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Original Article

INHIBITION OF MULTI-TOXIC PHOSPHOLIPASE A_2 OF *DABOII RUSELLII PULCHELLA* VENOM BY THE ETHANOLIC EXTRACT OF *A. PANICULATA*

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ABSTRACT

Objective: *Andrographis paniculata* (*A. paniculata*) Nees is an important medicinal plant found in the tropical regions and has been traditionally used in Indian and Chinese medicinal systems. *A. paniculata* is found to exhibit anti-snake venom properties; however its inhibitory potential on multi-toxic Phospholipases A_2 and its associated inflammatory reactions is not clearly understood. Henceforth, in this study we evaluate the inhibitory/neutralizing potential of alcoholic extract of *A. paniculata* on the isolated multi-toxic PLA_2 (of *Daboii rusellii pulchella* and *Naja naja*) and its induced edema in Swiss albino mice.

Methods: The multi-toxic-$svPLA_2$-VRV-PL-V from the venom of *Daboii rusellii pulchella* was purified according established methods. The inhibition of enzymatic activity and edema inducing activity was carried out according to established methods.

Results: *A. paniculata* extract dose dependently inhibited the multi-toxic $svPLA_2$ enzymatic activity with an IC_{50} value of 12.4±0.6 µg/ml. Further, the extract dose dependently inhibited the edema formation, when co-injected with the enzyme indicating a strong correlation between lipolytic activity and pro-inflammatory activity.

Conclusion: The ethanolic extract of *A. paniculata* effectively inhibited the multi-toxic $svPLA_2$ and its associated edema inducing activities, which substantiate their anti-ophidian properties. Further study is interesting to develop them into potent anti-ophidian agents.

Keywords: Snake venom, Neutralization, Drug, Antivenom, Cocktail of inhibitors.

INTRODUCTION

Snake bite is a public health problem causing considerable high morbidity and mortality around the world, particularly in the tropics. Snake bite is now recognized as a highly Neglected Tropical Disease (NTD) by the World Health Organization (WHO) [1, 2]. The World Health Organization (WHO) estimates that, globally, at least 4, 21, 000 envenoming and 20,000 deaths occur each year due to snake bite [1]. Snake venom is a cocktail of biologically active components, which are able to interfere with the biological processes in both prey and human [3, 4]. The pathophysiological effects observed in snake bite is due to the combine action of several enzymatic proteins and peptides, that includes phospholipases A_2, 5`Nucleotidase, hemorrhagic metalloproteases, proteolytic enzymes, proteins acting on coagulant components, neurotoxins, cytotoxins, cardiotoxins etc. [3, 4].

Snake venom phospholipases A_2 ($svPLA_2$s) are the principal component of snake venoms and have been extensively investigated as they have a wide range of biological effects of the wide variety of physiological and pathological effects [5-7]. In addition to their possible role in the digestion of prey, snake venom $sPLA_2$s exhibit a wide spectrum of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, anticoagulant, anticancer effects *etc.* [5, 6]. Due to the prominent role played by PLA_2s in the snake envenomation, there is pharmacological interest in search of $svPLA_2$ inhibitors [8, 9]. Further, considering the limitations of anti-serum therapy [10, 11], researches are focusing on development of alternative treatments and in this regard finding inhibitors of the multi-toxic $svPLA_2$s from medicinal plants have gained much interest [7, 12, 13]. In this context, many $svPLA_2$ inhibitors have been isolated from various medicinal plants [8, 9]. Several snake venom PLA_2 inhibitors are demonstrated to bring down the toxic and lethal effects of several venoms, and thereby help in management of snake bite [11].

Andrographis paniculata Nees (Acanthacae) commonly known as king of bitters, is widely distributed medicinal herb in tropical Asian countries including India. It has been diversely used as the medicinal herb in traditional medicinal systems of India, China, Thailand and Europe [14]. As evident from Indian Pharmacopoeia, *A. paniculata* is known to prominent in 26 ayurvedic formulations; while in traditional Chinese medicine, due to its important "cold property" is used to release body heat in fever [15]. The species is therapeutically well explored and effectively used for treatment of asthma, gonorrhea, piles, dysentery and dyspepsia, influenza, gastric complaints, diarrhea, pharyngitonsillitis, fever, myocardial ischemia, common cold, diabetes, respiratory tract infections, jaundice among others [14, 15]. It is reported to possess antimicrobial, hypotensive, anti-hyperglycaemic, oxygen radical scavenging, anti-atherosclerotic, anti-malarial, anti-HIV, antiplatelet aggregation, hepatic lipid peroxidation protective, hepatoprotective, choleretic, and anticancer activities. The plant extracts has also been reported to exhibit anti-typhoid, antifungal, antifertility, anti-nematicidal, anti-hepatitic, anti-hypogycemic, anti-ulcerogenic, anti-thrombogenic properties, anti-inflammatory and anti-snake venom activities [14-16]. In regard to treatment of snake bite, from long time back *A. paniculata* has been used in traditional herbal medicine [17-21]. It is used by tribes to treat victims of snake bites in rural areas of southern India especially in Andhra Pradesh and Tamilnadu [22-24]. It is observed that it had potent neutralization effect on the lethal effects of Indian Cobra (*Naja naja*), King Cobra (*Ophiophagus hannah*), and Rattlesnake (*Crotalus adamenteus*) venoms [25-27]. It was also shown the extract completely inactivated the neurotoxin of the *C. adamenteus* venom [25]. In this study, investigations were

carried to evaluate the modulatory effect of ethanolic extract of *A. paniculata* on multi-toxic PLA_2 (VRV-PL-V phospholipase A_2 enzyme) from Indian Russell's viper (*Daboia russelii pulchella*) venom to substantiate their anti-ophidian properties.

MATERIALS AND METHODS

Materials

Venom of *Daboia russelii* (Russell's viper) was purchased from Irula Co-operative Society Ltd., Chennai, India. Urosolic acid was purchased from Sigma chemicals, USA. All other reagents and chemicals used were of all analytical grades purchased from Sisco Research Laboratories (SRL), Bangalore, India.

Collection of plant and extraction of crude extract

Andrographis paniculata plant was collected from nearby areas of Thanjavur district, Tamil Nadu. The plant was authenticated at the Centre for Research in Indian Systems of Medicine *(CRISM),* SASTRA University, Thanjavur, Tamil Nadu, where a voucher specimen (SASTRA/CRISM/PL/182) was deposited. The plant collected of free of disease and injury, was washed several times with distilled water and shade dried. The powdered plant (40g dry weight) was extracted using 95% ethanol (400 ml) in soxhlet extractor solvent and extract was concentrated using rotary vacuum evaporator and the residue obtained (Dark brown in color), was dried, weighed and was preserved in airtight glass container and kept at 4 °C until further applications.

Animals

Swiss Wister albino mice weighing about 20-25 g were obtained from the central animal house facility. All protocols of animal experiments have been approved by the SASTRA University-Institutional Animal Care and Use Committee (IACUC). Animal care and handling were conducted in compliance with the national regulations for animal research.

Isolation of multi-toxic (VRV-PL-V) secretary phospholipase A_2

The multi-toxic $svPLA_2$ from the venom of *Daboii rusellii pulchela* (Southern region) was purified up to homogeneity as described previously by the method of Kasturi & Gowda [28], and as modified by Srinivasan, [29]. These proteins were further used for evaluating the anti-inflammatory potential of ethanolic extract of *A. paniculata*. The protein concentration was estimated according to the method of Lowry *et al.* [30] using BSA as the protein standard.

Inhibition of multi-toxic $svPLA_2$ activity by *A. paniculata* ethanolic extract

The Phospholipase A_2 assay was carried out according to the method as described by Bhat and Gowda, [31]. Phosphatidyl choline (PC) was diluted with petroleum ether (60–80°C) to get a concentration of 1000 nmoles/50 ml. The reaction mixture containing VRV-PL-V (3 µg) was made up to 680 ml with water. To the reaction mixture, 200 µl of ether, 100 µl of Tris-HCl buffer (0.05 M, pH 7.5), and 20 µl of $CaCl_2$ (500 mM) were added. The total reaction mixture was incubated at 37 °C for 60 minutes. After incubation, 0.5 ml of Doles mixture (Isopropanol: Pet ether: $1NH_2SO_4$, 40:10:1) was added, mixed and centrifuged at 1000 rpm for 3 min. To the organic phase 0.5 ml of $CHCl_3$: Pet ether (1:5) was added, mixed and centrifuged at 1000 rpm for 3 min. To the upper phase cobalt reagent [1.35 vol. of Triethanolamine made up to 10 ml with solution A (6 g of CO $(NO_3)_2$.-$6H_2O$+0.8 ml glacial acetic acid) and 7 ml of solution B (Saturated Na_2SO_4)] was added, mixed and centrifuged 1000 rpm for 3 min. The upper organic phase was carefully transferred and 0.75 ml of α-nitroso-β-naphthol reagent (0.4% α-nitroso-β-naphthol in 96% ethanol) was added. The intensity of the orange colour is directly proportional to the amount of cobalt present. After 30 min 2 ml of ethanol was added to dilute the contents and absorbance was read at 540 nm. The amount of free fatty acid released was estimated using standard linolenic acid curve. The enzyme activity was expressed as n moles of fatty acid released/min/mg of protein.

For inhibition studies, VRV-PL-V (3 µg) was pre incubated with or without different concentrations of ethanolic extract of *A. paniculata* (0-25 µg/ml) at 37 °C for 15 min. Appropriate controls were carried and further experiments were carried out as described above. The inhibition is expressed as percentage taking activity of venom alone as 100%. IC_{50} values were calculated using Graphpad version 5.0.

Neutralization of edema inducing activity by *A. paniculata* ethanolic extract

The procedure as modified by Vishwanath *et al.* [32] was followed. VRV-PL-V (5 µg) was pre-incubated without or with different concentration of ethanolic extract of *A. paniculata* (0-100 µg/ml) in a total volume of 20 µl saline. The reaction mixture was injected into intra plantar surface of right hind footpad of mice weighing 20–25 g. The left footpad that received 20 µl of saline served as control. After 45 min the mice were sacrificed by giving anaesthesia (Pentobarbitone, 30 mg/kg, i. p.) and both hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to oedema is expressed as the ratio of the weight of an oedematous limb to the weight of normal (sham injected) limb x 100. Minimum edema dose is defined as the microgram of protein causing an edema ratio of 120%. Injecting a fixed dose protein into mice footpads and sacrificing them at the regular period of time obtained the time course curve of edema inducing activity. Edema ratio was calculated and expressed as %.

Statistical analysis

The IC_{50} values were calculated using Graph Pad version 5.0. Inhibition percentages were calculated from the difference between inhibitor-treated group and control animals, which received the vehicle. Student's *t*-test for comparisons of unpaired data was used for statistical evaluation.

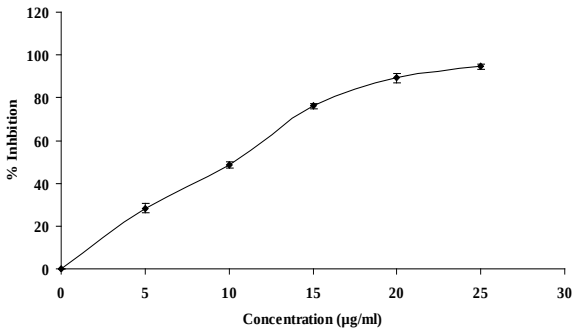

Fig. 1: Dose dependent inhibition of $svPLA_2$ (VRV-PL-V) activities by ethanolic extract of *A. paniculata*

Briefly, Phosphatidyl choline (PC) corresponding to 1000 nmoles/ml was made up to 680µl with VRV-PL-V (3 µg), with or without ethanolic extract of *A. paniculata* at various concentrations (0-25 µg/ml) and was incubated with other reaction mixture at 37 °C for 60 min and color developed was read at 540 nm. The results shows±SEM for n=3.

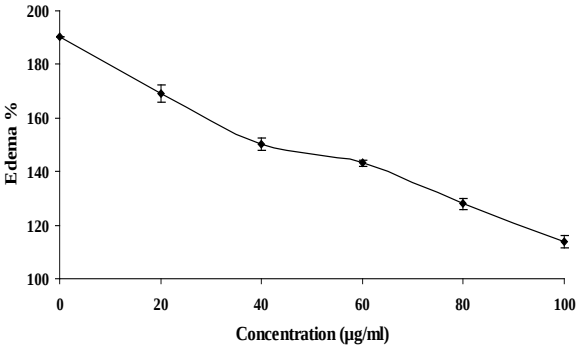

Fig. 2: Dose dependent neutralization of edema inducing activity of $svPLA_2$ (VRV-PL-VIIIa) activities by ethanolic extract of *A. paniculata*

The reaction mixture 30 µl containing VRV-PL-V (5 µg) was incubated for 30 min with increasing concentration of ethanolic extract of *A. paniculata* (100 µg/ml) of *M. indica*. Saline (30 µl) injected into the mouse foot-pad served as control. Data represents±SEM for n=3.

Table 1: Specific activity of VRV-PL-V and IC_{50} values

$sPLA_2$	Specific activity[a]	IC_{50}[b]
VRV-PL-V	142.1±4.3	12.4±0.6 µg/ml

[a]nmoles of fatty acid released/mg of protein/min at 37 °C, [b]IC_{50} value is defined as the amount of extract (µg/ml) required to inhibit 50% of enzyme activity in the given reaction mixture

RESULTS AND DISCUSSION

It is observed that effective inhibitors of $svPLA_2$ are known to suppress the toxic and lethal effects, which includes inflammation and its associated process [8]. In these line of studies, when evaluated anti-PLA_2 potential of *A. paniculata* against multi-toxic PLA_2 of *Daboii rusellii pulchella* i.e., VRV-PL-V, it was observed that the ethanolic extract of *A. paniculata* inhibited the enzymatic activity in a concentration dependent manner and the extent of inhibition was>95% at 25 µg/ml (fig. 1), with an IC_{50} value of 12.4±0.6 µg/ml (table. 1).

It is observed that $svPLA_2$s are known to induce edema when injected into mouse footpad as demonstrated before [32]. It is also observed that there are many inflammatory mediators which participate in the production of edema in a variety of inflammatory conditions [33]. Among others, histamine, prostaglandins, kinins and leukotrienes, could be implicated in the resulting edema due to PLA_2s in the case of snake venoms [34, 35]. The edema induced by snake venom PLA_2s, follows the classical two phases, which is characterized by a rapid initial first phase produced by mediators such as histamine and serotonin, and a delayed second phase mediated by prostaglandins that were as described in mice [34, 35]. Several $svPLA_2$ inhibitors are demonstrated to exhibit the concomitant inhibition of both enzyme activity and edema-inducing activity [7, 8]. From our earlier studies, based on the dose-response edema responsive reaction of multi-toxic-VRV-PL-V, a challenge dose of 5 µg was selected; due to its effective inflammatory result without damaging the overall physical integrity of animal, and help in the determination of the anti-inflammatory activity of *A. paniculata* [7]. It was observed that, when the ethanolic extract of *A. paniculata* was co-injected with VRV-PL-V, the extract significantly in a dose dependent manner reduced the edema formation (fig. 2). In addition**,** *A. Paniculate* extracts at the tested dose alone did not induce edema when injected into mice footpads. Urosolic acid inhibited the edema at all times after $svPLA_2$ injection (results not shown). Since the extract significantly inhibits edema formation it is most likely that the compounds in these extracts are directly interacting with PLA_2s activity and thus, acts on the first and second phases of this inflammatory response, as reported by others [34, 35]. *A. paniculata* has already been reported to inhibit carrageenan induced oedema showing statistically significant anti-inflammatory activity [36]. Andrographolide, which is one of the therapeutically important constituents of *A. paniculata is* known to significantly, inhibit carrageenan-; kaolin-and nystatin-induced paw oedema [37]. Further, it is reported that dehydroandrographolide, andrographolide and neoandrographolide exhibited strong anti-inflammatory activity by inhibiting *COX-1* expression in ionophore A23187-induced human platelets; LPS stimulated *COX-2* activity in human blood. Also, andrographolide is known to modulate the level of LPS-induced TNF-α, IL-6, IL-1β and IL-10 secretion in human blood [37, 38]. Our study further confirms the anti-inflammatory potential of *A. paniculata* using *Daboii rusellii pulchella* venom multi-toxic PLA_2 (VRV-PL-V) as inflammation inductor. In an earlier study, the terpeinoids fractions of *A. paniculata* was shown to neutralized the rattle snake (*Crotalus adamanteus*) venom activities [25]. Further, andrographolide which is a labdane diterpenoid and the main bioactive component of *A. paniculata* was shown to inhibit *O. hannah* venom activity [27]. From docking studies, it has been observed that andrographolide having significant binding activity against disintegrins, aggretin, echicetin, acutolysin C, denmotoxin and haditoxin [39]. Henceforth, it can be assumed that this active molecule might be the responsible for inhibiting the multi-toxin-VRV-PL-V PLA_2 activities.

Although at this point the mechanism of action of the extract is unclear and based on the finding that no visible change was detected in electrophoretic pattern of VRV-PL-V when incubated with extracts (data not shown), excludes the proteolytic degradation as a potential mechanism [10], and the most likely mechanism for anti-PLA_2 and its associated inflammatory activities by this extract could be due to the direct binding of the constituents of the extract with $svPLA_2$s active site, as it was observed that their was concernment inhibition of enzymatic and edematigenic activity of VRV-PL-V. Further, the ethanolic extract of *A. paniculata* inhibiting both *in vitro* PLA_2 enzymatic activity and *in vivo* edema inducing activity of VRV-PL-V, suggests a strong correlation between lipolytic activity and pro-inflammatory activity inhibition. It can be viewed that *A. paniculata* ethanolic extract can be developed for topical application, as effective anti-ophidian formulation, which contains potent anti-PLA_2 molecules.

CONCLUSION

The ethanolic extract of *A. paniculata* effectively inhibited the multi-toxic PLA_2 of *Daboii rusellii pulchella* i.e., VRV-PL-V and its associated inflammatory activities like edema. It was found that there is a strong correlation between lipolytic activity and pro-inflammatory activity inhibition. Therefore, the study suggests that the extract possess potent anti-ophidian agents that could be developed as a potential therapeutic agent against snake bite and also against inflammatory related diseases. This study also substantiates their anti-inflammatory properties by neutralizing the edema inducing activities of multi-toxic PLA_2 i.e., VRV-PL-V. Further in-depth studies on compounds present and the mechanism responsible for the anti-PLA_2 activity *viz* anti-inflammatory activity will be interesting, to development them as a new class of anti-ophidian and anti-inflammatory agents for therapeutic applications.

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CONFLICT OF INTERESTS

The authors declare that they don't have any conflict of interest

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