Antimutagenicity and Cytotoxicity of the Constituents from the Aerial Parts of *Rumex acetosa*

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Four anthraquinones isolated for the first time from the aerial parts of *Rumex acetosa* (Polygonaceae), a Korean and a Japanese medicinal plant, and two synthetic derivatives were examined for their cytotoxicities against five cultured human tumor cell lines, *i.e.* A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nerve system) and HCY15 (colon), using the Sulfrhodamine-B method *in vitro* and antimutagenic activities by Ames test with *Salmonella typhimurium* TA98 and TA100 and SOS chromotest with *E. coli* PQ37. Among the tested compounds, emodin strongly inhibited the proliferation of each examined tumor cell line with IC_{50} values ranged from 2.94 to 3.64 μg/ml and showed potent antimutagenic activities with 71.5% and 53.3% at the concentration of 0.1 mg/plate against the mutagens, NPD and sodium azide, respectively. Its antigenotoxic activity was also very effective at the final concentration of 10 μg/reaction tube against the mutagens, MNNG and NQO by SOS chromotest, reducing the induction factors by 19.6% and 43.5%, respectively. The structure–activity correlation study suggests that an additional OH group at C-6 position in the anthraquinone nucleus may play an important role for their cytotoxicities and an introduction of OH– or OCH_3 group at C-6 position is necessary for their antimutagenicities.

Key words *Rumex acetosa* L. Polygonaceae; anthraquinone; cytotoxicity; antimutagenicity

Rumex acetosa L. (Polygonaceae) is a perennial plant widely distributed in Korea and Japan. In Korea, this dioecious herb (garden sorrel) is officially listed in the Korean Food Code (Korea Food & Drug Administration) as one of the main food materials and has been used in folk medicine as a mild purgative and also for the treatment of cutaneous diseases.[1)]

The rhizomes of this plant have been reported to show antioxidant activity[2)] and their polysaccahrides mixture revealed the antitumor action in sarcoma 180-bearing mice.[3)] However, the effects of other parts of this plant have not been reported.

From its rhizomes, five anthraquinones and their glycosides have been isolated[4)] and its aerial parts are known to contain several flavonoids.[5)]

As a part of our continuing search[6,7)] for antimutagenic and cytotoxic agents from natural sources, we have examined the antimutagenic activity and cytotoxicity of the crude methanolic extracts and their fractions of the aerial parts of this plant.[8)] This previous investigation showed that the methylene chloride fraction exhibited most potently the antimutagenicity and cytotoxicity. In this study, we isolated four known anthraquinones from the methylene chloride fraction and synthesized two derivatives, of which antimutagenic and cytotoxic activities were evaluated and their structure–activity correlation was suggested.

MATERIALS AND METHODS

General Human tumor cell lines used in the experiment, *i.e.*, A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (melanoma), XF498 (central nervous system), and HCT-15 (colon) were obtained from the National Cancer Institute (NCI) in the USA, and are currently used in the NCI's *in vitro* anti-cancer drug screening. *E. coli* PQ37 (*sfi*A::Mud(Ap *lac*)*cts lac*ΔU169 *mal*+, *uvr*A, *gal*E *gal*Y, PhoC, *rfa*) and *S. typhimurium* TA98 and TA100 were used as the test strains for antigenotoxicity (SOS chromotest) and for antimutagenicity (Ames test), respectively. The mutagens MNNG (1-methyl-3-nitro-1-nitrosoguanidine), NQO (4-nitroquinoline-1-oxide), NPD (4-nitro-*o*-phenylenediamine) and sodium azide were purchased from Sigma Co. (St. Louis, MO, U.S.A.). Melting points were measured on an Electrothermal IA9100 apparatus (Reallabware Co., Watford Herts, U.K.) and are uncorrected. NMR spectra were recorded on a Varian GEMINI-200 (Varian Inc., Palo Alto, CA, U.S.A.) spectrometer using DMSO-d_6 or CD_3OD as a solvent. Mass spectra were obtained on a Finnigan MAT95 (Thermo Electron Corporation, West Palm Beach, FL, U.S.A.). HPLC analysis was carried out with a Shimadzu LC-10AD system (Kyoto, Japan) equipped with an Eclipse XDB-C18 column (4.6 mm×25 cm) (Agilent, Palo Alto, CA, U.S.A.) using a linear gradient of methanol (flow rate: 1 ml/min) at 254 nm at room temp.

Plant Material The aerial parts of *Rumex acetosa* were collected from the suburbs of Ulsan city, Korea in May, 2004 and identified by Prof. Byung-Soo Kang, College of Oriental Medicine, Dongguk University, Gyeongju, Korea.

Extraction and Isolation The powdered aerial part of the plant (300 g) were extracted with 80% methanol (2×1 l) under reflux. The extract were concentrated to dryness *in vacuo* at 40 °C to give a brown oily extract (75.8 g), which was successively fractionated with 200 ml of each of *n*-hexane (6.4 g), CH_2Cl_2 (15.2 g), EtOAc (12.5 g) and *n*-BuOH (7.2 g) in sequence to yield the corresponding dried extracts. The CH_2Cl_2 fraction was chromatographed on a silica gel column (5—40 μm, 5×70 cm) (Merck & Co., Inc., Whitehouse Station, NJ, U.S.A.). Elution was initiated with

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CH_2Cl_2, providing the mixture of two compounds, further elution with CH_2Cl_2 : MeOH (v/v 9 : 1) gave two separated compounds. The mixture of two compounds was rechromatographed using above column with CH_2Cl_2 : pet. ether (v/v 1 : 1) to furnish pure compounds individually, which were identified to be chrysophanol (**1**) and physcion (**2**). Above two separated compounds were identified to be emodin (**3**) and emodin-8-*O*-β-D-glucopyranoside (**4**), respectively. The structure of all isolated components **1**—**4** was confirmed by the comparison with the known spectral data.[4)]

The purity of compounds **1**—**4** was analyzed by reverse-phase HPLC to be 98.8% (t_R=32.2 min), 98.5% (t_R=45.2 min), 95.8% (t_R=17.1 min) and 97.5% (t_R=3.0 min), respectively.

Preparation of the Derivatives Emodin (50 mg, 0.186 mM), the most active component, was dissolved in acetone (300 ml) and refluxed with potassium carbonate (0.06 mg) and dimethylsulfate (60 μl, 0.6 mM) for 16 h. After evaporation of the reaction mixture *in vacuo*, the residue was separated by silica gel column chromatography with CH_2Cl_2 : MeOH (v/v 9 : 1) to afford two pure compounds, which were proved to be 1,8-dimethoxy-6-hydroxy-3-methyl-9,10-anthraquinone (**5**)[9)] and 1,6,8-trimethoxy-3-methyl-9,10-anthraquinone (**6**)[10)] (Fig. 1) by the instrumental analyses.

Cytotoxicity Assay Cytotoxicity after treatment of the tumor cells with the test materials was determined using the SRB (sulfrhodamine-B) method, currently adopted in the NCI's *in vitro* anti-cancer drug screening,[11)] *i.e.*, estimating the inhibition rate of cell proliferation after continuous exposure to test materials for 48 h. All samples were tested in triplicate and the mean IC_{50} values (μg/ml), a concentration that caused 50% inhibition of cell proliferation, and the S.E.M. were calculated, respectively.

Antimutagenic Activity Assay The antigenotoxicity test was performed by the method of Quillardet and Hofnung[12)] using 600 μl of bacterial suspension, 10 μl of mutagens (MNNG and NQO; each 100 μg/ml of DMSO), and 10 μl of compounds (1 mg/ml of DMSO). Genotoxicity is expressed as an induction factor (SOS induction by a mutagen),[13)] which is defined as the value of the enzyme activity ratio of β-galactosidase activity and alkaline phosphatase with sample and/or a mutagen divided by the enzyme activity ratio without sample or mutagen (spontaneous). The antimutagenicity test was performed essentially as described by Maron and Ames[14)] using 0.1 ml of bacterial suspension, 0.1 ml of mutagens (NPD 10 μg/plate, NaN_3 1 μg/plate) and 0.1 ml of compounds (0.1 mg/plate). The inhibition ratio of revertant colony forming unit (CFU) per plate was calculated by the following equation: % inhibition ratio=[1−(CFUsm−CFUp)/(CFUm−CFUp)]×100, where CFUsm is CFU with mutagen and sample, CFUm is CFU with mutagen, and CFUp is a spontaneous CFU with no mutagen or sample. The number of histidine revertants induced by the mutagen without any sample was given as 100%.

Statistics Inter-group comparisons of data were made by ANOVA, followed by *post hoc* multiple comparison analysis.

RESULTS AND DISCUSSION

Cytotoxicity The isolated constituents from the methylene chloride fraction of *Rumex acetosa* and their synthetic derivatives were estimated for their cytotoxicities against five cultured human tumor cell lines, *i.e.* non-small cell lung, ovary, melanoma, central nerve system and colon, using the SRB method *in vitro*. Among the tested compounds, emodin (**3**) showed the best cytotoxicity (IC_{50}=2.92—3.6 μg/ml) against all tumor cell lines and chrysophanol (**1**) and physcion (**2**) were less cytotoxic than emodin (Table 1). Upto 8.0 μg/ml of the final concentration, no cytotoxic effect of emodin was found in normal human liver cell (Chang cell) by XTT and MTT assays. Considering that emodin possesses a OH group at C-6 position, differently from compounds **1** and **2**, this group may play an important role for its cytotoxicity. Compound **4** (8-*O*-glucoside of emodin) containing a OH group at C-6 position as like emodin, however, revealed the lowest activity, which suggested that the additional OH group at C-8 is necessary for their cytotoxicities. This structure–activity correlation may be supported by the relatively weak cytotoxicity of compounds **5** and **6**, which have two or three OCH_3 groups instead of OH groups at the corresponding carbon atoms (Fig. 1).

The most active emodin (**3**) has been reported to exhibit the anticancer effect by a nucleotide excision repair[15)] and to

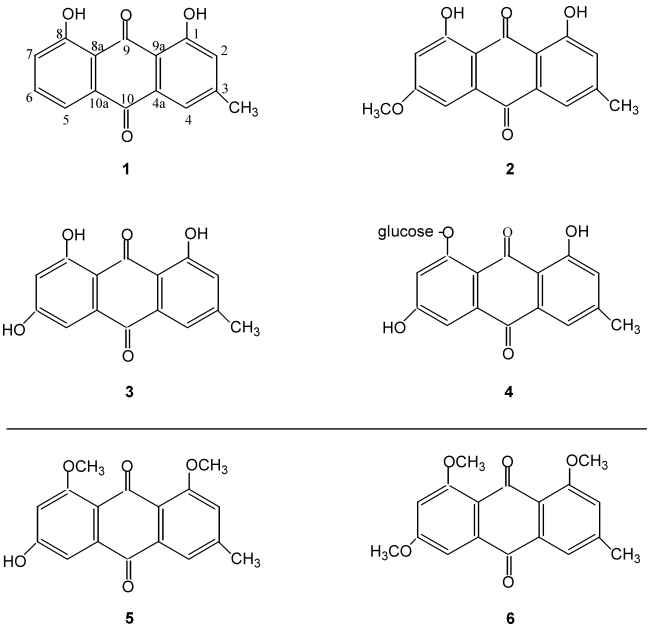

Fig. 1. Chemical Structures of the Isolated Components and Synthetic Derivatives

Table 1. Inhibition of Human Tumor Cell Proliferation by the Isolated Components **1**—**4** and the Synthetic Derivatives **5** and **6**

	IC_{50} (μg/ml)				
	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	24.76	7.28	5.83	>30.0	>30.0
2	25.16	22.41	26.07	26.40	26.88
3	3.32	2.94	3.64	2.98	3.10
4	>30.0	>30.0	>30.0	>30.0	>30.0
5	>30.0	21.34	22.06	>30.0	>30.0
6	>30.0	12.30	18.92	28.61	>30.0
Cisplatin	1.4	0.9	0.8	0.9	2.2

be effective against mouse leukemia L1210.[16] Moreover, this compound inhibited the *HER-2/neu* tyrosine kinase activity to exhibit the antitumor effect against *HER-2/neu*-overexpressing breast cancer cell,[17] however, exerted no cytotoxicity against ovarian cancer cell.[18] Our new results on five human tumor cells propose that further studies including an animal model test of emodin are needed for thorough evaluation of its effectiveness.

Antimutagenicity In our previous work[8] the methylene chloride fraction of *Rumex acetosa* revealed the most antimutagenic activity. Now its constituents **1**—**4** and the two derivatives **5** and **6** were investigated for their antimutagenic and antigenotoxic properties by the Ames test and the SOS chromotest. For antimutagenic activity, the inhibitory effect of the test samples was examined in a plate incorporation assay against mutagens NPD and NaN_3 using *Salmonella typhimurium* test strains TA98 and TA100, respectively, without S9 activation. No mutagenic activities of the tested compounds or the solvent DMSO were detected (data not shown), indicating that these are not mutagenic. Although DMSO is reported to block the mutagenic and carcinogenic activity of benzene,[19] this solvent exhibited no antimutagenicity in our assay system. Among the tested compounds, emodin (**3**) showed the strongest effect at a dose of 0.1 mg/plate in the Ames test revealing 71.5% and 53.3% inhibition ratios ($p<0.01$) of revertant CFU (colony forming unit) per plate against NPD and NaN_3, respectively (Table 2).

Chrysophanol (**1**) having no substituent at C-6 position exhibited the least antimutagenic activity, contrary to the results of cytotoxicity assay (Table 1), which may explain that an introduction of any group at C-6 position is necessary for the antimutagenicity, even though the important OH groups are bonded at C-1 and C-8 positions. The antimutagenicity of various anthraquinones was reported with their structure–activity relationship using *S. typhimurium* TA98 against 2-amino-3-methylimidazo[4,5-*f*]quinoline as a mutagen.[20,21] However, except emodin (**3**), the other compounds (**1**, **2**, **4**, **5**, **6**) were not tested in the above reports.

In order to evaluate the antigenotoxic activities of compounds **1**—**6** and to confirm the antimutagenicity of emodin, we performed the SOS chromotest using the test strain *Escherichia coli* PQ37. When a final concentration of 10 μg per reaction tube of the compounds was applied to *E. coli* PQ37, emodin (**3**) revealed much more effective antigenotoxic activity against both mutagens used (MNNG and NQO) in comparison with that of other components (Fig. 2). The induction factors (IF=2.54, 1.48) of emodin were reduced by 19.6% and 43.5% against the mutagens MNNG and NQO, respectively. The structurally related pattern of the antigenotoxic activity of the tested compounds was similar with that of their antimutagenic activity. The effect of all samples was not compared with that of a positive control, because no standard compound is yet known, and the above experiments showed only that each sample inhibits the induction of genotoxicity by the mutagens (negative control).

It has been reported that emodin, a kind of mycotoxin, contains mutagenicity in the *Salmonella typhimurium* TA1537 strain,[22] but no mutagenicity in the TA98 and TA100 strains,[23] as like in our assay system. Emodin is known to inhibit the mutagenicity of some mutagens, *e.g.*, 3-amino-1-methyl-5*H*-pyridol[4,3-*b*]indole,[24] benzo[*a*]pyrene,[25] 2-amino-3-methylimidazo [4,5-*f*]quinoline[20,21,23] or 1-nitropyrene[26] in TA98 strain. The present work demonstrates that emodin is also antimutagenic against the mutagens, NPD or NaN_3 in the TA98 and TA100 strains. Furthermore, the antigenotoxic activity of emodin has not yet been reported by the mutagen-based SOS chromotest, a more general antimutagenicity assay with accuracy and rapidity. Although emodin has been introduced to possess a marginal genotoxicity in the SOS chromotest without addition of any mutagen,[27] our results apparently prove that emodin significantly reduces genotoxicity of the mutagens MNNG and NQO.

Emodin diminished the induction factor increased by both mutagens in a dose-dependent manner, however, at doses of over 4 μg/assay the effect was almost unchanged (Fig. 3). When emodin was added to LB broth to give a final concentration up to 20 μg/ml, the cell growth of *E. coli* PQ37 or *S. typhimurium* after 24 h was not inhibited, which suggests that its antimutagenic effects are not due to a bactericidal effect of the compound in the presence of a mutagen. However, because the present results were obtained from an *in vitro* assay using a bacterial strain, emodin (**3**) would not directly be ap-

Table 2. Inhibitory Effect of the Isolated Components **1**—**4** and the Synthetic Derivatives **5** and **6** on the Mutagenicity Induced by NPD in *S. typhimurium* TA98 and Sodium Azide in *S. typhimurium* TA100

Treatment	Revertants per plate	
	TA98	TA100
Spontaneous	11±1	143±3
NPD	786±119[a]	—
NaN_3	—	445±38[a]
1	687±8 (12.8)	350±41 (31.4)
2	509±8[b] (35.7)	310±1[b] (44.7)
3	231±6[c] (71.5)	284±2[c] (53.3)
4	546±2[b] (30.9)	323±23 (40.4)
5	584±10 (26.1)	332±17 (37.4)
6	638±8[b] (19.1)	338±12 (35.4)

Values represent mean±S.E. of three independent experiments. The values in parentheses are the inhibition rates (%). *a*) Significantly different from the spontaneous group, $p<0.05$. *b*) Significantly different from the NPD- or NaN_3-treated group, $p<0.05$. *c*) Significantly different from the NPD- or NaN_3-treated group, $p<0.01$.

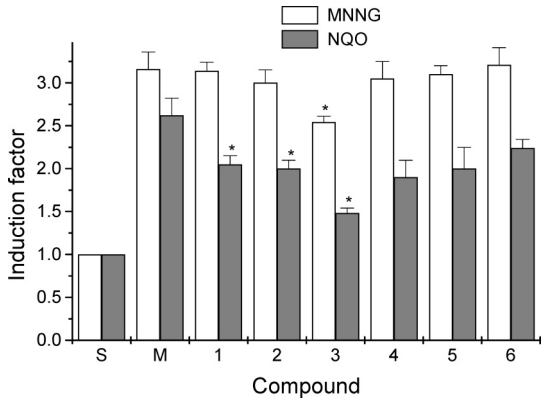

Fig. 2. Antigenotoxic Activity of the Tested Components against the Mutagens, MNNG and NQO (Negative Controls) Using *E. coli* PQ37 as a Test Strain

Level of statistical significance: $*p<0.05$ with respect to mutagen values from three experiments. S: spontaneous, M: mutagen.

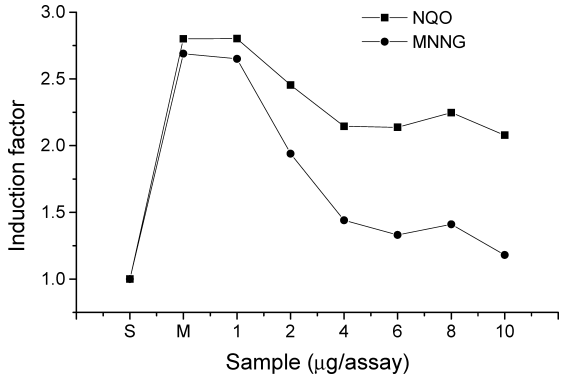

Fig. 3. Effect of Concentration of Emodin on Induction Factor of SOS Chromotest

S: spontaneous, M: mutagen.

plied to mammals as an anticancer agent, but should further be estimated in an *in vivo* test using animal models to prove their clinical values.

In conclusion, four known anthraquinones were isolated for the first time from the aerial parts of *Rumex acetosa* and investigated their cytotoxicities and antimutagenicities together with two synthetic analogues in a view of the structure–activity correlation, which are the new results on the *in vitro* cytotoxicity against five tumor cell lines and the antimutagenicity using *S. typhimurium* TA98 and TA100 against mutagens NPD and NaN_3, also the antigenotoxicity using *E. coli* PQ37 against mutagens MNNG and NQO. Among the tested compounds, emodin showed the potent cytotoxic and antimutagenic effects, which need further studies for its clinical application.

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