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Author Affiliation:
[1]Department of Biochemistry, Faculty of Life Sciences, Abdullahi Fodio University of Science and Technology, Aliero, Nigeria
[2]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria

[*]**Corresponding Author**
Abdulhamid Zubairu,
Department of Biochemistry, Faculty of Life Sciences, Abdullahi Fodio University of Science and Technology, Aliero, Nigeria,
E-mail and Phone No.: mrconfirmre@gmail.com, +2348062794160

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Bioactivity-Guided Isolation and *In Silico* Molecular Studies of Anti *Bitis arietans* Venom from *Faidherbia albida* (Delile) A. Chev Root-Bark Extracts

Ibrahim Sani[1], Angela Nnenna Ukwuani-Kwaja[1], Amina Yusuf Jega[2], Abdulhamid Zubairu[1]*, Fatima Bello[1]

ABSTRACT

Snake venoms contain several life-threatening toxins, while conventional therapies are available for the snake envenoming. The limitations of these antivenins have driven recent research toward the isolation and characterization of plant-derived antivenin compounds that complement synthetic antivenins. This research is designed to conduct bioactivity-guided isolation and *in silico* molecular studies of anti-*Bitis arietans* venom from *Faidherbia albida* root-bark extracts. Solvent fractionation, column, and thin-layer chromatography were used for the extraction and isolation of the plant compound. For the *in-vivo* studies, Albino rats were grouped into eight (8) groups of four (4) animals each: group I: normal control, group II: induced control, group III: antivenin control, groups IV, V, VI, VII, and VIII received crude methanol extract, hexane, ethyl-acetate, butanol, and aqueous fractions respectively. Each at 300mg/kg body weight. Chromatographic techniques (column and thin-layer), GC-MS, FTIR and UV were used to isolate and identify the most potent fraction. Standard procedures were employed to determine venom phospholipase A_2 and metalloproteinase inhibition in identifying the most active chromatographic fraction. *In silico* studies were used to conduct molecular docking. *F. albida* root aqueous fraction exhibits a significantly ($P<0.05$) higher average survival time compared to other fractions. Twenty-one (21) pooled fractions were obtained from the column and thin-layer chromatography of the most potent fraction. Pool chromatographic fraction 18 (PCF-18) exhibited the highest inhibitory activity against *B. arietans* venom phospholipase A_2 and metalloproteinase. GCMS, FTIR and UV of the PCF-18 revealed that alpha-methyl cinnamic acid chloride is the principal constituent of the fraction. *In silico* studies showed that alpha-methyl cinnamic acid chloride has strong binding affinities of -4.3 and -6.3 with phospholipase A_2 and metalloproteinase respectively. Hence, this study isolated and identified the antivenom compound from the aqueous extract of *F. albida* root-bark and documented enzyme inhibition as one of the possible mechanisms of action by which the compound; alpha-methyl cinnamic acid chloride exerts its antivenom activity against *B. arietans* venom.

Keywords: *Bitis arietans*, Venom, *Faidherbia albida*, Bioactivity-guided isolation, *In Silico*

1. INTRODUCTION

Puff adder (*Bitis arietans*) is a clinically essential snake species found across several countries, they are highly venomous viper species which are widely spread around sub-Saharan African countries (Dawson *et al*., 2024). The venom contains certain venom enzymes, such as L-amino acid oxidase which produces hydrogen peroxide and result to the formation of damaging reactive oxygen species. However, documenting the accurate National prevalence is difficult due to poor data reporting. The puff adder is frequently recognized among the three deadliest venomous snakes in Nigeria (Dawson *et al*., 2024).

Although the sole approved, effective and clinical therapy for acute snake envenomation is conventional anti-venom, a fundamental approach to search for natural compounds as antidotes for venom therapies to tackle prolonged inflammation, oxidative stress and tissue damage is advancing (Oliveira *et al*., 2022). Recently, studies are exploiting the isolation and characterization of plant-derived venom neutralizing agent that target several mechanism such as antioxidants, enzyme inhibition and anti-inflammation to complement the limitations of conventional antivenom therapy (Sadek *et al*., 2024).

A significant number of medicinal plants contribute vitally to the management of several diseases (Dalir *et al*., 2025). The *Faidherbia albida* plant is utilized in traditional system to treat various diseases like dermatitis, wound healing, stomach pain, etc. Pharmacologically, *F. albida* has been reported to have anti-inflammatory, anti-pyretic and antioxidant properties (Abdelwahab *et al*., 2024; Bashir *et al*., 2025). Recent survey documents the utilization of *F. albida* root in treating of snakebite victims by herbalist in Northern Nigeria (Mohammed *et al*., 2024). Therefore, proper isolation and Characterization of this plant can play a significant role in the identification of potent, affordable, broad-spectrum, less-toxic plant-based antivenom compounds.

2. MATERIALS AND METHODS

Experimental Animals

The Animals model (albino rats) used in this research were purchased from Usman Danfodiyo University, Sokoto, in February 2024. The rats are kept in Animal House, Faculty of Life Science, Abdullahi Fodio University of Science and Technology, Aliero, in well-ventilated cages. Before the trial started, the rats are kept in a clean cage and given fourteen (14) days to acclimatize. The rats are given free access to animal feed and water.

Standard Anti-Snake Venom

Snake venom (polyvalent) antisera with batch number 8904012480039, produced in November 2022, with an expiration date of October 2026, produced by Bharat Serums and Vaccines Limited, India, was used as the standard anti-venom.

Collection, Milking, and Preparation of *B. arietans* Venom

The Collection of *B. arietans* snake species, milking, and preparation of its venom are conducted, adapting the same procedures reported in a previous study by Sani *et al*. (2025).

Methanol Extraction

One hundred and fifty grams (150g) of the semi-powder of *Faidherbia albida* root was soaked in 1 liter of 99.8% methanol for 72 hours (3 days), with stirring every morning using a spatula and resealing the container with masking tape to prevent methanol from escaping. The sample is subsequently filtered using muslin fabric. The filtrate is concentrated using rotator evaporator, after which the concentrated crude is exposed to allow the remaining methanol to evaporate. The solid extract is preserved in a fridge for further use (Dupont *et al*., 2002).

Solvent-fractionation of *Faidherbia albida* Root Crude Methanol Extract

The *Faidherbia albida* root crude methanol extract was separated into n-hexane, ethyl acetate, butanol, and water fractions by liquid-liquid extraction. Fifty grams (50 g) of the methanol extract is diluted in 50ml of water in a 500ml separating funnel, and was then mixed (partitioned) with 50ml of hexane, ethyl acetate, butanol, and water; these solvents were introduced progressively in order of increasing polarity to obtain hexane, ethyl acetate, butanol, and aqueous fractions, respectively. The solvents were evaporated, and the fraction yields were preserved at -4°C for future use (Kupchan, 1962).

Anti-venom Activity Screening of *Faidherbia albida* Root Crude Methanol Extract and its Fractions

Animals are randomly distributed into eight (8) groups containing four (4) rats per group. Thereafter, extract and venom administrations were conducted as follows: group I: only received distilled water orally and served as normal control, group II: Received intraperitoneally (i.p.) injection of the snake venom LD_{100} and termed venom control. Group III (standard control): is injected with snake venom LD_{100} (i.p.), and treated with antivenin (i.v.) 1ml/0.45 mg venom. Group IV, V, VI, VII, and VIII also received snake venom LD_{100} (i.p.), followed by 30 minutes post-treatment with *F. albida* root crude methanol extract, hexane, ethyl acetate, butanol, and aqueous fractions at 300mg/kg b. wt respectively. The duration and frequency of rat survival are recorded within 24 hrs (Premendran *et al*., 2011).

Column Chromatography

Silica gel sized (80-120 mesh) slurry prepared using N-hexane is gently introduced inside the column, and the slurry is allowed to stabilize to form an unbroken packing. Then, the excess N-hexane in the column was collected through the stop-cock of the column. A flat bed made of cotton wool is arranged on top of the silica gel. About (3g) of dry powdered crude fractionated aqueous extract is added to the cotton bed and covered with another cotton bed, and allowed to be activated for 30minutes. Thereafter, the column was successfully eluted with a gradient solvent system comprising N-hexane, ethyl acetate, methanol, and water, either singly or in various ratios.

Thin Layer Chromatography

A TLC plate about 4.0cm wide and 10cm long was used. A small amount of each column chromatographic fraction ($CCF_{1\text{-}156}$) is spotted on a TLC plate. Then, the plates are placed into a TLC chamber containing the solvent system (methanol and water in specified ratios) to a depth of 1cm, and the chamber is covered for a few minutes. Spot are visualized by dipping the plate into a covered container containing a solution of 10% V/V H_2SO4 and 5% W/V vanillin in methanol. The retardation factor (Rf) is calculated using the formula below, and bottle fractions with similar RF values are pooled together (Hao *et al*., 2004).

$$Rf\,\text{Value} = \frac{\text{Distance moved by the molecule (spot)}}{\text{Distance moved by the mobile phase (Solvent Front)}}$$

Phospholipase A_2 Inhibition Assay

The acidimetric method of Tan and Tan (1988) was employed to measure venom phospholipase A_2 activity. The substrates in constant volumes consisting of calcium chloride (18mM), egg yolk, and 8.1 mM sodium deoxycholate were merged and mixed continuously for 10min to obtain a suspension of homogenous egg yolk. One (1 M) NaOH is used to adjust the pH to 8.0 of the mixture. The process of hydrolysis was initiated by adding snake venom (3 mg/ml) (15 ml), and a test tube containing normal saline only serves as a control. A decline in pH of the mixture was recorded after two (2) minutes using a pH meter. One (1) unit decline in pH is proportional to one hundred and thirty-three (133) µmole of fatty acid released. Enzyme activity is reported as µmole of fatty acid released per minute. The catalytic and percentage enzyme activity are calculated using the formulae below:

$$\text{Enzyme Activity (µmole FA/min)} = \frac{\text{µmole of FA released}}{\textit{time taken in minutes}} \quad \text{Where: FA = Fatty acid}$$

$$\%\ \text{Activity} = \frac{\text{Enzyme Activity of the test sample}}{\text{Enzyme Activity of venom control}} \times 100$$

Proteinase Inhibition Assay

The venom proteinase activity is assayed by adapting the protocol established by Greenberg *et al*. (1995). The reaction mixture, which included 0.5% casein, Tris–HCl buffer (pH 8.0), and crude venom at 2.5mg/ml in distilled water, was incubated for 4 hours at 37 °C in an incubator. After incubating, trichloroacetic acid (TCA) was introduced to halt the reaction and subsequently filtered. Thereafter, (1.0 ml) of the filtrate was utilized for protein quantification employing the method described by Lowry *et al*. (1951). L-tyrosine served as the standard. In this investigation, a unit of enzyme activity is defined as the quantity that produced 0.02 µmole of tyrosine per hour.

Gas Chromatography Mass Spectroscopy Analysis

The oven temperature is programmed to increase from 80°C to 280°C at 30°C/min. The injection temperature is maintained at 250°C in split mode. Helium gas (99.9%) served as the carrier gas at a flow rate of 1.58 ml/min, and the total GC-MS analysis time was 27 minutes. Ionization for mass spectrometric analysis is performed at 70 eV. Mass spectra are captured over a range of 40 to 600 m/z for a total of 28 minutes. Standard mass spectra from National Institute of Standards and Technology (NIST-LIB 0.5) libraries are used to identify the individual components from the sample, which are integrated into the GCMS software (Wiley GC-MS-2007) alongside relevant literature data. The various polyphenols present in the crude extract are effectively separated via the gas chromatography column. Upon separation, each compound that exited the GC is introduced into the Mass Spectrometer (MS) for ionization. The resulting MS ionization spectrum was recorded and subsequently compared to the MS spectra of known compounds within the NIST library. Each compound's identity was assessed based on a percentage score derived from both reverse and forward spectrum comparisons (Ferrer and Thurman, 2013).

Fourier Transform Infrared Spectroscopy

The FTIR analysis of PCF18 was performed using standard light sources and a TGS detector, with a resolution of 4 cm−1. A precise 1 ml solution of the sample was freeze-dried and dispersed, then covered with 100 mg potassium bromide (KBr) to create a thin, translucent sample disc for FT-IR analysis. This disc is then positioned in a sample cup equipped with a diffuse reflectance accessory. Investigations are conducted using an IRPrestige-21 (Shimadzu). The scanning absorption range extended from 400 to 4000 cm−1.

Ultraviolet-visible Spectroscopy

The PCF18 underwent UV-VIS scanning analysis. The sample was diluted 30-fold with the appropriate extraction solvent (methanol) at room temperature. Absorbance spectra are captured across 200 to 800 nm wavelengths with the aid of a spectrophotometer (UV-VIS).

Molecular Docking Studies

Molecular docking is performed utilizing the docking algorithm protocols of AutoDock Vina Tools 1.5.7 (ADVT) (MGLTools 1.5.7) through the conversion of ligands and protein structure into (pdbqt) format. Polar hydrogen was incorporated into the protein receptors, non-polar hydrogen is merged, and Gasteiger charges are subsequently calculated, followed by the assignment of AutoDock (ADVT 1.5.7) atom types. Python scripts from ADVT and custom scripts are used to assign torsions to the ligands automatically. The search area was also defined using ADVT 1.5.7. Initially, the native ligand is re-docked into the selected *B. arietans* receptors to reproduce the original poses; subsequently, the ligands are docked into each of the major enzyme receptors of *B. arietans*, replicating the parameters used for the native ligand re-docking. For the docking experimental procedure, both the protein and the ligands are loaded into ADVT 1.5.7 (Trott and Olson, 2010; Toyang and Verpoorte, 2013).

Data Analysis

The results of this study were presented in figures, plates, and tables of mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Duncan's Multiple comparison Test were used for statistical analysis of data and $P<0.05$ was considered significant using SPSS software (version 20).

3. RESULTS

The neutralization activity of *Faidherbia albida* root solvent fractions against *Bitis arietans* venom are presented in Table 1. The result revealed non-significant ($P>0.05$) differences in mean survival time between the antivenin control and the normal group. There were no significant ($P>0.05$) differences between the mean survival time of *Faidherbia albida* root hexane and ethylacetate fractions compared to the induced control. Meanwhile, the mean survival time of *Faidherbia albida* root butanol and aqueous fraction significantly ($P<0.05$) increased compared to the induced control. However, a higher activity was observed in the *Faidherbia albida* root aqueous fraction and is selected for further studies.

Table 1: Anti-venom Effect of *Faidherbia albida* Root Solvent Fractions against *Bitis arietans* Venom LD_{100}

Treatment	Survival / No. of Animals Used	% Survival	Mean Survival Time (h)
Normal Control (0.5ml normal saline IP)	$\frac{4}{4}$	100	24.00±0.00[d]
Negative Control-	$\frac{0}{4}$	0	2.75±0.19[a]
Positive Control (polyvalent antivenim) 1ml/0.45mg Venom	$\frac{4}{4}$	100	24.00±0.00[d]
Faidherbia albida Root Crude Extract 300mg/kg	$\frac{0}{4}$	0	17.39±1.22[b]
Faidherbia albida Root Hexane Fraction 300mg/kg	$\frac{0}{4}$	0	3.99±0.38[a]
Faidherbia albida Root Ethyl acetate Fraction 300mg/kg	$\frac{0}{4}$	0	4.37±0.79[a]
Faidherbia albida Root Butanol Fraction 300mg/kg	$\frac{1}{4}$	25	16.01±0.79[b]
Faidherbia albida Root Aqueous Fraction 300mg/kg	$\frac{3}{4}$	75	21.27±1.19[c]

Values are presented as mean ± SEM (n = 4). Values having different superscripts are significant ($P<0.05$). Analyzed with ANOVA through Duncan multiple comparison test using SPSS version 20.0.

Column Chromatographic Fractions

A total of one hundred and fifty-seven (157) eluted fractions are collected from the column chromatography. Based on the thin-layer chromatography, fractions with similarities in retention factor (R_f) values are pooled together Table 2.

Table 2: TLC-guided Pooled Chromatographic Fractions

Pooled Fractions	Fractions Composition	Weight of Fraction (mg)	Yield (%)	Rf values
PCF1	F1-F2	10.1	0.34	0.88
PCF2	F3-F12	35.31	1.18	0.84
PCF3	F13-F14	6	0.2	0.94
PCF4	F15-F16	16	0.5	0.86
PCF5	F17-F36	24	0.8	0.93
PCF6	F37-F39	7.2	0.24	0.89
PCF7	F40-F48	10.2	0.34	0.82
PCF8	F49-F53	100	3.33	0.81
PCF9	F54-F67	270	9	0.97
PCF10	F68-F77	90	3	0.91
PCF11	F78-F91	93	3.1	0.84

PCF12	F92-F94	15	0.5	0.94
PCF13	F95-F108	110	3.67	0.91
PCF14	F109-F118	50.1	1.67	0.99
PCF15	F119-F123	150	5	1
PCF16	F124-F131	95	3.17	0.82
PCF17	F132-F134	56	6.9	0.88
PCF18	F135-F142	207	1.87	0.94
PCF19	F143-F146	28	0.93	0.97
PCF20	F147-F149	12	0.4	0.93
PCF21	F150-F157	201	6.7	0.99

PCF= Pooled Chromatographic Fraction, F= Fraction

Inhibition of *B. arietans* Venom Phospholipase A_2 by Pooled Chromatographic Fractions of *F. albida Root*

Venom Phospholipase A2 inhibition by pooled fractions of *F. albida* root is presented in Table 3. All the pooled fractions (PCF1-PCF21) revealed significant ($P<0.05$) reductions in enzyme activity (µmole FA/min) compared to venom control. All the pooled fractions (PCF1-PCF21) significantly ($P<0.05$) increases in Phospholipase A_2 activity (µmole FA/min) compared to antivenin control, except fraction 18 which significantly ($P<0.05$) decreases in enzyme activity (µmole FA/min) compared to antivenin control. Additionally, only antivenin control, TLC-pooled fractions (PCF 11, and 18) revealed a slight decrease in pH indicating greater inhibitory activity.

Table 3: Inhibition of *B. arietans* Venom Phospholipase A_2 by Pooled Chromatographic Fractions

Venom (1mg/ml) + Test Material (1mg/ml)	Initial pH	pH changed after 3min	pH Difference	Fatty acid Released (µmole)	Enzyme Activity (µmole FA/min)
Venom	8	5.95	2.05	272.65	90.88 ± 0.51^{m}
Venom + Antivenin	8	7.32	0.68	90.44	30.14 ± 0.08^{b}
Venom + FAAFE	8	6.97	1.03	136.99	45.66 ± 0.38^{d}
Venom +PCF1	8	6.07	1.93	256.69	85.56 ± 0.57^{kl}
Venom +PCF2	8	6.08	1.92	255.36	85.12 ± 0.07^{kl}
Venom +PCF3	8	6.07	1.93	256.69	85.56 ± 0.57^{kl}
Venom +PCF4	8	6.10	1.90	252.70	84.23 ± 0.06^{jk}
Venom +PCF5	8	6.09	1.91	254.03	84.68 ± 0.57^{kl}
Venom +PCF6	8	6.24	1.76	234.08	78.03 ± 1.15^{e}
Venom +PCF7	8	6.17	1.83	243.39	81.13±0.08fg
Venom +PCF8	8	6.17	1.83	243.39	81.13±0.58fg
Venom +PCF9	8	6.19	1.81	240.73	80.24 ± 0.58^{efg}
Venom +PCF10	8	6.20	1.80	239.40	79.80 ± 3.38^{efg}
Venom +PCF11	8	7.03	0.93	129.01	43.00 ± 0.28^{c}
Venom +PCF12	8	6.07	1.93	256.69	85.56 ± 0.23^{kl}
Venom +PCF13	8	6.15	1.85	246.05	82.02 ± 0.01^{hij}
Venom +PCF14	8	6.12	1.88	250.04	83.35 ± 0.12^{hijk}
Venom +PCF15	8	6.15	1.85	246.05	82.02 ± 0.01^{hij}
Venom +PCF16	8	6.15	1.85	246.05	82.02 ± 0.01^{hij}
Venom +PCF17	8	6.22	1.78	236.74	78.91 ± 0.01^{ef}
Venom +PCF18	8	7.24	0.76	61.18	20.39 ± 0.05^{a}
Venom +PCF19	8	6.20	1.80	239.4	79.80 ± 0.09^{efg}
Venom +PCF20	8	6.18	1.82	242.06	80.69 ± 0.12^{fg}

Venom +PCF21	8	6.18	1.82	242.06	80.69±0.05^{fg}

FARAFE= *F. albida* root aqueous fractionated extract; PCF=Pooled chromatographic fraction. Values for enzyme activity are presented as mean ± SEM (n = 3) values in columns having the same superscript are not significantly different from the standard drug at (*P*>0.05) using One-Way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0. A decrease of pH by one (1) unit corresponded to 133 µmole of fatty acid released.

Inhibition of *B. arietans* Venom Proteases by Pooled Chromatographic Fractions of *F. albida* Root

The inhibition of *B. arietans* venom proteinases by TLC-pooled fractions of *F. albida* root is presented in Table 4. All the TLC-pooled fractions (PCF1-PCF21) revealed significant (P<0.05) decreases in enzyme activity compared to venom control, while all the TLC-pooled fractions (PCF1-PCF21) significantly (P<0.05) increases in enzyme (U/l) compared to antivenin control, except fraction 18, which showed insignificant (P>0.05) increase in enzyme activity compared to antivenin control.

Table 4: Inhibition of *B. arietans* Venom Proteases by Pooled Chromatographic Fractions of *F. albida Root*

Venom (1mg/ml) + Test Material (1mg/ml)	Absorbance	L-tyrosine Concentration (mg/ml)	Enzyme Activity (U/l)
Venom	0.705	2.25	0.0450±0.00^{o}
Venom + Antivenin	0.388	0.88	0.0176±0.00^{a}
Venom + FAAFE	0.400	0.93	0.0186±0.00^{bc}
Venom +PCF1	0.658	1.99	0.0398±0.00^{m}
Venom +PCF2	0.666	2	0.0400±0.00^{m}
Venom +PCF3	0.601	1.8	0.0360±0.00^{j}
Venom +PCF4	0.534	1.5	0.0300±0.00^{h}
Venom +PCF5	0.664	2.09	0.0418±0.00^{n}
Venom +PCF6	0.607	2.08	0.0416±0.00^{n}
Venom +PCF7	0.633	1.9	0.0380±0.00^{l}
Venom +PCF8	0.634	1.9	0.0380±0.00^{l}
Venom +PCF9	0.606	1.83	0.0366±0.00^{jk}
Venom +PCF10	0.632	1.9	0.0380±0.00^{l}
Venom +PCF11	0.412	0.95	0.0190±0.00^{cd}
Venom +PCF12	0.512	1.35	0.0270±0.00^{f}
Venom +PCF13	0.630	1.9	0.0380±0.00^{l}
Venom +PCF14	0.625	1.85	0.0370±0.00^{k}
Venom +PCF15	0.486	1.32	0.0264±0.00^{f}
Venom +PCF16	0.523	1.45	0.0290±0.00^{g}
Venom +PCF17	0.470	1.25	0.0250±0.00^{e}
Venom +PCF18	0.390	0.09	0.0180±0.00^{ab}
Venom +PCF19	0.508	1.35	0.0270±0.00^{f}
Venom +PCF20	0.408	0.99	0.0198±0.00^{d}
Venom +PCF21	0.541	1.55	0.0310±0.00^{i}

FARAFE= *F. albida* root aqueous fractionated extract; PCF=Pooled chromatographic fraction. Values for enzyme activity are presented as mean ± SEM (n = 3). Columns with the same superscript are not significantly different from the standard drug (P > 0.05) using One-Way ANOVA, followed by Duncan multiple comparison test with SPSS version 20.0.

Identification of Compounds from Pooled TLC Fraction 18

GC-MS Analysis of Pooled TLC Fraction 18

GC-MS analysis of pooled chromatographic fraction 18 identified 10 distinct peaks, suggesting the presence of 10 potential compounds (Figure 1). All the compounds and their library similarity index are presented in Table 5.

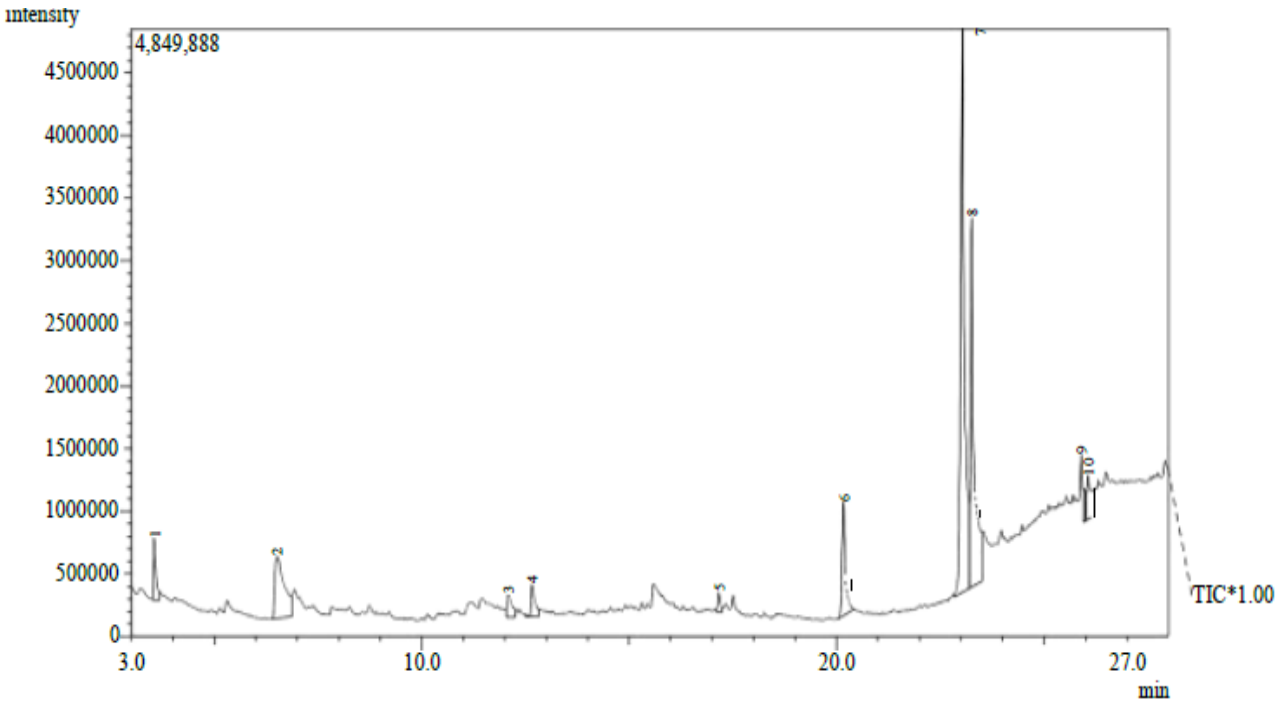


Figure 1: GC-MS Spectra for Pooled Chromatographic Fraction 18

Table 5: GC-MS Analysis of Pooled Chromatographic Fraction 18

Peaks	Possible compounds	Similarity Index
1	2,3-Butanediol	98
2	glycerin,	93
	glyceraldehyde	84
	1, 2,3,4 butanetetrol	83
3	2-Cycloocten-1-one,	77
	4-Oxatricyclo[4.3.1.1(3,8)]undecan-5-one	76
	3-Cyclohexene-1-carboxylic acid	76
4	alpha.-Methylcinnamic acid,	84
	2-Indanecarboxylic acid	79
	Benzocyclobutene	79
5	Ethyl linalool	77
	2-Hexadecen-1-ol	76
	2-Hydroxy-1,1,10-trimethyl-6,9-epidioxydecalin	80
	13-Methylpentadec-14-ene-1,13-diol	76
6	Octadecanoic acid	91
	Tetradecanoic acid	90
	Pentadecanoic acid	91
	n-Hexadecanoic acid	94
7	Oleic Acid	93
	Erucic	90
	Cyclopentadecanone	89
8	Octadecanoic acid	96
9	1,2-15,16-Diepoxyhexadecane,	86

	9-Hexadecenal,	86
	7-Tetradecenal,	86
	13-Octadecenal	86
	Cyclododecene epoxide	85
10	Docosanoic anhydride	70
	Docosanoic acid	70

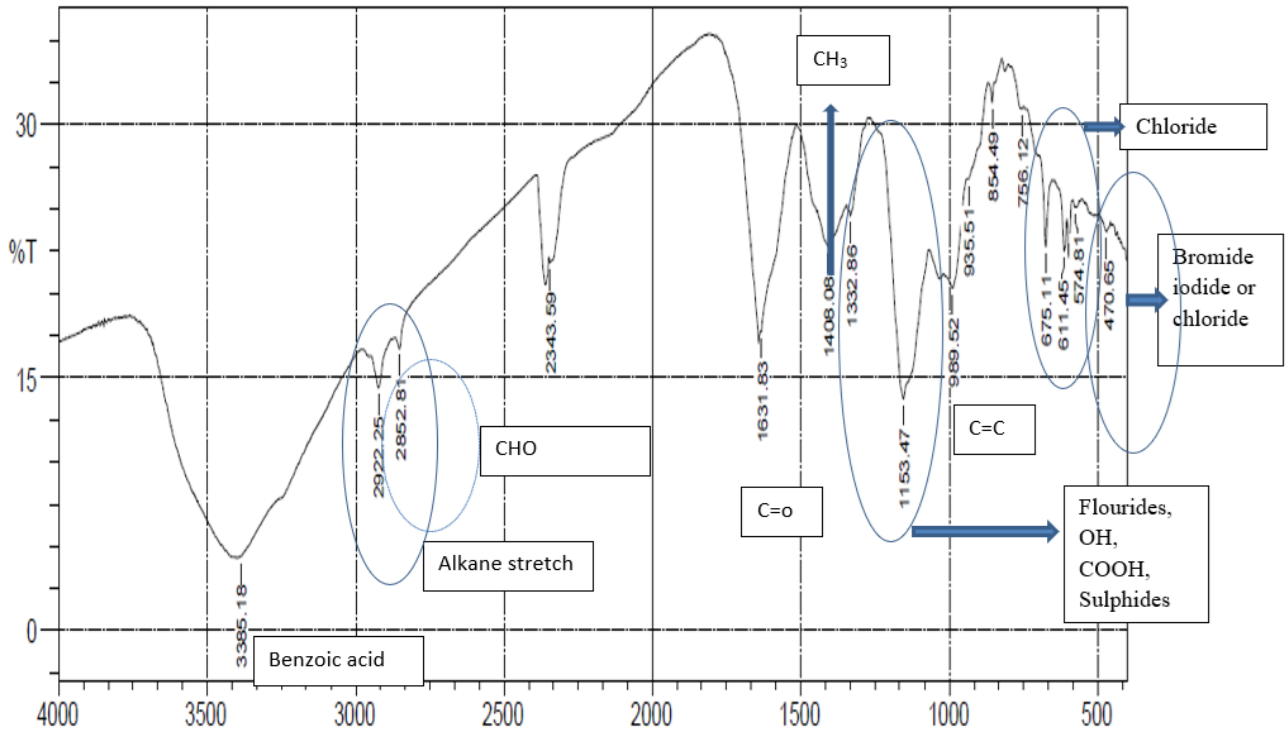


Figure 2: FTIR Spectra for Pooled Chromatographic Fraction 18
Frequency range for FTIR: <667= bromide, iodide, chloride, 600-800= Chloride, 900= Alkenes, 1000-1400= Hydroxyl/carboxylic groups, 1375-1450=Alkyl, 1640-1670= Alkenes, ketones, 2800-2900= Aldehyde, 2850-3000= Alkanes, 2400-3400= Phenyl-carboxylic acids

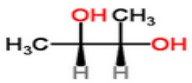
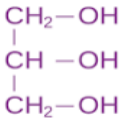
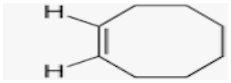
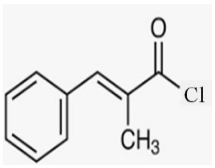
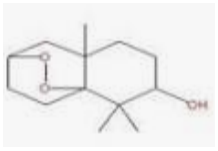
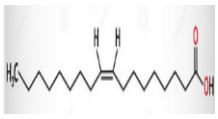
Characterization of Possible Compound in Pooled Chromatographic Fraction 18

The Characterization of possible compounds present in pooled chromatographic Fraction 18 is conducted based on three criteria, including structural similarities of compounds obtained from GCMS compared to FTIR spectra, polarity of the compounds obtained from GCMS, and UV wavelength of compounds obtained from GCMS (Table 6). Although, 2, 3 butanediol and di-glyceraldehyde are all polar compound however, they don't have phenyl and carboxyl groups as seen in FTIR spectra, also their wavelengths are lower than the UV spectra obtained in Figure 4. 2-cyclooctenone, oleic acid, octadecanoic acid, 1,2-15,16-Diepoxyhexadecane and Docosanoic acid were all non-polar compound and also lack the phenyl and carboxyl groups seen in FTIR spectra. While alpha.-Methylcinnamic acid been a polar compound, containing the major functional groups in FTIR spectra and has a UV Spectra of 270nm close to 274.50 obtained at peak six (6) in UV results of pooled chromatographic fraction 18 (Figure 4). However, because of the abundant Cl^- in pooled chromatographic fraction 18, as observed in the FTIR spectra, the possible compound might be alpha.-Methylcinnamic acid chloride, also known as alpha.-Methylcinnamoyl chloride.

UV Spectrophotometry of Pooled Chromatographic Fraction 18

The UV spectrophotometric analysis of pooled chromatographic fraction 18 revealed six absorption Peaks at wavelengths of 665, 607, 535.5, 409.5, 330.5, and 274.5nm. Among the ten possible compounds identified through GC-MS and FTIR analysis, only α-methylcinnamic acid chloride exhibited a wavelength (270 nm) closely matching the UV absorption peak at 274.5 nm (Figure 3). The structure of α-methylcinnamic acid chloride is shown in Figure 4.

Table 6: Characterization of Possible Compound in Pooled Chromatographic Fraction 18

Compound Name	Compound Structure	FTIR Similarity	Wavelength (nm)	Polarity
2,3-Butanediol		CH_3, OH	210	Polar
dl-Glyceraldehyde		CH_3, OH, CHO	245	Polar
3-Cyclohexene-1-carboxylic acid		Phenyl, COOH	245	Non-polar
alpha.-Methylcinnamic acid chloride		Phenyl, COOH, CH_3,C=C, Cl, C-C	270	Polar
2-Hydroxy-1,1,10-trimethyl-6,9-epidioxydecalin		OH, CH_3	<200	Non-polar
n-Hexadecanoic acid		CH_3,COOH,	213	Non-polar
Oleic acid		CH_3,COOH, C=C	230	Non-polar
Octadecanoic acid		CH_3, COOH,	<200	Non-polar
1,2-15,16-Diepoxyhexadecane		CH_3,	-	Non-polar
Docosanoic acid		CH_3,COOH,	<200	Non-polar

***In silico* Molecular Docking of Alphamethyl-cinnamic Acid Chloride against Phospholipase A_2 and Metalloproteinase**

Molecular interaction between ligand (Alpha methyl- cinnamic acid chloride) and target protein (phospholipase A_2 and metalloproteinase) are summarized in Table 7. Phospholipase A_2 showed an energy-binding affinity of -4.3 for alphamethyl-cinnamic acid chloride at three (3) amino acid residues (glycine, glutamine, and tyrosine) via conventional hydrogen, carbon-hydrogen, and Pi-Pi T-shaped bonds, respectively (Figure 5). In contrast, metalloproteinase showed a binding energy of -6.3, interacting with five (5) amino acids (Asparagine, aspartate, tryptophan, tyrosine, and leucine) via conventional hydrogen bond, carbon hydrogen bond, Pi-Pi T-shaped, and Pi-alkyl (Figure 6).

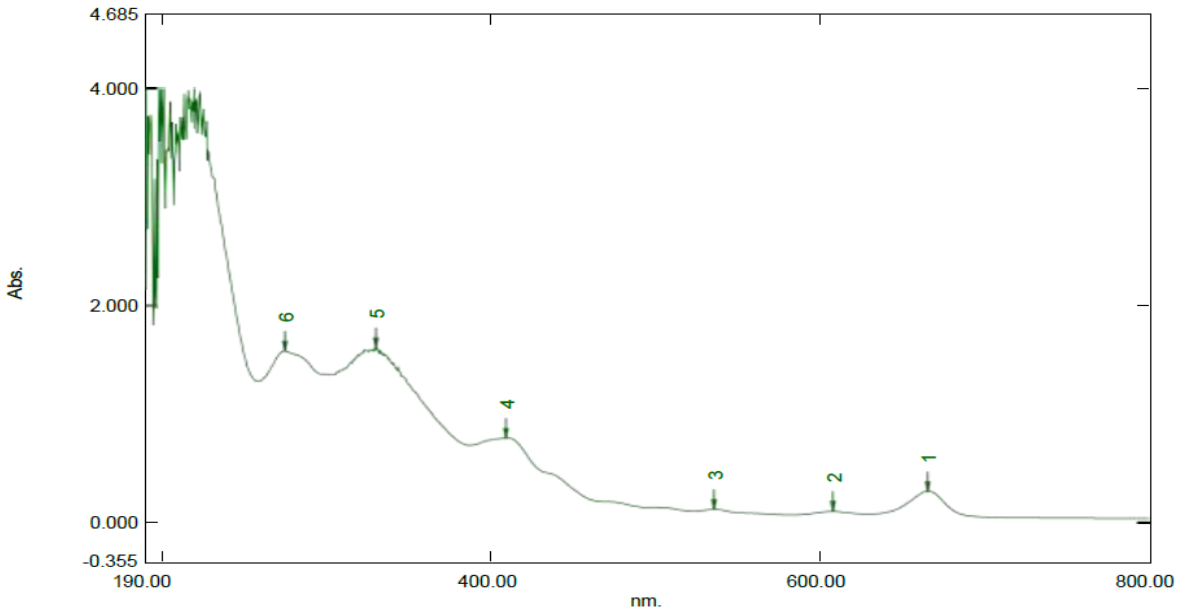


Figure 3: UV Spectrophotometric Analysis of Pooled Chromatographic Fraction 18

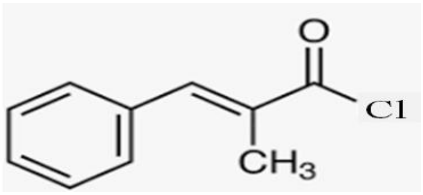


Figure 4: Structure of Possible Compound (alpha-Methyl-cinnamic acid chloride)

Table 7: *In silico* Molecular Docking interaction of Alphamethyl-cinnamic Acid Chloride against *B. arietans* venom major Enzymes

Target proteins	Energy Binding Affinity	Interacting Residues	Molecular weight (kDa)	Type of Interactions
Phospholipase A	-4.3	Glycine, glutamine, and tyrosine	14,000	Conventional hydrogen bond, carbon-hydrogen bond, and Pi-Pi T-shaped
Metalloproteinase	-6.3	Asparagine, aspartate, tryptophan, Tyrosine and leucine	82	Conventional hydrogen bond, carbonhydrogen bond, Pi-Pi T-shaped and Pi- alkyl

4. DISCUSSION

According to Srivastava *et al*. (2021), solvent fractionation of plant extracts is conducted to separate and isolate specific bioactive compounds based on their polarity, facilitating further purification and exposure of the compounds. The present study revealed that the aqueous fraction was the most effective in neutralizing *B. arietans* venom, indicating that the compounds responsible for the anti-venom activity are mostly water-soluble (polar). It was reported that water-soluble phytochemicals such as flavonoids and phenolic acids and saponins neutralize snake venom toxins. Hence the increased antivenom potency observed in *F. albida* aqueous fraction might be attributed to flavonoids, phenolic acids and saponins availability in the extract.

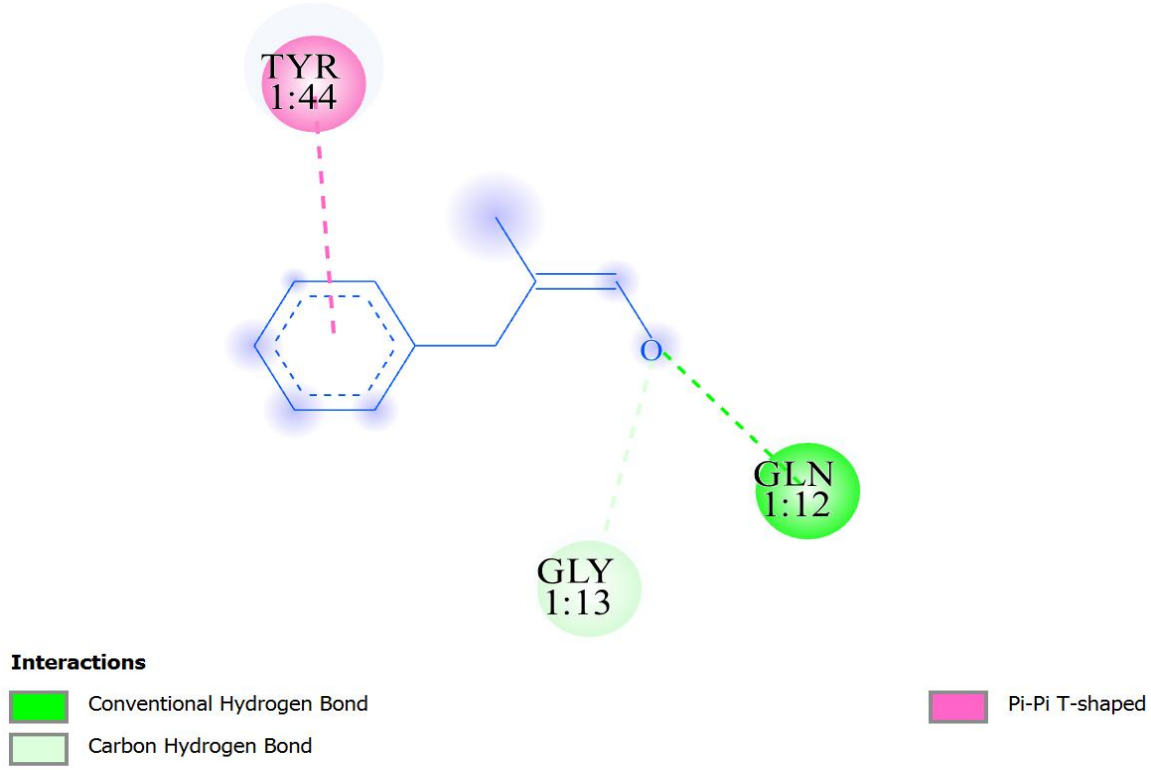


Figure 5: *In silico* Molecular Docking for Alphamethyl-cinnamic Acid Chloride against Phospholipase A_2

Column-chromatography is among the most frequently, widely and popularly used separation techniques for the isolation of bioactive compounds from plants suggesting its potential importance in chemical analysis of plant complex materials (Aguilar-Meza *et al*., 2022). Thin layer chromatographic technique on the other hand is a technique used to separate, purify, and analyse the composition of compounds such as extracts or formulations based on retention factor values (Kowalska and Sajewicz, 2022). According to David and Moldoveanu (2024), two or more different fractions having the same Rf (retention factor) value under specific conditions, especially if they have similar polarity and structure, and are run in the same chromatographic system, are considered to be the same compound. In the present study, several isolated biologically active compounds showed similar retention factor values, and this criterion was employed to combine the 157 fractions obtained from column chromatography into 21 TLC pooled fractions. This study is similar to the report of Baldé *et al*. (2021), who also isolated several bioactive compounds from the aqueous fraction of *albida* root.

The potent inhibitory activities exert by TLC pooled fraction 18 (PCF18) against *B. arietans* venom major enzymes (Proteinases and phospholipase A_2) might be attributed to the fraction ability to interfere with the catalytic activities possibly through binding to active or allosteric site of both *B. arietans* venom proteinases and phospholipase A_2 thereby neutralizing the venom toxic effect this is supported by the findings of Adrião *et al*., (2022) who reported that plant based anti-venom derivatives works by binding to venom components (enzymes) thereby neutralizing their toxins..

GC-MS is a technique primarily used to separate and identify volatile and semi-volatile compounds in plant extracts. FTIR spectroscopy is a rapid, non-destructive approach that provides information about the chemical bonds and functional groups present in samples such as plant extracts. UV-Vis spectroscopy is an analytical technique used to identify compounds based on their absorption characteristics. In the present study, GC-MS FTIR and UV-Vis analysis of PCF18 identified α-methyl cinnamoyl chloride or α-methyl cinnamic acid chloride as the primary component of the isolate. This aligns with findings by Kumar *et al*. (2025), which demonstrated that cinnamic acid derivatives exhibit significant anti-venom activity, particularly by inhibiting phospholipase A2 (PLA2) toxins in snake venom. Thus, the observed anti-venom effects of PCF18 are likely due to α-methyl cinnamoyl chloride.

In-silico molecular docking studies are computerized techniques used to predict molecular interaction between bioactive ligand (compounds) towards their target proteins (enzymes) either of known two- or three-dimensional structure (Agamah *et al*., 2020). Liu *et al*., (2023) reported that in molecular docking, strong ligand-protein interactions are achieved through a combination of complementary shapes, various chemical interactions like hydrogen bonds and hydrophobic interactions, and sometimes, induced fit where the protein

adapts its conformation to better accommodate the ligand. In the present study, the observed hydrogen bond, carbon hydrogen bond and pi alkyl bond links suggests that PCF18 (alpha metyl cinnamic acid chloride) has stronger binding interactions with proteinases. This is supported by Yusoff *et al*., (2024) who also reported that the binding of cinnamic acid derivatives and enzyme active sites are generally governed by a combination of non-covalent interactions (hydrogen bond, pi stalk, and hydrophobic interactions).

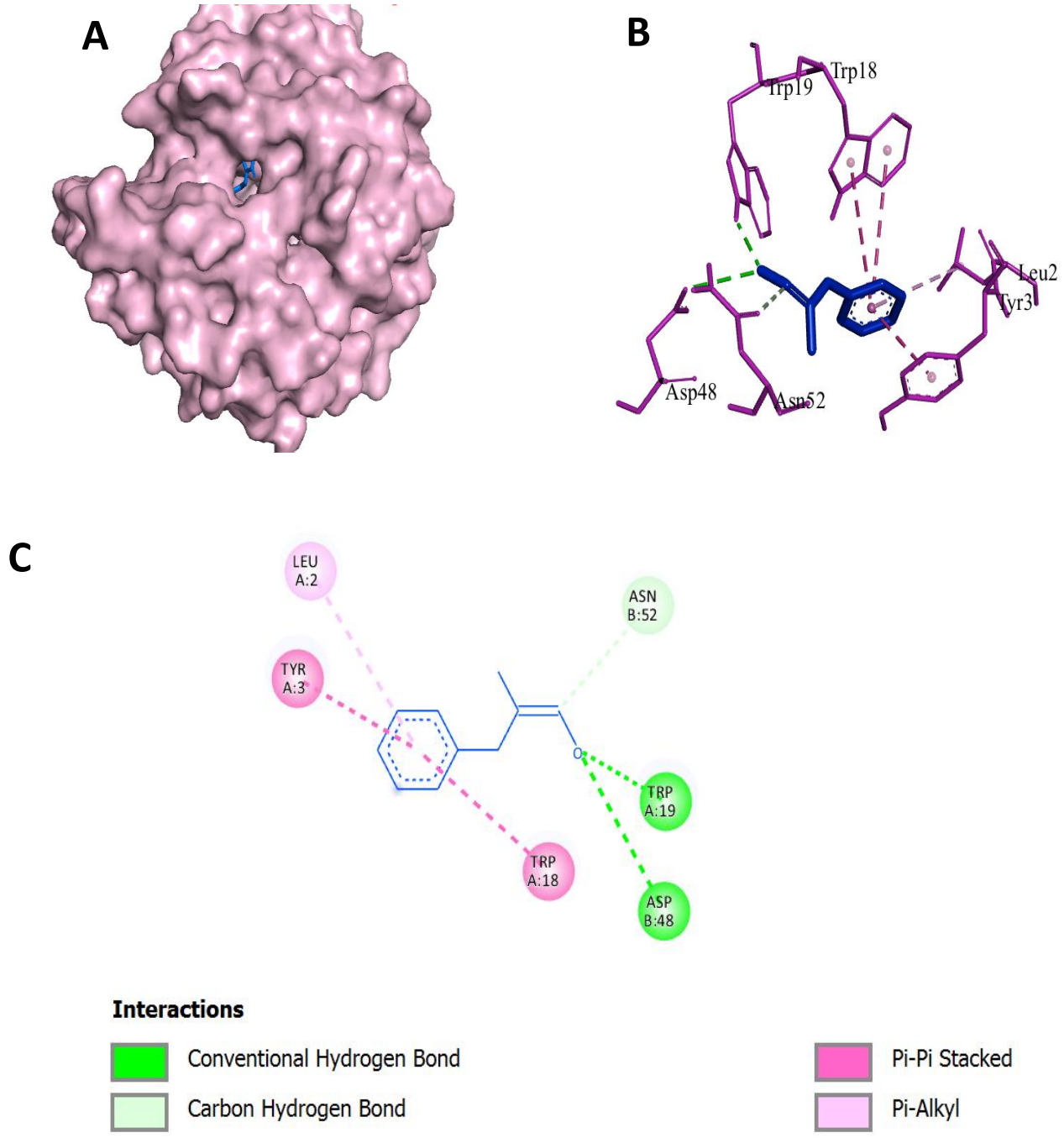


Figure 6: *In silico* Molecular Docking for Alphamethyl-cinnamic Acid Chloride against Metalloproteinase

Additionally, Owoloye *et al*. (2022) reported that a negative binding affinity score indicates a favorable, strong interaction between a protein and a ligand, with more negative values indicating a stronger interaction. This is consistent with the findings of the present study, as interactions between PCF18 (alphamethyl cinnamoyl chloride) and *the* venom proteinase and phospholipase A_2 enzymes of B.

arietans indicate a negative binding affinity. This conforms with the findings of Peperidou *et al*. (2014), who documented the binding affinities of cinnamic acid derivatives and some protein targets between the range of -6.3 to -7.0 kcal/mol.

5. CONCLUSION

The present study revealed that the *F. albida* root aqueous fraction was the most effective in neutralizing *B. arietans* venom in an *in vivo* assay. A hundred and fifty-seven (157) column chromatographic fractions were obtained, which were pooled into 21 pooled chromatographic fractions using thin-layer chromatography. Pooled chromatographic fraction 18 (PCF18) exhibits the most potent inhibitory effect against *B. arietans* venom Proteinases and phospholipase A_2 enzymes. GC-MS, FTIR, and UV-Vis analysis of PCF18 identified α-methyl cinnamoyl chloride or α-methyl cinnamic acid chloride as the primary component of the isolate. *In silico* studies showed that α-methyl cinnamic acid chloride has negative binding affinity of -4.3 and -6.3 with phospholipase A_2 and proteinases, respectively. Hence, this study isolates and identifies an antivenom compound from the aqueous extract of F. albida roots. The study documents the mechanism of action (enzyme inhibition) by which the compound exerts its antivenom efficacy against *B. arietans* venom.

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Author Contributions:

Ibrahim Sani: Sourced the snake, milked and prepared the venom. Then supervised the entire work.
Angela Nnenna Ukwuani-Kwaja: Evaluated the snake venom potency and supervised the work.
Amina Yusuf Jega: Performed the statistical analysis and compiled the manuscript
Abdulhamid Zubairu: Conducted the antivenom screening of the plants.
Fatima Bello: Collected, prepared the plant sample, and performed the extraction.

Ethical Approval

In this article, the animal regulations & plant guidelines are followed as per the ethical committee guidelines of Department of Biochemistry, Faculty of Life Sciences, Abdullahi Fodio University of Science and Technology, Aliero, Nigeria & Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria, respectively; the authors observed the Bioactivity-Guided Isolation and *In Silico* Molecular Studies of Anti *Bitis arietans* Venom from *Faidherbia albida* (Delile) A. Chev Root-Bark Extracts. The Animal ethical guidelines are followed in the study for observation, identification & experimentation. Also, the ethical guidelines for plants & plant materials are followed in the study for observation, identification & experimentation.

Informed Consent

Not applicable.

Conflicts of interests

The authors declare that they have no conflicts of interests, competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Data and materials availability

All data associated with this study will be available based on the reasonable request to corresponding author.

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