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Bio-active marvels of *Indigofera aspalathoides*: Unveiling antioxidant power and snake venom neutralization abilities through *In-vitro* exploration

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Abstract

Significant health hazards are posed by oxidative stress and snake venom envenomation, especially in areas with poor access to healthcare. In this work, the antioxidant and snake venom-neutralizing qualities of the traditional medicinal herb *Indigofera aspalathoides* are investigated. The phytochemical composition of the plant's extracts was examined. With remarkable DPPH and nitric oxide scavenging capabilities, the ethyl acetate extract demonstrated strong antioxidant activity. This extract substantially suppressed PLA_2 activity and decreased haemolysis caused by snake venom, according to in vitro tests. This extract successfully suppressed PLA_2 activity and decreased haemolysis brought on by snake venom, according to in vitro tests. A number of bio-active substances that contribute to these effects were found by GC-MS analysis. According to these results, *Indigofera aspalathoides* has significant antioxidant qualities and the ability to neutralise the venom of the Russell's viper (*Daboia russelii*), which supports its traditional use in folk medicine and emphasises its potential as a natural therapeutic agent against oxidative stress and snake venom envenomation.

Keywords: Traditional medicine, Antioxidant, Anti-snake venom, Snake venom, *Indigofera aspalathoides*

Introduction

The pathophysiology of chronic diseases like cancer, diabetes, neurodegenerative disorders, and cardiovascular complications is significantly influenced by oxidative stress, which is caused by an imbalance between reactive oxygen/nitrogen species (ROS/RNS) and antioxidant defences. DNA, proteins, and lipids are among the biomolecules that can be harmed by free radicals, which are produced during regular metabolic processes. By scavenging free radicals, antioxidants lessen these effects and maintain the integrity of cells. Redox balance is maintained by both endogenous enzymatic antioxidants and dietary sources, especially plant-derived polyphenols, flavonoids, and vitamins [1-3].

Another serious issue is snakebite envenomation, which is a significant public health risk in tropical and subtropical areas. More than 5 million snakebite cases are reported worldwide each year, with a high mortality and morbidity rate, particularly in areas with limited resources [4,5]. A complex mixture of proteins, peptides, enzymes, and toxins, snake venom can cause coagulopathies, haemorrhage, tissue necrosis, and neurotoxicity. Despite being the cornerstone of treatment, anti-snake venom serum (ASV) has drawbacks, including restricted availability, high expense, negative side effects, and need for cold storage[6,7]. As an alternative or supplementary treatment for envenomation, this has raised interest in plant-based therapies[8,9].

Indigofera aspalathoides (Family: Fabaceae), traditionally known as "Sivanar Vembu," has been extensively used in Siddha medicine for its anti-inflammatory, anti-leprotic, and wound-healing properties, and notably in the management of snakebite[10,11,12]. Phytochemical investigations have revealed the presence of flavonoids, alkaloids, saponins, phenols, steroids, and β-

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sitosterol, which may contribute to both antioxidant and anti-venom activities [13,14,15].

The current study intends to extract, isolate, and characterise bioactive fractions from *Indigofera aspalathoides* based on its ethnomedicinal relevance. It utilises *In vitro* assays like DPPH, nitric oxide scavenging, PLA_2 inhibition, and direct haemolytic assays to assess the bioactive fraction's antioxidant potential and snake venom neutralisation activities.

Materials and Methods

Collection and authentication of Plant material

The whole plant of *Indigofera aspalathoides* was collected from Sattankulam, Thoothukudi district, and authenticated by Mr. V. Chelladurai, Retd. Research Officer (Botany), CCRAS, Govt. of India, Tirunelveli. Russell's viper (*Daboia russelii*), venom was collected at muttukadu by Irula Snake Catcher's Cooperative Society. The Institutional Ethical Committee clearance (*Ref: CSP-III/24/JUN/06/195*) was obtained for using blood sample in the study.

Physicochemical and phytochemical extraction

The collected plant material was subjected to physicochemical analysis, including the determination of total ash, water-soluble ash, and acid-insoluble ash values. Extractive values were assessed using both water and alcohol as solvents. Additionally, the percentage of loss on drying was determined. All procedures were carried out in accordance with the standard methods prescribed in the Indian Pharmacopoeia (IP).

It was sequentially extracted with n-hexane, chloroform, ethyl acetate, and methanol (Sucessive solvent extraction) by maceration method[16]. The extracts were concentrated under reduced pressure using a rotary evaporator and stored for further analysis.

Phytochemical evaluation

Preliminary Phytochemical screening: The hexane, chloroform, ethyl acetate and methanolic extracts of *Indigofera aspalathoides was* subjected to qualitative phytochemical screening using standard procedures[17,18,19,20]. Major classes of secondary metabolites, including alkaloids, flavonoids, glycosides, tannins, saponins, phenols, terpenoids, identified based on characteristic colour reactions.

Quantitative Estimation of Phytoconstituents

The n-hexane, chloroform, ethyl acetate and methanolic extracts was subjected to quantitative estimation of phytoconstituents.

a. Total Phenolic Content (TPC): Determined using the Folin–Ciocalteu method. Gallic acid (1mg/ml) was used as the standard, and absorbance was measured at 765 nm. The Results were expressed as mg gallic acid equivalents (GAE)/g dry extract [20, 21, 22].

b. Total Flavonoid Content (TFC): Estimated using aluminium chloride colorimetric assay with quercetin(1mg/ml) as the standard. Absorbance was recorded at 510 nm and results expressed as mg quercetin equivalents (QE)/g dry extract [22, 23].

c. Total Tannin Content: Measured using the Folin–Ciocalteu method, with tannic acid (1mg/ml) as the standard. Absorbance was recorded at 700 nm and tannin content expressed as mg tannic acid equivalent (TAE)/g dry extract [24].

d. Total Alkaloid Content: Estimated via a colorimetric method using bromocresol green (BCG) reagent and phosphate buffer (pH 4.7), with atropine (1mg/ml) as the standard. Absorbance was measured at 470 nm, and results were expressed as mg atropine equivalents/g extract [25].

In vitro anti-oxidant evaluation of extracts

The n-hexane, chloroform, ethyl acetate and methanolic extracts were subjected to all *in vitro* tests.

DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical Scavenging Activity [19,26,27]: The antioxidant potential for all the four extracts was

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evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. A 0.1 mM DPPH solution was prepared in methanol and stored at –20 °C. All four extracts were prepared in methanol at concentrations ranging from 50 to 1000 µg/mL. One milliliter of DPPH solution was mixed with 1 mL of each concentration and incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a UV spectrophotometer. Ascorbic acid(1mg/ml) was used as the reference standard with concentration ranging from 50 to 1000 µg/mL. The percentage of inhibition was calculated using the formula:

%Inhibition=(AC-AO)/AC×100

where AC is the absorbance of the control and AO is the absorbance of the sample. IC_{50} values were determined from concentration–inhibition curves. All measurements were conducted in triplicate.

Nitric oxide Scavenging activity [27, 28]: The nitric oxide scavenging activity of the four extracts was determined using sodium nitroprusside and Griess reagent. Sodium nitroprusside (10 mM) was prepared in phosphate-buffered saline (PBS, pH 7.4). Test samples were prepared at concentrations ranging from 100 to 1000 µg/mL.

A reaction mixture containing 1 mL of sodium nitroprusside and 1 mL of test sample was incubated at 30 °C for 3 hours. After incubation, 1 mL of freshly prepared Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, and 2% orthophosphoric acid) was added to each tube. The absorbance of the resulting chromophore was measured at 550 nm using a UV spectrophotometer. Ascorbic acid (1mg/ml) served as the reference standard.

The nitric oxide scavenging activity was expressed as percentage inhibition using the following formula:

%Inhibition=(AC-AO)/AC×100

where AC is the absorbance of the control and AO is the absorbance of the sample. IC_{50} values were determined from concentration–inhibition curves. All measurements were conducted in triplicate.

***In vitro* Snake venom neutralizing activity for all four extracts**

PLA_2 Inhibition Assay [29,30,31]: The phospholipase A_2 (PLA_2) inhibitory activity of the four extracts was assessed using *Daboia russelii* venom. The venom stock (1 mg/mL) was prepared in sodium phosphate buffer (pH 7.4) and diluted to a working concentration of 10 µg/mL. All the four extracts were prepared at concentrations ranging from 50 to 1000 µg/mL in the same buffer.

Reaction mixtures were prepared in 96-well microtiter plates containing PLA_2 enzyme, lecithin (substrate), venom, and varying concentrations of extracts. Controls included: (i) Enzyme + substrate (negative control), and (ii) enzyme + substrate + venom (positive control). All reactions were incubated at 37 °C for 30 minutes.

The reaction was stopped by adding 0.1 M HCl, and mixtures were centrifuged at 1000 rpm for 10 minutes. The absorbance of the supernatant was measured at 425 nm using a UV spectrophotometer to quantify fatty acid release.

The percentage inhibition of PLA_2 activity was calculated using:

%Inhibition=(AC-AT)/AC×100

where AC is the absorbance of the positive control and AT is the absorbance of the test sample. IC_{50} values were determined by plotting inhibition percentage against concentration.

Direct Hemolytic Assay[30,32]: The hemolytic activity was evaluated using a 1% human red blood cell (HRBC) suspension. Whole blood was collected from a healthy volunteer and anticoagulated using heparin. RBCs were isolated by centrifugation at 1000 rpm for 10 minutes and washed three times with phosphate-buffered saline (PBS). The final RBC suspension was adjusted to 1% in PBS.

Snake venom (1 mg/mL) and four extracts, 50–1000 µg/mL were prepared in PBS. Equal volumes of HRBC suspension, venom, and test sample were mixed and incubated at 37 °C for 30 minutes.

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Post-incubation, the mixtures were centrifuged at 1000 rpm for 5 minutes. The absorbance of the supernatant was measured at 540 nm to determine the degree of hemolysis. Controls included venom-only (positive control) and PBS-only (negative control).

The percentage inhibition of hemolysis was calculated as:

%Inhibition=(AC-AT)/AC×100

where AC is the absorbance of the positive control and AT is the absorbance of the test sample. IC_{50} values were determined by plotting inhibition percentage against concentration.

The extract exhibiting the highest biological activity and phytochemical content was selected for further analysis. Phytochemical profiling of this bio-activity enriched extract was carried out using Gas Chromatography- Mass Spectrometry (GC-MS) to identify major constituents.

Isolation of Compounds via Column Chromatography: The extract exhibiting the highest bioactivity, as determined by preliminary studies, was subjected to column chromatography for the isolation of active constituents. Ten grams of the ethyl acetate extract were loaded onto a silica gel-packed glass column. Elution was performed using a gradient of solvents with increasing polarity, ranging from non-polar to polar (n-hexane, Chloroform, ethyl acetate and methanol), at increasing polarity of 10% was followed to ensure effective separation[33, 34].

The eluents were collected in fractions, which were monitored and analyzed by thin-layer chromatography (TLC) to assess the presence of distinct phytoconstituents. Fractions exhibiting similar TLC profiles were pooled, concentrated under reduced pressure, and further purified using recrystallization technique [33, 34].

Characterization of Isolated Compounds: The isolated compounds were characterized using Fourier-transform infrared (FTIR) spectroscopy to identify functional groups based on characteristic absorption bands.

Subsequently, the bioactivity of the isolated compounds was assessed through relevant *In vitro* biological assays to support their pharmacological significance.

***In vitro* Evaluation of Isolated Compound**

The purified compound was assessed for bioactivity using a number of recognized *In vitro* tests to confirm its therapeutic potential after it had been successfully isolated and structurally characterized. Antioxidant activity was assessed by DPPH radical scavenging, nitric oxide (NO) scavenging, enzyme suppression was assessed by phospholipase A_2 (PLA_2) inhibition, and direct haemolytic assay. To ensure consistency in comparative analysis, all assays adhered to the same standardized protocols that had been used for the crude extracts. Graded concentrations of 10, 20, 40, 80, and 120 µL were used to test the isolated compound. The percentage of inhibition for each assay was calculated from the results, and the biological efficacy of the compound was quantified by determining the corresponding IC_{50} values.

Results and Discussion:

Physicochemical evaluation:

The physicochemical properties of *Indigofera aspalathoides* are presented in (Table 1). The total ash content was 2.63%, indicating low inorganic residue, while the water-soluble and acid-insoluble ash values were 8.223% and 2.06%, respectively.

Table 1: Physico-chemical evaluation of *Indigofera aspalathoides*

S. No	Parameters	Percentage (% W/W)
I	Ash Value	
1.	Total ash	2.63
2.	Water soluble Ash	2.22
3.	Acid Insoluble Ash	2.06
II	Extractive Value	
1.	Water soluble Extractive	7.00
2.	Alcohol Soluble Extractive	4.40
III	Loss on drying	4.23

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Extractive values were found to be 7.00% for water and 4.4% for alcohol, reflecting the presence of predominantly polar constituents. The recorded loss on drying was 4.23%, suggesting notable moisture content that may influence storage stability. These parameters serve as essential markers for crude drug standardization and help ensure the identity, purity, and quality of the plant material.

Preliminary Phytochemical Screening:

Preliminary screening across various solvent extracts (hexane, chloroform, ethyl acetate, and methanol) indicated the presence of Phytochemicals in each extract (Table 2).

Quantitative estimation of Phytoconstituents

Quantitative estimation showed that ethyl acetate extract had the highest concentration of total phenolics (1.30±0.006 mg GAE/g), tannins (1.10±0.227 mg TAE/g), flavonoids (3.50±0.001 mg QE/g), and alkaloids (5.60±0.459 mg/g) (Table 3). These bioactive compounds are known for their role in antioxidant and anti-inflammatory activity, justifying the extract's further use in isolation and activity assays.

Table 2: Phytochemical screening of all four extracts of *Indigofera aspalathoides*

S. No	Phytochemical Test	HE	CE	EAE	ME
1.	Carbohydrate	+	+	+	+
2.	Alkaloids	-	-	+	+
3.	Saponin	+	+	+	+
4.	Phenols/ Tannins	-	-	-	+
5.	Flavanoids	-	+	+	+
6.	Glycoside	+	+	+	+
7.	Phytosteroids/ Steroids	+	+	+	+
8.	Protiein and Amino acid	-	-	-	+
9.	Gums and Mucilage	-	-	-	-
10.	Volatile oil/ Oils and Wax	+	+	+	-

(+) Indicates presence of compounds (-) Indicates absence of compounds
Note: HE= Hexane Extract, CE= Chloroform Extract, EAE= Ethyl Acetate Extract, ME= Methanol Extract.

In vitro* Anti-Oxidant Assay for all extracts of *Indigofera aspalathoides

DPPH Radical scavenging Assay for all four extracts of *I. aspalathoides*

The DPPH assay revealed that the ethyl acetate extract of *Indigofera aspalathoides* exhibited the highest antioxidant activity, with 88.9 ± 0.70% inhibition at 1000 µg/mL and an IC_{50} of 71.09 ± 0.52 µg/mL. The methanol extract also showed notable activity (68.9 ± 3.10%, IC_{50} = 99.39 ± 1.31 µg/mL), followed by hexane and chloroform extracts. The superior activity of the ethyl acetate fraction suggests the presence of potent antioxidant effect (Fig. 1).

Nitric Oxide radical scavenging assay for all four extracts of *I. aspalathoides*

The nitric oxide scavenging assay demonstrated that the ethyl acetate extract of *Indigofera aspalathoides* exhibited the highest activity at 1000 µg/mL (65.9 ± 0.7%), with an IC_{50} of 68.66 ± 0.54 µg/mL. The methanol extract showed moderate inhibition (37.4 ± 0.6%, IC_{50} = 95.38 ± 0.38 µg/mL), while

Table 3: Quantitative estimation of Phytochemicals of all extracts of *Indigofera aspalathoides.*

Quantitative Estimation	HE	CE	EAE	ME
TPC (mg GAE /g)	0.20.035	0.30.011	1.30.006	0.80.011
TTC (mg TAE /g)	-	-	1.10.227	0.30.006
TFC (mg QE /g)	1.7	4.40.000	3.50.001	1.3 0.001
TAC (mg/ g)	1.30.006	2.4 0.009	5.60.459	2.60.005

Each value represents mean ± S.D of triplicates(n=3)
Note: TPC= Total Phenolic Content, TTC= Total Tannin Content, TFC= Total Flavanoid Content, TAC= Total Alkaloid Content

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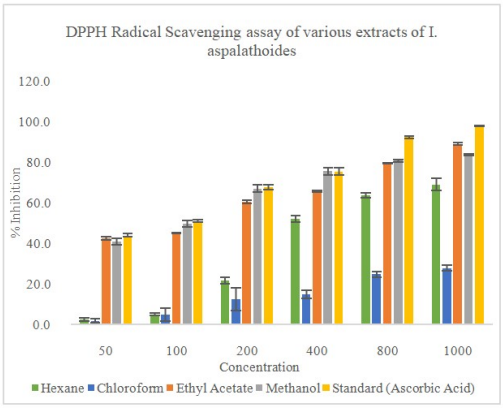

Fig. 1: DPPH Radical Scavenging assay of various extracts of *Indigofera aspalathoides*

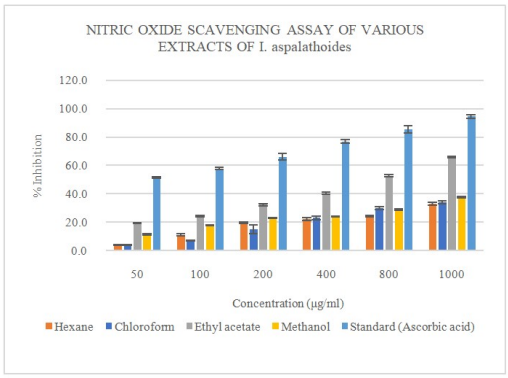

Fig. 2: Nitric oxide scavenging assay of all four extracts of *I. aspalathoides*

hexane and chloroform extracts displayed comparatively lower activity (Fig. 2). The ethyl acetate extract's significant nitric oxide scavenging capacity at higher concentrations suggests the presence of compounds capable of inhibiting nitric oxide generation or neutralizing reactive nitrogen species, aligning with its strong DPPH activity.

PLA_2 Inhibition assay for all four extracts of *I. aspalathoides*

The PLA_2 inhibition assay showed that the ethyl acetate extract of *Indigofera aspalathoides* exhibited the most pronounced

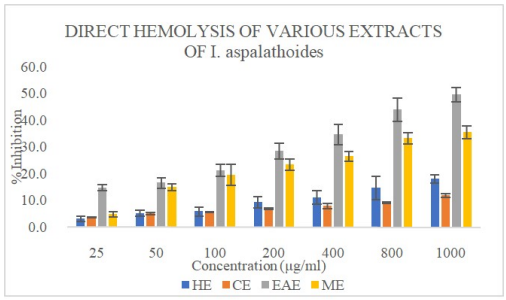

Fig. 3: PLA2 Inhibition activity of various extracts of *I. aspalathoides*

activity at 1000 µg/mL (50.9 ± 2.6%), with an IC_{50} of 70.4 ± 2.63 µg/mL. The methanol extract showed moderate inhibition (37.3 ± 2.3%, IC_{50} = 93.74 ± 2.02 µg/mL), whereas hexane and chloroform extracts were less active. Among all extracts, the ethyl acetate fraction demonstrated the strongest enzyme inhibition, suggesting the presence of bioactive compounds capable of suppressing phospholipase A_2 activity, which is relevant in anti-inflammatory and anti-venom contexts (Fig. 3)

Direct Hemolysis for all four extracts of *I. aspalathoides*

In the direct hemolysis assay, the ethyl acetate extract of *Indigofera aspalathoides* demonstrated the highest venom neutralizing activity at 1000 µg/mL (49.7 ± 2.7%), with an IC_{50} of 75.26 ± 2.7 µg/mL. The methanol extract showed moderate inhibition (35.6 ± 2.4%, IC_{50} = 98.64 ± 2.1 µg/mL), while hexane and chloroform fractions exhibited comparatively weaker activity. The superior performance of the ethyl acetate extract reinforces its potential in neutralizing hemolytic effects of venom, likely through inhibition of cytotoxic phospholipase or membrane-active components (Fig. 4).

Based on the outcomes of preliminary phytochemical screening, quantitative estimation of phytoconstituents and *In vitro* bioassays, the ethyl acetate extract of *Indigofera aspalathoides* which exhibited the

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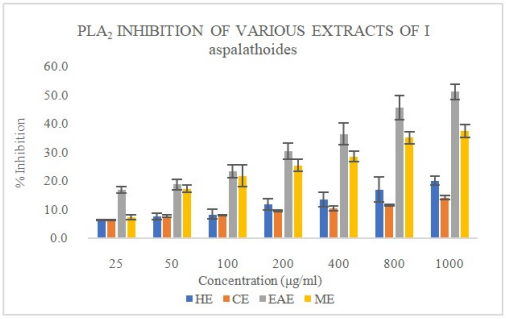

Fig. 4: Direct Heamolysis of Various extracts of *I. aspalathoides*

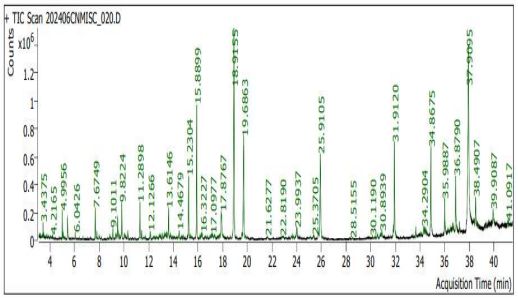

Fig. 5: GC-MS Spectra of Ethyl acetate extract of *I. aspalathoides*

most significant biological activity was selected for further phytochemical investigation using gas chromatography, followed by isolation and structural characterization of the active constituents.

GC-MS Analysis of Ethyl acetate extract of *Indigofera aspalathoides*

GC-MS analysis identified multiple phytocompounds in the ethyl acetate extract, including D-limonene, p-cymene, phytol, dibutyl phthalate, and 2,4-di-tert-butylphenol (Table 4). These compounds are known for their antioxidant and venom-neutralizing properties. Notably, dibutyl phthalate (13.58%) and 2-bromo-4,6-di(tert-butyl) phenol mesylate (17.08%) were present in significant quantities, highlighting their potential contribution to the observed biological activities (Fig. 5).

Isolation of Bio-active Compounds from *Indigofera aspalathoides*:

The ethyl acetate extract of *Indigofera aspalathoides*, selected based on its superior bioactivity, was subjected to column chromatography using a gradient elution system with increasing polarity. Solvent mixtures ranged from non-polar to polar, beginning with 100% hexane and gradually increasing the proportion of chloroform, ethyl acetate and methanol in 10% increments. This stepwise polarity gradient facilitated the differential separation of phytoconstituents based on their relative affinities for the stationary and mobile phases.

Thin Layer Chromatography (TLC) was employed to monitor the collected fractions. Visualization was performed under UV light and in an iodine vapor chamber. Fractions displaying similar Rf values were pooled and designated collectively as EAIA (Ethyl Acetate fractions of *Indigofera aspalathoides*). In total, eight pooled fractions were obtained. These were subsequently evaluated for their antioxidant and venom-neutralizing activities to identify the bioactive constituents.

Characterization of Bio-active Compounds from *Indigofera aspalathoides*:

Ultra-Violet (UV) Spectroscopy: The UV-Visible spectra of isolated fractions (EAIA 1–8) from the ethyl acetate extract of *Indigofera aspalathoides* showed λmax values between 328.95 nm and 378.98 nm, indicating conjugated systems. EAIA 1 and 2 had single peaks (336.04 nm and 330.15 nm), EAIA 3 showed multiple bands (328.95, 355.15, 378.98 nm) suggestive of extended conjugation, and EAIA 7–8 exhibited broad peaks typical of complex chromophores. These features point to aromatic systems, conjugated dienes/polyenes, heteroaromatic rings, and conjugated carbonyls. Fractions EAIA 1, 2, 3, and 5 were selected for further FTIR analysis.

Fourier Transform- Infra Red (FTIR): Fractions EAIA 1, 2, 3, and 5, selected based on their distinct UV absorption

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Table 4: GC-MS Spectral data of ethyl acetate extract of *I. aspalathoides*

S.No	Compound	Molecular Formula	Molecular Weight (g/mol)	Retention Time (min)	Peak Area%
1.	1-Decene	$C_{10}H_{20}$	140.2	3.4375	0.23
2.	D-Limonene	$C_{10}H_{16}$	136.1	3.7301	0.06
3.	Octane, 2,4,6-trimethyl-	$C_{11}H_{24}$	156.2	3.8579	0.11
4.	Nonanal	$C_9H_{18}O$	142.1	4.2165	0.11
5.	p-Cymene	$C_{10}H_{14}$	134.1	4.4391	0.03
6.	1-Dodecene	$C_{12}H_{24}$	168.2	4.9956	0.69
7.	Benzaldehyde, 3,4-dimethyl-	$C_9H_{10}O$	134.1	5.4201	0.87
8.	Undecane, 2-methyl-	$C_{12}H_{26}$	170.2	6.0426	0.17
9.	Tetradecane	$C_{14}H_{30}$	198.2	7.7944	0.26
10.	Nonane, 2-methyl-5-propyl-	$C_{13}H_{28}$	184.2	8.8579	0.23
11.	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{20}O_2$	220.1	9.1011	0.46
12.	Hexadecane	$C_{16}H_{34}$	226.3	9.3236	0.17
13.	Trichloroacetic acid, 2-ethylhexyl ester	$C_{10}H_{17}Cl_3O_2$	274.0	9.3690	0.23
14.	Dodecane, 2,7,10-trimethyl-	$C_{15}H_{32}$	212.3	9.4803	0.84
15.	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.2	9.8224	1.50
16.	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	198.2	10.3088	0.28
17.	2-Bromo dodecane	$C_{12}H_{25}Br$	248.1	12.1266	0.42
18.	Tridecane, 6-methyl-	$C_{14}H_{30}$	198.2	13.0046	0.21
19.	3-Octadecene, (E)-	$C_{18}H_{36}$	252.3	15.2304	2.57
20.	Isopropyl myristate	$C_{17}H_{34}O_2$	270.3	15.8899	6.16
21.	Tetradecane, 1-iodo-	$C_{14}H_{29}I$	324.1	16.3227	0.45
22.	Phytol	C20H40O	296.3	16.9864	0.22
23.	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.2	18.9155	13.58
24.	2-Bromo-4,6-di(tert-butyl) phenol mesylate	$C_{15}H_{23}BrO_3S$	362.1	37.9095	17.08
25.	Octadecane, 1-(ethenyloxy)-	$C_{20}H_{40}O$	296.3	39.9087	1.00

profiles, were further subjected to structural characterization using Fourier Transform Infrared (FTIR) spectroscopy to identify key functional groups present within the active constituents (Figs. 6-9).

FTIR analysis of EAIA-1 revealed functional groups indicative of a complex organic structure. Key absorption peaks included C–Cl and C–Br stretches (531.71 and 570.73 cm^{-1}), aromatic C–H bending (723.58–966.51 cm^{-1}), C–O and C–N stretches (1029.41–1239.88 cm^{-1}), aliphatic C–H bending (1374.09 and 1460.35 cm^{-1}), and a strong carbonyl (C=O) stretch at 1738.91 cm^{-1}. CH□ and CH□ stretching at 2851.56 and 2918.95 cm^{-1} further supported the presence of aliphatic chains. These features suggest EAIA-1 is a halogenated aromatic ester with additional nitrogen- and oxygen-containing functional groups.

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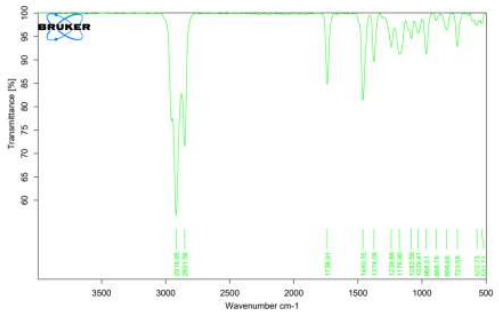

Fig. 6: FTIR spectrum of EAIA 1

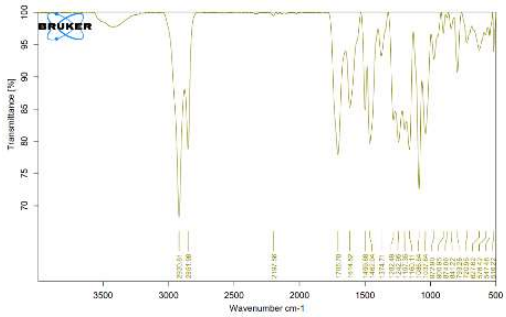

Fig. 7: FTIR spectra of EAIA 5

FTIR analysis of EAIA-2 revealed a diverse array of functional groups. Characteristic peaks for halogenated groups (C–Cl at 548.65 cm^{-1} and C–Br at 600.06 cm^{-1}), aromatic C–H bending and C=C stretching (722.09–1614.58 cm^{-1}), alcohols and ethers (C–O stretching at 1029.64 and 1083.72 cm^{-1}), and amines/amides (C–N stretch at 1166.40 cm^{-1}) were observed. Additional bands indicated aliphatic C–H vibrations, a strong carbonyl stretch (C=O at 1711.46 cm^{-1}), a nitrile group (C≡N at 2206.26 cm^{-1}), and broad O–H stretching (3403.86 cm^{-1}). These spectral features suggest EAIA-2 is a structurally complex, halogenated aromatic compound containing polar functional groups such as alcohols, carbonyls, amines, and nitriles.

FTIR spectral analysis of EAIA-5 revealed the presence of multiple functional groups. Peaks corresponding to C–Cl (516.22 cm^{-1}) and C–Br (547.48 cm^{-1})

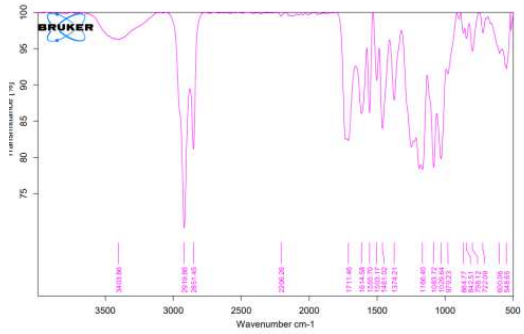

Fig. 8: FTIR spectra of EAIA 2

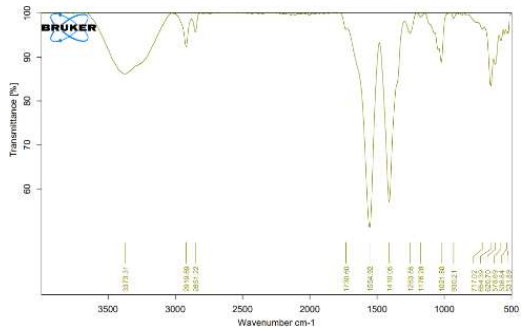

Fig. 9: FTIR Spectra of EAIA 7

confirmed halogenation. Aromatic C–H bending and C=C stretching appeared prominently between 576.42 and 1614.52 cm^{-1}, while aliphatic C–H vibrations and C–H bending were noted from 972.90 to 1462.04 cm^{-1}. Strong absorptions due to C–O (1037.64, 1086.64 cm^{-1}), C–N (1160.11, 1197.35 cm^{-1}), and C=O (1705.70 cm^{-1}) indicated the presence of alcohols, amines/amides, and carbonyl-containing compounds. A nitrile group was identified at 2197.56 cm^{-1}, and characteristic CH_2 and CH_3 stretches were observed at 2851.98 and 2920.61 cm^{-1}. These findings suggest that EAIA-5 is a halogenated aromatic compound with rich functional diversity, including polar and aliphatic features.

FTIR analysis of EAIA-7 revealed characteristic absorptions for halogenated groups, including C–Cl (531.89 cm^{-1}) and C–Br (536.84 cm^{-1}), along with prominent

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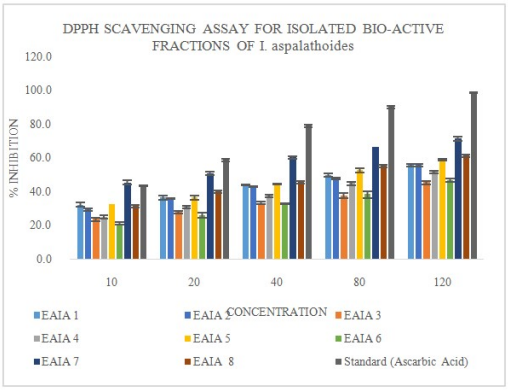

Fig. 10: DPPH Radical Scavenging assay isolated fraction from Ethyl acetate extract of *I. aspalathoides*

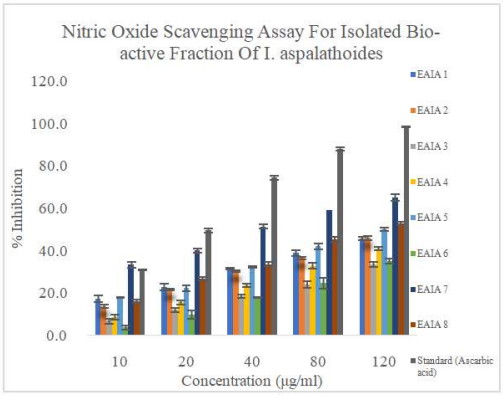

Fig. 11: Nitric oxide scavenging activity of isolated fractions from ethyl acetate extract of *I. aspalathoides*

aromatic C=C stretching and C–H bending bands between 578.69 and 1554.92 cm^{-1}. Aliphatic C–H bending (930.21, 1410.05 cm^{-1}), C–O stretching (1021.80, 1176.28 cm^{-1}), and C–N stretching (1253.85 cm^{-1}) indicated the presence of alcohols, ethers, and amines. A strong carbonyl stretches at 1730.60 cm^{-1} confirmed the presence of ketones, aldehydes, or esters. Additional CH_2 and CH_3 stretches were noted at 2851.22 and 2919.89 cm^{-1}, and broad O–H stretching at 3373.31 cm^{-1} suggested alcohol or phenolic groups. These features collectively point to a multifunctional aromatic compound with halogenated, aliphatic, and polar moieties.

In vitro Anti-Oxidant Assay for Isolated fractions EAIA

DPPH Radical scavenging Assay for Isolated Bio-active fractions

The antioxidant potential of the EAIA fractions was evaluated using the DPPH radical scavenging assay. Among the tested fractions, EAIA-7 exhibited the highest activity at 120 µg/mL, with 71.3 ± 1.25% inhibition and an IC_{50} of 45.26 ± 0.84 µg/mL. Fractions 8 and 5 showed moderate activity (61.1 ± 0.64% and 59.0 ± 0.56%, respectively). The IC_{50} values for other fractions ranged between 58.64 ± 0.63 and 94.09 ± 0.95 µg/mL, with EAIA-3 being the least active. These findings indicate that fraction 7 contains the most potent antioxidant constituents, reinforcing the efficacy of the ethyl acetate extract and validating the phytochemical separation approach (Fig. 10).

Nitric oxide scavenging Assay for Isolated bio-active fractions

The nitric oxide scavenging assay revealed that among the EAIA fractions, EAIA-7 exhibited the strongest activity at 120 µg/mL, with 64.9 ± 1.52% inhibition. Fractions 8 and 5 showed moderate effects, recording 52.5 ± 0.79% and 49.9 ± 0.68%, respectively. The IC_{50} values of the fractions ranged from 75.27 ± 0.77 to 106.0 ± 1.01 µg/mL, with EAIA-3 exhibiting the lowest activity (IC_{50} = 114.10 ± 1.16 µg/mL). These results confirm that EAIA-7—and to a lesser extent, EAIA-5—retain potent nitric oxide scavenging activity, supporting their role as key antioxidant constituents within the ethyl acetate extract (Fig. 11).

In-vitro snake venom neutralizing activity for isolated fractions from ethyl acetate extract of *I. aspalathoides*

PLA2 Inhibition assay

The phospholipase A_2 (PLA_2) inhibition assay demonstrated that EAIA-7 had the highest venom-neutralizing activity at

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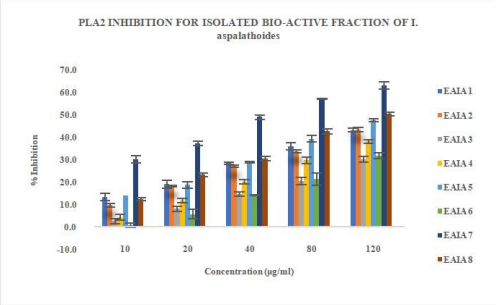

Fig. 12: PLA2 inhibition activity for isolated fractions from ethyl acetate etract of *I. aspalathoides*

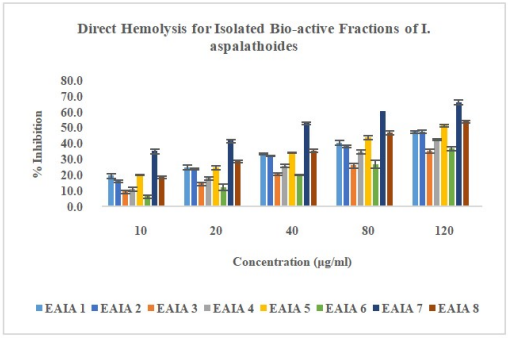

Fig. 13: Direct Haemolysis of isolated fractions from ethyl acetate extract of *I. aspalathoides*

120 µg/mL, with 63.4 ± 1.59% inhibition and an IC_{50} of 55.38 ± 1.07 µg/mL (Fig. 12). Fractions 8 and 5 also showed moderate inhibition at the same concentration (50.4 ± 0.82% and 47.8 ± 0.71%, respectively), with corresponding IC_{50} values ranging from 110.43 ± 0.90 to 185.04 ± 1.43 µg/mL. Fraction 3 exhibited the lowest activity (IC_{50} = 203.22 ± 1.21 µg/mL). These findings suggest that EAIA-7 possesses significant PLA_2 inhibitory potential, supporting its role in neutralizing venom-related enzymatic activity.

Direct Heamolysis activity

In the direct hemolysis assay, EAIA-7 displayed the strongest venom-neutralizing activity at 120 µg/mL, achieving 65.9 ± 1.48% inhibition with an IC_{50} of 49.79 ± 0.99 µg/mL. Fractions 8 and 5 showed moderate inhibition (53.91 ± 0.76% and 51.4 ± 0.66%, respectively), with IC_{50} values of 98.73 ± 0.84 µg/mL and within the range of 108.44 ± 0.75 to 229.52 ± 0.99 µg/mL for the other fractions (EAIA 1–5). These results further highlight the potent anti-venom activity of EAIA-7, likely due to the presence of active constituents that inhibit membrane lysis and enzymatic hemotoxicity (Fig. 13).

When compared to the other fractions, EAIA-7, EAIA-8, and EAIA-5 from the ethyl acetate extract of *Indigofera aspalathoides* demonstrated notable *In vitro* antioxidant and venom-neutralizing activities, particularly at higher concentrations.

Conclusion

The present study identified the ethyl acetate extract of *Indigofera aspalathoides* as a rich source of phenolics, flavonoids, tannins, and alkaloids, exhibiting strong *in vitro* antioxidant activity and moderate venom-neutralizing potential. This justified its fractionation and bioactivity-guided analysis, which revealed EAIA-7 as the most potent antioxidant fraction with notable anti-venom activity. Preliminary UV–Visible and FTIR analyses of four active fractions (EAIA-1, EAIA-2, EAIA-3, and EAIA-5) indicated conjugated and aromatic systems, suggesting distinct bioactive compounds and warranting further NMR and mass spectrometric studies for complete characterization. The findings support the therapeutic potential of the extract and its fractions, particularly EAIA-7, in managing oxidative stress-related disorders and as adjuncts to conventional antivenoms, especially for snakebite and possibly insect venom toxicity. Future work should focus on isolating individual compounds, elucidating mechanisms of action, and evaluating safety and efficacy in in vivo and clinical models, positioning *Indigofera aspalathoides* as a promising candidate for novel antioxidant and anti-venom phytotherapeutics.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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