Inhibition of Group IIA Secretory Phospholipase A₂ and its Inflammatory Reactions in Mice by Ethanolic Extract of *Andrographis paniculata*, a Well-known Medicinal Food

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ABSTRACT

Andrographis paniculata Nees is an important medicinal plant found in the tropical regions of the world, which has been traditionally used in Indian and Chinese medicinal systems. It is also used as medicinal food. *A. paniculata* is found to exhibit anti-inflammatory activities; however, its inhibitory potential on inflammatory Group IIA phospholipases A₂ (PLA₂) and its associated inflammatory reactions are not clearly understood. The aim of the present study is to evaluate the inhibitory/neutralizing potential of ethanolic extract of *A. paniculata* on the isolated inflammatory PLA₂ (VRV-PL-VIIIa) from *Daboii rusellii pulchella* (belonging to Group IIA inflammatory secretory PLA₂ [sPLA₂]) and its associated edema-induced activities in Swiss albino mice. *A. paniculata* extract dose dependently inhibited the Group IIA sPLA₂ enzymatic activity with an IC₅₀ value of 10.3 ± 0.5 µg/ml. Further, the extract dose dependently inhibited the edema formation, when co-injected with enzyme indicating that a strong correlation exists between lipolytic and pro-inflammatory activities of the enzyme. In conclusion, results of this study shows that the ethanolic extract of *A. paniculata* effectively inhibits Group IIA sPLA₂ and its associated inflammatory activities, which substantiate its anti-inflammatory properties. The results of the present study warranted further studies to develop bioactive compound (s) in ethanolic extract of *A. paniculata* as potent therapeutic agent (s) for inflammatory diseases.

Key words: Disease, drug, inflammation, inhibition, neutralization, plants

SUMMARY

- This study emphasis the anti-inflammatory effect of *A. paniculata* by inhibiting the inflammatory Group IIA sPLA2 and its associated inflammatory activities such as 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 processes potent anti-inflammatory agents, which

could be developed as a potential therapeutic agent against inflammatory and related diseases.
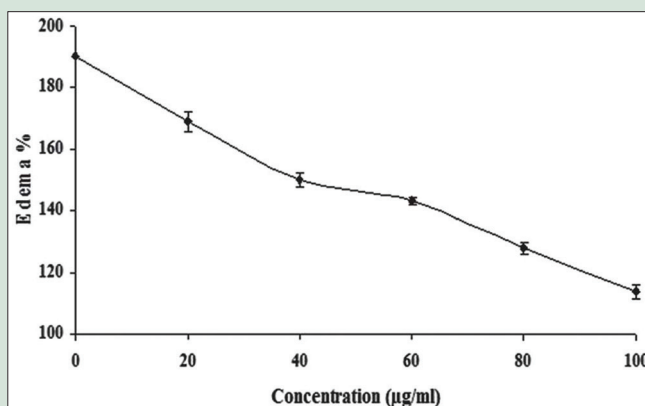
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INTRODUCTION

Secretory phospholipases A₂ (sPLA₂s) are known to regulate the arachidonic acid pathway by which pro-inflammatory mediators of inflammation are released.[1] In snake venoms, two groups of sPLA₂s (GI and GII) have been identified. Group I includes the snake venom PLA₂ (svPLA₂s) from *Elapinae* and *Hydrophiinae* venoms with 115–120 amino acid residues and these svPLA₂s are homologous to mammalian pancreatic GIB sPLA₂. Group II comprises the svPLA₂s from *Crotalinae* and *Viperidae* venoms with 120–125 amino acid residues and homologous to mammalian nonpancreatic Group IIA sPLA₂.[2] These groups of svPLA₂s are known to exhibit a wide variety of physiological and pathological effects that includes induction of inflammatory reactions.[3] Several studies have demonstrated that sPLA₂ inhibitors are efficient suppressors of inflammatory processes and thus help in the management of inflammatory diseases.[1,4,5] Due to the central role of sPLA₂s in the inflammatory process and considering the drawbacks and

severe side effects exhibited by present anti-inflammatory therapeutic agents, which includes the nonsteroidal anti-inflammatory drugs that inhibit either lipoxygenase or cyclooxygenase (COX)-½ enzymes,[6] there is renewed pharmacological interest in search of potent and specific sPLA₂ inhibitors from diverse sources.[1,5] In this context, many plant extracts and its constituents are reported for their anti-inflammatory

activity through the inhibition of sPLA$_2$s.[1,5,7] However, effective and specific inhibitors of sPLA$_2$ are still not available to date.

Andrographis paniculata Nees (Acanthaceae), commonly known as king of bitters, is a widely distributed medicinal herb in tropical Asian countries including India. It has been used as medicinal herb in traditional medicinal systems of India, China, Thailand, and Europe.[8] As evident from Indian Pharmacopoeia, *A. paniculata* is prominently known in 26 ayurvedic formulations; while in traditional Chinese medicine, due to its important "cold property," it is used to release body heat in fever.[9] This species is therapeutically well explored and effectively used for the treatment of asthma, gonorrhea, piles, dysentery and dyspepsia, influenza, gastric complaints, diarrhea, pharyngotonsillitis, fever, myocardial ischemia, common cold, diabetes, respiratory tract infections, and jaundice among others.[8,9] Further, it used as a medicinal food to treat various disorders.[8,9] It is reported to possess anti-microbial, hypotensive, anti-hyperglycemic, oxygen radical scavenging, anti-atherosclerotic, anti-malarial, anti-HIV, anti-platelet aggregation, hepatic lipid per-oxidation protective, hepatoprotective, choleretic, and anticancer properties.[8-10] In addition, the plant extract has also been reported to exhibit anti-typhoid, anti-fungal, anti-fertility, anti-nematicidal, anti-hepatitis, anti-hypoglycemic, antiulcerogenic, anti-snake venom, anti-thrombogenic, and anti-inflammatory properties.[8-10] In an earlier study, it was observed that the stem chloroform extract of *A. paniculata* is effective against carrageenan-induced edema showing statistically significant anti-inflammatory activity.[11] In this study, investigations were carried to evaluate the modulatory effect of whole plant ethanolic extract of *A. paniculata* on Group IIA sPLA$_2$ (i.e., purified VRV-PL-VIIIa PLA$_2$ enzyme from *Russell's Viper* venom), to further substantiate its anti-inflammatory properties.

MATERIALS AND METHODS

Materials

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

Collection of plant and extraction of crude extract

A. paniculata plant was collected from nearby areas of Thanjavur District, Tamil Nadu, India. The plant was authenticated at the Centre for Research in Indian Systems of Medicine (CRISM), SASTRA University (SU), Thanjavur, Tamil Nadu, where a voucher specimen (SASTRA/CRISM/PL/182) was deposited. The whole plant collected of free of disease and injury was washed several times with distilled water and shade dried. The whole plant was crushed into fine powder by using electric grinder. The powdered plant (40 g dry weight) was extracted using 95% ethanol (400 ml) in soxhlet extractor solvent and the extract was concentrated using rotary vacuum evaporator and the residue obtained (dark brown was dried, weighed, and was preserved in an airtight glass container. Further, until use, it was kept inside the refrigerator at 4°C.

Animals

Swiss Wister albino mice weighing about 20–25 g were obtained from the central animal house facility. The animal experiments protocol of this study were carried out after reviewing the protocols by the Animal Ethical Committee of the University and have been approved by the SU-Institutional Animal Care and Use Committee-(IAEC No. SU/IAEC/PROTOCOL No. 14/2013). Animal care and handling were conducted in compliance with the national and international regulations for animal research.

Isolation of Group IIA (VRV-PL-VIIIa) secretary phospholipase A$_2$

sPLA$_2$ belonging to Group IIA-VRV-PL-VIIIa from the venom of *Daboii russelli pulchella* (Southern region) was purified up to homogeneity as described previously by the method of Kasturi and Gowda,[12] and as modified by Srinivasan.[13] The enzyme was 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.*[14] using bovine serum albumin as protein standard.

Inhibition of phospholipase A2 activity by Andrographis paniculata ethanolic extract

The PLA$_2$ assay was carried out according to the method as described by Bhat and Gowda.[15] Phosphatidylcholine was diluted with petroleum ether (60–80°C) to get a concentration of 1000 nmoles/50 ml. The reaction mixture containing VRV-PL-VIIIa (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 min. After incubation, 0.5 ml of doles mixture (Isopropanol: Pet ether: 1NH$_2$SO$_4$, 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$_2$O + 0.8 ml glacial acetic acid] and 7 ml of solution B [saturated Na$_2$SO$_4$]) were added, mixed, and centrifuged at 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 color 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 nmoles of fatty acid released/min/mg of protein.

For inhibition studies, VRV-PL-VIIIa (3 μg) was preincubated 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, considering the activity of venom alone as 100%. IC$_{50}$ values were calculated using GraphPad version 5.0.

Neutralization of edema-inducing activity of Andrographis paniculata ethanolic extract

To examine the neutralization effect of ethanolic extract of *A. paniculata* on the VRV-PL-VIIIa induced edema, the procedure as modified by Vishwanath *et al.*[16] was followed. Here, the minimum edematic dose (MED) which is defined as the amount of protein concentration required to cause an edema ratio of 120% is used. Mice weighing between 20 and 25 g were used and distributed to seven groups each containing three mice. VRV-PL-VIIIa causing MED at 5 μg was preincubated without or with different concentrations of the extract (0–100 μg/ml) in a total volume of 20 μl saline and injected. Group – I was with VRV-P-VIIIa (5 μg) alone; Group-II with VRV-PL-VIIIa + 20 μg/ml of extract; Group-III with VRV-PL-VIIIa + 40 μg/ml of extract; Group-IV with VRV-PL-VIIIa + 60 μg/ml of extract; Group-V with VRV-PL-VIIIa + 80 μg/ml of extract; Group-VI with VRV-PL-VIIIa + 100 μg/ml of extract; and Group-VII with VRV-PL-VIIIa + ursolic acid. The reaction mixtures were injected into intra plantar surface of right hind footpad of mice and the left footpad that received saline (20 μl) served as control. After 45 min, the mice were sacrificed by giving anesthesia (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 edema is expressed as the ratio of the weight of edematous limb to the weight of normal (sham injected) limb ×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 regular intervals of time obtained time course curve of edema-inducing activity. Edema ratio was calculated and expressed as percent.

Statistical analysis

The IC$_{50}$ values were calculated using GraphPad 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.

RESULTS AND DISCUSSION

It is observed that effective inhibitors of sPLA$_2$ are known to suppress the inflammation and its associated processes.[1] Although *A. paniculata* is found to exhibit anti-inflammatory activities, its inhibitory potential on inflammatory Group IIA phospholipases A$_2$ and its associated inflammatory reactions are not clearly understood. In these line of studies, when analyzing the anti-inflammatory potential of *A. paniculata* for inhibiting inflammatory PLA$_2$ belonging to Group IIA, i.e., VRV-PL-VIIIa, 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 [Figure 1], with an IC$_{50}$ value of 10.3 ± 0.5 μg/ml [Table 1]. Most of the inflammatory sPLA$_2$s are known to induce edema when injected into mouse footpad as demonstrated before.[16] It is also observed that there are many inflammatory mediators which participate in the production of edema in a variety of inflammatory conditions.[17] Among others, histamine, prostaglandins, kinins, and leukotrienes could be implicated in resulting edema due to the action of PLA$_2$s as in the case of snake venoms.[18,19] The edema induced by snake venom PLA$_2$s belonging to Group IIA, 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.[18,19] Several sPLA$_2$ inhibitors are demonstrated to exhibit the concomitant inhibition of both enzyme activity and edema-inducing activity.[1,20] From

our earlier studies, based on the dose-response edema responsive reaction of VRV-PL-VIIIa, a challenge dose of 5 μg was selected. This dose was selected because its effective inflammatory result without damaging the overall physical integrity of the animal and helping in the determination of the anti-inflammatory activity of the drugs.[20] It was observed that when the ethanolic extract of *A. paniculata* was co-injected with VRV-PL-VIIIa (5 μg), the extract significantly reduced the edema formation in a dose-dependent manner [Figure 2]. In addition, *A. paniculata* extract at the tested dose alone did not induce edema when injected into mice footpads. Further, it was observed that ursolic acid which was used as standard when co-injected with VRV-PL-VIIIa, significantly reduced the edema formation (results not shown). Since the extract significantly inhibits edema formation, it is most likely that the compounds present in these extracts are directly interacting with PLA$_2$s activity. The study shows that the extract acts on the first and second phases of the inflammatory response, as reported by others.[18,19] *A. paniculata* has been reported to inhibit carrageenan-induced edema showing statistically significant anti-inflammatory activity.[11] Andrographolide, which is one of the therapeutically important constituents of *A. paniculata*, *is* known to significantly inhibit carrageenan, kaolin- and nystatin-induced paw edema.[21] Further, it is reported that dehydro andrographolide, andrographolide, and neo andrographolide exhibited strong anti-inflammatory activity by inhibiting COX-1 expression in ionophore A23187-induced human platelets and lipopolysaccharide (LPS) stimulated COX-2 activity in human blood. In addition, andrographolide is known to modulate the level of LPS-induced tumor necrosis factor-α, interleukin (IL)-6, IL-1 β, and IL-10 secretion in human blood.[21,22] It was also shown that *A. paniculata* extract neutralized the inflammatory and lethal effects induced by Indian Red Scorpion.[23] Our study further confirms the anti-inflammatory potential of *A. paniculata* by inhibiting Group IIA sPLA$_2$.

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 the electrophoretic

Table 1: Specific activity of VRV-PL-VIIIa and IC$_{50}$ values

sPLA$_2$	Specific activity[a]	IC$_{50}$[b]
VRV-PL-VIIIa	123.4±4.7	10.3±0.5 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. sPLA$_2$: Secretory phospholipase A2
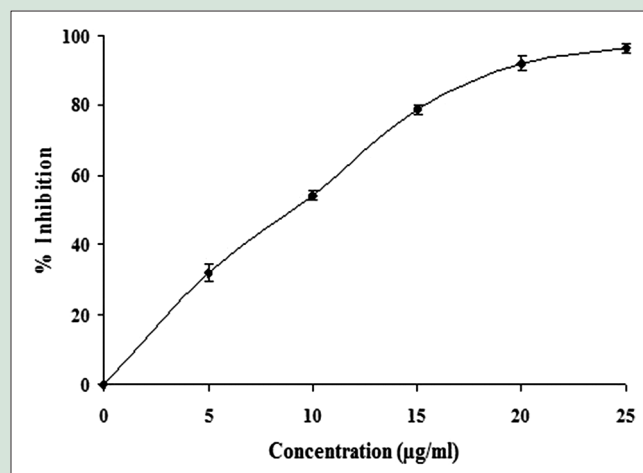
Figure 1: Dose-dependent inhibition of secretory phospholipase A$_2$ (VRV-PL-VIIIa) activities by ethanolic extract of *Andrographis paniculata*. Briefly, phosphatidylcholine corresponding to 1000 nmoles/ml was made up to 680 μl with VRV-PL-VIIIa (3 μg), with or without ethanolic extract of *Andrographis 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 show ± standard error of the mean for *n* = 3
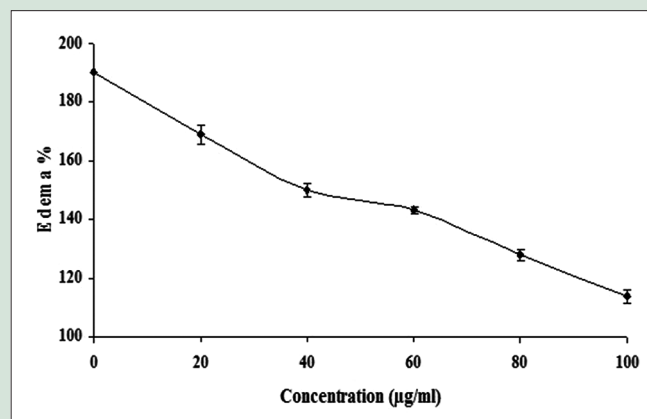
Figure 2: Dose-dependent neutralization of edema-inducing activity of secretory phospholipase A$_2$ (VRV-PL-VIIIa) activities by ethanolic extract of *Andrographis paniculata*. The reaction mixture 30 μl containing VRV-PL-VIIIa (5 μg) was incubated for 30 min with increasing concentration of ethanolic extract of *Andrographis paniculata* (100 μg/ml) of *Mangifera indica*. Saline (20 μl) injected into the mouse foot-pad served as control. Data represent ± standard error of the mean for *n* = 3

pattern of VRV-PL-VIIIa when incubated with extracts (data not shown), it excludes the proteolytic degradation as a potential mechanism,[20] and the most likely mechanism for anti-inflammatory activities by this extract could be due to the direct binding of the constituents of the extract with sPLA$_2$s active site, as it was observed that there was concernment inhibition of enzymatic and edematigenic activity of VRV-PL-V. 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, which contains potent anti-inflammatory molecules, can be developed for topical application as an effective anti-inflammatory formulation.

CONCLUSION

The ethanolic extract of *A. paniculata* effectively inhibited the inflammatory Group IIA sPLA$_2$ and its associated inflammatory activities such as 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 processes potent anti-inflammatory agents, which could be developed as a potential therapeutic agent against inflammatory and related diseases. This study also substantiates their anti-inflammatory properties by inhibiting the Group IIA sPLA$_2$. Further, in-depth studies on compounds present in the ethanolic extract of *A. paniculata* and the mechanism responsible for their anti-inflammatory activity will be investigated to develop them as a new class of anti-inflammatory agents for therapeutic applications.

Conflicts of interest

There are no conflicts of interest.

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