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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.1096.1102

Research Article
Evaluation of Acute Toxicity and Semi-chronic Toxicity of Extract from *Celastrus hindsii* Benth

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Abstract

Background and Objective: *Celastrus hindsii* Benth. has been used for generations in Northern Vietnam, for the treatment of disease relating to ulcers, tumors and inflammation without safety evidence. This study's goal is to evaluate the safety of the aqueous extract of leaves of *C. hindsii* through an acute and semi-chronic toxicity oral administration. **Materials and Methods:** In the acute study, a single oral dose (1000, 3000, 5000 and 15000 mg kg^{-1}) of the aqueous of *C. hindsii* extract were administered to mice and observed for seven days. In the semi-chronic study, rabbits were administered daily with 1000 and 3000 mg kg^{-1} of the extract for 35 days. Hematological and biochemical analyzes were carried out on blood and serum samples collected. **Results:** A single oral administration of 15000 mg kg^{-1} per day for white mice did not determine the LD_{50} dose. At doses of 1000 and 3000 mg kg^{-1} for 35 days, the extract from *C. hindsii* induced neither clinical symptoms of rabbits nor significant changes in hematological parameters such as; total blood cells, hemoglobin concentration, white blood cells and platelets. The quantity of aspartate transaminase (AST or GOT), alanine transaminase (ALT or GPT) of rabbits in the experimental and control group did not differ ($p > 0.05$). Liver and kidney organizations were also not affected adversely. **Conclusion:** The results indicate that the oral administration of *C. hindsii* extract did not produce any significant toxicity in mice, therefore, it is recommended to be used safely for traditional medical practices and modern pharmaceutical applications.

Key words: Safety evaluation, oral administration, hematological parameters, biochemical parameters, toxicity

Citation: Thanh Loan Pham, Van Huy Nguyen, Thi Tam Tien Ha, Thi Le Thu Hoang, Chi NghiaPhan and Thi Quyen Nguyen, 2020. Evaluation of acute toxicity and semi-chronic toxicity of extract from *Celastrus hindsii* benth. Pak. J. Biol. Sci., 23: 1096-1102.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The species, *Celastrus hindsii* Benth. belongs to the genus *Celastrus*, which includes species of aromatic herbaceous perennials, almost exclusively native to Asia. The plants have been used for generations in Northern Vietnam, particularly by Muong people for the treatment of diseases relating to ulcers, tumors and inflammation. Additionally, leaves of *C. hindsii* have been used for the manufacturing of tea products as a healthy drink[1]. Over the last decades, a large number of secondary metabolites exhibiting a wide range of bioactivity have been extracted from *C. hindsii*. In addition to numerous terpenoids, including a diverse array of sesquiterpenoids, various bioactive alkaloids and flavonoids have also been isolated[2]. In particular, celahinine A, a sesquiterpene pyridine alkaloid and the related known polyester A which showed potent cytotoxicity against Hepa-2 (hepatoma), Hela (cervix carcinoma), COLO-205 (colon carcinoma) and KB (nasopharynx carcinoma) cells *in vitro* were isolated from dried stems of *C. hindsii* by Kuo *et al*[3]. In their continuous research of 2 years afterward, 4 new triterpene compounds, celasdin-A, celasdin-C, anti-AIDS celasdin-B and cytotoxic maytenfolone-A were also isolated from the dried stems of *C. hindsii*[4,5]. Of those 4, maytenfolone-A demonstrated cytotoxicity against hepatoma and nasopharynx carcinoma and celasdin-B were found to exhibit anti-HIV replication activity in H9 lymphocyte cells. In another similar study, Huang *et al*.[6] has isolated a new sesquiterpene, celadin D and other six related derivatives. Of these, ermarginatine-E exhibited cytotoxicity against KB and COLO-205 with ED_{50} = 1.7 μg mL^{-1} and ED_{50} = 4.1 μg mL^{-1}, respectively. In contrast, the ED_{50} for other compounds all exceeded 10 μg mL^{-1}. Recently, Hu *et al*.[7] isolated 3 new diphenylpropanes named Hindsiipropane A, B, C, together with one known arypropyl quinone Griffithane D, which is initially obtained in the genus *Celastrus*. All these isolated compounds showed modest cytotoxicity against 4 human tumor cell lines (A549, HCT116, MDA-MB-231 and BEL7404) with IC_{50} values in the range of 10.95-62.19 μg mL^{-1}. In a later study by Hu *at el*.[2], a new macrocyclic lactone named Hindsiilactone A, a new 5,8-quinoglavan named Hindsiiquinoflavan B and three known compounds combretastatin D-2, combretastatin D-3 and isocorniculatolide A were isolated from the stems of *C. hindsii*. An increasing number of publications indicated that *C. hindsii* is a candidate for large-scale cultivation. The cultivation of this aromatic and medicinal plant will aim to utilize its essential secondary metabolites such as; flavonoids and alkaloids as well as their bioactivities which are important to the species physiology and human being[1–3,6]. It is believed that traditional use of medicinal plant resources might provide new beneficial compounds, however, it probably counters toxic effects of the existing medicines for consumers. Due to limited information available on the ingestion of *C. hindsii* at high doses, the system approach in evaluating the safety properties and efficacy is required. Therefore, the present study was conducted to evaluate the safety of *C. hindsii* leaves extract with acute toxicity study in mice, semi-chronic toxicity tests, hematological and biochemical analyses in rabbits.

MATERIALS AND METHODS

Study area: The study was carried out at the Institute of Applied Research and Development, Hung Vuong University and at the Testing Centre, Biochemistry Department, General Hospital in Phu Tho, Vietnam, from February, 2019-September, 2019.

Preparation of plant extract: The extract of *C. hindsii* was produced by continuous hot extraction (twice) at boiling temperature using 70% ethanol. The extract was then concentrated under reduced pressure through the rotary evaporator. The dark brown extracts were fully dissolved in water then preserved in a desiccator until used for further studies at Hung Vuong University, Vietnam.

Tested animals: Healthy and standardized white mice weighing 20-30 g and New Zealand rabbits with an average weight of 2500 g were used and kept in the animal house of the Institute of Applied Research and Development, Hung Vuong University, Vietnam.

Acute toxicity examination: The oral acute toxicity experiments using an ethanolic extract from *C. hindsii* deployed the Litchfield-Wilcoxon method followed by the safety regulation of traditional medicine efficacy by the Ministry of Medicine guidelines 1996 (SARTEM)[8,9].

Before being administrated orally, all-white mice were weighed and kept at 18 h fasting with free access to water. These mice were divided randomly into five groups, each comprised of 10 mice. The first group served as a negative control with distilled drinking water only, while the 2nd, 3rd, 4th and 5th were considered as tested groups and orally received *C. hindsii* (dissolved in water) extract at the dose of 1000, 3000, 5000 and 15000 mg kg^{-1}, respectively (the dose was calculated according to the body weight). Distilled water and extracts were directly taken into the mice's stomach by a curved head needle with a volume of 0.2 mL/10 g b.wt., per time.

The mice were observed for any clinical signs such as; respiratory pattern, mortality for the first 72 h to determine the highest dose, which did not cause death and the lowest dose of 100% mortality. Further observations of general behavioral changes, skin, fur and feces of mice were carried out after the 7th day of the first oral administration. LD_{50} on white mice through oral administration was determined by the method of Litchfield-Wilcoxon[8,9].

Semi-chronic toxicity examination: The oral semi-chronic toxicity examination was conducted according to Abraham[10] method. The experiments used 18 New Zealand rabbits aged one-month-old, divided into three groups, each comprised of six rabbits with a male:female rate 1:1. The first group served as a negative control with distilled water drink only, while the 2nd and 3rd were considered as tested groups received *C. hindsii* (dissolved in water) extract orally at the dose of 1000 and 3000 mg kg^{-1} b.wt., respectively. The tested mice were weighed before treatment and every week during the procedure and the post-treatment period of 35 days. These three groups were kept separate to avoid cross-infection. Oral administration of the plant extract was carried out with 5 mL kg^{-1} b.wt., per day, twice a day in the morning and the late afternoon for 35 consecutive days.

Observation parameters

Clinical manifestations: Behavioral changes and other parameters such as; food intake, water intake, body weight changes in fur, Hematological parameters such as; red blood cells, hemoglobin concentration, white blood cells and platelet count of the experimental animals from control and plant treated groups were determined. Biochemical examinations were conducted on serum after centrifugation of sampled blood and vital parameters such as; aspartate transaminase (AST or GOT), alanine transaminase (ALT or GPT), total bilirubin (T-BIL) and urea were determined for both treated groups and control, at 2 periods of time (the 15th day from the experiment started and 35 days when finished). After collecting the blood, some vital organs such as; the liver and kidney were observed and compared to the control group to determine if any lesion occurred. These hematological and biochemical parameters were analyzed at PhuTho Provincial General Hospital.

Statistical analysis: Statistical analysis was performed as Mean of Variance±S.E.M (n = 10 for mice, n = 6 for rabbits) followed by ANOVA test using Graph Pad Prism and multiple comparison tests among the groups. A probability level of $p<0.05$ was statistically significant.

RESULTS

Acute toxicity of *C. hindsii* extract: The acute toxic effect of ethanolic extract was determined as per the Litchfield-Wilcoxon method and SARTEM guidelines, where the maximum test dose of 15000 mg kg^{-1} was applied. After 72 h of oral administration of the *C. hindsii* extract, no mortality was observed for any tested groups which ranged from 1000, 3000, 5000 to 15000 mg kg^{-1} b.wt. The general behavioral observation results showed that there were not any drug-related changes in breathing, food and water consumption and digestive system. At the highest dose of 15000 mg kg^{-1}, although tested mice showed hypoactivity compared to the control group, no mortality was recorded. Therefore, the study has not determined the LD_{50} of the test plant extract, which seems to be safe at a dose level of 15000 mg kg^{-1} on the experimental mice. The parameters observed for acute toxicity study after the administration of the *C. hindsii* extract and control group are presented in Table 1.

Semi-chronic toxicity study of *C. hindsii* extract on experimental rabbits: Daily oral administration of *C. hindsii* leaves extract to rabbits did not induce any clinical symptoms

Table 1: Acute toxicity of *C. hindsii* extract

Groups	No. of tested mice	Dosage (mg kg^{-1}b.wt)	Alive/death after 72 h
1	10	Distilled water	10/0
2	10	1	10/0
3	10	3	10/0
4	10	5	10/0
5	10	15	10/0

Table 2: Effect of *C. hindsii* extract on hematological parameters

	15th day of the experiment			After 35th day of the experiment		
Parameters	1000 mg kg^{-1} extract	300 mg kg^{-1} extract	Control group	1000 mg kg^{-1} extract	300 mg kg^{-1} extract	Control group
Total RBC (x10^{12}/L)	004.66±00.73	004.34±0.62	004.76±01.02	005.46±00.86	005.84±01.05	005.67±00.57
Hemoglobin (g L^{-1})	100.05±15.14	100.33±8.26	102.67±10.44	115.50±03.99	118.50±16.15	108.67±13.05
WBC (×10^9/L)	007.50±00.51	007.65±0.72	007.52±00.82	007.63±00.49	007.74±00.58	007.55±00.97
MPV (g L^{-1})	374.30±26.90	401.70±27.50	393.70±35.20	388.33±17.15	408,80±51.90	402.50±54.20

Values are expressed as Mean±S.E.M. (n = 6). $p>0.05$ when compared to the normal control group, $p<0.05$ when compared between the 15th day and the 35th of the experiment for RBC and HGB. RBC: Red blood cell, HGB: Hemoglobin, WBC: White blood cell, MPV: Mean platelets volume

Table 3: Effect of C. *hindsii* extract on biochemical parameters of experimental rabbits

			Plant extract-treated group						
			1000 mg kg^{-1} extract		3000 mg kg^{-1} extract		Control		
Parameters	Physio-biological limit	Day	Mean	SD	Mean	SD	Mean	SD	p-value
Bilirubin (mmol L^{-1})	0.4-3	D15	0.75	0.07	0.83	00.08	0.75	0.07	p>0.05
		D35	1.22	0.08	01.87	00.12	1.28	0.17	p>0.05
		$P_{(D15-D35)}$	p<0.05		p<0.05		p<0.05		
Urea (mmol L^{-1})	10-33	D15	3.50	0.30	03.45	00.29	3.60	0.28	p>0.05
		D35	10.91	2.19	14.82	01.44	10.73	1.02	p>0.05
		$P_{(D15-D35)}$	p<0.05		p<0.05		p<0.05		
GOT (AST) (U L^{-1})	10-120	D15	31.87	12.23	22.73	04.32	28.47	9.24	p>0.05
		D35	34.02	4.64	34.60	07.54	32.17	4.52	p>0.05
		$P_{(D15-D35)}$	p>0.05		p<0.05		p>0.05		
GPT (ALT) (U L^{-1})	10-80	D15	67.12	12.16	69.50	10.71	64.67	6.54	p>0.05
		D35	56.02	10.23	63.73	08.44	56.43	7.17	p>0.05
		$P_{(D15-D35)}$	p>0.05		p>0.05		p>0.05		

Values are expressed as Mean±SEM, n = 6, GOT (AST): Aspartate transaminase, GPT (ALT): Alanine transaminase, D15: Data collected on the 15th day of the experiment course, D35: Data collected on the 35th day of the experiment course

Table 4: Changes in the lesions of liver and kidney on rabbits after oral administration of *C. hindsii* extract

Experimental groups	Organ	No. of samples	Level of lesion
Group I (1000 mg kg^{-1} extract)	Liver	3	No lesion
	Kidney	3	No lesion
Group II (mg kg^{-1} extract)	Liver	3	No lesion
	Kidney	3	No lesion
Control	Liver	3	No lesion
	Kidney	3	No lesion

of toxicity or mortality up to the highest dose of 3000 mg kg^{-1}. All experimental rabbits observed with normal behavioral activities in food and water intake, smooth fur and dry feces. Further hematological and biochemical studies on rabbits were carried out and the results were presented in Table 2-4.

Effect of *C. hindsii* extract on hematological parameters: The results of the hematological tests presented in Table 2 showed that total red blood cell, hemoglobin, white blood cell and platelets were within ordinary limits at all tested groups, including control one[11]. According to Smith[11], the total white blood cells and platelets in healthy rabbits should be in the range of 5.2-16.5×10 $^{9/l}$353-821 g L^{-1}, respectively. Although, there was a difference in hemoglobin and red blood cell between group one and the control group, this was not statistically significant (p>0.05). Generally, there were no significant differences marked between the control and plant extract treated groups for the hematological parameters measured at 2 periods (at 15th day and after 35th day, p>0.05).

However, it was noted that there was a statistically significant difference in RBC and HGB between the 15th day observation and when finishing (the 35th day) within tested group 2 (3000 mg kg^{-1}). This variation indicated that *C. hindsii* extract might increase RBC and HGB levels when the oral administration duration was extended.

Effect of *C. hindsii* extract on biochemical parameters of experimental rabbits: The impact of *C. hindsii* extract on biochemical tests on the treated rabbit group and control group are summarized in Table 3. The orally administrated dose of 1000 and 3000 mg kg^{-1} did not induce any statistically significant change in serum biochemical properties such as; bilirubin, urea, GOT and GPT when compared to the control group (p>0.05).

However, it was noted that the bilirubin and urea content were significantly different between the 15th day and the 35th day period when these two parameters were measured in all three tested groups, including the control group (p<0.05). It has been generally accepted that this change was the result of normal physiobiological functions of the liver and kidney on mice due to their different stages of development. These increases were still within the physiobiological limit and therefore were probably not induced by the oral administration of *C. hindsii* extract. Only at the dose of 3000 mg kg^{-1}, GOT measured on the 35th day of the

experiment were significantly higher than that on the 15th day. However, this change was still within range of the physiobiological limit and no significant difference was found in the 1000 mg kg^{-1} and control groups.

Effect of *C. hindsii* extract on the lesions of liver and kidney on experimental rabbits: The data from Table 4 showed that all liver and kidney samples from all tested groups and the control group were observed without any lesion. There were no marked changes in the kidney, no signs of congestion, inflammation or cholestasis in the liver were observed in rabbits of any group. Therefore, the oral administration of *C. hindsii* extract did not adversely impact the functions of the rabbit's kidney or liver.

DISCUSSION

Medicinal plants with pharmaceutical importance are globally recognized and have been validated scientifically as the sources of direct therapeutic agents and destination for new synthetic drugs[12]. The herbal products have advantages over the synthetics because they have been widely proven to be safer for human health and the environment. The *C. hindsii* contains a large number of secondary metabolites exhibiting a wide range of bioactivities[13], which are confirmed to have potent cytotoxicity against many cancer cells[2,3,6]. The study of acute toxicity of *C. hindsii* leaves extract was conducted on white mice at a broad spectrum of doses starting from 1000, 3000, 5000 up to 15000 mg kg^{-1} b.wt. The LD_{50} was not determined even at the high dose of 15000 mg kg^{-1}, which is several folds higher than that of other species plant extracts such as; *Pericampylus glaucus*, *Myrianthus arboreus*, *Lauridia tetragona*[14-16]. Generally, LD_{50} of oral plant extract higher than 1000 mg kg^{-1} could be considered as safe and low toxic in herbal and medicinal product consumption. The present acute toxicity result indicates that the *C. hindsii* extract can be used as a non-toxic orally administrated source. The result probably explained why *C. hindsii* tea leaves have been used in Vietnam for many generations as a safe and pharmaceutically beneficial source as it can avoid the risk of overdose uses in the treatment of several chronic disorders. The study of semi-chronic toxicity of *C. hindsii* conducted on both male and female rabbits showed no behavioral, respiratory patterns and no mortality during the treatment and the 25 days post-treatment check-up. The result suggested that oral administration of C. *hindsii* extract neither intervened in the healthy growth of rabbits nor induce any gender-based differences.

To confirm the safety of multiple-dose use, the study examines whether *C. hindsii* extract causes any signs of hepatotoxicity or nephrotoxicity. There were no effects observed for all recorded parameters, which were consistently similar between treated groups and control one ($p>0.05$). The blood cells produced from the bone marrow are supposed to be affected by some phytochemicals isolated from plants[17]. Because of no significant changes in the levels of red blood cell, hemoglobin, white blood cell and platelets, the plant extract treated groups appeared that is not harmful for bone marrow. The results justified the fact that at all doses of *C. hindsii* extract by oral administration is safe for cells and organs. The significant difference in RBC and HGB between 2 points of time during the experiments in only one test group (3000 mg kg^{-1} in rabbits) probably explained the changes by different development stages of animals, but may not because of the concentration of the plant extracts.

It is suggested that some herbal remedies which may adversely affect the bone marrow and subsequently cause an elevation in biochemical parameters. The 2 sensitive biomarkers (AST and ALT) of tissue damage are proven to be increased in the presence of injuries of the kidney, cardiac muscle and liver inflammation[17]. The result from the present study showed no significantly induced changes in these biomarkers in the liver and kidney of experimental rabbits between all treated groups and the control. As a result, it is probably related to the absence of alteration in liver and kidney after semi-chronic administration of *C. hindsii* extract of treated groups compared to control. The result supported the fact that *C. hindsii* leaves have been used for years in forms of tea products and or integrated into traditional folk remedies for health protection and treatment of some diseases, particularly ones related to the liver. This organ is considered the main target of bioactive compounds as it is exposed to foreign substances[12,18].

It was notable that the bilirubin and urea concentration was significantly increased in the 35th day of the experiment at all three tested groups. The total bilirubin and urea increasing is often the consequence of hepatic and cirrhosis diseases[19]. However, this significant elevation still occurred in the normal range of physiological limit[11], even for the control group with distilled water intake only and the plant extract may not be considered the cause of the increase of the total bilirubin and urea and any toxicity. In contrast to the insignificant increase of liver hepatic enzymes (AST and ALT) between plant extract-treated group and control group, only a significant increase of AST at 3000 mg kg^{-1} was observed in the 35th day compared to the 15th day of the semi-chronic

experiment, but not seen at 1000 mg kg^{-1} and control group. The result was in line with the present acute toxicity study when RBC and hemoglobin also increased as the experiment finished only at the dose of 3000 mg kg^{-1}. Also, all these increases still fell within the biological limitation and may not be toxicologically influenced. However, further toxicity assessments are essential to provide more in-depth insight into the effect of the *C. hindsii* extract on organs such as; the liver and kidney.

In addition, with usual histological features in the liver and kidney (Table 4), significantly higher levels of AST, RBC and hemoglobin at the point time of the 35th day may relate to higher biological activities of rabbits at the later stage of their life development[11] rather than associate with chronic inflammation, congestion or liver infections. Some bioactive compounds found in *C. hindsii* have had a profound effect in the treatment of inflammation, antibacterial, aging prevention, slowing down cancer cell proliferation, detoxification and preventing liver deterioration[6,7,18,20]. Therefore, the vital role of C. *hindsii* tea and medicinal products in supporting the liver and kidney functions of humans is quite clear.

CONCLUSION

The oral dose of 15000 mg kg^{-1} extract from leaves of *C. hindsii* has not determined the LD_{50}. Daily oral administration of *C. hindsii* extracts maintained the normal hematological and biochemical parameters. However, the increase in the level of RBC, hemoglobin and AST at the highest dose suggests cautions need when repeating the oral administration of *C. hindsii* extract. Nevertheless, it is recommended that *C. hindsii* extract its tea products should be considered as a safe source of toxicity for utilization in therapeutic and pharmaceutical applications.

SIGNIFICANCE STATEMENT

This study discovered that the oral administration of *C. hindsii* extract did not produce any significant toxicity in mice. Therefore, it is recommended to be used safely for traditional medical practices and modern pharmaceutical applications. This study will help the researchers to uncover the critical areas of pharmacology that many other researchers have not been able to explore. This also helps the producers to facilitate their commercial production of *C. hindsii* to meet the global demand for medicinal plant materials.

ACKNOWLEDGMENT

The authors are grateful to the Institute of Applied Research and Development, Hung Vuong University, Vietnam and scientific and technological project "Preliminary production of liver detoxification tea from *Celastrus hindsii* leaves at Hung Vuong University" for financial and technical assistance during the experiments. My Research Project was fully sponsored by the Department of Science and Technology with grant number 07/DA-KHCN.PT/2018.

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