



## A Toxicological Safety Assessment of a Standardized Extract of *Eulophia macrolulbon* in Rodents

Watcharaporn Preedapirom<sup>1</sup>, Kanokwan Changwichit<sup>2</sup>, Warisaraporn Tangchang<sup>3</sup>,

Kornkanok Ingkaninan<sup>2</sup> and Pornnarin Taepavarapruk<sup>1,3\*</sup>

<sup>1</sup>Department of Physiology, Faculty of Medical Science, Naresuan University, Phitsanulok, 65000, Thailand

<sup>2</sup>Department of Pharmaceutical Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok 65000, Thailand

<sup>3</sup>Center for Animal Research, Naresuan University, Phitsanulok 65000, Thailand

\* Corresponding author: E-mail address: taepavap@yahoo.com, pornnarint@nu.ac.th

Received: 31 January 2018; Accepted: 10 April 2018

### Abstract

The tuber of *Eulophia macrolulbon* (EM) has been traditionally used in Thailand to relieve pain and fatigue. Recently, it has been reported that EM extract could provide benefits such as anti-inflammatory, and antioxidant that may be used as herbal remedy in the pharmaceutical or food industries. Cytotoxic properties of EM extract on cancer cell lines were also reported *in vitro*. However, as yet, no safety data has been investigated. Our study is the first one in which the acute and chronic toxicity of the standardized extract of EM has been tested. The OECD Guideline No. 420 (limit test) was used for the acute toxicity test in which a single oral dose of 2000 mg crude extract/kg body weight was administered to Wistar rats. Observations were conducted during the 2-week period after administration. Clinical signs and mortality were used as parameters to evaluate the acute toxicity. The results showed no toxicity in terms of general behavior change, mortality, or change in gross appearance of internal organs. Chronic toxicity was studied by following OECD Guideline No. 452. EM extract was administered orally at doses of 5, 50 and 500 mg/kg for 6 months. Mortality, clinical signs, body weights, biological and hematological parameters, organ weights, as well as macro- and microscopic observation of several organs were evaluated at the end of the test period. Our tests showed that EM extract did not induce death or adverse effects, and nor did produce any abnormality of the hematological and biochemical parameters or histopathological changes.

Overall, our test results have indicated that the oral Lethal Dose 50 (LD<sub>50</sub>) of EM extract is more than 2,000 mg/kg, and oral administration of EM for long period up to 6 months is safe. Based on established literature on health benefits of EM, it is important to focus attention on its active constituents and therapeutic effects in order to further develop EM as herbal remedy for treating of inflammations associated diseases as well as erectile dysfunction.

**Keywords:** *Eulophia macrolulbon*, Toxicological safety assessment, Acute and Chronic toxicity, rodents

### Introduction

*Eulophia macrolulbon* (EM) is a terrestrial orchid which has a large tuber-like pseudobulb carrying oblong to elliptic lanceolate, acuminate leaves that blooms in the spring. The orchid is typical found in south-east Asia, especially in Thailand, Cambodia, Laos, Vietnam, the Eastern Himalayas and Northern Myanmar, at about 700 meters elevation. The genus *Eulophia* (E.) includes about 230 species of orchids (Thomas, 1998), several species of which, such as *E. campestris* (Medhi & Chakrabarti, 2009), *E. millsoni* (Tuchinda et al., 1988) and *E. nuda* (Jagdale, Shimpi, & Chachad, 2009) amongst others, are used as aphrodisiac herbs. In Thailand, the tuber of EM has been traditionally used to relieve pain and fatigue (Chuakul, 2010).

The ethanolic extract of EM roots has been tested *in vitro* for its bioactivity, and was found to show notable cytotoxic effects, as well as anti-inflammatory and antioxidant effects (Schuster et al., 2017).



Recently, a novel phenanthrene, [1] 9,10-dihydro-4-(4'-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol and three known phenanthrenes such as [2] 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol, [3] (9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol and [4] 1,5,7-trimethoxyphenanthrene-2,6-diol), were isolated from the EM and tested for phosphodiesterase 5 (PDE5) activity (Temkitthawon et al., 2017). The most powerful PDE5 inhibitor ( $IC_{50} = 1.67 \pm 0.54 \mu M$ ) was [2] whereas [1] showed mild activity ( $IC_{50} = 62.3 \pm 3.3 \mu M$ ). PDE5 is present in variety of organs such as lung, heart (Carson & Lue, 2005) and especially in penis which represents a target for erectile dysfunction (ED) (Corbin, 2004). Ethanolic extract of EM and its main compound [2] showed vasorelaxant effect on rat isolated mesenteric (Wisutthathum et al., 2018) and pulmonary artery (Wisutthathum et al., 2018). Furthermore, preliminary studies on mating behavior and erectile function tests showed that EM extract at doses of 15, 150 and 450 mg/kg BW, when administered orally to aged male rats for 21 days, could enhance sexual activity and increase the erectile response (Preedapirom, unpublished data). Notwithstanding that this medicinal orchid has been claimed to have therapeutic uses in several disorders, there are no studies on its toxic effects. The single-dose acute oral toxicity test is usually the initial step in evaluating the toxic properties of a test substance. Chronic toxicity test is to determine the cumulative adverse effects on target organs as the result of long term exposure to a test substance. (Antonelli-Ushirobira, et al., 2015). Therefore, the purpose of the present study was to evaluate the safety of EM extract in animals by performing a single dose oral toxicity test and chronic repeated dose oral toxicity tests, in rodents. Since tuber of EM has been traditionally used for pain and fatigue management of Thai people for a long time (Chuakul, 2010), it is possible to hypothesize that administration of EM extract, either a single high dose or a repetitive dosage for 6 months, would not lead to mortality or certain health effects.

## Methods and Materials

### ***Eulophia macrobulbon* (EM) crude extracts preparation**

EM was assembled from Prachinburi Province, Thailand. Plant identification was made by Asst. Prof. Dr. Anupan Kongbangkerd, Faculty of Sciences, Naresuan University. Sample has been lodged in the Herbarium of the Biology Department, Faculty of Science, Naresuan University, Thailand, under the following number No. 002716. The standardized ethanol extract of EM was prepared as described previously by Temkitthawon et al., (2017). Briefly, the fresh pseudo-bulbs were washed and air-dried at room temperature, then sliced into small pieces. They were dried by a hot air oven at 55 °C and grinded into fine powder. The dried powder was macerated for 3 days/time (3 times) with 95% ethanol. After that, it was filtered and evaporated under vacuum until dryness to give the crude ethanolic extract. The EM crude extract was stored in a sealed glass jar and kept at -20 °C freezer until use. The content of standard marker in the ethanolic extract of EM used in this study yielding as 0.52%.

### **Animals and Housing**

Adult male and female Wistar rats, 8-weeks old, were obtained from the National Laboratory Animal Center, Mahidol University, Nakhonpathom, Thailand. The experiments were approved by the Naresuan University Animal Care and Use Committee (NUACUC) (approval no. 56 04 0064) and followed the ethical guidelines of the National Research Council of Thailand. The animals were housed in an animal room at the



Naresuan University Center for Animal Research (NUCAR), which earned AAALAC International accreditation since 2014. All animals were housed under controlled conditions of temperature  $22\pm 1^{\circ}\text{C}$ , a relative humidity of  $55\pm 10\%$  and a 12:12 h light/dark cycle. They were fed with commercial food pellets (Rodent pellets, G082, C.P. Thailand) and clean reverse osmosed water *ad libitum* unless otherwise stated. To minimize any stressful conditions, all animals were allowed to adjust to the new environment for at least 7 days after they have arrived at NUCAR.

#### **Acute oral toxicity study**

The acute oral toxicity study was conducted in compliance with the OECD guideline No. 420 (OECD, 2001). Ten male and ten female rats were randomly divided into a control group and a test group, each with 5 males and 5 female rats. All the animals were fasted overnight (~12 h) and weighed. The control group received propylene glycol (PG) as vehicle while the test group received EM crude extract at a single dose of 2,000 mg/kg B.W. by oral gavage. The animals were regularly and individually observed for behavioral changes and general toxicity signs (changes of gait and posture, changes to the skin, fur texture, eyes, mucous membranes central nervous system, autonomic nervous system, motor activities, reflexes, respiratory patterns and cardiovascular system) during the first 24 h after dosing, with special attention being paid during the first 4 h. Observations continued daily for a total of 14 days. Each animal's body weight was recorded once a week throughout the study period. On day 15, the animals were euthanized using an overdose intraperitoneal injection of 100 mg/kg B.W. sodium pentobarbital (Nembutal®). The gross morphology of their internal organ was observed.

#### **Chronic oral toxicity study**

This study followed the OECD Guideline No. 452 (OECD, 2009). Eighty rats, 40 male and 40 female, were randomly divided into a control group, 10 males and 10 female rats, and 3 test groups, each with 10 males and 10 female rats. The control group orally received PG while one test group was administered 5 mg/kg/day, the 2<sup>nd</sup> group received 50 mg/kg/day, and the third test group was administered 500 mg/kg/day at the volume of 10 ml/kg B.W., once a day over a period of 6 months. Each rat had its body weight recorded weekly, and mortality and signs of toxicity were observed and recorded every day. At the end of the 6-month treatment period, all animals were subjected to overnight fast before euthanasia, using an overdose intraperitoneal injection of sodium pentobarbital. Blood samples were collected via cardiac puncture and transferred into tubes with or without anticoagulant (ethylenediamine tetraacetate) for the hematological and biochemical analyses. The visceral organs were then isolated and weighed before fixing with 10% neutral formalin solution for further histopathological examination.

#### **Hematological and biochemical analyses of blood samples**

Blood samples mentioned above were sent to a BioLab Clinic (Phitsanulok Province, Thailand) for hematological and biochemical analyses. The anticoagulated blood samples were examined for hematological parameters including the white blood cell (WBC), red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), red cell volume distribution (RDW).

The blood samples without anticoagulant were determined for biochemical parameters [creatinine, total protein, albumin, globulin, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), alkaline phosphatase (ALP) and blood urea nitrogen (BUN)].



### **Organ weight and histopathological examination**

The major internal organs were collected, rinsed in cold normal saline and weighed. Relative organ weight was calculated using the following formula: relative organ weight (kg) = (organ weight (g)/body weight (g)) x 1000. The major internal organs; heart, lung, liver, kidney, spleen, adrenals and gonads, were then preserved in 10% neutral formalin solution. All fixed tissue samples were processed routinely, embedded in paraffin wax, cut in 5 µm serial sections and stained with hematoxylin and eosin (H&E). Sections were examined for morphological alterations under light microscopy (Olympus, BX43F, Japan) with 20X magnification provided with cellSens standard software.

### **Statistical Analyses**

Results are expressed as the mean value ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons using the Graph prism 7.04, Software, inc., USA. A level of p value less than 0.05 was considered statistically significant.

## **Results**

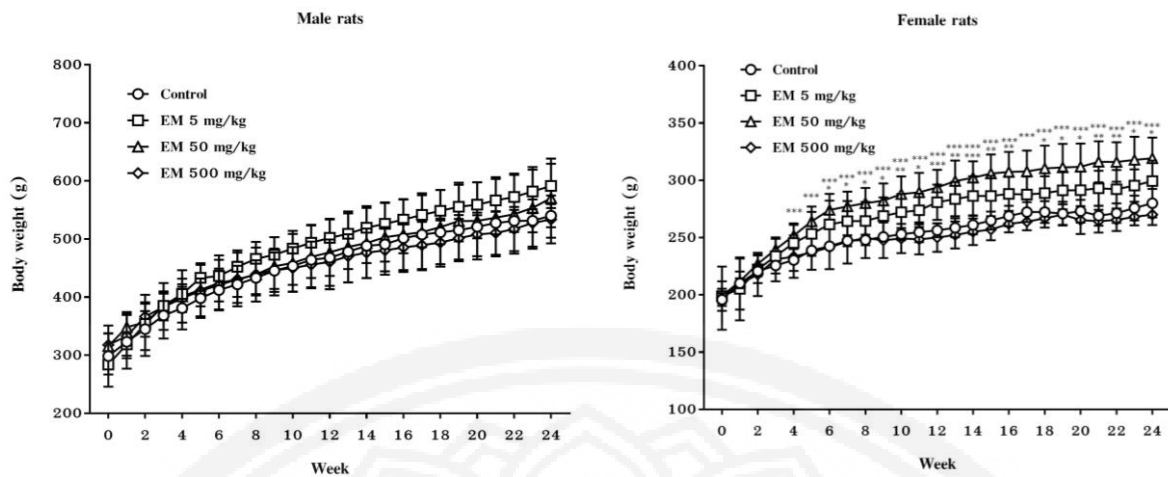
### **Mortality and Clinical Signs**

The administration of a single oral dose of 2000 mg/kg of the EM extract produced neither mortality nor any abnormal signs or behaviors in animals during the 14-day observation period. There were also no significant differences in the weight gain at the end of 14-day treatment between the treated and the vehicle control group. The relative organ weights of the treated group showed no statistical difference to that of the control group. In addition, necropsy revealed no gross pathological signs in any organs. Therefore, the LD<sub>50</sub> of EM extract in rats was estimated to be higher than 2000 mg/kg.

None of the male and female rats, in the chronic toxicity study, that had been administered with 5, 50, 500 mg/kg B.W. of EM for 6 months exhibited symptoms of toxicity, and no deaths occurred in any of the animals during that 6-month period. However, one male in the 500 mg/kg EM extract group and three female rats (one female in the control group and two female in the 500 mg/kg EM extract group) got sick after feeding routines. Their health were monitored closely. Since they lose weight rapidly, the veterinarian decided to euthanize them. Their gross necropsy examination revealed the tissue damage of either the esophagus or trachea which not related to the substance administered.

### **Changes in Body Weight and Relative Organs Weights**

The body weight of male rats in all groups increased normally during the study period, and there were no significant differences in the body weight of the male rats in the treated groups when compared with the control group (Figure 1). However, significant increases in body weights were observed in the female rats that had been administered 5 mg/kg EM crude extract group, from weeks 6-16 and 18-24. Also, significant increases were observed in the body weight of female rats in the 50 mg/kg EM extract group, in weeks 4-24.



**Figure 1** Mean body weights of the males (Control; n= 10, EM 5 mg/kg; n= 10, EM 50 mg/kg; n= 10 and EM 500 mg/kg; n= 9) and the females (Control; n= 9, EM 5 mg/kg; n= 10, EM 50 mg/kg; n= 10 and EM 500 mg/kg; n= 8) given daily doses of EM extract for 6 months. The values are expressed as mean  $\pm$  S.D., \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  compared with the control group.

The relative organ weights of both the female and male rats treated with EM doses (5, 50 and 500 mg/kg B.W.) are presented in Table 1 and 2, respectively. For male rats, no significant difference between the treated and control groups was observed (Table 1). For the female rats, significant decreases in the relative organ weights of the liver, right kidneys, spleen and ovary + uterus were noted in female rats treated with EM 5 and 50 mg/kg when compared to control group. There were no differences in the all relative organ weights between extract treated 500 mg/kg group and the control group (Table 2).

**Table 1** Relative organ weight of male rats in chronic toxicity study of EM extract

Organ	Control (n=10)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=9)
Final body weight (g)	540.20 $\pm$ 19.81	591.50 $\pm$ 38.15	570.40 $\pm$ 68.08	533.89 $\pm$ 41.25
Brain	0.38 $\pm$ 0.03	0.36 $\pm$ 0.02	0.38 $\pm$ 0.04	0.40 $\pm$ 0.02
Heart	0.27 $\pm$ 0.03	0.27 $\pm$ 0.03	0.26 $\pm$ 0.03	0.29 $\pm$ 0.02
Lung	0.33 $\pm$ 0.06	0.29 $\pm$ 0.05	0.31 $\pm$ 0.05	0.33 $\pm$ 0.03
Liver	2.89 $\pm$ 0.11	2.84 $\pm$ 0.23	2.89 $\pm$ 0.47	2.93 $\pm$ 0.14
Spleen	0.17 $\pm$ 0.02	0.18 $\pm$ 0.03	0.17 $\pm$ 0.03	0.19 $\pm$ 0.03
Stomach + Small intestines	0.48 $\pm$ 0.04	0.49 $\pm$ 0.04	0.43 $\pm$ 0.04	0.52 $\pm$ 0.07
Left kidney	0.24 $\pm$ 0.01	0.23 $\pm$ 0.02	0.22 $\pm$ 0.02	0.24 $\pm$ 0.02
Right kidney	0.24 $\pm$ 0.02	0.24 $\pm$ 0.03	0.23 $\pm$ 0.02	0.26 $\pm$ 0.03
Left adrenal glands	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
Right adrenal glands	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
Prostate + seminal vesicle	0.52 $\pm$ 0.06	0.51 $\pm$ 0.08	0.53 $\pm$ 0.11	0.52 $\pm$ 0.09
Left testis	0.39 $\pm$ 0.03	0.38 $\pm$ 0.04	0.36 $\pm$ 0.05	0.39 $\pm$ 0.04
Right testis	0.39 $\pm$ 0.02	0.38 $\pm$ 0.05	0.36 $\pm$ 0.05	0.37 $\pm$ 0.06
Left epididymis	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01
Right epididymis	0.06 $\pm$ 0.01	0.05 $\pm$ 0.00	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01
Left vas deferens	0.03 $\pm$ 0.01	0.03 $\pm$ 0.02	0.02 $\pm$ 0.01	0.02 $\pm$ 0.00
Right vas deferens	0.03 $\pm$ 0.01	0.02 $\pm$ 0.00	0.02 $\pm$ 0.01	0.02 $\pm$ 0.00

Values are mean  $\pm$  SD, \*  $p < 0.05$  compared with the control group

**Table 2** Relative organ weight of female rats in chronic toxicity study of EM extract

Organ	Control (n=9)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=8)
Final body weight (g)	280.10 ± 12.48	294.90 ± 21.96*	319.20 ± 18.17*	270.13 ± 9.19
Brain	0.67 ± 0.04	0.67 ± 0.05	0.63 ± 0.04	0.71 ± 0.04
Heart	0.34 ± 0.02	0.32 ± 0.02	0.31 ± 0.02	0.34 ± 0.03
Lung	0.42 ± 0.05	0.41 ± 0.05	0.40 ± 0.03	0.45 ± 0.04
Liver	3.17 ± 0.37	2.67 ± 0.44*	2.56 ± 0.21*	3.21 ± 0.27
Spleen	0.26 ± 0.02	0.22 ± 0.03*	0.21 ± 0.03*	0.25 ± 0.03
Stomach + Small intestines	0.81 ± 0.14	0.70 ± 0.09	0.69 ± 0.09	0.80 ± 0.15
Left kidney	0.28 ± 0.03	0.27 ± 0.02	0.27 ± 0.01	0.29 ± 0.02
Right kidney	0.31 ± 0.03	0.28 ± 0.02*	0.26 ± 0.02*	0.30 ± 0.03
Left adrenal glands	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
Right adrenal glands	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Ovaries + Uterus	0.30 ± 0.04	0.22 ± 0.06*	0.24 ± 0.05*	0.31 ± 0.05

Values are mean ± SD, \* p < 0.05 compared with the control group

### Effect of EM extract administration on biochemical and hematological parameters

After feeding the rats EM extract for 6 months, the males treated with EM 5 mg/kg BW showed increased levels of HGB and MCHC but decreased levels of MCV when compared with the control group. The male rats treated with EM 50 mg/kg BW showed decreases in the level of HGB and HCT. The group of males treated with 500 mg/kg also showed increases in the levels of WBC, HGB, MCH and MCHC (Table 3). Additionally, the female rat treated with 5 mg/kg showed increases in the levels of MCHC but decreases in MCV level. The group treated with 50 mg/kg BW showed decreases in the level of MCV and MCH but increases in MCHC level. The group treated with EM 500 mg/kg BW showed decreased levels of MCV (Table 4).

**Table 3** Hematological parameters of male rats in chronic toxicity study of EM extract

Parameter	Control (n=10)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=9)
WBC (/mm <sup>3</sup> )	2500 ± 620	3120 ± 697	2690 ± 1031	3600 ± 983*
RBC (cells/cu.mm)	8.88 ± 0.30	9.15 ± 0.46	8.38 ± 0.69	8.90 ± 0.23
Hemoglobin (g/dl)	15.52 ± 0.38	16.23 ± 0.68*	14.85 ± 0.54*	16.39 ± 0.48*
Hematocrit (%)	50.06 ± 2.27	48.10 ± 2.18	46.70 ± 1.57*	50.32 ± 2.89
MCV (fl.)	56.30 ± 2.41	52.40 ± 1.26*	55.10 ± 1.91	56.44 ± 3.24
MCH (pg.)	17.49 ± 0.44	17.74 ± 0.43	17.43 ± 0.65	18.38 ± 0.72*
MCHC (g/dl)	31.06 ± 0.87	33.85 ± 0.59*	31.75 ± 0.78	32.57 ± 0.97*
Platelet (/mm <sup>3</sup> )	507800 ± 51860	547000 ± 53435	566300 ± 93903	573444 ± 62245
RDW (%)	13.58 ± 0.56	12.85 ± 0.63	16.00 ± 6.22	13.79 ± 1.38

Values are mean ± SD, \* p < 0.05 compared with the control group

**Table 4** Hematological parameters of female rats in chronic toxicity study of EM extract

Parameter	Control (n=9)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=8)
WBC (/mm <sup>3</sup> )	2111 ± 860	1350 ± 476	1460 ± 766	2550 ± 1182
RBC (cells/cu.mm)	7.66 ± 0.16	7.85 ± 0.64	7.97 ± 0.22	7.74 ± 0.39
Hemoglobin (g/dl)	14.96 ± 0.34	15.02 ± 1.12	14.90 ± 0.51	14.94 ± 0.89
Hematocrit (%)	45.54 ± 1.42	43.40 ± 3.60	43.20 ± 1.32	44.41 ± 2.54
MCV (fl.)	59.44 ± 2.07	55.20 ± 1.32*	54.20 ± 1.32*	57.50 ± 1.41*
MCH (pg.)	19.56 ± 0.56	19.15 ± 0.43	18.68 ± 0.36*	19.31 ± 0.90
MCHC (g/dl)	32.91 ± 0.56	34.70 ± 0.37*	34.43 ± 0.37*	33.75 ± 1.34
Platelet (/mm <sup>3</sup> )	486222 ± 62757	463300 ± 175638	527500 ± 59985	523250 ± 86052
RDW (%)	10.86 ± 0.36	10.86 ± 0.42	10.98 ± 0.30	11.44 ± 0.60

Values are mean ± SD, \* p < 0.05 compared with the control group

**Table 5** Biochemical values of male rats receiving EM extract for 6 months

Parameter	Control (n=10)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=9)
Creatinine (mg/dl)	0.55 ± 0.08	0.56 ± 0.12	0.45 ± 0.08	0.62 ± 0.14
Total protein (g/dl)	5.79 ± 0.23	5.62 ± 0.29	5.41 ± 0.19*	5.68 ± 0.44
Albumin (g/dl)	2.72 ± 0.11	2.74 ± 0.13	2.86 ± 0.10*	2.92 ± 0.16*
Globulin (g/dl)	3.09 ± 0.18	2.88 ± 0.26	2.55 ± 0.20*	2.76 ± 0.32*
AST(SGOT) (Units)	154.5 ± 60.07	195.40 ± 61.33	93.1 ± 22.22*	165.22 ± 53.33
ALT(SGPT) (Units)	61.70 ± 20.92	66.40 ± 27.84	40.40 ± 7.89*	46.67 ± 14.34
ALP (I.U.)	61.60 ± 11.44	70.30 ± 12.92	71.20 ± 12.23	63.33 ± 16.05
BUN (mg/dl)	24.97 ± 2.75	18.46 ± 1.98*	23.52 ± 2.06	26.92 ± 3.76
Total Bilirubin (mg/dl)	0.16 ± 0.07	0.06 ± 0.01*	0.05 ± 0.01*	0.08 ± 0.05*

Values are mean ± SD, \* p < 0.05 compared with the control group

**Table 6** Biochemical values of female rats receiving EM extract for 6 months

Parameter	Control (n=9)	Dose of EM extract (mg/kg body weight per day)		
		5 (n=10)	50 (n=10)	500 (n=8)
Creatinine (mg/dl)	0.64 ± 0.11	0.55 ± 0.11	0.55 ± 0.08	0.5 ± 0.21
Total protein (g/dl)	5.61 ± 0.17	5.8 ± 0.29	5.47 ± 0.37	5.76 ± 0.45
Albumin (g/dl)	3.00 ± 0.09	3.12 ± 0.18	3.02 ± 0.20	2.96 ± 0.18
Globulin (g/dl)	2.61 ± 0.14	2.68 ± 0.16	2.45 ± 0.28	2.8 ± 0.32
AST(SGOT) (Units)	111.22 ± 24.41	168.4 ± 120.62	112.5 ± 9.78	163.38 ± 90.66
ALT(SGPT) (Units)	46.11 ± 7.74	55.5 ± 11.92	45.1 ± 10.79	52.25 ± 21.14
ALP (I.U.)	46.56 ± 20.89	38.1 ± 23.25	34.1 ± 16.31	56.75 ± 17.10
BUN (mg/dl)	22.18 ± 2.69	18.12 ± 3.59*	22.74 ± 2.55	23.93 ± 4.43
Total Bilirubin (mg/dl)	0.16 ± 0.05	0.06 ± 0.01*	0.06 ± 0.01*	0.13 ± 0.10

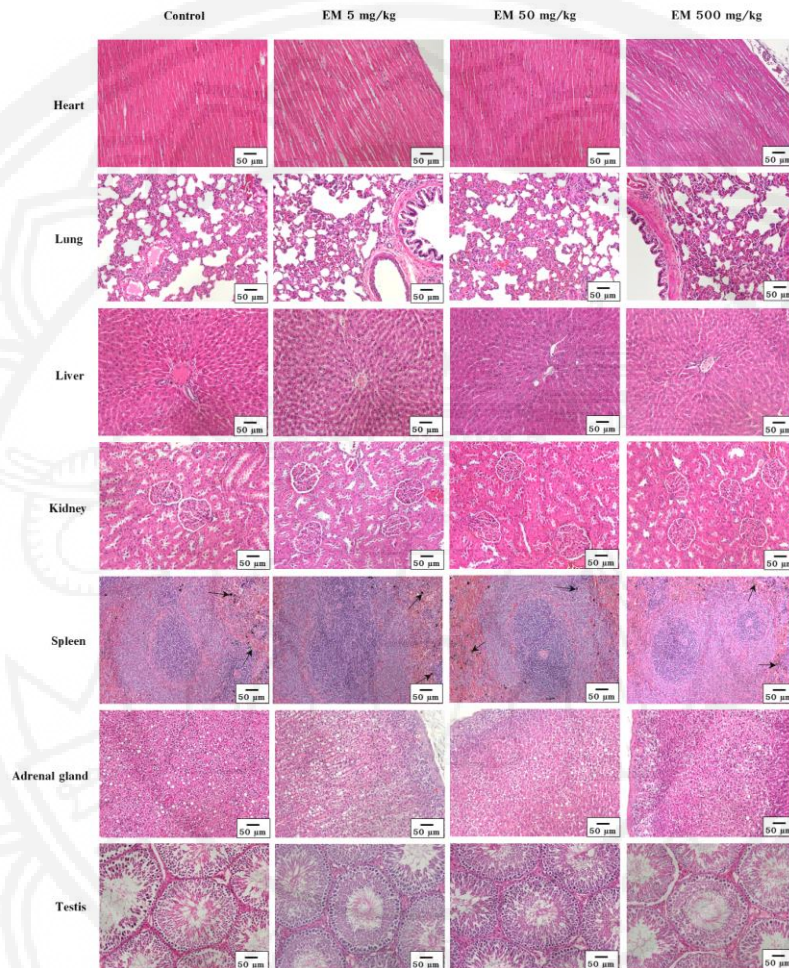
Values are mean ± SD, \* p < 0.05 compared with the control group

Results showed some alterations in biochemical parameters following the 6-month period of administration of the EM extract. The male rats treated with EM 5 mg/kg BW showed decreased BUN and total bilirubin levels compared to the control group, whereas EM 50 mg/kg BW caused decreases in the levels of total protein, globulin, AST, ALT and total bilirubin, and increases in albumin levels (Table 5). The male rats treated with EM 500 mg/kg BW also showed an increase in albumin levels but decreased globulin and total bilirubin levels. In the female rats, the administration of EM 5 mg/kg BW decreased the BUN level. Decreased total bilirubin were also observed in female rats treated with EM 5 and 50 mg/kg BW when compared to the control group (Table 6).



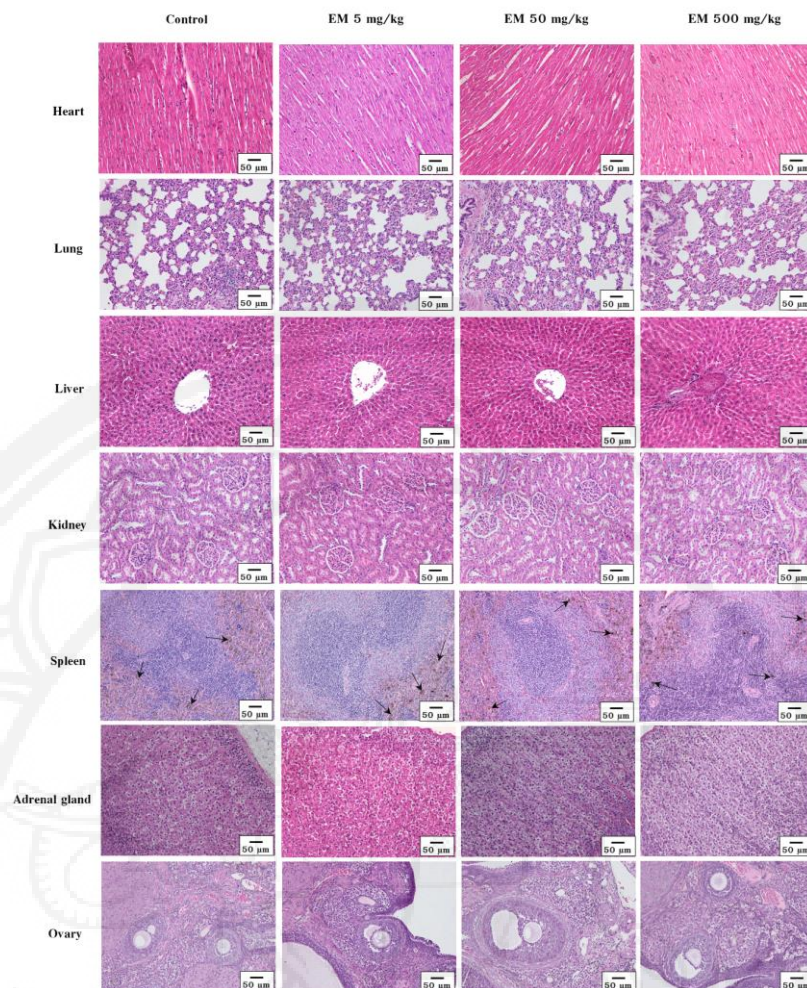
### Histopathological evaluation of rats receiving EM extract for 6 months

From the histopathological analysis of the vital organs (the heart, lungs, liver, kidneys, spleen, adrenal glands, and gonads), there were no remarkable lesions in these organs of either sex that could be attributed to the EM extract. Therefore, the changes in the relative organ weights of both male and female rats given the EM extract were considered unrelated to the treatment. Multifocal melanosis in the spleen were observed in all rats of the control group and the treatment groups, none which was statistically significant (Figure 2 and 3).



**Figure 2** Histological sections of internal organs at 20x of the male rats treated with vehicle and EM extract (5, 50, 500 mg/kg BW)





**Figure 3** Histological sections of internal organs at 20x of the female rats treated with vehicle and EM extract (5, 50, 500 mg/kg BW)

### Discussion

The nontoxic nature of EM extract and its oral safety at a single high dose of 2000 mg/kg was confirmed in acute toxicity study of both sexes. Neither sign toxicity nor death of any animals was observed during the period of the experiment, which indicated that the mean lethal dose ( $LD_{50}$ ) of the EM extract is considered to be greater than 2000 mg/kg (OECD, 2001). Furthermore, no significant differences were noted in body and internal organ weights between the control and the treated groups of both sexes. Pathologically, neither gross abnormalities nor histopathological changes were observed. Thus, it can be concluded that EM crude extract is absent of the acute oral toxicity. Furthermore, no macroscopic changes in of the vital organs of treated animals were observed, therefore not needing of histopathological examination (Andrade, Albuquerque, Maraschin & Silva, 2012). The chronic 6-month toxicity study showed that EM extract did not induce abnormal behavioral change, adverse effects and mortality in male and female rats. Since, changes in body weight has been used to evaluate the adverse effects of compounds and chemicals (Li et al., 2015).

Significant increase in body weight but decrease in relative organ weights of ovary and uterus in female rats treated with 5 and 50 mg/kg EM extract observed in this study may be one explanation for the appetite



inducing effect. The reduction in ovary weight can cause the reduction in estrogen production. Since estrogen is an appetite suppressant (Geary, 1998), therefore it is possible that reduced estrogen could induce appetite resulting in weight gain. Besides, the increase in weight may result from the appetite-increasing effect of this plant itself. Since several studies reported of the use of related species i.e. *E. campestris* (Chanda, Mohanti, Bhuyan, Kar & Nath, 2007) and *E. nuda* (Rao, 2004) as appetizers. Nevertheless, the appetite-inducing properties of EM have not been reported yet. In the present study, the body weight and relative organs weights in the EM extract treated groups were significantly different from those of the control groups, however, all of these changes may not be of clinical significance because the alterations were minor and did not decrease/increase in relation to doses ingested. In addition, the differences may due to the variation in size of internal organs and/or body weight of the animals (Bailey, Zidell & Perry, 2004; Levine, 2002). This finding was further confirmed by histopathological examination. The results showed no changes in organ morphology across the dose groups. Therefore, our study suggests that chronic treatment with EM extract did not produce any toxicity to internal organs examined. Unfortunately, in the current study, we did not record food and water consumption as well as analyze biochemical parameters such as plasma glucose and lipid profile that can be determined the increasing of body weight.

The hematopoietic system is one of the most susceptible targets to toxic compounds and is an important parameter for evaluating the physiological and pathological status in both animals and humans (Li et al., 2010). Although some parameters in both the male and female rats observed in this study showed a statistical difference in the groups treated with EM extract, these data are still within the reference values for normal rats (Giknis & Clifford, 2008).

Clinical blood chemistry determinations to examine major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed and under certain circumstances may provide useful information. Some of the biochemical markers such as AST, ALT and bilirubin can be used as indicative of hepatocellular damage (OECD, 2009) and others as biomarkers of diagnostic tools for kidney injury such as creatinine and BUN (Lameire, Van Biesen & Vanholder, 2005). Interestingly, the decrease in AST and ALT levels in male rats treated with 50 mg/kg/day were observed. Clearly, the possible AST and ALT lowering effect of EM require further investigation. Some parameters in both the male and female rats (total protein, globulin, AST, ALT, BUN, total bilirubin) were statistically different when compared to the control group. As for the changes of blood chemistry parameters, they were still within the normal range for healthy rats at this age (Giknis & Clifford, 2008; Hematological data of NLAC-MU laboratory animals, n.d.), and not dose-associated or reflected by any alterations in other parameters. However, albumin in male rats receiving 50 and 500 mg/kg EM extract was significantly higher than that in control group, but the same phenomenon seen in males was not observed in females. These results indicated that giving EM extract orally at these specific doses and duration investigated may result from the individual difference and is not a sign of toxicity. Furthermore, this finding occurred with a lower incidence and did not show a dose-dependent correlation.

### Conclusion

Our findings demonstrate that oral administration of standardized EM extract is safe in both sexes of Wistar rats, with no adverse effects on the functions of the major organs, and posed no health risk in the acute and 6-



month chronic oral toxicity study. The LD<sub>50</sub> of EM extract were determined as more than 2000 mg/kg/day regardless of sex. These results of non-clinical safety tests, especially the repeated dose toxicity, are necessary for human health risk assessment and are required for submission to Regulatory Authorities for registration and licensing of substance developed to be herbal remedy for treating of inflammations associated diseases as well as erectile dysfunction.

#### Acknowledgements

This study was financially supported by Thailand Agricultural Research Development Agency (ARDA). Our thanks to Mr. Roy I. Morien of the Naresuan University Graduate School for his assistance in editing this paper for correct grammar and English expression.

#### References

- Andrade, F., Albuquerque, C. A., Maraschin, M., & Silva, E. L. (2012). Safety assessment of yerba mate (*Ilex paraguariensis*) dried extract: results of acute and 90 days subchronic toxicity studies in rats and rabbits. *Food and Chemical Toxicology*, *50*, 328-334.
- Antonelli-Ushirobira, T. M., Blainski, A., Fernandes, H. G., Moura-Costa, G. F., Costa, M. A., Campos-Shimada, L. B., ... Mello, J. C. (2015). Acute toxicity and long-term safety evaluation of the crude extract from rhizomes of *Limonium brasiliense* in mice and rats. *Journal of Ethnopharmacology*, *174*, 293-298.
- Bailey, S. A., Zidell, R. H., & Perry, R. W. (2004). Relationships between organ weight and body/brain weight in the rat: what is the best analytical endpoint?. *Toxicologic pathology*, *32*, 448-466.
- Carson, C. C., & Lue, T. F. (2005). Phosphodiesterase type 5 inhibitors for erectile dysfunction. *BJU International*, *3*, 257-280.
- Chanda, R., Mohanti, J. P., Bhuyan, N. R., Kar, P. K., & Nath, L. K. (2007). Medicinal plants used against gastrointestinal tract disorders by traditional healers of Sikkim Himalayas. *Indian Journal of Traditional Knowledge*, *6*, 606-610.
- Chuakul, W. (2010) Thai traditional herbs for relief pain and fatigue. *Thai Pharmaceutical and Health Science Journal*, *1*, 1-13.
- Corbin, J. D. (2004). Mechanisms of action of PDE5 inhibition in erectile dysfunction. *International Journal of Impotence Research journal*, *1*, 4-7.
- Geary, N. (1998). The effect of estrogen on appetite. *Medscape Womens Health*, *3*(6), 3. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/9878925>
- Giknis, M. L., & Clifford, C. B. (2008). *Clinical Laboratory Parameters for CrI: WI (Han)*. Wilmington, MA: Charles River Laboratories.
- Hematological data of NLAC-MU laboratory animals (n.d.). Retrieved from <http://www.nlac.mahidol.ac.th/acth/index.php/quality-control/health-control#Hemato>
- Jagdale, S. P., Shimpi, S., & Chachad, D. (2009). Pharmacological studies of Salep. *Journal of Herbal Medicine and Toxicology*, *3*, 153-156.



- Lameire, N., Van Biesen, W., & Vanholder, R. (2005). Acute renal failure. *Lancet*, 365, 417–447.
- Levine, B. S. (2002). *Animal Clinical Pathology* (2nd ed.). USA: CRC Press.
- Li, F., He, X., Niu, W., Feng, Y., Bian, J., & Xiao, H. (2015). Acute and sub-chronic toxicity study of the ethanol extract from leaves of *Aralia elata* in rats. *Journal of ethnopharmacology*, 175, 499–508.
- Li, X., Luo, Y., Wang, L., Li, Y., Shi, Y., Cui, Y., & Xue, M. (2010). Acute and subacute toxicity of ethanol extracts from *Salvia przewalskii* Maxim in rodents. *Journal of ethnopharmacology*, 131, 110–115.
- Medhi, R., & Chakrabarti, S. (2009). Traditional knowledge of North-East people on conservation of wild orchids. *Indian Journal of Traditional Knowledge*, 8, 11–16.
- OECD GUIDELINE FOR THE TESTING OF CHEMICALS: 420 Acute Oral Toxicity–Fixed Dose Procedure (2001). Retrieved from [https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oced/oced\\_gl420.pdf](https://ntp.niehs.nih.gov/iccvam/suppdocs/feddocs/oced/oced_gl420.pdf)
- OECD GUIDELINE FOR THE TESTING OF CHEMICALS: 452 Chronic Toxicity Studies (2009). Retrieved from <https://www.oecd-ilibrary.org/docserver/9789264071209-en.pdf?expires=1525850043&id=id&accname=guest&checksum=C1584032F70B64DEEF0475FCBBA8F1A2>
- Rao, A. N. (2004). Medicinal orchid wealth of Arunachal Pradesh. *ENVIS Newsletter*, 1, 1–5.
- Schuster, R., Zeindl, L., Holzer, W., Khumpirapang, N., Okonogi, S., Viernstein, H., & Mueller, M. (2017). *Eulophia macrobulbon* – an orchid with significant anti-inflammatory and antioxidant effect and anticancerogenic potential exerted by its root extract. *Phytomedicine*, 24, 157–165.
- Temkitthawon, P., Changwichit, K., Khorana, N., Viyoch, J., Suwanborirux, K., & Ingkaninan, K. (2017). Phenanthrenes from *Eulophia macrobulbon* as Novel Phosphodiesterase-5 Inhibitors. *Natural Product Communications*, 12, 79–82.
- Thomas, S. A. (1998). A Preliminary Checklist of Genus *Eulophia*. *Lindleyana*, 13, 170–202.
- Tuchinda, P., Udchachon, J., Khumtaveeporn, K., Taylor, W. C., Engelhardt, L. M., & White, A. H. (1988). Phenanthrenes of *Eulophia nuda*. *Phytochemistry*, 27, 3267–3271
- Wisutthathum, S., Chootipa, K., Martin, H., Ingkaninan, K., Temkitthawon, P., Totoson, P., & Demougeot, C. (2018). Vasorelaxant and Hypotensive Effects of an Ethanolic Extract of *Eulophia macrobulbon* and Its Main Compound 1-(4'-Hydroxybenzyl)-4,8-Dimethoxyphenanthrene-2,7-Diol, *Front. Pharmacol*, 9, 1–12. <https://doi.org/10.3389/fphar.2018.00484>
- Wisutthathum, S., Demougeot, C., Totoson, P., Adthapanyawanich, K., Ingkaninan, K., Temkitthawon, P., & Chootipa, K. (2018). *Eulophia macrobulbon* extract relaxes rat isolated pulmonary artery and protects against monocrotaline-induced pulmonary arterial hypertension. *Phytomedicine*, 50(15), 157–165. <https://doi.org/10.1016/j.phymed.2018.05.014>