H, s, C3′—CH_3), 2.00~2.28(4H, m, —CH_2—CH_2—), 4.60(2H, d, J=7.0, C1′—H_2), 5.10(1H, t, J=7.0, C6′—H), 5.48(1H, t, J=7.0, C2′—H), 6.25(1H, d, J=9.5, C3—H), 6.88(2H, d, J=6.5, C6, C8—H), 7.39(1H, d, J=8.5, C5—H), 7.66(1H, d, J=9.5, C4—H).

Hydrolysis of the active substance

The substance (100 mg) was hydrolyzed in a mixture of acetic acid (1 ml) and sulfuric acid (0.1 ml) by standing for 2 hours at room temperature. The reaction mixture was diluted with water (10 ml) and extracted two times with ethyl ether (40 ml). The ether extract showed two spots on TLC, Rf values of which are identical with those of geraniol and umbelliferone [0.31 and 0.13 in benzene/acetone (9:1)], respectively. After the elimination of the solvent, the residue was directly crystallized from the mixture of ethyl acetate and n-hexane. White needles (35 mg), mp 221°C. Its physical data are identical with those of the authentic umbelliferone. The mother liquor was dried and dissolved in n-hexane (10 ml). The hexane solution was washed with 0.1N sodium hydroxide and water. After evaporation of n-hexane, the residue was chromatographed on a silica gel column (300×20 mm dia.) with benzene/acetone (9:1) to give geraniol (37 mg).

Methylation and allylation of umbelliferone

Umbelliferone (100 mg) was methylated or allylated with dimethyl sulfate (100 mg) or allyl chloride (120 mg) under the presence of anhydrous potassium carbonate (200 mg) in anhydrous acetone (10 ml) to give herniarin (90 mg) or 7-allyloxycoumarin (95 mg), respectively. The physical properties of the synthesized substances were identical with the reported ones[8].

RESULTS AND DISCUSSION

In order to accumulate the active principle(s), we firstly made a methanol extract of powdered fruit which was further extracted with petroleum ether, ethyl ether and ethyl acetate in sequence. Bioassaying these fractions on L1210 cell has shown that the activity was concentrated in the petroleum ether fraction. Its ED_{50} value was 12.5 μg/ml. Running a silica gel column of this fraction with n-hexane/acetone (9:1) gave 7 subfractions. The subfractions 3, 4 and 5 exhibited the cytotoxicities. The amount of the

ıbfraction 3 and 4 were too small to perform ırther management for the present study Гable I).

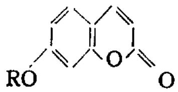
Crystallization of the subfraction 5 with n-exane gave white needles (mp 71°C). Its D_{50} value was 10.2 μg/ml. The isolated ıbstance showed IR absorptions at 1610 and ;05 cm^{-1} from a benzene moiety and at 1720 n^{-1} from an unsaturated carbonyl, indicating ıat it has a molecular structure similar with that : cinnamic acid derivatives or simple coumarins. s NMR spectrum contains all the necessary ɜaks for a 7-etherified coumarin.

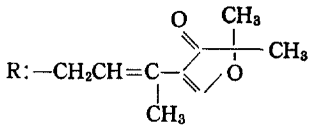
From comparison of its mp, NMR and IR ɔectra with those of the coumarin derivatives hich had been isolated from *P. trifoliata*[4,5,6], ıe substance was proven to be identical with ɹrapten, 7-geranyloxycoumarin[4].

In order to search for the cytotoxic structral moiety, the active substance was hydrlyzed, and found to give umbelliferone and ɜraniol. The etherification of umbelliferone ·ith dimethyl sulfate and allyl chloride gave -methoxy and 7-allyloxycoumarin, respectively. 'hese substances were tested on L1210 cells ɔr the cytotoxicities, and as shown in Table umbelliferone and its methyl or allyl ethers ·ere not cytotoxic. Geraniol, with ED_{50} value f 6.5 μg/ml, was the only active substance nong the hydrolytic products.

It was reported that some of coumarin derivtives have antitumor activities[9,10,11,12,13,14,15]. or example, geiparvarin, which is a kind of mbelliferone ether and has the same number f side chain carbons and double bonds as that f aurapten, showed a significant cytotoxicity gainst KB cells[12]. Geiparvarin showed an ED_{50} alue less than 5 μg/ml against L1210 cells in ıis experiments(Table I). The active structural

Table I. The structures and ED_{50} values of coumarin derivatives



Compounds	Structures	ED_{50} values (μg/ml)
Umbelliferone	R:—H	>20
Herniarin	R:—CH_3	>20
7-allyloxy-coumarin	R:—$CH_2CH=CH_2$	>20
Aurapten	R:—$CH_2CH=C(CH_3)CH_2CH_2CH=C(CH_3)CH_3$	10.2
Geiparvarin		<5

moiety of geiparvarin has not been studied, but the cytotoxicity of geiparvarin against L1210 cells may be due to the presence of the side chain, since umbelliferone moiety was found to be inactive, as above mentioned.

Nonetheless, in the in vivo sysyem of L1210, the coumarin moiety may play an important role in transporting the active geraniol moiety as do the nitrogen mustard-coupled coumarins[10,15].

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