

## Callus Induction and Determination of Iridoid Glycosides from *Barleria prionitis* Linn Leaf Explants

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**Abstract:** Callus cultures of *Barleria prionitis* Linn were established from leaf explants in Murashige and Skoog (MS) medium supplemented with various combinations of  $\alpha$ -Naphthalene acetic acid (NAA) and 6-benzylaminopurine (BA). The explants developed compact greenish calluses in the presence of NAA (1.0 mg/L) and BA (0.5 mg/L). The growth of calluses was measured over 60 days. Maximum dry biomass, 0.143 g, was obtained at 50 days. Calluses were extracted with methanol to examine the presence of the iridoid glycosides having biological activity in intact plants. The methanol extracts were further separated by solvent-solvent extraction to give dichloromethane and *n*-butanol solubles or *n*-hexane, chloroform and *n*-butanol solubles. Each soluble was analyzed by TLC (thin layer chromatography) and HPLC (high pressure liquid chromatography). The *n*-hexane and chloroform solubles or dichloromethane solubles did not contain glycosides, but the *n*-butanol solubles showed the existence of glycosides on the TLC plate sprayed with *p*-anisaldehyde-sulfuric acid reagent. However, the *n*-butanol solubles in the methanol extracts of callus contained neither of the iridoid glycosides (8-O-Acetyl shanzhiside methyl ester (barlerin) and shanzhiside methyl ester) present in intact plants. However, a glucoside was detected in the *n*-butanol solubles from the methanol extracts of callus by TLC. This study also attempted to determine the anticancer activity of methanol extracts from *B. prionitis* L. However, the methanol extracts were found to be inactive.

**Key words:** *Barleria prionitis* Linn, callus cultures, Murashige and Skoog (MS) medium, iridoid glycosides

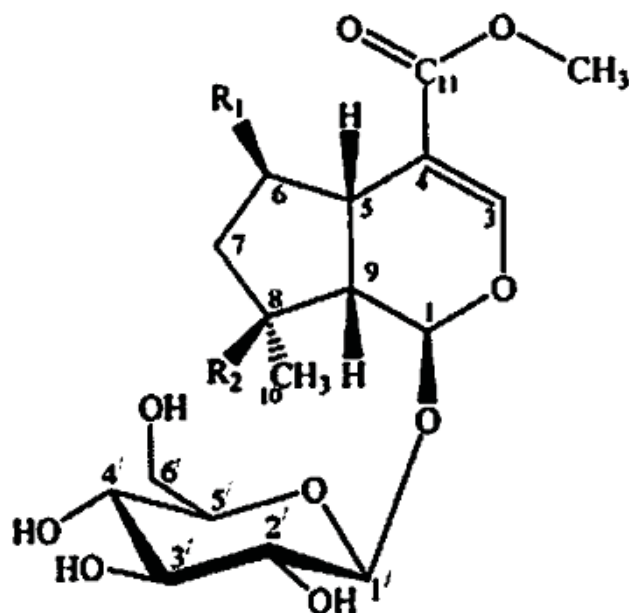
### INTRODUCTION

*Barleria prionitis* Linn belongs to the Acanthaceae (Smitinand, 2001). Known as “Vajradanti” in Hindi, and Ang-karb-Nuu in Thai, the plant is native to tropical areas of east Africa and Asia and reported to have broad medicinal properties. The leaves are chewed to relieve toothaches, and tribal communities in India use hot water extracts of the leaves to control irritation. A paste of its roots is used to disperse boils and gradual swelling. Moreover, the plant is used to treat stiffness of the limbs, enlargement of the scrotum and sciatica (Ambasta, 1986; Jain and Defillip, 1991; Chopra *et al.*, 1996; Gupta *et al.*, 2000; Singh *et al.*, 2003). When root methanol extracts of the plant were fed to male rats at 100 mg/kg for 60 days, spermatogenesis was reduced without any affect on general body metabolism (Verma *et al.*, 2005). Singh *et al.* (2003) reported that the aerial parts are used to treat inflammation. The *n*-butanol and aqueous fractions of whole plant extracts showed anti-inflammatory activity against carragenan-induced oedema in rats. Petroleum ether extracts of *Barleria* sp. had therapeutic potential as anti-bacterial and anti-inflammatory agents (Amoo *et al.*, 2008). Teneja (1975) isolated two iridoid glycosides, barlerin and acetyl barlerin, from *B. prionitis* L. Subsequent investigation

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clarified that barlerin and acetyl barlerin are 8-O-acetyl shanzhiside methyl ester and 6,8-di-O-acetyl shanzhiside methyl ester (Damtoft *et al.*, 1982). Ata *et al.* (2009) isolated phenylethanoid glycoside, barlerinoside, and six iridoid glycosides, shanzhiside methyl ester, 6-O-trans-p-coumaroyl-8-O-acetylshanzhiside methyl ester, barlerin, acetylbarlerin, 7-methoxydideroside, and lupinoside, from *B. prionitis* Linn. Barlerinoside has shown antioxidative activity with an IC<sub>50</sub> of 0.41 µg/mL. The active ingredients in *Barleria* species were identified as barlerin and acetylbarlerin (leaf and stem) and scutellarin-7-neohesperidoside (flower) (Chen *et al.*, 1998).

*B. prionitis* L is an attractive species because of its broad biological activities. In recent decades, plant cell and tissue cultures have become considerable potential sources of biopharmaceuticals. Advances in biotechnologically, particularly methods for culturing plant cells, should provide alternative sources of valuable chemicals (Misawa *et al.*, 1985). The aim of this study is to establish callus cultures of *B. prionitis* L and to clarify whether the calluses produce the iridoid glycosides (Fig.1) present in the intact plant.



R<sub>1</sub> = R<sub>2</sub>, Acetyl, Acetyl barlerin

R<sub>1</sub> = OH, R<sub>2</sub> = OAc, Barlerin

R<sub>1</sub> = R<sub>2</sub> = OH, Shanzhiside Methyl Ester

**Fig. 1:** Iridoid glucosides produced in *Barleria prionitis* Linn

## MATERIALS AND METHODS

Research to establish callus cultures of *Barleria prionitis* Linn and to determine the presence of 8-O-Acetyl shanzhiside methyl ester (barlerin) and shanzhiside methyl ester in the callus cultures of *B. prionitis* L was carried out at the Faculty of Agriculture, Natural resources and Environment, Naresuan University, Thailand during March 2007 to December 2008 and at the Faculty of Agriculture, Ehime University, Japan during January-March 2009, respectively.

### **Plant Material:**

*B. prionitis* L specimens were collected in the Phitsanulok province of Thailand during summer and authenticated by office of the forest herbarium. The plants were grown in a green house of the Faculty of Agriculture, Natural Resources and Environment, Naresuan University.

### **Induction of Callus Cultures:**

Calluses were generated from leaves and nodal segment of *B. prionitis* L. After surface sterilization with Clorox containing 3% active sodium hypochlorite for 5 min and two cleanings in sterilized distilled-water for

10 min, young leaves and buds were cut and transferred to Murashige and Skoog (MS) medium with sucrose (30 g/L) and agar (10 g), pH 5.6 and supplemented with 25 combinations of NAA and BA. Both the auxin and cytokinin concentrations were varied (0, 0.5, 1.0, 2.0, and 5.0 mg/L). Fifty explants were used for each combination. The callus was subcultured every month and incubated at an intensity of 1,000-3,000 lux, with a 16-h light period and 8-h dark period, at 24 °C.

#### **Determination of Callus Growth:**

Callus growth was expressed as fresh weight (gram) and dry weight (gram). Callus (0.5-0.6 g of fresh weight) was transferred to MS medium supplemented with NAA (1 mg/L) and BA (0.5 mg/L), then weighed after 10, 20, 30, 40, 50 and 60 days.

#### **Determination of Phytochemical Compounds:**

##### **Preparation of Extracts:**

Fresh leaves (4.13 g) were dried overnight at 105 °C to give 1 g of dry mass. Fresh callus (8.33 g) was lyophilized to give 1 g of dry weight. Each 1 g of sample of leaves and callus was ground to a powder, extracted with MeOH (50 ml), and concentrated dry under reduced pressure. The concentrated methanol extracts were suspended in water (50 ml) and extracted with dichloromethane (2x25 ml) and then *n*-butanol (2x25 ml), (Yalcin *et al.*, 2008). The *n*-butanol soluble was evaporated to dry and dissolved in a small amount of methanol to spot it on the TLC plate.

Furthermore, callus was lyophilized and the dried callus was powdered with a pestle and mortar to give a powder. Part of the powder (2.0g of dry weight) was extracted for 18 hours with methanol in a soxhlet extractor. The methanol solution was evaporated under reduced pressure to give methanol extracts (104.9 mg). Water was added to the extracts to make a suspension, and extracted with *n*-hexane, then chloroform and finally *n*-butanol. Yields were as follows; *n*-hexane solubles (25.0 mg), chloroform solubles (13.8 mg), *n*-butanol solubles (32.5 mg) and the residue (28.6 mg). Each soluble was subjected to TLC and spots were detected by spraying with *p*-anisaldehyde-sulfuric acid, a coloring reagent for glycosides.

##### **TLC Screening:**

*p*-Anisaldehyde-sulfuric acid, a reagent for visualization, was prepared as follows; a solution of *p*-anisaldehyde (1ml) and 97% sulfuric acid (1ml) in 18 ml of ethanol was mixed just before use. Developing solution system: methanol: water (50:50 v/v). *N*-Butanol solubles, glucose (standard sample), and authentic samples (barlerin and shanzhiside methyl ester) were examined by TLC (precoated plates of silica gel 60 F<sub>254</sub> Merck) (Yalcin *et al.*, 2008). Detection: *p*-anisaldehyde-sulfuric acid reagent was sprayed on a TLC plate and then heated for 10 min at 110 °C.

##### **HPLC of Methanol Extracts of Callus and N-butanol Solubles in the Extracts:**

Methanol extracts of callus and *n*-butanol solubles in the extracts were run on a reversed-phase column (5 µm) (Shiseido CAPCELL Pak C-18 4.6x250 mm, Japan) in a Shimadzu LC-10A liquid chromatograph with SPD-10A UV (ultraviolet-visible light detector wavelength: 245 nm) by isocratic elution with H<sub>2</sub>O: CH<sub>3</sub>OH (1:1 v/v) as the mobile phase. The column temperature was kept at 40°C. The flow rate was 0.5 ml/min and all chromatograms were plotted at an absorption wavelength of 245 nm. The iridoid glucosides, barlerin and shanzhiside methyl ester, in the methanol extracts and *n*-butanol solubles in the extracts were checked by comparing retention times with those of authentic barlerin and shanzhiside methyl ester isolated from *Lamium* sp. (Yalcin *et al.*, 2008).

##### **Determination of Anticancer Activity:**

KB (Human epidermis carcinoma of cavity, ATCC CCL-17), MCF7 (Human breast adenocarcinoma, ATCC HTB-22) and NCI-H 187 (Human small cell lung carcinoma, ATCC CRL-5804) were determined by resazurin microplate assay (REMA) with a modified fluorescent dye for mammalian cell cytotoxicity according to the method of O'Brien *et al.* (2000). Ellipticine and doxorubicin were used as positive controls. DMSO and sterile distilled water were used as negative controls. Briefly, cells at a logarithmic growth phase were harvested and diluted to 10<sup>5</sup> cells/ml in fresh medium and gently mixed. Test compounds (*B. prionitis* L methanol extracts) were diluted in culture medium at ratio of 1:2 giving 8 concentrations. Five microliters of test sample and 45 microliters of cells were put into 384-well microtiter plates in a total volume of 50 µl/well. Plates were incubated at 37°C in 5% CO<sub>2</sub> for 72 hours for KB and MCF, and 5 days for NCI-H187. After the incubation period, 12.5 microliters of resazurin solution was added to each well and the plates were incubated

at 37 °C for 4 hours. The plates were then processed for optical density absorbance measurements using a Victor 3 Microplate reader at dual wavelengths of 530 and 590 nm (O'Brien *et al.*, 2000).

## RESULTS AND DISCUSSIONS

### Induction of Callus Cultures:

Recently, a broad array of biological activity of extracts of *B. prionitis* Linn has been reported. We were interested in establishing callus cultures of *B. prionitis* L. This report is the first to describe the induction of callus cultures and observations of growth. Effects of explants on the generation of callus were also studied. Leaf explants were found to be suitable for the production of callus cultures and maintaining growth. The effect of various combination of  $\alpha$ -naphthalene acetic acid (NAA) and 6-benzylaminopurine (BA) on calluses induction of *B. prionitis* L. from leaf explants were studied. The optimum response was found in MS supplemented with NAA (1.0 mg/L) and BA (0.5 mg/L). The highest rate of induction was 100 % in 30 days. Greenish compact calluses were obtained after explants were placed on the medium for 23 days. MS medium without BA (0 mg/L) or with a high concentration of BA (5 mg/L) totally inhibited the formation of calluses. Among 25 combinations of BA and NAA, 1.0 mg/L of NAA and 0.5 mg/L of BA was found to be the best for the induction of callus after 35 days (Table 1).

**Table 1:** Effect of NAA and BA on the formation calli from leaves of *B. prionitis* L in 35 days of cultivation.

Concentration of BA (mg/L)	Concentration of NAA(mg/L)				
	0	0.5	1.0	2.0	5.0
0	-	+	++	+	+
0.5	+	++	++++	++	-
1.0	++	++	++	+++	+
2.0	+	++	+	++	-
5.0	-	+	+	++	-

Notes: ++++: highest induction ratio; +++: higher induction ratio; ++: medium induction ratio; +: lower induction ratio; -: no induction.

### Growth of Callus Cultures:

Each 0.5-0.6 g of fresh callus was transferred onto MS medium supplemented with NAA (1 mg/L) and BA (0.5 mg/L) and cultivated at 24 °C for 60 days. To measure the callus biomass, the callus was harvested at day 10, 20, 30, 40, 50 and 60. The weight of dried callus was obtained from lyophilized cultures. The callus was a compact greenish mass 0-30 days into the culture period, and had turned brownish by day 60. The time course of growth is shown in Table 2, Fig.2. Callus cultures grew slowly during 0-20 days, but their growth accelerated from day 30 onwards and tended to decline on day 60. Maximum fresh weight was 1.533 g, which was 2.3 fold that of the initial callus cultures. The highest dry weight was 0.143 g, 2 fold that of the initial callus.

### Determination of Iridoid Glycosides:

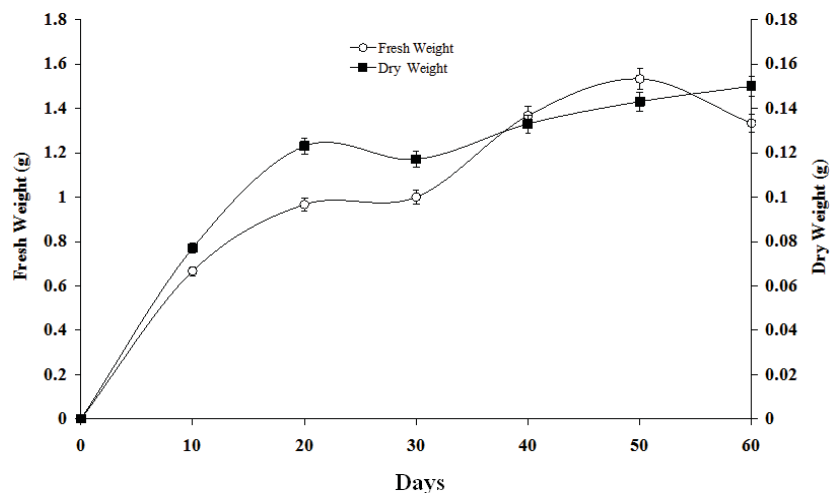
#### TLC Screening:

Dichloromethane and *n*-butanol solubles or *n*-hexane, chloroform and *n*-butanol solubles in the methanol extracts of callus were dissolved in a small amount of methanol to spot them on TLC plates. Each soluble was developed with the solvent system: methanol: water (50:50 v/v). The plate was observed under both short and long UV light after the solvent was dried with a dryer. A mixture of *p*-anisaldehyde-sulfuric acid was sprayed onto the plate and heated at 110 °C for a few minutes with a dryer. The glucose used as a control appeared as a light red spot at  $R_f = 0.7$ . The *n*-butanol solubles in the methanol extracts of *B. prionitis* showed only one large clear band, a dark red spot at  $R_f = 0.7$ . The *n*-butanol solubles in the methanol extracts from callus gave three spot; a greenish yellow spot at  $R_f = 0.8$ , a pink spot at  $R_f = 0.74$ , and on gray spot at  $R_f = 0.68$  (Fig.3). The screening revealed the color of the spots and  $R_f$  values to differ between *n*-butanol solubles in the methanol extracts of the intact plant and extracts of the callus. The TLC profile of *n*-butanol solubles in the methanol extracts of intact plants contained of iridoid glycosides as determined using two authentic samples (barlerin and shanzhiside methyl ester) (courtesy by Dr. Yalcin). The results are shown in Fig.4. In contrast, the *n*-butanol solubles in the methanol extracts from callus showed no target glycosides. However, the *n*-butanol extracts exhibited the presence of glucosides on TLC. The chemical structure as well as biologically activity of these glucosides needs to be examined in the future.

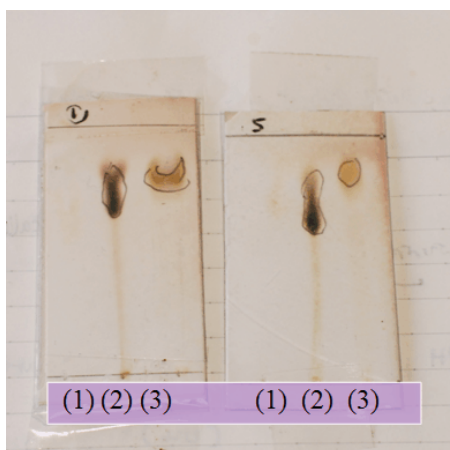
**Table 2:** Fresh and dry weight of callus cultures of *B. prionitis* L.

Biomass (g)	Cultivation time (days)					
	10	20	30	40	50	60
Fresh weight	0.667(±0.067)	0.967(±0.067)	1.000(±0.200)	1.367(±0.167)	1.533(±0.088)	1.333(±0.120)
Dry weight	0.077(±0.018)	0.123(±0.019)	0.117(±0.200)	0.133(±0.007)	0.143(±0.018)	0.150(±0.023)

\*Data presented in the table correspond to the mean ± standard deviation of three parallel measurements.



**Fig. 2:** Time course of callus growth of *B. prionitis* L in fresh and dry weight.

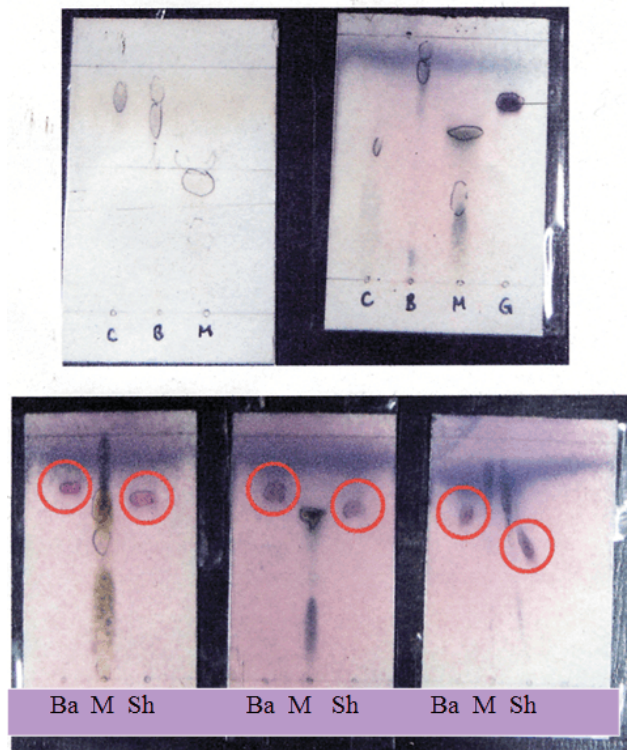


**Fig. 3:** TLC chromatogram of *n*-butanol (*n*-BuOH) soluble in the methanol extracts of intact plant and callus cultures of *B. prionitis* L. Notes: Developing solvent: methanol: water (50:50 v/v); (1) glucose; (2) methanol extracts of *B. prionitis* L (intact plant); (3) methanol extracts of callus cultures generated from the intact plant.

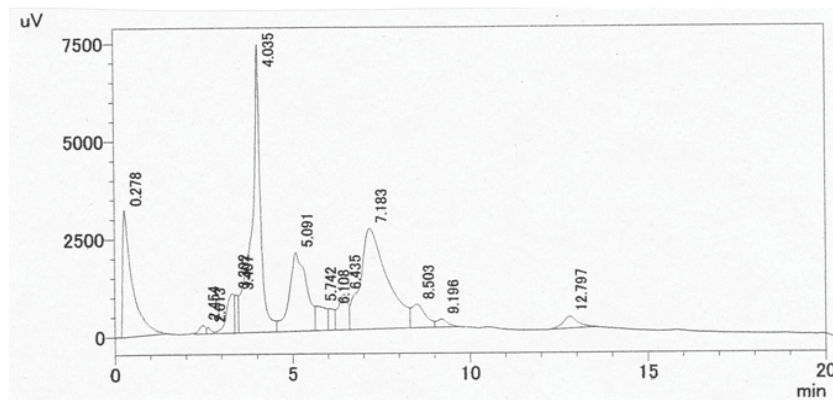
**HPLC Analysis:**

Furthermore, HPLC of the methanol extracts of callus and *n*-butanol solubles in the methanol extracts was conducted using authentic samples. Shanzhiside methyl ester and barlerin were eluted with retention times of 6.4 and 9.1 min, respectively. The HPLC profile showed neither of the authentic sample to be present the methanol extracts of callus cultures (Fig.5-6). These results showed that two authentic iridoid glycosides present in the intact plant, *B. prionitis*, were not produced in the calluses induced from the leaves under the conditions used in this experiment. However, a glucoside was produced by the callus cultures of the plant.



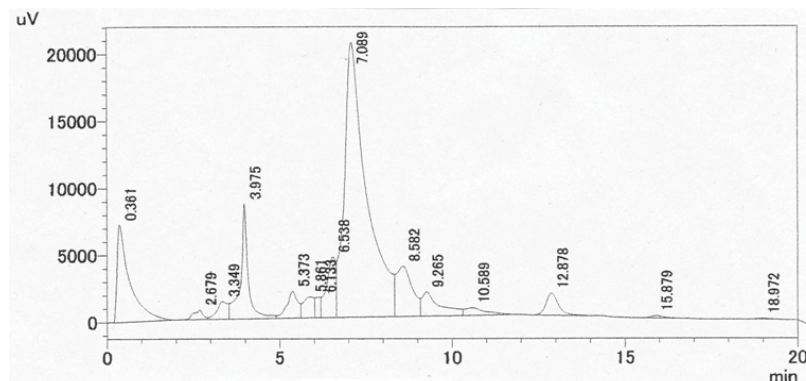


**Fig. 4:** TLC chromatogram of *n*-butanol solubles in the methanol extracts of callus cultures and two authentic samples of iridoid glycosides. Notes: Developing solvent: methanol: water (50:50 v/v); Coloring reagent: *p*-anisaldehyde-sulfuric acid; C: Chloroform solubles; B: *n*-Butanol solubles; M: Methanol extracts; G: Glucose, Ba: Barlein; M: Methanol extracts; Sh: Shanzhiside methyl ester.



**Fig. 5:** HPLC chromatogram of methanol extracts of callus from *B. prionitis* L.

In conclusion, we proposed callus induction of *B. prionitis* and detected that it was not produced two iridoid glycoside, barlerin and shanzhiside methyl ester, but the *n*-butanol extracts showed other iridoid glycosides in callus. This results demonstrated that *B. prionitis* has potentiality to be a new source of renewable therapeutic plant –derived drugs in the future after biotechnological process for the production of these compounds have to be further explored. From the findings here, it is necessary to investigate the culture conditions by manipulating the medium, growth regulators, and parts of explants to facilitate callus growth and enhance iridoid glycoside production.



**Fig. 6:** HPLC chromatogram of *n*-butanol solubles in the methanol extracts of callus cultures of *B. prionitis* L.

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