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Brine Shrimp Lethality of the Compounds from *Phryma leptostachya* L.

SangMyung Lee, ByungSun Min, and YungHee Kho
Korea Research Institute of Bioscience and Biotechnology, Daejon 305-600, Korea

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Brine shrimp assay-guided fractionation and isolation of the EtOAc soluble fraction of *Phryma leptostachya* L. (Phrymacaceae) gave two active compounds, phrymarolin II (**1**) and ursolic acid (**2**), which were identified by physicochemical and spectroscopic methods. Compound **1** exhibited potent lethality with LD_{50} value of 0.0013 μg/ml, whereas **2** showed moderate lethality with LD_{50} value of 27.0 μg/ml against brine shrimp. The cytotoxic activities of **1** and **2** were also evaluated against one murine and five human cancer cell lines employing the sulforhodamin B (SRB) method. Compound **2** exhibited cytotoxic activity against L1210 and SK-MEL-2 cells with ED_{50} values of 3.70 and 9.27 mg/ml, respectively, whereas **1** was devoid of any cytotoxic activity against all cancer cells tested.

Key words: Phryma leptostachya L., Brine shrimp, Cytotoxicity, Phrymarolin II, Ursolic acid

INTRODUCTION

The brine shrimp lethality test has been reported to be useful in predicting biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities (Anderson *et al.*, 1991; Fatope *et al.*, 1993; Solis *et al.*, 1993; Zani *et al.*, 1995; Ozala *et al.*, 1999). Recently, there has been interest in the brine shrimp lethality assay as a means of detecting ion regulation or ion-channel activity such as that involving Na^+-K^+-ATPase or calcium channels (Borowitz *et al.*, 1992; Watts *et al.*, 1996). Its convenience and reliability have also made it an efficient technique in performing bioassay-guided isolation (McLaughlin *et al*, 1991).

In the course of searching for bioactive compounds from natural sources, we have isolated one lignan and triterpenoid from *Phryma leptostachya* L. The herbaceous perennial plant *P. leptostachya* has been traditionally used as a natural insecticide in East Asia. From the extract of this plant, various lignans of the 3,7-dioxabicyclo[3,3,0]octanes (furofuran) have been isolated (Taniguchi *et al.*, 1969; Taniguchi *et al.*, 1972). Phrymarolins I and II, and haedoxane A are a representative member of the lignans isolated from this plant and shown to exhibit significant insecticidal activity against various insect species (Ishibashi *et al.*, 1998). The present investigation describes the isolation, brine shrimp lethality, and cytotoxicity of the active constituents from *P. leptostachya* L.

Correspondence to: YungHee Kho, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-600, Korea
E-mail: yhkho@mail.kribb.re.kr

MATERIALS AND METHODS

General procedures

Melting point was determined on an Electrothermal Melting Point Apparatus 9100. UV/VIS spectrum was measured on an UV-260 (Shimadzu). IR spectrum was measured on an IR Report-100 infrared spectrophotometer (JASCO). FABMS spectrum was measured on a JMS 700 Mass (JEOL). ^{1}H-NMR spectrum was recorded on an AC 300 MHz (BRUKER). The chemical shifts were represented as part per million (ppm) referenced to the residual solvent signal. Column chromatography was carried out using Kieselgel 60, 400-230 mesh (Merck). TLC was performed on aluminium backed Kieselgel 60 GF_{254} plates (Merck).

Plant material

P. leptostacha L. was collected from various regions in Korea and identified by Prof. K. H. Bae. A voucher specimen (CNU 1080) was deposited in the herbarium of the College of Pharmacy, Chungnam National University, Korea.

Extraction and isolation

The dried aerial part of *P. leptostacha* (354 g) was extracted with MeOH by refluxing. The MeOH extract (40 g) was suspended in water and then partitioned successively with hexane, EtOAc, and BuOH. Among the solvent fractions, the EtOAc-soluble fraction exhibited significant activity with LD_{50} value of 2.1 ± 0.6 mg/ml in brine shrimp assay. Accordingly, the EtOAc fraction (13 g) was subjected to column chromatography on silica gel (5 × 30 cm, 70-230 mesh) eluting with hexane/acetone (4:1). Five fractions were obtained based on their TLC (silica gel) pattern. Among the fractions, the fraction 2 (Fr. 2, 0.9 g) had the most significant activity with an LD_{50} value of 0.8 ± 0.01 μg/ml. Florisil column chromatography (1 × 20 cm, 70-230 mesh) of the Fr. 2 was carried out using a mobile phase of hexane/acetone (9:1) to yield six subfractions (Fr. 1′-6′). Compound 1 (80 mg) was recrystallized from fr. 2′-3′ in hexane/EtOAc. Compound 2 (94 mg) was isolated from fraction 4 (Fr. 4, 0.3 g) on florisil column chromatography (1 × 20 cm, 70-230 mesh), eluting with $CHCl_3$/MeOH (13:1).

Compound **1** (phrymarolin II)- colorless prism (EtOAc/hexane), mp 160-161°C, UV λ_{max} nm (EtOH): 297, 236. IR ν_{max} (KBr) cm^{-1}: 3000, 2760, 1745, 930, 1630~1650, FABMS (*m/z*): 459 $[M+H]^+$, ^{1}H-NMR (300 MHz, $CDCl_3$,): δ 3.80 (1H, d, *J* = 11.6 Hz, H-1β), 4.60 (1H, d, *J* = 11.6 Hz, H-1α), 5.70 (1H, s, H-3) 7.05 (1H, s, H-6), 6.53 (1H, s, H-9), 4.29 (1H, dd, *J* = 11.6, 6.8 Hz, H-10α), 4.06 (1H, br d, *J* = 11.6 Hz, H-10β), 2.84 (1H, t, *J* = 6.8 Hz, H-11), 4.87 (1H, d, *J* = 6.8 Hz, H-12), 6.58 (1H, d, *J* = 2.0 Hz, H-14), 6.69 (1H, d, *J* = 8.4 Hz, H-17), 6.48 (1H, dd, *J* = 8.4, 2.0 Hz, H-18), 5.94 (2H, s, H-19), 5.92 (2H, s, H-20), 3.77 (3H, s, OCH_3), 2.14 (3H, s, $OCOCH_3$).

Compound **2** (ursolic acid)- white amorphous powder (MeOH), mp. 261-263°C, IR ν_{max} (KBr) cm^{-1}: 3350, 2910, 1450, 1360, ^{1}H-NMR (300 MHz, $CDCl_3$ + DMSO-d_6) δ: 5.17 (1H, t, *J* = 3.5 Hz, H-12), 3.04 (1H, dd, *J* = 10.9, 4.8 Hz, H-3), 1.07 (3H, s, H-23), 0.94 (6H, s, H-26, 27), 0.90 (3H, s, H-24), 0.84 (3H, d, *J* = 6.4 Hz, H-30), 0.77 (3H, d, *J* = 9.6 Hz, H-29), 0.72 (3H, s, H-25).

Brine shrimp lethality assay

The assay was performed as described previously (Meyer *et al.*, 1982) using brine shrimp (*Artemia salina* Leach) nauplii. The eggs (San Francisco Bay Brand. USA) were placed in brine and hatched within 48 h. Each extracts and compounds (2 mg) were dissolved in 20 ml CH_2Cl_2/MeOH (1:1) to prepare a stock solution of 0.1 mg/ml concentration. From the stock solution, 200, 20, and 2 μl were transferred in triplicate to vials and the solvents were allowed to evaporate. After evaporation, 5 ml of brine was added to each vial (30 shrimps per concentration). The number of survivors at each concentration was recorded and the LD_{50} values (p, 0.05) were calculated using the Finney computer program.

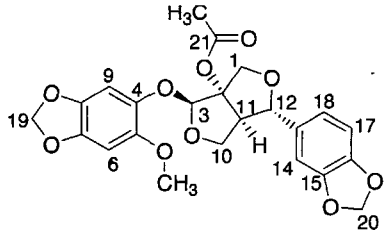

Fig. 1. Structure of phrymarolin II(**1**)

In vitro cytotoxicity

In vitro cytotoxicity was measured with murine (L1210, leukemia) and human cancer cells. Five different human cancer cell lines, A549 (lung adenocarcinoma), SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (CNS), and HCT15 (colon) were purchased from National Cancer Institute (NCI) in U. S. A. The cytotoxicity evaluation was carried out by sulforhodamine B (SRB) method (Skehan *et al.*, 1990) as described previously (Kwon *et al.*, 1998). The ED_{50} value was determined graphically by plotting the viability versus the concentration of the test sample.

RESULTS AND DISCUSSION

An EtOAc-soluble fraction of MeOH extract of *P. leptostachya* showed significant activity against brine shrimp with LD_{50} value of 2.1 μg/ml. Repeated silica gel and florisil column chromatography of the EtOAc-soluble fraction of *P. leptostachya* let to the isolation of compounds **1** and **2**. Compound **1** had a molecular weight of 458 based on the positive ion FAB-MS ($[M + H]^+$ at *m/z* 459). The characteristic ^{1}H-NMR signals at δ 3.08 (1H, d, *J* = 11.6 Hz), 4.60 (1H, d, *J* = 11.6 Hz), 4.29 (1H, dd, *J* = 11.6, 6.8 Hz), and 4.06 (1H, br d, 11.6 Hz) were indicative of H-1β, H-1α, H-10α, and H-10β, respectively, of furofuran moiety. In addition, the ^{1}H-NMR spectrum indicated aromatic protons at 7.05 (1H, s), 6.53 (1H, s), 6.58 (1H, d, *J* = 2.0 Hz), and 6.69 (1H, d, *J* = 8.4 Hz). The presence of two signals at δ 5.94 (2H, s), 5.92 (2H, s) were indicative of two

Table I. Brine shrimp lethality of extracts and isolates from *P. leptostachya*

	[a]LD_{50} (mg/ml)
MeOH extract	10.2 ± 5.4
Hexane extract	7.3 ± 3.1
EtOAc extract	2.1 ± 1.2
Butanol extract	98.2 ± 10.1
Phrymarolin II (**1**)	0.0013 ± 0.0001
Ursolic acid (**2**)	27.0 ± 3.9

[a]LD_{50} values were calculated by Finny program using three different concentrations in triplicate experiments.

Table II. Cytotoxic effects of phrymarolin II (**1**) and ursolic acid (**2**)

Compound	ED_{50} values (μg/ml)[a, b]					
	L1210	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
Phrymarolin II (**1**)	>20	>20	>20	>20	>20	>20
Ursolic acid (**2**)	3.70 ± 0.20	10.72 ± 3.21	12.19 ± 2.19	9.27 ± 1.10	10.98 ± 1.89	11.12 ± 3.11
Cisplatin	3.25 ± 1.01	0.91 ± 0.19	1.32 ± 0.20	0.89 ± 0.03	0.72 ± 0.13	3.17 ± 1.01

[a]ED_{50} is defined as the concentration which resulted in a 50% decrease in cell number.
[b]Results are means ± S.D. of 3 to 5 independent replicates.

[1,3] dioxole and the signals at δ 3.77 (3H, s), 2.14 (3H, s) confirmed the presence of a methoxy and an acetoxy group, respectively. Thus, the structure of **1** was determined to be 1-acetoxy-6-(2-methoxy-4,5-methylene-dioxyphenyl)-2-(3,4-methylenedioxyphenoxy)-3,7-dioxabicyclo[3.3.0]octane (Fig. 1), which was verified as phry-malorin II on comparison of the physicochemical and spectral data with those reported (Taniguchi *et al.*, 1969, 1972). Compound **2** was identified as ursolic acid on comparison of their spectral data with authentic samples (Min *et al.*, 2000).

Among the MeOH extract, hexane-, EtOAc-, and BuOH soluble fractions, EtOAc-soluble fraction showed significant lethality with LD_{50} value of 2.1 ± 0.6 μg/ml in the brine shrimp lethality assay. Phrymarolin II (**1**) and ursolic acid (**2**) from EtOAc fraction, showed brine shrimp lethality with LD_{50} values of 0.013 ± 0.0001 μg/ml and 27 ± 3.9 μg/ml, respectively. The cytotoxicities of **1** and **2** were evaluated *in vitro* against six cancer cell lines that were originated from murine (L1210) and human (A549, SK-OV-3, SK-MEL-2, XF498, and HCT15) cancers. As indicated in Table 2, **2** showed cytotoxic activity against L1210 with ED_{50} values of 3.70 μg/ml, while **1** was inactive against all cancer cell lines tested.

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