



PHYTOCHEMICAL STUDIES AND ANTISNAKE VENOM ACTIVITY OF THE
CRUDE METHANOL EXTRACT OF *INDIGOFERA NUMMULARIIFOLIA*

Haruna A^{*1}., Musa A. M²., Sani Y. M²., Danmusa U. M¹., Muhammed A¹., Idris A¹.,
Kassim A. A¹. And Aliyu B³.

¹Department of Pharmaceutical And Medicinal Chemistry, Kaduna State University, Kaduna

²Department of Pharmaceutical And Medicinal Chemistry, Ahmadu Bello University, Zaria

³Department of Immunology, Usmanu Danfodiyo University, Sokoto

Corresponding Author: harunaanas55@gmail.com, Tel: +2348167356490

Abstract

Medicinal plants remain an important source of therapeutic agents, especially in communities where traditional medicine continues to play a central role in healthcare. *Indigofera nummulariifolia* (Fabaceae), widely used in ethnomedicine for the management of snakebites, has received limited scientific evaluation despite its notable traditional relevance. This study examined the phytochemical constituents of the aerial parts of *I. nummulariifolia* and assessed the antisnake venom potential of its crude methanol extract (CME) against *Naja nigricollis* venom.

The plant material was extracted with methanol and successively fractionated using standard solvent partitioning techniques. Purification of the hexane fraction through repeated gel filtration yielded a white crystalline compound (AH). Spectroscopic analyses (IR, UV, ¹H- and ¹³C-NMR, including 2D NMR) identified AH as stigmaterol. Acute toxicity assessment in Swiss albino mice, following OECD guideline 425, indicated that the extract is relatively safe with an oral LD₅₀ greater than 2000 mg/kg. Meanwhile, the LD₉₉ of *N. nigricollis* venom was determined to be 6.53 mg/kg. In prophylactic studies, preincubation of CME (100–400 mg/kg) with venom conferred complete protection, resulting in 100% survival and significantly prolonged survival times ($p < 0.05$) compared with untreated controls. Curative administration after envenomation also extended survival time, though eventual mortality occurred in all groups.

These findings provide scientific validation for the traditional use of *I. nummulariifolia* in snakebite management. The crude extract's antisnake venom effects, highlights the plant as a potential source of promising lead compounds for further drug development.

Key words: *Indigofera nummulariifolia*, phytochemical, stigmaterol, anti-venom, of *N. nigricollis*

Introduction

Medicinal plants have long served as a cornerstone of traditional healthcare systems worldwide, providing a rich source of bioactive compounds with diverse therapeutic properties. The continued scientific exploration of these natural resources is essential for discovering novel drugs and validating traditional medicinal

practices (Parveen, 2023; Abubakar *et al.*, 2024). Among medicinal plant genera, *Indigofera* (family Fabaceae) is widely distributed and recognized for its extensive ethnomedicinal applications across Africa and Asia (Okafor & Noah, 2019).

Indigofera nummulariifolia (L.) Livera ex Alston is a tropical and subtropical species

traditionally used to manage liver disorders, inflammatory conditions, pain, and snakebites. Its widespread traditional use highlights the need for systematic pharmacological investigation to substantiate its therapeutic claims. Previous studies on related *Indigofera* species, including *Indigofera linnaei* and *Indigofera cordifolia*, have demonstrated significant anti-inflammatory and analgesic activities (Giri *et al.*, 2024), supporting the genus as a promising source of bioactive compounds.

Phytochemical analyses of *Indigofera nummulariifolia* have revealed the presence of flavonoids, tannins, saponins, glycosides, steroids/triterpenoids, and alkaloids (Okafor & Noah, 2019; Parveen, 2023). These secondary metabolites are frequently associated with anti-inflammatory, analgesic, and anti-snake venom activities. The crude methanol extract, being a broad-spectrum solvent extract, is likely to contain a diverse array of these bioactive constituents and therefore warrants comprehensive pharmacological evaluation.

Snakebite envenoming remains a neglected tropical disease and a major public health concern, particularly in rural regions of developing countries where access to conventional antsnake venom therapy is limited (Gutiérrez *et al.*, 2017; Afroz *et al.*, 2024). Consequently, many communities rely on traditional herbal remedies for snakebite management, despite limited scientific validation of their safety and efficacy (Opiyo & Njoroge, 2024; Kumar *et al.*, 2025). Although *Indigofera nummulariifolia* is traditionally employed in snakebite treatment, there is currently limited pharmacological evidence supporting its antsnake venom potential.

Therefore, this study aims to investigate the phytochemical constituents and evaluate the antsnake venom activity of the crude methanol extract of *Indigofera nummulariifolia*.

Methodology

Collection, Identification and preparation of Plant materials

The plant material *Indigofera nummulariifolia* was collected in September 2020 from Galadimawa village, Giwa Local Government of Kaduna State, Nigeria. It was identified by U.S Gallah at the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University, Zaria by comparing with herbarium reference voucher specimen (No. 2194).

The aerial parts collected were cleaned, shade dried and pulverized using pestle and mortar, the pulverized material was labelled and stored in an airtight container at room temperature for use.

Preparation and partitioning of the extract

The powdered aerial parts (1500 g) was extracted with methanol using maceration method. The extract was evaporated in-vacuo using rotary evaporator at 40°C to yield a reddish-brown residue (180.6 g) subsequently referred to as the crude methanol extract (CME).

One hundred gram (100 g) of CME was suspended in distilled water, filtered and partitioned successively with n-hexane, dichloromethane, ethylacetate and n-butanol to obtain hexane fraction (HF, 1.7 g), dichloromethane fraction (DF, 6.38 g), ethylacetate fraction (EF, 8.5 g), n-butanol fraction (BF, 17.6 g) and the residual aqueous fraction (AF, 20 g), respectively.

The HF portion was subjected to further phytochemical investigations.

Chromatographic Separation

The HF (1.7g) was gradiently eluted in a silica gel packed column starting with n-hexane (100%), hexane: ethylacetate (9:1) to hexane: ethylacetate (1:1). Eluates were collected in 30ml portion. A total of 80 fractions were collected and pooled based on their TLC profile to give (10) major fractions coded M1- M10. Fraction M5 (0.18 g) was further subjected to gel filtration chromatography using sephadex (LH-20) eluting with acetone to give a white crystalline powder (12 mg) coded AH, which crystallizes on addition of methanol.

TLC analysis of AH using two solvent systems, viz; hexane: ethylacetate (3:1) and (9:0.5) and sprayed with 10% sulphuric acid revealed single homogenous spot with *R_f* values of 0.74 and 0.69 respectively. The compound (AH) was further subjected to chemical test and spectroscopic analysis (UV, IR, 1D and 2D-NMR) to elucidate its structure.

Experimental animals

Swiss albino mice of either sex weighing (18-24 g) were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. They were fed with laboratory diet and water ad libitum and maintained under standard conditions (12 h light and 12 h dark cycle) in propylene cages at room temperature. All experimental procedures were approved by the Animal Right and Ethics Committee of the University.

Snake venom

The venom of an adult *N. nigricollis* was collected by the milking method of

Macfarlane (Macfarlane, 1967) at Venom, Antivenom and Natural Toxins Research Centre (VANTRAC) Ahmadu Bello University, Zaria. It was pooled, lyophilized and stored at 4 °C until required. The venom was subsequently referred to as the *N. nigricollis* venom.

Anti-snake venom

Lyophilized polyvalent snake venom antiserum sourced from VINS Nioproduct Limited, Andra Pradesh, India was used for the study.

Extract preparation for biological studies

The CME 1000 mg was dissolved in 2 ml distilled water which gave a concentration of 500 mg/ml used for median lethal dose determination, whereas for the antsnake venom studies, a stock concentration of 40 mg/ml was prepared by dissolving 80 mg of the CME in 2 ml of distilled water, the stock was serially diluted to give 20 mg/ml and 10 mg/ml for 200 mg/kg and 100 mg/kg treatment doses respectively.

Toxicity studies

Median lethal dose (LD₅₀) of the crude methanol extract

The OECD 425 up and down procedure (OECD, 2008) was employed and a total of three (3) mice were used for this study. One mouse was dosed orally with 2000 mg/kg body weight of the crude methanol extract of *Indigofera nummulariifolia* and then monitored for any signs of toxicity and mortality for 24 hours. With no signs of toxicity, the other two mice were given the same dose and then monitored for any signs of toxicity and mortality. All the mice were observed at 0.5, 1, 2, 4, 24 hours, 48 hours and 14 days post treatment for signs of toxicity and mortality. Body weights were also recorded prior to administration and at days 7 and 14 observation period.

Lethal dose 99 (LD₉₉) of Naja nigricollis venom

The LD₉₉ of the venom was determined according to the method of Meier and Theakston (1986). The snake venom (1.73 g) was dissolved in 3 ml distilled water (DW) to give a stock concentration of 0.576 mg/ml, four groups of mice (n=5) were injected intraperitoneally with different doses of venom ranging from 0.36–2.88 mg/kg body weight. The animals were observed for 24 hours after venom injection.

Prophylactic anti-snake venom activity of the extract

The method described by Abubakar *et al.*, (2000) was employed for this protocol. The mice were grouped into four groups (n=5). The first group (control group) received via intraperitoneal injection 0.2 mL of previously incubated (37°C for 30 min) mixture of 6.53 mg/kg LD₉₉ of *Naja nigricollis* venom and distilled water. Groups 2, 3 and 4 (treatment groups) received via intraperitoneal injection 0.2 mL of previously incubated (37°C for 30 min) mixture of 6.53 mg/kg LD₉₉ of *Naja nigricollis* venom and 100, 200 and 400 mg/kg of CME respectively. The number of deaths within 24 hours were recorded.

Curative anti-snake venom activity of the extract

Curative antisnake venom activity was determined using the method of Ibrahim *et al.*, (2020), the mice were grouped into four groups (n=5). Initially all the groups received via intraperitoneal injection 0.2 ml equivalent to 6.53 mg/kg LD₉₉ of *Naja nigricollis* venom. Five minutes later, group 1 received via intraperitoneal injection 0.2 ml DW while groups 2, 3 and 4 received 100, 200 and 400 mg/kg of crude methanol extract, respectively. The number of deaths within 24 hours were recorded.

Statistical Analysis

The statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software version 23. The results were presented as mean ± standard error of mean (SEM) as well as percentages in form of tables. Kaplan Meier Survival analysis was used to calculate the occurrence of death, while log rank pairwise comparison was used to calculate the level of difference between the groups. Values having $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Compound AH

Repeated gel filtration of the hexane fraction resulted in the isolation of a white-crystalline solid coded AH (12 mg). The uncorrected melting point of compound AH was 137 – 138 °C, and it gave a positive test with Liebermann-Burchard reagent for a steroidal nucleus, consistent with standard phytosterol characterization methods (Khan *et al.*, 2020).

The IR spectrum of compound AH (in KBr) exhibited simple absorptions suggesting the high purity of the compound. It showed a broad absorption band at ~3470 cm⁻¹, characteristic of O–H stretching, typical for a free hydroxyl group of phytosterols. The bands at ~2950–2925 cm⁻¹ were attributable to aliphatic C–H stretching, and the absorption at ~1650 cm⁻¹ was due to C=C olefinic stretching in the steroid nucleus (Takale *et al.*, 2023). The absorption at ~1045 cm⁻¹ indicates C–O or cycloalkane vibrations, consistent with literature for steroidal alcohols. These IR absorptions closely resemble those reported for stigmasterol in recent spectral studies (Sathish *et al.*, 2021; Takale *et al.*, 2023).

In the ^1H -NMR spectrum of AH (400 MHz, CDCl_3), three olefinic resonances were observed at δ 5.35, δ 5.10, and δ 5.03, typical of olefinic protons of stigmasterol side-chain and ring unsaturation. A resonance at δ 3.52 corresponded to a proton on an oxygenated carbon (C-3), and a cluster of aliphatic signals between δ 2.27 and δ 0.69 suggested the complex steroidal framework. These trends match closely with published stigmasterol ^1H data (Khan *et al.*, 2020; Akwada *et al.*, 2020; Tordzagla *et al.*, 2024).

The ^{13}C -NMR (125 MHz) and DEPT experiments established that the compound contained 29 carbons, consisting of 6 methyl (CH_3), 9 methylene (CH_2), 11 methine (CH), and 3 quaternary (C) carbons, a pattern characteristic of phytosterols (Tordzagla *et al.*, 2024). The downfield resonances at δ 140.76 (C-5), δ 121.71 (C-6), δ 138.31 (C-22), and δ 129.28 (C-23) indicate olefinic carbons, and shifts at δ 21.08 (C-19) and δ 12.05 (C-18) are consistent with angular carbon atoms found in stigmasterol (Hodge & Sterner, 2005; Isa *et al.*, 2015; Kankara *et al.*, 2023; Muhammad *et al.*, 2023). The resonance at δ 71.8 (C-3) corresponds to the C-3 β -

hydroxyl carbon, further supporting identification as stigmasterol (Khan *et al.*, 2020; Sathish *et al.*, 2021; Takale *et al.*, 2023; Tordzagla *et al.*, 2024).

The ^1H - ^1H COSY spectrum revealed correlations between protons in contiguous spin systems: δH 5.35 (H-6) with δH 2.27 (H-4); δH 5.03 (H-22) with δH 2.02 (H-20); δH 3.52 (H-3) with δH 1.85 (H-1); and δH 3.52 (H-3) with δH 2.27 (H-4), consistent with the typical coupling observed in the steroid nucleus (Khan *et al.*, 2020; Akwada *et al.*, 2020; Sathish *et al.*, 2021).

The HMBC spectrum further established long-range connectivities, such as H-4 (δ 2.27) with C2, C3, and C6, H-6 (δ 5.35) with C5 and C7, and H-22 (δ 5.03) with C20 and C24, consistent with the standard HMBC correlations for stigmasterol (Khan *et al.*, 2020; Akwada *et al.*, 2020; Sathish *et al.*, 2021).

The results of the 1D and 2D NMR data, as summarized on (Table 1) establish the structure of AH as stigmasterol. Comparison with a reference NMR data (Akwada *et al.*, 2020) showed a good match and further confirmed the proposed structure.

Table 1: ^1H & ^{13}C -NMR data of AH (Stigmasterol) compared with Akwada *et al.* 2020

Position	δC (AH)	δH (AH)	δC (Reference)	δH (Reference)
1	37.26		37.27	
2	31.67		31.68	
3	71.81	3.52 m	71.84	3.54 m
4	42.31		42.31	
5	140.76		140.8	
6	121.71	5.35 brs	121.74	5.38 brs
7	31.90		31.90	
8	31.90		29.71	
9	50.16		50.16	

Position	δC (AH)	δH (AH)	δC (Reference)	δH (Reference)
10	36.51		36.5	
11	21.21		24.32	
12	39.68		39.80	
13	42.22		42.3	
14	56.87		56.79	
15	24.36		24.32	
16	28.92		28.93	
17	55.96		56.08	
18	12.05	1.47 (s)	12.06	1.53 (s)
19	21.08	0.69 (s)	19.41	0.71 (s)
20	40.49		39.80	
21	23.07	1.70	21.2	1.86 (d)
22	138.31	5.03	138.33	5.04
23	129.28	5.10	129.30	5.18
24	51.24		51.25	
25	29.15		36.16	
26	18.98	0.78 (dd)	21.10	0.73 (d)
27	19.40	0.82 (dd)	21.23	0.95 (d)
28	25.40		25.42	
29	12.25	0.80	11.86	1.04

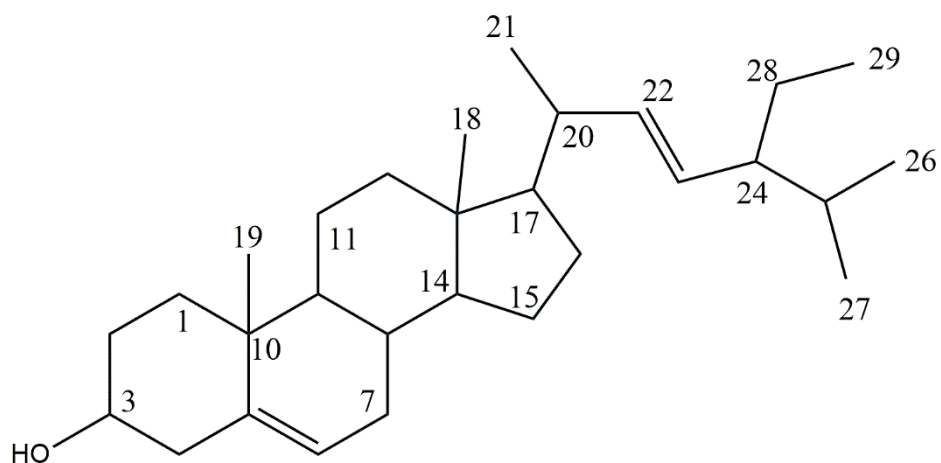


Figure 1: Stigmasterol

Median lethal dose (LD₅₀)

The oral median lethal dose (LD₅₀) of the CME was found to be greater than 2000 mg/kg.

Lethal dose 99 (LD₉₉) of *Naja nigricollis* venom

The LD₉₉ of the *Naja nigricollis* venom was estimated to be 6.53 mg/kg body weight (Table 2).

Prophylactic antisnake venom activity of *Indigofera nummulariifolia* CME against *Naja nigricollis* venom

All the envenomed mice treated with the CME survived more than twenty-four hours compared with the untreated mice, that all died (Table 3).

Kaplan Meier survival analysis log rank pairwise comparison showed a significant ($p < 0.05$) increase in the mean survival time of the extract treated groups compared to the untreated group (Table 3).

Curative antisnake venom activity of *Indigofera nummulariifolia* CME against *Naja nigricollis* venom

Although the envenomed mice treated with *I. nummulariifolia* CME died, however, Kaplan Meier survival analysis showed a significant ($p < 0.05$) increase in the mean survival time as compared to the envenomed untreated mice (Table 4).

Table 2: Lethal Dose 99 (LD₉₉) of *Naja nigricollis* Venom

Venom Dose (µg/kg)	Log Dose	% Death	Corrected %	Probit
360	2.56	0	5	3.36
720	2.86	0	5	3.36
1440	3.16	40	40	4.75
2880	3.46	100	95	6.64

Corrected formula for 0 and 100% mortality $\frac{0.25}{n} * 100$ and $\frac{n-0.25}{n} * 100$ respectively.

Venom Lethal Dose 99 calculations (Probit analysis)

$$y = 4.885 + 3.0x$$

if y (Probit) = 7.33 (99%), what will be the value of x (Log dose)

$$x = \frac{7.33 - 4.885}{3.00}$$

$$x = 0.815$$

$$\text{Antilog } 0.815 = 6.53$$

$$\text{LD}_{99} = 6.53 \text{ mg/kg}$$

Table 3: Prophylactic Antisnake venom Activity of *I. nummulariifolia* CME Against *Naja nigricollis*

Treatment (n=5)	Mean Survival Time (hour)	% Survival	% Mortality
LD ₉₉ + DW	0.57	0	100
LD ₉₉ + CME 100 mg/kg	> 24*	100	0
LD ₉₉ + CME 200 mg/kg	> 24*	100	0
LD ₉₉ + CME 400 mg/kg	> 24*	100	0

*Significant $p < 0.05$ Kaplan Meier Survival analysis Log rank pairwise comparison
DW- distilled water, LD₉₉ – Lethal dose 99

Table 4: Curative Antisnake venom Activity of *I. nummulariifolia* CME Against *Naja nigricollis* Venom in Mice

Treatment (n=5)	Mean Survival Time \pm SEM (min)	% Survival	% Mortality
LD ₉₉ + DW	32.8 \pm 1.83	0	100
LD ₉₉ + CME 100 mg/kg	48.2 \pm 3.34*	0	100
LD ₉₉ + CME 200 mg/kg	59.8 \pm 1.02*	0	100
LD ₉₉ + CME 400 mg/kg	59.4 \pm 2.94*	0	100

* Significant $p < 0.05$ Kaplan Meier Survival analysis log rank pairwise comparison, SEM - Standard error mean, DW – distilled water, LD₉₉ – Lethal dose 99

The LD₅₀ of the methanol crude extract of *I. nummulariifolia* was found to be above 2000 mg/kg in Swiss albino mice. Dose of 2000 mg/kg was not found to cause any mortality and signs of toxicity. This finding indicates the relative safety of the plant (Hodge & Sterner, 2005).

The Lethal Dose (LD₉₉) of the *Naja nigricollis* Venom is found to be 6.53 mg/kg body weight, other studies in the literature reported varying doses as the LD₉₉ of *Naja nigricollis*. Higher values of 9.55 mg/kg, 6.0 mg/kg, 9.7 mg/kg and 10 mg/kg were reported by (Abubakar *et al.*, 2000; Ode & Asuzu, 2006; Isa *et al.*, 2015; Kankara *et al.*, 2023) respectively. While lower values of 4.6 mg/kg, and 5.57 mg/kg were however reported by (Yusuf *et al.*, 2019; Muhammad *et al.*, 2023) respectively. The differences observed in the LD₉₉ of the venom might be as a result of variability of toxins in the venom composition due the geographical factors such as location, season, age of the snake and handling and storage (Chippaux & Goyffon, 1991; Isa *et al.*, 2015). Lethality of the same snake venom might vary with geographical location and diet (Premendran *et al.*, 2011).

In the prophylactic antisnake venom study, the intraperitoneal administration of pre-incubated mixture of varying concentration of the extract with *Naja nigricollis* venom (LD₉₉) significantly increased the survival time across the treated groups. The anti-venom potential of ethnomedicinal plants that are widely used against snake venom induced lethality and mortality could be due to the presence of various secondary metabolites (Raghavamma *et al.*, 2016) that bind directly to venom components and neutralizes their toxicity (Alam, 2014). The major targets of these phytochemicals are the venom components responsible for the severe effects of venom toxicity (Murakami *et al.*, 2005; Panfoli *et al.*, 2010; Bala *et al.*, 2018). However, the mechanism of neutralization of snake venom lethality is not well understood, but many hypotheses have been putting forward, such as proteolytic degradation, protein precipitation, enzyme inactivation, metal chelation, antioxidant action, and a combination of these mechanisms (Felix-Silva *et al.*, 2017).

In the curative antisnake venom study, the administration of varying doses of the crude methanolic extract of *I. nummulariifolia* as

therapy against the already administered LD₉₉ (6.53mg/kg) of the *Naja nigricollis* venom, showed a non-dose dependent increase in survival time of the envenomed animals across the treated groups, with animals in group 3 and 4 showing highest survival time.

Lethality of the venom is a combined action of toxic components present in the venom. The venom of *N. nigricollis* consists primarily

of cytotoxins, neurotoxins and cardiotoxins. Neurotoxins act at neuromuscular junction either post-synaptically or pre-synaptically. The probable mechanism of inhibition of neurotoxic effect by *I. nummulariifolia* may be by interfering with the acetylcholine receptor sites, thereby antagonizing the action of neurotoxic substances in the venom at the acetylcholine receptor sites (Shrikanth *et al.*, 2018). Cytotoxins in *Naja nigricollis* venom often act by inducing oxidative stress in tissues, causing cellular damage, lipid peroxidation, and necrosis (Ajisebiola *et al.*, 2024). Plant extracts rich in antioxidants, such as tannins, flavonoids, and polyphenols, counteract the oxidative stress by neutralizing free radicals generated by the venom, protecting tissues from damage and inflammation (Hano & Tungmunnithum, 2020). Studies on *Indigofera hirsuta* and *Parkia biglobosa* show that plants antioxidant components protect tissue by reducing oxidative damage and inflammation in envenomed animal models (Muhammad *et al.*, 2023). Another possible mechanism is by venom enzyme inhibition potential of some plant extracts to neutralize the lethality. *Naja nigricollis* venom contains enzymes such as

phospholipase A₂ (PLA₂), proteases, and hyaluronidases, which contribute to local tissue damage, inflammation, and hemotoxicity. Some plant extracts contain natural PLA₂ inhibitors that bind to and inhibit the active sites of venom enzymes, reducing their ability to damage cell membranes, degrade connective tissue, and disrupt blood clotting mechanisms (Adeyi *et al.*, 2023).

Conclusion

In summary, Stigmasterol was isolated from the hexane fraction of *Indigofera nummulariifolia* and its chemical structures was elucidated. To the best of our knowledge, this is the first report of isolation of this compound from the aerial parts of *Indigofera nummulariifolia*

We have also shown that CME of *I. nummulariifolia* possessed antisnake venom activity. Although the antisnake venom activity of the isolated stigmasterol could not be evaluated due to paucity of the material, effort is ongoing in our laboratory to further isolate this compound in sufficient quantity to allow for detailed bioactivity profiling of stigmasterol and to evaluate its potential as lead in the development of novel antisnake venom therapeutic agent.

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Conflict of interest

No conflict of interest is associated with this work

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