*Original Article*

# Dermal Toxicity Studies of an Herbal Cream Contained *Zingiber officinale* Roscoe and *Phyllanthus amarus* Extracts in Sprague-Dawley Rats

Maharan S, PhD[1], Muchimapura S, BSc, PhD[2,3], Wattanathorn J, BSc, PhD[2,3], Thukhummee W, BSc, PhD[2,3], Thong-Un T, MD[2,3], Wannanon P, MD[2,3]

[1] Department of Physiology (Neuroscience Program) and Graduate School, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[2] Department of Physiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand
[3] Research Institute for Human High Performance and Health Promotion, Khon Kaen University, Khon Kaen, Thailand

***Background:*** *Z. officinale* and *P. amarus* are famous herbs and used as a traditional medicine for a long time. Both herbs have anti-inflammatory and antioxidant properties. However, there is a few data on the development of herbal cream contained both *Z. officinale* and *P. amarus* extract (the combined extract) for dermal application.

***Objective:*** The present study aims to investigate the acute and sub-acute dermal toxicity test of an herbal cream contained the combined extract in male and female Sprague-Dawley rats.

***Material and Methods:*** The rats were randomly divided into the following three group: 1) control naive, 2) vehicle, and 3) 2,000 mg/kg. BW of the combined cream extract groups. The acute study, a single dose of 2,000 mg/kg.BW was applied at the dorsal of the back and determined the sensitivity and toxicity signs at 3 mins, 1 hr, 4 hr, 24 hr, 48 hr, 72 hr and at 14$^{th}$ day after a single application; the sub-acute study, repetitive treatments were applied 5 times/week, continually applied for 4 weeks. At the end of experiments, blood, skin, liver and kidneys were collected to evaluate hematological and histopathological effects.

***Results:*** The herbal cream contained the combined extract did not cause skin irritation, inflammation and others abnormal signs at the contact area. Moreover, the herbal cream did not affect the growth of rats. No abnormalities were found in the organs and no adverse reactions were seen. The LD toxicity of both acute and sub-acute dermal test is greater than 2,000 mg/kg.BW.

***Conclusion:*** The herbal cream contained the combined extract is highly safe for transdermal application for muscle injury and inflammation.

***Keywords:*** Dermal toxicity test, *P. amarus, Z. officinale*

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*Zingiber officinale (Z. officinale)* or ginger and *Phyllanthus amarus (P. amarus*) are the famous herbal plants which used in traditional medicine for a long time. Previous studies have demonstrated that the active ingredients of ginger possess biological and pharmacological effects ranging from anti-inflammation to anti-cancer[1,2]. Ginger is generally known for its anti-inflammatory property[3,4]. Additionally, it has also been shown that Ginger can inhibit chronic inflammation[5]. Moreover, several researches have confirmed the analgesic and antipyretic of ginger in mice and rat models[6]. Kiuchi et al (1982) found that ginger has anti-inflammatory actions via the inhibition of prostaglandins synthesis[7]. Furthermore, it has been demonstrated that ginger contains many ingredients such as shogaols that have pharmacological properties mimicking dual-acting non-steroidal anti-inflammatory drugs (NSAIDs) in intact human leukocytes *in vitro*[8].

*Phyllanthus amarus*, a plant in family of Euphorbiaceae, is one of the common medicinal plants which claimed for treating several conditions such as disturbance of hepatitis, kidney and diabetes in Ayuravedic medicine[9]. In addition, the extract compounds isolated from Phyllanthus species including *P. amarus* have shown a wide spectrum of pharmacological activity including anti-fibromyalgia effects[10]. Recent study has reported that the aqueous leaves extract of *P. amarus* at dose of 200 mg/kg exhibited analgesic and anti-inflammatory effects in both thermal and chemical models of pain in rats[11]. Moreover, the administration of 75% methanolic extract of whole plant of *P. amarus* at a dose of 250 mg/kg p.o. markedly inhibited paw edema induced by carrageenan, bradykinin, serotonin and prostaglandin E1[12].

**Correspondence to:**

Muchimapura S.

Department of Physiology, Faculty of Medicine and Integrative Complementary Alternative Medicine Research and Development Center in Research Institute of Human High Performance and Health Promotion, Khon Kaen University, Khon Kaen 40002, Thailand.

**Phone:** +66-43-348394, **Fax:** +66-43-348394

**E-mail:** supmuc@kku.ac.th

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Furthermore, the administration of both the aqueous and methanolic extracts of leaves and stems of *P. amarus* at the doses of 500, 250 and 100 mg/kg significantly inhibited paw edema up to 8 hours in female Balac/c mice[13].

In this point, the data show that both *Z. officinale* and *P. amarus* are potential plants thus evaluating the proper ratio of these two plants to develop as health product to relieve a condition in medical health is interesting. Unfortunately, the evidence about the combined extract is still less especially for dermal test. Therefore, the objective of this study was set up to determine the acute and sub-acute dermal toxicity test of ethanolic extract of the combined extract in male and female Sprague-Dawley rats.

## Materials and Methods

### *Experimental animals*

24 male, 24 female Sprague-Dawley rats weighting between 200 to 350 g. were randomly distributed into 3 groups to evaluate the acute and sub-acute dermal toxicity test. There are control naive, vehicle and 2,000 mg/kg.BW of the combined extract treated groups with equally divided as to sex. The animals were housed in group of 4 per cage in standard metal cages at 22±2°C on 12: 12 h light:dark cycle. All animals were given access to food and water ad libitum. The experiments were performed to minimize animal suffering and the experimental protocols were approved by faculty of Medicine, Khon Kaen University, Thailand and the Committee (No. AE MDKKU 3/2559).

In acute dermal toxicity test, a single dose of substance was tested by using either vehicle or 2,000 mg/kg.BW of the combined extract at the dorsum of back at day 1. All rats were observed and evaluated the effect of the acute dermal toxicity at a various time interval at 3 minutes, 1 hour, 4 hours, 24 hours, 48 hours, 72 hours and 14 days after a single dose was applied by using the grading of skin reaction-acute dermal irritation as described by Draize in 1959 and general observation[14]. Moreover, the pharmacologic signs and mortality rate were also evaluated. All animals which died during the study were subjected to gross necropsy examination. While the sub-acute dermal toxicity test, a repetitive dose of vehicle and 2,000 mg/kg of the combined extract were applied 5 days per week and applied it continually for 4 weeks. All rats were observed and investigated the effect of the dermal toxicity at various time intervals throughout 28 days by using the grading of skin reaction and general observation. The pharmacologic signs, mortality rate and necropsy have been also observed same as in acute dermal irritation test.

### *Preparation of the combined cream extract*

The rhizome of *Z. officinale* and the aerial part of *P. amarus* were collected from Khon Kaen province, Thailand. Fresh herbal plants were cleaned; cut to small pieces and dried it at 60°C continually for 72 hours with in the oven. Then dried plants were grounded to fine powder and maceration extract with 50% hydro-ethanol (1: 1 (v/v) = distilled water: ethanol) for 48 to 72 hours. The final volume of both extractions was filtered with Whatman No. 1 filter paper, evaporated, freeze dried and prepared as a combined extract at the ratio 3: 4 which shown the highest potent on anti-inflammation and anti-oxidant effect. The percent yielding of *Z. officinale* and *P. amarus* extract is 19.26 and 15.39 respectively.

### *Skin preparation for dermal toxicity studies*

All rats were acclimatized to the environment for 5 days. Then, 24 hours before test, the rats were anesthetized with 50 mg/kg sodium pentobarbital and gentle manual shaved at both left and right sides of back area, which not less than 10% of the body surface area based on the OECD guideline 402 and 410 for using as the site of tested[15,16].

### *Skin irritability grading*

Both acute and sub-acute dermal toxicity tests, the level of skin irritation was determined as described by Draize in 1959, which classified the score into I to V level by Grade I, the score between 0 to 8 mean no different than control naive; Grade II, the score between 9 to 28 mean mild irritable; Grade III, the score between 29 to 64 mean moderate irritable; Grade IV, the score between 65 to 80 mean strong irritable and Grade V, the score between 81 to 100 mean extreme irritable[14].

### *Necropsy*

All rats were deeply anesthetized with 60 mg/kg of Nembutal and then transcardially perfused with 0.9% normal saline.

### *Histopathology*

After clearly perfusion with 0.9% NSS, the skin (area of application cream), liver and kidneys samples were collected and immediately fixed with 10% formalin for 48 hours. All samples were cut into a small piece about 1.5 cm thicknesses, embedded with paraffin. The tissue samples were trimmed and sectioned with myotome at 4 um thickness. Then, sectioned tissues were deparaffinized by xylene, rehydrated by ethanol, rinsed in tap water and finally stained with H&E method. The slides were observed the histology changed via light microscopy at 5x, 10x, 20x and 40x magnifications.

### *Blood electrolyte and biochemistry*

Blood samples were collected at the end of experiment from the left abdominal venous blood to determine the complete blood count (CBC), electrolyte, the function of kidney (BUN, creatinine) and the function of liver (ALT, AST, total protein, albumin, globulin). All parameters of blood sampling were determined by the well-trained staff of Academic Clinical Research office (ACRO-KKU) with certificate via Good Laboratory Clinical Practice Guideline and HA.

### *Statistical analysis*

The data obtained were statistically analyzed by

using SPSS version 19. The values were expressed as mean ± standard error of mean for different parameters. The differences between groups were tested via analysis of variance (ANOVA) follow by Tukey *post-hoc* test. The significant values were regarded significant at *p*-value <0.05.

## Results

### *Mortality rate and skin irritability response of acute and sub-acute dermal toxicity*

Mortality rate of male and female rats after a single dose (acute) and repetitive doses (sub-acute) of the combined extract was zero. During the acute test, the irritable response of dermal tissue revealed that the combined extract shows no significant change of the grading score when compared with naive control (score is 0 both male and female).

While, the irritabilities response of dermal tissue of sub-acute toxicity showed that the combined extract did not show any significant change of the grading score when compared with naive control in both male and female rats. The irritable response grading score of erythema were grade I (score = 1.100), oedema grade I (score = 0.650) in male rats and the erythema and oedema score were 0.00 in female rats. Moreover, the data in the vehicle group also showed the irritable grade I in both male and female groups.

The histopathology of acute and sub-acute toxicity test showed that the topical combined extract did not affect on the thickness of skin in stratum spinosum and stratum granulosum layer both male and female rats as showed in Table 1. There was no adverse effect on any of the three groups.

### *General signs and behaviors of the rats*

During the period of acute dermal toxicity test, both male and female rats in the combined extract, vehicle and naive control groups showed the normal skin, fur, eyes, and behavioral patterns. There was no sign of coma and tremor responses. Moreover, there was no sign or symptoms of toxicity in either sex during the single dose study.

Similarly, the sub-acute test found that both male and female rats had the normal appearance in all aspects of general observation when evaluated before and after repeated dose of the combined extract.

### *Body weight and organ weight*

In acute dermal test, it was found that the body weight at baseline, 7 days and 14 days in both sexes showed no significant change of body weight when compared with naive control group.

Moreover, organ weight was also determined at the end of experiment. It was found that the vital organ weight of brain, heart, lung, liver and kidney of both male and female showed no significant change when compared with naive control and vehicle group respectively.

In sub-acute toxicity test, the changes of body weight (% weight change) were evaluated in every week within 1 month. There was no significant difference between groups in both male and female rats after exposure to repetitive dose of the combined extract.

Moreover, the mean vital organ weight of the combined extract and vehicle group showed no significant change in both male and female rats.

### *Blood electrolyte evaluation*

The blood electrolytes including sodium, potassium, chloride and calcium were evaluated at the end of acute toxicity test. In male rats, there was no significant changes in all parameters of electrolyte in naive control, vehicle and 2,000 mg/kg of the combined extract, all values were lay within the normal range.

In sub-acute toxicity test, it was found that neither male nor female rats had any significant changes in blood electrolyte after repetitive dose of the combined extract.

### *Blood serum biochemistry for determine Liver and renal function test*

#### *Liver function test on acute toxicity test*

ALT and AST are the two main important biomarkers in enzymatic system for detecting abnormal liver function. In this studied the male combined extract group showed no significant change in either ALT or AST levels when compared with naive control group. All enzymes were within the normal range.

In female rat, the combined extract group showed a significant reduction in total protein and albumin ($p = 0.000$; $p = 0.000$). The production of total protein, albumin and

**Table 1.** Comparison of the skin thickness (stratum spinosum and stratum granulosum) between group in male and female rat after single (acute test) and repetitive dose (sub-acute test) of 2,000 mg/kg.BW of the combined extract

| Items | The thickness of skin (micrometers) | | | |
|---|---|---|---|---|
| | Acute toxicity test | | Sub-acute toxicity test | |
| | Female | Male | Female | Male |
| Naive control | 408.20±3.65 | 579.60±5.20 | 496.52±5.74 | 576.55±10.90 |
| Vehicle | 401.44±5.08 | 576.48±3.78 | 515.68±8.64 | 553.82±4.71 |
| 2,000 mg/kg.BW of the combined extract | 418.74±5.00 | 560.40±6.92 | 517.52±7.33 | 519.99±5.73 |

globulin in vehicle group was also show significantly decreased when compared with control naive ($p = 0.001$; $p = 0.002$; $p = 0.042$).

### *Liver function test in sub-acute toxicity test*

In male rat, the repetitive dose of the combined extract for 4 weeks shows the significantly decrease of AST when compared with control naive ($p = 0.002$). For the production of proteins, the combined extract and vehicle had also significantly decrease in globulin when compared with control naive ($p = 0.026$; $p = 0.026$) respectively.

For female rat, the repetitive dose of the combined extract did not affect on liver functions when evaluated with the enzymatic test (ASL, AST) and protein production (total protein, albumin, globulin and A/G ratio).

### *Renal function test*

Renal function tests after a single dose of the combined extract reveal that the BUN/creatinine ratio significantly decrease when compared with control naive ($p = 0.005$) in male rat. In contrast with female rat, the combined extract was also showing the significant change in BUN production ($p = 0.004$).

Regarding renal function tests in sub-acute testing, the combined extract had significant increase in BUN and creatinine in male rat ($p = 0.023$; $p = 0.041$).

### *Histopathology examination*

#### *Acute test and Sub-acute test*

In this part, after single and repetitive dose of the topical combined extract was applied in male and female rat; the histology of kidney, liver and skin showed no significant change.

## Discussion

In acute and sub-acute dermal toxicity test, male and female rats were received the single and repetitive administration of limit dose (2,000 mg/kg) of the combined extract topical cream test. The results showed that the mortality was 0% and the acute and sub-acute toxicity test had the $LD_{50}$ more than 2,000 mg/kg.BW in both sexes.

All rats in acute and sub-acute dermal toxicity test were present in normal general appearance, behavioral pattern with no observation of the clinical sign such as coma and tremor during the periods of test. The body weight and vital organ weight had showed no significant change when compared with naive control group both male and female rats of acute and sub-acute tests. These results implied that the combined extract had no effect on food and drink consumption during the period of test. Support for these findings by the information from Klaassen et al the extracts did not induce the decrease in appetite which led to no disturbance of nutrients in the rat[18]. The relative and absolute body weight and organ weight in naive control, vehicle and the combined extract showed no significant different in either sex. In general, the adverse effects of drugs or chemicals will be noted a significant change of the body weight loss more than 10% of the initial weight[19]. The liver, kidney and spleen are the primary organs affected by metabolic reaction caused by the toxicant[20]. In the present study, the gross appearances of the selected organs both sexes were normal while the absolute organ weight in the combined extract group show no significant differences.

However, the toxicity effect of the plants on blood and histopathology-related change was also evaluated for higher safety level. The result from blood serum test disclosed that in this study for acute toxicity test, the female rat was slightly more sensitive than the male rat when observed with blood serum test. In detail of acute test, the liver function test and renal function test: female rat response to the combined extract via the significant decrease of AST, albumin and total protein production showed the same pattern with vehicle group. These data reveal that the combined extract which had the bioactive substances of Phyllanthin and 6-gingerol can adjust the higher level of AST, albumin and total protein to the lower level while the male response for the combined extract had its effect only in total protein production. Related with the previous finding found that both ginger and *P. amarus* have the high potent on anti-oxidative and anti-inflammation effect in living tissue[3,4]. Moreover, in Ayurvedic line, Phyllanthin which is the main phyto-constituent substance of Phyllanthus species especially in *P. amarus* was used to treat with the hepatitis or liver function abnormal patient for a long time. Therefore, it is no surprise that the combined extract was used in the present study (ginger and *P. amarus*) and had the effect on AST liver function test. The change of serum blood was also observed in female renal function test via a significant decrease in BUN when compared with control naive group, while in the male rat one could observe the changes in only BUN/creatinine.

Contrasting with sub-acute test, the male rat shows higher sensitivity than female rat after repetitive doses of the combined extract. It was found that AST and albumin significantly decreased in male rats but they had no effect on female rats' liver function test. Interestingly, while the liver function of male rat was improved by the combined extract but the renal function test had showed the increasing significant of BUN and creatinine filtration. However, the combined extract used in this study also showed the dominant effect on the decreasing AST enzymatic production in liver same as in acute toxicity test with contrast as sex response.

There was no necrosis, inflammatory reaction, fibrosis, or local fatty degeneration or disarrangement of the hepatocytes observed in liver while the glomeruli and Bowman's capsules also showed a normal microphotograph display with no adverse effects.

## Conclusion

The results show that the $LD_{50}$ toxicity of both acute and sub-acute dermal is more than 2,000 mg/kg.BW. Thus, the herbal cream contained the combined extract of *Z. officinale* and *P. amarus* and is very safe for transdermal application.

## What is already known on this topic?

*Z. officinale* and *P. amarus* are generally used as a traditional medicine for a long time. Both herbs have anti-inflammatory and antioxidant properties.

## What this study adds?

The combined extract of *Z. officinale* and *P. amarus* is very safe for transdermal application.


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## Potential conflicts of interest

The authors declare no conflicts of interest.

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