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Antioxidant Activity, Cytotoxicity, and DNA Information of *Glossogyne tenuifolia*

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This study investigates the antioxidant activity and cytotoxicity of *Glossogyne tenuifolia* extract on various cancer cell lines. The 5.8s DNA of *G. tenuifolia* was isolated, and the species of this plant was confirmed by NCBI's DNA database. *G. tenuifolia* was then extracted with ethanol and separated into several fractions using the partition procedure with water, *n*-butanol, and ethyl acetate (EA). Among these, the EA fraction most significantly affected the activity of DPPH• and superoxide anion scavenging. Additionally, only the EA fraction exhibited cytotoxicity on breast cancer cells (MCF-7 and MDA-MB-231) and liver cancer cells (Hep G2 and Hep 3B). Next, the EA fraction was further separated by column chromatography, and 15 fractions were obtained. Three effective components were isolated and identified separately from the active fractions: oleanolic acid (OA) from fraction 6, luteolin from fractions 8−10, and luteolin-7-glucoside from fraction 12. The test of these three compounds on scavenging activity of DPPH• and superoxide anion indicates that luteolin had the highest antioxidant activity, whereas the effect of OA was negligible. Additionally, a synergistic effect between luteolin and luteolin-7-glucoside was observed. Kick-out experiments showed that the activities were vanished or decreased. Especially on MDA-MB-231 and MCF-7 cells, the cytotoxicity completely disappeared when luteolin was eliminated from fractions 8−10. These findings demonstrate that luteolin plays a crucial role in the inhibition of the growth of hepatoma cancer cell lines. Fraction 3, which did not contain luteolin, luteolin-7-glucoside, and oleanolic acid, had cytotoxicity on MDA-MB-231, MCF-7, Hep G2, Hep 3B, and A549, which implies that this fraction contained some other effective ingredients and requires further study. The investigation is currently underway in our laboratory.

KEYWORDS: ***Glossogyne tenuifolia*; flavonoids; antioxidant activity; cytotoxicity**

INTRODUCTION

Flavonoids are a diverse group comprising more than 4000 compounds, mostly isolated from plants, including vegetables, fruits, and herbal medicines. Many studies have indicated that flavonoid compounds, such as quercitin, luteolin, rutin, and catechin, are good free radical scavengers (*1*−*3*) and, thus, may be employed as healthy food ingredients to prevent inflammation and some diseases including tumors, cancer, coronary heart disease, and thyroid disease (*4*−*7*).

Several investigations have indicated that the phenolic compounds in vegetables, fruits, and herbal medicines can scavenge free radicals (*2*, *8*−*10*). Such compounds generally have a certain effective dosage for scavenging free radicals. If any other compounds with similar scavenging effects are added simultaneously, they may have a synergistic effect; that is, the combination of different compounds can strengthen or weaken their effects as compared with that of one compound alone. Such synergistic effects were detected in different antioxidants including tocopherol, ascorbic acid, proanthocyanidins, flavonoids, herbal medicines (*11*−*13*), and affected antioxidant activity, anticancer activity, proliferation inhibition, cytotoxicity, and apoptosis of human tumor or cancer cell lines (*13*−*16*).

Glossogyne tenuifolia is a perennial herb with stems woody at the base, 20−30 cm long, and somewhat tufted. Radical leaves are persistent, glabrous, 4.5−9.0 cm long, somewhat undivided and linear, and generally pinnately parted, with segments of two or three pairs. The plant is distributed mainly in southern Asia, Australia, and New Caledonia, as well as in exposed coastal areas, occasionally on raised coral reefs, and in southern Taiwan. The plant's origin is Penghu Island, which is a small island in Taiwan (*17*). *G. tenuifolia* is used to make a traditional healthy food and drink consumed in Penghu. Wu et al. (*18*) isolated some effective components from *G. tenuifolia* and

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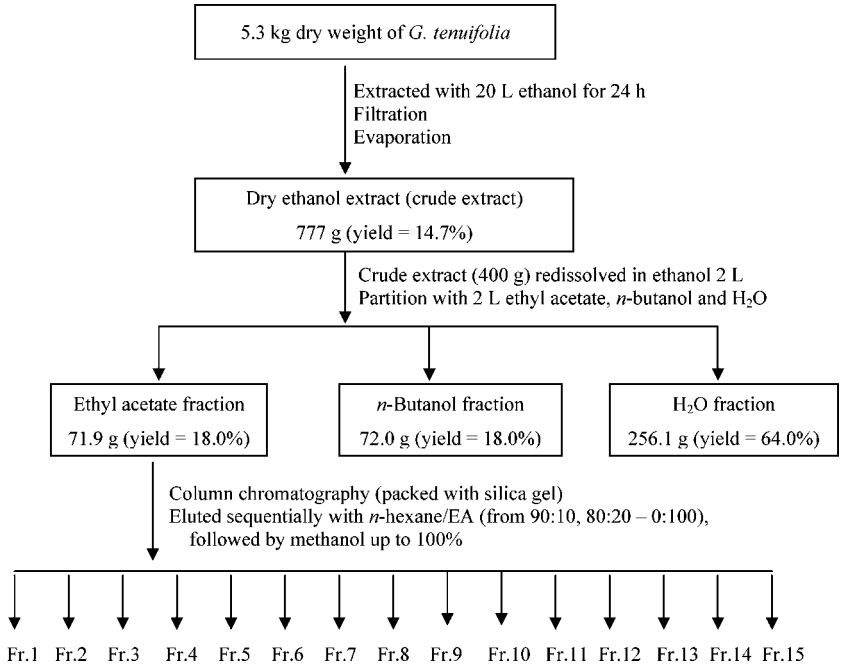

Figure 1. Flowchart of the extractive operation of *G. tenuifolia*.

investigated the actions of these compounds on the inhibition of inflammatory mediator production in vitro. Additionally, to our knowledge, phytochemical components responsible for its activity and even their mechanism of certain actions have not been examined. Therefore, the study of the bioactivity of *G. tenuifolia* is still in its preliminary stage.

Due to the popularity of *G. tenuifolia* among the Taiwanese, this work attempts to develop it as a healthy food. The herb's potential for chemical prevention, cancer prevention, and cytotoxicity are considered in the experiments. This study examines the antioxidant activity and the cytotoxicity of *G. tenuifolia* extract on various cancer cell lines with the bioactivity-directed fractionation and isolation (BDFI) approach. The antioxidant activity is quantified from the scavenging ability of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) and superoxide anion by *G. tenuifolia* extracts. The cytotoxicity on various cancer cells is investigated upon the inhibition of cell proliferation on MDA-MB-231, MCF-7 (breast cancer), Hep G2, Hep 3B (hepatoma), and A549 (lung cancer) cell lines. The distribution of bioactivity of these plant extracts is also discussed.

MATERIALS AND METHODS

Materials and Chemicals. The raw materials of *G. tenuifolia* were bought from an herb store in Penghu Island and then later recognized by DNA sequence identification. DPPH, α-tocopherol, (+)-catechin, nitroblue tetrazolium (NBT), hypoxanthine (HPX), and xanthine oxidase (XOD) were purchased from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were of reagent or analytical grade.

Preparation of *G. tenuifolia* Extracts. Dry whole plant materials of *G. tenuifolia* (5.3 kg) were crushed and drenched in 20 L of ethyl alcohol for 1 day and then extracted three times with 20 L of ethyl alcohol. The flowchart of the operation is shown in **Figure 1**. After filtration by medical gauze, the filtrates were collected and concentrated with a vacuum evaporator. The weight of crude extract was 777 g, and the yield was ~14.7%. The crude extract (400 g) was further portioned by ethyl acetate (EA), *n*-butanol, and water. The dry weights of water, *n*-butanol, and EA fractions were 256.1, 72.0, and 71.9 g. Accordingly, the yields of these three fractions from the crude extract were 64.0, 18.0, and 18.0%, respectively. On the basis of the BDFI results, the EA fraction (61.5 g) was further separated by column chromatography [760 mm length, 120 mm diameter, packed with 2.5 kg silica gel (Si-60, 40−63 μm, Merck Co.)]. The samples were eluted sequentially with a 1.5 L gradient of *n*-hexane/EA (from 90:10, 80:20−0:100) and followed by methanol up to 100%. After the operation, 15 fractions were collected.

Ribosomal DNA Analysis. The genus of *G. tenuifolia* growing in Penghu was extracted and analyzed by a Polymerase Chain Reaction (PCR)-based phylogenetic inference proposed by Stacey and Isaac (*19*) with minor modifications. The ribosomal genome DNA sample was prepared by grinding the freshly freeze-dried whole plant (2−5 g) to a fine powder and then isolated by a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). The DNA concentration was determined by measuring the absorbance at 260 nm with a spectrophotometer (model DU-7400, Beckman Instruments, San Ramon, CA), and the purity of DNA was evaluated from the 260/280 nm UV absorption ratios. Pure DNA has an A_{260}/A_{280} ratio of 1.7−1.9.

The ITS-1 gene and the ITS-2 genes were used as the DNA amplification primers of *G. tenuifolia*. The sequence of the ITS-1 gene (262 bp) is as follows: 5′-TCGAAACCTGCGTAGCAGAACGACCAGCGAACAAGTAAGAACACCTGGCTTTGCGGGGGGGGTTTGAAGCATTTGCTTCAATCCTTGTAGAGCCTCGCCGATGCGCGTTCATTGTCGCCCTTTGGGGCGTCTTGAACGTCAAGTCGGCACAACGAACAACTCCGGCACAACACGTGCCAAGGACAACTAAACATAAAGGGTCGGTGTCATGATGCCCCGTCTACGGTGTGCGCATGGCAAGCGGCCTCTTTGTAAACCTAAAC-3′.

The sequence of the ITS-2 gene (233 bp) is as follows: 3′-CACGCATCACGTCGCCCCCACCAACCATCTCGTCTTGGGACGTGTTTTGGATTGGGGCGGATATTGGTCTCCCGTGCCCATAGGCATGGTTGGCCCAAACATGAGTCCCTTCGTGAGTGGCGCACGACTAGTGGTGGTTGATATGACTGTCTTCTCGTGTTGTGCGCTCGATTCATGCGGGTGAAGCTCTTTGAAGACCCTGATGCATTGTCTTGTGATGATGCTTCGATCGC-5′.

The DNA amplification was carried out in a PCR thermal cycler (Gene Amp PCR System 9600, Perkin-Elmer) following the LeRoy protocols (*20*) with minor alterations: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s, 65 °C for 30 s, 72 °C for 4 min, and finally incubation at 4 °C. The DNA amplified products were analyzed using polyacrylamide gel electrophoresis. The PCR fragment of ribosomal genome DNA had 763 bp, including the 18s DNA, ITS-1, the 5.8s DNA, ITS-2, and the 26s DNA (see **Figure 2**). These experimental results were compared with the DNA sequence of several plants in BLAST (the Basic Local Alignment Search Tool) software provided

18s

TTCCACTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAA

CCTGCGGAAGGATCATTGTCGAAACCTGCGTAGCAGAACGACCAGCGAACAAGTAAGAAC

ITS1

ACCTGGCTTTGCGGGGGGGGTTTGAAGCATTTGCTTCAATCCTTGTAGAGCCTCGCCGAT

GCGCGTTCATTGTCGCCCTTTGGGGCGTCTTGAACGTCAAGTCGGCACAACGAACAACTC

CGGCACAACACGTGCCAAGGACAACTAAACATAAAGGGTCGGTGTCATGATGCCCCGTCT

ACGGTGTGCGCATGGCAAGCGGCCTCTTTGTAAACCTAAACGACTCTCGGCAACGGATAT

CTCGGCTCACGCATCGATGAAGAACGTAGCAAAATGCGATACTTGGTGTGAATTGCAGAA

5.8s

TCCCGTGAACCATCGAGTTTTTGAACGCAAGTTGCGCCCGAAGCCATCCGGCTGAGGGCA

ITS2

CGTCTGCCTGGGAGTCACGCATCACGTCGCCCCCACCAACCATCTCGTCTTGGGACGTGT

TTTGGATTGGGGCGGATATTGGTCTCCCGTGCCCATAGGCATGGTTGGCCCAAACATGAG

TCCCTTCGTGAGTGGCGCACGACTAGTGGTGGTTGATATGACTGTCTTCTCGTGTTGTGC

GCTCGATTCATGCGGGTGAAGCTCTTTGAAGACCCTGATGCATTGTCTTGTGATGATGCT

26s

TCGATCGCGACCCCAGGTCAGGCGGGACTACCCGCTGAGTTTA

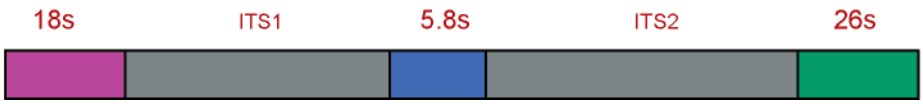

Figure 2. Sequence of 5.8s ribosomal DNA of *G. tenuifolia*.

by the National Center for Biotechnology Information (NCBI, U.S. National Library of Medicine; http://www.ncbi.nlm.nih.gov/).

Determination of Total Phenolic Content. The total amount of phenolic compounds was determined according to the method of Zielinski and Kozlowska (*21*). A 0.10 mL aliquot of the extract solution was mixed with 0.5 mL of a 0.2 M Folin−Ciocalteu reagent (Fluka, Buchs, Switzerland) and 0.4 mL of a 7.5% sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min and then measured at 760 nm with a spectrophotometer.

Determination of Scavenging Activity on DPPH Radical. The radical scavenging activity of *G. tenuifolia* extract against DPPH free radical was measured using the method of Chu et al. (*3*) with minor changes. An ethanolic solution of DPPH (0.25 mL of 0.5 M) was mixed with 1.0 mL of *G. tenuifolia* extract solution in an Eppendorf tube. The absorbance at 517 nm was measured after the solution had been allowed to stand in the dark for 30 min. The control was the measurement using ethanol to replace the *G. tenuifolia* extract in the reaction solution. The blank was measured by using ethanol to replace DPPH in the solution. The scavenging activity of the DPPH radicals by *G. tenuifolia* extract was calculated according to the following formula:

$$\text{DPPH}^{\bullet}\ \text{scavenging activity (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}})/A_{\text{control}}] \times 100\%$$

After the scavenging activity measurements had been performed under many different concentrations of samples, the EC_{50} value, that is, the concentration of sample required to cause 50% inhibition, was estimated from the plot of scavenging activity against the sample concentration.

Determination of Scavenging Activity on Superoxide Anion. Superoxide anion radicals were generated in a HPX−XOD system by HPX oxidation and assayed by NBT reduction (*22*). The solutions of NBT (300 μM), HPX (1.1 mM), and XOD (1.67 IU/mL) were prepared separately in a 0.1 M sodium phosphate buffer (PBS, pH 7.4). The HPX (760 μL) was mixed with 100 μL of NBT and 100 μL of *G. tenuifolia* extract solution in a 1-mL cuvette. Next, 40 μL of XOD was added. The decrease in absorbance at 560 nm was measured every 15 s for 6 min. The rate of decreasing (designated R_1) was estimated by enzyme kinetic function of the spectrophotometer. The control was the measurement using PBS (pH 7.4) to replace the *G. tenuifolia* extract in the reaction solution, and the decreasing rate of the absorbance is designated R_0. The amount of residual $O_2^{\bullet -}$ can be calculated by the following equation:

$$O_2^{\bullet -}\ \text{scavening activity (\%)} = (R_0 - R_1)/R_0 \times 100\%$$

Antiradical activity is defined as the amount of antioxidant necessary to reduce the initial $O_2^{\bullet -}$ concentration by 50% (efficiency concentration, denoted EC_{50}).

Cancer Cell Lines and Culture. MDA-MB-231, MCF-7, Hep G2, Hep 3B, and A549 cells were obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). These five cell lines were grown in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, and 1.5 g/L sodium bicarbonate.

Determination of Cytotoxicity of Cancer Cells. The cytotoxicity was determined according to the method of Zhang et al. (*23*). Cells (MDA-MB-231, 8000 cells; MCF-7, 1×10^4 cells; Hep G2, 1×10^4 cells; Hep 3B, 5×10^3 cells; and A549, 5×10^3 cells) were seeded in 96-well plates containing a DMEM-F12 medium with various concentrations of fractions/pure compounds and supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, and 1.5 g/L sodium bicarbonate. The cells were cultivated at 37 °C with 5% CO_2 and 95% air and in 100% relative humidity. After 72 h of cultivation, the medium

Table 1. Plants of the Compositae Family for Which the Fidelity of the 5.8s DNA Sequence with *G. tenuifolia* Is >99%

genus	species
Agoseris	*Agoseris grandiflora, A. heterophylla*
Arnaldoa	*Arnaldoa weberbaueri*
Aster	*Aster bellidiastrum*
Barnadesia	*Barnadesia pycnophylla*
Camptacra	*Camptacra gracilis*
Canadanthus	*Canadanthus modestus*
Chromolaena	*Chromolaena collina*
Chromolepis	*Chromolepis heterophylla*
Chrysothamnus	*Chrysothamnus viscidiflorus*
Coreocarpus	*Coreocarpus dissectus*, *C. parthenioides*, *C. sanpedroensis*, *C. sonoranus*, *C. arizonicus* var. *pubescens*, *C. arizonicus* var. *arizonicus*
Damnxanthodium	*Damnxanthodium calvum*
Dahlia	*Dahlia apiculata*, *D. australis*, *D. biflora*, *D. coccinea*, *D. imperialis*, *D. merckii*, *D. pinnata*, *D. rudis*, *D. sherffii*, *D. sorensenii*, *D. tenuicaulis*, *D. variabilis*
Ericameria	*Ericameria arborescens*
Erigeron	*Erigeron aliceae*, *E. annuus*, *E. barbellulatus*, *E. compositus*, *E. eatonii*, *E. lepidopodus*, *E. linearis*, *E. ochroleucus*, *E. vagus*
Hecastocleis	*Hecastocleis shockleyi*
Helianthella	*Helianthella uniflora*
Helianthus	*Helianthus verticillatus*
Huarpea	*Huarpea andina*
Hulsea	*Hulsea vestita* subsp. *parryi*
Lagenifera	*Lagenifera pumila*
Lasianthaea	*Lasianthaea helianthoides*
Lasthenia	*Lasthenia californica*
Microseris	*Microseris borealis*, *M. douglasii*, *M. laciniata*, *M. scapigera*, *M. sylvatica*
Nothocalais	*Nothocalais cuspidate*
Oreostemma	*Oreostemma alpigenum* var. *haydenii*
Rhodogeron	*Rhodogeron coronopifolius*
Rumfordia	*Rumfordia penninervis*
Sachsia	*Sachsia polycephala*
Saussurea	*Saussurea crispa*
Stebbinsoseris	*Stebbinsoseris decipiens*, *S. heterocarpa*
Uropappus	*Uropappus lindleyi*
Vernonia	*Vernonia populifolia*
Vigethia	*Vigethia mexicana*
Wyethia	*Wyethia scabra*, *W. reticulate*, *W. elata*
Xylorhiza	*Xylorhiza confertifolia*, *X. cronquistii*, *X. venusta*, *X. wrightii*, *X. glabriuscula* var. *glabriuscula*, *X. tortifolia* var. *imberbis*, *X. tortifolia* var. *tortifolia*

solution was removed. An aliquot of 100 μL of DMEM-F12 medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/mL) was reloaded to the plate, the cells were cultured for 1 h, and then the medium solution was removed. An aliquot of 100 μL of dimethyl sulfonate was added to the plate, which was shaken until the crystals dissolved. The cytotoxicity against each cancer cell was determined by measuring the absorbance of the converted dye at a wavelength of 550 nm in an ELISA reader (Bio-Rad model 550).

Statistical Analysis. All experiments were conducted for three to five independent replicates. The data are expressed in terms of mean and standard deviation. The experimental data were analyzed using Microsoft Excel software (Microsoft Software Inc.). Correlations between the antioxidant levels and activity in the extracts were calculated using Microsoft Excel's STDEV function.

RESULTS AND DISCUSSION

DNA Sequence Identification of *G. tenuifolia*. Plant species are generally characterized in terms of their appearance and from tissue slides of flowers, stems, roots, and seeds. Alternatively, DNA sequence analysis has become a more powerful tool in plant species identification. **Figure 2** shows the DNA sequence of *G. tenuifolia*. Compared with the data in NCBI's DNA database, the fidelity was ~100%. This implies that the specimen of *G. tenuifolia* used in this study strongly resembles the generally recognized *G. tenuifolia* plant. The 5.8s DNA sequence in **Figure 1** is ~99% similar to 35 genuses of plant (as shown in **Table 1**), all of which belong to the Compositae family. Compositae plants have various appearances and are difficult to identify by phytotaxonomists. This investigation found the 5.8s DNA sequence to be useful in the genetic identification and plant taxonomy of Compositae plants.

Table 2. EC_{50} Values of Different Fractions of *G. tenuifolia* Extract on DPPH• Scavenging Activity

sample	total polyphenol content (%)	EC_{50}[a] on DPPH (μg/mL)
crude	30.34 ± 2.24	15.66 ± 0.66
EA fraction	67.73 ± 1.51	9.23 ± 0.14
n-butanol fraction	56.62 ± 1.01	10.92 ± 0.67
water fraction	12.80 ± 0.70	38.00 ± 1.42
α-tocopherol	nd[b]	12.18 ± 0.78
catechin	nd	5.55 ± 1.22

[a] Results are expressed as mean ± SD of three experiments. [b] Not detected.

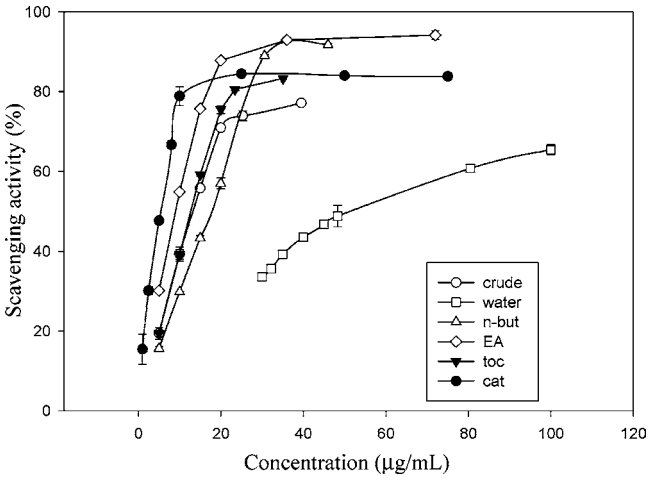

Figure 3. DPPH• scavenging activity of various partitions from *G. tenuifolia* extract at different concentrations.

Total Polyphenol Content and DPPH• Scavenging Activity. Several investigations have indicated that the phenolic compounds in vegetables, fruits, and herbal medicines can scavenge free radicals (*2*, *8*−*10*). The total polyphenol contents in crude extract, water, *n*-butanol, and EA fractions were measured (**Table 2**). Among them, the EA fraction contained more polyphenol than the others (67.73 ± 1.51%). The total polyphenol content followed the order EA fraction > *n*-butanol fraction > crude extract > water fraction.

The DPPH radical is a stable organic free radical with an absorption band in the range of 515−528 nm. The radical loses this absorption feature when accepting an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. Because the DPPH radical can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used to screen antiradical activities of fruit and vegetable juices or extracts (*2*). **Figure 3** shows the DPPH• scavenging activity of various partition fractions of *G. tenuifolia* extract at different concentrations. Because catechin (CAT) and α-tocopherol are well-known as good free radical scavengers (*24*, *25*), these two compounds were also examined for comparison. Generally, increasing concentrations of these

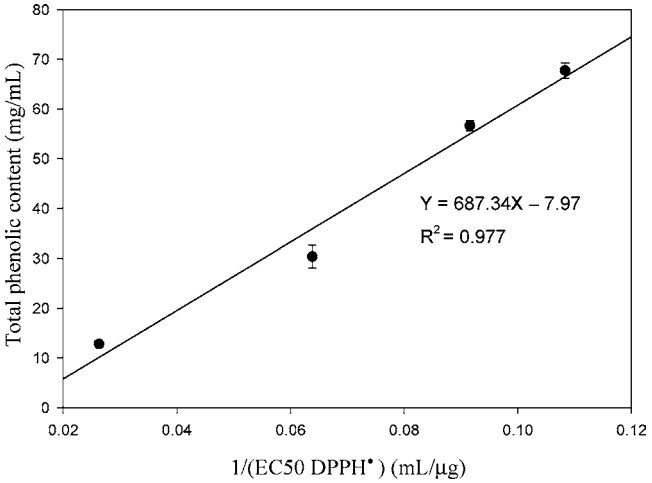

Figure 4. Correlation between total polyphenol content and 1/(EC_{50} DPPH).

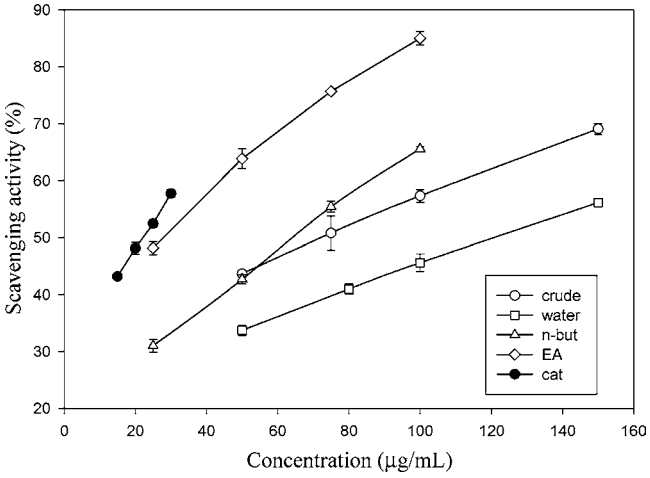

Figure 5. Superoxide anion scavenging activity of various partitions from *G. tenuifolia* extract at different concentrations.

samples exhibited a rising scavenging activity and then reached a plateau. The EC_{50} values of DPPH scavenging activity were calculated and are listed in **Table 2**. The DPPH• scavenging activity was found to decline in the order CAT > EA fraction > α-tocopherol > *n*-butanol fraction > crude extract > water fraction. Notably, the scavenging activity of the EA fraction of the *G. tenuifolia* extract was better than that of α-tocopherol.

The antioxidant activity of many edible plant extracts rises with the rising polyphenol content of the extract (*26*). A linear relationship between the reciprocal of EC_{50} value and the total polyphenol content of the four partitions was observed in this study (**Figure 4**), indicating that increasing the polyphenol content strengthens the antioxidant activity. This finding is similar to that reported by Katsube et al. (*25*).

Superoxide Anion Scavenging Activity. Superoxide anion is a major source of many free radicals, such as peroxyl, alkoxyl, hydroxyl, and nitric oxide, which are formed from superoxide anion through Fenton reaction and/or lipid oxidation or nitric oxidation (*27*). Hence, if a compound can scavenge the superoxide anion, it can reduce the production of many free radicals (*28*). As reported in the literature (*29*), the experimental results of this study have revealed that α-tocopherol is an efficient scavenger of lipid peroxyl radicals, but it cannot be a superoxide anion scavenger. Therefore, only catechin was used as the positive control in this experiment. The superoxide anion scavenging activities of the samples were measured and are presented in **Figure 5**. For all of the samples examined, within the concentration range tested, raising the concentration increases the superoxide anion scavenging activity. The EC_{50} values of these samples are shown in **Table 3**. The superoxide anion scavenging activity followed the order CAT > EA fraction > *n*-butanol fraction > crude extract > water fraction. This order is the same as the order for DPPH• scavenging activity. In conclusion, the EA fraction exhibited the highest antioxidant activity among the *G. tenuifolia* samples.

Cytotoxicity on Various Cancer Cell Lines. Cancer cell lines including MB-231, MCF-7 (breast cancer), Hep G2, Hep 3B (hepatoma), and A549 (lung cancer) were adopted to evaluate the cytotoxicity of *G. tenuifolia* extract. Doxorubicin is an effective anticancer drug, which was used as the positive control. The National Cancer Institute (NCI) indicates that the cytotoxicity of crude extract mixture is efficient if the IC_{50} is below 20 μg/mL. According to **Table 4**, the EA fraction was effective on MCF-7 cells (IC_{50} = 7.24 μg/mL) and had a minor cytotoxicity effect on MDA-MB-231 cells (IC_{50} = 19.12 μg/mL) and Hep G2 cells (IC_{50} = 20.01 μg/mL), but only a slight

Table 3. EC_{50} Values of Different Fractions of *G. tenuifolia* on Superoxide Anion Scavenging Activity

sample	EC_{50}[a] on O_2^- (μg/mL)
crude	71.87 ± 1.02
EA fraction	25.39 ± 0.78
n-butanol fraction	65.93 ± 0.36
water fraction	123.44 ± 0.95
catechin	22.67 ± 0.67

[a] Results are expressed as mean ± SD of three experiments.

Table 4. Cytotoxicity of Crude Extract, Water Fraction, *n*-Butanol Fraction, and EA Fraction on Various Cancer Cell Lines

	IC_{50} (μg/mL)				
cell line	crude extract	water fraction	*n*-butanol fraction	EA fraction	doxorubicin
MDA-MB-231	–[a]	–	–	19.16	1.21
MCF-7	–	–	–	7.24	0.73
Hep G2	–	–	–	20.01	0.15
Hep 3B	–	–	–	33.31	0.31
A549	–	–	–	–	0.92

[a] –, cytotoxicity can be neglected.

effect on Hep 3B cells (IC_{50} = 33.31 μg/mL), and no effect against A549 cancer cells. The IC_{50} values of doxorubicin ranged between 0.3 and 1.21 μg/mL toward the target cell lines. Accordingly, the EA fraction possessed an effective cytotoxicity on MDA-MB-231 cells. Conversely, the other fractions were inefficient (IC_{50} > 20 μg/mL) for the cancer cell lines tested. Therefore, the EA fraction was selected for further investigation.

Separation of the EA Fraction Using the Cytotoxicity BDFI Method. The EA fraction constituting a dry weight of 61.39 g was dissolved and loaded in a glass chromatography column. The sample was eluted sequentially with *n*-hexane, EA, and methanol. After the operation, 15 fractions were collected.

Cytotoxicity tests were performed on the 15 fractions on various cancer cell lines. The experimental data are depicted in **Table 5**. For MDA-MB-231 cells, fractions 3, 8, 9, and 10 showed significant effects. The cytotoxicity of fractions 6 and 12 were over 45 and 15%, respectively, at a concentration of 20 μg/mL. The other fractions were inactive. For MCF-7 cancer cells, fractions 3, 8−10, and 12 exhibited significant effects. For Hep G2 and Hep 3B cancer cells, fractions 3, 4, and 6−12 were active. *G. tenuifolia* is an herb traditionally used in hepatitis

Table 5. Cytotoxicity of the 15 Fractions Separated from the EA Fraction on Different Cancer Cell Lines

	IC_{50} (μg/mL)															
	EA fraction															
cell line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	doxorubicin
MDA-MB-231	–[a]	–	16.88	–	–	20 μg/mL, >45%	–	17.26	17.60	12.16	-	20 μg/mL, >15%	–	–	–	1.21
MCF-7	–	–	12.52	–	–	20 μg/mL, >20%	–	16.87	17.28	14.02	-	19.01	–	–	–	0.73
Hep G2	–	–	8.29	14.47	–	10.63	13.50	8.15	9.11	5.40	17.96	8.01	–	–	–	0.15
Hep 3B	–	–	6.05	16.69	–	9.82	16.32	3.97	7.48	3.80	15.27	15.80	–	–	–	0.31
A549	–	–	18.23	–	–	–	–	–	–	–	–	–	–	–	–	0.92

[a] –, cytotoxicity effect can be neglected.

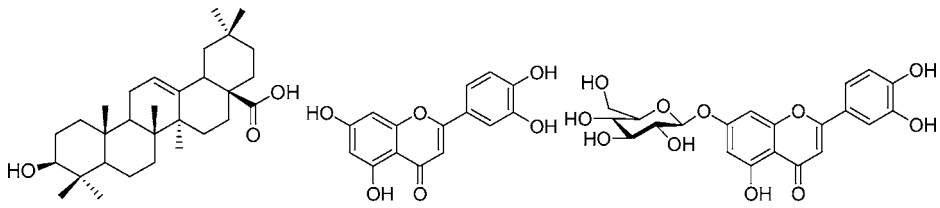

Figure 6. Structures of oleanolic acid, luteolin, and luteolin-7-glucoside.

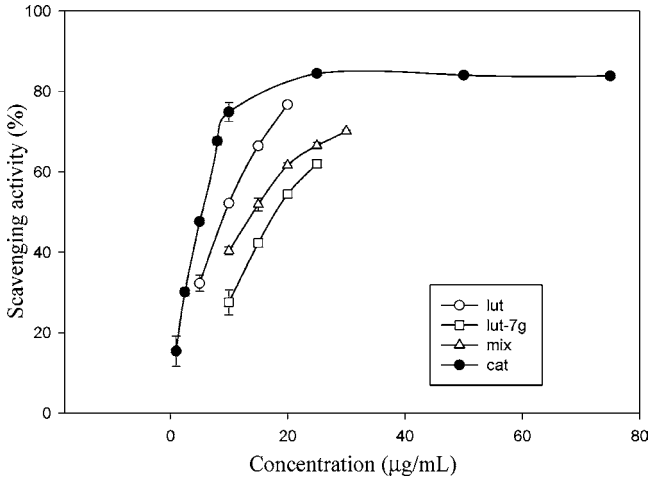

Figure 7. DPPH• scavenging activity of the effective ingredients from *G. tenuifolia* extract at different concentrations.

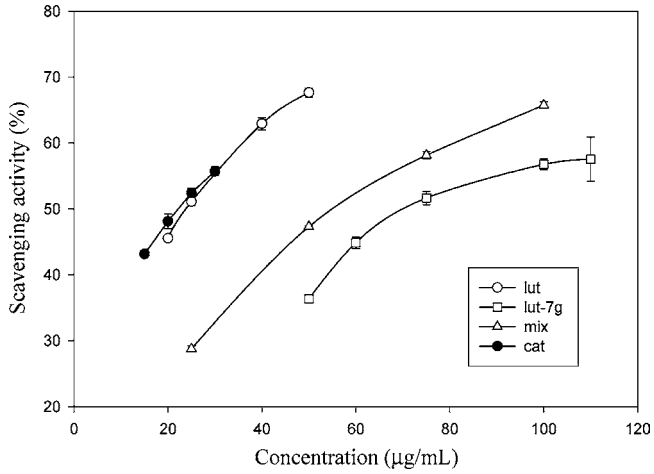

Figure 8. Superoxide anion scavenging activity of the effective ingredients from *G. tenuifolia* extract at different concentrations.

treatment, liver protection, and treatment of hepatic related diseases in Penghu Island. However, scientific evidence for these therapeutic effects on liver has not been found until this study. The results should be noted for further investigation. As for the A549 cancer cell, only fraction 3 was effective (IC_{50} = 18.23 μg/mL).

In summary, only fraction 3 had cytotoxicity on all five of these cancer cell lines. This fraction might contain certain effective ingredients that can inhibit the proliferation of these cancer cells.

Identification of the Main Ingredients in the EA Fraction. Next, the major active compounds were sought from the active fractions (**Figure 6**). Oleanolic acid (OA) was separated from fraction 6. The dry weight of OA was 6.13 g, and the yield was 9.99% based on the EA fraction. Luteolin was obtained from fractions 8−10, and its dry weights in these fractions were 2.31, 1.01, and 1.13 g, respectively. Thus, the total quantity of luteolin was 4.45 g, and the yield was 7.24%. Luteolin-7-glucoside was separated from fraction 12. The dry weight of luteolin-7-glucoside was 0.31 g, and the yield was 0.54%. Lin et al. (*30*) and Wu et al. (*18*) had isolated OA, luteolin, and luteolin-7-glucoside from *G. tenuifolia*. This study reveals the yields of these three compounds by extraction from *G. tenuifolia*, and their spectroscopic data are reported herein.

Luteolin ($C_{15}H_{10}O_6$)*:* EIMS, *m*/*z* 286.1 $[M]^+$; UV, λ_{max} (nm) (MeOH) 350, 292, 254; ^{1}H NMR (solvent DMSO) δ 7.39 (1H, dd, J = 1.4 and 8.3 Hz, H-6′), 7.37 (1H, d, J = 1.4 Hz, H-2′), 6.86 (1H, d, J = 8.3 Hz, H-5′), 6.42 (1H, d, J = 1.2 Hz, H-8), 6.66 (1H, s, H-3), 6.18 (1H, d, J = 1.2 Hz, H-6).

Luteolin-7-glucoside ($C_{21}H_{18}O_{12}$)*:* EIMS, *m*/*z* 462 $[M]^+$, 286 $[M - GluA]^-$; UV, λ_{max} (nm) (MeOH) 252, 347. ^{1}H NMR (solvent DMSO) δ 7.46 (1H, dd, J = 1.4 and 8.3 Hz, H-6′), 7.44 (1H, d, J = 1.4 Hz, H-2′), 6.92 (1H, d, J = 8.3 Hz, H-5′), 6.88 (1H, d, J = 1.2 Hz, H-8), 6.79 (1H, s, H-3), 6.44 (1H, d, J = 1.2 Hz, H-6), 5.22 (1H, d, J = 7.0 Hz, H-1 GluA), 4.14 (1H, d, J = 9.5 Hz, H-5 GluA), 3.68 (1H, J = 9.5 and 9.5 Hz, H-4 GluA), 3.59 (1H, dd, J = 7.0 and 9.5 Hz, H-2 GluA), 3.59 (1H, J = 9.5 and 9.5 Hz, H-3 GluA).

Oleanolic acid ($C_{30}H_{48}O_3$)*:* EIMS, $[M]^+$ *m*/*z* 455.3 $[M - H]^-$, 439.2 $[M - OH]^-$; ^{13}C NMR (solvent $CDCl_3$) δ 15.22 (C25), 15.49 (C24), 16.85 (C26), 18.25 (C6), 23.15 (C16), 23.50 (C11), 23.71 (C30), 26.01 (C27), 27.61 (C15), 27.96 (C2), 27.96 (C23), 30.60 (C20), 32.46 (C7), 32.75 (C22), 33.00 (C29), 33.77 (C21), 36.96 (C4), 38.32(C1), 38.62 (C10), 39.17 (C8), 41.06 (C18),

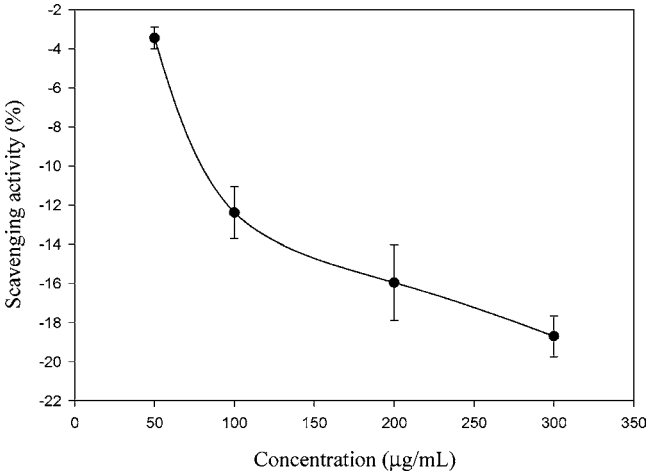

Figure 9. Measurement of superoxide anion scavenging activity of OA at different concentrations.

Table 6. EC_{50} Values of Different Compounds Obtained from *G. tenuifolia* on DPPH• and Superoxide Anion Scavenging Activity

	EC_{50}[a] (μg/mL)	
compound[b]	DPPH•	$O_2^{\bullet -}$
Lut	12.18 ± 1.22	24.52 ± 0.63
Lut-7-g	19.00 ± 0.18	76.91 ± 0.73
OA	–[c]	IA[d]
mix	12.99 ± 0.70	62.53 ± 0.42
catechin	5.55 ± 1.22	22.67 ± 0.67

[a] Results are expressed as mean ± SD of three experiments. [b] Lut, luteolin; Lut-7-g, luteolin-7-glucoside; OA, oleanolic acid; mix, Lut/Lut-7-g/OA = 1:1:1. [c] –, cytotoxicity effect can be neglected. [d] Inaccurate analysis.

41.62 (C14), 46.38 (C17), 46.38 (C19), 47.55 (C9), 55.15 (C5), 78.97 (C3), 122.29 (C12), 143.73 (C13), 181.65 (C28).

The identification of these known compounds was further confirmed by comparison with literature data (*18*).

Antioxidant Activity of OA, Luteolin, and Luteolin-7-glucoside. The experimental results of DPPH• and superoxide anion scavenging activity of OA, luteolin, and luteolin-7-glucoside are shown in **Figures 7** and **8**. Because some evidence shows that the antioxidants or the phenolic compounds have a synergistic effect (*11*−*13*, *31*), this study also examined a mixture of OA, luteolin, and luteolin-7-glucoside at a ratio of 1:1:1 (represented as "mix"). In the DPPH• scavenging test, the addition of OA to a rather high concentration of 500 μg/mL had no significant effect on DPPH• scavenging. This finding is similar to that reported by Gerhauser et al. (*32*), who measured the DPPH• scavenging activity of OA up to the concentration of 110 μg/mL and indicated that the DPPH• scavenging of OA was low. In contrast, the superoxide anion scavenging activity of OA decreased remarkably when the OA concentration was increased (**Figure 9**). In the experiment, a higher absorbance was observed when the OA concentration was increased, meaning that the increment of OA resulted in superoxide anion formation. This phenomenon might be caused by the activation effect of OA on XOD, because the superoxide anion in the assay solution was produced from the HPX−XOD system.

Despite the function of OA, **Figures 7** and **8** show that both of the other two compounds exhibited a concentration-dependent relationship in antioxidant activity. Both the DPPH• and superoxide anion scavenging activity following the order CAT > luteolin > mix > luteolin-7-glucoside. The EC_{50} values of DPPH• and superoxide anion scavenging of these compounds are listed in **Table 6**.

For the synergistic effect on DPPH• scavenging, the EC_{50} value of the mix (12.99 ± 0.70 μg/mL) was close to that of the pure luteolin (12.18 ± 1.22 μg/mL), but lower than that of the pure luteolin-7-glucoside (19.00 ± 0.18 μg/mL). Thus, the synergistic effect of the mix was clearly observed. In contrast, for the superoxide anion scavenging activity, the EC_{50} value of the mix (62.53 ± 0.42 μg/mL) was close to that of luteolin-7-glucoside (76.91 ± 0.73 μg/mL), but far from the activity of luteolin (24.52 ± 0.63 μg/mL). As the experimental data show, OA either was inactive or raised the superoxide anion production, thus lowering the scavenging activity of the mix.

Cytotoxicity of Main Ingredients on Cancer Cell Lines. Table 7 presents the cytotoxicity of luteolin, luteolin-7-glucoside, OA, and the mix on the five cancer cells. Although OA is an active component of *G. tenuifolia* on the inhibition of inflammation (*18*), the cytotoxicities of OA on these five cancer cells were all insignificant. The cytotoxicities of luteolin on the cancer cells tested were stronger than that of luteolin-7-glucoside. Additionally, the cytotoxicities of OA on MDA-MB-231, MCF-7, Hep G2, and Hep 3B cells showed weak activity at ∼20 μg/mL.

To avoid any loss of information about other effective ingredients in *G. tenuifolia*, kick-out experiments were carried out to remove OA, luteolin, and luteolin-7-glucoside in fractions 6, 8−10, and 12. The kick-out samples were prepared by removing the target compounds (OA, luteolin, and luteolin-7-glucoside) using a HPLC (VersaFlash, Supelco, Sigma-Aldrich Co.), equipped with a preparative RP-18 column (silica cartridge, 40 × 150 mm, Supelco, Sigma-Aldrich Co.). The portions other than the target compounds from the HPLC separation were collected and mixed together. The solvent was evaporated under vacuum, and the samples were dried by lyophilization. The cytotoxicities of these samples on various cancer cells are shown in **Table 7**. The cytotoxicities of the kick-out samples of fractions 8−10 on MDA-MB-231 and MCF-7 cells were absent. Because these three fractions contained mainly luteolin, the cytotoxicity of these fractions on these two cells must have

Table 7. Cytotoxicity Tests for Luteolin, Luteolin-7-glucoside, and OA, as well as for Some EA Fractions Which Had These Three Compounds Removed (Kick-out Experiment), on Various Cancer Cell Lines

	IC_{50} (μg/mL)									
						kick-out expt				
cell line[a]	Lut	Lut-7-g	OA	mix	doxorubicin	6	8	9	10	12
MDA-MB-231	7.37	16.73	–[b]	9.58	1.21	–	–	–	–	–
MCF-7	3.41	6.08	–	5.46	0.73	–	–	–	–	19.59
Hep G2	8.25	14.81	–	11.31	0.15	10.73	18.32	14.94	12.49	8.29
Hep 3B	13.95	–	–	22.20	0.31	15.25	17.67	17.04	14.52	15.96
A549	9.89	13.78	–	22.79	0.92	–	–	–	–	–

[a] Lut, luteolin; Lut-7-g, luteolin-7-glucoside; OA, oleanolic acid; mix, Lu/Lu-7-g/OA = 1:1:1. [b] –, cytotoxicity effect can be neglected.

resulted from the luteolin. Many studies have demonstrated that luteolin has bioactivity on melanocytes and endothelial cells and, thus, can be used to prevent the diseases of human breast, prostate, and thyroid cancers (*4*, *9*, *23*, *33*−*37*). For both prostate and breast cancer cells, luteolin has a flavonoid-induced inhibition effect on fatty acid synthesis, thus impeding the cell growth and inducing the apoptosis effect (*36*). This property should be why luteolin had strong cytotoxicity on MDA-MB-231 and MCF-7 cells.

As for fraction 12, which mainly included luteolin-7-glucoside, its cytotoxicity on MCF-7 cells was retained after the removal of luteolin-7-glucoside. Therefore, this fraction probably has other compounds that can affect the cytotoxicity on MCF-7 cells.

For the cytotoxicity of these five fractions on Hep G2 and Hep 3B cancer cells, whether with or without luteolin and luteolin-7-glucoside, the cytotoxicities on these two cell lines were all significant (**Tables 4** and **7**). Among them, for fractions 8−10, the potencies were higher when luteolin existed. Correspondingly, these fractions should contain other unidentified ingredients that can inhibit the proliferation of Hep G2 and Hep 3B cells.

G. tenuifolia is a traditional antipyretic and hepatic herb used in Penghu Island and may soon become an important economic healthy food. However, the information of effective ingredients was still rare and unclear until now. In this study, some active ingredients, including luteolin, luteolin-7-glucoside, and OA, were separated and identified according to the BDFI approach. The experimental results show that this plant should include other components that exhibit significant antioxidant activity and cytotoxicity. Moreover, the cytotoxicities of fraction 3 (isolated from the EA fraction) were significant on all the five cancer cells tested. This fraction did not contain luteolin, luteolin-7-glucoside, or OA. Therefore, the effective compounds included in fraction 3 are required to be studied further and have already been performed in our laboratory.

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