Antiproliferative Constituents in the Plant 8. Seeds of *Rhynchosia volubilis*[1)]

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The MeOH extract of the seeds of *Rhynchosia volubilis* (Leguminosae) showed antiproliferative activity against human gastric adenocarcinoma [MK-1, 50% growth inhibition (GI_{50}): 25 μg/ml], human uterus carcinoma (HeLa, GI_{50}: 30 μg/ml), and murine melanoma (B16F10, GI_{50}: 8 μg/ml) cells. Bioactivity-guided fractionation resulted in the isolation of gallic acid methylester (1), gallic acid (2), 7-*O*-galloylcatechin (3), 1,6-di-*O*-galloylglucose (4), 1-*O*-galloylglucose (5), and trigalloylgallic acid (6), and their antiproliferative activity was estimated. All showed much stronger inhibition against B16F10 cell growth than against HeLa and MK-1 cell growth. Compound 2 and its tetramer (6) with a free carboxyl group showed higher activity than those which did not have a free carboxyl group. In relation to the gallic acid tetramer (6), two gallic acid dimers (ellagic acid and dehydrodigallic acid) and trimers (tergallic acid dilactone and flavogallonic acid dilactone) were tested for their activity, and compared with those of the isolates.

Key words *Rhynchosia volubilis*; antiproliferative activity; polyphenol; gallic acid and related compound

The plants of *Rhynchosia genus* (Leguminosae) are herbaceous, commonly climbing or trailing, sometimes woody, with a woody rootstock.[2)] There are two hundred species throughout the tropics and subtropics with the greatest number in Africa. Some species have attractive seeds, which have been used as beads. The seeds of *R. volubilis* LOUR. have been used as an expectorant in Japanese folk medicine (Tankiri-Mame).

In the course of our continuing studies on the antiproliferative principles of higher plant origin, we found that the MeOH extract of these seeds inhibits the growth of human gastric adenocarcinoma [MK-1, 50% growth inhibition (GI_{50}): 25 μg/ml], human uterus carcinoma (HeLa, GI_{50}: 30 μg/ml), and murine melanoma (B16F10, GI_{50}: 8 μg/ml) cells. These results led to our interest in the investigation of the chemical constituents of the MeOH extract. This paper deals with the isolation and identification of the active constituents.

MATERIALS AND METHODS

Instruments and Reagents The instruments and reagents used in this study are the same as those described in the previous paper.[3)]

Materials The seeds of *R. volubilis* were collected from Ogawa Island, Saga Prefecture, Japan, in October, 1999. The test samples of ellagic acid , dehydrodigallic acid, flavogallonic acid dilactone, and tergallic acid dilactone were those previously isolated by two authors (Tanaka and Nonaka) from *Nuphar japonicum*[4)] and *Mallotus japonicus*.[5)]

Extraction and Isolation The seeds (323 g) were powdered and extracted with MeOH under reflux. The extract was partitioned between EtOAc and 40% MeOH. After evaporation, the 40% MeOH extract was subjected to Diaion HP-20 column chromatography using H_2O and MeOH. Both fractions were further purified by Sephadex LH-20 (50% MeOH, MeOH) chromatography, and the antiproliferative activity was monitored. The MeOH fraction (1.0 g) provided gallic acid methylester (**1**) (130 mg) and 1,6-di-*O*-galloylglucose (**4**) (30 mg), and from the H_2O fraction (7.0 g), gallic acid (**2**) (140 mg) and 1-*O*-galloylglucose (**5**) (70 mg) were isolated. The EtOAc layer was concentrated and the extract (14 g) was subjected to Sephadex LH-20 column chromatography ($CHCl_3$: MeOH : H_2O = 5 : 5 : 1) to afford four fractions (Fr. I—IV). Since the latter three fractions showed antiproliferative activity, they were further purified by the same resin chromatography using different solvent systems. Compound **5** (240 mg) from Fr. II (1.4 g, solvent; 50%MeOH), compounds **1** (2.4 g), **2** (570 mg) and **5** (130 mg) from Fr. III (4.4 g, solvent; MeOH), and trigalloylgallic acid (**6**) (50 mg) and 7-*O*-galloylcatechin (**3**) (280 mg) from Fr. IV (0.4 g, solvent; EtOH) were isolated. Compounds **1**—**6** were identified as gallic acid methylester, gallic acid,[6)] 7-*O*-galloylcatechin,[7)] 1,6-di-*O*-galloylglucose,[6)] 1-*O*-galloylglucose,[8)] and trigalloylgallic acid,[9)] respectively, by comparison of their physical data with those reported.

Cells MK-1 was provided from Dr. M. Katano, Faculty of Medicine, Kyushu University. HeLa and B16F10 were supplied from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University.

Determination of Antiproliferative Activity The inhibition of the cellular growth was estimated using the MTT assay described by Mosmann.[10)] The detailed procedure is shown in the previous paper.[11)]

RESULTS AND DISCUSSION

Bioactivity-guided fractionation of the MeOH extract of the seeds of *Rhynchosia volubilis* has resulted in the isolation of six polyphenols, **1**—**6** (Fig. 1).

The antiproliferative activity of each isolate was determined by the MTT assay, and their GI_{50} values are listed in Table 1.

All isolates inhibited the growth of three tumor cells, and were more potent against B16F10 than against HeLa and MK-1. Compound **6** showed the highest activity of all, and **2** followed next. The activity of **1** and three other esters (**3**—**5**) were less potent than that of **2**. Esterification of the carboxyl

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group of gallic acid reduced the activity. A similar result has already been reported against rat hepatoma (dRLh-84) cells by Inoue *et al*.[12] We have also observed the phenomenon that the antiproliferative activity of caffeic acid is lowered by esterification.[1]

In spite of structural differences, **5** and **3** showed similar activity. Since the activity of catechin was less potent than that of **3** (not shown), the presence of the galloyl group would be a key factor for antiproliferative activity.

The GI_{50} values of **4** are slightly smaller than those of **5**. Furthermore, the GI_{50} value of **6**, which was a tetramer of **2**, was about one-half that of **2**. These data suggest that the number of the galloyl group would be one factor for enhancing the activity, although not directly proportional.

Inoue *et al*. elucidated that three adjacent phenolic hydroxyl groups of gallic acid should be essential to the cytotoxicity, since the methylation (syringic acid, 4-*O*-methyl gallate) or deletion (protocatechuic acid, 3,5-dihydroxybenzoic acid, *p*-hydroxybenzoic acid) of phenolic hydroxyl groups completely removed the cytotoxicity. And a carboxyl group is likely to be necessary in order for gallic acid to show selectivity to cancer cells, since pyrogallol killed not only dRLh-84 cells, but also primary cultured rat hepatocytes.[12] Furthermore, they also disclosed that the cell death induced by gallic acid was accompanied by internucleosomal DNA fragmentation characteristic of apoptosis.[13]

In order to clarify in more detail the structure-antiproliferative activity relationship of gallic acid derivatives, we tested for the antiproliferative activity of ellagic acid and three related phenol carboxylic acids (Fig. 2) which are the non-saccharide parts of hydrolyzable tannins. We checked the activity of two dimers of gallic acid, ellagic acid (EA) and dehydrodigallic acid (DA), and two trimers, flavogallonic acid dilactone (FAD) and tergallic acid dilactone (TAD). All inhibited B16F10 cell growth, were less active against MK-1 cells, and almost inactive toward HeLa cells.

Against our first conjecture that the number of the galloyl group and the presence/absence of the free carboxyl group influenced the activity, EA, a gallic acid dimer with neither a free carboxyl group nor three adjacent phenolic hydroxyl groups, showed almost similar activity to that of **2** against B16F10 cells, and the activity of the other three derivatives with the free carboxyl group were less than EA, except against HeLa cells.

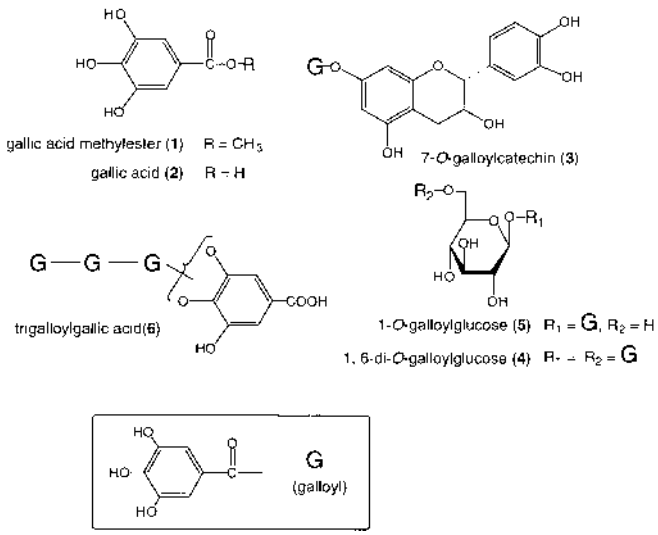

Fig. 1. Antiproliferative Polyphenols Isolated from *Rhynchosia volublis*

Table 1. Antiproliferative Activity (GI_{50}, μM) against B16F10, HeLa and MK-1 Cell Lines

Compounds	B16F10	HeLa	MK-1
1	18	43	65
2	7.1	22	19
3	9.0	38	41
4	8.1	29	39
5	15	45	60
6	2.9	9.3	10
EA	10	>100 μg/ml	43
DA	12	89	47
FAD	26	>100 μg/ml	110
TAD	17	117	53

Values are the means of four determinations. EA: ellagic acid, DA: dehydrodigallic acid, FAD: flavogallonic acid dilactone, TAD: tergallic acid dilactone.

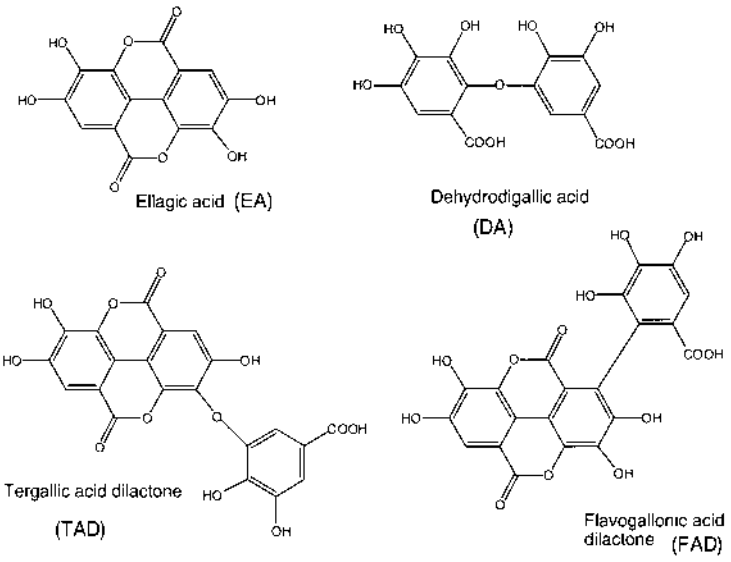

Fig. 2. Ellagic Acid and Related Phenol Carboxylic Acids

There might be some factors other than the numbers of the galloyl group and the presence/absence of the free carboxyl group for polyphenols to exhibit antiproliferative activity. Since Inoue *et al*. reported that the mechanism of apoptosis by **2** was different than that by its ester derivative (GD-1: 3,4-methylenedioxyphenyl 3,4,5-trihydroxybenzoate),[13,14] it would be necessary to accumulate the activity data of a variety of polyphenols in order to discuss the structure-activity relationship.

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