Toxicological and clastogenic evaluation of pinocembrin and pinostrobin isolated from *Boesenbergia pandurata* in Wistar rats

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ABSTRACT

Pinocembrin (5, 7-dihydroxyflavanone) and pinostrobin (5-hydroxy-7-methoxy flavanone) are flavanones present in some natural products including the rhizome of *Boesenbergia pandurata*. Many reports showed their *in vitro* biological benefits without safety indication. The toxicity and mutagenicity of both flavanones in male Wistar rats were examined. There were no deaths observed following a single oral administration of 500 mg/kg of both compounds. Body weight, vital organ weights and blood biochemistry values of treated rats were not significantly different compared with those of the control group. The mutagenicity of both flavanones using liver micronuclei in male Wistar rats was assessed. After feeding rats with 1-100 mg/kg for 7 days, the liver micronucleus formation and mitotic cells in hepatocytes were analyzed. Neither phytochemical affected micronucleus formation or mitotic index. Our results indicated that pinocembrin and pinostrobin are non toxic and are not mutagenic to male Wistar rats within the 1-100 mg/kg interval.

Keywords: Toxicity, Boesenbergia pandurata, Clastogenicity, Micronucleus, Pinocembrin, Pinostrobin

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การประเมินความเป็นพิษและฤทธิ์ทำลายสารพันธุกรรมของสารพิโนเซมบรินและสารพิโนสโตรบินที่แยกได้จากเห้ากระชายในหนูขาว

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บทคัดย่อ

พิโนเซมบรินและพิโนสโตรบินเป็นสารในกลุ่มฟลาวาโนนในเห้ากระชายที่มีฤทธิ์ทางชีวภาพอย่างกว้างขวาง อย่างไรก็ตามการศึกษาดังกล่าวล้วนเป็นผลการวิจัยในหลอดทดลอง ดังนั้นจึงได้ทำการศึกษาความเป็นพิษและฤทธิ์การทำลายสารพันธุกรรมในหนูแรท ผลการทดลองพบว่าสารพิโนเซมบรินและพิโนสโตรบินไม่ก่อให้เกิดการตายในหนูแรทที่ได้รับสารดังกล่าวความเข้มข้น 500 มิลลิกรัมต่อน้ำหนักตัว อีกทั้งการวิเคราะห์ความเป็นพิษต่ออวัยวะสำคัญและค่าทางชีวเคมีของเลือดบางชนิดไม่พบความผิดปกติเมื่อเทียบกับกลุ่มควบคุม จากการประเมินฤทธิ์ทำลายสารพันธุกรรมโดยการให้สารพิโนเซมบรินและพิโนสโตรบินความเข้มข้น 1-100 มิลลิกรัมต่อน้ำหนักตัวแก่หนูแรทเป็นเวลา 7 วัน พบว่าสารทั้งสองชนิดไม่เหนี่ยวนำให้เกิดการแตกหักของแท่งโครโมโซมและไม่มีผลต่อดัชนีการแบ่งตัวของเซลล์ตับ ดังนั้นสารพิโนเซมบรินและพิโนสโตรบินจึงไม่มีความเป็นพิษในหนูแรทและไม่มีความเป็นพิษต่อสารพันธุกรรมในเซลล์ตับหนูในช่วงความเข้มข้น 1-100 มิลลิกรัมต่อน้ำหนักตัว

คำสำคัญ: ความเป็นพิษ กระชาย ฤทธิ์ทำลายสารพันธุกรรม ไมโครนิวเคลียส พิโนเซมบริน และ พิโนสโตรบิน

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Introduction

Boesenbergia pandurata (Roxb.), currently known as *Boesenbergia rotunda*[1] or locally called Krachai in Thailand, is a perennial herb of the ginger family (Zingiberaceae). The fresh rhizome is used as a food ingredient, an aphrodisiac Thai folk medicine, and for the treatment of colic disorder. Several biological functions including antimutagenicity, antioxidant and antibacterial activity of the components of fingerroot have been demonstrated.[2, 3]

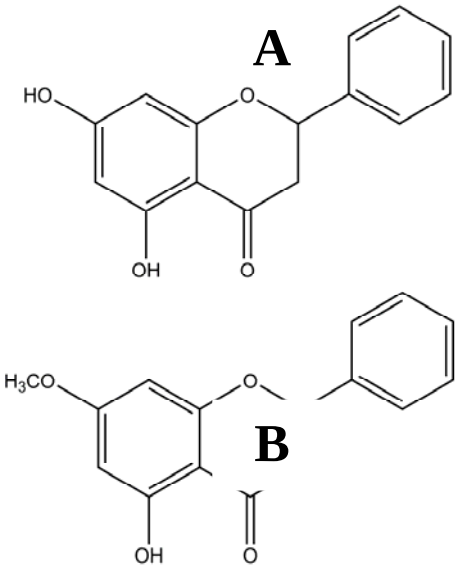


Figure 1 Chemical structure of pinocembrin (A) and pinostrobin (B)

Pinocembrin and pinostrobin, (Figure 1) are the biologically active flavanones found in the rhizome of *B. pandurata*. There are many reports indicating their biological benefits. Pinocembrin has displayed antibacterial and antifungal activity in vitro.[4-6] Recently, it has been shown to prevent glutamate-induced apoptosis in SH-SY5Y neuronal cells via decrease of bax/bcl-2 ratio,[7] and was capable of protecting the rat brain from oxidation and apoptosis induced by ischemia-reperfusion both in vitro and in vivo.[8] It has also exhibited antimutagenicity in Ames tests,[9] and triggered Bax-dependent mitochondrial apoptosis in colon cancer cells.[10] Pinostrobin has extremely high quinone reductase activity in murine hepatoma.[11] Trakoontivakorn reported that pinostrobin had antimutagenic effect toward pyrolysate product, Trp-P-1 in *Salmonella typhimurium* TA 98 (9). In addition, it has been reported that pinostrobin inhibited breast cancer cell (MCF-7) growth and induced cell cycle arrest (G2/M phase) in prostate cancer cells (LNCaP and PC3).[12, 13] To date, extensive studies have demonstrated the health improvement and disease prevention, and anticancer effects of phytochemicals.[14, 15] Little is known, however, concerning either *in vivo* mutagenicity or *in vivo* antimutagenicity against mutagens for both of the above mentioned compounds.

Well-known tests for cytogenetic damage include the bacterial mutation test, micronucleus test, and chromosome aberration test. However, application of the right test a suitable target organ should be considered for obtaining reliable conclusions.[16, 17] It is well known that the liver is extremely important for risk and mutagenic assessment due to the predominantly high penetration of almost all compounds to this organ and its metabolizing capacity.[18] The liver

micronucleus test provides a predictive assay of mutagenicity and early events in carcinogenesis, and has further usage in the screening of compounds for clastogenicity and mutagenicity.[19, 20]

In this study, we evaluated the acute toxicity and clastogenicity of both pinocembrin and pinostrobin isolated from rhizomes of *Boesenbergia pandurata* in male Wistar rats.

Materials and methods

Materials

Collagenase type IV prepared from *Clostridium histolyticum* (CAS no. 9001-12-1) and 4'-6-Diamidino-2-phenylindole (DAPI) as fluorescent dye (CAS no. 28718-90-3) were purchased from Invitrogen Corp. (CA, USA). Diethylnitrosamine (CAS no. D0516) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Japan). N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid (HEPES, free acid) (CAS no. 7365-45-9) and ethylene glycol tetraacetic acid (EGTA) as chelating agents (CAS no. 67-42-5) were purchased from Amresco, Inc. (Ohio, USA). Tween 80 (CAS no. 9005-65-6) was purchased from VWR International (Pennsylvania, USA).

Plant materials

B. pandurata rhizomes were collected from Lampang province, Thailand. The plant was identified by Prof. Dr. Thawatchai Santisuk and the voucher specimen (BKF 68909) deposited at the Forest Herbarium, National Park, Wildlife, and Conservation Department, Bangkok, Thailand.

Animals and housing

Six-week old male outbred Wistar rats (190-200 g) were obtained from the National Laboratory Animal Center (Nakorn-Prathom, Thailand). Three rats per cage were acclimatized for at least seven days prior to the start of the study. Rats were housed in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. They were maintained at 25˚C, with 12 hr alternate light and dark cycles. Commercial pellet diet and chlorinated tap water were available *ad libitum* throughout the experiment. All rats were housed and treated under ethical committee permission.

Preparation of pinocembrin and pinostrobin from rhizome of Boesenbergia pandurata

The air-dried powder (1 kg) of *B. pandurata* rhizomes was percolated with *n*-hexane for 9 days, at room temperature. Then, the residue was subsequently percolated with ethyl acetate for 15 days. The filtrate was evaporated to dryness under reduced pressure to obtain an ethyl acetate crude extract (79.99 g).

To obtain pinocembrin and pinostrobin, the ethyl acetate extract was firstly subjected to coarse separation on a silica column chromatography. Gradient elution was conducted initially with *n*-hexane, gradually enriched with ethyl acetate, followed by increasing amounts of methanol in ethyl acetate

and finally with methanol. Six fractions were collected. The active fraction, fraction 5, was rechromatographed on a silica column, eluting with the various proportions of ethyl acetate: *n*-hexane, followed by increasing amounts of methanol in ethyl acetate and finally with methanol. Fractions were collected and combined on the basis of their thin layer chromatography behavior. The subfraction 4 and 5 were eluted by ethyl acetate and *n*-hexane. The obtained solid was recrystalized from ethanol to obtain pinostrobin (95.99 mg) and pinocembrin (69.32 mg). Finally, structural confirmation using UV, FTIR, ^{1}H NMR, ^{13}C NMR, MS was performed. The data obtained were compared to the those in previously reported literatures.[21-23] The purity of both compounds was more than 95%.

Toxicity study of pinocembrin and pinostrobin

The toxicity of pinocembrin and pinostrobin were evaluated in rats using the modified guidelines established by the organization for economic cooperation and development.[24]

Rats were randomly divided into three groups, 5 rats per group. The treatment groups were given a single dose of 500 mg/kg bw of pinocembrin or pinostrobin which was derived from air-dried fingerroot about 1.4 kg for pinocembrin and 1.0 kg for pinostrobin. The control group was received 5% Tween-80. Rats were observed individually after dosing periodically during the first 24 hr, with special attention given during the first 4 hr, and daily thereafter for 14 days. Additional observations, including changes in skin and fur, eyes, mucous membranes, respiration, and general behavior pattern were checked during the experiment.

All the rats were weighed once before the commencement of the dosing and then twice a week. The amount of food and water consumption was measured twice a week, and at the end of the experiment. At the end of the study, blood was taken from the abdominal vein under anesthesia and was collected in centrifuge tubes for analysis of biochemical values. The serum was separated by centrifugation at 5,000 rpm for 20 min at room temperature, and transferred into collecting tube for further analysis. The rats were sacrificed with an overdose of ether. Examination of the organs including liver, kidneys, testis, spleen, lungs, heart, and thymus gland were carried out. Each organ was removed and weighed.

Parameters for blood biochemical value

Liver function markers, including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase were determined by commercial kits purchased from Biotech Co., Ltd (Bangkok, Thailand). Determination of albumin/globulin ratio was determined using a bromcresol green assay.[25] The oxime method for determining urea concentration is based on a reaction described by Fearon (1939). Finally, total protein was determined according to the Lowry method (1951).

Clastogenicity study of pinocembrin and pinostrobin

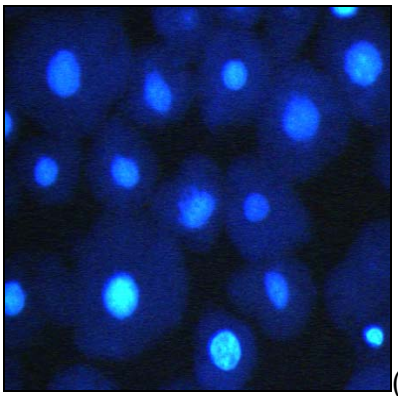
Rats were orally administered by 1, 10 and 100 mg/kg bw of pinocembrin or pinostrobin for seven days. (6 rats per group) Five percent of Tween-80 was used as a negative control and 30 mg/kg bw of diethylnitrosamine was used as a positive control. After that, all rats were subjected to partial hepatectomy (PH) according to the method of Higgins and Anderson[26] on day 8 and left for four days for compensatory proliferation of hepatocytes. At day 12, sacrifice was done by two-step collagenase perfusion as described below.

Isolation of hepatocytes by two-step collagenase perfusion

Isolation of hepatocytes was performed according to Puatanachokchai's method.[27] In brief, anesthetized rats were firstly perfused with preperfusion medium containing 0.01 M HEPES and 0.5 mM EGTA, pH 7.4, 37 °C, to remove blood. Secondly, collagenase medium containing 0.05% collagenase type IV and 0.01 M HEPES, pH 7.4, 37 °C was continuously perfused to isolate hepatocytes. Then, the isolated hepatocytes were washed with phosphate buffer saline, pH 7.4 followed by 10% buffered formalin, pH 7.4 3 times. The hepatocytes were suspended in 10% buffered formalin, pH 7.4 and kept at 4 °C until micronucleus analysis.

Micronucleated hepatocyte analysis

Prior to micronucleus analysis, hepatocyte suspensions were mixed with 20 µg/ml 4´, 6-diamidino-2-phenylindole dihydrochloride (DAPI) stain solution for fluorescent microscopy. Stained hepatocytes were dropped onto a glass slide and covered with a coverslip. The number of micronucleated hepatocytes was counted and recorded based on analysis of 2,000 hepatocytes from each animal under a fluorescent microscope (x400) equipped with an ultraviolet system. Criteria for micronucleated hepatocyte scoring was the presence of a round or distinct micronucleus stained with the same color as the nucleus, with diameter of ¼ or less than that of the main nucleus.[28] Mitotic index (MI), indicative of mitotic activity, was calculated from mitotic cells in 2,000 hepatocytes. Diagrams of normal hepatocytes and micronucleated hepatocytes are illustrated in Fig. 2.


(A)

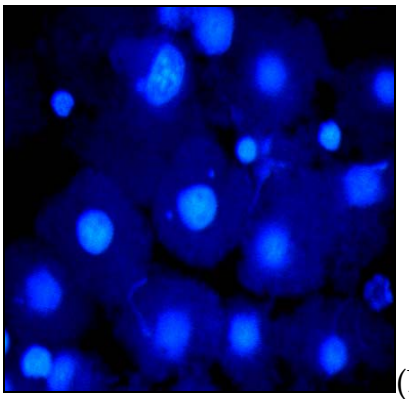

(B)

Figure 2
Normal hepatocytes (A)
and Micronucleated hepatocytes (B)
obtained from fluorescent microscopy
(x400 magnification)

Table 1 Body weight change, water intake and food consumption of control and pinocembrin- and pinostrobin-treated rats.

Parameters	Control	Pinocembrin	Pinostrobin
Initial body weight (g)	204 ± 13.87	205 ± 10.00	193 ± 7.58
Final body weight (g)	280 ± 23.18	281 ± 12.94	290 ± 11.73
Body weight change (%)	37.29 ± 7.58	37.29 ± 8.27	50.46 ± 8.88
Average water intake (ml/rat/day)	43.75 ± 9.22	46.75 ± 16.34	37.50 ± 7.77
Average food intake (g/rat/day)	22.00 ± 2.16	22.75 ± 2.22	21.75 ± 2.36

Values expressed as mean ± standard deviation, n = 5

Treated groups were fed with pinocembrin or pinostrobin while the control group was fed with 5% Tween 80.

Initial and final body weights were recorded on day 1 and day 14, respectively. Percentages of body weight change were calculated on day 14.

Table 2 Relative organ weight of control and pinocembrin- and pinostrobin-treated rat obtained from acute toxicity study

Organs	Relative organ weight (g/100 g bw)		
	Control	Pinocembrin	Pinostrobin
Lungs	0.44 ± 0.04	0.44 ± 0.04	0.46 ± 0.06
Liver	3.41 ± 0.29	3.06 ± 0.52	3.71 ± 0.28
Spleen	0.20 ± 0.02	0.21 ± 0.12	0.21 ± 0.01
Heart	0.31 ± 0.04	0.32 ± 0.03	0.31 ± 0.03
Kidneys	6.60 ± 1.11	7.25 ± 1.72	5.65 ± 0.33
Testis	1.20 ± 0.08	1.21 ± 0.14	1.21 ± 0.03
Thymus gland	0.28 ± 0.04	0.29 ± 0.14	0.25 ± 0.03

Values expressed as mean ± standard deviation, n = 5

Treated groups were fed with pinocembrin or pinostrobin while the control group was fed with 5% Tween 80.

Organs were removed and weighed from all rats on day 14.

Table 3 Effect of pinocembrin and pinostrobin liver on blood biochemical values in male rat

Parameter	Control	Pinocembrin	Pinostrobin
AST (U/L)	105.0 ± 11.6	101.1 ± 6.3	92.6 ± 22.9
ALT (U/L)	50.9 ± 10.2	55.9 ± 3.3	51.8 ± 16.5
ALP (U/L)	122.8 ± 45.8	104.5 ± 7.36	132.4 ± 14.2
Total protein (g/L)	100.4 ± 11.1	112.9 ± 18.5	103.4 ± 10.3
A/G ratio	1.0 ± 0.3	0.8 ± 0.3	0.8 ± 0.1
Urea (mmol/L)	12.7 ± 2.2	10.7 ± 2.4	13.9 ± 1.1

Values expressed as mean ± standard deviation, n = 5

Treated groups were fed with pinocembrin or pinostrobin while the control group was fed with 5% Tween 80. AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase, A/G ratio = albumin/globulin ratio

Table 4 Clastogenicity study of pinocembrin in male rat liver

Pinocembrin (mg/kg)	Initial bw (g)	Final bw (g)	MNH/1,000	Mitotic index (%)
0	190.7 ± 9.9	226.4 ± 13.5	1.28 ± 1.11	1.03 ± 0.28
1	191.7 ± 8.2	238.3 ± 14.7	2.32 ± 1.74	0.99 ± 0.43
10	192.1 ± 8.6	212.1 ± 25.3	1.11 ± 1.44	0.74 ± 0.25
100	188.0 ± 4.5	229.0 ± 16.4	0.70 ± 0.80	1.36 ± 0.24

Values expressed as mean ± standard deviation, n = 5 for control group and n = 6 for treated group

MNH/1,000: number of micronucleated hepatocytes per 1,000 hepatocytes (based on 2,000 hepatocytes per animal) Treated groups were fed with 1, 10, and 100 mg/kg of pinocembrin or pinostrobin for 7 days while the control group was fed with 5% Tween 80. PH was carried out on day 8. The number of micronucleated hepatocytes in a positive control group which was received 30 mg/kg bw of diethylnitrosamine were 31.84 ± 8.98.

Table 5 Clastogenicity study of pinostrobin in male rat liver

Pinostrobin (mg/kg)	Initial bw (g)	Final bw (g)	MNH/1,000	Mitotic index (%)
0	205 ± 6	237 ± 12	2.02 ± 1.28	1.02 ± 0.17
1	204 ± 6	228 ± 7	1.92 ± 1.14	1.13 ± 0.33
10	210 ± 8	244 ± 13	2.03 ± 1.37	1.23 ± 0.24
100	206 ± 5	243 ± 8	2.05 ± 1.26	1.16 ± 0.17

Values expressed as mean ± standard deviation, n = 5 for control group and n = 6 for treated group

MNH/1,000: number of micronucleated hepatocytes per 1,000 hepatocytes (based on 2,000 hepatocytes per animal) Treated groups were fed with 1, 10, and 100 mg/kg of pinocembrin and pinostrobin for 7 days while the control group was fed with 5% Tween 80. PH was carried out on day 8. The number of micronucleated hepatocytes in a positive control group which was received 30 mg/kg bw of diethylnitrosamine were 31.84 ± 8.98.

Statistical analysis

Data expressed as means ± standard deviation of each parameter for each group were firstly tested for normality using a Kruskal-Wallis test. The significance of difference between groups was analyzed using analysis of variance (ANOVA) with Bonferroni error level adjustment for a post hoc test (SPSS 15.0 software package). *P* value <0.05 was regarded as significant.

Results and discussion

Body weights of rats administered a single 500 mg/kg dose of pinocembrin or pinostrobin increased throughout the study period. Water intake and food consumption did not show any significant change as compared to the control group in the period of time studied. Water, but not food intake by pinostrobin treated rats was lesser than in control rats. Other toxicity tests, such as sub-chronic or chronic toxicity tests, might be performed to confirm this effect. There were no adverse effects or behavioral changes observed between control and treated rats. There were no deaths, and the LD_{50} of both phytochemicals is more than 500 mg/kg for the Wistar strain.

The effects of both phytochemicals on organ weights and biochemical values were measured. The relative organ weights, including liver, lungs, kidneys, spleen, heart, testis, and thymus gland of the treated rats showed no significant change compared with control rats (Table 2). As shown in Table 3, parameters such as aspartate

aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, albumin, albumin-globulin ratio (A/G), and urea concentration were unaffected by both phytochemicals. However, more toxicity tests should be conducted to confirm that daily intake of fingerroot or any other plant containing these compounds is not toxic.

In vivo clastogenicity of pinocembrin or pinostrobin was examined by a micronucleus assay in regenerating livers of rat. Four days after PH, the body weights of treated rats were not differed from those of the control group, and mean number of micronucleated hepatocytes/1,000 hepatocytes (MNH/1,000) similar to mitotic index were not different in rats treated with 1 (low dose), 10 (medium dose), and 100 (high dose) mg/kg of pinocembrin or pinostrobin compared with vehicle control rats (Table 4 and 5). Accordingly, an experiment is considered as positive when significantly induced the number of micronucleus increase dose dependently and exceed the corresponding control level at least 1.5 times.[29] In this experiment, a positive control group which was injected by 30 mg/kg bw of diethylnitrosamine induced clastogenicity as indicated by the significantly increase the number of micronucleated hepatocytes (31.84 ± 8.98) compared with a negative control group. The number of micronucleated hepatocytes of pinocembrin and pinostrobin treated groups were not different from their negative control. Correspondingly, the threshold of 1.5 times over the control level is based on the variations of micronucleus frequencies in negative controls and on the observation that micronucleus frequencies recorded with non-mutagens or non-carcinogens were generally below this level.[29] It might be suggested that pinocembrin and pinostrobin did not present clastogenicity in rat liver.

In conclusion, both pinocembrin and pinostrobin exhibited non-toxic and non-genotoxic to male Wistar rats. Since, there are many reports concerning the *in vitro* antimutagenicity[2, 9] and induction of phase II chemopreventive and antioxidant properties of these compounds,[21] they may be promising agents for cancer chemoprevention.

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