

Antiproliferative Activity of the Main Constituents from *Phyllanthus emblica*

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Eighteen main compounds, including four norsesquiterpenoids (1–4) and 14 phenolic compounds (5–18) isolated previously from *Phyllanthus emblica*, together with a main constituent, proanthocyanidin polymers (19) identified at this time from the roots, were estimated for their antiproliferative activities against MK-1 (human gastric adenocarcinoma), HeLa (human uterine carcinoma), and B16F10 (murine melanoma) cells using an MTT method. All of the phenolic compounds including the major components 5–8 from the fruit juice, 8, 9, and 12 from the branches and leaves, and 19 from the roots showed stronger inhibition against B16F10 cell growth than against HeLa and MK-1 cell growth. Norsesquiterpenoid glycosides 3 and 4 from the roots exhibited significant antiproliferative activities, although their aglycon 1 and monoglucoside 2 showed no inhibitory activity against these tumor cells.

Key words *Phyllanthus emblica*; Euphorbiaceae; antiproliferative activity; MK-1 cell; HeLa cell; B16F10 cell

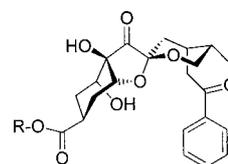
The plants of the genus *Phyllanthus* (Euphorbiaceae) are widely distributed throughout tropical and subtropical countries. They have been used in China, India, the Philippines, Nigeria, East and West Africa, the Caribbean, and Central and South America by traditional medicinal practitioners for the treatment of different types of diseases.^{1,2} Several therapeutic properties such as antipyretic, antibacterial, antiparasitic, antinociceptive, and antiviral activities have been attributed to this genus.³ Crude extracts of species such as *Phyllanthus amarus* and *Phyllanthus emblica* have been reported to provide antioxidant and/or antigenotoxic protection.^{2,4} Moreover, phyllanthostatins 1–6, which exhibited potent antineoplastic activity, have been isolated from the roots of *Phyllanthus acuminatus*.⁵

P. emblica L. is a shrub or tree growing in the tropical and subtropical parts of Southeast Asia, particularly in southern China, India, Indonesia, and the Malay Peninsula. It has been used widely for its antiinflammatory and antipyretic effects in many local traditional medicinal systems, such as Chinese herbal medicine, Tibetan medicine, and Ayurvedic medicine. The fruit is edible and the fruit juice is produced as a beverage in Yunnan Province, People's Republic of China. The air-dried fruit is also used for the treatment of cancer in Tibetan and Egyptian medicines.⁶ The active principles or extracts of *P. emblica* have been shown to possess several pharmacologic actions, e.g., antitumor, and cytotoxic activities.^{2,7}

Our previous chemical investigation of this plant led to the isolation of several novel sesquiterpenoids from the roots,^{8,9} new organic acid gallates and polyphenols from the fruit juice,^{10,11} and new ellagitannins and flavonoids from the branches and leaves.^{11,12} Among them, phyllaemblic acid (1) and its glycosides phyllaemblicins A–C (2–4) were the major sesquiterpenoids from the roots, and organic acid gallates, L-malic acid 2-*O*-gallate (5), and mucic acid 2-*O*-gallate (6) together with hydrolysable tannins, 1-*O*-galloyl- β -D-glucose (7), corilagin (8), and chebulagic acid (9) were found to be the major phenolic constituents of fruit juice. Elaecarpusin (10) and putranjivain A (11) were the other two main

ellagitannins obtained from the fruit juice. Moreover, seven other tannins and flavonoids, geraniin (12), phyllanemblinins C and E (13 and 14), prodelphinidin B₁ (15), prodelphinidin B₂ (16), (–)-epigallocatechin 3-*O*-gallate (17), and (*S*)-eriodictyol 7-[6-*O*-(*E*)-*p*-coumaroyl]- β -D-glucoside (18) were the main phenolic compounds isolated from the branches and leaves of the plant (Figs. 1, 2). Among these compounds, 1–6, 13, 14, and 18 are newly isolated from *P. emblica*.

Continuous studies on the 60% aqueous acetone extract of the roots of *P. emblica* led to the isolation of a major component, proanthocyanidin polymer phyllemtannin (19). Although many studies have so far been carried out on the chemical components of *P. emblica*,^{13–18} its detailed active principles are generally not well known. This paper deals with the inhibitory activity of 19 together with the 18 main constituents 1–18 isolated previously from *P. emblica* on the proliferation of B16F10 (murine melanoma), HeLa (human uterine carcinoma), and MK-1 (human gastric adenocarcinoma) cells to determine their roles in the anticancer uses of this plant. The determination of proanthocyanidin polymer, phyllemtannin (19), from the roots is also described.



Phyllaemblic acid (1) R=H
Phyllaemblicin A (2) R=glc
Phyllaemblicin B (3) R=glc²-glc
Phyllaemblicin C (4) R=glc²-glc²-ara

Fig. 1. Structures of Norsesquiterpenoids 1–4

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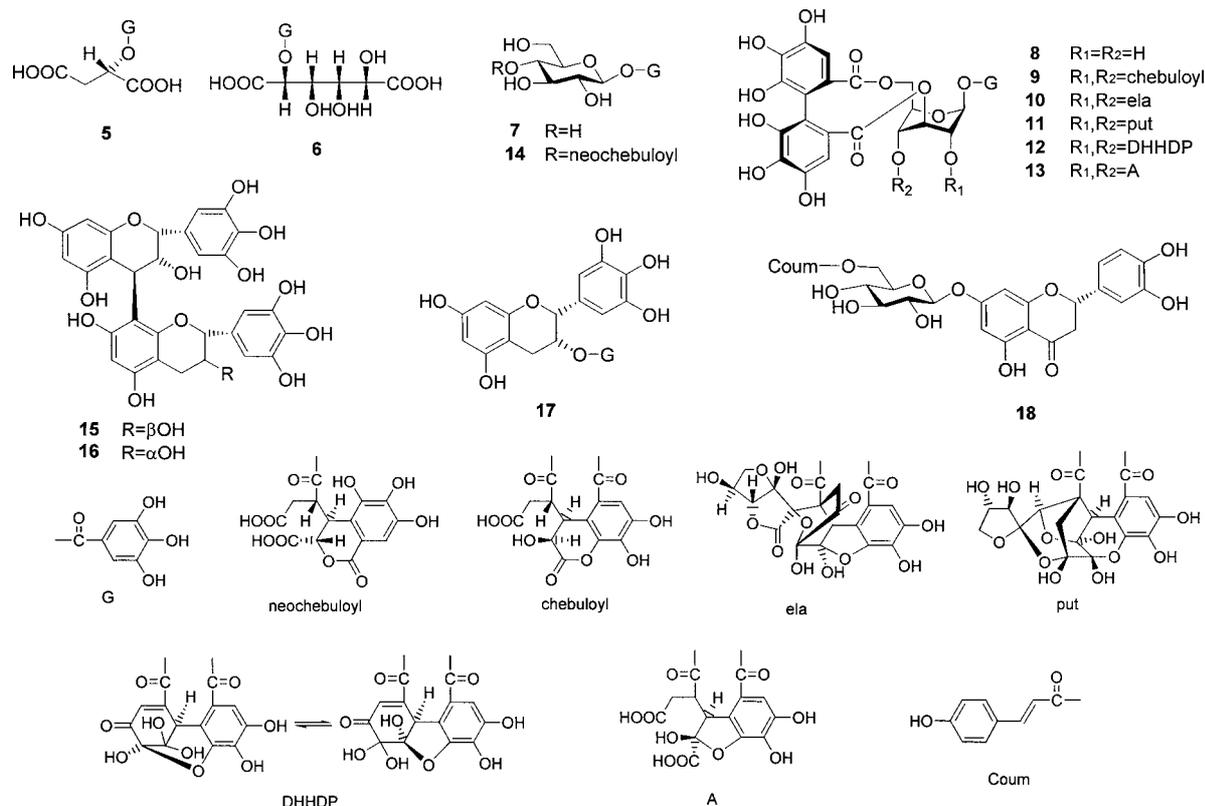


Fig. 2. Structures of Polyphenolic Compounds 5—18

RESULTS AND DISCUSSION

Identification of Phyllemntannin (19) The 60% aqueous acetone extract of the air-dried roots of *P. emblica* was extracted successively with EtOAc and *n*-BuOH, followed by repeated reprecipitation of the water layer with H₂O/MeOH to give a tannin fraction designated as phyllemntannin (19).

Phyllemntannin (19), a brown amorphous powder, is highly astringent and strongly positive (a dark blue spot) to the FeCl₃ reagent. It could not be developed with the benzene/ethyl formate/formic acid system by TLC analysis. Treatment of 19 with ethanolic HCl gave a deep red color, which may be attributable to the formation of an anthocyanidin pigment,¹⁹ thus suggesting 19 is a proanthocyanidin polymer. The IR spectrum of 19 showed strong absorption bands due to ester (1680—1700 cm⁻¹) and hydroxyl (3200—3400 cm⁻¹) groups.

To determine the structure of 19, the thiol-promoted degradation reaction using mercaptoethanol was carried out, followed by HPLC analysis. When 19 was treated with mercaptoethanol in the presence of acetic acid,¹⁹ it yielded catechin (19a), epicatechin (19b), epigallocatechin (19c), epicatechin 4-(2-hydroxyethyl)thio ether (19d), and epigallocatechin 4-(2-hydroxyethyl)thio ether (19e), while in the presence of HCl it gave 19a—e, and galocatechin (19f), epicatechin 3-*O*-gallate 4-(2-hydroxyethyl)thio ether (19g), and epigallocatechin 3-*O*-gallate 4-(2-hydroxyethyl)thio ether (19h)²⁰ as the products (Fig. 3). Products 19a—c and 19f were derived from the lower terminal unit of the polymer, and 4-(2-hydroxyethyl)thio ethers, 19d, 19e, 19g, and 19h were formed from the extension unit (upper unit). From these spectral and chemical findings, 19 is suggested to be a mix-

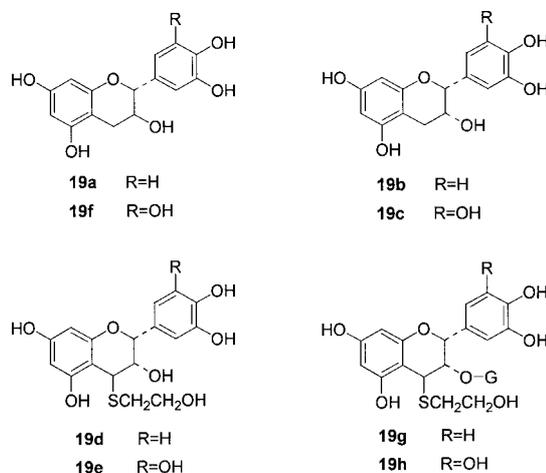


Fig. 3. Structures of the Thiol-Promoted Degradation Products (19a—h) of 19

ture of proanthocyanidin polymers composed of epicatechin, epigallocatechin, epicatechin 3-*O*-gallate, and epigallocatechin 3-*O*-gallate in the chain extension part, and of catechin, epicatechin, galocatechin, and epigallocatechin units in the chain termination part. Furthermore, judging from the occurrence of 4,8'-linked proanthocyanidin dimers in the root, 19 would be expected to consist predominantly of C₄ to C₈-linked epicatechin, epicatechin 3-*O*-gallate or epigallocatechin 3-*O*-gallate polymers with catechin, epicatechin, galocatechin, or epigallocatechin units in the terminal portion (Fig. 4). The different thiol-promoted degradation results of 19 revealed that the cleavage ability of acetic acid and HCl is different and the interflavanoid bonds of proanthocyanidin

oligomers or polymers with the galloyl group in the molecule could not be cleaved in the presence of acetic acid.

Antiproliferative Activity of Compounds 1–19 Compounds 1–19, as the main constituents obtained at this time or previously from *P. emblica*,^{8,10–12} were divided into four groups based on their structural characters, and their yields from the plant are listed in Table 1. Among them, 19 was the major component and 1–4 were the main sesquiterpenoids of the root. Compounds 5–9 were the major phenolic compounds of the fruit juice, while 8, 9, and 12 were the major tannins of the branches and leaves. Compounds 7, 9, and 11 were obtained from both fruit juice and the branches and leaves, and 8 was found in all the three parts (fruit juice, roots, and branches and leaves) of the plant.

The antiproliferative activity of these main constituents (1–19) was determined by a 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, using three tumor cell lines, MK-1, HeLa, and B16F10 cells. Their 50%

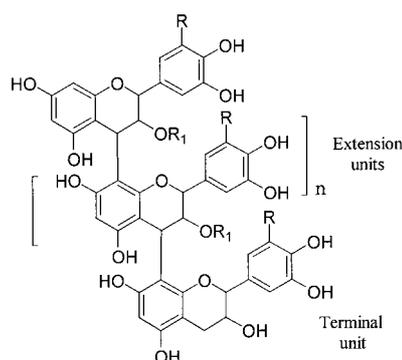


Fig. 4. Structure of 19

growth inhibition (GI_{50} , $\mu\text{g/ml}$) values are shown in Table 1.

Norsesquiterpenoid glycosides, phyllaemblicins B (3) and C (4), exhibited significant inhibitory effects on B16F10 (GI_{50} at 2.0, 3.5 $\mu\text{g/ml}$, respectively), HeLa (GI_{50} at 3.0, 12.0 $\mu\text{g/ml}$, respectively), and MK-1 (GI_{50} at 7.0 $\mu\text{g/ml}$ for both compounds) cell proliferation. However, their aglycon (1) and monoglucoside (2) showed no cytotoxicity at a concentration lower than 100 $\mu\text{g/ml}$. Norsesquiterpenoids 1–4 possessed a highly oxygenated norbisabolane skeleton, which is similar to those of the potent antineoplastic phyllanthostatin 1–6, the less polar bisabolane glycosides isolated previously from *P. acuminatus* by Pettit and coworkers.⁵ The different inhibitory effects of 1–4 on the growth of tumor cells revealed that the sugar moieties in the norbisabolane molecule should play an important role in their activities.

All phenolic compounds 5–19 showed inhibitory activity against the growth of the three tumor cell lines at a concentration of less than 68 $\mu\text{g/ml}$ and were more potent against B16F10 than against HeLa and MK-1 cells (Table 1). Among them, the major phenolic compounds corilagin (8) and geraniin (12) as well as hydrolysable tannin elaeocarpusin (10) and condensed tannin prodelphinidins B₁ and B₂ (15, 16) showed the highest activity (GI_{50} at 2 $\mu\text{g/ml}$ for 12, 15, 16, and 3 $\mu\text{g/ml}$ for 8, 10) against B16F10, while 15 and 16 had the highest inhibitory activity (GI_{50} 9 $\mu\text{g/ml}$) against HeLa, and (–)-epigallocatechin 3-*O*-gallate (17), known as the active constituent in green tea, showed the highest activity (4 $\mu\text{g/ml}$) against MK-1.

Compounds 5–7, 9–17, and 19, with a galloyl or pyrogallol group in the molecule, showed higher activity than the flavonoid 18. These data are consistent with the previous reports and suggest that three adjacent hydroxyl groups (gal-

Table 1. Yields of Compounds 1–19 from *P. emblica* and Their Inhibitory Activity against the Growth of Tumor Cells (GI_{50} , $\mu\text{g/ml}$)

Test sample	Roots ^{a)} (%)	Fruit juice ^{b)} (%)	Leaves and branches ^{c)} (%)	B16F10	HeLa	MK-1
Positive control						
Cisplatin				4.5	0.8	5
5-Fluorouracil				0.4	3.1	12
Norsesquiterpenoids						
Phyllaemblic acid (1)	0.043	—	—	>100	>100	>100
Phyllaemblicin A (2)	0.002	—	—	>100	>100	>100
Phyllaemblicin B (3)	0.0054	—	—	2	3	7
Phyllaemblicin C (4)	0.018	—	—	3.5	12	7
Organic acid gallates						
L-Malic acid 2- <i>O</i> -gallate (5)	—	0.092	—	12	20	35
Mucic acid 2- <i>O</i> -gallate (6)	—	0.16	—	15	24	41
Hydrolyzable tannins						
1- <i>O</i> -Galloyl- β -D-glucose (7)	—	0.39	0.0015	5	12	20
Corilagin (8)	0.0015	0.17	0.03	3	19	8
Chebulagic acid (9)	—	0.2	0.066	8	15	21
Elaeocarpusin (10)	—	0.013	—	3	12	11
Putranjivain A (11)	—	0.002	0.0026	7	15	25
Geraniin (12)	—	—	0.023	2	11	9
Phyllanemblinin C (13)	—	—	0.0013	6	12	20
Phyllanemblinin E (14)	—	—	0.0018	11	32	45
Flavonoids and condensed tannins						
Prodelphinidin B ₁ (15)	—	—	0.0004	2	9	8
Prodelphinidin B ₂ (16)	—	—	0.0002	2	9	9
Epigallocatechin 3- <i>O</i> -gallate (17)	0.0013	—	0.0002	5	17	4
(<i>S</i>)-Eriodictyol 7-(6'- <i>O</i> - <i>trans</i> -coumaroyl)- β -D-glucoside (18)	—	—	0.0011	16	30	68
Phyllemntannin (19)	1.84	—	—	11	26	30

a) Air-dried roots (8 kg). b) Fruit juice powder (960 g). c) Fresh leaves and branches (15 kg). —, not isolated. GI_{50} values are the means of four determinations.

loyl or pyrogallol group) in the molecule is a key factor for enhancing the activity.^{21,22} Moreover, **5**—**7** are all simple structures with the presence of a galloyl group in the molecule; however, the activity of **7** with glucose in the molecule was much higher than that of **5** and **6**. The results suggest that there might be some factors other than the presence of a galloyl group for polyphenols to exhibit antiproliferative activity.

In this work, the antiproliferative activity of 19 main constituents from different parts of *P. emblica* was determined against MK-1, HeLa, and B16F10 cells. L-Malic acid 2-*O*-gallate (**5**), mucic acid 2-*O*-gallate (**6**), 1-*O*-galloyl- β -D-glucose (**7**), corilagin (**8**), and chebulagic acid (**9**) in the fruit juice exhibited certain levels of cytotoxicity against the tumor cells. The antiproliferative activity of these compounds against B16F10 cells, some of which were comparable with those of the positive controls, was stronger than against that HeLa and MK-1 cells. These major components are possibly responsible for the folk anticancer uses of this plant. Moreover, the norsesquiterpenoid glycosides phyllaemblicins B (**3**) and C (**4**) showed significant antiproliferative activity against tumor cells, even though their yields from the roots were not high. These compounds are worthy of consideration as a potential cancer chemopreventive and/or anticarcinogenic agents after additional biological evaluation *in vivo*.

Research showed that the antitumor activity of polyphenols might be linked to their antiinflammatory properties.²³ Since *P. emblica* has been used widely for its antiinflammatory and antipyretic effects by local people in its growing areas, we have also investigated the inflammatory and antioxidative activity of the polyphenols isolated from this plant. The results will be published elsewhere.

MATERIALS AND METHODS

IR spectra were measured with a JASCO FT/IR-410 spectrometer. Thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F254 plates, 0.2 mm thick (Merck), with benzene/ethyl formate/formic acid (1 : 7 : 1, v/v), and spots were detected by spraying with 2% ethanolic FeCl₃, anisaldehyde-H₂SO₄, or 10% sulfuric acid reagents followed by heating. Analytical high-pressure liquid chromatography (HPLC) was performed on a Cosmosil 5C₁₈-AR II, 250×4.6 mm i.d. column (Nacalai Tesque, Kyoto, Japan) with gradient elution from 10 to 20% (30 min) of CH₃CN in 50 mM H₃PO₄ at a flow rate of 0.8 ml/min, equipped with a JASCO UV-970 detector and a JASCO 880-PU HPLC pump.

Chemicals and Materials Compounds **1**—**18** were respectively isolated from air-dried roots (8.0 kg), powdered fruit juice (960 g), and fresh branches and leaves (15 kg) of *P. emblica* as described in detail elsewhere.^{8,10—12}

The materials and reagents used for the bioassay were described in a previous paper.²⁴

Isolation of 19 As described in a previous paper, the 60% aqueous acetone extract (450 g) of air-dried roots (8.0 kg) of *P. emblica* was suspended in H₂O and successively partitioned with EtOAc and *n*-BuOH.⁹ The water layer was concentrated and filtered to obtain a filtrate and a precipitate, and the latter was repeatedly reprecipitated with H₂O-MeOH to give a brown amorphous powder (**19**) (147.5 g). IR [diffu-

sive reflection (DR)] ν_{\max} cm⁻¹: 3200—3400 (OH), 1680—1700 (—COO—).

Thiol-Promoted Degradation of 19 with Mercaptoethanol-CH₃COOH A mixture of **19** (5.0 g), mercaptoethanol (10 ml), CH₃COOH (10 ml), and EtOH (100 ml) was stirred at 50 °C for 1 h. After removing the solvent, the reaction mixture was applied to a Sephadex LH-20 (60→100% MeOH) column to afford a fraction (0.22 g) positive to iron(III) chloride and anisaldehyde-sulfuric acid reagents, which was analyzed by reverse-phase HPLC. The peaks corresponding to catechin (**19a**) (13.26 min), epicatechin (**19b**) (16.48 min), epigallocatechin (**19c**) (11.1 min), epicatechin 4-(2-hydroxyethyl)thio ether (**19d**) (20.8 min), and epigallocatechin 4-(2-hydroxyethyl)thio ether (**19e**) (15.55 min) were detected and confirmed by co-HPLC with authentic samples.

Thiol-Promoted Degradation of 19 with Mercaptoethanol-HCl A mixture of **19** (0.01 g), mercaptoethanol (1 ml), concentrated HCl (0.5 ml), and EtOH (5 ml) was heated at 50 °C for 1 h. As described above, the reaction mixture was applied to a Sephadex LH-20 column and then analyzed by reverse-phase HPLC. The peaks corresponding to **19a** (13.23 min), **19b** (16.11 min), **19c** (10.78 min), **19d** (20.31 min), **19e** (15.32 min), galocatechin (**19f**) (7.90 min), epicatechin 3-*O*-gallate 4-(2-hydroxyethyl)thio ether (**19g**) (26.46 min), and epigallocatechin 3-*O*-gallate 4-(2-hydroxyethyl)thio ether (**19h**) (20.03 min) were detected and confirmed by co-HPLC with authentic samples.

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

Measurement of Antiproliferative Activity against Tumor Cell Lines The inhibition of the cellular growth was determined using an MTT assay on 96-well microplates as described by Mosmann.²⁵ The detailed procedure was described in a previous paper.¹⁸ The GI₅₀ values (μ g/ml) are the means of four determinations. The antitumor drugs cisplatin and 5-fluorouracil were used as the positive controls.

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