Indian Journal of Ecology (2025) 52(2): 426-431
DOI: https://doi.org/10.55362/IJE/2025/4515

Manuscript Number: 4515
NAAS Rating: 5.38

Evaluation of Acute and Chronic Toxic Effect of *Calotropis procera* Leaf Extract on *Bandicota bengalensis*

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Abstract**:** *Calotropis procera* has nephroprotective and hepatoprotective properties. Its hay is used for increasing the meat quality. Besides being medicinal plants, also have suggested its pesticidal effects. For the present investigation, three types of baits were prepared (two from dry leaf powder alcoholic extract and one from fresh leaves juice) and given in bi-choice condition to field rat, *Bandicota bengalensis* for three days to observe its acute toxicity. Treatment period was also extended to 30 days to see the chronic toxic effect on kidney through histological studies and biochemical analysis. *C. procera* dry leaf powder alcoholic extracts and fresh leaves juice based baits had no acute toxicity against *B. bengalensis* when given under bi-choice condition for three days. Chronic toxicity test also revealed no histopathological alteration in kidneys. Total soluble protein content of kidney of treated rats decreased significantly compared to untreated rats. However, a non-significant biphasic trend was recorded in the antioxidant enzymatic activity in treated groups in comparison to untreated group. Thus *C. procera* leaf extracts based baits have neither acute nor chronic toxicity effects. Thus, these cannot be used as pesticide against rodents.

Keywords**:** *Calotropis procera*, *Bandicota bengalensis*, Histological studies, Biochemical analysis

The herbal medicines hold prominent place right from ancient period in Indian Ayurveda. *Calotropis procera* is one of the most valuable medicinal plant. It is also found in other countries like Malaysia, Sri Lanka, Bangladesh, Pakistan, Bhutan, Nepal and belongs to family Ascalpidaceae and has both therapeutic and pesticidal properties. It is a xerophytic plant found in tropical, subtropical areas and also extended into temperate regions (Nasser et al., 2012). Morphologically, it has simple slightly leathery leaves with simple stem and white and purple coloured flowers (Joseph et al., 2013). It is a laticiferous plant and grows up to 2.5 m (Moustafa and Sarah 2017). Arial parts of the plants contain a sticky substance or a milk sap called latex.

All parts of the *C. procera* such as leaves, roots, flower, bark, latex are used for various purposes and for the treatment of various diseases all over the world since antiquity. Total 547 types of formulations are formed from *Calotropis procera*, which is treating around 58 diseases (Kale et al., 2022). These plant parts are used for the treatment of toothache, earache, fungal infection like ringworm, stings, rheumatism, to relieve pain, epilepsy, skin infections, leprosy, diarrhoea, malaria, ulcers, mental disorders, spleen, liver problems, wound-healing, as analgesic, pro-coagulant (Abeysinghe 2018), and also having anti cancerous properties (Chandekar et al., 2020). It was also used as nephroprotective and hepatoprotective agent. In Andhra Pradesh, Bagata tribes of Vishakhapatnam district use dried roots and latex as an antidote for poisonous snake biting (Seema and Anitha 2015). Phytochemicals such as carbohydrate, tannins, cardiac glycoside, flavonoids, terpenoid, saponin and saponin glycoside are present in *C. procera* and have therapeutic values (William et al., 2015). Hay from *C. procera* was also reported to be a good animal food because it has high protein content and is highly digestible (Madruga et al., 2008). Besides being medicinal plant and its use in increasing the meat quality, several authors also have suggested its toxic and harmful effects. In rats and sheeps cardiotoxic and hepatotoxic effects were observed (Lima et al., 2011). In mice, LD_{50} value with dry latex was 3 g/kg body weight. However, in rodents, no lethal effect was recorded at 165 to 830 mg/kg body weight (Dewan et al., 2000). *C. procera* is being widely used both for its medicinal and poisonous effects, depending upon the dose and mode of use. Even an acute poison act as a drug if it is taken in prescribed manner. Earlier studies reported the beneficial medicinal value of this plant at low doses and toxicity at high doses (William et al., 2015). During present study, baits using high doses of *C. procera* extract were prepared to utilize the toxic property of *C. procera* against rodents pests.

MATERIAL AND METHODS

Experimental site and preparation of *C. procera* extracts and juices: This study was conducted at Rodents Research Laboratory and Animal House of Punjab Agricultural University, Ludhiana, Punjab, India. *C. procera* leaves were collected from barren land surrounds Ladhowal Seed Farm, Punjab Agricultural University, Ludhiana. Both stock *C. procera* dry leaf powder alcoholic extract (CLAE-60ml) and fresh leaf juice (FLJ-50ml) were prepared using 100g *C. procera* dry leaves powder and fresh leaves respectively. The

known quantity of stock *C. procera* leaf alcoholic extract was mixed in known amount of WSO (Wheat:Sugar:Oil; 96:2:2) based plain bait for the preparation of treated baits 1 and 2. Fresh leaf juice was extracted by grinding fresh leaves in grinder, which were then hand squeezed to collect the fresh leaves juice, mixed in WSO bait to prepare treated bait 3.

Preparation of treated baits: Treated bait 1 was prepared by mixing 20 ml of CLAE in 100 g WSO bait to prepare 33.3% dried leaf powder treated WSO bait. Treated bait 2 was prepared by mixing 30 ml of CLAE in 100 g WSO bait to prepare 50% dried leaf powder treated WSO bait. Treated bait 3 was prepared by mixing 50 ml of fresh leaves juice (from 100 g fresh leaves) in 100g WSO bait.

Collection and maintenance of animals: The lesser bandicoot rats, *Bandicota bengalensis* were trapped from grocery shops and were divided into 4 groups (n = 3 of each group). Before the start of the experiment, rats were acclimatized. For acclimatization, rats were kept in individual laboratory cages of size 36 × 23 × 23 cm with food and water provided *ad libitum* for 10-15 days. Cracked wheat, powdered sugar, and vegetable oil (WSO bait) were mixed in a ratio of 96:2:2 to prepare the bait for rats. Approval for the usage of animals was taken from the Institutional Animal Ethics Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (vide memo no. GADVASU/2019/IAEC/49/04 dated: 18, April 2019, during XLIX Meeting of IAEC). Guidelines on the regulation of scientific experiments on animals were followed (Pandey and Sharma 2011). Proper hygienic conditions were maintained. Plastic trays were kept under each laboratory cage for collection and disposal of animal faeces and urine.

Acute and chronic toxicity: Group I rats were fed on plain WSO bait. Rats of groups II, III and IV were fed on treated baits 1, 2 and 3 respectively for three days in bi-choice to record their consumption and acute toxicity. After that same groups of rats were fed on *C. procera* treated baits under bi-choice conditions for 30 days to determine the chronic toxic effect of the same baits on kidneys.

Histomorphological and biochemical studies: Kidneys were collected from both treated and untreated rats after 30 days of treatment period and a piece of kidney was fixed in 10% NBF (natural buffer formalin) and processed as per standard method for histological studies (Humason 1966). Bowman's capsule (BC), proximal convoluted tubules (PCT), distal convoluted tubules (DCT), ascending limbs (AL), descending limbs (DL) in the transverse sections of kidneys were identified (Victor, 2017). Number of BC, PCT, DCT, AL and DL in the transverse section of kidneys of different groups of rats was determined. The known weight of kidney was also homogenized in phosphate buffer saline and then centrifuged at 3000 r.p.m. for 10 min. The supernatant was used for quantative estimations of total soluble protein (Lowry et al., 1951) and to determine the specific activity of antioxidants such as superoxide dismutase (Marklund and Marklund 1974), glutathione peroxidase (Hafeman et al., 1984), glutathione-S-transferase (Habig et al., 1974) and glutathione reductase (Carlberg and Mannervik 1985).

Statistical analysis: SPSS 16.0 and SAS 9.3 software were used for statistical analysis.

RESULTS AND DISCUSSION

Acute toxicity: The pre-treatment consumption signified that all the rats were healthy and can be used for treatment. The overall consumption of all the three treated baits was significantly less than the untreated bait indicating that all the treated baits have less palatability as compared to the untreated bait. Percent acceptance (%) of treated baits was non-significantly different among treated groups with ranging from 33.36% (bait 3) to 56.03% (bait 1). The bait 1 having minimum dose of CLAE has maximum palatability and percent acceptance was significantly higher than bait 3. Active ingredient (CLAE and FLJ) consumed per day was also non-significantly different among groups with values ranging from 1.22 g (bait 3) to 1.77 g/100 g b. wt. (bait 1) indicating consumption of active ingredient was maximum with treated bait 1. Although consumption of active ingredients (*C. procera* leaf) with three treated baits/day ranged from 1.22 to 1.77 g/100 g b. wt. with percent acceptance in comparison to plain bait being 33.36 to 56.03% for a period of three days but mortality was nil with all the three treated baits indicating *C. procera* dry leaf extract based baits and fresh leaf juice based bait had no lethal effects (Table 1). Earlier acute toxicity test conducted in albino rats reported that LD_{50} value of *C. procera* leaf extract is 774 mg/kg b. wt. (William et al., 2015). No toxicity observed during present study might be due to the less bioavailability of secondary metabolites when mixed in bait because of reduction in absorption of dietary fats, protein and fibres (Bushra et al., 2011). The average daily consumption of all the treated baits was lowest on first day of treatment period. However with increase in duration of their exposure, rats developed habituation to all the three treated baits and consumption of treated baits increased with time. However, there was a non-significant difference in the consumption of treated bait among days. On day one, initially for about 2 hours, rats were inactive and showed neophobic behaviour towards the treated baits. But after that rats were normal and active and consumed treated bait along with plain bait; although percent acceptance of all the treated baits was less than the plain bait. Therefore acute toxicity test of baits

treated with alcoholic extract of *C. procera* dry leaf powder and fresh leaf juice on *B. bengalensis* revealed that all the three treated baits containing 33.3 to 50% dry leaf powder and fresh leaves have no lethal effect on rats. Therefore, these baits in present form can't be used as rodenticides against dominating crop field rats, lesser bandicoot rats. There is a need to develop a formulation using CLAE to increase the bioavailability of treated bait.

C. procera products contain natural secondary metabolites called glycosides. These glycosides are toxic and poisonous in nature. Glycosides present in the leaves of *C. procera* include calotropin, calotopagenin, calotoxin, calactin, uscharin, mudarine (bitter yellow acid) and resin. *C. procera* is well known for its pesticidal properties. It has been successfully used against many pests and has been reported as one of the most promising plants which have potential as alternative to chemical pesticides (Begum et al., 2013,Eisa and Yassin 2016,). It was hypothesized that it can be applied in fields against rodents as bait. However during present investigation, toxic symptoms after ingestion of treated baits 1 - 3 were not recorded. Therefore these baits in present form cannot be used as rodenticide against predominant rodent pest, *B. bengalensis*. Earlier study also reported that administration of single high dose i.e. 3 g/kg ethanolic extract of *C. procera* did not cause any mortality or visible toxicity symptoms. However, treatment for long period (90 days) caused significant mortality (Ahmed et al., 2005). Acute toxic (behavioural and neurological responses) effects were however not recorded in albino rats after administration of up to 2500 g/kg body weight of latex, ethanolic, chloroform and aqueous leaves extract of *C. procera* for 7 days (Ismaiel and Alrheam 2015). Death and acute toxicity was also not observed in male Wistar rats after 48 hours when fresh latex was injected intra-peritoneally at doses ranges from 0.1 to 0.6 ml of latex/kg of b. wt. (Lima et al., 2011). No mortality was recorded in mice with dry latex of *C. procera* administered orally at doses ranging from 165-830 mg/kg (Dewan et al., 2000).

However, an earlier study reported 20- 80.5% mortality in Norway rats administered with 21.5- 215 mg /kg of *C. procera* leaf extract respectively (Eisa and Yassin 2016). For male albino rats, LD_{50} value was 95.52 mg/kg (El-Shafey et al., 2011) while for Wistar rats, LD_{50} value was 993 mg/kg (Manivannam et al., 2011). Seema and Anitha (2015) also reported that stem and roots of *C. procera* is toxic than *C. gigantica* and even more hazardous than the cobra's venom. Latex is irritant and having neurotoxic and anticholinergic properties which cause toxicity and sometime death. The fatal or death period varies from 30 min to 8 hr. (Seema and Anitha 2015). However, during present investigation mortality was not recorded even after consumption of very high dose of *C. procera* dry leaf extract and fresh juice. Toxicity of dry leaf extract and fresh leaf juice are reduced when mixed in bait. Therefore, there is a need to develop a formulation/ bait with enhanced bioavailability of secondary metabolites of *C. procera* leaf extract.

Effect on histomorphology of kidney: A chronic toxicity test was also conducted for a period of 30 days to access the effect of same *C. procera* dry leaf powder and fresh leaf juice based treated baits on histomorphology and activity of antioxidant enzymes in kidneys of treated rats. During treatment period of 30 days, percent acceptance of different treated baits/day ranged from 44.07 to 69.30%. There was no effect of treatment on histomorphology of kidneys. No significant pathological effect of treatment was observed on the numbers and histoarchitecture of Bowman's capsule, proximal convoluted tubules, distal convoluted tubules in cortical region and loop of henle's, descending limb and ascending limb in medullary region of kidney (Table 2, Fig. 1). These results signified that *C. procera* did not cause any

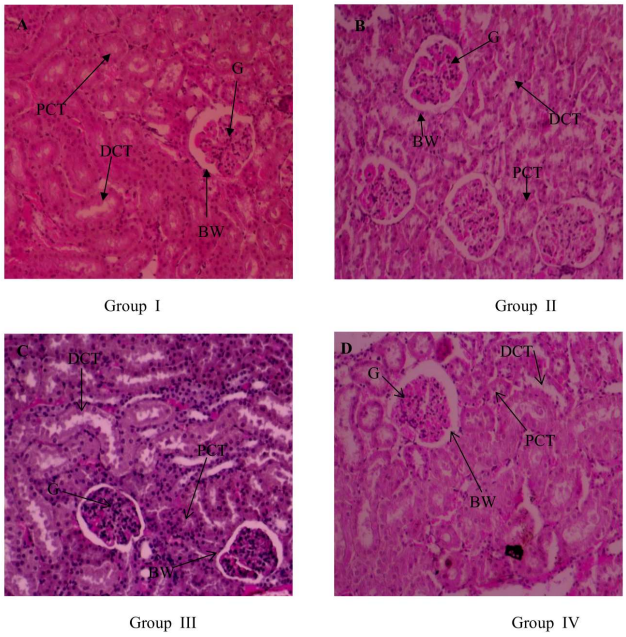
Table 1. Consumption (g/100 g b wt.), percent acceptance and active ingredient consumed per day of *C. procera* leaf based treated baits by *B. bengalensis*

Treatments (n=3)	Body weight (g)	Consumption of baits (g/100 g b wt./day)			Acceptance of treated bait (%)	Active ingredient consumed per day (g/100 g b wt.)	Mortality (%)
		Pre-treatment period	Treatment period				
			Plain	Treated			
Treated bait 1 (CLAE : 33.3% DLP)	270 [a]	10.50 [a]	6.31 [b]	3.64 ± 0.13[b]	56.03 ± 0.95[a]	1.77 ± 0.03[a]	Nil
Treated bait 2 (CLAE:50% DLP)	273 [a]	10.53 [a]	7.52 ± 0.57[ab]	3.27 ± 0.30[ab]	44.60 ± 6.33[ab]	1.64 ± 0.15[a]	Nil
Treated bait 3 (FLJ: 50%FL)	258 [a]	10.51 [a]	8.05 ± 0.31[a]	2.68 ± 0.42[a]	33.36 ± 5.43[b]	1.22 ± 0.24[a]	Nil

Different superscripts indicates significant difference ($p \leq 0.05$) in consumption between plain and treated baits along the rows as well as among baits along the columns

CLAE: *Calotropis procera* leaf alcoholic extract, DLP: Dry Leaf powder; FLJ: Fresh leaf juice, FL: Fresh leaves

chronic toxic effect on kidney of rats. However, earlier study reported that administration of ethanolic leaf extract (1/20 of LD_{50}) of *C. procera* for 4 weeks begins reduction in luminal space of Bowman's capsule and this space was completely vanished with increase in concentration of *C. procera* ethanolic leaf extract (Fahim et al., 2016). Level of urea, uric acid and serum creatinine were increased with the consumption of *C. procera* latex or ethanolic leaf extract which interfered in renal activities or damaged tubular epithelial cells (Ahmad et al., 2014). Ethanolic extract (200-400 mg/kg) of *C. procera* roots was also documented to have hepatoprotective and nephroprotective properties (Prakash et al., 2011). The aqueous extract of *C. procera* flower have nephroprotective effect on rabbits when co-ingested with gentamicin (Javed et al., 2014).



BW: Bowman's capsule, G: Glomerulus, PCT: Proximal convoluted tubules, DCT: Distal convoluted tubules, DL: Descending limb, AL: Ascending limb
Group 1: Untreated rats; Group II: Rats fed on treated bait 1; Group III: Rats fed on treated bait 2; Group IV: Rats fed on treated bait 3

Fig. 1. T.S of kidney of different groups of rats (100X, H & E)

Effect on anti-oxidants: The total soluble protein content of kidney of group II treated rats decreased significantly as compared to untreated rats. However activities of anti-oxidants were non-significantly different in all the treated groups as compared to the untreated group. Specific activity of superoxide dismutase, glutathione reductase and glutathione-S-transferase decreased slightly in treated groups in comparison to untreated group. However, the specific activity of glutathione peroxidase remained same in untreated and treated groups (Table 3). Earlier studies also reported that the dichloromethane, hexane and ethyl acetate crude extracts of *C. procera* are toxic at 250 and 500 µg/mL, on the other hand, methanol and aqueous extracts were less toxic even at > 2000 µg/mL but the lower concentrations

Table 2. Effect of *C. procera* leaf based treated baits on cortex and medullary region of kidney of *B. bengalensis*

Treatments	Number/mm^2 in cortical region			Number/mm^2 in medullary region	
	Bowman's capsule	PCT	DCT	DL	AL
Group I (Untreated bait)	2.01 ± 0.03^a	5.18 ± 0.12^a	1.37 ± 0.15^a	5.45 ± 0.06^a	5.40 ± 0.08^a
Group II (Treated bait 1)	2.0 ± 0.04^a	5.11 ± 0.08^a	1.36 ± 0.07^a	5.35 ± 0.05^a	5.47 ± 0.02^a
Group III(Treated bait 2)	2.00 ± 0.03^a	5.14 ± 0.06^a	1.38 ± 0.11^a	5.39 ± 0.09^a	5.48 ± 0.03^a
Group IV (Treated bait 3)	1.97 ± 0.02^a	5.17 ± 0.07^a	1.27 ± 0.08^a	5.41 ± 0.04^a	5.52 ± 0.02^a

Similar superscripts indicate non- significant difference ($p \leq 0.05$) along the columns
Untreated bait: WSO mixed plain bait
Treated bait 1: *C. procera* alcoholic leaf extract (CLAE) = 33.3% Dry Leaf Powder (DLP) in WSO bait
Treated bait 2: *C. procera* alcoholic leaf extract (CLAE) = 50% Dry Leaf Powder (DLP) in WSO bait.
Treated bait 3: Fresh leaf juice (FLJ) = 50% Fresh leaves (FL) in WSO bait.
PCT: Proximal convoluted tubules, DCT: Distal convoluted tubules, DL: Descending limb, AL: Ascending limb

Table 3. Effect of *C. procera* leaf extract based treated baits on total protein content and specific activity of different antioxidants

Treatments	Soluble protein content (mg/g)	SOD (U/mg protein)	GP_x (U/mg protein)	GR (µ moles of NADPH conjugate / min/ mg protein)	GST (µ moles of GSH-CDNB conjugate formed / min /mg protein)
Group I (Untreated bait)	11.28 ± 0.07^c	25.46 ± 4.77^a	0.62 ± 0.038^a	0.158 ± 0.02^a	0.361± 0.064^a
Group II (Treated bait 1	10.70 ± 0.03^b	22.09 ± 2.10^a	0.60 ± 0.045^a	0.069 ± 0.01^a	0.346 ± 0.015^a
Group III (Treated bait 2)	9.67 ± 0.15^a	20.42 ± 0.36^a	0.61 ± 0.040^a	0.081 ± 0.027^a	0.303 ± 0.040^a
Group IV (Treated bait 3)	10.11 ± 0.18^a	23.00 ±3.25^a	0.63 ± 0.069^a	0.115 ± 0.039^a	0.327 ± 0.079^a

Different superscripts indicate significant difference ($p \leq 0.05$) along the columns
Untreated bait: WSO mixed plain bait
SOD: Superoxide dismutase, GP_x: Glutathione peroxidase, GR: Glutathione reductase, GST: Glutathione-S-transferase

(100-12.5 µg/mL) had no toxic effects and did not cause any morphological changes in cells. Calo-protein (1000 µg/mL) from stem bark of *C. procera* also did not produce any toxic or harmful effect on skin cells (Samy and Chow 2012). *C. procera* have beneficial medicinal value at low doses (118 mg/kg b. wt.) and toxicity at high doses (774 mg/kg b. wt.). Therefore, recommended that shouldn't be used for medicinal purposes at higher concentration (William et al., 2015). However during present investigation, no toxicity was recorded even at high dose. *C. procera* leaf extracts have neither acute nor chronic toxic effects when mixed in bait. Earlier studies reported that it can be safely used for its medicinal properties as well as can be fed to domestic animals for increasing meat quality but cannot be used as rodenticide (Eisa and Yassin 2016).

CONCLUSIONS

C. procera leaf extracts have no (acute and chronic) toxic effects when mixed in bait against *B. bengalensis* and it shows no lesions and inflammation on kidney. So it can be used as medicinal herbs and as fodder for domestic animals but cannot be used as rodenticides.

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Received 09 December, 2024; Accepted 20 March, 2025