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# Isolation Purification and Partial Characterization of Antisnake Venom Plant Peptide (BRS-P19) from *Bauhinia rufescens* (LAM FAM) Seed as Potential Alternative to Serum-Based Antivenin

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## Abstract

Several studies have been reported on active peptides isolated from some medicinal plants, which were effective inhibitors against snake venom induced toxicities. Hence, the aim of this research work was to isolate, purify and characterize an antisnake venom plant peptide from *Bauhinia rufescens* seed that can serve as potential alternative to serum-based antivenins. *B. rufescens* seed was collected, duly identified, authenticated and processed. The peptide was isolated from the seed and purified using gel filtration chromatography and SDS-PAGE and then named as BRS-P19. Venom Phospholipase A2 (VPLA2) was used for the study and was isolated from *Naja nigricollis* venom. Albino mice of both sexes were used for in vivo experiments. They were divided into seven (7) groups of three (3) mice each. Group 1 served as normal control, group 2 were injected with VPLA2 only, group 3 and 4 were injected with VPLA2 then treated with BRS-P19 at doses of 0.2 and 0.4 mg/kg b.w. respectively, while mice in group 5 were injected with VPLA2 then treated with standard antivenin, group 6 and 7 were injected with VPLA2 followed by administration of ascorbic acid and α-tocopherol respectively. In all the groups, hepatic and renal levels of reactive oxygen species (ROS), lipid peroxidation (MDA) and activities of antioxidant enzymes were determined. The results showed that, the BRS-P19 has molecular weight of ~19kD. Its percentage in vitro inhibitory effect against VPLA2 was 91.85 ± 0.32%. For the *in vivo* study, the animals treated with 0.4 mg/kg b.w. of the BRS-P19 showed a significant (P<0.05) decrease in the hepatic and renal ROS and MDA levels when compared with the VPLA2 untreated group. But, the activities of the antioxidant enzymes in all the treated groups were significantly (P<0.05) increased by the BRS-P19 at 0.4 mg/kg b.w. when compared to the VPLA2 untreated group. Based on these findings, it has been established that, BRS-P19 has antisnake venom effect through inhibition of VPLA2 and antioxidant activity as the possible mechanisms of action.

**Keywords:** Plant peptide; Venom phospholipase A2; *Bauhinia rufescens*; BRS-P19; Antioxidant.

## 1. Introduction

Snakes have fascinated mankind since prehistoric times. They are one of the few living organisms which evoke a response; positive or negative when one hears a hissing or rattling sound or even a mere mention of the word 'snake'. This intense fascination probably arises from the deadly effect of their venoms, which when injected into the victim cause a variety of physiological reactions such as paralysis, myonecrosis and often death [1].

Snake venoms have evolved into complex mixtures of pharmacologically active proteins and peptides that exhibit potent, lethal and debilitating effects to assist in prey capture. Snakes use their venoms as offensive weapons in incapacitating and immobilizing their prey (the primary function), as defensive tools against their predators (the secondary function) and to aid in digestion. Biochemically, snake venoms are complex mixtures of pharmacologically active proteins and polypeptides. All of them in concert help in immobilizing the prey.

The most important and abundant toxic component in snake venom is phospholipase A2 (PLA2). This enzyme hydrolyzes the sn-2 ester bond of membrane glycero-phospholipids generating fatty acids such as, arachidonic acid and lysophospholipids, which are precursors of eicosanoids such as prostaglandins, thrombaxanes, leukotrienes and lipoxins participating in inflammatory process [2]. Oxidative metabolism of arachidonic acid generates potentially

toxic reactive oxygen species (ROS) including superoxide and hydroxyl free radicals [3]. An imbalance between the excessive generation and poor removal of ROS causes lipid peroxidation leading to cellular damage [4].

PLA2 plays a prominent role in inflammatory pathway and local tissue damage, the effect which in many cases cannot be neutralized even by antivenoms [5]. In severe cases, the local effects may lead to permanent tissue loss, disability or amputation. Therefore, snake venom PLA2 can be an attractive target in neutralizing the pharmacological effects and inhibiting the generation of reactive oxygen species leading to inflammation and tissue necrosis in victims [6].

Application of medicinal plants with antisnake venom activities might be useful in treating victims of snakebites, which is particularly important in rural areas where antivenins are not readily available [7]. However, antivenins have some disadvantages, thus limiting their efficient use. For example they can induce adverse reactions ranging from mild symptoms to serious anaphylaxis and in addition, they do not neutralize the local tissue damage [8]. Therefore, complementary therapeutics needs to be investigated, with plants being considered as major sources that could be used in the future as potent alternatives to serum-based antivenins.

Interest in bioactive peptides (BPs) has considerably increased in the last decade, and since 2014 more than 800 publications have been reported each year (www.scopus.com). BPs are protein fragments, usually 2–20 amino acids long, and thus they can be very heterogeneous, but the lowest common denominator is some functions which affects and benefits human health [9].

Apart from the better characterized glycoproteins from snakes and mammals, which exhibit inhibitory properties against toxins from snakes and their toxic properties, special mention is made of a newly described glycoprotein isolated from *Withania somnifera*, a medicinal plant reputed to have multiple pharmacological effects as an anti-ulcer, anti-hepatotoxic, anti-inflammatory, anti-tumor, immune-modulator, and also reported to be active against scorpion sting envenomation [10].

Aside from a wide array of well-documented non-protein chemical compounds with antivenom properties from plants and even marine organisms [11], the anti-toxic protein of *W. somnifera* represents the first known plant protein to inhibit toxic effect of snake venom. Early studies Mishra, *et al.* [12] have shown that aqueous extracts of this plant neutralized the toxic activity of *Naja naja* venom, and the active antivenom glycoprotein named WSG was isolated from this extract.

# 2. Materials and Methods

## 2.1. Study Area

The research was conducted from November, 2019 to March, 2020. It was performed in Biochemistry Research Laboboratory, Department of Biochemistry, Faculty of Life Sciences, Kebbi State University of Science and Technology, Aliero, Nigeria.

## 2.2. Plant Materials

The seeds of *Bauhinia rufescens* Lam. Fam were collected in the month of November, 2019 at Jiga Birni, Aliero Local Government Area, Kebbi State, Nigeria. The seeds were authenticated at the Department of Plant Science and Biotechnology, Kebbi State University of Science and Technology, Aliero, where a voucher specimen (VN: 253) was deposited.

## 2.3. Experimental Animals

Adult Wister albino mice of both sexes aged 2 – 4 months and weighing between 20 – 30 g were used for the experiments. They were purchased from National Veterinary Research Institute, Vom, Nigeria and kept under standard laboratory conditions (22–24$^{o}$C; 12:12 h dark/light cycle). The animals were allowed free access to both food (commercial rodent's pellets) and water *ad libitum* [13], they were allowed to acclimatize for 2 weeks. Weight of each mouse was taken before the commencement of the experiment. All animal experiments were conducted in accordance with the guidelines for the use and care of experimental animals [14].

## 2.4. Standard Snake Venom Antiserum (Antivenin)

The lyophilized polyvalent snake venom antiserum (Batch No.: 01AS83659, Man. Date: March, 2018, Exp. Date: February, 2021) was used as standard to compare with the efficacy of the isolated plant peptide. It was produced by a standard pharmaceutical company (VINS Bioproducts Limited, Andhra Pradesh, India).

## 2.5. Collection of Snakes

The snakes; black-necked spitting cobra (*Naja nigricollis*), carpet viper (*Echis ocellatus*) and puff adder (*Bitis arietans*) were captured and housed in wooden cages with the help of snake charmers. After collection, they were duly identified by a Zoologist.

## 2.6. Milking of Venoms

The venoms were collected at 5.30 pm, in a low light condition at ambient temperature according to the method of Goswami, *et al.* [15] by using a short-acting general anesthesia; halothane (Piramal Healthcare Limited, U.K.). The glands below the eyes of each snake were compressed to release the stored venom into a clean and sterilized container.

## 2.7. Preparation of Venom

After milking, each venom was lyophilized using a freeze-dryer (Millrock Technology, USA) and kept inside a refrigerator (HR135A, Haier-Thermocool, Lagos, Nigeria) in a light resistant and air-tight container. All the venoms were mixed and the mixture was used for isolation of venom phospholipase A2.

## 2.8. Isolation and Purification of Venom Phospholipase A2

The venom mixture (20 mg) was dissolved in 1 ml equilibration buffer (0.05 M Tris-HCl, pH 6.8), and then loaded on Sephadex G-50 column (2.6 × 50 cm). The sample-dissolving buffer was used for equilibration of the Sephadex column, and elution of the loaded samples. Fractions of 4 ml were collected at a flow rate of 48 ml/hr using fraction collector. Fractions with phospholipase A2 (PLA2) activity recovered were pooled and directly applied to a CM-Sephadex C-25 column (1.6 × 25 cm) pre-equilibrated with the same buffer and eluted with a linear KCl gradient from 0 to 1.3 M in the same buffer. The enzymatic PLA2 activity was detected by the indirect hemolytic assay according to Al-Abdulla, et al. [16]. The active fractions were pooled, freeze-dried and stored as venom PLA2.

## 2.9. Isolation of the Plant Peptide

Crude protein extraction from the plant seed and its purification were carried out using the method described by Rehman and Khanum [17]. Briefly the plant material was washed under running tap water, after which 200g was crushed using mortar and pestle then blended in an electric blender with 500ml of cold extracting buffer (0.05M Tris-base 6.5 g/L; 0.007M citric acid (monohydrate) 1.5 g/L; 0.1% cysteine hydrochloride 1 g/L; 0.1% ascorbic acid 1 g/L; 1.0% polyethylene glycol 10.0 g/L; 1 mM 2-mercaptoethanol 0.08 mL/L; 2 Mm EDTA; 5% glycerol) with final pH 8.0. The homogenates was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was precipitated out with 80% ammonium sulphate solution. The precipitated solution was then centrifuged at 10,000 rpm for 30 min at 4°C. The pellet was dissolved in deionized water and then screened for the venom PLA2 inhibition.

## 2.10. Purification of the Isolated Peptide
## 2.10.1. Gel-Filtration Chromatography

The isolated peptide was purified by gel-filtration chromatography using column of Sephadex G-50 with 0.02M sodium acetate buffer (pH 5) [18]. The column was eluted with the same buffer at the flow rate of 1 ml/min. The fractions obtained were then tested for inhibitory effect on the venom phospholipase A2. Further G-50 Peak-I was fractionated on Sephadex G-100 column by eluting with Tris–HCl buffer (20 mM, pH 7.4) to arrive at homogenous preparation.

## 2.10.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The fraction having the highest venom PLA2 inhibition from ion-exchange chromatography was further purified. The homogeneity and molecular mass of pooled fractions were run on 15% SDS-gel [19] along with crude extract and dialyzed ammonium sulphate precipitate. The separating gel was placed in Coomassie blue R-250 staining solution on a shaker for 3 h. Excess dye was removed by de-staining with methanol : acetic acid : water (30:60:10 v/v). The approximate molecular mass of the fraction having the venom PLA2 inhibition was determined using molecular weight of standard proteins.

## 2.11. Protein Estimation

The protein concentration of all fractions of both isolated peptide and VPLA2 was estimated using the method of Bradford [20] applying bovine serum albumin as a standard and concentration was expressed in mg/ml.

## 2.12. *In Vitro* Inhibitory Effect of the Isolated Peptide on VPLA2

The venom phospholipase A2 activity was determined acidimetrically using the method of Tan and Tan [21] with slight modification. Constant volumes of substrate comprising calcium chloride (18 mM), 1% tween-80 and egg yolk were stirred for 10 min to get homogenous egg yolk suspension. By addition of sodium hydroxide (1 M), pH of the suspension was adjusted to 8.0. Venom PLA2 (3 mg/ml) was added to the above mixture (15 ml) to initiate the process of hydrolysis and saline (0.9%) was used as control. A decrease of 1 pH unit corresponded to 133 μmole of fatty acid released. Enzyme activity was expressed as μmole of fatty acid released per minute [22]. For the inhibition studies, venom PLA2 was pre-incubated with the peptide for 30 min at 37°C.

$$\text{Enzyme Activity (μmole FA/min.)} = \frac{\text{μmole of FA released}}{\text{Time taken in minutes}}$$

Where: FA = Fatty acid

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

## 2.13. Oxidative Organ Damage of VPLA2 and Antioxidant Effect of the Isolated Peptide

Twenty one (21) albino mice were randomly divided into seven (7) groups of three (3) mice each.
Group 1: Received 0.9% normal saline intravenously (i.v) and served as normal control.

Group 2: Injected intraperitoneally (i.p) with 0.1mg/ml/kg b.w. of the venom phospholipase A$_2$ (VPLA$_2$) and served as negative control.

Group 3 and 4: Served as treatment groups and were injected (i.p) with 0.1mg/ml/kg b.w. of the venom phospholipase A$_2$ (VPLA$_2$) then administered (i.v) with the peptide at the dose of 0.2 and 0.4 mg/kg b.w. respectively.

Group 5: Injected (i.p) with 0.1mg/ml/kg b.w. of the venom phospholipase A$_2$ (VPLA$_2$) then administered (i.v) with standard antivenin at the dose of 1ml/0.6mg venom.

Group 6: Injected (i.p) with 0.1mg/ml/kg b.w. of the venom phospholipase A$_2$ (VPLA$_2$) then administered (i.v) with vitamin C (Ascorbic acid) at the dose of 15mg/kg b.w.

Group 7: Injected (i.p) with 0.1mg/ml/kg of the venom phospholipase A$_2$ (VPLA$_2$) then were administered (i.v) with vitamin E (α-tocopherol) at the dose of 10mg/kg b.w.

After 24 hours, the animals were sacrificed by cervical decapitation after anaesthetizing with chloroform. Liver and kidneys from each mouse were collected and weighed, tissue homogenates were prepared by weighing 1g of tissue and mince with sterile laboratory sand then homogenized with 10ml of 10 mM ice cold phosphate buffer (pH 7.4). The resultant mixtures were centrifuged at 8000 rpm for 10 min at 4$^{\circ}$C.

## 2.13.1. Quantification of Reactive Oxygen Species (ROS)

The method of Vrablic, *et al.* [23] was used. A 0.2 ml nitroblue tetrazolium (NBT) (1 mg/ml) was added to the hepatic and renal homogenates, followed by additional incubation for 1h at 37$^{\circ}$C. The solution was then treated with 0.1 mL KOH (2 M). The absorbance at 570 nm was measured spectrophotometrically and expressed as mmol NBT reduced/g tissue.

## 2.13.2. Determination of Lipid Peroxidation (MDA Level)

The level of malondialdehyde (MDA), an index of lipid peroxidation was determined using the method of Buege and Aust [24]. A 1.0 ml of the supernatant was added to 2ml of (1:1:1) TCA-TBA-HCl reagent (Thiobarbituric acid 0.7%, 0.24N HCl and 15% TCA) then boiled at 100$^{\circ}$C for 15 min, and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed and the absorbance was read at 532nm against a blank. MDA level was calculated using molar extinction coefficient for MDA-TBA complex of 1.56 x 10$^5$ M$^{-1}$ cm$^{-1}$.

$$MDA = \frac{\Delta A \times VT}{\sum \times Vs}$$

Where: ΔA= Change in Absorbance, V$_T$ = Total volume, Vs= Sample volume, ∑= Molar extinction (1.56 x 10$^5$ M$^{-1}$ cm$^{-1}$)

## 2.13.3. Superoxide Dismutase (SOD) Activity Assay

A 200 µl of the diluted radical detector (containing 250µl of a tetrazolium) and 10 µl of normal standard were added to the SOD standard wells. A 200 µl of the diluted radical detector and 10 µl of sample were added to the sample wells. The reaction initiated by adding 20 µl of diluted xanthine oxidase to all the wells. The precise initial time was noted and then the xanthine oxidase was added quickly. Carefully, the 96-well plate was shook for a few seconds to mix. The plate was covered with it cover and then incubated on a shaker for 20 minutes at room temperature. The absorbance was read at 460 nm using a plate reader. The SOD activity was calculated using the formula below:

$$\text{SOD Activity (U/ml)} = \left[ \left( \frac{Sample\ LR - y - intercept}{Slope} \right) \times \frac{0.23\ ml}{0.01\ ml} \right] \times sample\ dilution$$

Sample LR = Linearized rate (Abs Std/Abs Sample).

## 2.13.4. Catalase (CAT) Activity Assay

This enzyme activity was measured according to the method of Clairborn [25]. This procedure is based on decomposition of H$_2$O$_2$ to water and oxygen in the presence of CAT. An aliquot (50 µL) of clear supernatant from the tissue homogenate was mixed with 1.95 mL of 50 mM potassium phosphate buffer (pH 7.0) and 1.0 mL of 20 mM H$_2$O$_2$. The change in the absorbance at 240 nm was recorded immediately and after every 30s for 3min. CAT activity was determined using the rate of decomposition of H$_2$O$_2$, which is proportional to the reduction of the absorbance at 240 nm. One (1) unit of CAT activity was defined as the amount of CAT decomposing 1.0 µM H$_2$O$_2$ per min and was calculated using the molar extinction coefficient of H$_2$O$_2$ (43.6 M$^{-1}$ cm$^{-1}$ at 240 nm).

$$H_2O_2 \text{ Conc.} = \frac{(\Delta A/min) \times VT}{\sum \times Vs}$$

Where: ΔA/min = change in Absorbance per minute, V$_T$= Total volume, Vs= Sample volume, ∑= Molar extinction (43.6 M$^{-1}$ cm$^{-1}$ at 240 nm).

## 2.13.5. Glutathione Peroxidase (GPx) Activity Assay

A 120 µl of assay buffer (containing; 50mM Tris-HCl, pH 7.6 and 5 mM EDTA) was added to the background or non-enzymatic wells and 50 µl of co-substrate mixture to three (3) wells. A 100 µl of assay buffer was added to positive control wells (bovine erythrocyte GPx), 50 µl of co-substrate mixture and 20 µl of diluted GPx (control) to three wells. A 100 µl of assay buffer was added to sample wells, 50 µl of co-substrate mixture and 20 µl of sample to three (3) wells. The samples were diluted with sample buffer. The mixture was mixed by shaking the plate and the

reaction initiated by adding 20 μl of Cumene hydroperoxide to all the wells used. The absorbance was read once every minute at 340 nm using a plate reader to obtain 5 times point. The following formulae for the calculation of GPx activity were used. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of a 0.00373 $\mu M^{-1}{}_{CM}{}^{-1}$. One (1) unit is defined as the amount of enzyme that causes the oxidation of 1.0 nmol of NADPH to NADP per minute.

$$GPx\ activity = \frac{\Delta A340/min.}{0.00373\ \mu M-1} \times \frac{0.19\ ml}{0.02\ ml} \times Sample\ dilution = nmol/min/ml$$

$$\Delta A_{340}/min = \frac{A340\ (Final\ Time\ )-A340\ (Initial\ Time)}{Final\ Time\ (min)-\ Initial\ Time\ (min)}$$

$\Delta A_{340}$/min. = Change in absorbance at 340 nm per minute

## 2.14. Statistical Analysis

The data collected were subjected to statistical analysis. The significance of the difference between the means was determined using one way analysis of variance (ANOVA) and $P<0.05$ was considered as significant. Duncan Multiple Comparison test was used to test the differences using SPSS (Version 25) statistical software.

# 3. Results
## 3.1. Purification of the Isolated Peptide

Fractionation of the extract on Sephadex G-50 column resulted in five (5) peaks; peak-I, peak-II, peak-III, peak-IV and peak-V (Figure 1). Peak-III which showed maximum VPLA2 inhibitory activity (83.4%) yielded single major peak on Sephadex G-100 (Figure 2). The isolated peptide run as a single band on SDS-PAGE and showed approximate molecular weight of 19 kDa (Figure 3). This peptide was named as "*Bauhinia rufescens* seed peptide with MW 19 kDa" (BRS-P19). Table 1 shows the summary of purification of BRS-P19. Crude water extract had highest protein concentration (83.02 mg/ml). After gel-filtration through sephadex G-50 and G-100 the protein concentrations were 1.07 mg/ml and 1.04 mg/ml respectively.

## 3.2. Partial purification of the Isolated VPLA2

Fractionation of the venom on Sephadex G-50 column resulted in four (4) peaks; Peak-I, peak-II, peak-III and peak-IV (Figure 4). Peak-III displayed maximum VPLA2 activity of 85.31 μmole FA/min and was further fractionated on Carboxymethyl-Sephadex C-25 column, which yielded single major peak (Figure 5). This major peak was regarded as venom PLA2. Table 2 shows the summary of purification steps of the venom PLA2.

## 3.3. Inhibitory Effect of BRS-P19 on VPLA2

The results for the *in vitro* inhibitory effect of BRS-P19 on the VPLA2 are presented in Table 3. The VPLA2 activity of 96.59 ± 0.03 μmole FA/min observed was significantly decreased to 7.86 ± 0.30 μmole FA/min after addition of BRS-P19.

## 3.4. Antioxidant Effect of BRS-P19

The results for the antioxidant effect of BRS-P19 are presented in Figures 6 7 8 9 10. The levels of hepatic and renal reactive oxygen species (ROS) and malondialdehyde (MDA) in the treated and untreated albino mice are presented in Figures 6 and 7 respectively. The activities of the hepatic and renal antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are presented in Figures 8, 9 and 10 respectively.

After VPLA2 administrations to the animals, a single IV injection of BRS-P19 at 0.4 mg/kg b.w. significantly decreased the levels of ROS in mice liver and kidneys to 0.16 ± 0.02 mmol/g tissue and 0.05 ± 0.07 mmol/g tissue respectively when compared to the VPLA2 administered mice liver and kidneys (3.84 ± 0.20 mmol/g tissue and 3.06 ± 0.08 mmol/g tissue respectively) (Figure 6).

There was also significant reduction in the levels of MDA in liver and kidneys of albino mice treated with BRS-P19 at 0.4 mg/kg b.w. (1.17 ± 0.07 mmol/g tissue and 1.09 ± 0.04 mmol/g tissue respectively when compared with VPLA2 administered albino mice liver and kidneys (6.91± 0.18 mmol/g tissue and 6.01 ± 0.14 mmol/g tissue respectively (Figure 7).

There was significant decrease in the SOD activities in the VPLA2 administered mice liver and kidneys (11.13 ± 0.11 U/g tissue and 7.12 ± 0.20 U/g tissue. The activities of this enzyme significantly increased to 55.73 ± 0.57 U/g tissue and 48.39 ± 1.80 U/g tissue, respectively in the BRS-P19 treated animals (Figure 8).

The CAT activities in VPLA2 administered mice liver and kidneys were 6.23 ± 0.10 U/g tissue and 9.50 ± 0.50 U/g tissue respectively, but in the BRS-P19 (0.4 mg/kg b.w.) treated mice, the activities of the enzyme significantly increased to 37.06 ± 0.24 U/g tissue and 30.54 ± 0.60 U/g tissue for the liver and kidneys respectively (Figure 9).

The GPx activities in VPLA2 administered mice liver and kidneys were 23.01 ± 0.32 U/g tissue and 31.09 ± 1.87 U/g tissue respectively. In the BRS-P19 (0.4 mg/kg b.w.) treated mice, the GPx activities were significantly increased to 145.12 ± 1.07 U/g tissue and 125.11 ± 0.20 U/g tissue, respectively (Figure 10).

**Figure-1.** Sephadex G-50 elution profile of *Bauhinia rufescens* seed VPLA2 = Venom Phospholipase A2
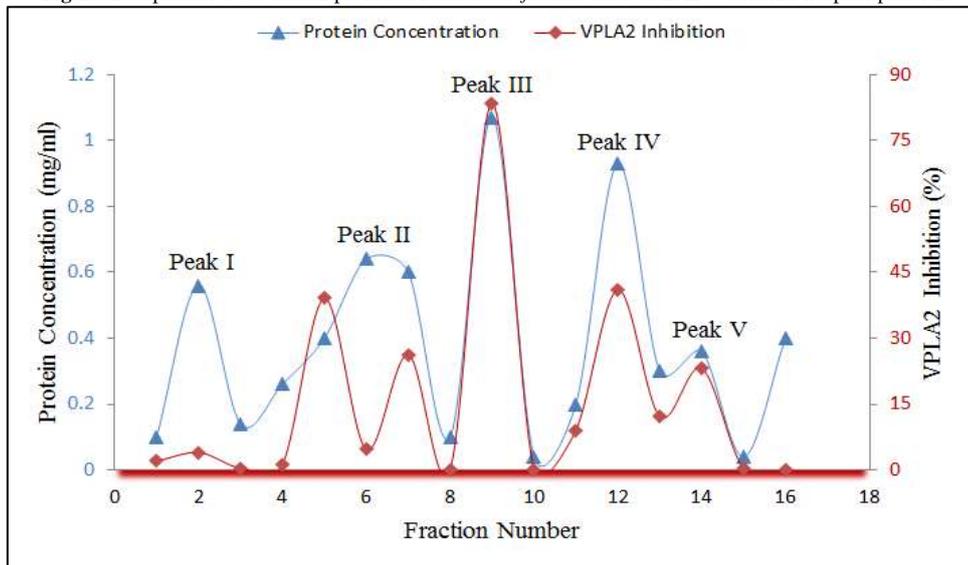


**Figure-2.** Sephadex G-100 elution profile of G-50 peak III VPLA2 = Venom Phospholipase A2
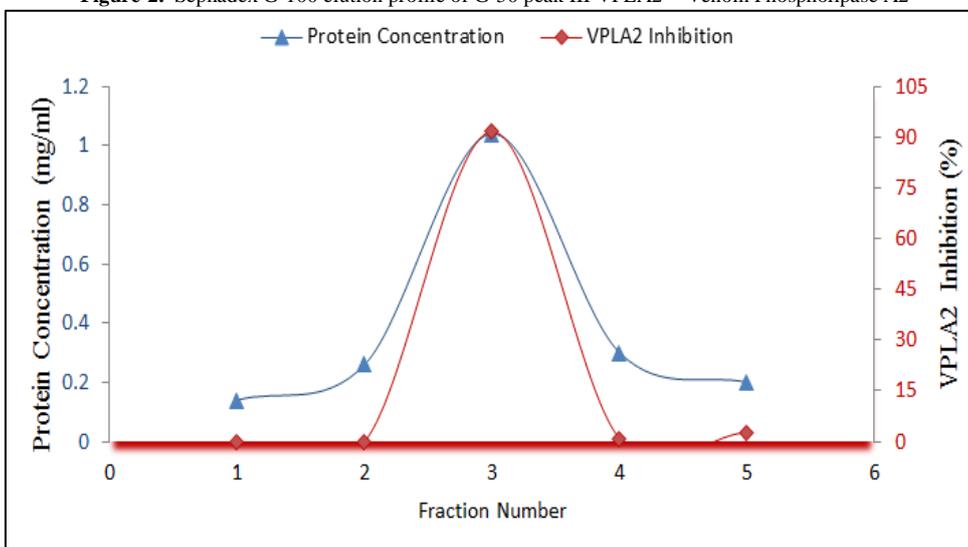


**Figure-3.** SDS-PAGE of BRS-P19 Lane 1 (crude protein extract, Lane 2 (protein content after ammonium sulphate precipitation), Lane 3 (dialized protein content), Lane 4 (1$^{st}$ stage of gel filtration chromatography), Lane 5 (purified BRS-P19 through 2$^{nd}$ stage of gel filtration chromatography). Arrow showed approximate molecular weight of 19 kDa.
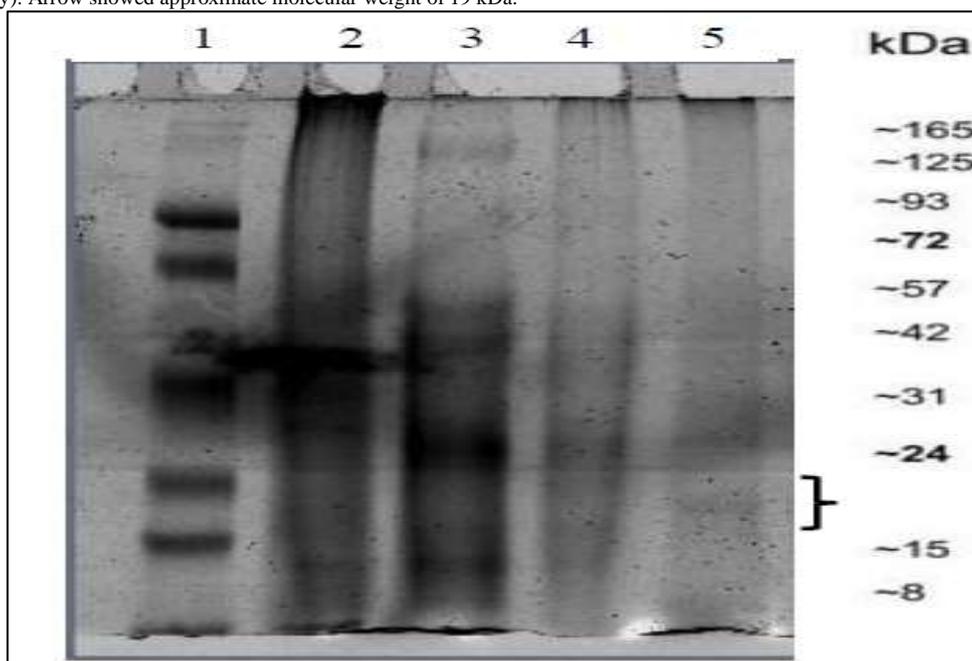
**Table-1.** Purification Steps of the Isolated Peptide

| Purification Step | Protein Concentration (mg/ml) | Yield (%) |
|---|---|---|
| Crude Extract | 83.02 | 100 |
| 80% Ammonium Sulphate | 34.17 | 41.16 |
| Dialysis | 16.08 | 19.37 |
| Gel-Filtration | | |
| Sephadex G-50 | 1.07 | 1.28 |
| Sephadex G-100 | 1.04 | 1.26 |

**Figure-4.** Sephadex G-50 elution profile of the venom Fractions were analyzed for VPLA2 activity and fraction 7 (peak III) was observed to have the VPLA2 activity. VPLA2 = Venom Phospholipase A2
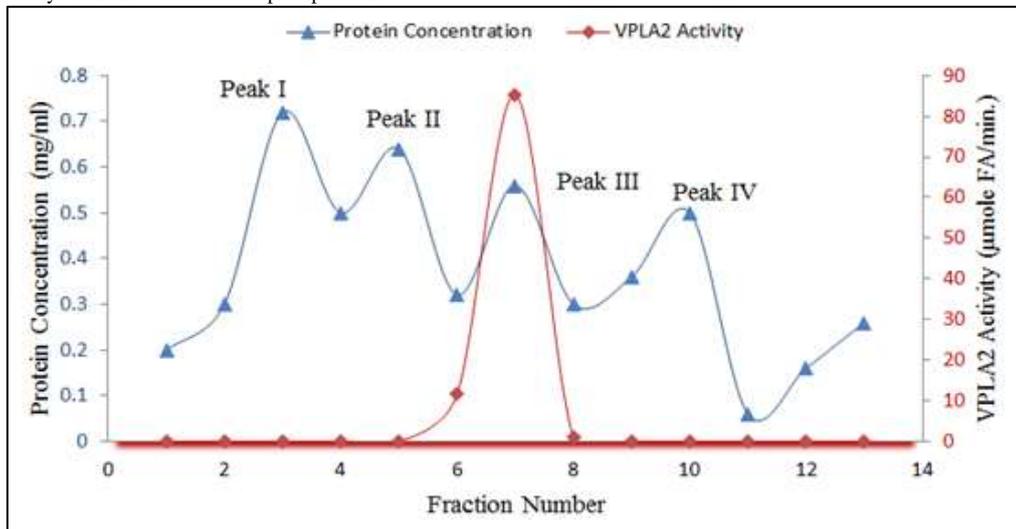


**Figure-5.** Carboxymethyl-sephadex C-25 elution profile of peak-III with VPLA2 activity
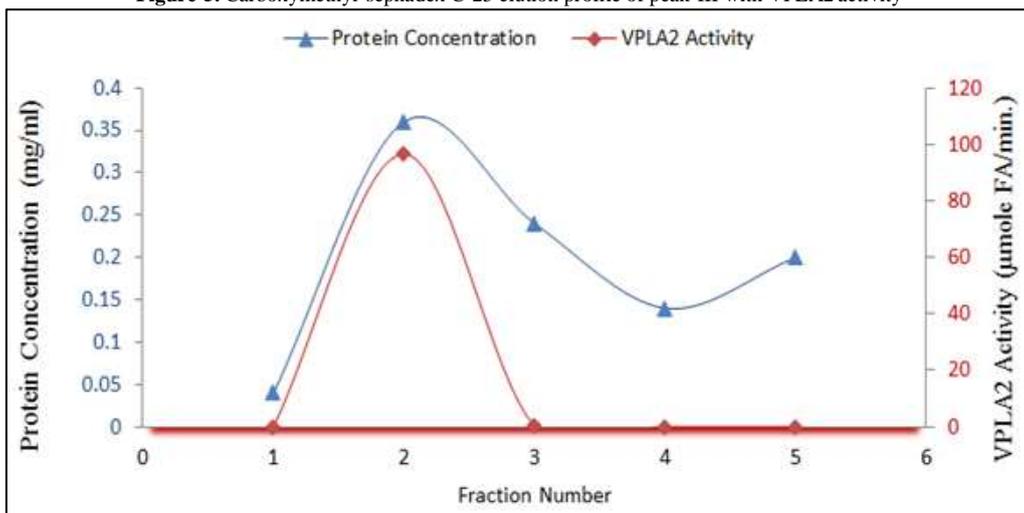


**Table-2.** Purification Steps of the Venom Phospholipase A2 (VPLA2)

| Purification Step | Protein Concentration (mg/ml) | VPLA2 Activity (µmole FA/min) | Yield (%) |
|---|---|---|---|
| Crude Venom | 41.14 | 53.08 | 100 |
| Sephadex G-50 (PeakIII) | 0.56 | 85.31 | 1.36 |
| Sephadex C-25 | 0.36 | 96.57 | 0.88 |

**Table-3.** Venom Phospholipase $A_2$ Inhibition Assay of BRS-P19

| Test Material | Enzyme Activity (µmole FA/min) |
|---|---|
| $VPLA_2$ + Normal Saline | $96.59 \pm 0.03^c$ |
| $VPLA_2$ + BRS-P19 | $7.86 \pm 0.30^a$ |
| $VPLA_2$ + Antivenin | $25.92 \pm 0.49^b$ |

Values are expressed as Mean ± S.D of two different preparations. Values carrying different superscripts on the same column are significantly different (P<0.05).

**Figure-6.** Level of Reactive Oxygen Species (ROS) in Treated and Untreated Albino mice Similar Bars carrying different superscripts are significantly different (P<0.05)
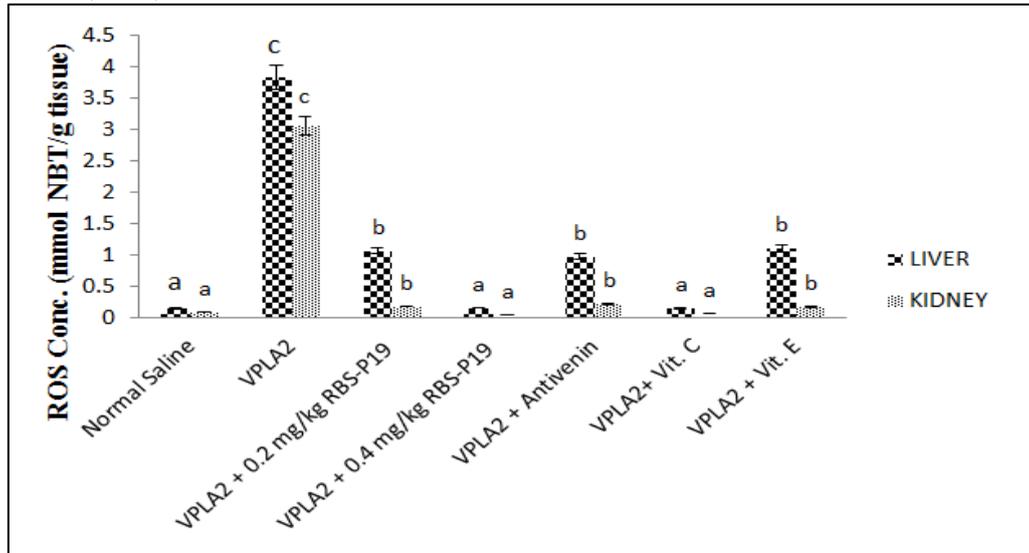


**Figure-7.** Level of Lipid Peroxidation (Malondialdehyde) in Treated and Untreated Albino mice Similar Bars carrying different superscripts are significantly different (P<0.05)
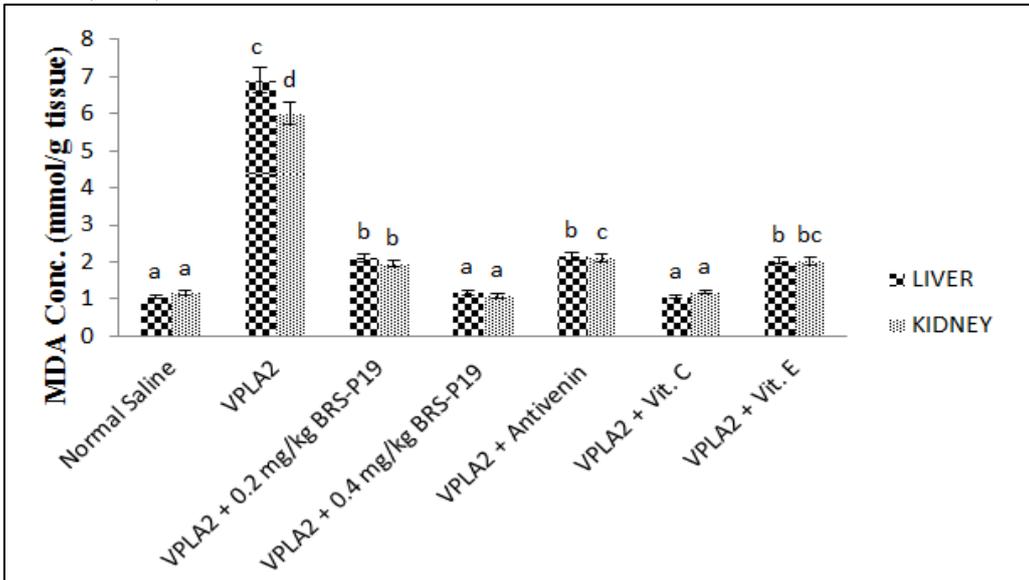


**Figure-8.** Superoxide Dismutase (SOD) Activity in the Treated and Untreated Albino mice Similar Bars carrying different superscripts are significantly difference (P<0.05)
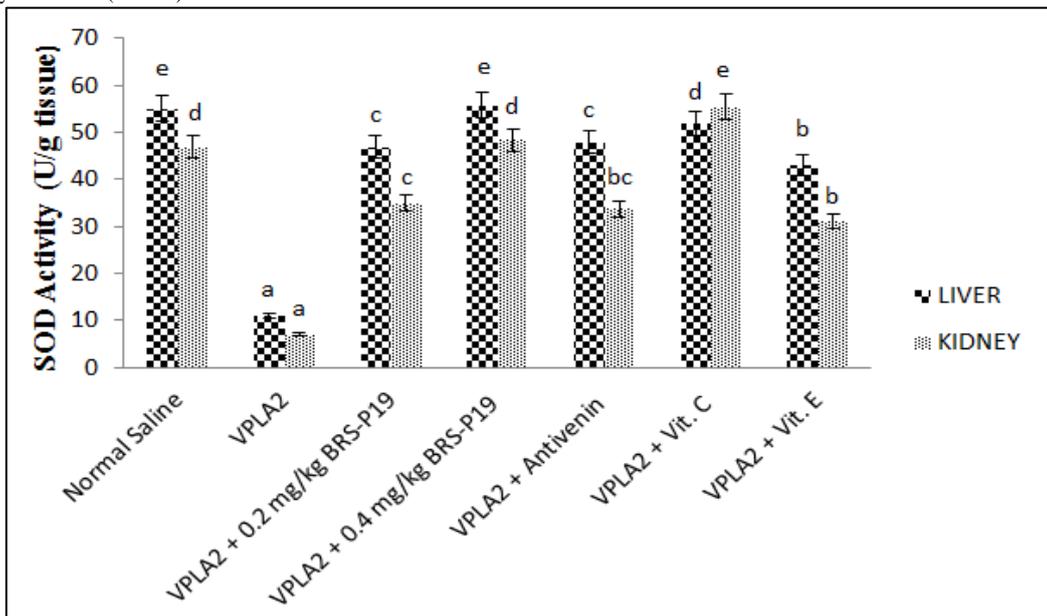
**Figure-9.** Catalase (CAT) Activity in the Treated and Untreated Albino mice Similar Bars carrying different superscripts are significantly different (P<0.05)
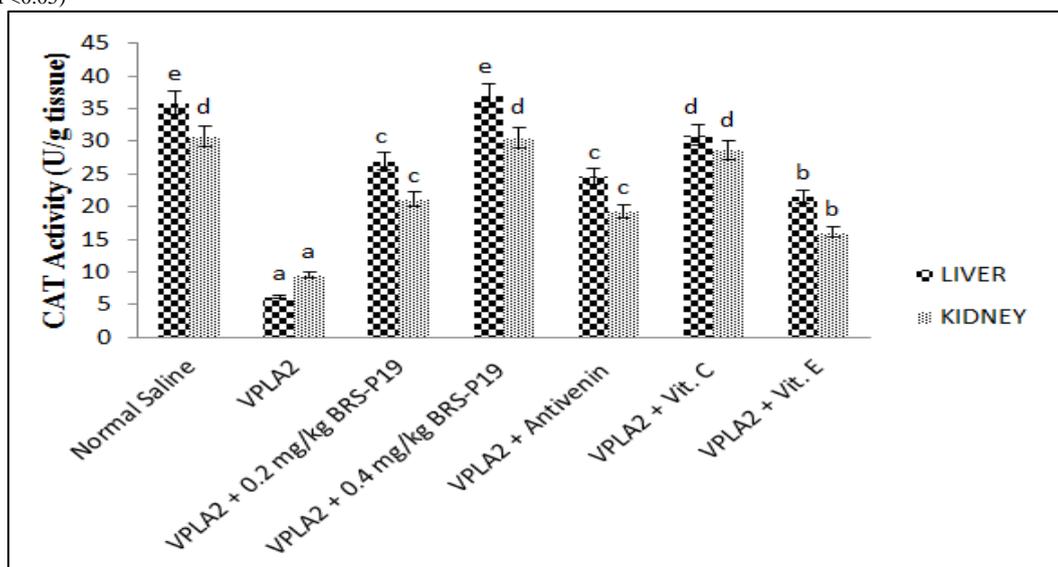


**Figure-10.** Glutathione Peroxidase (GPx) Activity in the Treated and Untreated Albino mice Similar Bars carrying different superscripts are significantly different (P<0.05)
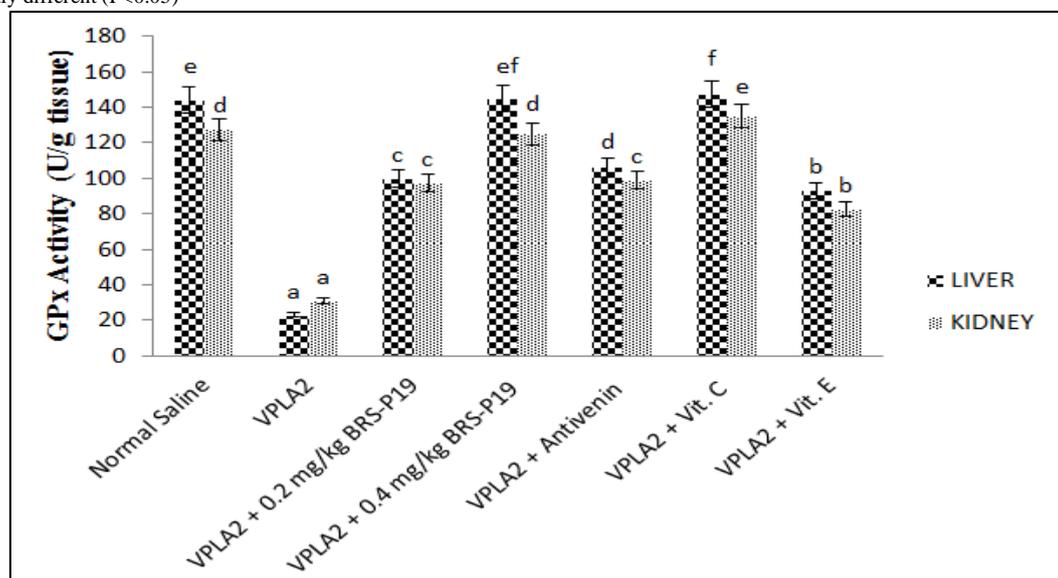


## 4. Discussion

### 4.1. The Isolated BRS-P19

The identified molecular weight of BRS-P19 was shown to be ~19 kDa. The molecular weight is within the range of earlier reported molecular weights of some isolated peptides; Tumerin (~14 kDa) from Chethankumar and Srinivas [26], WSG (~27 kDa) from *Withania somnifera* Deepa and Gowda [10] and MP-4 (~20.9 kDa) from *Mucuna pruriens* Kumar, *et al.* [27]. Cai, *et al.* [28], reported that, the high activity against lipid peroxidation in a linoleic acid model system of BNH-P7 was due to the small size of peptides. Therefore, appropriately low molecular weight can exert a significant effect on the antioxidant activities of peptides [28]. Hence, BRS-P19 with low molecular weight (~19 kDa) had significant inhibitory effect on the VPLA2 and on the organ oxidative damage.

### 4.2. Inhibitory Effect of BRS-P19 on the VPLA2

BRS-P19 was studied for its inhibitory effect on the venom phospholipase $A_2$ in comparison with standard antivenin. The results showed that BRS-P19 is a potent inhibitor of VPLA2 as compared to the standard antivenin. Previously, Many isolated plant peptides with anti-snake venom properties have shown inhibitory effects on the VPLA2 [27, 29, 30]. Deepa and Gowda [10], also reported a glycoprotein isolated from *Withania somnifera* L. as an inhibitor of toxic phospholipase A2 from *Naja naja* venom and demonstrated physical interaction of both the toxic phospholipase $A_2$ and the inhibitor. Hence, based on this finding, it was suggested that the inhibitory effect of BRS-P19 could be by direct interaction with the toxic PLA2.

### 4.3. Antioxidant Effect of BRS-P19

Venom phospholipase A2 induces oxidative organ damage by hydrolyzing cellular phospholipids, resulting in the release of arachidonic acid. Hence, oxidative metabolism of arachidonic acid generates potentially toxic reactive oxygen species (ROS) and excessive generation of these ROS and their accumulation causes lipid peroxidation leading to cellular damage [4]. The accumulation of this species leads to the low activities of natural antioxidant enzymes [31]. Hence the reason for the reduction in activities of tissues' SOD, CAT and GPx in group administered with VPLA2 only.

BRS-P19 treatment resulted to a significant inhibitory effect in the activity of VPLA2 and scavenging the free ROS leading to prevention in the generation of lipid peroxidation. In comparison with standard antioxidants, BRS-P19 at lower dose (0.2 mg/kg body weight) significantly increases the activity of SOD, CAT and GPx leading to the reduction in the levels of ROS and MDA, hence, preventing lipid peroxidation. The extent of activation of SOD, CAT and GPx and reduction in the level of ROS and MDA was in the following order; BRS-P19 > ascorbic acid > α-tocopherol > antivenin. This could be either by inhibiting the activity of VPLA2 or increasing the activities of the natural antioxidant enzymes. This finding corresponds with the studies reported by Smitha, et al. [32], Chethankumar [33] and Ramadas and And Srinivas [29]. According to Chethankumar [33], an isolated peptide; Turmerin has been investigated for its ability to prevent oxidative organ damage against Naja naja venom phospholipase A$_2$ (NV-PLA2) in Male Swiss Wistar mice. It was also reported that β-turmerin is effective in preventing lipid peroxidation at very low concentration of 0.125 mM [32]. Ramadas and And Srinivas [29], reported that, BGS-Haridrin scavenged hydroxyl, Diphenyl2picrylhydrazyl (DPPH) radicals, superoxide radicals and inhibited lipid peroxidation 78% at a maximum dosage of 0.9 mM concentration when compared to Butylated hydroxyanisole (BHA), Curcumin (0.4 mM) and α-tocopherol (0.4 mM).

The mechanism of antioxidant action by BRS-P19 could probably be by counteracting/quenching of the reactive oxygen species, thereby reducing the potential of prooxidants to attack cellular components (lipids or proteins). Houghton, et al. [34], reported that, the inhibitory mechanism of RW-12 is brought about by inhibition of snake venom cardiotoxins through formation of complex between the RW-12 and the venom cardiotoxins. Hence, the mechanism of antioxidant action of BRS-P19 could be by inhibition of venom phospholipase A2 (VPLA2) through formation of complex between BRS-P19 and the VPLA2. These features can make BRS-P19 to serve as an antioxidant agent due to either ROS scavenging activity or inhibitory effect of VPLA2.

## 5. Conclusion

Plant peptides have been identified with antioxidant activities acting as promising agents in combating the local toxicity induced by snakebite envenomation. Based on the findings of this research, it was established that the peptide BRS-P19 from Bauhinia rufescens seed has a potential inhibitory effect on the VPLA2 isolated from the mixture of Naja nigricollis, Echis ocellatus and Bitis arietans venoms, thus preventing organ damage in albino mice. This can serve as lead for the development of safe, readily available and affordable plants based antivenoms that can substitute the use of conventional serum-based antivenins.

## 6. Funding

## Conflict of Interest

No conflicting interest exists.

## References

[1]      Menez, A., 1998. "Functional architectures of animal toxins: a clue to drug design?" Toxicon, vol. 36, pp. 1557–1572.

[2]      Code, C., Mahalka, A. K., Bry, K., and Kinnunen, P. K., 2010. "Activation of phospholipase A2 by 1-almitoyl-2-(9-oxo-nonanoyl)-sn-glycero-3-phosphocholine in vitro." Biochim Biophys Acta, vol. 1798, pp. 1593–1600.

[3]      Tariq, M., Elfaki, I., Khan, H. A., Arshaduddin, M., Sobki, S., and Al-Moutaery, M., 2006. "Bromophenacyl bromide a phospholipase A2 inhibitor attenuates chemically induced gastro duodenal ulcers in rats." World J. Gastroenterol, vol. 12, pp. 5798-5804.

[4]      Adibhatla, R. M., Hatcher, J. F., and Dempsey, R. J., 2003. "Phospholipase A2 hydroxyl radicals and lipid peroxidation transient cerebral ischemia." Antioxide Redox Signal, vol. 5, pp. 647-654.

[5]      Sani, I., Umar, R. A., Hassan, S. W., Faruq, U. Z., Abdulhamid, A., Bello, F., and Fakai, I. M., 2019. "Major enzymes from snake venoms: Mechanisms of action and pharmacological applications." Asian J. Biol. Sci., vol. 12, pp. 396-403.

[6]      Sani, I., Umar, R. A. H., S. W., and Faruq, U. Z., 2018. "Antisnake venoms and their mechanisms of action: A review." Saudi J. Med. Pharm. Sci., vol. 4, pp. 512-520.

[7]     Faruq, U. Z., Zuru, A. A., Abdulrahman, F. W., and Bilbis, L. S., 2002. "Preliminary screening of sclerocarya birrea bark as snake venom antidote against naja nigricollis." *Nig. J. Biochem. Mol. Biol.,* vol. 17, p. 1.

[8]     Gutierrez, J. M., Calvete, J. J., Habib, A. G., Harrison, R. A., Williams, D. J., and Warrell, D. A., 2017. "Snakebite envenoming." *Nat. Rev. Dis. Primers.,* vol. 3, p. 17063.

[9]     Sani, I., Nwaogu, J., Obafemi, J. I., Bello, F., Abdulhamid, A., Aminu, H., Sulaiman, A., and Yanah, Y. M., 2019. "Antimicrobial phyto-peptides: Distribution, structural diversity and mechanisms of action." *World J. Pharm. Res.,* vol. 8, pp. 58-75.

[10]    Deepa, M. and Gowda, T. V., 2002. "Purification and characterization of a glycoprotein inhibitor of toxic phospholipase from Withania somnifera." *Arch. Biochem. Biophys.,* vol. 408, pp. 42–50.

[11]    Middleton, E. J., 1998. "Effect of plant flavonoids on immune and inflammatory cell function." *Adv. Exp. Med. Biol.,* vol. 439, pp. 175–182.

[12]    Mishra, L. C., Singh, B. B., and Dagenais, S., 2000. "Scientific basis for the therapeutic use of Withania somnifera (ashwagandha): a review." *Altern. Med. Rev.,* vol. 5, pp. 334–346.

[13]    Aboubakar, O. B. F., Bella, N. M. T., Ngo lemba, T. E., Bilanda, D. C., and Dimo, T., 2012. "Antihypertensive activity of jateorhiza meacrantha (menispermaceae) aqueous extract on ethanol-induced hypertension in Wister Albino Rats." *Intern. J. Pharm. Sci.,* vol. 4.

[14]    AUCC, 2009. "Guide to the use and care of experimental animals." In *Animal use and Care Committee, National Veterinary Research Institute, Vom, Nigeria.*

[15]    Goswami, P. K., Samant, M., and Srivastava, R. S., 2014. "Snake venom, anti-snake venom and potential of Snake Venom." *Inter. J. Pharm. Pharmec. Sci.,* vol. 6, pp. 4-7.

[16]    Al-Abdulla, I. H., Sidki, A. M., and Landon, J., 1991. "An indirect haemolytic assay for assessing antivenoms." *Toxicon,* vol. 29, pp. 1043-1046.

[17]    Rehman, S. and Khanum, A., 2011. "Isolation and characterization of peptide (s) from pisum sativum having antimicrobial activity against various bacteria." *Park. J. Bot.,* vol. 43, pp. 2971– 2978.

[18]    Deutscher, M. P., 1990. "Maintaining protein stability." *Methods Enzymol.,* vol. 182, pp. 83-89.

[19]    Laemmli, U. K., 1970. "Cleavage of structural proteins during the assembly of head of bacteriophage T4." *Nature,* vol. 227, pp. 680-685.

[20]    Bradford, M. M., 1976. "Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye-binding." *Anal. Biochem.,* vol. 72, pp. 248-254.

[21]    Tan, N. H. and Tan, C. S., 1988. "Acidimetric assay for phospholipase A2 using egg yolk suspension as substrate." *Anal. Biochem.,* vol. 170, pp. 282–288.

[22]    Yap, M. K. K., Tan, N. H., and Fung, S. Y., 2011. "Biochemical and toxinological characterization of Naja sumatrana (Equatorial spitting cobra) venom." *J. Venom. Anim. Toxins incl. Trop. Dis.,* vol. 17, pp. 451–459.

[23]    Vrablic, A. S., Albright, C. D., Craciunescu, C. N., Salganik, R. I., and A., W. D., 1995. "Clinical toxicology of snakebite in africa and the middle east arabian peninsula in: Meter. J. And white j. (eds). Handbook of clinical txicology of animal venoms and poisons." *CRC press. Florida,* pp. 433-492.

[24]    Buege, J. A. and Aust, S. D., 1978. "Microsomal lipid peroxidation." *Methods Enzymol,* vol. 15, pp. 302-310.

[25]    Clairborn, A., 1995. *Catalase activity in: Greewald a. R., editor. Handbook of methods for oxygen radical research.* Boca Raton: CRC press. pp. 237-242.

[26]    Chethankumar, M. and Srinivas, L., 2008. "Gangliosides as potential inhibitors of Naja naja venom PLA2 (NV-PLA2) induced human erythrocyte membrane damage." *Afr J Biochem Res.,* vol. 2, pp. 8–14.

[27]    Kumar, A., Gupta, C., Nair, D. T., and Salunke, D. M., 2016. "MP-4 contributes to snake venom neutralization by Mucuna pruriens seeds through an indirect antibody-mediated mechanism." *J. Biol.,* vol. 291, pp. 11373–11384.

[28]    Cai, L. Y., Wu, X. S., Zhang, Y. H., Li, X. X., Ma, S., and Li, J. R., 2015. "Purification and characterization of three antioxidant peptides from protein hydrolysate of grass carp (Ctenopharyngodon idella) skin. ." *J. Funct. Foods,* vol. 16, pp. 234–242.

[29]    Ramadas, D. and And Srinivas, L., 2011. "Antioxidant effects of 28kda antioxidant protein from turmeric (curcuma longa l)." *Asian J. Pharm. Clin. Res.,* vol. 4, pp. 119-123.

[30]    Zare-Zardini, H., Tolueinia, B., Hashemi, A., Ebrahimi, L., and Fesahat, F., 2013. "Antioxidant and cholinesterase inhibitory activity of a new peptide from ziziphus jujuba fruits." *Am. J. Alzheimer's Dis. Other Dem.,* vol. 28, pp. 702-709.

[31]    Sharma, K. V. and Sisodia, R., 2009. "Evaluation of free radical scavenging activity and radio protective efficacy of Grewia asiatica fruit." *J. Radio Prot.,* vol. 29, pp. 429-443.

[32]    Smitha, S., Dhananjaya, B. L., Dinesha, R., and Srinivas, L., 2009. "Purification and characterization of a w34 kDa antioxidant protein (β-turmerin) from turmeric (Curcuma longa) waste grits." *Biochimie,* vol. 91, pp. 1156-1162.

[33]    Chethankumar, M., 2010. "Turmerin, a protein from Curcuma longa L. prevent oxidative organ damage against Naja naja venom phospholipase A2 in experimental animal." *J. Curr. Pharm. Res.,* vol. 3, pp. 29-34.

[34]    Houghton, P. J., Osibogun, I. M., and Bansal, S., 1992. "A peptide from Schumanniophyton magnificum with anti-cobra venom activity." *Planta Medica,* vol. 58, pp. 263–265.