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Original Article

EVALUATION OF ACUTE AND SUB-ACUTE ORAL TOXICITY OF *CLINACANTHUS NUTANS* LEAVES EXTRACT IN MICE

TRAN THI LINH GIANG[1], LE KIM THACH[2], LE NGUYEN TU LINH[1], VU QUANG DAO[1], TRINH THI BEN[1], BUI DINH THACH[1]

[1]Institute of Tropical Biology, Vietnam Academy of Science and Technology, [2]Nong Lam University Ho Chi Minh City
Email: thachdinhbui@yahoo.com.vn

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ABSTRACT

Objective: This study aimed to evaluate acute and sub-acute oral toxicity of ethanol extract of *Clinacanthus nutans* leaves in Swiss mice.

Methods: Acute oral toxicity study was performed as per OECD-423 guidelines. Sub-acute oral toxicity study was performed as per OECD-407 guidelines. The extract was dissolved in 10% dimethyl sulfoxide and administered orally, while the control group received only the vehicle.

Results: The acute oral toxicity test on mice showed that this extract was well tolerated up to LD_{50} 5000 mg/kg body weight/day oral dosage level and non-toxic to mice under the present experimental conditions. The sub-acute toxicity study was carried out on mice with the oral dosage of the extract from 100 mg/kg–500 mg/kg body weight/day and 5000 mg/kg body weight/day for 28 d. The results showed that this extract did not induce death or adverse effects in activity, feed consumption or body weight gain. There were not significant changes in heamotological and biochemical parameters between control and experiment groups.

Conclusion: Thus, *Clinacanthus nutans* leaf has a very low toxicity value.

Keywords: Acute oral toxicity, *Clinacanthus nutans*, Extract, Sub-acute oral toxicity, Swiss mice

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INTRODUCTION

The plant-derived herbal compounds have a long history of clinical use, better patient tolerance and acceptance. They are freely available natural compounds that can be safely used to prevent various ailments. Plants became the basis of the traditional medicine system throughout the world for thousands of years and continue to provide mankind with new remedies [1]. Here, we present a research study on a medicinal plant, Clinacanthus nutans, a native of Southeast Asia.

C. nutans belongs to the family of Acanthaceae, is a small shrub that native to tropical Asia countries. It is commonly consumed in the form of herbal tea for the treatment of diabetes mellitus, fever, diarrhoea and dysuria [2]. In recent years, the pharmacological properties of *C. nutans* such as anti-viral, anti-oxidant, anti-inflammatory, anti-cancer have been previously reported [3-8].

The purpose of toxicity studies is, 'to determine the effect of an action on a biological system which can be used later to extrapolate the doses and effects on humans' [9]. This data is essential to identify the optimal therapeutic dose and the highest dose up to which the extract can be given, above which lethality would be expected.

Previous studies reported that them ethanolic extract of *C. nutans* at the highest dose of 2500 mg/kg did not cause any toxic effect on the liver and kidney of mice, thus suggesting its no-observed-adverse-effect level (NOAEL) to be greater than the tested dose [10]. Therefore, the objective of this study is to investigate the possible subacute oral toxicity effect of *C. nutans* ethanolic extract in mice at dose up to 5000 mg/kg bodyweight for 28 d.

MATERIALS AND METHODS

Animals

Outbred Swiss albino mice weighing 18-20 g of either sex, bred in Pasteur Institute, Ho Chi Minh City were procured and used for the study. The animals were allowed food pellets (Pasteur Institute, Ho Chi Minh City) and water *ad libitum*. Animals were maintained in control conditions (12 h: 12 h dark and light cycle and room temperature).

Preparation of the extract

Leaves of *C. nutans* (1 y old) were collected from the Institute of Tropical Biology, Vietnam Academy of Science and Technology. Our Department of Botanical Museum has identified the plant. They were cut into small pieces and dried in Laboratory drying oven at 50 °C. They were then crushed into a course powder using a laboratory grinding mill with ring sieve, size 0.25 mm. Maceration extraction of *C. nutans* in ethanol solvent followed by drying at 50 °C resulted in an average extract efficacy of 13%. The extract was stored at-20 °C.

Acute toxicity studies

Acute oral toxicity study was performed as per OECD-423 guidelines [11]. The mice were divided into two groups: a control group and a treatment group (n = 10). The extract was dissolved in 10% dimethyl sulfoxide and administered orally at a single dose of 5000 mg kg-1 body weight, while the control group received only the vehicle. Mice were fasted prior to conducting the experiment (only food but not water was withheld for 4 h). Following the period of fasting, the mice were weighed and the test substance was administered orally at a single dose, after which the food may be withheld for a further 1-2 h. A dose of 5000 mg/kg body weight was given to the first mouse then the clinical signs (changes in physical appearance, skin, pain, stress, abdominal contraction) and mortality were observed throughout the first hour, then every hour for 3 h and finally periodically until48 h. If the animal survived, additional animals will be given the same 5000 mg/kg dose sequentially at 48 h intervals. All of the experimental animals were monitored for apparent signs of toxicity for the 14 consecutive days, while the number of died mice within the study period was noted and subjected to necropsies. All mice were weighed on the 7th and 14th days after administration.

Sub-acute toxicity studies

Sub-acute oral toxicity study was performed as per OECD-407 guidelines [12]. The mice were divided into seven groups (n = 10), and their weights were measured. The extract was dissolved in 10%

DMSO, and administered orally at single doses of 100, 200, 300, 400, 500 and 5000 mg/kg/d for 28 d, at a dosing volume of 10 ml/kg body weight, while the control group received only the vehicle. The animals were observed daily for their physiological and behavioral changes (i.e. signs of toxicity, mortality and the bodyweight changes). The body weights of the animals were recorded once a week throughout the study period. Blood samples were obtained from tail incision, transferred into EDTA-containing and non-heparinized tubes. Blood with EDTA-containing tube was used immediately for hematological parameters while for a non-heparinized tube, it was allowed to clot under room temperature for 15-30 min before being centrifuged at 3000 x g at 4 °C for 10 min using a centrifuge machine. The serum obtained was stored at-20 °C until further analysis of biochemical parameters. The animals were then sacrificed while organs such as spleen, heart, liver, kidneys, lungs were removed, rinsed in 0.9% saline and weighed individually.

Hematological and biochemical analysis

Hematology and biochemical analysis were performed at Medic Medical Center-Hoa Hao Medic Company Limited, HCMC, Vietnam. The hematological parameters, which comprised of hemoglobin (Hb), hematocrit (Hct), total red blood cell (RBC) count, total white blood cell (WBC) count, total platelet count (PLT) were performed using an automated hematology analyser (Siemens Advia® 2120i,Siemens, Germany).

The biochemical analysis tests were performed using an automated chemistry analyser (Abbott™ Alinity™ Systems, Abbott, US), for biochemical enzymes 'level (i.e. alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), creatinine, total bilirubin, total protein, albumin, glucose and cholesterol).

Statistics

All data were expressed as mean±SD (n = 10). The data were analyzed by Student's t-test and one way ANOVA using stat graphics Centurion XV. Differences between groups were considered to be statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Acute toxicity

All the mice that received 5000 mg/kg of ethanol leaves extract of *C. nutans* did not show any toxic signs and abnormal behavioural changes post 72 h of *C. nutans* treatment and during 14 d observation duration. In additional tothat, *C. nutans* treated mice in this study showed no significant effect on body weight after 14 d observation period (table 1). From the results obtained, the exact LD_{50} value could not be determined due to no mortality was observed. However, it is believed that the LD_{50} of ethanol leaves extract of *C. nutans* is greater than 5000 mg/kg in mice. Based on the classification of chemical toxicity as described in the OECD 423 guideline, the toxic profile of *C. nutans* is classified as closely to category 5 which is low acute toxicity hazard [11]. Our study was not different from the toxicological study on *C. nutans* carried out by Zakaria *et al.* in LD_{50} value [10].

Table 1: Effect of ethanol leaves extract of *C. nutans* on body weight changes and mortality in mice

Group (mg/kg)	Body weight (g)			% Mortality
	0D	7D	14D	
Control	21.17±3.25	32.00±2.10	33.67±1.75	0
Extract (5000)	22.00±1.67	29.67±2.42	32.33±2.42	0

Value = mean±standard deviation; n=10. Analyzed using LSD's test

Sub-acute toxicity

General observations

Throughout the 28 d feeding study, there were no clinical signs or effects on survival that could be attributed to the administration of ethanol leaves extract of *C. nutans* in mice.

Body weights

Any alteration in body weight is believed to be associated with adverse effects of drugs and chemicals [13]. The reduction in body weight exceeding 10% of initial body weight and internal organ weights are considered as a simple and sensitive index of toxicity following exposure to potentially toxic substances [10]. There was no reduction of body weights were observed between the treated and control groups (table 2). Thus evidencing that *C. nutans* leaves extract didn't cause metabolism reduction in the test animals.

Organ weights

In this study, the results displayed non-significant changes for organ/body weight ratios of mice in all treated groups as compared to the control (table 3). In general, any change in the liver and kidney weight might be related to the organs injury, including swelling, atrophy or hypertrophy [14]. However, the results still need to be further validated by biochemical analysis assessment to confirm on the findings.

Hematological analysis

Analysis on hematological parameters are often employed to determine the safety profile and influence of foreign compounds, including plant extracts, on the blood constituents as well as blood-related functions in humans. Such assessment on the blood is crucial and the results thereof can be harnessed to establish how pharmacologically safe an agent is, on the well being of humans. The parameters investigated in this study are useful indices in ascertaining the toxic potentials of botanicals in living systems [15]. There were no statistically significant changes in hematology parameter in mice (tables 4). Data showed that hematology parameters, such as Hb, RBC, WBC and PTL were not significantly affected by ethanol leaves extract of *C. nutans* ($P>0.05$).

Table 2: Sub-acute (28 d treatment) effect of ethanol leaves extract of *C. nutans* on body weights in mice

Group (mg/kg)	Bodyweight (g)				
	0D	7D	14D	21D	28D
Control	18.80±1.30	19.00±1.22	19.60±1.52	20.40±1.82	20.80±1.48
Extract(100)	18.20±0.45	18.60±0.89	19.60±1.14	20.20±1.48	21.80±1.64
Extract (200)	18.80±1.09	18.80±0.84	20.00±0.71	20.40±1.52	21.40±2.30
Extract (300)	19.00±1.00	20.67±2.31	22.00±1.00*	23.30±1.15*	26.00±2.00**
Extract (400)	19.80±2.94	21.00±2.55	22.00±2.65*	23.20±1.79*	25.60±1.34**
Extract (500)	19.60±2.70	21.20±3.11	22.80±3.11*	23.60±2.88*	25.80±2.77**
Extract (5000)	19.40±1.14	20.00±1.87	20.40±2.07	21.60±2.30	24.40±2.97**

Value = mean±standard deviation; n=10. Analyzed using LSD's test. Significantly different from control: *p<0.05, **p<0.01

Table 3: Sub-acute (28 d treatment) effect of ethanol leaves extract of *C. nutans* on organ weights in mice

Relative organ weights		Control	Extract (mg/kg body weight)					
			100	200	300	400	500	5000
Organ/body weight ratio (%)	Liver	4.52±0.31	4.40±0.36	4.45±0.79	3.89±0.57	4.00±0.56	3.87±0.18	4.45±0.49
	Kidney	1.20±0.14	1.19±0.14	1.18±0.18	1.02±0.13	1.02±0.15	1.01±0.15	1.06±0.13
	Heart	0.50±0.05	0.49±0.06	0.48±0.08	0.44±0.03	0.44±0.03	0.44±0.02	0.46±0.03
	Spleen	0.69±0.38	0.77±0.45	0.86±0.23	0.56±0.14	0.66±0.25	0.64±0.23	0.96±0.44
	Lung	0.85±0.44	0.66±0.13	0.87±0.34	0.67±0.06	0.81±0.29	0.77±0.17	0.80±0.14

Value = mean±standard deviation; n=10. Analyzed using LSD's test

Table 4: Sub-acute (28 d treatment) effect of ethanol leaves extract of *C. nutans* on hematological parameters in mice

Parameters	Control	Extract (mg/kg body weight)					
		100	200	300	400	500	5000
WBC ($x10^9$/l)	12.18±1.21	10.84±1.07	12.72±1.33	11.48±1.40	10.90±1.42	11.73±1.96	11.71±1.82
RBC ($x10^9$/l)	8.09±0.40	7.93±0.13	8.16±0.39	8.02±0.43	8.09±0.42	8.06±0.36	8.05±0.42
PLT ($x10^9$/l)	913.00±153.68	888.60±142.47	841.20±84.03	1000.40±160.80	941.20±197.98	811.40±203.52	879.60±160.64
Hemoglobin (%)	13.78±0.66	11.80±0.98	14.28±1.21	13.66±1.90	13.16±2.30	12.56±3.29	12.40±3.52
Hematocrit (g/dl)	42.76±12.03	40.08±5.69	49.24±4.50	47.04±4.71	45.02±3.58	42.30±8.74	42.20±7.81

Value = mean±standard deviation; n=10. Analyzed using LSD's test

Biochemical analysis

Liver and kidney are the two main organs for investigation in oral toxicity study. For the liver function test, four serum hepatic biochemical parameters, namely alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and total bilirubin were analyzed in this study. AST and ALT are common markers used to diagnose hepatocyte integrity. On the other hand, increases in the serum ALP and bilirubin levels would indicate the presence of cholestasis [16]. An increase in the level of serum proteins is a sign of tissue injury. The determination of serum proteins such as albumin can act as a criterion to assess the synthetic capacity of the liver since nearly all are synthesized in hepatocytes. A reduction in serum proteins, therefore, tends to reflect an occurrence of chronic damage [10].

Table 5: Sub-acute (28 d treatment) effect of ethanol leaves extract of *C. nutans* on biochemical parameters in mice

Parameters	Control	Extract (mg/kg body weight)					
		100	200	300	400	500	5000
AST (U/l)	189.16±6.93	187.44±9.59	188.30±12.00	191.32±8.63	192.82±9.44	190.84±9.31	190.12±12.19
ALT (U/l)	62.33±5.69	61.88±5.17	66.60±5.18	60.15±5.06	61.57±7.43	66.87±7.38	62.06±8.27
ALP (U/l)	147.40±25.95	136.40±19.79	140.20±30.73	145.40±25.79	151.40±18.02	156.20±23.31	134.20±31.58
Total bilirubin (mg/dl)	0.16±0.01	0.16±0.01	0.16±0.01	0.16±0.01	0.15±0.00	0.16±0.01	0.15±0.00
Total protein (g/dl)	6.88±0.39	6.94±0.76	7.46±0.67	7.29±0.90	6.87±0.11	7.02±0.60	6.96±0.37
Albumin (g/dl)	4.48±0.20	4.41±0.24	4.39±0.25	4.54±0.31	4.32±0.59	4.54±0.29	4.39±0.40
Glucose (mmol/l)	8.83±1.20	8.21±1.57	7.45±2.24	7.29±0.52	8.02±1.86	7.97±0.89	9.00±0.62
Cholesterol (mmol/l)	3.96±0.51	3.75±0.30	4.09±0.27	4.20±0.55	3.70±0.71	3.81±0.49	3.71±0.35
Creatinin (mg/dl)	0.32±0.04	0.33±0.04	0.33±0.02	0.35±0.01	0.34±0.03	0.33±0.03	0.32±0.06

Value = mean±standard deviation; n=10. Analyzed using LSD's test

For kidney function tests, serum renal biochemical parameter, namely creatinine, was analysed in this study. Blood serum contains a number of organic constituents such as glucose, protein, bilirubin, cholesterol and enzymes whose concentrations vary with changes in the physiological state. Serum enzymes, especially ALT and AST, are often considered as sensitive indicators to adverse drug effects or to the presence of disease [17]. From the results obtained, all doses of ethanolic extract of *C. nutans* leaves ranging from 100 to 500 mg/kg bw and 5000 mg/kg bw showed no significant influence on all serum biochemical parameters when comparing all treated groups to the control group (table 5), indicating that *C. nutans* leaves showed no substancial toxic effect on mice liver and kidney. These results are in agreement with some previous reports [2, 10, 18].

CONCLUSION

The acute and sub-acute toxicity studies showed no treatment-related signs of toxicity or mortality. Sub-acute toxicity did not induce any biochemical, hematological, anatomica signs of toxicity. Therefore, the LD_{50} for the acute toxicity study was greater than 5000 mg/kg while for the sub-acute toxicity study and the NOAEL was greater than 5000 mg/kg/day.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declare none

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