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A ONE HEALTH APPROACH TO SNAKEBITE: UNLOCKING THE ANTIVENOM POTENTIALS OF *INDIGOFERA HIRSUTA* AERIAL PARTS THROUGH PHYTOCHEMICAL ANALYSIS OF ITS CHLOROFORM FRACTION AGAINST *NAJA NIGRICOLLIS*

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Abstract

Phytoconstituents have been reported to have protective activity against snake venom. This research work is aimed at identifying the components using two spectroscopic techniques Fourier Transform- Infrared (FTIR) and Gas Chromatography-Mass Spectrometry (GCMS) and scientifically test their antivenin potential via *in vivo* model. Preliminary phytochemical screening conducted on chloroform fraction obtained through liquid-liquid partition of crude methanol extract revealed the presence of secondary metabolites including alkaloids, steroids, terpenes and phenols. FTIR spectrum of the column fraction (E) showed sharp absorption band at 29.22.2 cm^{-1} characterized by C-H stretching, weak diagnostic peak at 2853.3cm^{-1} for methoxy group and appearance of peak at 1459.3cm^{-1} due to C-C stretching for aromatic molecule. GCMS analysis identified various components at different retention time including hispidulin triacetate (22.302min) as the major components while other constituents detected include squalene (23.401min), fumaric acid (20.00min) heptafluro butyric acid (12.0314min) and linoelainic acid (14.177min). Chloroform fraction (at 125, 250 and 500mg/kg) was able to protect mice lethality induced by *Najo nigricollis* venom in dose dependent manner (40, 40 and 60% respectively) while pooled fraction (E) offered 100% protection only 15mg/kg. The observed biological activity might be attributed to the identified phytoconstituents. Therefore, further work on isolation of pure compound needs to be done to ascertain the component(s) responsible for this activity.

Keywords: Phytochemicals, *I. hirsuta, N. nigricollis*, spectroscopy

Introduction

Snakebite envenoming still remain a potentially life threatening health problem which occurred as a result of toxins in the bite of venomous snake. This envenoming can also be caused by having venom sprayed into the eyes of certain species of snakes that have the ability to spit venom as a defense measure (Ref). Available data worldwide showed 4.5 - 5.4 million people get bitten by snakes annually (WHO, 2024) Snake bites incidences commonly found in rural communities of Nigeria recorded mainly in the middle belt region comprising plateau, Benue, Kwara, Kogi (Glolade, 2021). Four families of venomous snakes found in Nigeria are *elapidae*, *colubridae*, *Viperidae*, and *actraspididae*, but two most important snakes associated with envenomation in Nigeria are carpet viper (*Echis Coellatus*) and black necked spitting cobra (*Naja nigricollis*) (Habib and Abubakar, 2011; WHO, 2007).

Snake venoms are soluble in water, acidic and have a specific gravity of 1.03. The lethality and composition of the venoms vary with the specie and age of the snake, geographical location and envenoming snake's diet (Goswani *et al.,* 2014). These snake venoms consists of complex mixture of enzymatic and toxic proteins such as necleotidases, phospholipases A2, acetylcholinesterase nitrate and other proteolytic enzymes (Kang *et al.,* 2011; Gomez-Betancur *et al.,* 2019). Which are injected to immobilize the victim (Janardhan *et al.,* 2014). These toxins cause haemotoxicity-damage to blood vessels resulting in spontaneous systematic and muscle paralysis, myolysis, arrhythmias, renal and cardiac failure (Omara *et al.,* 2020).

The elementary therapy for envenomation involves intravenous infusion of antisnake venom. Though antivenom effectively counteracts the general impact of a poisonous bite, but many studies have shown that it is often ineffective in neutralizing low molecular mass toxins responsible for nicrosis, hemorrhage and neportoxicity (Chippaux, 2017). Antivenoms work by boosting our immune response after a snake bite. They are made by immunizing donor animals such as horses or sheep with snake venoms. These animals have robust immune system, and produce powerful antibodies that can bind to snake venom component, enabling our own immune defence to eliminate these toxins (WHO, 2017). Preminum antivenom and Echis Tab Plus – ICP are examples of antisnake venom authorized in Nigeria, but immunological and biochemical variations of snake venom components lead different challenges in manufacturing appropriate venom (Bala *et al.,* 2022). Other factors contributing to the severity of this health problem include low supply to the rural locations, limited knowledge about the treatment and affordability (Harrison *et al.,* 2009) similarly due massive doses antivenom precipitate allergic reaction (Gupta and Peshin, 2012) thereby limiting its usage. Therefore, alternative plant based antivenom is necessary.

Medicinal plants have been reported in Nigeria to have protective potential against snake venom (Gblolade, 2021). *Indigofera hirsuta* (rough hairy indigo) is a specie of flowering plant in the fabaceae family, a native of nearly all the trophics. It is an erect or spreading herbaceous legume growing 60-230cm tall, with spreading and ascending branches (Burkill, 1995). *I hirsuta* have several medicinal

uses, in Nigeria, leaf sap is taken internally for liver complaints and leaf decoction is used for jaws, ulcer, epilepsy or convulsion in infants. It is used in treatment of diabetes, leprosy, infections, snake bites, and management of malaria (Burkill, 1995). Some biological activities of the plant reported includes anti-inflammatory activity of butanol fraction of *I. hirsuta* (Abbas *et al.,* 2013). Furthermore, antisnake activity has been reported on *I. hirsuta* extracts, the result showed significant (*P<0.05*) and dose dependent activity (Muhammad *et al.,* 2022).

Identified antivenom constituents from the plants through GCMS, spectroscopy include phenol, kaempterol luteolin (Gopi et al., 2015). Other constituents that showed promising activity against snake venom include lupeol acetate, *β-Sitosterol*, stigmastrol quercertin (Shabbir *et al.,* 2014). This research work will therefore, purify *I. hirsuta* chloroform extract using column chromatography, characterize the resulting fraction using FTIR and GCMS spectroscoping and evaluate pooled the fraction for antivenin activity.

Materials and Methods

Plant Collection and Identification

Freshly aerial parts (whole part) of *Indigofera hirsuta* was obtained from Sabon Gari Local Government Area of Kaduna State, Nigeria. The sample was identified and authenticated at the Herbarium Unit, Department of Botany, Faculty of Life Science, Ahmadu Bello University Zaria, Nigeria where a voucher specimen number V/NABU0503 was obtained.

Plant Preparation

The freshly aerial part (whole parts) of *Indigofera hirsuta* was properly cleaned and washed carefully with distilled water (4 liters) but not excessive and shade dried, pulverized, labelled and stored at room temperature for further use.

Plant Extraction

The pulverized plant material (1500g) was extracted using maceration method with 7 liters of methanol for seven (7) days with occasional shaking, the extract was filtered using Whatman No.1 filter paper and solvent was evaporated using rotary evaporator. The resulting crude extract was dried and subsequently weighed to determine the percentage yield. The crude methanol extract (130g) was successively subjected to liquid-liquid extraction with Hexane (1000cm^3), Chloroform (1000cm^3) and Ethyl acetate (1000cm^3) in sequential order of polarity, afforded Hexane (HF), Chloroform (CF) and Ethyl acetate (EF) fraction respectively (Kalsi and Tagtap, 2013).

Qualitative Phytochemical Screening

Each extract (2 g) was subjected to a preliminary phytochemical screening to detect for the presence of secondary metabolites according to the procedures described by Trease and Evans (2008); Sheikh and Patil (2020)

Chromatographic Procedure

Thin-layer Chromatography (TLC)

Thin-layer chromatography was carried out using TLC pre coated plates (Silica gel 60 F_{254}) by using ascending technique as described by Shashank and Shreya (2022) spotting was carried out manually using capillary tubes and developed in an air-tight chromatographic tank, at room temperature. Preliminary TLC of the chloroform fraction was conducted. Different solvent systems were used to develop the plates, including;

i. Hexane: Ethyl acetate (1:1, 3:1, 2:1, 12:1)
ii. Chloroform: Ethyl acetate (5:1, 3:1, 7:2)

Developed chromatograms were air dried and visualized;

i. Under normal day light
ii. Under ultra violet light (254 and 366 nm)
iii. By spraying with 10 % sulphuric acid followed by heating at 104 ^{o}C for at least 5 minutes in an oven.

Column Chromatography of Chloroform extract

Chloroform fraction (CF) (3g) was dissolved in chloroform, then absorbed on 3g silica gel and allowed to dry. The dried sample was chromatographed over a silica gel (60g) packed column. It was then eluted continuously using hexane and mixtures of hexane and ethyl acetate solvents, starting with hexane 100%, and then followed by the addition of ethyl acetate in different ratio (95:5, 90:10, 95,15, 80:20, 70:30, 60:40, 50:50) and finally ethyl acetate 100%. Total of 106 collections were made and pooled based on similarity in their TLC profile to offered eleven pooled fractions (A-K)

Fourier Transform Infrared Spectroscopic Analysis of fractions from Column Chromatography of CF

The fraction (CF) were subjected to FTIR analysis. To create the sample disc, 0.1g of dry CF fractions (CF) was premixed with a potassium bromide (KBr) pellet and pressed into an FTIR spectrophotometer (Agilent) disk. The IR radiations were allow to pass through the disk and scanned between 4000 and 600 cm^{-1}. The sample was averaged from 200 scans with a spectral resolution of 4 cm^{-1}. It took three minutes to record. Then, for the specified materials, a final average spectrum was computed (Kumar and Ramaswamy, 2014).

GC – MS Analysis of the Fractions (CF)

The dried fraction (CF) was dissolved in chloroform and analyzed using a GC-MS model QP2010 plus SHIMADZU with a detector and slit injection system. The temperature was initially set to 60^{o}C for 3 minutes before progressively increasing to 250^{o}C. For analysis, 1.6µL of solution was injected at a temperature of 250^{o}C during the experiment. Hellium gas (99.999%) was used as the carrier gas, with a constant flow rate of 1ml/min and an injection volume of 2l (split ratio of 10:1); injector temperature 2500^{o}C, ion-source temperature 2800^{o}C. The oven temperature was programmed from 1100^{o}C (isothermal for 2 minutes) with an increase of 10^{o}C/min at 2000^{o}C, then 50^{o}C/min at

2800^{o}C, and ending with a 9-minute isothermal at 2800^{o}C. Mass spectra were taken at 70ev, with a scan interval of 0.5 second and fragments ranging from 45 to 450Da. The total GC running time was 36 min. Each component's relative percentage amount was computed by dividing its average peak area by the overall area. The program used to handle mass spectra and chromatograms was turbo mass. The identification of components was based on comparison of their mass spectra with those present in the National institute for standard Technology computer data bank (NIST 2009 Library)

Experimental Animals

Locally bred adult Swiss Albino mice of either sex (16-30 g body weight) and were acquired from Animal House Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Science, Ahmadu Bello University, Zaria, Nigeria. The animals were fed with laboratory diet and water *ad libitum* and maintained under standard conditions in clean cages under normal 12 hours light, 12 hours dark circle at Department of Pharmacology and Toxicity Usmanu Dnafodiyo University Sokoto. The animals were acclimatized for one week prior to the commencement of the experiment.

Acute toxicity Studies (Median Lethal Dose LDso)

The method described by Lorke (1983) was employed and route of administration was intra-peritoneal. In the first phase, exactly nine mice of either sex were divided into three groups containing three mice each. The first, second and third groups received 10, 100 and l000mg/kg respectively. In the second phase, four mice were used based on the outcome of the first phase. Each of the four mice received different doses of the chloroform fraction which are 200, 400, 800 and 1600mg/kg. The median lethal dose was calculated as follows:

$LD_{50}\ \sqrt{minimum\ lethal\ Dose\ (MLD) x\ Maximum\ Survial\ Dose\ (MSD)}$

Lethality Assay of the Venom LD_{99}

The LD_{99} of the venom was determined using the method described by Theakson and Reid (1983); the *Naja nigricollis* venom was reconstituted with normal saline and concentrations ranging from 1 to 12 mg/cm^3 were obtained. Eighteen mice were divided into six groups of three mice each and were injected *intraperitoneally* (*ip*) with different doses of the reconstituted venom. The control group received only normal saline (0.2 ml each) (*ip*). The LD_{99} was estimated from the death of the mice within 24h of venom injection (Finney, 1977).

***In vivo* Snake Venom Detoxifying Effects**

The method described by Theakston and Reid (1983) was employed. Thirty five mice were divided into seven groups (n=5). Group 1 (control) received 5.75 mg/kg of the venom only. Groups 2, 3 and 4 received 1.25, 250 and 500 mg/kg of the ethyl acetate fraction respectively (*i.p*) while group 5, 6 and 7 received 5, 10 and 15 ml/kg of the isolated compound (*ip*). The test groups were then injected with 5.75 mg/kg of the venom 30 minutes after injecting the samples. The route of administration was *intraperitoneal*. All animals were observed for mortality for 24hours.

Statistical Studies

Statistical analysis of differences between means was performed by one-way analysis of variance (ANOVA), followed by multiple comparisons of values in control versus treatment groups using post hoc Dunnett's test. The results were considered significant at $p \leq 0.05$. All the analysis was done using statistical package for the social sciences (SPSS) version 20.0.

Results and Discussion

Qualitative phytochemical screening conducted on the chloroform fraction obtained through liquid – liquid partition chromatography revealed the presence of secondary metabolites including flavonoids, alkaloids, steroids, terpenoids, and phenols etc. These observed constituents were reported to have antivenin potentials (Gopi *et al.,* 2015; Liaqat *et al.,* 2022).

The median lethal dose (LD_{50}) was estimated to 3807 mg/kg (Table 2-3) indicating that the chloroform fraction is relative less toxic (Lorke, 1983) and safe at administered dose. Minimal lethal dose (LD99) of *Naja nigricollis* venom is 4.04 mg/kg. Larger values were reported (5.75mg) and 9.55 mg/kg as reported by Muhammad *et al*. 2022 and Dauda *et al.* (2025) respectively. This variability observed in the minimum lethal dose of the venom might be due to the difference in venom composition which is attributed to seasonal and geographical variations and snake species (Gopi *et al.,* 2015).

Table 2: Results of Acute toxicity (LD_{50}) *I. hirsuta* chloroform fraction (First phase)

Dose (mg/kg)	Number of mice used	Mortality
10	3	0/3
100	3	0/3
1000	3	0/3

Table 3: Second phase of acute toxicity study of *I. hirsute* chloroform fraction

Dose (mg/kg)	Number of mice used	Mortality
1600	1	0/1
2900	1	0/1
5000	1	1/1

The minimum lethal dose (LD_{99}) of *N. nigricollis* venom was calculated to be 4.04 mg/kg as shown in Tables 3. The LD_{99} (lethal dose for 99% of a population) of snake venom is an important parameter to determine before conducting *in vivo* antisnake venom studies. It helps determine the proper dose of test samples needed to successfully neutralize venom, which is crucial for therapeutic applications, and it guarantees that the venom dose used in tests is constant and repeatable (Casewell *et al.,* 2014). The LD_{99} of *N. nigricollis* (Table 3) demonstrated that, these venoms is highly toxic to human and animals.

Table 3: Result of the lethality assay of the *N. nigricollis* (LD_{99}) Venom

Dose (mg/kg)	Dose (mg/ml)	Dead/Total	Death%
1	1.0	0/3	0
2	2.5	0/3	0
3	4.0	3/3	100
4	5.0	3/3	100
5	7.5	3/3	100
6	12	3/3	100

In vivo detoxifying potentials of chloroform fraction and column pooled fraction (E) was observed. The chloroform fraction showed protection (at 125, 250 and 500mg/kg) in a dose dependent pattern with 40, 40 and 60% respectively while pooled fraction (E) demonstrated significant ($P<0.05$) activity only at 15mg/kg graded dose with 100% survival rate. The discovered activity might be attributed to the components identified. Phenolic compounds such as catechin, and chalcones were reported by Yusuf *et al.* (2020), and Isah *et al*. (2022) research teams.

Table 4: *in vivo* dotoxifying effect of *I.hirsuta* of CF and pooled fraction (E)

Samples	**Control group**	**Treatment % Survival (24h)**		
	LD_{99}	125mg/kg	250mg	500mg/kg
Chloroform Fraction (CF)	4.04	40	40	60
Column Fraction (E)	4.04	0*	0*	100*

*Column Fraction (E): Dose = 5mg/kg, Dose = 10mg/kg, Dose = 15mg/kg

The *I. hirsuta* CF was subjected to upon column chromatography using silica gel and mixture of hexane: ethyl acetate as mobile phase. In this chromatography, one hundred and six (106) fractions (Table 5) were collected and pooled based on the TLC profile to obtained nine (9) fractions. Fraction E was selected and analyzed for compounds identifications using FTIR and GCMS.

Table 5: Column chromatography of chloroform fraction

Collections	**Hexane : Ethyl acetate**	**Pooled fractions**
1-10	98:4	A
11-15	95:5	B
16-49	90:10	C
51-60	80:20	D
61-69	**80:20**	**E**
70-88	70:30	F

89-93	60:40	G
93-99	50:50	H
100-106	Ethyl acetate (100%)	I

Fourier transform infrared spectroscopy (FTIR) is a technique used to identify the various functional groups in the plant sample by using the beam of infrared radiations (Khan et al., 2018). The spectral data of the column pooled fraction (E) indicate sharp absorption peak at 2922.2cm^{-1} which is characterized by C-H stetching and a weak diagnostic band at 2853.3cm^{-1} for methoxy group. It also revealed the appearance of peak at 1459.3cm^{-1} due to C-C stretching vibration band for aromatic molecule, while peak at 1725.1cm^{-1} represent C=O stretching as shown in (Figure 1).

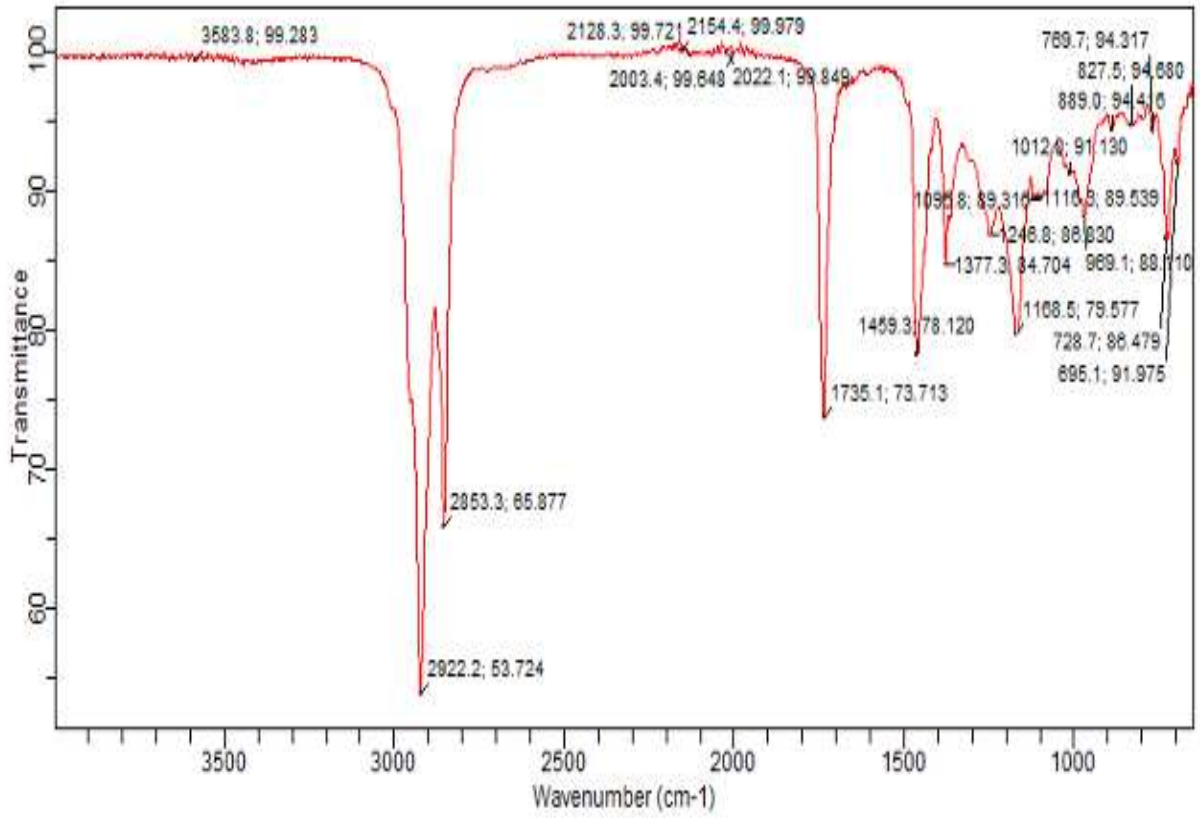


Figure 1: FTIR Spectrum of Column Fraction (E)

GCMS analysis of *Indigofera hirsuta* column fraction (E) revealed the presence of flavonoids as the major compound identified as 4, 5, 7-trihydroxy 6-methoxy flavone acetate, also known as Hispidulin triacetate at retention time of 22.305min (figure 2) Other constituents detected in the fraction at various retention time of the chromatogram include squalene (23.401min), fumaric acid (20.00min) heptafluoro butyric acid (12.0314min) and linoelainic acid (14.177min)

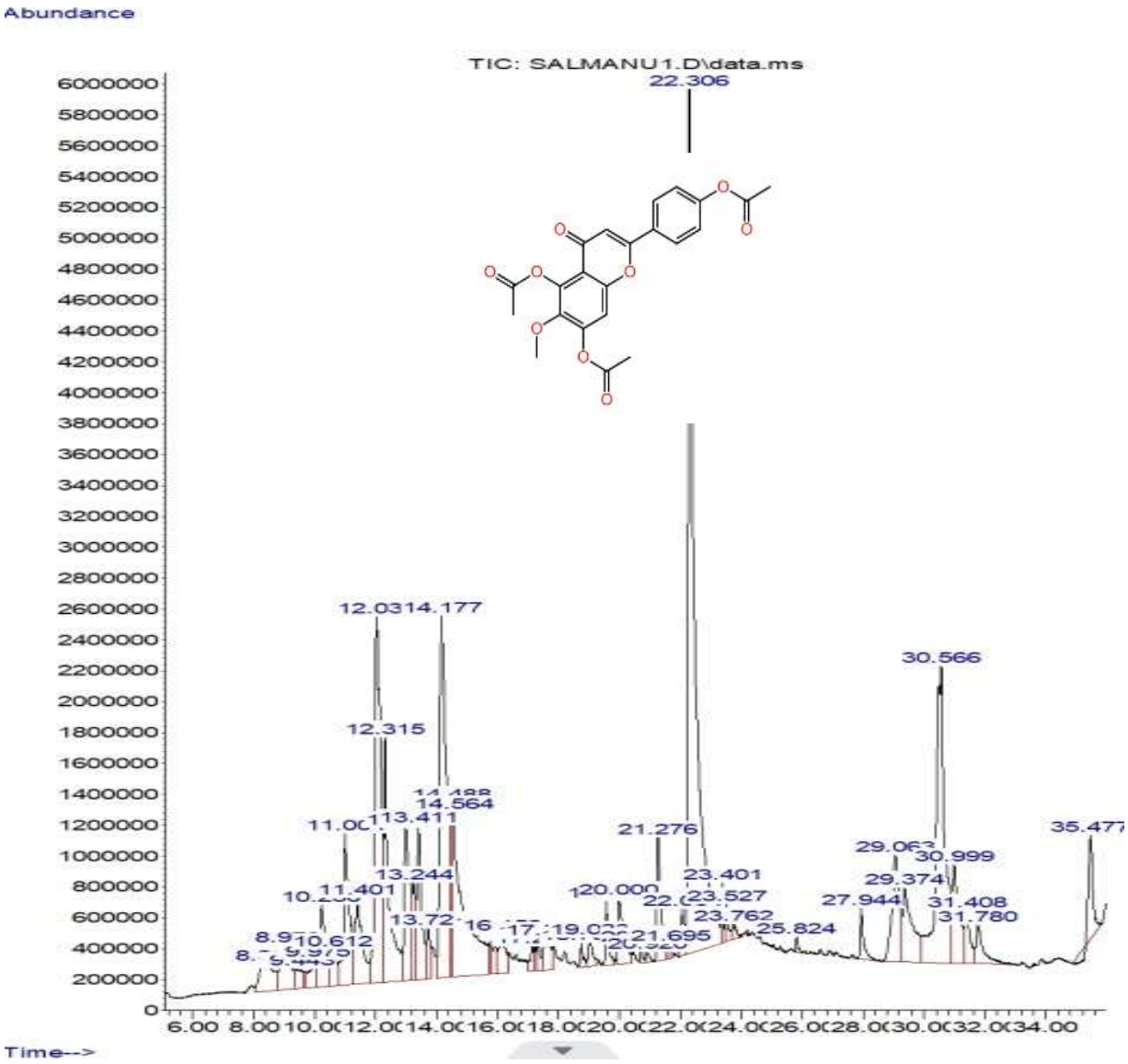


Figure 2: GCMS spectra of Column Pooled Fraction (E)

Flavonoids have anti-oxidative and mucosal protective effect (Abebaw *et al.,* 2017; Stoclet and Shini-kerth, 2011) they are characterized by their good bioavailability and hence, constant dietary consumption of flavonoids has been reported to give pharina cologically relevant plasma concentrations in humans (CaO *et al.,* 2010). Additionally, flavonoids such as chalcones, catachin have been reported for their antivenin activity (Isah *et al.,* 2022; Yusuf *et al.,* 2020). Quercetin, Kaempferol, luteolin, ellagic acid and chlorogenic acid were identified through GC-MS and reported for antivenin potential (Gopi *et al.,* 2015). Terpenes such as squalene have significant importance as intermediates in cholesterol and sterol synthesis and possess antioxidant, moisturizing and anti-inflammatory properties.

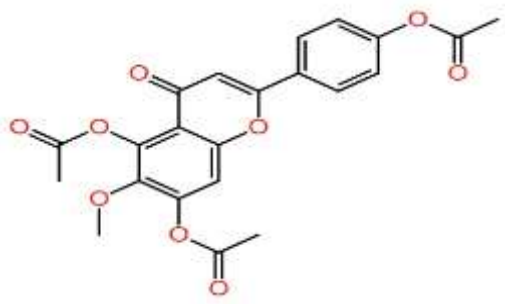
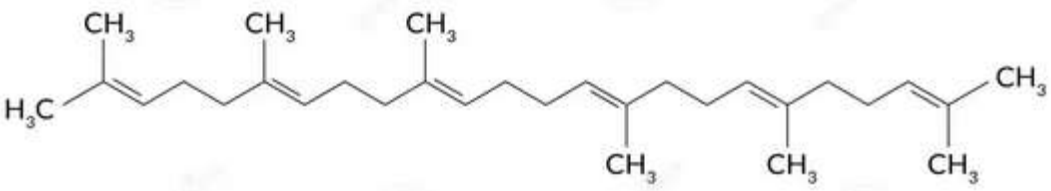
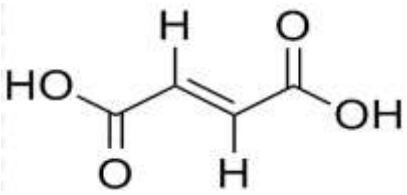


Figure 3: Hispidulin triacetate (major phytoconstituent)

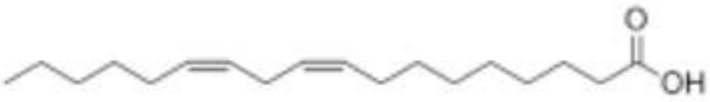


Squalene



Fumaric acid

Heptafluoro butyric acid



Linoelainic acid

Figure 4: GCMS identified compounds from column fraction (E)

Conclusion

The secondary metabolites from the plant extract was identified and the fraction's acute toxicity was evaluated for acute toxicity while column chromatographic analysis of CF results to FTIR and GC-MS of the active pooled fraction (E). The fraction (E) is rich with bioactive compounds from which Hispidulin acetate was identified as the major phytoconstituents. Additionally, yhe fractions CF and column pooled fraction (E) demonstrated significant ($p<0.05$) *in vivo* antivenin activity against the venom of *N. nigricollis*. Thus, this work highlight the potential of *Indigofera hirsute* for the management of snakebite envenomation therapy.

Ethical Approval

All authors hereby declare that principle of laboratory Animals cares was taken from (ABU/AUC), with approval No. ABUCAUC/2021/055.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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