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ACUTE AND SUBACUTE ORAL TOXICITY EVALUATION OF THE WATER EXTRACT OF TRIPHALA FORMULATION IN RATS

PRATYA PHETKATE[1], TULLAYAKORN PLENGSURIYAKARN[2], KESARA NA-BANGCHANG[2], PRASOB-ORN RINTHONG[3], SOMBOON KIETINUN[1], KUSUMA SRIYAKUL[1*]

[1]Graduate Program in Integrative Medicine, Chulabhorn International College of Medicine, Thammasat University, Pathum Thani 12120, Thailand

[2]Center of Excellence in Molecular Biology and Pharmacology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Thammasat University, Pathum Thani 12120, Thailand

[3]Pharmaceutical Chemistry and Natural Products Research Unit, Faculty of Pharmacy, Mahasarakham University, Maha Sarakham 44150, Thailand

***Corresponding Author: Kusuma Sriyakul: E Mail: kusuma_cat@yahoo.com; Phone number**: +66811718221

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ABSTRACT

Background: Triphala is the one formulation widely used for many diseases in Thailand, India, and throughout the Southeast Asia in many dosage forms, such as powder, liquidand extract. However, the Triphala water extract have not been tested for toxicity test. **Methods:**Aim of this study is to evaluate the acute and subacute toxicity of Triphala recipes extract in male and female rats according to the Organization for Economic Cooperation and Development (OECD) Guideline. The chemical composition of the extract was performed by HPLC. In the acute study, the dose administered was 5000 mg/kg, and signs of toxicity and mortality was observed. In the sub-acute study, the extract was administered at doses of 5,000, 3,000 and 1,000mg/kg for 28 days for any clinical changes associated with toxicity from the drugs. Serum chemistry, haematological effects, and selected histological tissue samples of each animal immediately after euthanasia were analyzed at the end of the

study.**Results:** In acute and subacute toxicity study, there was no significant difference ($p > 0.05$) observed in the relative organs, body weights, hematological, biochemical parameters, and gross abnormalities, compared to the control. No mortality was recorded. **Conclusion:** Administration of Triphala water extract did not cause relevant toxic effects to rats in both acute and subacute toxicity.

Keywords: Triphala, acute and subacute toxicity, toxicology, animal study, preclinical study

INTRODUCTION

The study of medicinal plants has been very important in the development of new drugs. In Thai traditional medicine, there are many formulation from ancient textbook. Triplala is one of famous formula originate from India and widely used in traditional treatment of a variety of chronic diseases in Thailand and other Southeast Asian countries. Triphla is composed of 3 fruits; *Teminaria chebulai*, *Termanalia bellirica* and *Phyllantus emblica*in equal proportion. Triphalais used for many diseases such as cardiovascular disease and hypertension disease, also it's used for digestive problem and constipation [1]. The formula has been prescribed for chemical rejuvenation and for important health tonic [2].In Thailand, it is used traditionally as adjustment of the physiological functions of the body according to climate change for one's strength and health; basically it is used for balancing all four elements of the body [3].Based on several scientific studies, it contains tannin, beta-sitosteroland phenolic compound such as gallic acid, ellagic acid, and chebulagic acid, etc [4]. Triphalahas showed multiple pharmacological effects and clinical studies such as antihyperlipidemic effect [5], immunomodulatory effect [3-4,6], antiinflamatory and antiarthritic effect [7], anticancer activity [8], antioxidant [9], antimicrobial [10], antifungal [11],etc.However, tests for determining the safety of using this formula are few reports in term of raw material or crude drug. But in term of extract, it has not been reported yet. Therefore, the present study aimed to evaluate acute and subacute oral toxicity of the water extract of Triphala in experimental animals.

MATERIALS AND METHODS

Chemicals and reagents: HPLC grade methanol and distilled water were purchased from Fisher Scientific (Leicestershire, UK). Ethanol was obtained from Labscan Asia Co. Ltd. (Bangkok, Thailand). Tween-80 was purchased from Sigma-Aldrich (MO, USA). Neutral buffered formalin (NBF, 10%) was purchased from Bio-Optica (Milano, Italy). Isoflurane for euthanasia was purchased

from MinradInc. (PA,USA). All other chemicals and reagents were high purity grade obtained from commercial suppliers.

Plant materials: The fresh fruits of *T. Chebula*, *T. belerica* and *P. emblica* were collected from Nadoon district, MahaSarakham, Thailand in February, 2016. Botanical authentication of plant material was confirmed through macroscopic and microscopic characters as previously described [12].All voucher specimens were deposited at Faculty of Pharmacy, Mahasarakham University, MahaSarakham, Thailand. Pulp of eachfruitwasdried in hot air oven at 60ºC for 48 hrs. The dried pulps were blended using blender and sieved through mesh no.14.

Preparation of Triphala Extract: Fifteen grams of each *T. chebula*, *T. Belerica* and *P. Emblica* powders were thoroughly mixed and boiled with 1 L of distilled water for 1 hr. The liquid part was filtered through a Whatman filter paper No. 1. The filtrate was reduced to 50% (v/v) in a low pressure distillation system at 50°C and it was converted to fine powder using spray drying technique. Spray dried condition was prepared by Lab plant spray dryer SS-06AG under the following operating conditions established by Rinthong et.al. (2017): inlet temperature, 180°C; feed rate, 5 mL/min and spraying pressure, 2 bar. The Triphala extract spray dried powders was kept in closed container and light protection at -20ºC until using [13].

The extract was standardized for extraction efficiency and quality control using high performance liquid chromatography (HPLC) with slightly modified method from Sato et.al. (2017) [14].Gallic acid and ellagic acid were employed as marker compounds. HPLC quantitative analysis was performed on a Shimadzu SCL-10A VP equipped with LC-10 AD binary pumps and SPD-M20A photodiode array detector (Kyoto, Japan). Data analysis was carried out using Class-VP version 6.1. Separation was performed on an Eclipse XDB-C18 column (250×4.6 mm, 5 µm) with Eclipse XDB-C18 guard column (12.5×4.6 mm, 5 µm) at ambient temperature. The HPLC mobile phase consisted of 0.05% trifluoroacetic acid (A) in water and acetonitrile (B) with flow rate of 1 mL/min. The gradient elution was programmed at 0-1 min, 5% B; 1-4 min, 5-10% B; 4-12 min, 10-15% B; 12-26 min, 15-30%B;26-30 min, 30-100% B; 30-40 min, 100% B;40-43 min, 100-5% B; 43-50 min, 5% B. The sample was injected to HPLC system at 20 µL and the absorbance was detected at 270 nm.

Animals: The study was conducted at the Laboratory Animal Center, Thammasat University under the AAALAC

International-certified laboratory. Wistar rats (6 weeks, 150-180 g) were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. Animals were quarantined 1 week before the start of the study and then randomly assigned to each group. Rats were allowed free access to food (standard pellet) and water ad libitum throughout the experimental period. The temperature of the room was maintained at 22 ± 2°C with relative humidity of 30-70%. Good hygiene was maintained by constant cleaning and removal of feces and spilled feed from cages twice a week. Effort was made to minimize animal suffering during the experimental period. The animals were handled according to the International Guidelines for Animal Welfare and the study protocol was approved by the Ethics Committee for Animal Research of Thammasat University, Thailand (Ethical approval number 019/2559).

***In vivo* toxicity (acute and subacute) evaluation:** Acute and subactute toxicity tests were done according to the protocols under the Acute Oral Toxicity-Fixed Dose Procedure (OECD Test No. 420) [15] and Repeated-Dose 28-day Oral Toxicity Study in Rodents' (OECD Test No. 407) [16], respectively. Male and female rats (n = 5 per group per sex) were given the mixture of Triphala extract (resuspended in a mixture of distilled water and Tween-80 4:1, v:v) by gavage starting from the highest dose of 5,000 mg/kg bodyweight. The control animals were fed with a mixture of distilled water and Tween-80. Animals were closely observed for signs of toxicity during the first 30 minutes, periodically during the first 24 hours, and then daily for 14 days (acute toxicity) or 28 days (subacute toxicity).

Clinical Observation and Mortality: Each rat was weighed at the beginning of the study, weekly during the administration and recovery periods, and at necropsy and observed daily for clinical signs and mortality. Following 14 and 28 days of Triphala extract exposure and a recovery period, all animals were euthanized by isoflurane under euthanasia machine.

Hematology and Blood Biochemistry Examination: At the end of the study, the animals were fasted overnight prior to blood collection. The fasting rats were anesthetized, and blood was collected from the posterior vena cava. Red blood cell count (RBC), white blood cell count (WBC), haemoglobin (Hb), hematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), reticulocyte count (RET), platelet count (PLT), platelet distribution width (PDW), mean platelet

volume (MPV), percentage of neutrophils, and percentage of other leukocytes (including lymphocytes, monocytes, eosinophils, and basophils) were analyzed using an automatic blood analyser (IDEXX ProCyte DX, MA, USA). All blood biochemistry examination were analyzed using an automatic chemical analyser (Cobas c311, Mannheim, Germany) including; total protein (TP), albumin (ALB),alkaline phosphatise (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Crea), glucose (GLU), cholesterol (CHO), triglyceride (TG), uric acid (UA), total bilirubin (TB), sodium (Na), potassium (K),and chloride (Cl).

Histopathological Examination: A complete necropsy was conducted on all animals. The necropsies included examination of the external surface; all orifices; and the cranial, thoracic, abdominal, and pelvic cavities including viscera. At the time of necropsy, the following tissues and organs were collected and placed in 10% neutral buffered formalin: Liver, kidneys, adrenals, testes, epididymides, whole prostate, thymus, spleen, brain, heart, lung, stomach, urinary bladder, and uterus with vagina and cervix.All samples were sectioned and stained with hematoxylin and eosin.The morphological changes within the tissues of the control and treated groups of mice were observed under binocular compound light microscope with camera (Leica Microsystems, Wetzlar, Germany) at 100x (oil immersion) magnification.

Statistical Analyses: Data were expressed as mean ± SD and analyzed by SPSS 11.5 statistical software (SPSS Inc., USA). The threshold for significance was set at $P < 0.05$, comparing each test group to the control group by sex.

RESULTS

Triphala extract and marker identification: The water extract of Triphala formulation was a semi-solid oily solution, with fruity odor and amber color. The extract was evaluated for content of gallic acid and ellagic acid. HPLC analysis of the extract showed a peak at the same retention time of 12.45 and 32.24 min, respectively (Figure 1). The content of gallic acid and ellagic acid in Triphala extract was calculated from a calibration curve of standards and it was quantitated to be 24.59 ± 0.80 and 133.80 ±2.94 mg/g extract, respectively

***In vivo* toxicity of Triphala formulation extract**

Acute toxicity test

Rat receiving the water extract of Triphala and untreated control (20% tween-80) showed no sign of toxicity (general

behavior, body weight change, and histopathology of vital organs) at a single highest dose level of 5,000 mg/kg body weight, with no death during the observation period of 14 days.

Subacute toxicity test

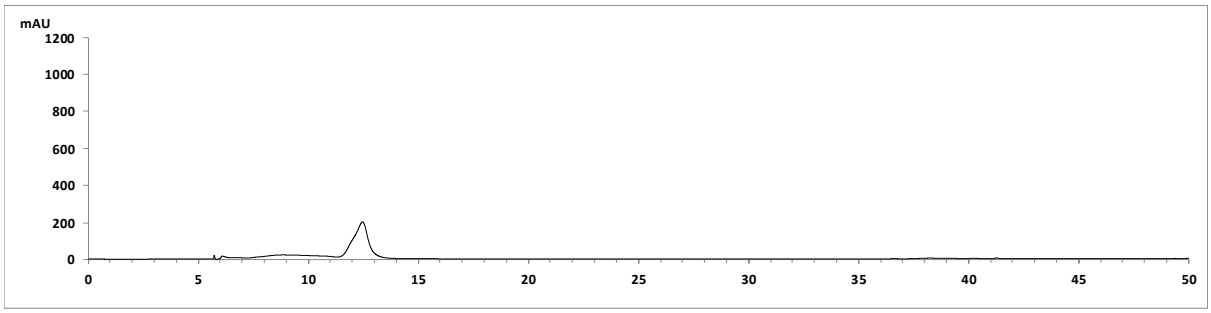
Mortality and Clinical Signs: All rats survived to the end of the study, and no treatment-related serious adverse clinical appearances were found during the 28-day study. Compared with the controls, the rat in high dose group treatment (5,000 mg/kg body weight) showed stomach irritation and general CNSdepressant signs such as delayed locomotion andreduced response to touch and balance. Stomach irritation was observed in all animalsimmediately after feeding the extract with the highest dose but the symptom subsided within 1 hoursof administration.

Hematology: Most of the hematological parameters were not significantly different between treatment groups and the controlgroups; however, significant differences were observed in some parameters, including decrease in WBC and RET at the highest dose (5,000 mg/kg body weight) and increase in hemoglobin and hematocrit in the low dose group (1,000 mg/kg body weight) both in male and female rats (Table 1).

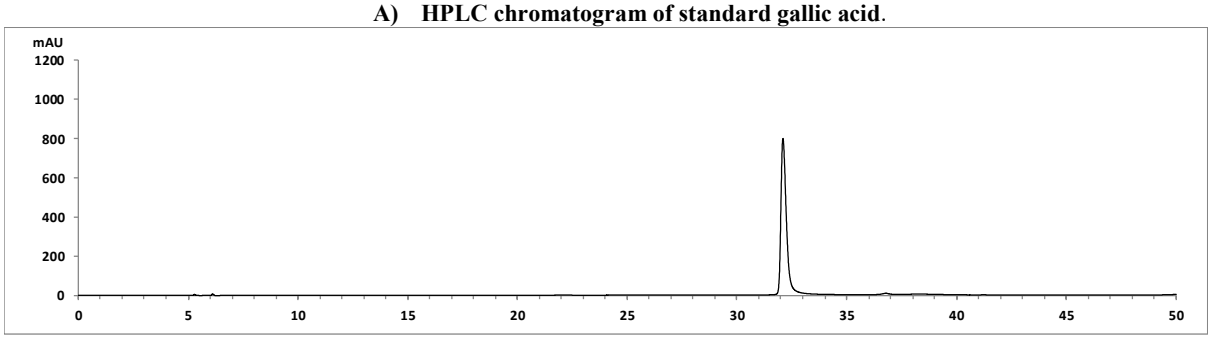
Blood Biochemistry: Compared with controls, there were some significant differences in blood biochemistry, including a decrease in cholesterol and triglycerides at doses level of 5,000 mg/kg body weight both male and female rats. There were no significant differences in any other parameters (Table 2).

Histopathology: Most of the histopathological findings in all treatment groups were comparable with those in the controls (Table 3). Observations included hepatocyte potty necrosis, renal interstitial inflammation, cortical hyperplasia, lymphocytic infiltration and dacryoadenitis, spleen lymphoid hyperplasia, diffuse degeneration of the cerebral white matter in brain, myocardial cell necrosis, pulmonary macrophage accumulation and foreign body granuloma, gastric ulcer, testicular atrophy, and the absence of mature sperm in the epididymal duct in individual male specimens, and vaginitis in individual female specimens. As shown in Table 3, pulmonary macrophage accumulation and chronic renal interstitial inflammation were observed in moderately level of the rat-treated on highest dose group (5,000 mg/kg body weight). In addition, mild level toxicity of hepatocyte spotty necrosis, gastric ulcer and lymphocytic infiltration and dacryoadenitis of thymus cells were observed in rat-treated with the water extract of Triphala formulation at the dose level of 5,000 and 3,000 mg/kg body

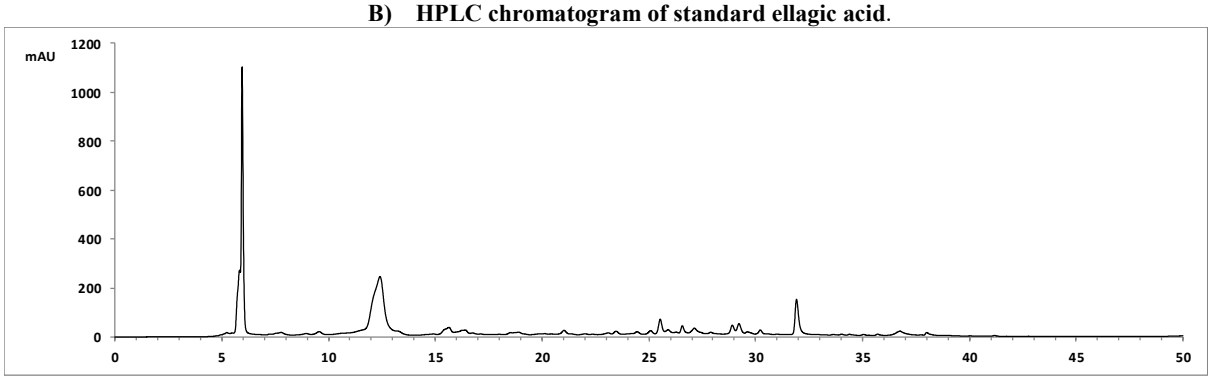
weight, while vaginitis and epithelial necrosis was observed in the 5,000 and 3,000 mg/kg body weight treatment group in individual female specimens. On the other hands, minimal level of testicular atrophy, and the absence of mature sperm in the epididymal duct was observed in the 5,000 mg/kg body weight in individual male samples. No abnormalities were detected in other organs and tissues.



A) HPLC chromatogram of standard gallic acid.



B) HPLC chromatogram of standard ellagic acid.



C) HPLC chromatogram of Triphala extract.

Figure 1: HPLC chromatogram of chemical markers (A,B) and Triphala extract (C)

Table 1: Hematological findings in rats after administration of the water extract of Triphala formulation with three doses for 28 days (n = 5 per group; mean ± SD)

Parameters	Sex	Groups			
		Triphala(mg/kg body weight)			Untreated control
		5,000	3,000	1,000	
Hb(g/dl)	M	16.4 ± 1.9	15.9 ± 1.7	18.7 ± 1.4*	16.8 ± 1.3
	F	15.6 ± 1.2	15.8 ± 1.1	18.9 ± 0.9*	15.7 ± 1.4
RBC (10^6/µl)	M	9.9 ± 0.8	9.6 ± 0.5	9.3 ± 0.7	9.3 ± 0.7
	F	8.4 ± 0.7	8.1 ± 0.4	8.3 ± 0.5	8.2 ± 0.6
HCT (%)	M	51.2 ± 3.5	52.3 ± 4.6	57.5 ± 3.8*	51.9 ± 4.3
	F	49.0 ± 3.1	48.4 ± 2.5	56.3 ± 2.6*	49.4 ± 3.6
MCV (fl)	M	55.0 ± 2.4	55.7 ± 2.5	56.1 ± 2.3	56.7 ± 2.7

Parameters	Sex	Groups			
		Triphala(mg/kg body weight)			Untreated control
		5,000	3,000	1,000	
	F	52.6 ± 2.6	53.1 ± 2.2	52.0 ± 1.3	52.5 ± 3.5
MCH (g/dl)	M	18.4 ± 1.1	18.7 ± 0.9	17.9 ± 0.8	18.5 ± 1.2
	F	18.0 ± 0.9	18.5 ± 0.6	18.5 ± 0.8	18.7 ± 1.3
MCHC (g/dl)	M	33.5 ± 2.2	33.8 ± 2.1	33.3 ± 1.8	33.3 ± 1.8
	F	33.1 ± 2.5	33.7 ± 2.2	33.4 ± 2.0	32.8 ± 2.0
RDW (%)	M	22.0 ± 1.0	21.7 ± 0.8	21.5 ± 1.1	21.1 ± 1.3
	F	21.7 ± 1.5	20.8 ± 1.4	20.9 ± 0.9	21.2 ± 1.3
RET (%)	M	3.2± 0.3*	4.3 ± 0.4	4.2 ± 0.5	4.1 ± 0.5
	F	2.9 ± 0.3*	3.9 ± 0.4	4.0 ± 0.6	3.8 ± 0.4
PLT (10^3/µl)	M	884 ± 75	903 ± 89	925 ± 67	860 ± 55
	F	829 ± 68	798 ± 48	813 ± 55	806 ± 59
PDW (fl)	M	10.6 ± 1.1	10.2 ± 0.6	10.4 ± 0.6	11.0 ± 1.2
	F	10.9 ± 0.9	11.2 ± 0.8	10.6 ± 0.7	11.2 ± 1.1
MPV (fl)	M	8.6 ± 0.7	8.8 ± 0.6	8.9 ± 0.9	8.5 ± 0.8
	F	8.2 ± 0.8	8.1 ± 0.9	7.9 ± 0.7	8.4 ± 0.9
P-LCP (%)	M	16.2 ± 0.9	15.8 ± 0.5	16.7 ± 0.8	16.7 ± 0.8
	F	15.9 ± 0.6	16.2 ± 0.7	15.5 ± 0.5	16.3 ± 0.9
WBC (10^3/µl)	M	11.9 ± 1.5*	14.2 ± 1.3	14.4 ± 1.4	14.1 ± 1.4
	F	11.6 ± 1.2*	12.9 ± 1.4	13.1 ± 1.3	13.2 ± 1.5
N (10^3/µl ,%)	M	2.6 ± 0.4	2.5 ± 0.3	2.3 ± 0.3	2.8 ± 0.5
	F	2.4 ± 0.3	2.2 ± 0.2	2.3 ± 0.2	2.5 ± 0.3
L (10^3/µl ,%)	M	10.1 ± 1.1	11.0 ± 1.2	10.5 ± 0.9	10.3 ± 0.9
	F	10.2 ± 0.8	10.5 ± 0.7	10.6 ± 0.6	10.4 ± 0.7
M (10^3/µl ,%)	M	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
	F	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
E (10^3/µl ,%)	M	0.2 ± 0.05	0.2 ± 0.04	0.2 ± 0.04	0.1 ± 0.03
	F	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.03
B (10^3/µl ,%)	M	0.01 ± 0.005	0.01 ± 0.006	0.01 ± 0.004	0.01 ± 0.004
	F	0.01 ± 0.005	0.01 ± 0.005	0.01 ± 0.007	0.01 0.005

Note Hb, hemoglobin;RBC, red blood cell; HCT, Hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; RET, reticulocyte count; PLT, platelet count; PDW, Plateletdistributionwidth; MPV, Mean platelet volume; WBC, white blood cell;N, neutrophils; L, lymphocytes; M, monocytes; E, eosinophils; B, basophils; M, male; F, female. *$P < 0.05$, significantly different from controls

Table 2:Blood biochemistry of rats after administration of the water extract of Triphala formulation with three doses for 28 days (n = 5 per group; mean ± SD)

Parameters	Sex	Groups			
		Triphala (mg/kg BW)			Untreated control
		5,000	3,000	1,000	
TP (g/dl)	M	7.8 ± 0.3	8.1 ± 0.5	7.5 ± 0.4	8.0 ± 0.6
	F	8.0 ± 0.5	7.8 ± 0.3	7.6 ± 0.4	7.5 ± 0.3
ALB (g/dl)	M	5.1 ± 0.4	5.3 ± 0.5	5.2 ± 0.3	5.2 ± 0.4
	F	5.3 ± 0.5	5.2 ± 0.4	5.0 ± 0.4	5.5 ± 0.5
ALP (U/L)	M	119 ± 17	122 ± 18	116 ± 15	122 ± 17
	F	86 ± 8	79 ± 7	88 ± 11	82 ± 7
AST (U/L)	M	100 ± 9	106 ± 12	110 ± 14	103 ± 18
	F	93 ± 14	88 ± 8	98 ± 11	95 ± 15
ALT (U/L)	M	53 ± 4	55 ± 6	50 ± 5	51 ± 6
	F	47 ± 5	51 ± 7	49 ± 6	45 ± 5
BUN (mg/dl)	M	25.2 ± 4.1	24.3 ± 3.2	23.7 ± 3.5	26.1 ± 3.9
	F	25.0 ± 2.8	25.6 ± 2.3	25.5 ± 2.4	26.2 ± 3.8
Crea(mg/dl)	M	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
	F	0.3 ± 0.05	0.3 ± 0.05	0.3 ± 0.05	0.4 ± 0.1
GLU (mg/dl)	M	334 ± 45	327 ± 35	311 ± 28	344 ± 39
	F	324 ± 35	309 ± 22	298 ± 25	309 ± 26
CHO (mg/dl)	M	85 ± 7*	86 ± 8	88 ± 7	97 ± 14
	F	89 ± 11*	90 ± 10	93 ± 13	111 ± 22
TG (mg/dl)	M	99 ± 15*	103 ± 14	105 ± 17	114 ± 25
	F	87 ± 12*	89 ± 15	92 ± 16	102 ± 23
UA (mg/dl)	M	7.9 ± 1.1	8.0 ± 1.0	8.2 ± 1.3	8.3 ± 1.4

	F	**7.7 ± 1.2**	**8.3 ± 1.3**	**8.6 ± 1.4**	**9.0 ± 1.5**
TB (mg/dl)	**M**	**1.1 ± 0.2**	**1.0 ± 0.1**	**1.2 ± 0.2**	**1.0 ± 0.2**
	F	**0.8 ± 0.1**	**0.9 ± 0.1**	**0.9 ± 0.1**	**1.2 ± 0.3**
Na (mmol/L)	**M**	**140.2 ± 5.4**	**145.2 ± 6.7**	**143.2 ± 4.1**	**142.3 ± 6.2**
	F	**138.9 ± 3.8**	**135.6 ± 4.8**	**145.7 ± 5.9**	**142.5 ± 4.4**
K (mmol/L)	**M**	**4.5 ± 0.6**	**4.3 ± 0.4**	**4.2 ± 0.4**	**4.6 ± 0.5**
	F	**4.4 ± 0.5**	**4.2 ± 0.3**	**4.0 ± 0.3**	**4.5 ± 0.6**
Cl (mmol/L)	**M**	**104.2 ± 8.7**	**110.7 ± 9.9**	**113.5 ± 9.8**	**109.5 ± 7.9**
	F	**105.8 ± 8.5**	**110.2 ± 8.9**	**113.6 ± 10.9**	**108.3 ± 8.4**

Note: TP, total protein; ALB, albumin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, Blood urea nitrogen; Crea, creatinine; GLU, glucose; CHO, cholesterol; TG, triglyceride; UA, uric acid; TB, Total bilirubin; Na, sodium; K, potassium; Cl, chloride; M, male; F, female;. *$P < 0.05$*, significantly different from controls.

Table 3: Histopathology examination of rats after administration of the water extract of Triphala formulation

Microscopic Lesions		**Doses (mg/kg body weight)**					
		5,000		**3,000**		**1,000**	
		Male	**Female**	**Male**	**Female**	**Male**	**Female**
Liver	**Spotty necrosis**	**2** (+)	**2** (+)	**2** (+)	**1** (+)	-	-
Kidney	**Renal interstitial inflammation**	**2** (++) **3** (+)	**3** (++) **2** (+)	**4** (+)	**3** (+)	-	-
Adrenals	**Cortical hyperplasia**	**1** (+)	**1** (+)	-	-	-	-
Testis	**Testicular atrophy**	**1** (+)	-	-	-	-	-
Epididymis	**No mature sperm**	**1** (+)	-	-	-	-	-
Prostate	**Prostatitis**	**2** (+)	-	**1** (+)	-	-	-
Harderian Gland	**Lymphocytic infiltration, dacryoadenitis**	**1** (+)	**1** (+)	-	**1** (+)	-	-
Spleen	**Lymphoid hyperplasia**	-	-	-	-	-	-
Brain	**Diffuse degeneration of the cerebral white matter**	-	-	-	-	-	-
Heart	**Myocardial cell necrosis**	-	-	-	-	-	-
Lung	**Macrophage accumulation**	**4** (++)	**5** (++)	**2** (+)	**3** (+)	-	-
	Foreign body granuloma	-	**1** (+)	-	-	-	-
Stomach	**Ulcer**	**4** (+)	**5** (+)	**1** (+)	**3** (+)	-	-
Urinary bladder and uterus	**Cystitis and lesions**	-	-	-	-	-	-
Vagina and cervix	**Epithelial necrosis**	-	**2** (+)	-	**2** (+)	-	-

Note: Tissues from 10 animals were examined in each group. Numbers of + in the brackets indicate the degree of histopathologic changes: minimal (+);mild (++);moderate (+++);severe (++++). Numbers before the brackets indicate the incidence of histopathologic changes. '-' means no observed changes.

DISCUSSION

Triphala formulation was evaluated for content of gallic acid and ellagic acid using HPLC analysis. The content of gallic acid and ellagic acid in Triphala extract was calculated basis on the calibration curve of standards which overall content was respectivelyquantitated to be 24.59 ± 0.80 and 133.80 ±2.94 mg/g extract. Similar to the previous research, gallic acid is a common phyto-constituent present in all three herbsin Triphala formulation [17]. *P. emblica* might be the major source of gallic acidin Triphala formulation [18]. In term of ellagic acid, the Triphala formulation was also showed to be a high source of ellagic acid. In accordance with the previous study, Triphala formulation was reported as an enormous amount of tannins such as ellagic acid and gallic acid [19].

Toxicity and dose optimization: In acute and sub-acute toxicity testing, Triphala formulation at the dose of 1,000, 3,000 and 5,000 mg/kg body weight showed the non-mortality effect on the observation period of 14 days and also showed the normal general behavior, body weight change. All ratssurvived until the end of the study, and no treatment-related serious adverse clinical appearances were found during the 28-days study when compared with the controls, rat in 5,000 mg/kg body weight maybe showed mildstomach irritation and general CNS depressant signs but does not indicate toxicity or seriousness. In accordance with the previous research, *T. chebula* showed non-toxic in oral toxicity study (2000 mg/kg), and also showed non-mortality or abnormal lesions in the internal organs of rats [20]. *P. emblica* was also previously investigated for acute and chronic toxicity. The water extract of *P. Emblica* was examined as a single oral dose of 5000 mg water extract/kg body weight. The results showed no toxicity in terms of general behavior change, mortality, or change in gross appearance of internal organs with LD50 more than 5,000 mg/kg.

Hematology, Blood Chemical and Histopathology Analysis: The hematopoietic system is animportant index of the physiological and pathological status for both animals and humans [21].In haematological examinations,significant decrease in haemoglobin and haematocrit were observed for 1,000mg/kg/day dose. However, these values were also within the normal range indicating that Triphala formulation does not affecthaematopoiesis in rats [22]. Clinical blood chemistry examination was performed in order to evaluate any toxiceffects on the pancreas, kidney and liver function. In general, if the clinical blood chemistry valuesdiffer more or less than one fold from the normal values, abnormality of kidney, liver and pancreasfunctionshould be noted [23]. The results in Table 2 and Table 3 show non-significant differences among the experimentalgroups in all tests. The liver enzymes were near to thenormal range as compared to the control group. Moreover, kidney parameters of male and female rats of both treatment and control groups showed that the drug almost same as that of control group. While, the results of lipid profile of treatment groups showedthe alleviation effect on cholesterol and triglyceride level at5,000mg/kg. All lipid profiles of male and female rats showed that some values were significantly decreased but remained within the normal range. The histopathological examination of kidney, the normal distill and proximal tubule along with glomerulus was observed and compared to control group. Triphala

formulation at the dose of 5,000 mg/kgshowed mild tubulointerstitial injury and minimal inflammationaccounted for 8.33 percent, but the dose of 3,000 mg/kg showed only minimal level damage in some ratsaccounted for 11.67 percent. In addition, the liver of minority male and female rats were also examined and found that the minimal spotty necrosis may be found in 5,000and 3,000 mg/kg accounted for 11.67 percent of the total. In lung examination, macrophage accumulation may obscurelyfound in 5,000 mg/kg. In summary, most of the results inhistopathological examination showed the values were slightly different,which is not a toxic or severe tissue damage in animals. These may indicate thehealthy status organs in the treatment rats which similar to the previous study,*T. Chebula* has been examined for haematological, blood chemical and histopathological parameters. The water extract from dried fruits hold a promise to be safe in experimental animal with non-significant change in any parameters [22]. Together with, the extract from *P. emblica* and *T. bellerica* also showed the similar results to*T. chebula* [24-25].

CONCLUSION

The current acute and subacute oral toxicity study present the safety profile with maximum tolerate dose level at 5,000 mg/kg body weight of the water extract of Triphala formulation and may provide scientific data for the risk assessment of them.

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REFERENCES

[1] Mukherjee PK, Rai S, Bhattacharyya S, Debnath PK, Biswas TK, Jana U, *et al.*, 2006. Clinical study of'Triphala'-a well known phytomedicine from India.Iranian J Pharmacol Therap., 5:51-54.

[2] Peterson CT, Denniston K, Chopra D.,2017. Therapeutic Uses of Triphala in Ayurvedic Medicine. J Altern Complement Med., 23:607-14.

[3] Phetkate P, Kummalue T, Y UP, Kietinun S., 2012. Significant increase in cytotoxic T lymphocytes and natural killer cells by triphala: a clinical phase 1 study. Evid Based Complement Alternat Med., 2012:239856.

[4] Belapurkar P, Goyal P, Tiwari-Barua P., 2014.Immunomodulatory effects of Triphala and its individual constituents: a review.Indian J Pharm Sci., 76:467-475.

[5] Saravanan S, Srikumar R, Manikandan S, Jeya Parthasarathy N, Sheela Devi R., 2007.Hypolipidemic effect of Triphala in experimentally induced hypercholesteremic rats. Yakugaku zasshi., 127:385-388.

[6] Srikumar R, Jeya Parthasarathy N, Sheela Devi R., 2005. Immunomodulatory activity of Triphala on neutrophil functions. Biol Pharm Bull., 28:1398-1403.

[7] Rasool M, Sabina EP., 2007. Antiinflammatory effect of the Indian Ayurvedic herbal formulation Triphala on adjuvant-induced arthritis in mice.Phytother Res., 21:889-894.

[8] Baliga MS.,2010.Triphala, Ayurvedic formulation for treating and preventing cancer: a review. J Altern Complement Med., 16:1301-1308.

[9] Gupta SK, Kalaiselvan V, Srivastava S, Agrawal SS, Saxena R., 2010.Evaluation of anticataract potential of Triphala in selenite-induced cataract: *In vitro* and *in vivo* studies.J Ayurveda Integr Med., 1:280-286.

[10] Thomas S, Asokan S, John B, Priya G, Kumar S., 2017.Comparison of Antimicrobial Efficacy of Diode Laser, Triphala, and Sodium Hypochlorite in Primary Root Canals: A Randomized Controlled Trial.Int J Clin Pediatr Dent.,10:14-17.

[11] Gautam AK, Avasthi S, Sharma A, Bhadauria R., 2012.Antifungal potential of Triphala churna ingredients against Aspergillus species associated with them during storage. Pak J Biol Sci.,15: 244-249.

[12] Soonthon charoennon N, Sotanaphun U, Wongsinkongman P., 2008. TLC: a simple method for qualitative analysis of Thai crude drugs., Nonthaburi (Thailand) Ministry of Public Health.

[13] Rinthong P, Khunawattanakul W, Thongantang J, Chotikan T, Prayongpetch P., 2017. Physical Properties and Phytochemical Contents of Triphala Extract Prepared by using Spray Dry Technique. The 13th Mahasarakham University

Research Conference, Thailand. pp: 565-571.

[14] Satoa V H, Sungthong B, Nuamnaichatia N, Rinthong P, Mangmoola S, Satoa H.,2017. *In vivo* and *in vitro* evidence for the anti-hyperuricemic, anti-inflammatory and antioxidant effects of a traditional ayurvedic medicine, Triphala. Nat Prod Com., 12:1635-1638.

[15] OECD., 2001. OECD Guidelines for the Testing of Chemicals. Test No.420: Acute Oral Toxicity-Fixed Dose Procedure. Paris., OECD.

[16] OECD., 2008. OECD Guidelines for the Testing of Chemicals Test No.407 :Repeated Dose 28-day Oral Toxicity Study in Rodents. Paris., OECD.

[17] Prakash S, Shelke AU., 2014. Role of Triphala in dentistry. J Indian SocPeriodontol., 18:132-135.

[18] Vazirian M, Khanavi M, Amanzadeh Y, Hajimehdipoor H., 2011. Quantification of gallic acidin fruits of three medicinal plants. Iran J Pharm Res.,10:233-236.

[19] Jeganathan NS, Kanna K., 2008. HPTLC Method for Estimation of Ellagic Acid and Gallic Acid in Triphala churanam Formulations. Res J Phytochem., 2:1-9

[20] Kim JH, Koo YC, Hong Co, Yang SY, Jun W, Lee KW., 2012. Mutagenicity and oral toxicity studies of Terminalia chebula. Phytother Res., 26:39-47.

[21] Adeneye AA, Benebo AS., 2008. Protective effect of the aqueous leaf and seed extract of Phyllanthus amarus on gentamicin and acetaminophen-induced nephrotoxic rats. J Ethnopharmacol., 118: 318-323.

[22] Jacob Filho W, Lima CC, Paunksnis MRR, Silva AA, Perilhão MS, Caldeira M, *et al.*, 2017. Reference database of hematological parameters for growing and aging rats. Aging Male., 21:145-148.

[23] Caisey JD, King DJ., 1980. Clinical chemical values for some common laboratory animals. Clin Chem., 26:1877-1879.

[24] Jaijoy K, Soonthornchareonnon N, Lertprasertsuke N, Panthong A, Sireeratawong S., 2010.Acute and chronic oral toxicity of standardized water extract from the fruit of *Phyllanthus emblica* Linn. Int J Appl Res Nat Prod., 3:48-58.

[25] Sireeratawong S, Jaijoy K, Panunto W, Nanna U, Lertprasertsuke N, Soonthornchareonnon N., 2012.Acute and chronic toxicity studies of the water extract from dried fruits of *Terminalia bellerica* (Gaertn.) Roxb. In Sprague-Dawley rats. Afr J Rradit Complement Altern Med.,10:223-231.