RESEARCH COMMUNICATION

# Effects of Pinocembrin on the Initiation and Promotion Stages of Rat Hepatocarcinogenesis

**Charatda Punvittayagul[1], Wilart Pompimon[2], Hideki Wanibuchi[3], Shoji Fukushima[4], Rawiwan Wongpoomchai[1]***

## Abstract

**Pinocembrin (5, 7-dihydroxyflavanone) is a flavanone extracted from the rhizome of *Boesenbergia pandurata*. Our previous studies demonstrated that pinocembrin had no toxicity or mutagenicity in rats. We here evaluated its effects on the initiation and promotion stages in diethylnitrosamine-induced rat hepatocarcinogenesis, using short- and medium-term carcinogenicity tests. Micronucleated hepatocytes and liver glutathione-S-transferase placental form foci were used as end point markers. Pinocembrin was neither mutagenic nor carcinogenic in rat liver, and neither inhibited nor prevented micronucleus formation as well as GST-P positive foci formation induced by diethylnitrosamine. Interestingly, pinocembrin slightly increased the number of GST-P positive foci when given prior to diethylnitrosamine injection.**

**Keywords:** *Boesenbergia pandurata* - cancer chemoprevention - diethylnitrosamine - liver micronucleus test

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## Introduction

Cancer chemoprevention is defined as the use of chemical agents to reverse, suppress, or prevent multistage carcinogenesis (Surh, 2003). Nowadays, many dietary phytochemicals can be considered as chemopreventive agents because they have been shown to inhibit carcinogenesis (Debersac et al., 2001). The mechanism of chemical protection against the initiation stage involves the induction of phase I and phase II xenobiotic-metabolizing enzymes (Tan & Spivack, 2009). Moreover, the chemopreventive activity also influences cell proliferation, differentiation and apoptosis (Chen & Kong, 2004), preventing the accumulation of damaged cells.

Flavanones are a subclass of flavonoids that naturally occur in various plant species, including spices and condiments, cereals, vegetables and fruits. There have been many reports indicating their effects on multistep carcinogenesis (Galati & O'Brien, 2004). Hsiao et al. (2007) showed that flavanone and 2'-OH flavanone inhibited the invasion and metastasis of lung cancer cells in both *in vitro* and *in vivo* models. In 2009, Aranganathan and Nalini demonstrated that hesperetin had anti-carcinogenic potential against DMH-induced colon cancer. In addition, naringenin reduced tumor size and weight in N-methyl-N'-nitro-N-nitrosoguanidine-induced rat gastric carcinogenesis (Ekambaram et al., 2007), and also inhibited glial tumor cell proliferation in rat C6 glioma models (Sabarinathan et al., 2011).

Pinocembrin is a flavanone found in rhizomes of *B. pandurata* or "Kra-chai" in Thai (Jaipetch et al., 1982). The chemical structure of this compound is shown in Figure 1. Previous investigations have demonstrated that pinocembrin has various pharmacological activities, including anti-oxidant and anti-inflammatory (Pepeljnjak et al., 1985; Santos et al., 1998; Tuchinda et al., 2002; Hwang et al., 2003; Sala et al., 2003; Liu et al., 2008). Moreover, it exhibited a strong antimutagenic activity against mutagenic heterocyclic amines (Trakoontivakorn et al., 2001). Our previous study indicated that pinocembrin had no toxicity or mutagenicity in male rats (Charoensin et al., 2010). In addition, it could inhibit activities of P450 isozymes involved in carcinogen metabolism (Siess et al., 1995) and also induced the activity of heme oxygenase in rat liver (Punvittayagul et al., 2011).

Based on these observations, we hypothesized that pinocembrin may help protect against chemical-induced hepatocarcinogenesis. However, the in vivo

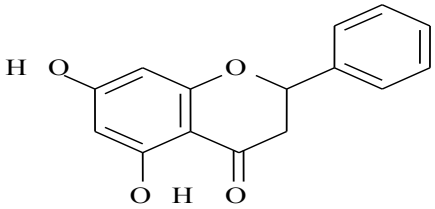


**Figure 1. Structure of Pinocembrin**

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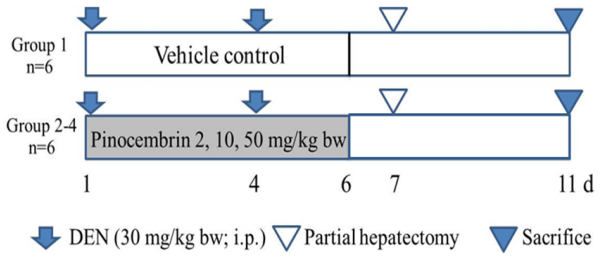


**Figure 2. The Protocol for Examining the Inhibitory Effect of Pinocembrin on DEN–Induced Initiation Stage of Rat Hepatocarcinogenesis**

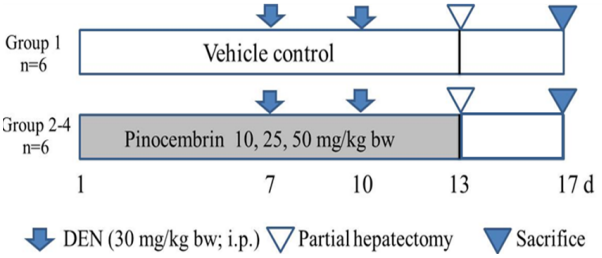


**Figure 3. The Protocol for Investigating the Preventive Effect of Pinocembrin on DEN–Induced Initiation Stage of Rat Hepatocarcinogenesis**

carcinogenic and anticarcinogenic effects of pinocembrin have not previously been investigated. Therefore, rat models are needed to determine whether administration of pinocembrin could inhibit hepatocarcinogenesis. Hence, the rat liver micronucleus and medium-term carcinogenicity tests were performed to determine the effect of pinocembrin on the initiation and promotion stages of rat hepatocarcinogenesis, respectively.

## Materials and Methods

*Animals*

Male Wistar rats were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn-Prathom, Thailand and were kept in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Rats were given an acclimatization period of one week before each experiment. They were housed at a maximum of three per cage with a light–dark cycle 12–12 hours, at temperatures of 21–25 °C and relative humidity 50–60% throughout the study. Each animal had free access to diet and tap water. The experimental protocols were approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University.

*Chemicals*

Pinocembrin was obtained from Assoc. Prof. Wilart Pompimon, Faculty of Science, Lampang Rajabhat University, Thailand; collagenase type IV and 4', 6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen, USA; diethylnitrosamine was purchased from Tokyo Kasei Kogyo Co. Ltd., Japan; diaminobenzidine was from Dojindo, Japan; primary rabbit polyclonal antibodies against rat GST-P was obtained from MBL, Japan; Vectastrain ABC kit was obtained from Vector

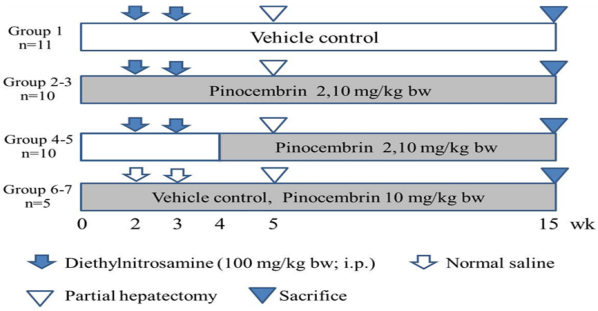


**Figure 4. Medium-Term Carcinogenicity Protocol**

Laboratories, Inc., USA.

*Short-term carcinogenicity test*

The first experiment investigated the inhibitory and preventive effects of pinocembrin on diethylnitrosamine (DEN) – induced initiation stage of rat hepatocarcinogenesis. This study was performed using 2 protocols. All rats were intraperitoneal (i.p.) injected with DEN on day 0 and day 3. In the first protocol, rats were divided into 4 groups orally receiving various concentrations of pinocembrin, 0, 2, 10, and 50 mg/kg bw for 6 days on day 0 to day 5. In the latter protocol, rats were classified into 4 groups receiving various dosages of pinocembrin, 0, 10, 25 and 50 mg/kg bw for 12 days, 6 days before DEN injection on day 6 of the experiment. The incidence of micronucleated hepatocytes was determined 4 days after partially hepatectomy, as shown in Figures 2 and 3, respectively. Hepatocytes were isolated from anesthetized rats by the 2-step collagenase perfusion method according to Puatanachokchai et al. (Puatanachokchai et al.,1996). Then hepatocyte suspensions were mixed with DAPI stain solution, and analyzed under a fluorescent microscope. The micronucleated hepatocytes (MNHEPs) and mitotic cells were recorded based on analysis of 2000 hepatocytes from each animal.

*Medium-term rat liver carcinogenicity test*

To determine the effect of pinocembrin on the promotion stage in DEN-induced hepatocarcinogenesis, a modified method of the medium term bioassay system of Ito based on the two-step model of hepatocarcinogenesis (Ito et al., 2003; Tsuda et al., 2010) was developed in our laboratory for detection the carcinogenic and anticarcinogenic activities of chemical compounds. In this experiment, male Wistar rats were divided into 7 experimental groups (Figure 4). At weeks 3 and 4 of the experiment, groups 1 to 5 were given a double i.p. injection of DEN to initiate hepatocarcinogenesis, while groups 6 and 7 were i.p. administered a normal saline solution. Before 2 weeks of injection, groups 2 and 3 received oral pinocembrin at 2 and 10 mg/kg bw, respectively. Groups 4 and 5 were fed with pinocembrin at 2 and 10 mg/kg bw, respectively, after injections for 1 week. Groups 1 and 6 were treated with a vehicle control, while group 7 was fed pinocembrin at 10 mg/kg. All animals were 2/3 partial hepatectomized at week 6 to stimulate the hepatocytes into mitosis using the technique described by Higgins and Anderson (1931) and were sacrificed at week 15. Blood samples were collected and analyzed for serum alanine aminotransferase, aspartate aminotransferase and alkaline

**Table 1. Inhibitory Effect of Pinocembrin on Diethylnitrosamine-Induced Micronucleus Formation in Rat Liver**

| Treatment | Body weight (g) | | MNHEPs/1,000 | Mitotic |
|---|---|---|---|---|
| (mg/kg bw) | Initial | Final | hepatocytes | index (%) |
| DEN | 197.7 ± 5.4 | 216.7± 7.5 | 31.8 ± 9.0 | 3.38±1.12 |
| DEN+PC2 | 206.3 ± 11.1 | 228.8± 8.5 | 27.9 ± 12.4 | 3.73±1.50 |
| DEN+PC10 | 200.0 ± 7.9 | 226.0± 13.9 | 28.5 ± 4.8 | 2.94±0.62 |
| DEN+PC50 | 201.7 ± 8.2 | 218.3± 12.5 | 27.6 ± 11.9 | 2.81±0.99 |

*Values expressed as mean ± SD, MNHEPs = micronucleated hepatocytes, DEN = diethylnitrosamine, 30 mg/kg bw; i.p.

**Table 2. Preventive Effect of Pinocembrin on Diethylnitrosamine-Induced Micronucleated Hepatocyte Formation in Rats**

| Treatment | Body weight (g) | | MN- | % Inhi- | Mitotic |
|---|---|---|---|---|---|
| (mg/kg bw) | Initial | Final | HEPs[a] | bition | index% |
| DEN | 176.0±8.2 | 248.0±18.2 | 26.8±5.3 | - | 2.1±0.4 |
| DEN+PC10 | 168.8±6.3 | 235.0±7.1 | 20.1±3.1 | 25.1 | 1.9±0.3 |
| DEN+PC 25 | 171.7±9.3 | 243.3±10.3 | 26.5±7.3 | 1.3 | 2.3±0.7 |
| DEN+PC 50 | 173.3±5.2 | 235.0±12.1 | 23.2±4.2 | 13.6 | 2.1±0.2 |

*MNHEPs = micronucleated hepatocytes, [a]MNHEPs/1,000 hepatocytes

**Table 3. Relative Organ Weight and Blood Biochemical Analysis of Rats in the Medium-Term Carcinogenicity Experiment**

| Treatment | Exposure period | Relative organ weight (%) | | | Enzyme activity (IU/L) | | |
|---|---|---|---|---|---|---|---|
| | of pinocembrin | Liver | Spleen | Kidney | AST | ALT | ALP |
| DEN | - | 2.75±0.14 | 0.20±0.02 | 0.55±0.02 | 101.4±18.6 | 53.1±11.1 | 122.0±20.6 |
| DEN[+]PC 2 mg/kg bw | week 1-15 | 2.95±0.21 | 0.19±0.02 | 0.56±0.06 | 81.4±14.8 | 42.6± 8.7 | 133.3±26.7 |
| DEN[+] PC 10 mg/kg bw | week 1-15 | 2.84±0.19 | 0.20±0.02 | 0.56±0.04 | 108.1±15.9 | 73.0±20.2 | 137.7±34.3 |
| DEN[→]PC 2 mg/kg bw | week 5-15 | 2.85±0.27 | 0.19±0.01 | 0.54±0.10 | 96.8±11.5 | 57.4±20.5 | 152.3±55.8 |
| DEN[→]PC10 mg/kg bw | week 5-15 | 2.78±0.23 | 0.19±0.02 | 0.52±0.04 | 92.2±13.1 | 41.8±10.2 | 139.3±32.4 |
| NSS | - | 2.68±0.37 | 0.20±0.04 | 0.51±0.04 | 103.0±16.0 | 47.6±13.2 | 140.4±61.3 |
| NSS+ PC10 mg/kg bw | week 1-15 | 2.78±0.34 | 0.20±0.03 | 0.52±0.04 | 99.6±24.3 | 42.2± 9.7 | 124.0±43.2 |

*AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase, Values expressed as mean ± SD, DEN = diethylnitrosamine, 100 mg/kg bw, 2 times; i.p.; NSS= 0.9% Normal saline solution, 2 times; i.p., [+]Pinocembrin treatment before 2 weeks of the first DEN injection (week 1-15 of experiment), [®]Pinocembrin treatment after 1 week of DEN injections (week 5-15 of experiment)

phosphatase activities. The liver samples were fixed in 10% formalin and embedded in paraffin. They were used for immunohistochemical examination of glutathione-*S*-transferase placental form (GST-P), which is the end point marker of rat hepatocellular carcinoma.

Immunohistochemical assessment of GST-P positive foci was performed using the avidin–biotin complex method according to Puatanachokchai et al. (Puatanachokchai et al., 2006). The numbers and areas of GST-P positive foci greater than 0.2 $mm^2$ in area and the total areas of the liver sections were measured using a color image processor to give values per $cm^2$ of liver section.

*Statistical methods*

Data are reported as means ± SD of each variable for each group. Differences between treated groups and control groups were determined by Welch's t-tests after application of a preliminary F-test for equal variance and $P<0.05$ was considered as significant.

## Results

*Effect of pinocembrin on initiation stage of rat hepatocarcinogenesis*

In the inhibitory study, rats treated with 2, 10 and 50 mg/kg bw of pinocembrin showed no significant effect on the number of micronucleus formation induced by DEN (Table 1), indicating that pinocembrin did not inhibit the micronucleus formation induced by DEN.

Due to the initial observation of pinocembrin lacking inhibitory effects, the next study was designed to increase the concentration of pinocembrin and duration of treatment. Rats were orally administered with 10, 25 and 50 mg/kg bw of pinocembrin 6 days before the first injection of 30 mg/kg bw of DEN. The number of micronucleated hepatocytes and mitotic index are summarized in Table 2. Ten mg/kg bw of pinocembrin showed a slight decrease in micronucleated hepatocytes, but there were no significant differences between groups. These finding suggested that pinocembrin did not prevent micronucleus formation induced by DEN in rat liver.

*Effect of pinocembrin on promotion stage of rat hepatocarcinogenesis*

Glutathione-*S*-transferase placental form formation in rat liver was used to evaluate the effect of pinocembrin on promotion stage in DEN – induced rat hepatocarcinogenesis. In this study, we evaluated the effect of pinocembrin concentrations of 2 and 10 mg/kg bw after and before diethylnitrosamine injections for 10 and 15 weeks, respectively. There were no significant differences in water and food intake among the investigated groups (data not shown). The general observations, including relative organ weights and the serum AST, ALT and ALP activities, are summarized in Table 3. There were no significant differences between groups, demonstrating that pinocembrin at concentrations 2 and 10 mg/kg bw had no toxic effects in rats.

The quantitative data for GST-P positive foci are summarized in Table 4. Pinocembrin 10 mg/kg bw did not induce GST-P positive foci formation. It is evident that pinocembrin did not present carcinogenicity in

**Table 4. Quantitative Values for GST-P Positive Foci of Rats in the Medium-Term Carcinogenicity Experiment**

| Treatment (mg/kg bw) | Exposure period of pinocembrin (week) | Body weight (g) Initial | Final | GST-P positive foci No./cm$^2$ | Area (mm$^2$/cm$^2$) |
|---|---|---|---|---|---|
| DEN | - | 65.6±1.7 | 436.7±32.2 | 3.17±1.15* | 0.23±0.09* |
| DEN+PC 2 | 1-15 | 67.5±2.6 | 429.5± 46.5 | 2.38±1.77 | 0.24±0.26 |
| DEN+PC 10 | 1-15 | 67.0±3.5 | 427.5±35.3 | 5.85±4.39 | 0.64±0.54 |
| DEN→PC 2 | 5-15 | 65.0±4.1 | 432.0± 28.8 | 4.34±3.09 | 0.41±0.35 |
| DEN→PC10 | 5-15 | 68.0±2.6 | 422.0±20.8 | 2.89±0.99 | 0.31±0.13 |
| NSS | - | 68.5±2.2 | 429.3±35.3 | 0.00±0.00 | 0.00±0.00 |
| NSS+PC10 | 1-15 | 69.4±7.9 | 421.3±41.6 | 0.06±0.14 | 0.00±0.00 |
| NSS+PC10 | 1-15 | 69.4±7.9 | 421.3±41.6 | 0.06±0.14 | 0.00±0.00 |

*significantly different from negative control group, $p<0.05$

rats. Moreover, rats treated with 2 and 10 mg/kg bw of pinocembrin received before or after DEN injection did not show a significant decrease in the number of GST-P positive foci. Interestingly, pinocembrin at 10 mg/kg bw slightly increased the number of GST-P positive foci relative to the positive control (84%) when administered before DEN injection. These results indicated that pinocembrin did not inhibit or promote the DEN-induced promotion stage of rat hepatocarcinogenesis.

## Discussion

Pinocembrin exhibited a strong antimutagenic activity against mutagenic heterocyclic amines in vitro using the Ames test (Trakoontivakorn et al., 2001). In this study, the carcinogenic and anticarcinogenic activities of pinocembrin on rat hepatocarcinogenesis were evaluated by short- and medium-term carcinogenicity tests using DEN as a hepatocarcinogen. We found that pinocembrin did not induce micronucleus formation. Moreover, it did not decrease the number of micronucleated hepatocytes in the DEN – induced initiation stage of hepatocarcinogenesis. We also found that 10 mg/kg bw of pinocembrin tended to decrease the number of micronuclei more than 25 and 50 mg/kg bw. Subsequently, an analysis of the reduction of mutagenic potency of DEN and prolonged administration of pinocembrin exposure were performed. Rats were orally fed with 10 mg/kg bw of pinocembrin for 21 days, 14 days before 20 mg/ kg bw of DEN injection. We found that, oral administration of 10 mg/kg bw of pinocembrin reduced micronucleus frequency by 30% in rat liver when compared to positive control, but the difference was not statistically significant (data not shown). The results of the present investigation clearly showed that pinocembrin did not present either mutagenic or antimutagenic potential on diethylnitrosamine-induced mutagenesis in rat liver.

In the promotion stage, pinocembrin at 10 mg/kg bw did not induce GST-P positive foci formation. Moreover, rats treated with 2 and 10 mg/kg bw of pinocembrin had no significant decrease in the number of GST-P positive foci for treatments given before or after DEN injection. These results are relevant to previous studies showing that propolis, which contains pinocembrin, did not protect against DEN-induced GST-P positive foci formation in rat liver (Said et al., 2010). Interestingly, pinocembrin at 10 mg/kg bw slightly increased the number of GST-P positive foci higher compared to positive control (84%) when administered before DEN injection. The present study clearly indicated that high doses of pinocembrin (10 mg/kg bw) promoted the development of preneoplastic lesions in the rat livers. Our results are relevant to previous findings that *Boesenbergia pandurata* significantly increased the number of GST-P positive foci (Tiwawech et al., 2000) in 2-amino-3, 8-dimethylimidazo (4, 5-f ) quinoxaline induced rat hepatocarcinogenesis. It should be emphasized that pinocembrin is one of compounds in *B. pandurata* that promoted hepatocarcinogenesis. In addition, Satoh et al. (2001) demonstrated that end-products of lipid peroxidation can induce the expression of GST-P in rat liver. In this study, we also found that administration of pinocembrin 10 mg/kg bw before DEN injection slightly induced lipid peroxidation relative to positive control (data not shown). This is one result supporting the suggestion that the promoting effect of pinocembrin might be due to lipid peroxidation.

In this study, extraction of 1 kg of dried *B. pandurata* yielded 69 mg of pinocembrin. Based on the average consumption, the doses of pinocembrin that we used in these experiments corresponded to dried *B. pandurata* 6 – 145 g/day in the short-term and 6 and 29 g/day in medium-term carcinogenicity tests. As a result, the concentrations of pinocembrin may not have been suitable for inhibiting DEN-induced rat hepatocarcinogenesis. In addition, pharmacokinetic study of pinocembrin in rats indicated that the plasma concentration of pinocembrin rapidly decreased due to either fast excretion and/or extensive metabolism (Yang et al., 2009). Thus pinocembrin might rapidly conjugate with either glucuronide or sulfate and then be excreted from the body. This may be one of the major reasons why pinocembrin did not present anticarcinogenic activity in rat liver.

Recently, our laboratory studied the effects of pinostrobin (5-hydroxy-7-methoxyflavanone), a flavanone compound found in *B. pandurata* rhizome, in DEN-induced initiation of rat hepatocarcinogenesis. We demonstrated that pinostrobin prevented the initiation stage of rat hepatocarcinogenesis induced by DEN (Charoensin, 2008). Even though pinostrobin inhibited hepatocarcinogenesis, pinocembrin did not; this may be associated with the structure and the position of functional groups of this compound. According to a previous study, free hydroxyl groups of the polyphenols are rapidly excreted from the body after conjugation with glucuronide and/or sulfate. In addition, flavonoids containing methoxyl groups in their structure may not only increase hepatic metabolic stability but also increase their intestinal absorption. These effects could be due to greatly increased oral bioavailability, and thus methoxylated flavonoids had greater chemopreventive potency than unmethylated flavonoids or polyphenols (Wen & Walle 2006; Walle et al., 2007).

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