Acute and subchronic oral toxicity assessment of extract from *Etlingera pavieana* rhizomes

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**ABSTRACT**

In Southeast Asia, the rhizome of *Etlingera pavieana* is commonly consumed and parts of the rhizomes have been used as a medicine for the treatment of several disorders. Its pharmacological effects have previously been reported. However, its potential toxicity has not been described. This study aimed to evaluate in vivo toxicity of *E. pavieana* rhizome extract (EPE) in Sprague Dawley rats. Acute toxicity testing of EPE at a single dose of 2,000 mg/kg produced no toxic effects in female rats after 14 days of treatment. Subchronic toxicity testing showed that all doses of EPE (500, 1,000, and 2,000 mg/kg/day) produced no sign of toxicity during 90 days of treatment. All biochemical and hematological values were within normal ranges. There were no significant histopathological differences in the internal organs among the tested groups. Therefore, the no-observed-adverse-effect level of EPE was 2,000 mg/kg/day in both male and female rats, thereby confirming the safety of EPE for use in traditional medicines.

**1. Introduction**

Zingiberaceae is a large family of plants of approximately 52 genera and 1587 species [1], including some that are currently used in alternative and complementary medicine [2]. Preclinical and clinical studies have found that some species of Zingiberaceae have pharmacological effects [3-6]. The pharmacological properties and safety of chemicals and natural substances must be confirmed prior to use as medicines and food additives. Generally, the toxicity of a plant derivative should be evaluated to ensure safety prior to use in clinical trials and subsequent distribution worldwide.

*Etlingera pavieana* (Pierre ex Gagnep) R.M.Sm., a plant in the Zingiberaceae family widely found in Southeast Asia [7]. The rhizome of *E. pavieana* is commonly consumed as an herbal medicine and spice [7]. In Thailand, *E. pavieana* rhizomes are traditionally used for the treatment of flatulence, diuresis, fever, and digestive disorders [7,8]. In Cambodia, the rhizome of *E. pavieana* is combined with *Amomum verum* Blackw, also a medicinal plant in the Zingiberaceae family, for treatment of pharyngitis and gastrointestinal disorders [7]. In southeastern Thailand, *E. pavieana* grows naturally and is cultivated [7].

Phytochemical studies of *E. pavieana* have identified several phenylpropanoids and the major components, trans-4-methoxycinnamaldehyde (MCD) and 4-methoxycinnamyl p-coumarate (MCC) [9,10], possess several pharmacological activities, including antioxidant [11], anti-inflammatory [10-15], antimicrobial [16], and anticancer [17] effects. Moreover, the single oral administration of MCC at a dose of 2,000 mg/kg body weight did not produce any acute toxic effects or mortality in mice, indicating the relative safety of this bioactive compound from *E. pavieana* rhizomes [15]. However, scientific data of potential toxic effects of extract from *E. pavieana* rhizomes are still needed.

**Keywords:**

Etlingera pavieana
Acute toxicity
Subchronic toxicity
Rats

**Abbreviations:** BW, body weight; NOAEL, no-observed-adverse-effect level; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; BUN, blood urea nitrogen; PLT, Platelet; NEU, Neutrophil; LYMP, Lymphocyte; MONO, Monocyte; EOS, Eosinophil; HCT Hematocrit; HGB, Hemoglobin.

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https://doi.org/10.1016/j.toxrep.2022.07.005

Received 23 January 2022; Received in revised form 26 January 2022; Accepted 6 July 2022
Available online 8 July 2022

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The current study aimed to investigate possible toxic effects of *E. pavieana* rhizomes using acute and subchronic oral toxicity tests in rats.

2. Material and methods

2.1. Plant material

*D. pavieana* (plant list record 244738) was harvested from Chanthaburi Province and authenticated by a plant taxonomist affiliated with the Department of Biology, Burapha University (Chonburi Province, Thailand). The plant specimen (voucher number KS-SCBUU-0012–2) was stored in a facility operated by the Faculty of Science of Burapha University.

2.2. Preparation of the ethanol extract of *E. pavieana* rhizomes (EPE)

Dried rhizomes of *E. pavieana* were powdered using a mechanical grinder. The rhizome powder was soaked in 95% ethanol (1:10, w/v) for 7 days with occasional agitation. The solution was filtered under pressure and the residue of plant powder was then re-soaked with 95% ethanol twice for seven days each time. The pooled extracts were evaporated under reduced pressure using a rotary evaporator. The obtained *E. pavieana* rhizome ethanol extract was designated as EPE. The extraction yield based on dry weight was 7.27%. The contents of MCC and MCD, the major active ingredients of EPE, were determined by high-performance liquid chromatography as described by Srisook et al. [18]. Quantitative analysis revealed that dried EPE contained 5.92 mg/g of MCC and 1.37 mg/g of MCD. For the oral toxicity testing in rats, EPE was found to be dissolved in 5% Tween 80, which was then used as a vehicle control. The extract was daily prepared at the concentration of 200 mg/mL, and was orally given to the rats in a final volume of 1 mL/100 g body weight of the animals.

2.3. Experimental animals

Female and male Sprague Dawley (SD) rats, 6–7 weeks old with body weight (BW) of 180–200 g, were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand) and kept in a room at temperature (24±1 °C) and light-controlled room with ad libitum access to drinking water and standard rat chow. The rats were adapted for 7 days prior to starting the experiments. All experimental protocols were approved by the Animal Ethics Committee of Chiang Mai University (approval no. 43/2561) and the Institutional Animal Care and Use Committee of Burapha University (approval no: IACUC 004/2562).

2.4. Acute oral toxicity study

The study protocol was performed followed the OECD Guidelines 420 [19]. The test group (5 female rats) was administered EPE at 2000 mg/kg. The vehicle control group (5 female rats) received 5% Tween 80. Toxic signs and mortality were monitored at 1, 2, 4, and 6 h after oral treatment of EPE and then once daily for 14 days. All surviving rats were sacrificed at the end of the experiment. Necropsy and pathological examinations were performed of major internal organs.

2.5. Repeated dose 90-day oral toxicity study

The subchronic oral toxicity study was performed in accordance with the OECD Test Guideline 408: repeated dose 90-day oral toxicity test [20] with minor modifications. Forty female and 40 male rats were separated into four groups (10 rats/sex/group): a control group (5% Tween 80) and three EPE-treated groups (500, 1000, or 2000 mg/kg/day). Satellite control and satellite EPE groups (5 females and 5 males/group) were orally administered 5% Tween 80 or EPE (2000 mg/kg/day) for 90 days and then housed for a further 28 days to observe insistence of toxic signs.

During the experimental period, the BW, consumption behavior, general behavior, and clinical signs of all the rats were assessed. At the end of the experiment, all rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After fasting for 16–18 h, blood was collected for hematological and biochemical analyses, respectively.

2.6. Hematological analyses

Blood samples in EDTA-containing tubes were used for hematological examination. WBC, RBC, and platelet counts, hemoglobin, hematocrit, MCV, MCH, MCHC, and differential leukocyte count were examined using a HumaCount Plus Hematology Analyzer (Gesellschaft für Biochemica und Diagnostica mbH, Germany).
and testis) were removed, weighed, and histopathologically examined to
Netherlands).
2.8. Histopathological examination
After sacrificing the animals, the internal organs (i.e., brain, lung, heart, liver, spleen, adrenal gland, kidney, ovary, uterus, epididymis, and testis) were removed, weighed, and histopathologically examined to detect any signs of damage.

2.9. Statistical analysis
Data are shown as the mean ± SD. Statistically significant differences between groups were analyzed by ANOVA and by the post hoc test for the subchronic toxicity study. A probability (p) value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Acute oral toxicity study
A single oral dose of EPE at 2000 mg/kg was not fatal to any of the female rats during the observation period and no signs of toxicity or general behavioral changes were observed. The BW gain between the treatment and control groups was not significantly different (Fig. 1).

3.2. Subchronic oral toxicity study
The results showed that oral administration of EPE at 500–2000 mg/ kg/day for 90 days was not fatal to any of the rats and none showed signs of toxicity at any time during the experiment. Food and water intake of the EPE-treated groups was not different from the control groups (data not shown). Fig. 2 showed the BW curves of female and male rats. There was no significant difference in the BW of female and male rats between the treatment and control groups or between the satellite treatment and control groups on day 118 (data not shown).

3.2.1. BW and clinical observations

3.2.2. Hematological analysis
Table 1 showed the hematological examination. There were slight, but significant, differences in RBC and MCV levels of the EPE-treated female rats (2000 mg/kg/day) as compared with those of the female control group. In male rats (Table 2), the average hematocrit and hemoglobin levels of the group treated with EPE at 2000 mg/kg/day were remarkably lower than those of the control group. The MCHC level of the male group that received EPE 1,000 mg/kg/day was slightly but remarkably lower than that of the male control group. In both female and male rats, there was no significant differences between the satellite treatment and control groups.

Table 1
Hematological analysis results of female rats in the subchronic toxicity test of EPE.

|          | Control a | EPE (500 mg/kg) b | EPE (1000 mg/kg) b | EPE (2000 mg/kg) b | Control a | EPE (2000 mg/kg) b |
|----------|-----------|-------------------|--------------------|--------------------|-----------|-------------------|
| PLT (×10^3/μL) | 8.37 ± 0.95 | 8.19 ± 1.10 | 8.39 ± 1.64 | 8.98 ± 1.06 | 8.38 ± 0.55 | 8.66 ± 0.50 |
| WBC (×10^3/μL) | 4.11 ± 1.36 | 3.44 ± 0.80 | 3.84 ± 1.47 | 3.59 ± 0.78 | 3.01 ± 1.31 | 2.49 ± 0.62 |
| NEU (%) | 22.71 ± 6.67 | 22.60 ± 6.75 | 23.54 ± 4.78 | 17.60 ± 4.53 | 22.61 ± 1.51 | 22.16 ± 4.40 |
| LYMP (%) | 66.09 ± 5.88 | 67.28 ± 7.69 | 66.76 ± 5.46 | 71.39 ± 7.95 | 68.78 ± 4.70 | 68.41 ± 5.57 |
| MONO (%) | 9.33 ± 7.90 | 8.14 ± 5.67 | 7.62 ± 4.42 | 9.55 ± 4.49 | 7.03 ± 3.56 | 7.81 ± 6.32 |
| EOS (%) | 1.88 ± 0.67 | 1.98 ± 0.78 | 2.09 ± 0.97 | 1.47 ± 0.81 | 1.58 ± 1.16 | 1.62 ± 0.97 |
| RBC (×10^6/μL) | 7.41 ± 0.29 | 7.16 ± 0.45 | 7.27 ± 0.45 | 7.04 ± 0.26 | 7.09 ± 0.31 | 7.09 ± 0.20 |
| HCT (%) | 41.54 ± 1.66 | 40.71 ± 2.45 | 41.08 ± 2.00 | 40.50 ± 1.77 | 39.90 ± 0.91 | 39.52 ± 1.20 |
| HGB (g/dL) | 13.73 ± 0.56 | 13.43 ± 0.71 | 13.60 ± 0.50 | 13.37 ± 0.54 | 13.03 ± 0.25 | 12.78 ± 0.30 |
| MCHC (g/dL) | 33.05 ± 0.69 | 32.99 ± 0.52 | 33.11 ± 0.66 | 33.03 ± 0.45 | 32.65 ± 0.60 | 32.34 ± 0.50 |
| MCH (pg) | 18.55 ± 0.42 | 18.75 ± 0.64 | 18.77 ± 0.66 | 18.98 ± 0.54 | 18.38 ± 0.60 | 18.04 ± 0.40 |
| MCV (fL) | 56.11 ± 0.82 | 56.80 ± 1.82 | 56.62 ± 1.21 | 57.53 ± 1.58 | 56.38 ± 1.61 | 55.74 ± 0.66 |

Values: Mean ± SD, n = 10
a: Group received 5 % Tween 80 or EPE for 90 days
b: Group received 5 % Tween 80 or EPE for 90 days with no further treatment for 28 days
*p < 0.05 vs control*
3.2.3. Biochemical analysis

Biochemical serum values of female rats are shown in Fig. 3. ALT levels of female groups treated with EPE at 1000 or 2000 mg/kg/day for 90 days were significantly less than those of the female control group. BUN levels of the male rats treated with EPE at 1,000 or 2,000 mg/kg/day were markedly less than those of the control group (Fig. 4). When compared with the control group, creatinine levels were significantly lower in the EPE-treated male group (2,000 mg/kg/day). The other biochemical values between the EPE-treated groups and control group did not show significant difference. The direct bilirubin level of the satellite EPE-treated group was markedly greater than that of the satellite control group.

3.2.4. Organ weights and histopathology

In female rats (Fig. 5), the brain weight of the groups treated with EPE at 500 or 2000 mg/kg/day for 90 days was significantly higher than
that of the vehicle-treated group. The heart weight of the EPE-treated female rats (2,000 mg/kg/day) was also significantly higher than that of the female control group. The organ weights of male rats are shown in Fig. 6. The brain and heart weights of the EPE-treated male rats were significantly higher than those of the male control group. The liver weight of the male groups treated with EPE at 500 or 1,000 mg/kg/day was also significantly greater than that of the male control group. However, these significant differences in organ weights were not dose-dependent. Moreover, the organ weights of the satellite treatment and control groups were not significantly different. There were no significant differences in the histopathological results of the internal organs of male and female rats between the high-dose treatment and control groups (Fig. 7).

4. Discussion

This is the first report of the acute and subchronic toxicity of *E. pavieana* rhizome extract (EPE) in rats. To date, evidence pertaining to toxicity of this extract or its potential active constituents has been reported in several different cell lines including mouse macrophages [10, 12, 14], mouse microglia [21], and human endothelial cells [11, 13]. One of the bioactive compounds in *E. pavieana* rhizomes, MCC, has been evaluated in acute oral toxicity study in mice, and found to be nontoxic after acute exposure [15]. To elucidate the conserved toxicity response to this plant extract in animals, we therefore performed delicate experiments in rats. *E. pavieana* rhizomes are widely used in traditional medicine and consumed as food [7, 8]. Several pharmacological studies of *E. pavieana* have been reported [10, 11, 13, 17, 21]. In this study, the
The highest dose of EPE was set at 2,000 mg/kg for both acute and subchronic toxicity tests, since *E. pavieana* rhizome is commonly used as food and a dose limit in traditional medicine has not yet been established.

Acute oral toxicity testing is commonly used to identify and evaluate the harmful short-term effects of a test agent given with a single dose or multiple doses administered within 24 h [19]. In the present acute toxicity experiment, oral treatment of a single dose of EPE at 2,000 mg/kg to the female rats did not result in death or any abnormalities in any of the groups throughout the observation period. BW and food as well as water intake of all rats administered with EPE were not significantly different from those of the vehicle-treated rats. No gross abnormalities were detected in the EPE-treated rats. The results indicate that EPE is relatively nontoxic and that the oral LD$_{50}$ of EPE is higher than 2,000 mg/kg.

Repeated dose 90-day oral toxicity testing is generally recognized as a validated method to assess the long-term effects of a test agent on various biological systems. In this study, the subchronic toxicity of EPE was evaluated in male rats over a period of 90 days. The biochemical analysis of serum samples showed that EPE did not significantly alter the levels of glucose, BUN, creatinine, total protein, albumin, and total bilirubin compared to the control group. These findings suggest that EPE is safe for consumption within the tested dosage range.

![Fig. 4. Effect of EPE on biochemical values of male rats in the subchronic toxicity study of EPE (n = 10). * p < 0.05 vs control, # p < 0.05 vs satellite control.](image-url)
an appropriate subchronic toxicity test model to afford general information on possible health hazards likely to arise after long-term treatment [22–24]. Evidence from subchronic toxicity studies includes major toxic effects, identification of affected organs, and the no-observed adverse-effect level (NOAEL) of the test sample [20]. This information is useful to select dosing levels for further chronic toxicity testing and to establish appropriate criteria regarding safe exposure levels in humans [22,25]. Observational post-treatment groups, including control satellite groups and the highest dose satellite groups of both sexes, are also useful to determine the reversibility and insistence of any toxic effects. In the present study, no mortality or toxic signs in rats of either sex were observed during the 90-day long-term oral administration of EPE at 500, 1,000, and 2,000 mg/kg/day.

In toxicity evaluations, analysis of hematology is commonly carried out to assess the effects of test agents on the hematopoietic system [23, 26–28]. It has long been known that hematological parameters are sensitive and reliable indicators to evaluate the harmful effects of toxic substances [29]. In this study, most of the hematological values of EPE-treated male and female rats were at the same level as those of the control rats with the exception of the RBC and MCV levels of the female group treated with high-dose EPE, as well as the hemoglobin, hematocrit and MCHC levels in some of the male EPE-treated groups. However, the differences in RBC, MCV, hemoglobin, hematocrit, and MCHC values in the EPE-treated groups were not clinically significant since all hematological values were within the normal ranges for SD rats [25,30].

Serum biochemical analysis is commonly used to assess the physiological functions of body systems [28,31]. Serum BUN and creatinine are important indices of kidney function [32]. Biochemical indices, such as
Fig. 5. Relative organ weights (g/100 g BW) of female rats in the subchronic toxicity study of EPE. (n = 10). * p < 0.05 vs control.
Fig. 6. Relative organ weights (g/100 g BW) of male rats in the subchronic toxicity study of EPE (n = 10). * p < 0.05 vs control.
AST, ALT and ALP, are reliable parameters of liver function \cite{33,34}. Our data showed that some of the biochemical values of the EPE-treated female and male groups were significantly different from the control groups. Notably, ALT levels were lower in some EPE-treated female groups and BUN and creatinine levels were lower in some EPE-treated male groups when compared with those of the respective control groups. However, these statistically significant differences may only represent normal variations since these biochemical values were still in the normal ranges for SD rats \cite{25,30}.

BW and internal organ weight are major determinants commonly used to assess the toxic effects of test substances \cite{23,24}. In the EPE-treated groups of both sexes, the weights of some organs, including the liver, lungs, brain, and heart, were significantly different than those of the control groups. However, these differences were very small and the weights of organs in each of the groups were still within the normal ranges for SD rats. Additionally, the histopathological results revealed no changes in any tissues of the internal organs of the EPE-treated rats.

The present toxicity study found no mortality and no toxic signs related to body weight or internal organ weight. Results of histopathological examination of internal organs as well as hematological and biochemical analyses were also negative, so the NOAEL of EPE can be considered to be greater than 2,000 mg/kg/day. However, prior to using EPE in clinical trials, further studies of the toxicology of EPE, e.g., chronic toxicity evaluation and toxicity testing in non-rodent species, should be performed.

5. Conclusions

A single high-dose or long-term (90 days) oral administration of EPE did not result in mortality or any signs of toxicity in either male or
female rats, indicating that EPE is well tolerated in these animals. Although further studies to confirm its safety in all aspects should be performed, the present toxicity study demonstrates the relative safety of EPE, suggesting its potential for pharmaceutical development as a natural medicinal product.

**CRediT authorship contribution statement**

Natthakarn Chiranthanut: Methodology, Formal analysis, Visualization, Data curation, Project administration, Writing – original draft. Nirush Lertprasertsuke: Investigation. Ekaruth Srisook: Methodology. Klaokwan Srisook: Conceptualization, Funding acquisition, Project administration, Methodology, Writing – review & editing.

**Declaration of Competing Interest**

The authors have no conflict of interest to declare.

**Acknowledgments**

This work was financially supported by a research grant from Burapha University through the National Research Council of Thailand (grant no. 2/2560); the Research Unit of Natural Bioactive Compounds for Healthcare Products Development; the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Higher Education, Science, Research and Innovation, Thailand. We would like to thank Dr. G. Lamar Robert for his assistance in editing the manuscript.
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