SUPPLEMENTARY MATERIAL

Biological activities of *Peristrophe bivalvis* extracts: promising potential anti-snake venoms against *Naja kaouthia* and *Trimeresurus albolabris* venoms

Jatuporn Phaopongthai^{a,*}, Jureeporn Noiphrom^b, Supat Phaopongthai^a, Narumol Pakmanee^b and Jirapast Sichaem^c

^a*Faculty of Science and Technology, Rajamungala University of Technology Thanyaburi, Thanyaburi, Pathum Thani, 12110, Thailand*

^b*Research and Development Department, Queen Saovabha Memorial Institute, Pathumwan, Bangkok, 10330, Thailand*

^c*Natural Products Research Unit, Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand*

*Corresponding author: Jatuporn Phaopongthai, Faculty of Science and Technology, Rajamungala University of Technology Thanyaburi, Thanyaburi, Pathum Thani, 12110, Thailand. Tel: +6681 870 1048. Email address: plek2121@yahoo.com

Abstract

The present study evaluates the *in vitro* anti-snake venom potential of *Peristrophe bivalvis* (PB) extracts against *Naja kaouthia* (NK) and *Trimeresurus albolabris* (TA) venoms, including inhibition of cytotoxic effects and enzymatic activities, and the binding-precipitation of extracts and venom proteins analysis. In addition, the antioxidant, cytotoxic and *in vivo* acute oral toxic activities of PB extracts were also reported. The *in vitro* cytotoxic and enzymatic analysis reveals that the ethanol extracts of stems and leaves of PB showed good anti-snake venom activity against NK and TA venoms. In addition, the antioxidant result indicated that only the ethanol extract of leaves exhibited weak DPPH radical scavenging activity. The ethanol whole plant extract of PB also showed no cytotoxicity against four cell lines. Moreover, the *in vivo* acute oral toxicity result of the ethanol whole plant extract reported that all treated rats did not exhibit abnormal toxic signs or deaths.

Keywords: *Peristrophe bivalvis*, anti-snake venom activity, cytotoxicity, antioxidant activity, acute oral toxicity

Experimental

Plant material

Fresh *Peristrophe bivalvis* were obtained from Damnoen Saduak District, Ratchaburi Province, Thailand in January 2014. They were identified and a voucher specimen (BK 37891) has been deposited at Sirindhorn Plants Museum Building, Kasetsart University in Bangkok, Thailand.

Preparation of plant extracts

Dried powder of stems (250 g), leaves (750 g) and a whole plant (2.5 kg) of *P. bivalvis* were macerated in various solvents using ethanol for stems, leaves and a whole plant, boiled water and rice whisky (40% ethanol) for leaves as the extracting solvent, at material/solvent ratio of 1 : 2 (w/v), for 24 h at room temperature. The extraction was performed four times. All extracts were filtered, combined and concentrated in a rotary evaporator at 40 °C. The extracts were evaporated under reduced pressure, and then freeze-dried to afford crude extracts with yields of ethanol extract of stems (Extr.1, 14 g), a whole plant (Extr.2, 180 g) and leaves (Extr.3, 29 g), rice whisky extract of leaves (Extr.4, 32.5 g) and boiled water extract of leaves (Extr.5, 25 g).

1. In vitro anti-snake venom assay of PB extracts

Venoms

Lyophilized *Naja kaouthia* (NK) and *Trimeresurus albolabris* (TA) venoms were obtained from the Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

Human Cell Culture

Fibroblasts originating from human skin were obtained from ATCC (CCD-25Sk, CRL-1474) and were maintained in completed DMEM and supplement with 10% fetal bovine serum. Cells were cultured in a humidify atmosphere with 5% CO₂ at 37 °C. The fibroblasts were seeded at 2×10^4 cells/well onto the 96 well plates.

1.1 Cytotoxicity assay (MTT assay)

Normal human skin fibroblast (ATCC, CRL-1474), in D-MEM supplemented with 10% fetal bovine serum, were seeded on 96-well plate at an approximate initial density of 2×10^4 cells/well and then treated with venoms at the various concentrations. After 72 hr, MTT was added to each well and incubated for 3 hr. Medium was removed and DMSO was added in the purpose of dissolving formazan crystals. The absorbance was read at 540 nm and results were expressed as percentage of cell survival. Assays were done in triplicate and performed on 3 separate instances. Cytotoxic activity of snake venoms was estimated by effective concentration ($\mu\text{g/well}$) which gave the result of 50% cell survival, EC₅₀. The effective concentration giving the approximate result of 10% cell survival, EC₉₀, was selected to use in the evaluation of PB extracts.

Venoms at the concentration of EC₉₀ were reacted with five PB extracts, to observe the inhibition of cytotoxic effect. The EC₉₀ of venoms ($\mu\text{g/well}$) was mixed with each extract

at equal volume and incubated, and incubated for 30 min at 37 °C. After centrifugation, the reaction (50 μ l) was added to 2×10^4 cells/well of CRL-1474 on 96-well plate and investigated using MTT assay after 72 hr incubation period. As the parallel experiment, each extract was added immediately following the venom adding to the cells. The concentration and volume of each venom or extract were same as above. Then, MTT assay was performed. The experiments were done in triplicate and performed on 3 separate instances. The percentage of inhibition by the extracts was reported.

Figure S1. Cytotoxicity of NK and TA venoms toward normal human skin fibroblast.

Figure S2. Inhibitory activity of all extracts against NK venom.

Figure S3. Inhibitory activity of all extracts against TA venom.

Figure S4. Inhibitory activity of all extracts toward normal human skin fibroblast.

Table S1 Inhibitory activity of PB extracts (Extr.1-5) against normal human cells, NK and TA venoms (mean±SD).

Crude extract (µg/well)		% Survival		% Inhibition activity			
		Normal human skin fibroblast		NK		TA	
		I ^a	F ^b	I ^a	F ^b	I ^a	F ^b
Extr.1	500	14.77±3.82	8.49±0.45	84.21±3.34	99.01±1.40	91.77±3.23	96.34±0.92
	250	77.25±2.65	12.30±3.69	67.02±12.41	92.63±2.69	25.11±10.49	89.37±3.96
	125	84.88±2.56	60.48±12.83	66.11±2.41	37.07±13.33	16.26±5.87	30.58±13.38
	25	89.01±9.80	82.05±4.03	18.29±4.71	17.93±2.01	9.42±5.73	10.99±3.36
Extr.2	500	41.47±2.29	25.53±7.08	66.15±10.40	88.12±15.38	74.75±3.31	93.14±10.55
	250	66.41±6.20	63.93±4.13	61.52±5.43	68.82±1.11	36.80±4.48	72.53±17.74
	125	67.96±9.73	67.25±5.34	55.52±6.10	60.55±10.17	35.47±2.23	45.19±10.49
	25	79.99±7.03	67.86±6.65	25.02±3.67	36.04±0.13	25.65±4.16	29.00±3.63
Extr.3	500	36.40±2.22	15.01±0.63	89.92±7.73	98.88±1.76	64.90±1.96	96.50±1.91
	250	83.29±7.71	15.82±2.03	52.13±14.93	97.50±1.37	23.44±7.17	93.77±3.16
	125	81.42±3.91	27.58±3.71	49.09±15.78	77.89±1.64	12.73±5.68	72.94±2.67
	25	81.05±6.16	52.86±4.26	33.50±15.65	49.33±3.28	12.47±8.25	44.07±4.62
Extr.4	500	79.94±3.32	71.71±1.60	52.89±6.38	57.09±2.39	30.70±5.34	18.05±3.83
	250	82.38±4.08	82.30±1.03	38.25±3.73	40.85±0.96	20.84±2.53	7.22±4.95
	125	87.66±0.27	91.18±1.03	20.26±1.32	21.35±1.56	10.55±0.76	3.43±4.26
	25	89.78±4.17	98.95±1.07	8.11±5.00	8.50±2.52	9.53±9.11	0.00±5.92
Extr.5	500	77.63±9.96	64.43±6.17	100.59±6.02	79.58±2.17	23.41±11.70	39.33±8.52
	250	85.11±6.04	73.83±6.14	82.87±3.08	56.42±3.31	22.25±4.53	28.79±8.97
	125	90.82±5.75	85.19±9.69	39.85±3.13	33.03±5.11	14.62±6.50	15.38±11.09
	25	93.64±5.81	99.42±1.01	6.63±5.81	2.42±2.62	9.25±8.01	0.00±8.45

^a Incubate and centrifuge

^b Follow immediately

1.2 Results

The cytotoxicity of NK and TA venoms toward normal human skin fibroblast (CCD-25Sk, ATCC, CRL-1474) is shown in Figure S1. This result exhibited those NK and TA venoms at concentrations of 4.46 and 2.23 µg/well, yielded 50% of cell survival, while the

concentrations of 6.10 and 3.06 $\mu\text{g}/\text{well}$, afforded 10% of cell survival. Therefore, we selected 6.10 and 3.06 $\mu\text{g}/\text{well}$ as suitable concentrations for the inhibitory studies.

In this experiment, the inhibitory activity of extracts (Extr.1-5) against both venoms was evaluated by two methods, including the incubation of each venom for 30 minutes at 37 °C and centrifuge, then the supernatant part was further tested with cells in the first method, or the addition of each extracts follow immediately after cells were treated by snake venoms in the second method. The results indicated that all extracts (Extr.1-5) of PB showed good activity against snake venoms (Figures S2 and S3). Although the ethanol leaf extract (Extr.3) tended to reduce the survival of normal skin fibroblast more than that of the ethanol extract of stem (Extr.1), both of them showed strong results of dose-dependent patterns in the inhibition of cell death induced by NK and TA venoms. On the other hand, boiled water (Extr.5) and 40% ethanol (Extr.4) extracts of leaves slightly inhibited cytotoxic effect of both snake venoms. The cytotoxicity of all extracts toward normal human skin fibroblast by two methods is also shown in Figure 4S. The result revealed that all extracts at concentrations of 25 $\mu\text{g}/\text{well}$, yielded > 50% of cell survival.

2. Analysis of NK and TA venom proteins (SDS-PAGE)

Venoms at the amount of 10, 20 and 40 μg were incubated with 25 μl of their antivenoms for 30 min, at 37 °C. After centrifugation, supernatants were analyzed by 12.5% gel electrophoresis (SDS-PAGE), slightly modified method of Laemmli (1970). Protein bands of the mixture were compared to those of venoms or antivenoms alone, with prestained protein ladder ranging from 10K to 170K.

Figure S5. The SDS-PAGE electrophoretograms of two venoms and five PB extracts (A), the supernatants of the venom-extract mixtures (B and C) and the supernatants of the venom-solvent mixtures (D).

2.1 Result

The SDS-PAGE electrophoretograms demonstrated the venom proteins remaining in supernatant after incubation with the extracts. Some of both venom proteins disappeared or faded when incubated with Extr.1-3. This indicated the precipitation of the venom proteins from the venoms incubated with these three extracts occurred (Figure S5). To examine the solvent effect, the mixtures of venom and solvent alone (ethanol or 40% ethanol) were tested. The results showed a gradual disappearance of some of both venom proteins, but no significant effect on the results. (Figure S5D).

3. *In vitro* tests for the inhibition of enzymatic activities

3.1 Phospholipase A₂ activity

Phospholipase A₂ activity was measured according to the method described by Holzer and Mackessyk (1996) using 4-nitro-3-(octanoyloxy) benzoic acid as substrate. NK or TA venom was incubated with the PB extracts at 37 °C for 30 min and then incubated with substrate for 20 min, at 37 °C. The reaction was stopped by placing on ice and terminated with Triton-X 100 then monitored at 425 nm. All experiments were done in duplicate. A change in absorbance of 0.10 AU at 425 nm was equivalent to 25.8 nmoles of chromophore release. The enzyme activity, expressed as the initial velocity (V_0) of the reaction (nmol/min/mg), was calculated considering the increase in absorbance after 20 min. The inhibition by PB extracts was calculated using enzyme activity of venom alone as 100%.

3.2 Proteolytic activity

Proteolytic activity was performed using modified method based on the method of Murzaeva (2000). NK or TA venom was incubated with the PB extracts at 37 °C for 30 min and then incubated with 1% casein, as substrate, for 90 min, at 37 °C. The reaction was stopped by 5% trichloroacetic acid. After centrifugation, the digested protein in the supernatant was estimated by optical absorption of Folin reagent-stained tyrosine at 670 nm. All experiments were done in duplicate. Standard tyrosine curve was used to determine units/ml of enzyme. The proteolytic activity of snake venom was expressed in term of units/mg. The inhibition by PB extracts was calculated using enzyme activity of venom alone as 100%.

Table S2. *In vitro* inhibition of PLA₂ and proteolytic activities of NK and TA venoms by PB extracts

Extract	Inhibition of PLA ₂ (%) ^a		Inhibition of Protease (%) ^a	
	NK	TA	NK	TA
Extr.1	49.90	26.12	59.44	26.86
Extr.2	7.00	5.20	50.70	33.10
Extr.3	39.90	35.18	50.70	27.94
Extr.4	17.06	9.62	- ^b	20.21
Extr.5	3.62	6.25	- ^b	8.14

^a using the same amount of venom and extracts at 250 µg.

^b uninterpretable results due to interference of the extract itself.

3.3 Results

Enzymatic and inhibition studies revealed that the ethanol extract of stems and leaves (Extr.1 and 3) showed good inhibition of PLA₂ and protease enzymes present in those venoms (Table S2) which rendered as active extracts. These extracts were able to inhibit the toxic enzymes of both venoms at 10 mg/ml concentration. Because of interference of the extract itself, the inhibition of Extr.4 and Extr.5 against protease enzyme of NK venom was unreadable. NK protease had lower activity than that of TA. Even after increasing the concentration of NK venom to 20 mg/ml, the results of Extr.4 and Extr.5 were still unreadable. However, these two extracts tended to be weak inhibitor for venom enzymes.

4. Cytotoxicity assay against Vero (African green monkey kidney), Caco2, HepG2 and HEp-2 cell lines

4.1 Sample preparation

The samples were weighed and dissolved in dimethylsulfoxide to make a stock concentration of 50 mg/ml and were then serial diluted in the culture medium of cells giving 8 concentrations of 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 µg/ml. Sample 5 was weighed and dissolved in dimethylsulfoxide to make a stock concentration of 10 mg/ml and was then serial diluted in the culture medium of cells giving 8 concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml.

4.2 Cell culture

The target cells were Vero cell line (African green monkey kidney; ATCC Cat. No. CCL-81), Caco2 cell line (human caucasian colon adenocarcinoma ATCC Cat. No. HTB-37), HepG2 cell line (human caucasian hepatocyte carcinoma ATCC Cat. No. HB-8065) and HEp-2 (Human larynx epithelial; ATCC Cat. No. CCL-23). The Vero, Caco2, HepG2 and HEp-2 cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acid, 1.0 mM sodium pyruvate, 100 unit/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37°C in a fully humidified, 5% CO₂ : air atmosphere.

4.3 MTT cytotoxicity test, a brief summary of method

This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). This MTT assay is a tetrazolium-dye based colorimetric microtitration assay. Metabolism- competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded in a 96-well plate at a density of 3,000 cells/well, and incubated for 48 hours. The sample at various concentrations were added to the cells and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested with MTT assay.

Briefly, 50 µl of MTT in PBS at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the wells,

and formazan solubilized with 200 μ L of DMSO and 25 μ L of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC₅₀ for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC₅₀) of the cells compared to controls.

Table S3 Cytotoxicity of PB extracts (Extr.1-5) against Vero, Caco2, HepG2 and HEp-2 cell lines.

Concentration (μ g/ml)	% Survival			
	Vero	Caco2	HepG2	HEp-2
500	64 $\pm$ 3	109 $\pm$ 2	65 $\pm$ 3	55 $\pm$ 17
250	101 $\pm$ 1	96 $\pm$ 2	102 $\pm$ 4	100 $\pm$ 5
125	106 $\pm$ 1	112 $\pm$ 9	102 $\pm$ 2	101 $\pm$ 8
62.5	106 $\pm$ 2	109 $\pm$ 4	101 $\pm$ 2	107 $\pm$ 2
31.25	101 $\pm$ 3	100 $\pm$ 4	101 $\pm$ 4	107 $\pm$ 1
15.6	107 $\pm$ 1	110 $\pm$ 7	101 $\pm$ 1	105 $\pm$ 2
7.8	107 $\pm$ 1	93 $\pm$ 1	102 $\pm$ 1	110 $\pm$ 1
3.9	109 $\pm$ 1	97 $\pm$ 5	100 $\pm$ 2	108 $\pm$ 2
IC ₅₀ (μ g/ml)	>500	>500	>500	>500

The indication of toxicity has been evaluated in 2 ranges:

- At % survival >50% will be evaluated for no toxicity
- At % survival $\leq$ 50% will be evaluated for toxicity with IC₅₀

4.4. Results

The cytotoxicity assay (Table S3) conforms to the published standard methods (BS-EN30993-5 and ISO10993-5) (Plumb et al. 1989) using Vero, Caco2, HepG2 and HEp-2 cell lines. The cells were exposed to the sample for 24 hours over the concentration ranges of 500-3.9 μ g/ml. The results were shown by % survival of cells at each concentration compared to control and IC₅₀ values. The ethanol whole plant extract of PB exhibited no cytotoxicity against these four cell lines (IC₅₀ > 500 μ g/ml).

4.5 Reference

Plumb JA, Milroy R, Kaye SB. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium based assay. Cancer Res. 1989 49:4435-4440.

5. DPPH radical scavenging activity

Radical scavenging activity was validated by the UV absorbing method [Yen, G.C., Hsieh, C.L., 1997. Antioxidant effects of dopamine and related compounds. Biosci.

Biotechnol. Biochem. 61, 1646–4649.] in 96-well microplate. Briefly, various concentrations of samples dissolved in MeOH (50 μ L) was added 0.3 mM DPPH solution (200 μ L). After 30 minutes incubation at room temperature in the dark, the absorbance of the resulting solution was measured at 517 nm with a spectrophotometer. All tests were run in triplicate and averaged.

Table S4 Antioxidant activity of PB extracts (Extr.1-5).

Extract	IC ₅₀ (mg/mL)
Extr.1	2.77 $\pm$ 0.52
Extr.2	No Test
Extr.3	1.61 $\pm$ 0.93
Extr.4	3.0 $\pm$ 1.18
Extr.5	5.05 $\pm$ 2.13
Ascorbic acid (standard)	0.02 $\pm$ 0.11

5.1 Result

All extracts were subjected to examination for potential free radical scavenging on DPPH. The result indicated that the ethanol extract of leaves (Extr.3) exhibited weak antioxidant activity at IC₅₀ value of 1.61 mg/ml. Other extracts were regarded as inactive (IC₅₀ > 2.0 mg/ml), while the standard reference (ascorbic acid) showed the IC₅₀ value of 0.021mg/ml (Table S4).

6. In vivo acute oral toxicity determination (LD₅₀) of a PB ethanol whole plant extract

6.1 Materials

6.1.1 Test material: “Ethanol extract of a PB whole plant”. Ethanol extract of PB was brownish crude extract with characteristic odor

6.1.2 Animals: Healthy male and female Wistar rats were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom province. Body weight ranges at start were 213 - 238 g for male and 191 - 211 g for female.

6.1.3 Food, Pokphand Animal Feed Co. Ltd., Thailand

6.1.4 Drinking water, filtered water

6.1.5 Distilled water

6.1.6 Balance, OHAUS Model E 02140, Switzerland

6.1.7 Surgical instruments

6.1.8 1-5 ml disposable syringe, Nippon Shoji Kaisha Ltd., Japan

6.1.9 Stomach tubes, Ogawa Seiki Co. Ltd., Japan

6.2 Method

The test was carried out following the Guideline No. 423: Acute oral toxicity - Acute toxic class method of the OECD Guidelines for Testing of Chemicals (2001) with a slight modification in term of increasing doses.

6.2.1 Preparation of the animals

Twenty Wistar rats were divided into two treatment groups (five of each sex per group) and ten Wistar rats (five of each sex) were kept as in a control group. They were acclimatized to the laboratory environment for one week prior to experimentation. Using the simple random sampling method, the rats were randomly selected and marked to indicate individual identification by tail labeling. The rats were fasted overnight for 16 hrs prior to dosing of the test sample while drinking water was available *ad libitum*. The animal protocol followed by the researchers was approved by the Animal Ethics Committee, Pharmaceuticals and Natural Products Department, the Thailand Institute of Scientific and Technological Research (ID TS-59001).

6.2.2 Preparation of the sample and the route of administration

On experimentation day, the test sample was dosed orally at 2,000 and 15,000 mg/kg body weight, respectively. The treated doses were calculated based on their body weights of rats after fasting. The rats in the control group were dosed with distilled water at the equivolume as the treatment group.

6.2.3 Observation

After dosing, food was withheld for 3-4 hrs but water was provided *ad libitum*. All treated rats were observed for any toxic signs immediately at ½, 1 and 3 hrs. The special care should be considered to rats that obviously showed toxic signs during the first 4 hrs after dosing and observed once daily thereafter for 14 days. The definite time, at which signs of toxicity appeared and recovered were recorded to determine the duration of recovery period.

6.2.3.1 Body weight

The body weight of each rat was recorded on days 1, 8 and 15 (at termination) or after death during experimentation period. The weight changes were recorded and calculated. At the end of the test, all survivors were weighed and humanely killed by CO₂ asphyxiation. The means of body weight gain of the rats in treatment groups were calculated in comparison to those of rats in the control group.

6.2.3.2 Gross pathology

All control and treated rats including those died were subjected to gross necropsy. Any gross pathological changes were recorded for each rat.

6.3 Results

The result showed that all treated rats did not exhibit abnormal toxic signs or deaths. No gross pathological lesions were observed on necropsy of the control and all treated rats.

The mortality data were shown in **Table S5**. The body weight of all rats of the control and treatment groups recorded during the observation period was shown in **Table S6**. The means of body weight gain of the rats of the control and treatment groups were shown in

Table S7. Results of necropsy finding at termination of control and all survival rats of treatment groups were shown in **Table S8**.

Table S5 Summary of mortality data of the control and treated rats.

Treatment / Dose	^a Mortality rate		
	Male	Female	Total
Control group Distilled water at the equivolume as the treatment group	0/5	0/5	0/10
Treatment group Ethanol extract of PB 2,000 mg/kg body weight	0/5	0/5	0/10
Treatment group Ethanol extract of PB 15,000 mg/kg body weight	0/5	0/5	0/10

^a Number of dead rats / number of rats tested.

Table S6 Values of body weight of the control and treated rats recorded during the observation period.

Treatment / Dose	Animal No.	Sex	Body weight (g)		
			^b Day 1	Day 8	Day 15
Control group Distilled water at the equivolume as the treatment group	1551	Male	224	260	301
	1552	Male	226	268	306
	1553	Male	228	265	297
	1554	Male	224	263	308
	1555	Male	230	274	304
	1556	Female	200	204	215
	1557	Female	211	250	260
	1558	Female	202	222	234
	1559	Female	203	218	227
	1560	Female	208	223	238
Treatment group Ethanol extract of PB 2,000 mg/kg body weight	1561	Male	220	266	292
	1562	Male	238	281	311
	1563	Male	233	283	312
	1564	Male	221	268	297
	1565	Male	226	278	306
	1566	Female	202	219	229
	1567	Female	201	222	234
	1568	Female	201	216	227
	1569	Female	210	228	238
	1570	Female	208	225	236
Treatment group Ethanol extract of PB 15,000 mg/kg body weight	1571	Male	218	272	302
	1572	Male	230	282	312
	1573	Male	230	276	304
	1574	Male	216	260	298
	1575	Male	213	255	287
	1576	Female	191	210	222
	1577	Female	192	202	218
	1578	Female	191	205	216
	1579	Female	204	216	227
	1580	Female	203	223	234

^b Recorded as initial body weight of rats after 16-hr overnight fasting time.

Table S7 Means of body weight gain of the control and treated rats recorded during experimentation and at termination.

Sex	Treatment / Dose	^c Mean of body weight gain (g)	
		Day 8	Day 15
Male	Control group Distilled water at the equivolume as the treatment group	39.60 ± 1.50	76.80 ± 2.55
	Treatment group Ethanol extract of PB 2,000 mg/kg body weight	47.60 ± 1.57	76.00 ± 1.58
	Treatment group Ethanol extract of PB 15,000 mg/kg body weight	47.60 ± 2.31	79.20 ± 2.15
Female	Control group Distilled water at the equivolume as the treatment group	18.60 ± 5.72	30.00 ± 5.58
	Treatment group Ethanol extract of PB 2,000 mg/kg body weight	17.60 ± 0.98	28.40 ± 1.21
	Treatment group Ethanol extract of PB 15,000 mg/kg body weight	15.00 ± 1.95	27.20 ± 1.62

^c Data are mean ± SEM

Table S8 Results of necropsy finding of the control and treated rats at termination.

Treatment / Dose	Animal No.	Sex	Gross pathological finding
Control group Distilled water at the equivolume as the treatment group	1551	Male	Normal
	1552	Male	Normal
	1553	Male	Normal
	1554	Male	Normal
	1555	Male	Normal
	1556	Female	Normal
	1557	Female	Normal
	1558	Female	Normal
	1559	Female	Normal
	1560	Female	Normal
Treatment group Ethanol extract of PB 2,000 mg/kg body weight	1561	Male	Normal
	1562	Male	Normal
	1563	Male	Normal
	1564	Male	Normal
	1565	Male	Normal
	1566	Female	Normal
	1567	Female	Normal
	1568	Female	Normal
	1569	Female	Normal
	1570	Female	Normal
Treatment group Ethanol extract of PB 15,000 mg/kg body weight	1571	Male	Normal
	1572	Male	Normal
	1573	Male	Normal
	1574	Male	Normal
	1575	Male	Normal
	1576	Female	Normal
	1577	Female	Normal
	1578	Female	Normal
	1579	Female	Normal
	1580	Female	Normal

6.4 Statistical Analysis

All values in the *in vivo* acute oral toxicity determination of a PB ethanol whole plant extract were expressed as mean $\pm$ standard error of the mean (S.E.M). Data from treated animals were compared with those of control group. Males and females were evaluated separately and statistical significances between the treated groups and the respective control groups were determined by two-tailed unpaired Student's t-test. Differences with $p < 0.05$ were considered statistically significant.

6.5 Summary

The acute oral toxicity determination (LD₅₀) of Ethanol extract of PB was carried out with a slight modification of the Test Guideline No. 423 : Acute oral toxicity - Acute toxic class method of the OECD Guidelines for Testing of Chemicals (2001).

Twenty Wistar rats of two treatment groups (five of each sex per group) were orally dosed with Ethanol extract of PB at 2,000 and 15,000 mg/kg body weight. All rats were observed for toxic signs at ½, 1 and 3 hrs after dosing and once daily thereafter for 14 days. The result showed that all treated rats did not exhibit abnormal signs or deaths. No significant gross pathological lesions were found on necropsy of the control and treated rats.

The oral LD₅₀ of the ethanol extract of PB in Wistar rats of both sexes was greater than 15,000 mg/kg body weight.

6.5 References

1. Auletta, C.S. (1995). Acute, Subchronic and Chronic Toxicity. In: CRC Handbook of Toxicology. Michale J. Derelanko and Mannfred A Holinger, eds, CRC Press, pp. 51-104.
2. Donald, J.E. (1997). The Basic of Toxicity Testing. 2nd Edition, CRC Press, Boca Raton, New York.
3. Litchfield, J.T. and Wilcoxon, F. (1949). *J. Pharmacol. Exp. Ther.* 96, 99-113.
4. Organization for Economic Co-operation and Development (2001). OECD Guidelines for Testing of Chemicals, Volume 2, Section 4: Health Effects. Acute Oral Toxicity - Acute Toxic Class Method. Test Guideline No. 423.
5. United State Environmental Protection Agency (EPA) (1998). Prevention, Pesticides and Toxic Substances (7101) Health Effects Test Guidelines, OPPTS 870-1100 Acute Oral Toxicity, EPA 712-6-96-190.