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/neutrallizing potential of ethanolic extract of A. paniculata on the isolated inflammatory PLA₂ (VRV-PlVIIIa) from Daboii russellii 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.

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, sPLA₂s from Elapinae and Hydrophiinae venoms with 115–120 amino acid residues and these sPLA₂s are homologous to mammalian pancreatic GIB sPLA₂. Group II comprises the sPLA₂s from Crotalinae and Viperidae venoms with 120–125 amino acid residues and homologous to mammalian nonpancreatic Group IIA sPLA₂.² These groups of sPLA₂s 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.⁴ 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 lipooxygenase or cyclooxygenase (COX)-1/2 enzymes,⁵ there is renewed pharmacological interest in search of potent and specific sPLA₂ inhibitors from diverse sources.⁶ In this context, many plant extracts and its constituents are reported for their anti-inflammatory activities.

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Cite this article as: Kishore V, Yarla NS, Zameer F, Nagendra Prasad MN, Santosh MS, More SS, et al. Inhibition of group IIA secretory phospholipase A₂ and its inflammatory reactions in mice by ethanolic extract of Andrographis paniculata, a well-known medicinal food. Phcog Res 2016;8:213-6.

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activity through the inhibition of sPLA₂. However, effective and specific inhibitors of sPLA₂ 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. 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. 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. Further, it is used as a medicinal food to treat various disorders. 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. In addition, the plant extract has also been reported to exhibit anti-typoidal, anti-fungal, anti-fertility, anti-nematicidal, anti-hepatitis, anti-hypoglycemic, antitumorogenic, anti-snake venom, anti-thrombogenic, and anti-inflammatory properties. 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. In this study, investigations were carried to evaluate the modulatory effect of whole plant ethanolic extract of A. paniculata on Group IIA sPLA₂ (i.e., purified VRV-PL-VIIa PLA₂ enzyme from Russell’s Viper venom), to further substantiate its anti-inflammatory properties.

**MATERIALS AND METHODS**

**Materials**

Venom of Viper 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 for future use). Further, until use, it was kept inside the airtight glass container. Further, until use, it was kept inside the airtight glass container. Further, until use, it was kept inside the airtight glass container.

**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-VIIa) secretary phospholipase A₂**

sPLA₂ belonging to Group IIA-VRV-PL-VIIa from the venom of *Daboii russelli pulchella* (Southern region) was purified up to homogeneity as described previously by the method of Kasturi and Gowda, and as modified by Srinivasan. 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. using bovine serum albumin as protein standard.

**Inhibition of phospholipase A₂ activity by Andrographis paniculata ethanolic extract**

The PLA₂ assay was carried out according to the method as described by Bhat and Gowda. Phosphatidylcholine was diluted with petroleum ether (60–80°C) to get a concentration of 1000 nmoles/50 ml. The reaction mixture containing VRV-PL-VIIa (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 CaCl₂ (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: Na₂SO₄ 40:10:1) was added, mixed, and centrifuged at 1000 rpm for 3 min. To the organic phase, 0.5 ml of CHCl₃; 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(NO₃)₂ -6H₂O + 0.8 ml glacial acetic acid] and 7 ml of solution B [saturated Na₂SO₄] 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-VIIa (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%. IC₅₀ 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-VIIa induced edema, the procedure as modified by Vishwanath et al. 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-VIIa 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-VIIa (5 µg) alone; Group-II with VRV-PL-VIIa + 20 µg/ml of extract; Group-III with VRV-PL-VIIa + 40 µg/ml of extract; Group-IV with VRV-PL-VIIa + 60 µg/ml of extract; Group-V with VRV-PL-VIIa + 80 µg/ml of extract; Group-VI with VRV-PL-VIIa + 100 µg/ml of extract; and Group-VII with VRV-PL-VIIa + 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...
Statistical analysis
The IC₅₀ 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 sPLA₂ are known to suppress the inflammation and its associated processes. Although A. paniculata is found to exhibit anti-inflammatory activities, its inhibitory potential on inflammatory Group IIA phospholipases A₂ 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 PLA₂, 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 IC₅₀ value of 10.3 ± 0.5 µg/ml [Table 1]. Most of the inflammatory sPLA₂s are known to induce edema when injected into mouse footpad as demonstrated before. It is also observed that there are many inflammatory mediators which participate in the production of edema in a variety of inflammatory conditions. Among others, histamine, prostaglandins, kinins, and leukotrienes could be implicated in resulting edema due to the action of PLA₂ as in the case of snake venoms. The edema induced by snake venom PLA₂s 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. Several sPLA₂ inhibitors are demonstrated to exhibit the concomitant inhibition of both enzyme activity and edema-inducing activity. 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. 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 mouse footpads. Further, it was observed that ursoic 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 PLA₂s activity. The study shows that the extract acts on the first and second phases of the inflammatory response, as reported by others. A. paniculata has been reported to inhibit carrageenan-induced edema showing statistically significant anti-inflammatory activity. Andrographolide, which is one of the therapeutically important constituents of A. paniculata, is known to significantly inhibit carrageenan, kaolin-and nystatin-induced paw edema. Further, it is reported that dehydroandrographolide, 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. It was also shown that A. paniculata extract neutralized the inflammatory and lethal effects induced by Indian Red Scorpion. Our study further confirms the anti-inflammatory potential of A. paniculata by inhibiting Group IIA sPLA₂.

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

| sPLA₂     | Specific activityᵃ | IC₅₀ [µg/ml]  |
|-----------|--------------------|--------------|
| VRV-PL-VIIIa | 123.4±4.7          | 10.3±0.5     |

ᵃ: moles of fatty acid released/mg of protein/min at 37°C. IC₅₀ value is defined as the amount of extract (µg/ml) required to inhibit 50% of enzyme activity in the given reaction mixture.

Figure 1: Dose-dependent inhibition of secretory phospholipase A₂ (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 A₂ (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, 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 sPLA₂ active site, as it was observed that there was concernment inhibition of enzymatic and edematogenic activity of VRV-PL-V. The ethanolic extract of A. paniculata inhibiting both in vitro PLA₂, 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 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. This study also substantiates their anti-inflammatory properties by inhibiting the Group IIA sPLA₂. 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.

Acknowledgments

Dr. Dhananjaya Bhadrupura Lakkappa (DBL) thanks Jain University for the constant encouragement given to progress in research. DBL and V. Kishore thank SU. DBL acknowledge the Department of Science and Technology, Government of India, for providing the grant INT/SLP/P-007/2012, dated 15th May, 2014. DBL also acknowledge SU for providing the T. R. Rajagopalan Fund. F. Zameer acknowledge University Grants Commission (UGC) for C. V. Raman Fellowship postdoctoral Fellowship 2014–15, Prof. Dr. K. V. Prabhakara, Principal, SBRR Mahajana First Grade College and the Management for their support and inspiration. N. S. Yarla and D. G. Rao are thankful to the UGC (F.No. 42-643/2013) for financial support.

Financial support and sponsorship

N. S. Yarla and D. G. Rao are thankful to University Grants Commission.

Conflicts of interest

There are no conflicts of interest.

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