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Original Article

Acute and Subacute Toxicities of the Ethanol Extract from the Fruits of *Terminalia belerica* (Gaertn.) Roxb.

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Abstract The study was carried out to evaluate acute and subacute toxicities of the ethanol extract from Terminalia belerica (Gaertn.) Roxb. A single oral administration of the ethanol extract at a dose of 5,000 mg/kg did not produce signs of toxicity, behavioral changes, mortality and differences on gross appearance of internal organs. In the subacute toxicity, all rats were received a repeated oral dose of 1,000 mg/kg of the ethanol extract over 14 days. The satellite group was given the ethanol extract in the same period but kept for further 14 days without dosing in order to detect the delayed effects or reversibility of toxic effects. The results showed that the extract did not cause changes in terms of general behaviors, mortality, weight gain, hematological or clinical blood chemistry parameters. The results of gross and histological examinations showed normal appearance of the internal organs when compared to those of the control group.

Keywords: acute toxicity, subacute toxicity, *Terminalia belerica*

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INTRODUCTION

Terminalia belerica (Gaertn.) Roxb., "Samor-pephek" (Thai name) or "Bahera" (Combretaceae) is a large deciduous tree. This plant is most commonly found in many countries. Bahera has been recommended for treatment of sore throat, pharyngitis, laryngitis, cough, catarrh, bronchitis, gastric ulcers, hemorrhoids, chronic diarrhea, dysentery, parasites, cholelithiasis, ophthalmia, headache, edema, rheumatism (topical), wounds (topical), alopecia and premature greying.[1-5] The fruits are largely used in Thailand for antipyretic.[6] Two polyphenolic fractions isolated from *T. belerica* are significantly effective against mutagenic effects in *Salmonella typhimurium*. The inhibitory effect likely results from interaction of the polyphenols with S9 proteins.[7] In addition, four lignans including (i) termilignan, (ii) thannilignan, (iii) hydroxy-3',4'-[methylenedioxy]flavan, and (iv) anolignan B from Bahera have been ascribed as antifungal activity, antimalarial activity and anti-human immunodeficiency virus 1 (HIV-1) *in vitro*.[8] Importantly, its HIV inhibition effect of the 95% ethanol extract has recently been reported.[9] However, the toxicity of *T. belerica* has not been intensively investigated. The aim of this study is to evaluate the safety of the 95% ethanol extract from *T. belerica* in rats by determining both oral acute and subacute toxicities.

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MATERIALS AND METHODS

Plant Material

Terminalia belerica was collected from Bangkok, Thailand in April 2002. The plant materials were identified in the Pharmaceutical Botany Mahidol (PBM) Herbarium, Faculty of Pharmacy, Mahidol University, Bangkok. The voucher specimen (Fansai 0008) was kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok and deposited in the PBM Herbarium, Faculty of Pharmacy, Mahidol University, Bangkok.

Preparation of the Extract

The fruits were cut into small pieces and dried in a hot air oven at 55ºC. The dried materials were ground and macerated in 95% ethanol for 3 days and filtered. The filtrate was evaporated under reduced pressure until dryness. The residue from the filtration was macerated in 95% ethanol again for 3 days and filtered. The filtrate was evaporated again and combined with the extract from the first extraction. Thin layer chromatography (TLC) fingerprints of the extract were recorded.

Experimental Animals

Male and female Sprague-Dawley rats (120-160 g) were obtained from the National Laboratory Animal Center, Nakorn Pathom, Thailand. All animals were kept in the room maintained under environmentally controlled conditions of 24 ± 1°C and 12-hour dark-light cycle. Before each experiment, the animals were fasted overnight with free access to water.

Acute Toxicity Study

According to the World Health Organization (WHO) guideline[10] and the Organization of Economic Co-operation and Development (OECD) guideline for testing of chemicals,[11] ten rats were randomly divided into two groups of five animals per sex. The 2,500 mg/ml ethanol extract in 10% dimethyl-sulfoxide (DMSO) was administered by oral intubation to a group of rats at single oral dose of 5,000 mg/kg body weight while vehicle was given to the second group of rats as a control group. The animals were observed at the first, second, fourth and sixth hours following once daily over 14 days for symptoms such as changes in rate and depth of breathing, color of body surfaces, frequency and nature of movement, marked involuntary contraction or seizures of contraction of voluntary muscle, and loss of reflex etc. The body weight of survival rats was measured daily and observed for clinical signs of toxicity for up to 14 days. After the experimental period, rats of both groups were sacrificed and their internal organs including heart, lungs, livers, kidneys, spleen, adrenals, sex organs and brain were examined.

Subacute Toxicity Study

The method was performed following the protocal described by the WHO guideline[10] and the OECD guideline.[12] According to the OECD guideline for testing of chemical, a dose of 1,000 mg/kg was chosen in the subacute toxicity test for daily administration to rats for 14 days unless the test substance at the dose of 5,000 mg/kg produces any signs of acute toxicity. Rats were assigned at randoms three groups of ten males and ten females. The ethanol extract was orally given to the rats at a dose of 1,000 mg/kg/day, while the control group received the vehicle under the same experimental condition. The satellite group was orally treated with the extract at daily dose of 1,000 mg/kg/day for 14 days, and no further treatment for the following 14 days to determine the reversibility of toxic effects. Animals were observed during the test period for body weight, clinical signs of toxicity and mortality.

At the end of the period of ethanol extract administration, all rats were fasted for 16 hours and anesthetized with ether for blood collection. Blood was collected from the common carotid artery for hematological studies (complete blood count, red blood cell count, platelet count and red cell indices). The serum was tested for the clinical blood chemistry such as the concentrations of glucose, blood urea nitrogen (BUN), creatinine, total protein, albumin, total bilirubin, direct bilirubin, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and alkaline phosphatase (ALP).

The positions, shapes, sizes and colors of internal organs were evaluated. Heart, lungs, thymus, livers, kidneys, spleen, adrenals, small intestine, stomach and duodenum, muscle with sciatic nerve, thoracic spines, brain, eyes, sex organs, uterus and epididymis were removed from all rats to visually detect gross lesions and weighed to determine relative organ weights. All tissues were fixed in 10% buffered formalin solution. After routine processing, the paraffin sections of each tissue were cut at 5 μm thickness and stained with haematoxylin and eosin for a microscopic examination.

Statistical Analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. The data obtained from acute toxicity studies was analyzed using Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

In both male and female animals, neither sign of toxicity nor death among the rats was observed during 14 days of experimental period after administration of a single oral dose at 5,000 mg/kg of the 95% ethanol extract from the fruits of *T. belerica.* Toxicity evaluation was further carried out by observing body weight gain and internal organ weights of the animals as summarized in Tables 1 and 2. The body weight of the male and female extract-treated rats on the seventh

Table 1. Body weights of rats in acute toxicity of the ethanol extract from the fruits of *Terminalia belerica* (5,000 mg/kg)

	Body weight (g)			Weight gain (g)
	Day 0	Day 7	Day 14	on day 14
Female				
Control	108.00 ± 8.00	145.40 ± 10.05	160.00 ± 5.10	52.00 ± 4.69
T. belerica	107.20 ± 1.20	134.00 ± 4.73	161.60 ± 3.71	54.40± 2.42
Male				
Control	126.40 ± 4.41	179.00 ± 8.07	218.80 ± 7.61	92.40 ± 4.11
T. belerica	124.00 ± 3.41	159.20 ± 4.72	208.40 ± 4.44	84.40 ± 1.72

Values are expressed as mean ± S.E.M., n = 5.
There were no significant differences at $p < 0.05$.

Table 2. Organ weights (in grams) of rats in acute toxicity of the ethanol extract from the fruits of *Terminalia belerica* (5,000 mg/kg)

	Control	*T. belerica*
Female		
Lung	1.01 ± 0.04	0.89 ± 0.05
Heart	0.76 ± 0.03	0.70 ± 0.05
Liver	7.74 ± 0.20	6.66 ± 0.27
Spleen	0.60 ± 0.04	0.55 ± 0.01
Adrenal	0.02 ± 0.00	0.03 ± 0.00
Kidney	0.88 ± 0.01	0.80 ± 0.03
Ovary	0.06 ± 0.00	0.04 ± 0.00
Male		
Lung	0.99 ± 0.03	1.02 ± 0.02
Heart	0.86 ± 0.02	0.79 ± 0.02
Liver	9.53 ± 0.40	9.27 ± 0.28
Spleen	0.77 ± 0.03	0.71 ± 0.02
Adrenal	0.02 ± 0.00	0.03 ± 0.00
Kidney	1.06 ± 0.05	1.01 ± 0.02
Testis	1.17 ± 0.05	1.07 ± 0.03

Values are expressed as mean ± S.E.M., n = 5.
There were no significant differences at $p < 0.05$.

day was slightly decreased, yet no significant change in the body weight gain was detected on the fourteenth day. There was no difference in gross and weight examinations of the internal organs. These results suggest that the 95% ethanol extract from the fruits of *T. belerica* is practically not toxic after an acute exposure. A significant decrease in the body weight gain of male extract-treated rats was detected at day fourteenth (Table 3), but the body weight of all animals was still within the standard values as recommended by the National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand. These results suggest the animals were normal or not harmed by the substances. Moreover, none of animals in the subacute toxicity study of the ethanol extract at the dose of 1,000 mg/kg/day exhibited any abnormal parameters such as animal behaviors, toxic signs. Next, macroscopic changes were not observed in the internal organs as comparable to the control group. In addition, Table 4 shows the effect of the ethanol extract

Table 3. Body weights of rats in subacute toxicity of the ethanol extract from the fruits of *Terminalia belerica* (1,000 mg/kg)

	Body weight (g)			Weight gain (g)
	Day 0	Day 14	Day 28	on day 14
Female				
Control	142.67 ± 13.74	173.00 ± 11.49	-	30.33 ± 3.55
T. belerica[a]	139.67 ± 20.19	165.33 ± 17.60	-	25.67 ± 4.11
T. belerica[b]	128.67 ± 15.94	158.33 ± 13.76	179.33 ± 8.00	29.67 ± 5.85
Male				
Control	142.67 ± 6.64	210.00 ± 10.26	-	67.33 ± 5.36
T. belerica[a]	144.25 ± 9.15	186.50 ± 11.08	-	42.25 ± 3.19*
T. belerica[b]	143.50 ± 7.09	191.50 ± 7.42	262.25 ± 5.60	48.00 ± 4.72*

Values are expressed as mean ± S.E.M., n = 10.
[a] A group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days.
[b] A satellite group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days followed by no treatment for 14 days. * Significantly different from control, $p < 0.05$.

Table 4. Organ weights (in grams) of rats in subacute toxicity of the ethanol extract from the fruits of *Terminalia belerica* (1,000 mg/kg)

	Control	*T. belerica*[a]	*T. belerica*[b]
Female			
Lung	1.05 ± 0.045	1.02 ± 0.04	1.16 ± 0.03
Heart	0.79 ± 0.03	0.77 ± 0.04	0.86 ± 0.03
Liver	5.67 ± 0.22	5.37 ± 0.20	6.48 ± 0.35*
Spleen	0.54 ± 0.02	0.60 ± 0.02	0.61 ± 0.04
Adrenal	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Kidney	0.82 ± 0.02	0.72 ± 0.03*	0.86 ± 0.03
Ovary	0.04 ± 0.00	0.05 ± 0.00	0.11 ± 0.05
Male			
Lung	1.22 ± 0.05	1.08 ± 0.03*	1.31 ± 0.04
Heart	1.10 ± 0.06	0.98 ± 0.06	1.18 ± 0.07
Liver	8.37 ± 0.42	6.80 ± 0.37*	9.42 ± 0.50
Spleen	0.77 ± 0.06	0.67 ± 0.05	0.80 ± 0.02
Adrenal	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Kidney	1.08 ± 0.04	0.95 ± 0.05*	1.23 ± 0.05*
Testis	1.23 ± 0.03	1.24 ± 0.05	1.63 ± 0.06*

Values are expressed as mean ± S.E.M., n = 10.
[a] A group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days.
[b] A satellite group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days followed by no treatment for 14 days. * Significantly different from control, $p < 0.05$.

on the internal organs' weight of the control and treated groups. The weights of some internal organs were found to be statistically different between the treated and the control group. However, they may be due to variation of the internal organ weight.[13]

To determine intravascular effect and bone marrow activity of the extract, the hematological parameters were evaluated as present in Tables 5 and 6. As compared with the control, an increase in the concentration of hematocrit was noticed in the female satellite group. The increased mean corpuscular hemoglobin concentration but decreased mean corpuscular volume was found in the male satellite group when compared to those of the control group. The differential white blood cell count in the female treated group (Table 6) gave a significant increase in neutrophils and a decrease in lymphocytes. However, all of the change remained within the normal range.[14] Moreover, the blood smear results revealed normal characteristic (data not shown). These results suggest that the extract did not cause hematological defect.

The total protein level is a combined measurement of two blood protein molecules, albumin and globulin. Albumin is normally produced by the liver. We often see albumin levels depressed when the animals have liver diseases. The bilirubin is by-product of the breakdown of hemoglobin. Therefore, bilirubin levels may be higher than normal when excessive numbers of red blood cells are breaking down, or if the liver is diseased. As summarized in Table 7, the results of clinical blood chemistry studies showed that the female treated groups had a significant increase in the levels of total protein, total bilirubin and direct bilirubin, but these values remained within the normal ranges.[15-18] Moreover, all of the rats did not have any the signs of the impaired function of liver as confirmed by the histopathological examination. The histopathological analysis of internal organs, livers and kidneys (Figure 1), heart, lungs, thymus, spleen, adrenals, small intestine, stomach and duodenum, muscle with sciatic nerve, thoracic spines, brain, eyes, sex organs, uterus and epididymis did not reveal pathological features in all groups. Thus, these results suggest that the 95% ethanol extract from

Table 5. Hematological values of rats in subacute toxicity of the ethanol extract from the fruits of *Terminalia belerica*

	Control	*T. belerica* [a]	*T. belerica* [b]
Female			
Red blood cell ($x10^6/\mu l$)	7.61 ± 0.25	7.16 ± 0.51	8.12 ± 0.21
Hemoglobin (g/dl)	14.71 ± 0.46	14.28 ± 0.91	15.37 ± 0.33
Hematocrit (%)	37.62 ± 3.69	41.00 ± 2.82	46.25 ± 4.88*
Mean corpuscular volume (fl)	57.76 ± 0.40	57.30 ± 1.12	56.55 ± 0.57
Mean corpuscular hemoglobin (pg)	19.27 ± 0.23	19.31 ± 0.15	18.94 ± 0.24
Mean corpuscular hemoglobin concentration (g/dl)	33.40 ± 0.41	33.77 ± 0.58	33.51 ± 0.30
Platelet ($x10^5/\mu l$)	7.83 ± 0.50	7.32 ± 1.41	7.97 ± 0.63
Male			
Red blood cell ($x10^6/\mu l$)	7.34 ± 0.34	7.84 ± 0.25	8.07 ± 0.20
Hemoglobin (g/dl)	14.24 ± 0.60	15.05 ± 0.60	15.37 ± 0.43
Hematocrit (%)	40.55 ± 4.54	47.75 ± 1.79	46.28 ± 1.19
Mean corpuscular volume (fl)	61.38 ± 0.29	60.71 ± 0.46	57.23 ± 0.79*
Mean corpuscular hemoglobin (pg)	19.38 ± 0.21	19.19 ± 0.30	19.17 ± 0.20
Mean corpuscular hemoglobin concentration (g/dl)	31.60 ± 0.32	31.56 ± 0.30	33.28 ± 0.37*
Platelet ($x10^5/\mu l$)	9.14 ± 0.79	9.91 ± 0.75	8.49 ± 1.17

Values are expressed as mean ± S.E.M., n = 10.
[a] A group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days.
[b] A satellite group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days followed by no treatment for 14 days. *Significantly different from control, $p < 0.05$.

Table 6. Differential white blood cell count values of rats in subacute toxicity of the ethanol extract from the fruits of *Terminalia belerica*

	Control	*T. belerica*[a]	*T. belerica*[b]
Female			
White blood cell ($x10^3/\mu l$)	2.57 ± 0.34	2.91 ± 0.42	2.44 ± 0.24
Neutrophil (%)	13.87 ± 1.08	25.57 ± 4.36*	13.00 ± 3.46
Lymphocyte (%)	82.50 ± 0.91	67.14 ± 4.31*	77.62 ± 4.49
Monocyte (%)	1.12 ± 0.51	2.57 ± 0.89	2.37 ± 0.86
Eosinophil (%)	2.50 ± 0.53	3.86 ± 0.63	4.62 ± 1.18
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Male			
White blood cell ($x10^3/\mu l$)	2.64 ± 0.29	2.83 ± 0.47	3.74 ± 0.50
Neutrophil (%)	19.22 ± 3.01	24.37 ± 3.84	13.57 ± 5.26
Lymphocyte (%)	76.67 ± 3.15	68.50 ± 3.51	84.57 ± 5.62
Monocyte (%)	3.00 ± 1.78	3.12 ± 1.31	0.71 ± 0.28
Eosiniphil (%)	0.78 ± 0.32	1.75 ± 0.72	0.86 ± 0.34
Basophil (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values are expressed as mean ± S.E.M., n = 10.
[a] A group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days.
[b] A satellite group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days followed by no treatment for 14 days. * Significantly different from control, $p < 0.05$.

Table 7. Clinical blood chemistry values of rats in subacute toxicity of the ethanol extract from the fruits of *Terminalia belerica*

	Control	*T. belerica*[a]	*T. belerica*[b]
Female			
Glucose (mg/dl)	95.50 ± 4.86	92.57 ± 1.41	100.75 ± 3.89
BUN (mg/dl)	18.25 ± 0.96	19.86 ± 1.39	20.25 ± 1.74
Creatinine (mg/dl)	0.39 ± 0.03	0.46 ± 0.03	0.41 ± 0.02
Total protein (g/dl)	5.29 ± 0.11	5.81 ± 0.15*	5.56 ± 0.22
Albumin (g/dl)	2.71 ± 0.11	3.03 ± 0.13	2.87 ± 0.15
Total bilirubin (mg/dl)	0.21 ± 0.06	0.66 ± 0.14*	0.46 ± 0.13
Direct bilirubin (mg/dl)	0.14 ± 0.07	0.62 ± 0.16*	0.38 ± 0.13
SGOT (U/l)	133.25 ± 15.17	135.71 ± 20.76	132.88 ± 18.66
SGPT (U/l)	29.75 ± 2.03	28.14 ± 1.56	32.75 ± 1.67
ALP (U/l)	101.63 ± 9.00	103.57 ± 13.60	94.00 ± 6.41
Male			
Glucose (mg/dl)	113.56 ± 5.89	109.00 ± 8.90	107.50 ± 15.26
BUN (mg/dl)	18.00 ± 3.21	17.38 ± 1.85	17.83 ± 1.08
Creatinine (mg/dl)	0.40 ± 0.05	0.40 ± 0.03	0.45 ± 0.02
Total protein (g/dl)	5.18 ± 0.31	5.65 ± 0.35	5.07 ± 0.13
Albumin (g/dl)	2.65 ± 0.32	2.92 ± 0.28	2.55 ± 0.11
Total bilirubin (mg/dl)	0.36 ± 0.19	0.33 ± 0.07	0.21 ± 0.06
Direct bilirubin (mg/dl)	0.24 ± 0.20	0.24 ± 0.09	0.12 ± 0.07
SGOT (U/l)	142.89 ± 22.25	151.50 ± 28.81	139.50 ± 8.68
SGPT (U/l)	34.67 ± 0.85	35.88 ± 3.29	39.50 ± 2.01
ALP (U/l)	160.33 ± 8.89	164.75 ± 10.17	154.33 ± 4.88

Values are expressed as mean ± S.E.M., n = 10.
[a] A group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days.
[b] A satellite group was given the ethanol extract from fruits of *T. belerica* at 1,000 mg/kg daily over 14 days followed by no treatment for 14 days. * Significantly different from control, $p < 0.05$.

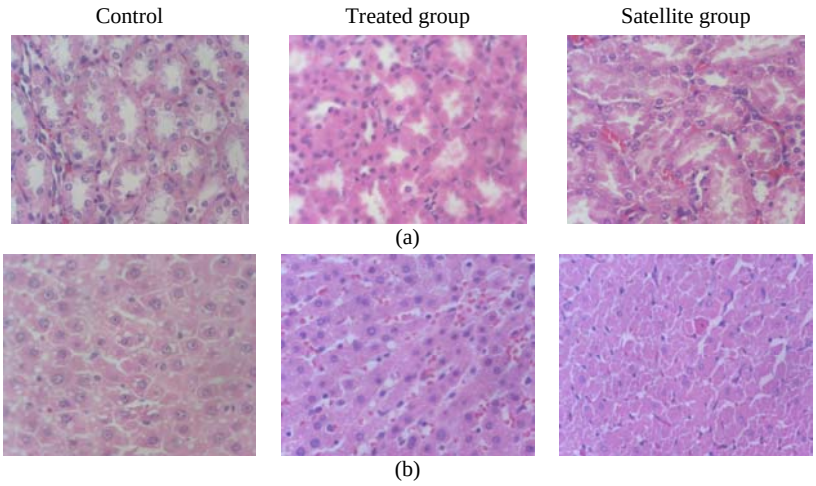

Figure 1. The histology of kidney (a) and liver (b). No significant damage was detected in any treatment groups.

the fruits of *T. belerica* does not produce signs of oral acute or subacute toxicity in rats. Further study is in progress in order to evaluate the chronic toxicity.

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