

Neutralization of Inflammation by Inhibiting *In vitro* and *In vivo* Secretory Phospholipase A₂ by Ethanol Extract of *Boerhaavia diffusa* L.

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ABSTRACT

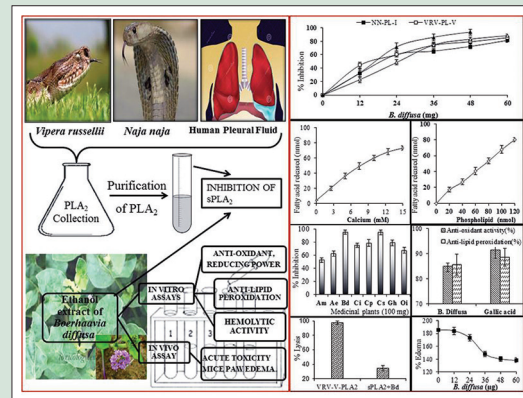
Background: Inflammation is a normal and necessary prerequisite to healing of the injured tissues. Inflammation contributes to all disease process including immunity, vascular pathology, trauma, sepsis, chemical, and metabolic injuries. The secretory phospholipase A₂ (sPLA₂) is a key enzyme in the production of pro-inflammatory mediators in chronic inflammatory disorders such as rheumatoid arthritis, coronary heart disease, diabetes, and asthma. The sPLA₂ also contribute to neuroinflammatory disorders such as Parkinson's, Alzheimer's, and Crohn's disease. **Aims:** The present study aims to investigate the inhibition of human sPLA₂ by a popular medicinal herb *Boerhaavia diffusa* Linn. as a function of anti-inflammatory activity.

Materials and Methods: The aqueous and different organic solvents extracts of *B. diffusa* were prepared and evaluated for human synovial fluid, human pleural fluid, as well as *Vipera russelli* and *Naja naja* venom sPLA₂ enzyme inhibition. **Results:** Among the extracts, the ethanol extract of *B. diffusa* (EEDB) showed the highest sPLA₂ inhibition and IC₅₀ values ranging from 17.8 to 27.5 µg. Further, antioxidant and lipid peroxidation activities of *B. diffusa* extract were checked using 2,2-diphenyl-1-picrylhydrazyl radical, thiobarbituric acid, and rat liver homogenate. The antioxidant activity of EEDB was more or less directly proportional to *in vitro* sPLA₂ inhibition. Eventually, the extract was subjected to neutralize sPLA₂-induced mouse paw edema and indirect hemolytic activity. The EEDB showed similar potency in both the cases. **Conclusions:** The findings suggest that the bioactive molecule/s from the EEDB is/are potentially responsible for the observed *in vitro* and *in vivo* sPLA₂ inhibition and antioxidant activity.

Key words: Ethanolic extract of *Boerhaavia diffusa* L., human pleural fluid, human synovial fluid, *Naja naja* phospholipase A₂, *Vipera russelli* phospholipase A₂

SUMMARY

- The present study aims to investigate the inhibition of human sPLA₂ by a popular medicinal herb *Boerhaavia diffusa* Linn. as a function of anti-inflammatory activity.



Abbreviation Used: EEDB: Ethanolic extract of boerhaavia diffusa, sPLA₂: Secretory phospholipase A₂, HSF: Human synovial fluid, HPF: Human pleural fluid, VRV-PLA₂-V: *Vipera russelli* phospholipase A₂, NN-PLA₂-I: *Naja naja* phospholipase A₂.

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INTRODUCTION

Inflammation is a normal and necessary prerequisite to heal the injured tissues.^[1] Inflammation contributes to all disease processes including immunity, vascular pathology, trauma, and sepsis. External force or internally disruptive chemical or biological agents also cause tissue injury, where inflammatory reactions are initiated with the results that blood vessels and other tissues in or near the injured area will exhibit dynamic complex cytological and histological reactions. These reactions are local in nature and are designed to cause the destruction or removal of injurious materials, such as bacteria, foreign matter, or chemicals, injured tissue and to promote morphological responses that lead to repair and healing.^[1]

However, inflammation is a double-edged sword, with undesirable consequences such as systemic shock and circulatory collapse, if prolonged leads to local tissue damage in many organs.^[2,3] Inflammatory response can destroy healthy tissue and cause more damage than the

original problem; as a result, uncontrolled inflammatory reactions is the biochemical basis for the origin of chronic inflammatory disease such as rheumatoid arthritis, coronary heart disease, diabetes, and asthma. Hence, inflammatory diseases contribute the greatest clinical suffering and provide major targets for improved treatment.

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Secretory phospholipase A₂

Inflammation is mainly mediated by secretory phospholipase A₂ (sPLA₂), which catalyzes the hydrolysis of membrane phospholipids at the second position. Number of studies has reported dramatic increase of sPLA₂ levels in biological fluids of patients with various inflammatory diseases. Initially, Vadas *et al.* showed that septic shock is associated with high levels of sPLA₂ in the plasma of patients.^[4] Later, it was shown that high level of sPLA₂ is present in the inflammatory exudates and plasma of patients with diseases such as rheumatoid arthritis, septic shock, asthma, Crohn's disease, and bronchoalveolar lavage fluids.^[5-7] The other functions of sPLA₂s are cell migration, apoptosis, reactive oxygen species (ROS) generation and cytotoxicity, coagulation,^[8-11] and several snake venom PLA₂ enzymes are responsible for local tissue damage such as edema and hemorrhage.^[12]

Pro-inflammatory mediators and reactive oxygen species

Elevated level of sPLA₂ enzymes in many inflammatory disorders plays a key role by releasing free arachidonic acid and lysophospholipid which are precursors for the production of pro-inflammatory lipid mediators and ROS. Cyclooxygenase-1/2 and lipoxygenase catalyze the synthesis of pro-inflammatory mediators such as prostaglandins, thromboxanes, and leukotrienes. Lysophospholipid is further converted into platelet activation factor (PAF) by acetyltransferase that continued to cause inflammation.^[13]

In addition, the arachidonic acid cascade is considered to be a significant source of ROS such as O₂⁻ and OH[•],^[11,14] which serves as a part of defensive mechanism by harming the invading pathogens. The generation of free radicals, if persisted after the defensive role, results in a more deleterious complication. In such conditions, regulation of these free radicals is equally important to that of the regulation of pro-inflammatory eicosanoids. However, there is a continuous production of pro-inflammatory lipid mediators along with existing free radicals cause augmentation of inflammatory disease by several folds. Thus, PLA₂ catalyzes the rate-limiting step in the eicosanoid pathway by generating pro-inflammatory lipid mediators and free radicals and is therefore regarded as a crucial enzyme in various inflammatory diseases.

Boerhaavia diffusa Linn. (Punarnava)

A perennial creeping weed belongs to the 4 o'clock family or *Nyctaginaceae*. *Boerhaavia diffusa* L. is commonly called hogweed, encountered in different terrestrial habitat, ranging from managed grasslands, wastelands, agroecosystems to large forest gaps.^[15] The plant *B. diffusa* has a long history of uses by indigenous and tribal people and in ayurvedic and leaves of *B. diffusa* are often used as a green vegetable in many parts of India. It is taken in herbal medicines for pain relief and other uses.^[16]

The *B. diffusa* roots have been widely used for the treatment of dyspepsia, inflammations, heart diseases, jaundice, abdominal pain, and enlargement of spleen.^[17] Pharmacological studies have demonstrated that extracts of *B. diffusa* possesses diuretic,^[15] anti-inflammatory,^[16] antifibrinolytic,^[18] anticonvulsant, and antibacterial properties.^[19] The *B. diffusa* contains secondary metabolites such as hypoxanthine 9-L-arabinofuranoside, boeravinone A to F,^[20-22] serratagenic acid, liriodendron,^[23] oleanolic acid,^[24] ursolic acid, and punarnavoside.^[25] All these properties have made this plant very interesting and the plant has played an important role in the treatment of human diseases. Hence, in the present study, the ethanolic extract of *Boerhaavia diffusa* (EEBD) was subjected for PLA₂ inhibition as an anti-inflammation.

MATERIALS AND METHODS

Materials

¹⁴C-oleic acid was obtained from PerkinElmer Life Sciences Inc., Boston, MA, USA. Fatty acid-free bovine serum albumin (BSA) fraction V was purchased from PAA Laboratories, GmbH Haidmannweg, Austria. Agar, beef extract, peptone, and lactose were obtained from HiMedia Laboratories Private Limited, Mumbai, India. Scintillation cocktail (Ultima Gold) was obtained from Packard Bioscience, USA. *Escherichia coli* (lyophilized cells of strain W [ATCC 9637]), Sephadex (G-25, 50, and 75), CM-Sephadex C-25, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]), thiobarbituric acid (TBA), gallic acid (GA), dimethyl sulfoxide (DMSO), oleanolic acid were purchased from Sigma-Aldrich Chemical Laboratories, St. Louis, MO, USA. Human pleural fluid (HPF) and synovial fluid were obtained from Princes Krishnammanni Tuberculosis and Chest Disease Hospital, Mysore, and Dr. Hegde Orthopaedic Clinic, Mysore, India. Blood samples for indirect hemolytic activity were obtained from the healthy volunteers, Department of Studies in Biochemistry, University of Mysore. All other chemicals and reagents used in this study were of analytical grade or better and solvents were redistilled before use.

Plant materials

Aegle marmelos, *Ailanthus excelsa*, *B. diffusa*, *Clerodendrum indicum*, *Clerodendrum phlomidis*, *Clerodendrum serratum*, *Gmelina arborea*, and *Oroxylum indicum* plants were collected from "Chandravana," the garden of medicinal plants maintained by Ayurvedic College, Mysore, India. These plants were authenticated with the help of botanist Prof. T. C. Shivashankarmurthy. The voucher specimens were deposited in the Herbarium, Department of Studies in Botany, University of Mysore, India.

Venom

Lyophilized powder of *Naja naja* and *Vipera russelli* snake venoms were purchased from Hindustan Park, Kolkata, India, and Irula Co-operative Society Ltd., Chennai, India, respectively.

Animal

Swiss albino mice and rats weighing 20–25 g and 150–200 g were obtained from Central Animal House Facility, Department of Studies in Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the National Regulations for Animal Research. The animal experiments were carried out after reviewing the protocols by the Animal Ethical Committee of the University of Mysore, Mysore, India.

Methods

Preparation of plant extracts

The plants were shade dried and powdered using conventional mixer. The pulverized powder of each plant was extracted hexane, benzene, chloroform, acetone, ethanol, methanol, and water using Soxhlet apparatus (100/750; w/v). The entire sample obtained was centrifuged at 5000 ×g for 15 min, and solvents in the supernatant were removed by vacuum rotary evaporator (Heidolph, HB Digit, Q-01). Aqueous extract was reduced to powder by lyophilization. The solid powder obtained was weighed to calculate the percentage yield from the initial weight. For the further studies, the extracts were dissolved in ethanol.

Preparation of ¹⁴C-oleate-labeled *Escherichia coli* substrate

¹⁴C-oleate-labeled *E. coli* substrate was prepared according to Patriarca *et al.*^[26] A single colony of *E. coli* was picked to prepare a miniculture using 10 ml of Luria-Bertani (LB) medium. The culture was grown

overnight in rotating water bath at rpm of 90 at 37°C. This miniculture was diluted 100 times with LB medium and continued the incubation. An absorbance of 0.6–0.9 at 520 nm indicates that the bacteria are in log phase of their growth. These cells were harvested by centrifugation at 1500 ×g for 5 min. The cell pellet was resuspended in 100 ml of LB medium. In a separate conical flask, 125 μCi of ¹⁴C-oleate was evaporated and was resuspended in 5 ml of 20% fatty acid-free BSA. The prepared *E. coli* culture was mixed with ¹⁴C-oleate and continued incubation at 37°C in rotating shaking water bath for 4 h at an rpm of 90. The labeled *E. coli* were washed 3–4 times with phosphate buffer saline (PBS) and autoclaved to inactivate endogenous PLA₂ activity. The labeled *E. coli* cells were diluted with PBS and similarly prepared autoclaved cold *E. coli* cells to get 10,000 cpm in 30 μl of diluted sample. This preparation was used further as substrate for PLA₂ enzyme activity.

Purification of secretory phospholipase A₂ from *Vipera russelli* venom

The sPLA₂ from *V. russelli* venom was purified to homogeneity according to modified method of Kasturi and Gowda.^[27] Briefly, *V. russelli* venom (110 mg) was fractionated on Sephadex G-75 column (1.5 cm × 110 cm) using 0.05 M phosphate buffer pH 7.0. The venom was resolved into three fractions. The second fraction, which constitutes about 30% of the total protein loaded, showed PLA₂ activity. This PLA₂ fraction was lyophilized and further subjected to CM Sephadex-C-25 column (2.5 cm × 20 cm) chromatography. The fractions were eluted stepwise using phosphate buffers of varying molarities and pH (0.05–0.2 M, pH 7.0–8.0). They were resolved into two fractions labeled as V and VIII, respectively. The above eluted two fractions are similar to V and VIII of Kasturi and Gowda protein profile. The lyophilized fractions V and VIII were next subjected to Sephadex G-50 column (1.0 cm × 40 cm) chromatography and eluted using 0.05 M phosphate buffer pH 7.0. Both the peaks were homogenous and showed PLA₂ activity. Homogeneity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)^[28] and reversed-phase high-performance liquid chromatography.

Purification of secretory phospholipase A₂ from human inflammatory fluids

The sPLA₂ from these inflammatory fluids, HPF, and human synovial fluid (HSF) is purified by the modification method previously described by Stefanski *et al.*^[28] Briefly, fluids were centrifuged at 1000 ×g for 15 min to sediment cells and debris. The supernatant was mixed with equal volume of ice-cold 0.36 N H₂SO₄ constant stirring at 4°C overnight. After centrifugation at 7500 ×g, the supernatant was dialyzed against 10 mM sodium acetate buffer, pH 4.5, and the dialysate was centrifuged at 7500 ×g for 5 min. The supernatant was incubated at 80°C for 5 min and centrifuged to yield a supernatant enriched in sPLA₂ activity. The above procedure was repeated several times to obtain considerable amount of sPLA₂ enriched source, which was lyophilized and stored. This lyophilized powder was dissolved in 0.5 M sodium acetate buffer, pH 5.0 and applied to CM-Sephadex C-50, which had been preequilibrated with the same buffer. The column was discontinuously eluted with 0.5 M sodium acetate buffer, pH 5.0; 0.3 M NaCl in 0.2 M Tris-HCl, pH 8.5; and 3 M NaCl in 0.2 M Tris-HCl buffer pH 8.5. The PLA₂ was eluted in the latter buffer. Fractions with PLA₂ activity were pooled, dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, and lyophilized. The lyophilized residue was dissolved in 0.05 M Tris-HCl, pH 7.5. This PLA₂ fraction was passed through Sephadex G-75 column using 10 mM Tris-HCl buffer, pH 7.5. The protein peak with PLA₂ activity from Sephadex G-75 column was further checked for purity on SDS-PAGE. This peak was further resolved into two protein bands in 12.5% SDS-PAGE. Both bands showed PLA₂ activity after electroelution. The fractions with PLA₂

activity were pooled, lyophilized, and further used as purified pleural fluid sPLA₂ (Group-IIA).

Inhibition of phospholipase A2 activity

Inhibition of PLA₂ activity was studied by previous method.^[29] Solutions of *B. diffusa* extracts were prepared by dissolving 1 mg in very small volume of DMSO (30 μl) and made up to 1 ml with 100 mM Tris-HCl buffer (pH 7.4). Inhibition was carried out with indicated concentrations of inhibitors for HSF, HPF, *Vipera russelli* phospholipase A₂ (VRV-PLA₂-V), and *Naja naja* phospholipase A₂ (NN-PLA₂-I) enzymes. The amount of enzyme was chosen such that a 60%–70% hydrolysis of substrate by concentration dependent assay at 37°C for 60 min. IC₅₀ concentration was calculated using software GraphPad version Prism 5.0 (GraphPad Software, San Diego California USA). Ursolic acid is well-known inhibitor of sPLA₂ and used as positive control. Control experiments were performed with DMSO (the highest concentration of DMSO used is 0.022%).

Effect of substrate and calcium concentration on secretory phospholipase A2 inhibition

The reaction mixture 350 μl containing VRV-PL-V alone or with IC₅₀ concentration of inhibitors in 100 mM Tri-HCl buffer, pH 7.4 and 5 mM calcium was used for PLA₂ assay. Addition of various concentration of substrate (20–120 nM) started the reaction. Calcium concentration ranging from 2.5 to 15 mM was added to PLA₂ assay mixture. The assay was carried out as described above.

Neutralization of indirect hemolytic activity

Indirect hemolytic activity was assayed as described by Boman and Kaletta.^[30] The substrate for the indirect hemolytic assay was prepared by suspending 1 ml of packed fresh human RBC and 1 ml fresh hen's egg yolk in 8 ml of PBS. Extracts and EEED were preincubated with 30 μg of VRV-PL-V for 30 min at 37°C. To this preincubated sample, 1 ml of substrate was added and allowed to react for 45 min at 37°C. The reaction was stopped by adding 9 ml of ice-cold PBS. The suspension was mixed and centrifuged at 1500 ×g for 20 min. The released hemoglobin was read at 530 nm. Sample with venom alone served as positive control.

Neutralization of edema-inducing activity

The modified procedure of Vishwanath and Gowda was followed.^[31] Mice weighing 20–25 g were injected with 5 μg of VRV-PL-V enzyme alone or mixed with different concentrations of EEED in a total volume of 20 μl saline into their intraplantar surface of right hind footpads. Respective left footpads received 20 μl of saline or vehicle and served as controls. After 45 min, the mice were sacrificed after giving anesthesia (pentobarbital, 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. Percentage increase of sham-injected control compared to the uninjected limb is 117 ± 4.

Determination of total phenolics by Folin–Ciocalteu assay

The concentration of total phenolics in the extracts of medicinal plants was determined by the method of Singleton *et al.*, with slight modification.^[32] Extracts were taken at 100 μg concentration by their dry weight and mixed with 500 μl of 5% sodium carbonate; 250 μl of Folin–Ciocalteu reagent (1:1 diluted with water) was added, mixed thoroughly, and incubated at room temperature for 1 h. After the incubation, the absorbance was measured at 725 nm using a spectrophotometer (Shimadzu 1601A). The phenolic contents of the extracts were determined from calibration curve generated using GA and the concentration was expressed as equivalents of GA (GAE/100 g).

Antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl radical method

Antioxidant activity of plant extracts was determined using DPPH as described by Blios.^[33] The medicinal plants extracts were taken at 100 µg concentration by their dry weight and mixed with 5 ml of 0.1 mM methanolic solution of DPPH[•] and incubated at 20°C for 20 min in darkness. The control was prepared as above without any extract and methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical scavenging activity was expressed as percentage activity using the following formula. Butylated hydroxyanisole and GA were used for competition.

Percentage radical scavenging activity = $[(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100$.

Antioxidant activity by thiobarbituric acid method

Antioxidant activity of the extracts was performed using TBA according to the protocol of Halliwell and Gutteridge.^[34] Lipid peroxidation induced by ferric chloride resulted in the production of malondialdehyde (MDA), lipid peroxide. TBA reacts with MDA to form a di-adduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm.

Preparation of rat liver homogenate for lipid peroxidation

The animal used was normal albino rats of Swiss Wistar strain and rats perfused liver were isolated, and 10% (w/v) homogenate was prepared using a Potter-Elvehjem homogenizer under ice-cold (0°C–4°C) condition. The homogenate was centrifuged at 1500 ×g for 5 min, and clear supernatant was used for lipid peroxidation analysis.^[35]

Plant extracts were taken at 100 µg concentration by their dry weight and mixed with 1.0 ml of 0.15 M KCl and 0.5 ml of rat liver homogenate. Peroxidation was initiated by adding 100 µl of 0.2 mM ferric chloride. After incubation at 37°C for 30 min, the reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 4% trichloroacetic acid (TCA) and 0.38% TBA. The reaction mixtures were heated at 80°C for 60 min. The samples were cooled and centrifuged, and the absorbance of the supernatants was measured at 532 nm. An identical experiment was performed in the absence of the extracts to determine the amount of lipid peroxidation obtained in the absence of extract. The anti-lipid peroxidative (ALP) activity is expressed as percentage ALP using the following formula:

Percentage ALP = $1 - \text{sample absorbance} / \text{control absorbance} \times 100$.

Determination of reducing power

The reducing powers of extracts were determined according to the method of Yen Duh.^[36] The extracts were taken at 60 µg concentration of their dry weight and mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 20 min. The reaction was terminated by adding 2.5 ml of 10% TCA and the mixture was centrifuged at 3500 ×g for 10 min. An aliquot of supernatant (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride to form a colored complex which is measured at 700 nm.

Acute toxicity

Acute oral toxicity study was performed as per the OECD-423 guidelines (acute toxic class method). Albino mice ($n = 6$) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for overnight providing only water after which the extracts were administered orally at the dose level of 5 mg/kg body weight by oral feeding needle and observed for 14 days. Mortality was not observed; the procedure was repeated for further higher dose such as 50, 300, and 2000 mg/kg body weight.^[37]

Statistical analysis

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. The experimental results were presented as mean ± standard deviation of three determinations. The IC₅₀ concentration was calculated by Boltzmann dose–response analysis using originpro 6.1 OriginLab, Northampton, MA. The statistical significance between different sPLA₂s at a given concentration was analyzed by Duncan's multiple range tests at $P < 0.05$ using ANOVA of SPSS software (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc.).

RESULTS

Inhibition of sPLA₂ is the intense pharmacological interest because the release of arachidonic acid from the *sn*-2 position of phospholipids is the rate-limiting step in the production of eicosanoid mediators of inflammation.^[38,39] The oxidative metabolism of arachidonic acid also produces ROS, which are implicated in many degenerative neurological conditions.^[40] Some medicinal plants such as *Andrographis paniculata* and *Tamarindus indica* show inhibition of sPLA₂ at less IC₅₀.^[41,42] The present study report the potency of ethanolic leaf extract of *B. diffusa* against enzymatic and pharmacological activities of secretory PLA₂ enzymes.

Initially, inhibition of sPLA₂ (VRV-PL-V) was carried out using the extracts of all the medicinal plants at 100 µg/350 µl concentration. The extracts of plants inhibit to the various extents. Out of the eight ethanol extracts, *B. diffusa* and *C. serratum* plants inhibited VRV-PL-V activity to the maximum extent (>90%). The extracts of *Aegie mermelosa*, *A. excelsa*, *C. indicum*, *Clerodendrum phlomidis*, *G. arborea*, and *O. indicum* were also showed more than 50% inhibition [Figure 1a]. The EEED showed highest inhibition VRV-PL-V [Table 1].

Inhibition of secretory phospholipase A2 enzymes

Further, inhibition of sPLA₂ of different source by the EEED was carried out. The sPLA₂s from HPF, HSF PLA₂, VRV-PL-V (belongs to Group-IIA), and NN-PL-I (belongs to Group-I) inhibition was carried out for the comparison. The extract which was in the range of 12–60 µg was incubated with sPLA₂ enzymes in standard set of conditions. The EEED inhibited sPLA₂ enzymes in concentration-dependent manner to the various extents. The extent of inhibition of HSF-PLA₂ was highest (96%) at the extract concentration of 48 µg, followed by HPF-PLA₂ (92.1%), VRV-PL-V (85.6%), and NN-PL-I (78.0%) at 60 µg

Table 1: Percentage inhibition of *Vipera russelli* venom phospholipase-V by different solvent extracts of medicinal plants. 75 g powder of each source was extracted sequentially using Soxhlet apparatus with increasing polarity of organic solvents at 50°C–70°C for 48 h each and inhibition of secretory phospholipase A₂ (*Vipera russelli* venom phospholipase-V) enzyme was studied by extracts at 100 µg concentration.

Plants/% inhibition	Hexane	Benzene	Chloroform	Acetone	Ethanol	Methanol	Water
<i>B. diffusa</i>	10.4	15.4	45.2	42.5	93.5	72.9	80.2
<i>C. serratum</i>	4.2	5.3	25.6	68.5	85.6	51.2	76.4

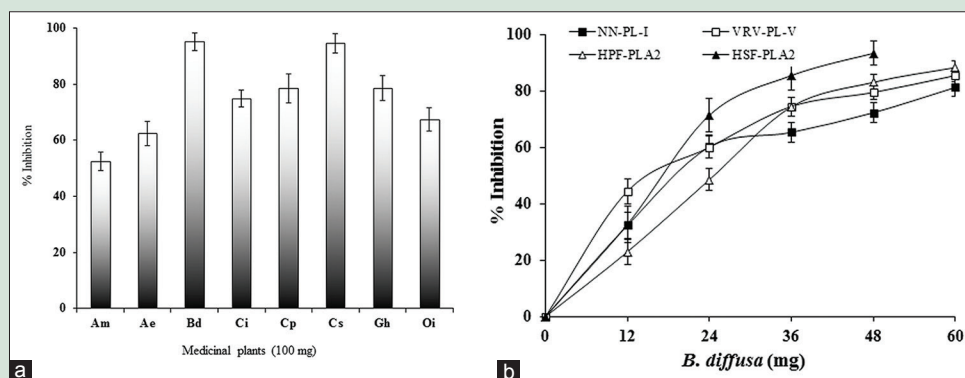


Figure 1: Inhibition of sPLA₂ (VRV-PL-V) enzyme by ethanol extracts of medicinal plants (a) and inhibition of sPLA₂ from different sources by ethanol extracts of *Boerhaavia diffusa* leaf (b). The reaction mixture, 350 ml contained VRV-PL-V (4 µg), 5 mM calcium, 100 mM Tris-HCl buffer, and 100 µg ethanolic extract of *Boerhaavia diffusa*. The reaction was initiated by adding 30 µl of substrate and incubated for 1 h at 37°C. Data represents mean ± standard deviation (n = 3). Plants: (Am: *Aegle marmelos*; Ae: *Ailanthus excelsa*; Bd: *Boerhaavia diffusa*; Ci: *Clerodendrum indicum*; Cp: *Clerodendrum phlomidis*; Cs: *Clerodendrum serratum*; Ga: *Gmelina arborea*; and Oi: *Oroxylum indicum*). sPLA₂: Secretory phospholipase A₂; VRV-PLA₂-V: *Vipera russelli* phospholipase A₂

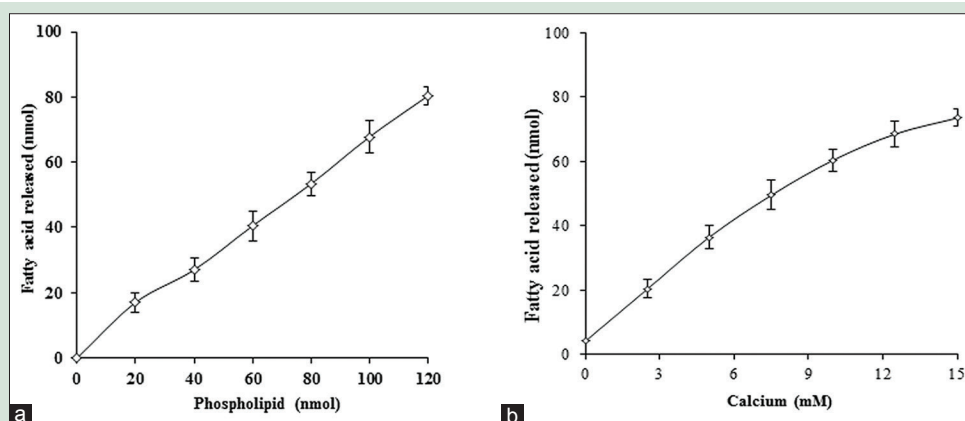


Figure 2: Effect of substrate (a) and calcium (b) concentration on inhibition of VRV-PL-V by the extracts at IC₅₀ concentration: The reaction mixture contains VRV-PL-V alone or with IC₅₀ concentrations of extracts in 100 mM Tri-HCl buffer pH 7.4 and 5 mM CaCl₂. The reaction was initiated by adding indicated concentration of substrate and incubated for 1 h. The data were represented as mean ± standard deviation (n = 4). VRV-PL-V: *Vipera russelli* phospholipase A₂

concentration [Figure 1b]. The oleonic acid is a well-known inhibitor of the Group-IA and Group-IIA PLA₂ enzymes taken as standard (data not shown). The linear regression analysis of linear portion of concentration-dependent inhibition curves used to calculate IC₅₀ values. IC₅₀ values of HSF-PLA₂ and HPF-PLA₂ enzymes are 17.8 and 22.5 µg, respectively, whereas VRV-PL-V and NN-PL-I are 26.4 µg and 27.5 µg, respectively [Table 2].

Effect of substrate and calcium concentration on inhibition

The mechanism of inhibition of PLA₂ enzymes by the EEBD was examined as a function of substrate concentration. The sPLA₂ (VRV-PL-V) activity was measured in the presence and absence of IC₅₀ concentration of EEBD. The substrate concentration was increased up to 120 nM. Activity of VRV-PL-V enzyme increases almost linearly with increased substrate concentration. The percentage of inhibition is in the range of 49%–52% and almost remained constant over the entire range of substrate concentration used [Figure 2a]. Over all the results, the inhibition of sPLA₂ by EEBD is independent of substrate concentration.

The sPLA₂ enzymes are calcium dependent and require calcium for optimum activity. The possibility of inhibition by metal ion chelation

was determined by VRV-PL-V inhibition of with IC₅₀ concentrations of extracts EEBD. The range of calcium concentration was 0–15 mM. Increasing calcium concentration from 0–15 mM increases sPLA₂ activity linearly but did not change the percentage of inhibition at IC₅₀ concentration of extracts [Figure 2b]. The inhibition sPLA₂ ranges 48%–54%. The inhibition of sPLA₂ is independent of calcium concentration and does not inhibit the activity by chelating calcium ions required for the enzyme activity.

Determination of total phenolics, antioxidant activity, and anti-lipid peroxidation

The extracts of *B. diffusa* inhibited sPLA₂s in the *in vitro* condition and the inhibition was independent of substrate and calcium concentrations. Further, the total phenolic content, antioxidant activity, and reducing power were estimated at 100 µg concentration. The result showed that extracts of *B. diffusa* contain 93% phenolics [Table 2]. The antioxidant activity was determined by DPPH method. The extract of *B. diffusa* showed the considerable antioxidant activity if compared to standard GA. ALP activity of the extracts was performed using TBA. The extract of *B. diffusa* showed 87.5% ALP activity [Figure 3a]. These data suggested that the percentage of PLA₂ inhibition by these sources is almost

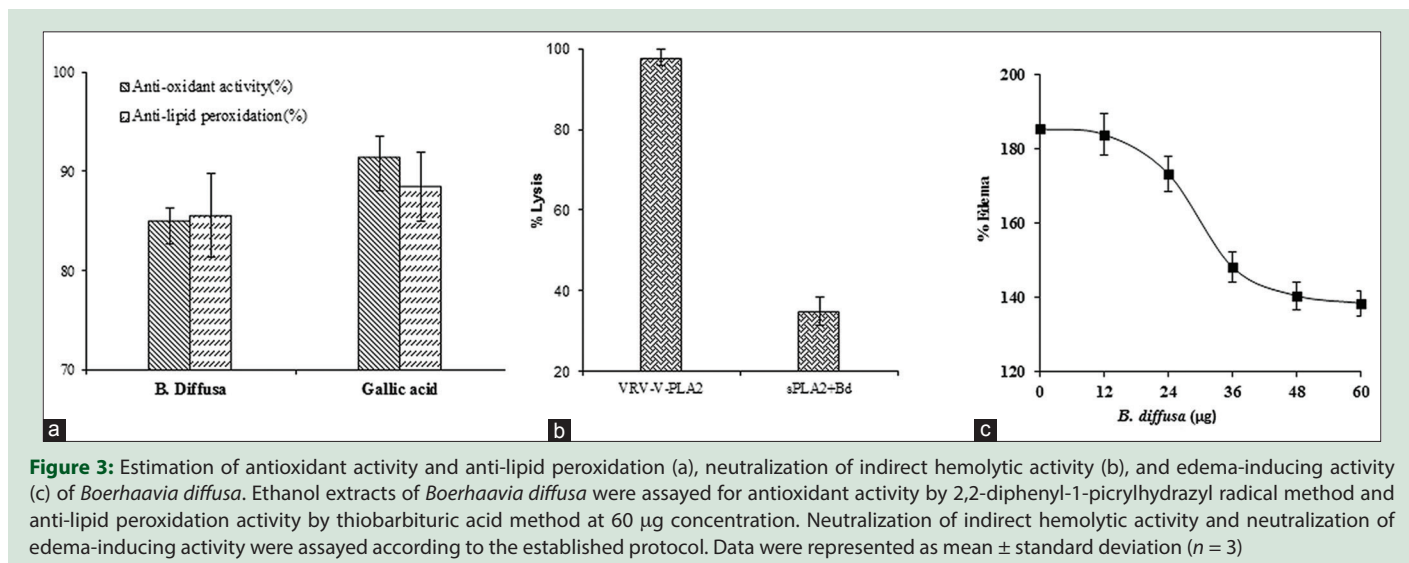


Table 2: Total phenolics, antioxidant, reducing power, and activity of *Boerhaavia diffusa* and IC₅₀ values for different secretory phospholipase A₂ enzymes. The linear regression analysis of the linear portion of the dose-dependent inhibition curve of each enzyme was used for the calculation of IC₅₀

Extract	Total phenolics (% w/w)	DPPH scavenging (% activity)	TBA (% activity)	Reducing power (A700 nm)
<i>B. diffusa</i> *	93.2	88.6	85	2.01
IC ₅₀ values for different sPLA ₂ sources				
Extracts	HSF-PLA ₂	HPF-PLA ₂	VRV-PL-V	NN-PL-I
<i>B. diffusa</i>	17.8	22.5	26.4	27.5

accordance of the amount of phenolic content, antioxidant, reducing power and anti-lipid peroxidative activity [Table 2].

In situ and in vivo studies

Since the extract inhibited sPLA₂ activity in the *in vitro*, the effect of these extracts on *in situ* hemolytic activity of sPLA₂ enzymes was examined. *In situ* hemolytic activity is an indirect way of measuring PLA₂ activity using egg yolk and washed erythrocytes as substrate. VRV-PL-V was subjected to inhibition by the extracts (60 µg) of *B. diffusa*. The reaction mixture without enzyme served as control. The extract of *B. diffusa* inhibited indirect hemolytic activity of VRV-PL-V. The maximum inhibition was achieved at 48 µg [Figure 3b].

The effect of extracts on edema-inducing activity of VRV-PL-V was determined. The sPLA₂ (VRV-PL-V) was injected into the mouse footpad; the edema ratio was >180% compared with controlled experiments. The enzyme was preincubated for 30 min at 37°C with the extracts of medicinal plants and legumes before the co-injection into mice footpads. The extracts inhibited edema formation in dose-dependent manner when co-injected with VRV-PL-V [Figure 3c]. The edema ratio was decreased to 137% by *B. diffusa* at 60 µg concentration. The apparent IC₅₀ for inhibition of edema was about 30 µg.

Thus, EEBD inhibited the activity of all sPLA₂ enzymes from both human and snake venom sources as well as neutralized the indirect hemolytic and edema-inducing activities of PLA₂ enzymes.

DISCUSSION

The extracellular Group-IIA sPLA₂ activity could be implicated in the control of wide range of physiological states, such as acute inflammation, pancreatitis, and rheumatoid arthritis.^[41] Thus, the relationship of sPLA₂ activation to the inflammatory response has been assumed an important manifestation. Inhibition of increased PLA₂ activity has tremendous scope in the development of pharmacologically potent molecules to control inflammation. Further, the PLA₂ inhibitors could minimize the tissue damage and other deleterious effects that take place secondary to inflammation.

In many countries, extracts of medicinal plants have been traditionally used for treatment of inflammation and related diseases. These plant extracts constitute rich source of novel compounds with a variety of pharmacological activities.^[43] Although biologically active phytochemicals have been reported to be present in a wide range of plants source, their efficacy in a wide range of plants, their efficacy in disease prevention has been clearly established only in case of some. The potency of a plant in preventing a disease is the sum of bioactivities of all phytochemicals present in that source with a common property. These phytochemicals are reported to prevent disease mainly through their functions as antioxidants detoxifiers, neuropharmacological and immunopotentiating agents, and source of dietary fiber.

The preliminary screening data with several folk medicinal plants that are extensively used against inflammation clearly inhibited sPLA₂ activity. In the present study, crude extracts of medicinal plants showed sPLA₂ inhibition, which inhibits enzymatic and toxic activities exerted by a variety of purified sPLA₂ enzymes from snake venoms and human inflammatory exudates. The extracts from Soxhlet method of extraction, the polar solvents, showed good inhibition compared to extracts from nonpolar solvents. The highest inhibition was achieved with ethanol extracts, followed by water and methanol extracts. The data indicate that *B. diffusa* containing more of hydrophilic inhibitors than hydrophobic inhibitors.

To check whether the extract inhibits other PLA₂ enzymes, extract of *B. diffusa*, was subjected to inhibit sPLA₂ enzymes from different sources. It inhibited sPLA₂ enzymes from human inflammatory fluids (HSF and HPF) and *V. russelli* snake venom (VRV-PL-V) which all belongs to Group-IIA sPLA₂. The result showed that human inflammatory PLA₂s are more sensitive to inhibition by these extracts than snake venom PLA₂ (VRV-PL-V). The extracts were further checked for their ability

to inhibit other group sPLA₂ enzyme, NN-PLA₂-I, which belongs to Group-IA, but percentage of inhibition is slightly less compared to Group-IIA sPLA₂s, indicating that these extracts are more competent to Group-IIA sPLA₂s either from snake venom or human inflammatory fluids. The extent of inhibition is differing from Group-I to Group-II PLA₂ enzymes. Although they exhibit more than 70% homology, many inhibitors inhibit these enzymes differentially.^[7] The variable extent to which these PLA₂ enzymes are inhibited may be due to the differential binding affinities at the active site.

Inhibition of sPLA₂ by binding of inhibitors to the phospholipid substrate and affecting the quality of interface that renders phospholipids inaccessible to the enzyme is doubt against sPLA₂ inhibitors potency. The steroid-inducible inhibitors of sPLA₂ such as lipocortin I and II are shown to inhibit sPLA₂s by nonspecific binding and by affecting the “quality of interface” of the membrane phospholipids. However, the inhibition by these agents was relieved when the substrate concentration was increased in the reaction mixture.^[43] Contrast to this, the extracts of *B. diffusa* retained the sPLA₂ inhibition potency even when the substrate concentration was increased by twofold. This observation suggests that the crude extracts of these plants do not compete for the substrate-binding site and may interact at a separate site strongly, and the inhibition cannot be affected by increased substrate concentration.

Some of the sPLA₂ inhibitors are shown to mediate displacement of catalytically essential calcium from the enzyme, and thus, inhibition by these agents appears to be dependent on calcium. Our investigation shows that inhibition of sPLA₂ by the extracts *B. diffusa* is independent of calcium concentration and not by chelation. Since the enzyme was inhibited to the extent of about 50% even when the calcium concentration was four times more than that of standard calcium concentration used in regular assay system. The involvement of molecules in extracts in binding to calcium ion hence leading to inhibition is ruled out. The above results indicated that the compounds in the crude extract directly interact with the enzyme and bring about irreversible inhibition.

The phenolic concentration, antioxidant activity, anti-lipid peroxidation activity, and reducing power of *B. diffusa* were not proportional to the phenolic concentration. The reducing power of the extract in terms of antioxidant activity was determined. The inhibition of sPLA₂ activity in the *in vitro* and *in vivo* condition by *B. diffusa* was more or less directly proportional to their antioxidant activity and anti-lipid peroxidation activity.

Probably, molecules of extract bind at the substrate-binding region of the enzyme and enzyme activity was inhibited irrespective of concentration of substrate provided for its activity. This is reflected in the inhibition of both *in vitro* sPLA₂ inhibition and indirect hemolytic activity where the substrate nature is totally different. The intact *E. coli* membrane was used as substrate in the *in vitro* assay and crude egg phospholipids mixture dispersed in the buffer as micelles in the indirect hemolytic assay. Because of this nature, these can be readily administered into *in vivo* model.

Injection of venom sPLA₂ or inflammatory fluid into joint of animal causes acute inflammatory response such as edema, swelling of synovial cell, and hyperplasia.^[44] The inhibitors on co-injection with sPLA₂ enzymes resulted in neutralization of edema-inducing activity in animal model.^[45,46] The extract of *B. diffusa* on co-injection with VRV-PL-V decreased the edema-inducing activity in dose-dependent manner. Since extracts inhibit sPLA₂ *in vivo* method, they are effective in preventing edema.

Conclusively, the juice of fresh leaves of *B. diffusa* reported to markedly reduces pain in mice.^[16] Ethanolic and aqueous extracts of *B. diffusa* effectively reduced the sPLA₂-induced paw edema. These effects

established some pharmacological evidence to support the folklore claim that it is used as anti-inflammatory agent. The EEBD has shown the higher potency in inhibiting sPLA₂ enzymes. This kind of herbs is reported to have higher concentration of compounds such as D-mannitol, oleanolic acid, ursolic acid, apigenin, stigmasterols, alpha-spinasterol, luteolin, baicalin, quercetin, and genistein. Since the extracts used in the assay were crude, there is always a possibility of single molecule or cumulative effect, which may be the other reason for sPLA₂ inhibition. However, several important questions remain open and further investigations are necessary to confirm which compound/s is/are responsible for the anti-inflammatory activity.

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Conflicts of interest

There are no conflicts of interest.

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