ACUTE AND SUBCHRONIC ORAL TOXICITY ASSESSMENTS OF BTL TEA IN EXPERIMENTAL ANIMALS

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Tobacco smoking remains a leading cause of preventable diseases and premature death in many countries. Many smokers want to quit smoking but are not offered the highly effective treatments available to manage tobacco dependency. There has been a current trend for researchers to find new natural ingredients that were safe and still effective in treating tobacco dependence. BTL tea was a herbal-derived product prepared from Herba Menthae, Pogos cablin (Blanco) Benth., Zingiber Officinale Rosc., Flos Chrysanthemi, Radix Glycyrrhizae, Pericarpium Citri deliciosa, and Flos Lonicera. At this time, the safety of this product has not been reported. Thus, this study aimed to evaluate BTL tea's acute and subchronic toxicity through oral administration in experimental animals. The acute toxicity was determined by Litchfield-Wilcoxon method in mice at the doses of 45.0 g/kg b.w/day to 112.5 g/kg b.w/day. The subchronic toxicity was evaluated following WHO and OECD's Guidelines in rats with oral doses of 1.08 g/kg b.w/day (equal to recommended human dose) and 3.24 g/kg b.w/day (three times as high as recommended human dose) for four consecutive weeks. As a result, in the acute toxicity test, the mice showed no abnormal sign or death. The subchronic toxicity test, hematological indexes, hepato-renal functions, and microscopic images of liver and kidney were unchanged. However, compared with the control group, there were significant differences in various indexes, including total WBC, lymphocytes, neutrophils, and AST level, but the levels were still safe. In conclusion, BTL tea does not appear to produce acute and subchronic toxicities in mice and rats.

Keywords: BTL tea, acute toxicity, subchronic toxicity, experimental animals.

I. INTRODUCTION

Tobacco use remains the leading preventable cause of disease, disability, and mortality in the world.[1] Despite a general awareness that smoking is harmful and widespread interest in smoking cessation, nearly 1.3 billion people worldwide continue to smoke. There was a range of novel pharmacological approaches for tobacco dependence treatment, including oral and pulmonary nicotine delivery and the non-nicotinic medications as antidepressants, an alpha2-noradrenergic agonist, opioid antagonists, GABAergic medications. However, these synthetic drugs caused many undesirable effects such as nausea, headaches, and gastrointestinal disorders…[2] Therefore, one of the most urgent missions of research was to find novel drugs derived from herbs to treat tobacco dependence supportively with limited side effects.

Nature has been a source of medicinal agents from ancient times, and medicinal plants become the basis of a wide variety of traditional medicines used in various countries worldwide.[3] The exclusive use of herbal drugs

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Received: 23/07/2021
Accepted: 06/09/2021

to manage various ailments continues due to easy access, better compatibility, and economic reasons. According to the World Health Organization (WHO), up to 80% of developing country populations use traditional medicine for their primary health care. However, the lack of evidence-based approaches and toxicological profiling of herbal preparations form the biggest concern of medicinal plant use. Thus, evaluating their toxicity plays a vital role in recognizing these effects, in order to characterize them, evaluate their risk for humans, and propose measures to mitigate the risk, particularly in early clinical trials.[4]

Toxicity refers to unwanted effects on biological systems. In order to evaluate biological toxicity, it is crucial to choose the correct system since no effects may otherwise be seen. Toxicity of a substance can be impacted by many factors, such as the route of exposure (skin absorption, ingestion, inhalation, or injection); the time of exposure (a brief, acute, subchronic, or chronic exposure); the number of exposures (a single dose or multiple doses for a while); the physical form of the toxin (solid, liquid, or gas); the organ system involved (cardiovascular, nephro-, hemo-, nervous-, or hematopoietic-system); and even the genetic makeup and robustness of the target cells or organisms.[5] Subchronic systemic toxicity is defined as adverse effects occurring after a test sample's repeated or continuous administration for up to 90 days or not exceeding 10% of the animal's lifespan.[6]

In 2018, the National Hospital Of Traditional Medicine created a novel product named CTL lozenges. CTL lozenges contained five ingredients: *Herba Menthae, Pogos cablin* (Blanco) Benth.*, Zingiber Officinale* Rosc.*, Flos Chrysanthemi*, and *Radix Glycyrrhizae*. After CTL lozenges were used for 60 patients with tobacco addiction, about 35% of patients successfully quitted smoking. However, patients usually complained about the unpleasant taste of CTL lozenges. As a result, the formulation of BTL tea was based on the compositions of CTL lozenges and added two ingredients (*Pericarpium Citri deliciosa,* and *Flos Lonicera)*. In addition, this product changed the form of lozenges to tea for convenient usage. So far, there have been no report available on the toxicity of the combined components in the world and Vietnam. Therefore, in this study, we aimed to validate the acute and subchronic toxicity of BTL tea in animals.

II. METHODS

1. The preparation of BTL tea

BTL tea was produced by the Pharmacy Department, National Hospital Of Traditional Medicine, Vietnam, STBA achieved. It was formulated in the form of teabags, and each bag contained 3g BTL tea, corresponding to 12g *Herba Menthae,* 10g *Pogos cablin* (Blanco) Benth.*,* 4g *Zingiber Officinale* Rosc.*,* 8g *Flos Chrysanthemi,* 4g *Radix Glycyrrhizae,* 4g *Pericarpium Citri deliciosa* and 8g *Flos Lonicera*. These ingredients were prepared following the Processing Method of Pharmacy Department, National Hospital Of Traditional Medicine (2016). They were steamed, dried or extracted in order to obtain tea powder; after that, tea powder was dried at 80^{o}C in a fluidized bed dryer until tea powder had less than 5% moisture content. The dosage in a patient was three sachets of BTL tea per day. BTL tea bags were soaked in boiled water for 2-3 minutes, dipping tea bags 5 times and then take them out before using.

2. Chemicals and laboratory machines

Kits for testing enzymes and metabolites in blood: ALT (alanin aminotransferase), AST

(aspartat aminotransferase), total bilirubin, albumin, total cholesterol, creatinine kits from Hospitex Diagnostics (Italy), and DIALAB GmbH (Austria) were used for Screen Master machine of Hospitex Diagnostics (Italy). Blood-testing solutions ABX Minidil LMG of ABX Diagnostics were used for Vet abcTM Animal Blood Counter. Chemicals for tests and histopathological examination.

3. Experimental animals

In this study, healthy Wistar rats (150 - 200g) and Swiss mice (20 - 22g) were used. The animals were housed in cages (groups of ten rats or mice/cage) in a room with access to a standard certified rodent diet and water ad libitum. They were acclimated to housing for at least one week before investigation at the Department of Pharmacology, Hanoi Medical University.

4. Acute toxicity study

Acute toxicity studies were carried out according to WHO Guidance and Organization for Economic Co-operation and Development guidelines (OECD guidelines).[5,6]

Group of mice (10 per group) were fasted for 12 hours and orally administered with BTL tea at ascending doses that mice could be tolerated. The general symptoms of toxicity and mortality in each group within 24 hours were recorded. The median lethal dose (LD50) was estimated by the Litchfield Wilcoxon method.[7] Animals that survived after 24 hours were further observed for seven days for signs of delayed toxicity.

5. Subchronic toxicity study

Subchronic toxicity studies were carried out according to WHO Guidance and OECD guidelines.[7,8]

The study was carried out in a continuous 4-week period. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control) was served as the distilled water control. Each rat was applied 1 ml distilled water/100 g/day by the oral route of administration;
- Group 2 was applied BTL tea at the dose of 1.08 g/kg/day (equivalent to the human recommended dose, conversion ratio 6);
- Group 3 was applied BTL tea at the dose of 3.24 g/kg/day (three times as high as the dose at group 2).

Animals were treated daily by the oral route of administration of distilled water and BTL tea with the volume of 10 mL/kg b.w once a day in the morning for four consecutive weeks and observed once daily to detect signs of toxicity. BTL tea was prepared every day following the instruction before giving to animals. This product was administrated by oral route through a feeding needle for rats.

The signs and indexes were checked during the study, including:

- General condition consists of mortality and clinical signs.
- Bodyweight changes.
- Hematopoietic function: red blood cells (RBC), hemoglobin (HGB), hematocrit, total white blood cells (WBC), WBC differentials, platelet count (PLT).
- Serum biochemistry: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, total cholesterol, and creatinine levels.

The parameters were checked at times: before treatment, two weeks after treatment, and four weeks after treatment at the laboratory of Department of Pharmacology, Hanoi Medical University. At the end of the experiment, all animals were subjected to a complete gross necropsy. Thirty percent of rats of each group will be removed liver and

kidney for histopathology examinations. The micro-histological examination was carried out at Center for Research and Early Detection of Cancer (CREDCA). Assoc. Prof. Le Dinh Roanh, Director of CREDCA gave results of pathological image analysis.

6. Statistical analysis

Data were analyzed using Microsoft Excel software version 2010. The significance levels between the experimental and control groups were made using the student's t-test and the Avant-après test. Data were shown as mean ± standard deviation. All data were considered significant at $p < 0.05$.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control group.

$^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment".

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, animals treated with BTL tea showed no mortality was observed up to the highest dose level (112.5 g/kg body weight) within 24 h and for seven consecutive days. Also, animals did not show signs of acute toxicity such as piloerection, lacrimation, or changes in locomotion and respiration (Table 1).

Table 1. Acute toxicity study of BTL tea

Group	n	Dose (ml/kg)	Dose (g/kg body weight)	The proportion of deaths (%)	Other abnormal signs
Group 1	10	30	45.0	0	No
Group 2	10	45	67.5	0	No
Group 3	10	60	90.0	0	No
Group 4	10	75	112.5	0	No

2. Subchronic toxicity study

General condition

Animals had normal locomotor activities and good feedings. None of the animals in all treated groups showed any macroscopic or gross pathological changes compared with the control group.

Body weight changes

Table 2. The effect of BTL tea on body weight changes

Time		Body weight (g)		
		Group 1	Group 2	Group 3
Before treatment		154.0 ± 11.7	153.0 ± 16.4	158.0 ± 20.4
After treatment (week)	2	152.2 ± 22.5	169.0 ± 17.9	168.0 ± 23.0
	4	165.0 ± 25.4	175.5 ± 31.3	177.0 ± 21.1

Table 2 showed that no significant differences were observed between groups treated with BTL tea and the control group (group 1) ($p > 0.05$).

Effect on hematological examination

Table 3. Effect of BTL tea on hematopoietic function

Parameters	Group	Before treatment	After treatment (week)	
			2	4
Red blood cells count (T/L)	Group 1	8.74 ± 0.82	8.56 ± 1.29	8.96 ± 0.55
	Group 2	8.71 ± 0.95	8.27 ± 0.34	8.13 ± 1.07
	Group 3	8.39 ± 0.60	8.22 ± 0.97	8.23 ± 0.82
Hemoglobin level (g/dL)	Group 1	12.32 ± 0.93	11.82 ± 1.89	11.73 ± 0.77
	Group 2	12.34 ± 1.07	11.47 ± 0.79	11.49 ± 0.72
	Group 3	11.83 ± 0.78	11.28 ± 1.08	11.07 ± 1.02
Hematocrit (%)	Group 1	45.42 ± 4.51	42.96 ± 7.01	43.95 ± 2.71
	Group 2	44.55 ± 4.15	41.27 ± 2.72	40.47 ± 5.30
	Group 3	42.52 ± 3.14	40.47 ± 4.97	40.60 ± 4.04
MCV (fL)	Group 1	47.39 ± 14.35	49.90 ± 1.73	49.11 ± 1.29
	Group 2	51.20 ± 2.25	49.60 ± 2.22	49.70 ± 2.11
	Group 3	50.8 ± 3.22	49.22 ± 3.42	50.00 ± 2.40
Platelet count (G/L)	Group 1	548.90 ± 86.21	481.70 ± 97.12	564.50 ± 25.01
	Group 2	581.60 ± 56.41	544.30 ± 64.22	563.60 ± 101.42
	Group 3	567.90 ± 114.10	559.80 ± 78.61	624.10 ± 92.13

MCV: Mean corpuscular volume

There was no significant difference in red blood cell count, hematocrit, hemoglobin level, MCV, and platelet count between groups treated BTL tea and group 1 ($p > 0,05$) (Table 3).

Table 4. Effects of BTL tea on total WBC count and WBC differentials

Parameters	Group	Before treatment	After treatment (week)	
			2	4
Total WBC count (G/L)	Group 1	13.11 ± 2.16	14.29 ± 2.79	13.03 ± 2.00
	Group 2	11.26 ± 2.03	6.51 ± 1.24***	13.41 ± 3.00
	Group 3	11.87 ± 2.18	7.72 ± 1.09***	11.59 ± 2.17
Lymphocytes (%)	Group 1	82.4 ± 3.8	76.3 ± 8.5	79.1 ± 3.3
	Group 2	78.5 ± 4.5	69.3 ± 6.2ΔΔ	75.0 ± 10.8
	Group 3	82.6 ± 3.8	64.9 ± 14.9ΔΔ	79.2 ± 3.7

Parameters	Group	Before treatment	After treatment (week)	
			2	4
Neutrophils (%)	Group 1	6.5 ± 1.0	9.0 ± 3.6	7.0 ± 1.8
	Group 2	7.7 ± 1.7	15.9 ± 4.5**$^{\Delta\Delta\Delta}$	15.9 ± 4.5
	Group 3	5.4 ± 1.6	15.8 ± 5.0**$^{\Delta\Delta\Delta}$	6.1 ± 1.4
Monocytes (%)	Group 1	11.1 ± 3.5	14.8 ± 6.0	13.1 ± 2.7
	Group 2	13.9 ± 3.6	14.8 ± 3.9	17.2 ± 6.6
	Group 3	13.0 ± 2.7	17.7 ± 9.5	14.7 ± 3.1

****p < 0.001 compared with group 1*

$^{\Delta\Delta}$p < 0.01, $^{\Delta\Delta\Delta}$p < 0.001 compared with the time point "before treatment"

WBC: white blood cells

Table 4 demonstrated that after two weeks of treatment, total WBC count and leukocytes at groups treated BTL tea decreased dramatically as compared with group 1 and the time point "before treatment" (p < 0.001). BTL tea at groups treated BTL caused a substantial increase of neutrophils compared with group 1 after two weeks of treatment. However, after four weeks of treatments, there was no significant difference in total WBC count, leukocytes, and neutrophils at groups treated BTL tea as compared with group 1 and the time point "before treatment" (p > 0.05)... No significant change was observed in monocytes between groups treated with BTL tea and group 1 (p > 0.05).

Effect on liver parameters

There were no significant differences in alanine aminotransferase (ALT) level, total bilirubin, albumin concentration, and total cholesterol concentration between groups treated BTL tea and the control group (p > 0.05). However, after 4 weeks of treatment, aspartate aminotransferase (AST) level increased substantially compared with group 1 and the time point "before treatment". The results were shown in table 5.

Table 5. Effects of BTL tea on liver parameters

Parameters	Group	Before treatment	After treatment (week)	
			2	4
AST level (UI/L)	Group 1	79.50 ± 8.07	79.00 ± 18.87	83.70 ± 15.90
	Group 2	74.00 ± 10.79	82.20 ± 7.42	102.20 ± 12.64**$^{\Delta\Delta\Delta}$
	Group 3	93.20 ± 16.10	81.70 ± 17.65	127.10 ± 25.87***$^{\Delta\Delta}$
ALT level (UI/L)	Group 1	37.80 ± 4.39	42.70 ± 6.82	38.00 ± 7.15
	Group 2	35.50 ± 7.50	45.50 ± 13.30	40.60 ± 7.00
	Group 3	37.70 ± 8.07	48.50 ± 15.24	43.20 ± 5.37
Total bilirubin (mmol/L)	Group 1	13.30 ± 0.63	13.51 ± 0.35	13.42 ± 0.31
	Group 2	13.56 ± 0.48	13.42 ± 0.41	13.51 ± 0.34
	Group 3	13.58 ± 0.46	13.43 ± 0.40	13.43 ± 0.34

Parameters	Group	Before treatment	After treatment (week)	
			2	4
Albumin concentration (g/dL)	Group 1	3.16 ± 0.19	3.07 ± 0.28	3.01 ± 0.16
	Group 2	3.28 ± 0.26	2.97 ± 1.06	3.18 ± 0.24
	Group 3	3.06 ± 0.23	3.30 ± 0.28	3.20 ± 0.26
Total cholesterol concentration (mmol/L)	Group 1	1.85 ± 0.40	1.56 ± 0.34	1.84 ± 0.73
	Group 2	1.91 ± 0.22	1.68 ± 0.28	1.95 ± 0.15
	Group 3	1.86 ± 0.57	1.62 ± 0.29	1.73 ± 0.15

***p < 0.01, ***p < 0.001 compared with group 1*

$^{\Delta\Delta}$p < 0.01, $^{\Delta\Delta\Delta}$p < 0.001 compared with the time point "before treatment"

Effect on kidney function

Table 6 illustrated that BTL tea caused no significant differences in serum creatinine level between the control group and groups treated with BTL tea (p > 0.05).

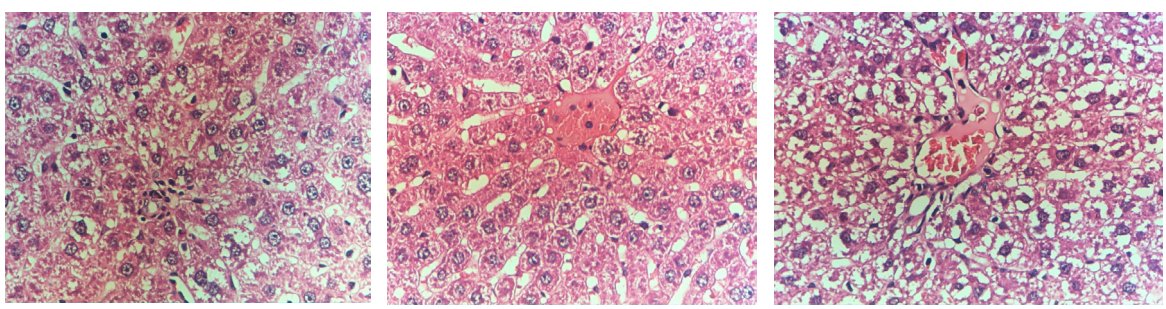
Table 6. Effects of BTL tea on serum creatinine level

Days		Creatinine level (mg/dl)		
		Group 1	Group 2	Group 3
Before treatment		0.75 ± 0.13	0.76 ± 0.16	0.75 ± 0.15
After treatment (week)	2	0.86 ± 0.13	0.87 ± 0.12	0.83 ± 0.15
	4	0.82 ± 0.14	0.89 ± 0.14	0.82 ± 0.14

Histopathological examination

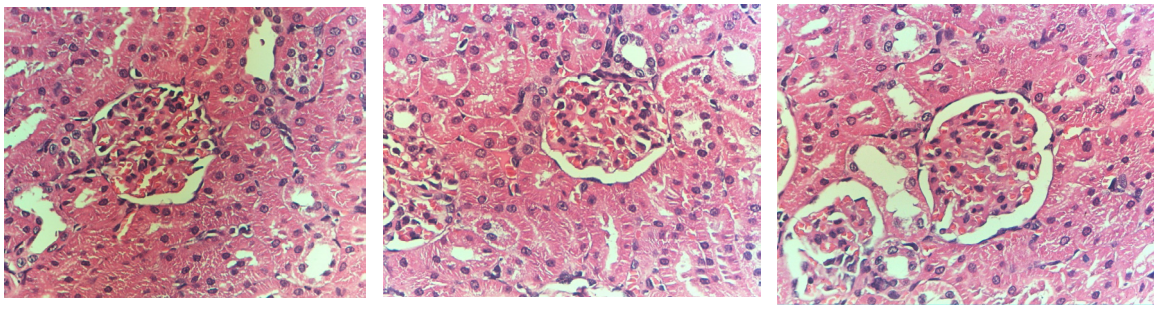
No gross lesions or changes in size were observed when subjected to all experimental rats to a full complete gross necropsy that examined the hearts, livers, lungs, kidneys, and abdominal cavities.

There was no significant difference in the liver and kidney histopathological examination between mice treated BTL tea and the control group after four weeks of treatment (Figures 1 and 2).



Group 1 Group 2 Group 3

Figure 1. Histopathological images of the liver (HE × 400)



Group 1 Group 2 Group 3

Figure 2. Histopathological images of the kidney (HE × 400)

IV. DISCUSSION

1. Acute toxicity of BTL tea

In this experiment, an acute oral toxicity test showed that BTL tea was tolerated up to 112.5 g/kg (approximately 52.08 times as high as recommended human dose). Moreover, no signs of toxicity and no mortality were observed for seven continuous days. As a result, oral LD50 of BTL tea was not determined in mice. As defined by WHO, BTL tea was a safe product derived from herbal medicine.

2. Subchronic toxicity of BTL tea

Toxicity is the degree to which a substance can harm humans or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g., renal or liver toxicity), or the whole organism. In order to determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animal models to predict the toxicity and provide guidelines for selecting 'safe' therapeutic doses in humans. A subchronic toxicity study provides information on the effects of repeated oral exposure and can indicate the need for further longer-term studies.[7,10] Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity.[11]

The body weight changes serve as a sensitive indicator of the general health status of animals.[11] Weights were observed in all animals treated with BTL tea. It can be stated that BTL tea did not interfere with the normal metabolism of animals, as corroborated by the nonsignificant difference from animals in the distilled water control group.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an essential index of physiological and pathological status in men and animals.[7,10] After two weeks of the treatment, there were substantial differences in total WBC, lymphocytes, and neutrophils between groups treated BTL tea with the control group, but this level was still in a normal range. Other hematological parameters including total red blood cells, hematocrit, hemoglobin level, and platelet count did not change as compared with the control group. So, it can be concluded that the administration of BTL tea did not affect the hematological profile and blood formation process. Furthermore, such analysis is relevant to risk evaluation as changes in the hematological system have higher predictive value for human toxicity when the data are translated from animal studies.

Analysis of kidney and liver is critical in the toxicity evaluation of drugs and plant extracts as they are both necessary for the survival of an organism. The clinical biochemistry analyses were carried out to evaluate the plant products' possible alterations in hepatic and renal functions.[12] The liver releases AST, ALT, and an elevation in plasma concentration indicates liver damage.[7] Despite the significant increase of AST level compared with the control group, this level was still in a normal range. Moreover, there was no substantial change in the ALT level between the group treated BTL tea and the control group. These results indicated that BTL tea had no deleterious effect on liver function.

Creatinine levels can be used in describing the function of the kidneys.[10] The blood biochemistry level of control and BTL tea in treated rats at various dose levels have presented no significance between groups that treated BTL tea with the control group ($p > 0.05$), so BTL tea did not affect the liver and kidney function.

Our study showed no significant difference in the liver, and kidney histopathological examination between the between-group treated BTL tea and the control group. The histopathological examination revealed the alteration in cell structure when viewed under the light microscope. Further, the histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of BTL tea.

Overall, the findings of this study indicated that no significant differences were observed in blood profile, biochemistry parameters, and histopathological observations of liver and kidney tissues between groups treated with BTL tea and the control group.

Our study was consistent with the results from previous reports about the toxicity of components in BTL tea. Ginger (*Zingiber officinale* Roscoe) powder at doses of 500, 1000, and 2000 mg/kg b.w for 35 days caused no overt organ abnormality in general conditions, growth, hematological, and blood biochemical parameters.[13] Following the study of Shen H (2011), single oral (that is, intragastric) administration of the *Flos Chrysanthemi Indici* extract in a dose of 15 g/kg b.w for rats produced zero acute toxicity over an observation period of two weeks. Macroscopic and microscopic studies of the internal organs revealed no pathological changes.[14]

V. CONCLUSION

No signs of toxicity and no mortality were observed in mice treated BTL tea at the dose of 112.5 g/kg (approximately 52.08 times as high as recommended human dose). Oral LD50 of BTL tea was not determined in mice.

For four consecutive weeks, BTL tea at oral doses of 1.08 g/kg/day and 3.24 g/kg/day did not produce toxic signs or evident symptoms of subchronic toxicity in rats. Despite the significant increase of various indexes including total WBC, lymphocytes, neutrophils, and AST level as compared with the control group, but this level was still in a normal range. Moreover, there was no change in histopathological structures of livers at groups treated BTL tea as compared with the control group. Thus, it can be concluded that BTL tea had no deleterious effect on liver function and hematopoietic function in rats.

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