Trop J Nat Prod Res, January 2024; 8(1):5779-5784 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Acute Toxicity and Lethal Dose (LD_{50}) of Ethyl Acetate Extract of Dandang Gendis (*Clinacanthus nutans* L) Leaves on Mice Using the Thompson-Weil Method

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ARTICLE INFO

Article history:
Received 10 September 2023
Revised 18 January 2024
Accepted 24 January 2024
Published online 01 February 2024

ABSTRACT

Dandang Gendis (*Clinachantus nutans* L.) is a plant commonly used in traditional medicine. Based on the empirical experience of people, dandang gendis leaves are believed to possess antidiabetic and antibacterial properties. This study aimed to determine the LD_{50} value to assess the toxicity level of the extract and the resulting toxic effects of dandang gendis leaves. The Thompson-Weil calculation method was employed in this study, utilising 25 mice divided into 5 groups of 5 mice each. Group one served as a control (Na-CMC 1%), while the other four groups were treated with graded concentrations of 2.5% w/v, 5% w/v, 10% w/v, and 20% w/v of the extract. The extract was orally administered only once at the beginning of the study period. After administration, the mice were observed at specific intervals: 0, 0.5, 1, 2, 3, 4, 12, and 24 hours. Subsequent observations were made after 24 hours, and the mice were monitored for delayed toxic effects over 14 days. From acute toxicity testing of the ethyl acetate extract of dandang gendis leaves, the LD_{50} value was 6.15 g/kg BW, showing the extract is not toxic at this oral dose in mice. Further research may be necessary to fully understand the potential health benefits and risks associated with dandang gendis, particularly as an antidiabetic agent.

Keywords: Acute Toxicity, Dandang Gendis Leaves, Ethyl Acetate Extract, LD_{50} Value

Introduction

The use of traditional medicines in treatment has long been practiced worldwide in both developing and developed countries. The history of medicine indicates that some traditional medicines were the forerunners of modern medicine.[1] As traditional herbal medicine becomes more popular, various studies are required to identify their active components and assess their effectiveness and safety. This necessitates a series of toxicological tests, including acute toxicity tests[2], to establish the uses and safety of traditional medicines.[3]

One plant commonly used in traditional medicine is the leaves of dandang gendis (*Clinacanthus nutans* L) from the Achanthaceae family. These leaves are effective in lowering blood glucose levels,[4] inhibiting the activity of the alpha-glucosidase enzyme[5,6] down-regulating Glut-2 expression in rats[6], and exhibiting anti-inflammatory activity.[7]

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Citation: Artati, Ahmad A, Karim A, Fajriah S, Karim H, Nuradi, Fajriah SN , Nasir M, Dewi R, Pratama R, Djasang S, Rafika, Armah Z, Hasan ZA, Budirman, Yulianingsih A, Hasanuddin P, Ridwan A, Nur A. Acute Toxicity and Lethal Dose (LD_{50}) of Ethyl Acetate Extract of Dandang Gendis (*Clinacanthus nutans* L) Leaves on Mice Using the Thompson-Weil Method. Trop J Nat Prod Res. 2024; 8(1):5779-5784. http://www.doi.org/10.26538/tjnpr/v8i1.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Dandang gendis leaves contain saponins, flavonoids and tannins.[8] The various parts of *Clinacanthus nutans* L plant contain components that can address various health issues with a low risk of side effects.[9] The half-maximal inhibitory concentration (IC_{50}) of ethyl acetate leaf extract of dandang gendis against alpha-glucosidase enzyme was reported to be 7.28 ppm.[6] This IC_{50} value shows that the extract was active in reducing blood glucose levels and could be utilised as an alpha-glucosidase enzyme inhibitor.[10]

The use of dandang gendis leaves in treating various diseases is gaining popularity. There is a need, therefore, to conduct acute toxicity tests to evaluate the safety and potential adverse effects of dandang gendis leaf before its full utilisation as an herbal drug. This critical step is necessary to ensure the leaf is safe for human consumption. Acute toxicity testing is a vital preclinical assessment used to evaluate the short-term toxic effects of a substance at a specific dose. The quantitative data obtained from this test is expressed as the LD_{50} (Lethal Dose 50), which categorises a compound's toxicity, ranging from extremely toxic to practically non-toxic. Additionally, qualitative data encompasses clinical observations, morphological changes, and the mechanisms of toxic effects.[11,12] It has been reported that the ethanol extract of dandang gendis leaves is essentially non-toxic when administered as a single oral dose, with an LD_{50} value exceeding 2000 mg/kg body weight.

The Thompson-Weil method, using the LD_{50} calculation list, is a frequently utilised approach for determining the toxicity level of a compound. This method was chosen due to its high confidence level, accurate results, and the advantage of not requiring many experimental animals. Using the LD_{50} calculation list enhances the accuracy of the results.[13] This method was employed in this study to evaluate the acute

toxicity of ethyl acetate leaf extract of dandang gendis (*Clinacanthus nutans* L.) in mice (*Mus musculus*) to establish its Lethal Dose (LD_{50}).

Materials and Methods

Materials

The materials used in this research were a syringe (oral sonde), beaker (Iwaki®), measuring cup (Iwaki®), watch glass (Pyrex®), mouse cage, mortar (Rofa), stamfer (Rofa), dropper (Pudak), spatula, vial, observation table, stopwatch, scissors (Yinglian), tweezers (Alfamed), and animal scales (Kitchen scale), ethyl acetate extract of Dandang Gendis Leaves (*Clinachantus nutans* L.), distilled water, NaCMC 1%, white male mice, mouse food (standard feed No.552 (Vital)), ethanol, n-hexane, butanol, and ethyl acetate.

Preparation of Experimental Animals

This research was conducted following the ethical guidelines, and approval was obtained from the Health Polytechnic of the Ministry of Health Makassar No: 0626/KEPK-PTMKS/X/2021.

Male white mice, weighing 20-30 grams and aged 6-8 weeks, were used as test animals. The experimental animals were divided into five groups of five mice each. One group served as control, while the other four were assigned for treatment. BALB/c strain, healthy mice exhibiting no significant changes in body weight, indicating normal behaviour, were used for this study. Before the treatment, the mice were acclimatized for one week with access to food and water *ad libitum*.[14]

Dosage Determination

Malone's dose escalation factor was used to determine the distance between doses:[15]

$$F = N\text{-}1 \times \sqrt{\frac{LD}{SD}} \qquad Dn = DK \times F\ N\text{-}1$$

Where:
F = dose multiple factor
N = number of desired dose levels
LD = largest dose
SD = smallest dose
Dn = dose to be given

Test Sample Preparation

To prepare and create a 1% w/v suspension of Na-CMC, 1 g of Na-CMC powder was placed in a mortar with 20 mL hot water. The mixture was thoroughly crushed until uniform and subsequently diluted with distilled water to a total volume of 100 mL. Additionally, ethyl acetate extracts of *Clinachantus nutans* L. leaves were prepared at varying concentrations: 0%, 2.5%, 5%, 10%, and 20% w/v. Each concentration was mixed with Na-CMC 1% w/v. The orally administered test preparation volume was adjusted to be 1% of the test animal's body weight.

Determination of LD_{50} Value

The LD_{50} value was determined by calculating the number of animal deaths within 24 hours due to administering the test extract at a specific dose level. LD_{50} is expressed as the dose causing the death of 50% of the experimental animals within 24 hours after injection.

Evaluation of Delayed Toxicity Effects for 14 Days

Visible symptoms of toxicity were observed for 24 hours to 14 days in the treatment groups. Parameters observed included deaths and the emergence of changes in autonomic effects such as urination, diarrhea, salivation, seizures, decreased movement activity, paralysis, and increased respiratory rate.[16]

Data Analysis

The obtained data was processed using the Thompson-Weil method to determine the LD_{50} value. This method utilises a list of LD_{50} calculations with a relatively high confidence level. The Thompson-Weil equation, calculated based on the biometric table, was employed.

$$\text{Log } M = \log D + d(f+1)$$

Where:
M = LD_{50} value
D = Smallest dose used
d = log r (dose multiple)
f = factor obtained from the Thompson-Weil table

Parameters such as urination, diarrhea, salivation, seizures, decreased movement activity, paralysis, and increased respiratory rate are presented as a description of the observed results.

Results and Discussion

In the acute toxicity study, each treatment group received dandang gendis leaf extract orally dissolved in distilled water using a probe with varying concentrations for the four treatment groups. The LD_{50} value was calculated using the Thompson-Weil method, which combined the number of animals that died spontaneously or in a moribund state to determine the LD_{50} value. The results of the acute toxicity test are presented in Table 1.

Results of the study showed that no deaths occurred in group 1 animals (concentration 2.5% w/v). In group 2 (concentration 5% w/v), there were two deaths (<50% deaths). Group 3 (concentration 10% w/v) had more than 50% mortality, with four mice dying. Group 4 (concentration 20% w/v) witnessed 100% mortality (all mice died), resulting in an order of death (r) of 0.2, 4.5. Based on the Weil table, the price r 0,2,4,5 has an f (factor) value of 0.30000. The mortality data were analysed using the Weil table, which shows an LD_{50} value of 6.15 g/kg BW for the ethyl acetate extract of dandang gendis (*Clinachantus nutans L.)* leaves.

Apart from determining the LD_{50} value, clinical symptoms or signs of acute toxicity following the administration of the test solution (ethyl acetate extract of dandang gendis leaves) were observed. The experimental animals were observed for 30 minutes, 1-4 hours, and 24 hours after dosing to detect any visible toxic symptoms and the number of mice that died in the experimental groups. The results of observations on toxic effects emerging after treatment are shown in Table 2. In group 1 (concentration 2.5% w/v), signs of cardiovascular toxicity were evident, characterised by a rapid heartbeat (increased respiratory rate) at the 4th hour. Weakness, marked by decreased movement activity, was observed in the 3rd hour, followed by paralysis (3rd and 4th hour), convulsions (4th hour), increased salivation (3rd and 4th hour), Diarrhea occurs in the 3rd and 4th hour, and, urination occurs in the 3rd and 4th hour.

Table 1: Acute toxicity test results (Lethal dose 50) of ethyl acetate extract of dandang gendis (*Clinachantus nutans* L.) leaves

No	Group	Concentration (% w/v)	Mice Number	Death	Annotation
1.	Control (-)	-	5	0	-
2.	Group 1	2.5	5	0	-
3.	Group 2	5.0	5	2	Death<50%
4.	Group 3	10.0	5	4	Death>50%
5.	Group 4	20.0	5	5	Death 100%

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Group 2 (concentration 5% w/v) exhibited signs of cardiovascular toxicity, including rapid heartbeats (increased respiratory rate) at the 2nd and 3rd hours. Weakness, indicated by decreased movement activity, was observed at the 3rd and 4th hours, followed by paralysis (3rd and 4th hours), convulsions occurred at the 3rd and 4th hours, increased salivation occurred in the 3rd and 4th hours, diarrhea (3rd, 4th, and 24th hours) and urination (3^{rd} and 24^{th} hours).

In group 3 (concentration 10% w/v), signs of cardiovascular toxicity were noted, including a rapid heartbeat (increased respiratory rate) at the 3rd and 4th hours. Weakness, characterised by decreased movement activity, was observed at the 4th and 24th hours, followed by paralysis (3rd, 4th, and 24th hours), seizures (4th and 24th hours), increased salivation (4th and 24th hours), diarrhea (hours 2, 3, 4, and 24), and urination (hours 3, 4, and 24).

Table 2: Observation results of toxic effects that occur after the administration of ethyl acetate extract of dandang gendis leaves

Observed Parameters	T	C	Group 1		Group 2				Group 3				Group 4			
			2.5%		5%				10%				20%			
			3	4	2	3	4	24	2	3	4	24	1	2	3	4
Urination	1	-	-	-	-	+	-	-	-	+	-	-	-	+	-	-
	2	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+
	3	-	+	-	-	+	-	+	-	+	+	-	-	+	+	-
	4	-	+	-	-	-	-	-	-	-	-	+	-	+	-	+
	5	-	-	-	-	+	-	+	-	+	-	-	-	+	-	-
Diarrhea	1	-	-	+	-	-	-	-	-	-	+	-	-	-	+	-
	2	-	+	-	-	+	-	-	+	-	-	-	-	+	-	-
	3	-	-	-	-	-	+	-	-	+	+	-	+	-	+	-
	4	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-
	5	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-
Salivation	1	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
	2	-	-	-	-	-	+	-	-	-	+	+	-	-	+	-
	3	-	-	-	-	+	-	-	-	-	+	-	-	-	+	-
	4	-	+	-	-	+	-	-	-	-	+	-	-	+	-	+
	5	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Seizures	1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	2	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-
	3	-	-	+	-	-	+	-	-	-	+	-	-	-	+	-
	4	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Decreased Movement Activity	1	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-
	2	-	-	-	-	+	+	-	-	-	+	+	-	+	-	-
	3	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
	4	-	-	-	-	+	-	-	-	-	-	+	-	-	+	+
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Paralysis	1	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-
	2	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-
	3	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-
	4	-	-	-	-	+	+	-	-	+	+	-	-	-	-	+
	5	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
Increased Respiratory Rate	1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	+	+	-	-	-	-	-	+	-	-	+	-	-
	3	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-

Description:
T: Number of mice observed
C: Control
Observation time: 1 hour, 2 hours, 3 hours, 4 hours, and 24 hours

Group 4 (concentration 20% w/v) animals displayed signs of cardiovascular toxicity, including a rapid heartbeat (increased respiratory rate) at the 1st, 2nd, and 3rd hours. Weakness, characterised by decreased movement activity, was observed at the 2nd, 3rd, and 4th hours, followed by paralysis (2nd, 3rd, and 4th hours), seizures occurred at the 2nd, 3rd, and 4th hours, increased salivation (4th and 24th hours), diarrhea (1st, 2nd, and 3rd hours) and urination occurred at 1st, 2nd, 3rd, and 4th hours. Based on the observed toxicity signs, it was concluded that a concentration of 20% w/v is considered harmful due to its induction of autonomic effects in mice over a short period.

Qualitative analysis of the observed toxic effects, considering the number of effects linked to the weighting factors for each effect category calculated in percentages for each group, showed that the highest percentage was parasympathomimetics or parasympathetic nerve stimulation (cholinergic effects). This occurs due to stimulation of the activation of the digestive tract and intestinal peristalsis, causing diarrhea, stimulating contraction of the bladder and ureters, facilitating the flow of urine (urination), and stimulating the central nervous system, leading to convulsions (Table 3 and 4). The analysis of mortality data using the Weil table indicates that the ethyl acetate extract from dandang gendis (*Clinachantus nutans* L.) leaves falls into the practically non-toxic category with a potential for acute toxicity in the range of 5-15 g/kg BW.[17]

The observed signs of toxicity in groups 1, 2, 3, and 4 likely occurred due to the use of a high dose of the dandang gendis leaf extract, which may contain alkaloids. According to existing literature, many alkaloids can be toxic to various organisms, with the degree of toxicity depending on both the organism and the specific chemical structure of the alkaloid.[18] In contrast, the control group showed no signs of toxicity, and no deaths were recorded among the test animals. This can be attributed to the fact that the animals in the control group were administered a non-toxic and non-irritating negative control agent, i.e., Na-CMC 1% suspension.[17] When orally administered, the ethyl acetate extract of dandang gendis leaves allows the absorption of active compounds in the digestive tract. Subsequently, these active substances undergo distribution and metabolism. Some resulting toxic metabolic by-products act as inhibitors for enzymes in subsequent metabolic stages. This inhibition, in turn, leads to reactions between the active substances and receptors in the effector organ, ultimately causing poisoning symptoms.[19]

Alterations in autonomic effects, particularly increased urination (resulting in higher urine volume), are linked to an elevated intake of drinking water. This increase in water consumption is triggered by an upsurge in the osmolarity of extracellular fluids, leading to a state of dehydration that prompts experimental animals to drink more water. The variations in water intake observed in the test animals can be attributed to the administration of the ethyl acetate extract of dandang gendis leaves. This may be due to the presence of saponins in the extract. According to a source,[20] saponins tend to impart a bitter taste. As a result, test animals consume relatively large quantities of water to alleviate this bitter taste when they ingest preparations containing these secondary metabolites.

Diarrhea was consistently observed in the experimental animals following the administration of the ethyl acetate extract from dandang gendis leaves across all concentrations. These symptoms arise due to the activation of the parasympathetic nervous system. Stimulation of the parasympathetic nerves can lead to increased digestive activity, heightened intestinal peristalsis, and increased secretion of gastric juice. Another consequence of parasympathetic nerve stimulation is the contraction of the bladder and ureters, facilitating the passage of urine. The animals treated with the extract at concentrations of 2.5, 5, 10, and 20% (w/v) exhibited signs of toxicity, including various changes in autonomic effects. These changes encompassed alterations in urination, salivation, diarrhea, increased respiratory rate, reduced motor activity, convulsions, and paralysis. These effects could be categorised as cholinergic effects, central nervous system stimulation, central nervous system depression, and muscle relaxation. The toxic effect of an elevated respiratory rate is classified as central nervous system stimulation. This occurs due to the activation of sympathetic nerves, leading to increased heart activity, resulting in a higher heart rate (tachycardia). Additionally, it causes an increase in the volume of blood ejected with each heart contraction and the dilation of the coronary arteries. This dilation is brought about by an enhanced depolarisation mediated by both norepinephrine (NE) and epinephrine, acting on NE receptors located on postsynaptic effector cells. The toxic effects characterised by salivation, urination, and diarrhea are classified as cholinergic effects and are more likely to manifest at low concentrations. These effects result from stimulating sympathetic nerves, leading to increased activity in the digestive tract, including peristalsis and secretions in the salivary glands. Additionally, the stimulation causes the contraction of the bladder and ureters, resulting in an increased urine output. Parasympathetic stimulation further contributes to these effects by releasing acetylcholine (ACh), which acts on ACh receptors in postsympathetic cells and all parasympathetic postganglionic nerve endings.

The onset of mortality typically commences with convulsions and paralysis. Convulsions result from the combined activity of the central nervous system parasympathetic and sympathetic nerves. On the other hand, paralysis is induced by the inhibition of sympathetic nerve activity. Acetylcholine released at the parasympathetic postganglionic nerve endings interacts with the muscle's nicotinic receptors at the nerve end plate on the skeletal muscle cell membrane. This interaction causes local depolarisation, generating a muscle potential and, subsequently, an action potential, triggering muscle contractions. However, the presence of substances that occupy the muscle's nicotinic receptors hinders the interaction with acetylcholine at the postsynaptic level.

Conclusion

The need to evaluate the toxicity potential of plant extracts is becoming increasingly valuable following the popularity of herbal products and different claims by marketers. Results of the *in vivo* acute toxicity investigation of the ethyl acetate extract of Dandang Gendis (*Clinacanthus nutans* L) leaves using the Thompson-Weil protocol showed that the plant extract at 6.15 g/kg BW was not essentially toxic. However, oral doses beyond this exhibited toxicity effects in the experimental animals, which were qualitatively categorized as parasympathomimetics, followed by central nervous system depression, muscle relaxation, and sympatholytic effects. Despite the biological potential and the use of this plant in treating various diseases, there is a need to exercise caution in its usage.

Table 3: Calculation results of the number of effects that arise with the weighting factor for each activity observed

Category	Concentration (% w/v)			
	2.5%	**5.0%**	**10%**	**20%**
Central nervous system depression	3.75	7.5	13.75	15
Central nervous system stimulation	2.5	6.6	6.6	11.6
Parasympathomimetics	6	9.5	13.5	15.5
Sympatholytic	2.5	7.5	12.5	15
Parasympatholytic	2.5	5	10	10
Sympathomimetics	2.5	5	10	10
Muscle relaxation	3.75	7.5	13.75	15

Table 4: Relationship between weighting factors, activities and categories

Activity	Weighting Factor	Category			
Urine	2.0		Parasympathomimetics		
Diarrhea	1.0		Parasympathomimetics		
Increased Respiratory Rate	2.0				Central Nervous System Stimulation
Seizures	1.0	Sympathomimetics	Parasympathomimetics	Parasympatholytic	Central Nervous System Stimulation
Decreased movement activity	1.0	Central Nervous System Depression		Sympatholytic	Muscle Relaxation
Salivasi	1.0		Parasympathomimetics		
Paralysis	1.0	Central Nervous System Depression			Muscle Relaxation

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

The authors express gratitude to Poltekkes Kemenkes Makassar for generously providing the facilities essential for conducting this study. This research was support by LPPM Hasanuddin University through the Second year of PDD 2022 fiscal year program with contract No.956/UN4.22/PT.01.03/2022.

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