EVALUATION OF ACUTE AND SUBCHRONIC TOXICITIES OF BTL LOZENGES ON EXPERIMENTAL ANIMALS

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ThIs research evaluated the acute and subchronic toxicities of BTL lozenges through oral administration in experimental animals. The acute toxicity was determined by Litchfield Wilcoxon method in Swiss mice. The subchronic toxicity was evaluated by the recommendation of WHO and OECD in Wistar rats with oral doses of 720 g/kg body weight/day (equal to recommended human dose) and 1440 g/kg body weight/day (2 times as high as recommended human dose) in 90 consecutive days. As a result, BTL lozenges at the highest dose used for mice (12 lozenges/kg body weight) did not express acute toxicity in mice. In terms of the subchronic toxicity test, after oral administration of BTL lozenges, hematological parameters, hepato-renal functions and microscopic images of liver and kidney at an equivalent to the human recommended dose were unchanged as compared with the control group. In conclusion, BTL lozenges with both doses 720 g/kg body weight/day and 1440 g/kg body weight/day did not produce acute and subchronic toxicities in Swiss mice and Wistar rats.

Keywords: BTL lozenges, acute toxicity, subchronic toxicity, polyherbal medicine, experimental animals.

I. INTRODUCTION

Herbal medicine is recognized as the most common form of alternative medicine. The World Health Organization (WHO) estimates that 80% of the world's population relies on these "alternative" plant-based medicines as their primary medical intervention, especially in developing and in developed countries where modern medicines are predominantly used.[1]

Over the years, the use of herbs in treating i medical conditions has been very successful and its historical usage has been useful in drug discovery development. Herbal remedies are considered safer and less damaging to the human body than synthetic drugs. Although herbal supplements may be considered safe, some are known to be toxic at high doses and others may have potentially adverse effects after prolonged use. However, the lack of evidence-based approaches and toxicological profiling of herbal preparations forms the largest concern of medicinal plant use. Thus, toxicity evaluation and characterization plays a vital role to recognize the negative effects, to evaluate their risk for humans, and to propose measures to mitigate the risk, particularly in early clinical trials.[2]

An acute toxicity test is used to evaluate any adverse effects appearing within a short time after a single large dose of the test substance or after multiple doses given within 24h. A subchronic toxicity study is typically conducted from 1 to 3 months because some substances that do not cause immediate toxicity may cause toxic effects after repeated exposure. Subchronic systemic toxicity is defined as adverse effects occurring after a test sample's repeated or continuous administration for up to

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12 weeks or not exceeding 10% of the animal's lifespan. The objective of subchronic toxicity studies is to determine the possible clinical adverse reactions caused by the substance, including the nature and degree of harm, the dose response and time response relationships, the effects on target organs or tissues, and the reversibility, and then predict the safe dose range for repeated drug use.[3–5]

BTL lozenge is intended for the treatment of smoking cessation. It was prepared from seven medicinal herbs ingredients including *Herba Menthae*, *Herba Pogostemi*, *Rhizoma Zingbiberis*, *Flos Chrysanthemi*, *Radix Glycyrrhizae*, *Flos Lonicera*, *Pericarplum Citri deliciosa*.The safety of each constituents of BTL lozenges have been extensively evaluated in the previous studies such as the BTL tea, CTL lozenges.[6,7] So far, there have been no report available on the safety of a combination product from these components. Therefore, the aim of this study was to evaluate the acute and subchronic toxicities of the polyherbal in BTL lozenges on experimental animals.

II. MATERIALS AND METHODS

1. The preparation of BTL lozenges

BTL lozenges, manufactured by Vietnam Materials Joint Stock Company. It was formulated in lozenge form, and each lozenge is a combination of seven medicinal herbs ingredients. Ingredients for each capsule 0.5 g include 1,2 g *Herba Menthae, 1,0 g Zingiberis rhizoma recens, 0,8 g Flos Chrysanthemi indici, 0,8g Flos Lonicerae, 0,4 g Pericarpium Citri reticulatae perenne, 0,4 g Herba Pogostemonis, 0,4 g Radix et Rhizoma Glycyrrhizae* and excipients. The expected dose in human: 12 times per day, one lozenge each time (equivalent to 60 g/day). The lozenges were dissolved with distilled water before giving orally for rats.

2. Experimental animals

Healthy *Wistar* rats of both sexes weighing between 180 - 220 g were provided by Dan Phuong Experimental Animal Center. Healthy *Swiss* mice of both sexes weighing between 18 - 22 g were provided by the National Institute of Hygiene and Epidemiology. The animals were housed in cages (groups of ten rats or mice per cage) in a room with access to a standard certified rodent diet and water. They were allowed to acclimatize for seven days to the laboratory conditions at the Department of Pharmacology - Hanoi Medical University before the experiment.

3. Acute toxicity study

Acute toxicity studies were carried out according to the Organization for Economic Cooperation and Development (OECD) guidelines 423 (OECD guideline) and WHO Guidance.[8,9]

A group of mice (10 per group) were fasted for 12 hours and orally administered with BTL lozenges at ascending doses that mice could be tolerated. The general symptoms of toxicity and the mortality in each group were observed within 72h. The median lethal dose (LD50) was detected by the Litchfield Wilcoxon method.[10] Animals that survived after 24 hours were further observed for seven days for signs of delayed toxicity.[8]

4. Subchronic toxicity study

Subchronic toxicity studies were carried out according to WHO Guidance and OECD guidelines.[8,9]

Wistar rats were divided into three groups (10 per group):

- Group 1 (control group) was administered orally of distilled water;

- Group 2 was administered orally BTL lozenges at the dose of 720g/kg body weight/

day (equivalent to the human recommended dose, conversion ratio 6);

- Group 3 was administered orally BTL lozenges at the dose of 1440 g/kg body weight /day (2 times as high as the dose at group 2).

Distilled water and BTL lozenges were administered using a curved, ball-tipped stainless steel feeding needle with a volume 10 mL/kg daily for a period of 90 days, and observed once daily to detect clinical signs and time points for laboratory tests. The composition in lozenges was dissolved with distilled water (the solvent of BTL) before giving orally to rats.

The signs and parameters were checked during the study including general conditions, mortality and clinical signs.

- Assessment of general health status, body weight were performed weekly [8]

- Hematopoietic function: red blood cells (RBC), hemoglobin (HGB), hematocrit, mean corpuscular volume (MCV), total white blood cells (WBC), WBC differentials, platelet count (PLT).

- Serum biochemistry test: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, total cholesterol and creatinine levels.

The parameters were checked before treatment and 30 days, 60 days, and 90 days after treatment. At the end of the experiment, all animals were subjected to a full gross necropsy. The livers and kidneys of 30% rats of each group will be taken for histopathology examinations. The micro-histological examination was carried out at the Center for Research and Early Detection of Cancer (CREDCA).

5. Statistical analysis

The research data were statistically processed by Student t-test and Avant – Apres test. The data are expressed as mean ± SD. The difference is significant when $p < 0.05$.

III. RESULTS

1. Acute toxicity study

In the acute oral toxicity test, groups were given BTL lozenges from 50 mL/kg (equivalent to 60 lozenges/kg body weight) to a maximum dose of 100 mL/kg (equivalent to 120 lozenges/kg body weight). Animals were treated with a concentrated solution of BTL lozenges times in 24 h, with no symptom, no abnormal symptoms, and no mortality at the highest dose level within 72 hours and for additional 7 hours. In addition, 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 lozenges

Group	n	Dose (lozenges/kg body weight)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	60	0	No
Group 2	10	90	0	No
Group 3	10	120	0	No

2. Subchronic toxicity study

General condition

General condition, food and water consumption were assessed. In the first two weeks after dosing, rats in both groups taking a BTL dose of 720g/kg body weight/day and 1440 g/kg body weight/day had diarrhea, poor

appetite, and reduced movement. Biological control mice eat and exercise normally, with no diarrhea. From the 3rd week onwards, rats in both groups taking a BTL dose of 720g/kg body weight /day and 2.88 lozenge body weight/kg / day no longer had diarrhea, eating better but still exercising less than the biological control group.

Body weight changes

Figure 1 showed that after 30 days, 60 days and 90 days, the body weight in all groups increased significantly compared with before the treatment. The weight gain of rats taking a BTL dose of 1440 g/kg body weight/day was lower than the weight gain of the biological control group and the group taking a BTL dose of 720g/kg body weight/day. Still, the difference was not statistically significant ($p > 0.05$).

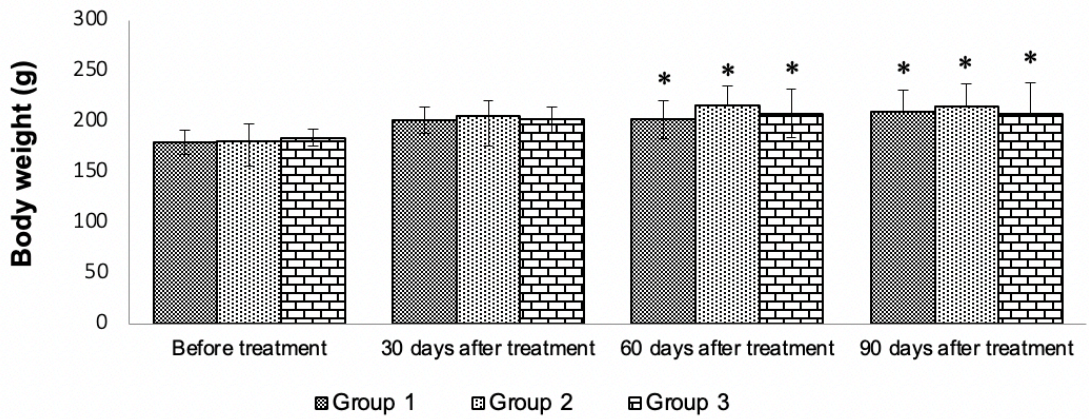


Figure 1. The effect of BTL lozenges on body weight changes

* $p < 0.05$ as compared with the time point Before treatment

The effect of BTL lozenges on the hematological system

There were no significant difference in red blood cell count, hematocrit, hemoglobin level, platelet count, mean corpuscular volume, total WBC count and WBC between BTL lozenges treated groups and control group ($p > 0.05$) (Tables 2 and 3).

Table 2. The effect of BTL lozenges on hematopoietic function

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
Red blood cells count (T/L)	Group 1	8.19 ± 1.34	9.20 ± 1.55	8.94 ± 1.49	7.64 ± 0.83
	Group 2	8.07 ± 0.86	9.47 ± 1.57	8.86 ± 1.17	8.11 ± 0.96
	Group 3	8.17 ± 1.30	8.96 ± 1.17	9.52 ± 1.98	7.92 ± 0.96
Hemoglobin level (g/dL)	Group 1	10.27 ± 1.11	10.92 ± 2.02	11.50 ± 1.24	9.72 ± 1.17
	Group 2	10.93 ± 0.89	11.65 ± 2.44	10.65 ± 1.32	10.76 ± 1.64
	Group 3	10.97 ± 1.24	10.71 ± 1.62	11.68 ± 1.39	10.01 ± 1.58

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
Hematocrit (%)	Group 1	42.77 ± 6.27	48.32 ± 5.62	48.40 ± 5.69	38.52 ± 5.45
	Group 2	44.17 ± 4.45	49.00 ± 6.60	44.07 ± 6.10	41.47 ± 6.24
	Group 3	42.92 ± 4.89	46.70 ± 5.64	48.65 ± 4.73	39.59 ± 5.40
MCV (fl)	Group 1	52.10 ± 4.15	50.90 ± 1.91	50.20 ± 2.66	50.30 ± 3.02
	Group 2	52.50 ± 2.51	51.90 ± 1.45	50.60 ± 3.17	51.70 ± 3.23
	Group 3	53.80 ± 2.70	52.20 ± 1.40	50.50 ± 2.51	51.00 ± 2.45
Platelet count (G/L)	Group 1	544.30 ± 123.38	517.40 ± 138.05	585.80 ± 152.92	546.80 ± 89.92
	Group 2	542.50 ±149.99	503.50 ± 118.39	586.20 ± 136.40	523.60 ± 126.81
	Group 3	629.20 ± 117.05	530.10 ± 156.84	609.90 ± 137.59	510.90 ± 149.95

MCV = Mean Corpuscular Volume. * p >0.05 *as compared with the control group.*

Table 3. The effects of BTL lozenges on WBC

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
Total WBC count (G/L)	Group 1	7.95 ± 1.78	9.41 ± 2.40	7.61 ± 1.88	8.50 ± 2.60
	Group 2	7.66 ± 1.85	9.71 ± 2.63	7.87 ± 1.91	7.82 ± 2.02
	Group 3	8.66 ± 2.10	9.39 ± 2.98	8.95 ± 2.52	8.69 ± 2.29
Lymphocytes (%)	Group 1	71.35 ± 5.64	71.00 ± 7.05	68.34 ± 9.24	74.94 ± 8.24
	Group 2	67.94 ± 4.57	70.05 ± 6.86	70.72 ± 8.51	71.40 ± 7.27
	Group 3	68.36 ± 6.49	69.87 ± 13.79	69.07 ± 8.56	77.15 ± 11.30
Neutrophils (%)	Group 1	15.99 ± 3.55	15.56 ± 4.32	18.31 ± 5.81	13.44 ± 4.80
	Group 2	16.41 ± 3.72	18.06 ± 5.10	16.65 ± 5.08	13.87 ± 4.02
	Group 3	18.22 ± 4.61	16.81 ± 11.23	18.62 ± 4.96	13.32 ± 4.14

* p >0.05 *as compared with the control group.*

The effect of BTL lozenges on liver functions

The liver functions of groups treated with BTL lozenges were within the normal physiological range. At the dose equivalent to the human recommended dose, after 90 days of treatment, the tested groups and the control group exhibited no significant difference when compared with before the treatment" (Table 4; p > 0.05). There were no significant difference in AST and ALT levels between BTL lozenges treated groups and the control group (p > 0.05). Data suggest that treatment with BTL lozenges has no discernible effect on liver function.

Table 4. The effect of BTL lozenges on liver functions

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
AST level (UI/L)	Group 1	85.00 ± 25.57	101.80 ± 27.34	82.90 ± 19.77	94.40 ± 12.21
	Group 2	83.80 ± 25.14	99.90 ± 25.75	86.70 ± 21.38	102.10 ± 24.16
	Group 3	84.80 ± 21.36	100.10 ± 24.74	102.50 ± 32.69	100.50 ± 17.75
ALT level (UI/L)	Group 1	36.10 ± 11.90	31.90 ± 5.82	31.90 ± 5.86	32.70 ± 9.93
	Group 2	31.40 ± 9.07	36.00 ± 7.70	32.60 ± 8.72	37.10 ± 8.80
	Group 3	33.00 ± 7.62	35.50 ± 9.24	34.20 ± 6.80	33.00 ± 5.73
Total bilirubin (mmol/L)	Group 1	9.90 ± 0.82	9.97 ± 0.40	9.38 ± 0.63	10.56 ± 1.64
	Group 2	10.02 ± 0.89	9.89 ± 0.96	9.41 ± 0.90	10.65 ± 1.28
	Group 3	10.20 ± 0.49	9.61 ± 0.81	9.41 ± 1.01	10.54 ± 1.12
Albumin concentration (g/dL)	Group 1	2.34 ± 0.31	2.63 ± 0.44	2.63 ± 0.60	2.57 ± 0.29
	Group 2	2.45 ± 0.24	2.66 ± 0.39	2.54 ± 0.58	2.86 ± 0.48
	Group 3	2.33 ± 0.28	2.62 ± 0.47	2.71 ± 0.47	2.62 ± 0.45
Total cholesterol (mmol/L)	Group 1	1.27 ± 0.16	1.24 ± 0.08	1.39 ± 0.14	1.33 ± 0.13
	Group 2	1.32 ± 0.20	1.29 ± 0.14	1.43 ± 0.18	1.34 ± 0.16
	Group 3	1.36 ± 0.16	1.34 ± 0.14	1.43 ± 0.29	1.37 ± 0.13

The effect of BTL lozenges on kidney functions

Figure 2 demonstrated that after 30 days, 60 days and 90 days of treatment, blood creatinine of rats of both treatment and control groups showed that of BTL lozenge with a dose of 720g/kg body weight/day and 1440 g/kg body weight/day remained almost same as that of control ($p > 0.05$)

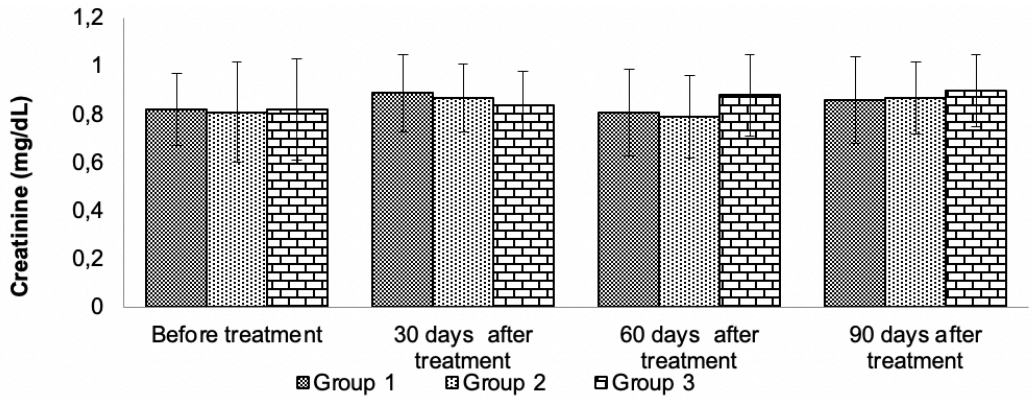
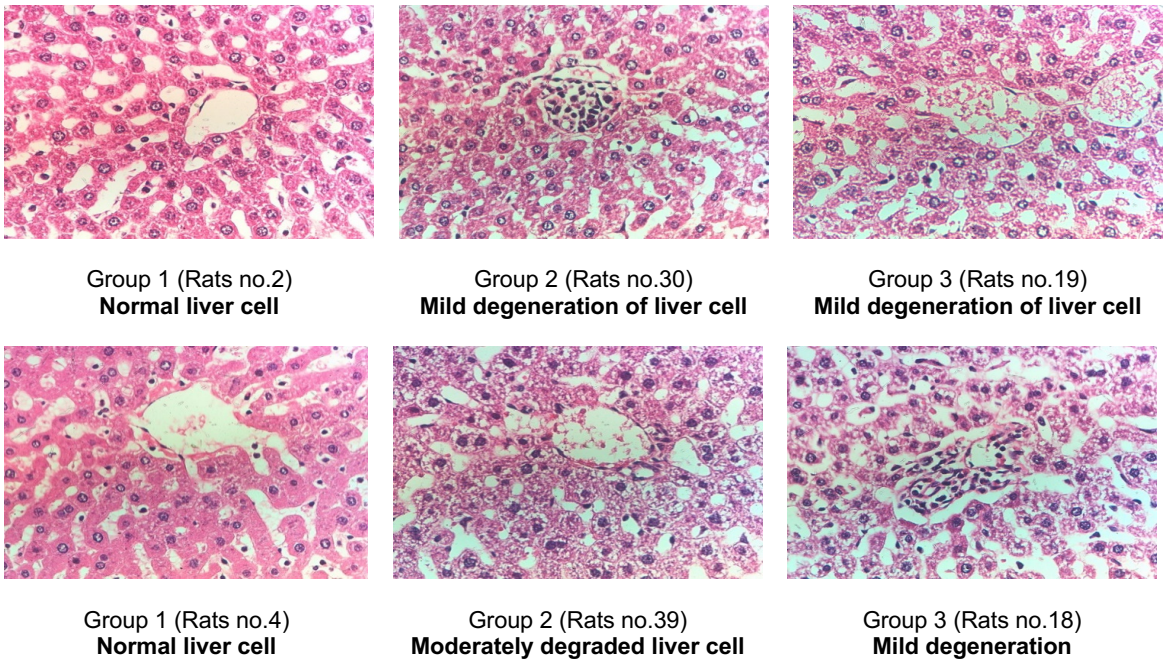


Figure 2. The effects of BTL lozenges on serum creatinine level

3. Histopathological examination

No gross lesion or change in size was observed when all experimental rats were subjected to a full gross necropsy which examined the hearts, livers, lungs, kidneys and abdominal cavities.



Group 1 (Rats no.2) **Normal liver cell**

Group 2 (Rats no.30) **Mild degeneration of liver cell**

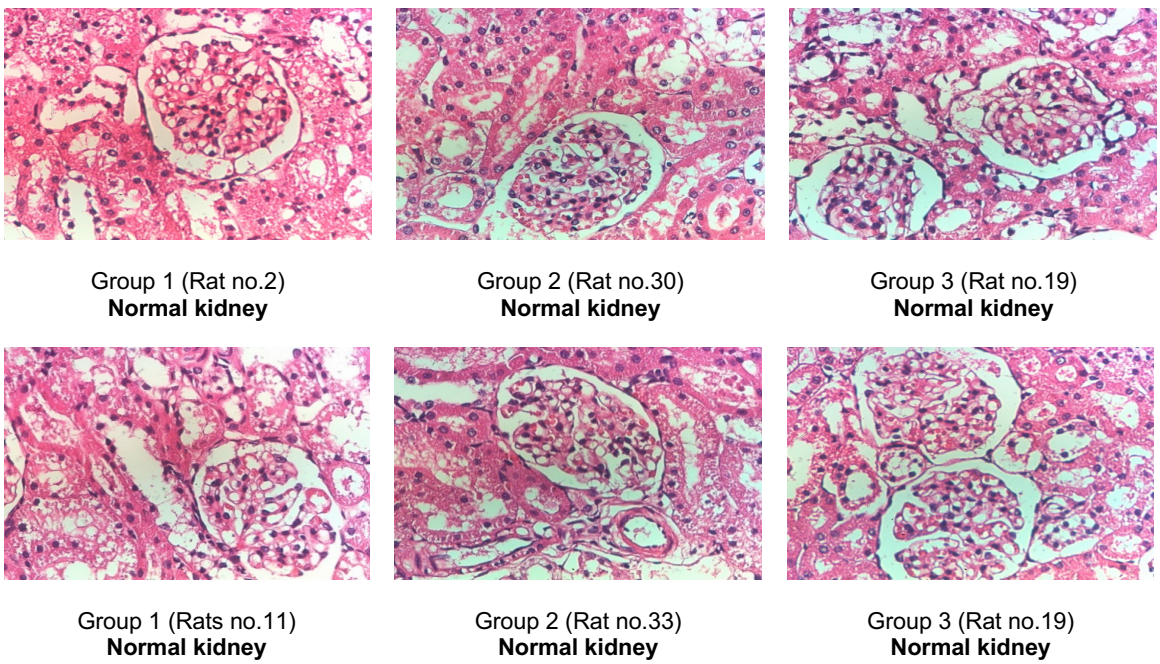
Group 3 (Rats no.19) **Mild degeneration of liver cell**

Group 1 (Rats no.4) **Normal liver cell**

Group 2 (Rats no.39) **Moderately degraded liver cell**

Group 3 (Rats no.18) **Mild degeneration**

Figure 3. Histopathological morphology of liver (HE × 400)



Group 1 (Rat no.2) **Normal kidney**

Group 2 (Rat no.30) **Normal kidney**

Group 3 (Rat no.19) **Normal kidney**

Group 1 (Rats no.11) **Normal kidney**

Group 2 (Rat no.33) **Normal kidney**

Group 3 (Rat no.19) **Normal kidney**

Figure 4. Histopathological morphology of kidney (HE × 400)

After 90 days of treatment, the histopathological examination showed no change in kidney morphology at the dose of 720g/kg body weight/day compared with the control group, while few glomeruli were observed. Endothelial cells degenerate at a dose of 1440 g/kg body weight/day. While the portal space and central vein were observed to have quite a lot of inflammatory cells, blood vessels with many fibrin fibers, and hepatocytes had mild to moderate degenerative foci in the liver at a dose of 720g/kg body weight /day and dose of 1440g/kg body weight /day (Figures 3 and 4).

IV. DISCUSSION

Acute toxicity of BTL lozenges

In the present study, an acute oral toxicity test showed that BTL lozenges were tolerated up to 120 lozenges/kg body weight (approximately 41.6 times as high as recommended human dose). Moreover, no sign of toxicity and no mortality occured for seven days consecutively. As a result, oral LD50 of BTL lozenges was not determined in mice. As defined by WHO, BTL lozenges herbal medicine were safe.

Subchronic toxicity of BTL lozenges

Traditional medicine has used popularity in developing countries. According to the World Health Organization (WHO), up to 80% of developing country populations use traditional medicine for their primary health care. However, the safety of herbal medicine use has recently been questioned due to reports of herbal medicine's toxicity.[11–13] Although many traditional herbal medicines are available, only a few have been verified by clinical trials. Subchronic studies assess the undesirable effects of continuous or repeated exposure to plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. A subchronic toxicity study provides information on target organ toxicity with longer-term use of herbal medicine.[8,9]

Changes in body weight, food and water ingestion are generally used as indicators of the harmful effects and the normal metabolism effects of drugs and chemicals. Both doses of BTL reagent did not affect the weight of the rats. In the first two weeks of taking the BTL reagent, mice had diarrhea, but from the 3rd week to the end of the study, mice no longer had diarrhea. There were no significant differences between BTL lozenges treated groups and control groups ($p > 0.05$). Thus, the findings of this study suggested that different doses of BTL lozenges (720g/kg body weight and 1440 g/kg body weight) orally administered to rats for 90 days had no significant effect on general behavior, mental state, or food intake.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an important parameter of physiological and pathological status in humans and animals.[8,9] 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. After 30 days, 60 days and 90 days of the treatment, there were no significantly difference in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count and WBC differentials between the BTL-treated groups with the control group, so it can be concluded that the BTL lozenges do not affect the hematological system.

The liver and kidneys are frequent targets of drug action because the liver is the primary organ for drug biotransformation, and the kidneys are the primary organs for drug excretion. Alanine amino transaminase (ALT) and Aspartate amino transaminase (AST) are largely used to assess

liver damage by drugs or any other hepatotoxin.[14] However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury.[15] The levels of ALT and AST in the research were within the normal physiological range, indicating that the BTL lozenges formula may not possess a hepatotoxic effect. Total protein measurements can reflect the nutritional status and may be used to screen and to assist kidney and liver diseases and many other conditions diagnosis. There were no significant change in total protein in rats treated with BTL lozenges, which suggested no sign of impaired renal function and liver function. The insignificant decrease observed in the level of total cholesterol in groups treated with BTL lozenges may be attributed to the presence of hypolipidemic agents in the polyherbal drug. Similarly, the drug had no adverse effect on the concentration of creatinine. This suggests there was no kidney damage specifically by renal filteration mechanism or BTL lozenges did not interfere with the renal capacity to excrete these metabolites. Therefore, it was evident that the drug at doses employed did not cause renal impairment or kidney damage. In the histopathalogical examination no change in liver and kidney morphology was seen at dose of 720 g/kg body weight/day, suggesting that the product is more appropriate to be prescribed at this dose. The safety of each ingredientss of BTL lozenges has been evaluated in the previous studies.[6,7]

Overall, the findings of this study indicated that no significant difference was observed in blood parameters, biochemistry parameters and histopathological observations of liver and kidney tissues between the BTL-treated groups and the control group.

V. CONCLUSION

No sign of toxicity and no mortality was observed in BTL-treated mice at the dose of 120 lozenges/kg body weight (approximately 41.6 times as high as recommended human dose). Oral LD_{50} of BTL lozenges was not determined in *Swiss* mice.

For 90 consecutive days, BTL lozenges at doses 720g/kg body weight/day and 1440 g/kg body weight/day did not generate any toxic sign or symptoms of subchronic toxicities in *Wistar* rats.

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