

# *Ixora coccinea* fruit inhibit the secretory phospholipase A2-IIA activity and exhibit antioxidant efficacy: An attempt of assessing anti-inflammatory function

M. Narayanappa<sup>1</sup>, Deepadarshan Urs<sup>1</sup>, Rajkumar S. Meti<sup>1,2</sup>, A. D. Sathisha<sup>3</sup>, K. K. Dharmappa<sup>1,2</sup>

<sup>1</sup>Inflammation Research Laboratory, Department of Studies and Research in Biochemistry, Mangalore University, Jnana Kaveri Post Graduate campus, Kodagu, Karnataka, India, <sup>2</sup>Department of Studies and Research in Biochemistry, Kodagu University, Jnana Kaveri campus, Chikka Aluvara, Kodagu, Karnataka, India, <sup>3</sup>Division of Biochemistry, School of Life Sciences, JSS Academy of Higher Education and Research, SS Nagar, Mysore, Karnataka, India

## Abstract

*Ixora coccinea* fruits found in Western Ghats are widely used in Indian folk medicine for treating a wide variety of diseases such as dysentery, ulcers, gonorrhoea, and inflammatory diseases. This study assessed the antioxidant and anti-inflammatory function of *I. coccinea* to validate the folklore usage for disease treatment. The fruit's phytoconstituents have been determined by the phytochemical screening, which contains phenolics, flavonoids, terpenoids, alkaloids, saponins, and alkaloids. Aqueous and different solvent extracts of *I. coccinea* fruit extracts were assessed for antioxidant efficacy. Among, the aqueous extract exhibited better antioxidant activity ( $81.12\% \pm 1.2$ ); further, it showed reducing power activity to  $71.14\% \pm 4.4$ . Further the *I. coccinea* fruit inhibited secretory phospholipase A2 enzyme by egg yolk plate method. The aqueous extract showed sPLA2-IIA inhibition to a good extent that was revealed by zone of inhibition (50%). The fruit extract neutralized sPLA2-IIA-induced hemolytic activity ( $IC_{50} 50.22 \mu\text{g}$ ) and showed the comparable effectiveness in the neutralization of PLA2-IIA-induced mouse paw edema ( $117.35\% \pm 2.74$ ) with the  $IC_{50}$  value of  $45.12 \pm 1.36 \mu\text{g}$ . Furthermore, the aqueous extract of *I. coccinea* prevents the denaturation of proteins, which is one of the aspects of the anti-inflammatory activity. Thus, the experimental findings justified the therapeutic applications of the *I. coccinea* fruit in the indigenous system of medicine.

**Key words:** Anti-inflammatory, antioxidant, edema, *Ixora coccinea*, secretory phospholipase A<sub>2</sub>, wild edible fruits

## INTRODUCTION

Inflammation is a pathophysiological process that occurs during tissue injury. Inflammation is a necessary prerequisite for lifesaving, if inflammation is not controlled, it can lead to chronic inflammatory diseases such as inflammatory bowel disease, allergies and asthma, rheumatoid arthritis (RA), atherosclerosis, psoriasis, and certain cancers.<sup>[1-4]</sup> Numerous research investigations have shown that human secretory phospholipase A2 enzymes-IIA (sPLA2-IIA) catalyze arachidonic acid synthesis and, thus, play an important role in many oxidative and inflammatory reactions. This pathway is responsible for the release of proinflammatory mediators such as prostaglandin, thromboxanes, prostacyclins, leukotrienes, as

well as platelet-activating factor. The sPLA2-IIA concentration in healthy conditions is  $\sim 3 \text{ ng/mL}$ . However, it substantially rises during infections and inflammatory reactions from  $250\text{--}500 \text{ ng/mL}$ . Supporting that, increased sPLA2-IIA concentration is observed in several inflammatory diseases such as arthritis, inflammatory bowel diseases, acute coronary syndrome, asthma, atherosclerosis, and acute respiratory

### Address for correspondence:

K. K. Dharmappa, Inflammation Research Laboratory, Department of Studies and Research in Biochemistry, Mangalore University, Jnana Kaveri Post Graduate campus, Kodagu, Karnataka, India.  
Tel: + 91-9008162862. E-mail: dharmappa@gmail.com

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distress syndrome. The sPLA2-IIA is also a biomarker for cardiovascular diseases, sepsis, and chronic graft failure.<sup>[5]</sup>

The reactive oxygen species (ROS) produced during oxidative stress and chronic inflammatory conditions in many degenerative neurological conditions, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and other brain dysfunctions ranging from brain injury to aging.<sup>[6-12]</sup> The interesting fact is, the ROS enhances sPLA2 activity,<sup>[13,14]</sup> and lipid peroxidation, which produces lipid peroxides that, in turn, activates sPLA2 enzyme. If radicals initiate lipid peroxidation and sPLA2 activation, then, the antioxidants' capacity to neutralize free radicals and sPLA2-IIA inhibition may explain their desirable therapeutic effect.

Since a long ago, non-steroidal anti-inflammatory drugs (NSAIDs) have been considered for treating inflammatory diseases. NSAIDs inhibit the cyclooxygenase (COX 1/2) enzymes and prevent the conversion of arachidonic acid into proinflammatory mediators. However, prolonged use of these drugs resulted in various side effects, including hepatic injury, gastrointestinal toxicity, and cardiovascular complications.<sup>[15-17]</sup> Over the decades, several exogenous and endogenous sPLA2-IIA inhibitors were studied, some of the drug clinical trials were also carried out. Unfortunately, the drugs failed to enter the market as effective anti-inflammatory drugs, due to either problems in their formulation or the cytotoxic properties. As a result, there is a need for a safe and effective sPLA2-IIA inhibitor as an anti-inflammatory molecule.<sup>[18]</sup>

The earliest medicines were medicinal herbs used to treat various diseases, which were practiced by all civilizations.<sup>[19]</sup> Many medicinal plants were employed in the traditional medical practises of Ayurveda, Siddha, and Unani to heal human illnesses.<sup>[20]</sup> The World Health Organization estimates that 80% of the world's population relies primarily on medicines derived from plants. Therefore, in the current investigation, we focused on the fruit of *Ixora coccinea*, which is a member of *Rubiaceae* family. Ayurvedic practitioners have long relied on this plant to treat a wide range of conditions and shown to have antioxidative, antibacterial, gastroprotective, hepatoprotective, anti-diarrhea, antinociceptive, antimutagenic, antineoplastic, and chemopreventive properties.<sup>[21]</sup> However, fruits of *I. coccinea* are underutilized even though which is rich in phytoconstituents such as phenolics, flavonoids, terpenoids, alkaloids, organic acids, sugars, and vitamin C.<sup>[22]</sup> Therefore, in our current study, we planned to assess the effectiveness of *I. coccinea* fruit for inhibiting the sPLA2-IIA enzyme and its antioxidant potential.

## EXPERIMENTAL

### Chemical Required

Glass wares, NaOH, Iodine solution, Conc. H<sub>2</sub>SO<sub>4</sub>, sodium thiosulfate, potassium iodide, iodine, HCL, Wagner's reagent,  $\alpha$ -naphthol, Fehling's A and B, chloroform, 10%

ammonia, copper sulfate, ethanol, Conc. nitric acid, sodium hydroxide, lead acetate, acetic-anhydride, ferric chloride, sodium chloride, gallic acid, acetylsalicylic acid ascorbic acid, 2,2-diphenyl 1-picrylhyorazyl (DPPH), and methanol were the materials used in the experiment.

### Collection of Fruits and Preparation of Extracts

*I. coccinea* fruit was collected from Western Ghats (Kodagu and Mangalore regions), India. Fruits were air dried under shade, mechanically powdered, and stored in an airtight container. The powder was extracted using a Soxhlet apparatus and ethanol, methanol, and aqueous water as a solvent, then refrigerated for later use.

### Phytochemical Analysis

#### Test for alkaloids

*Wagner's test:* Wagner's reagent was used to process the crude extract (Iodine in Potassium Iodide). A brown or reddish precipitate is produced once alkaloids are present.

#### Test for flavonoids

*Sulfuric acid test:* Conc. sulfuric acid should be added to the fruit extract. The presence of flavonoids is indicated by the orange color.

#### Test for terpenoids

To the 2 mL of fruit extract, 2 mL of chloroform and 3 mL of concentrated H<sub>2</sub>SO<sub>4</sub> were added. The appearance of terpenoids was indicated by the formation of a reddish layer at the interface.

#### Test for steroids

2 mL of chloroform was added with 3 mL of concentrated sulfuric acid to 2 mL of plant extract. The formation of the reddish layer at the interface indicated the presence of steroids.

#### Test for glycosides

*Liebermann's test:* Add 2 mL of acetic acid and 2 mL of chloroform for the fruit extract. After cooling, concentrated sulfuric acid was added. Green coloration indicated the presence of aglycone, a steroidal component of glycosides.

#### Test for tannins

*Ferric chloride test:* 0.1% ferric chloride was added to the fruit extract. Tannins were indicated by a brownish-green or black, blue coloration.

#### Test for phenols

*Ferric chloride Test:* 0.3% ferric chloride was added to the fruit extract. The existence of Phenols was attributed to the presence of green or blue coloration.

### Test for saponins

**Foam Test:** 5 mL of distilled water added to fruit extract, vigorously mixed; the existence of froth suggests the presence of saponins.

### Antioxidant activity

#### DPPH method radical scavenging assay

The *in vitro* antioxidant activity of *I. coccinea* fruit extract was assessed according to the method of Blois.<sup>[23]</sup> In a test tube, 1.5 mL of DPPH working solution (0.1 mM) was mixed with 0.5 mL of fruit extract or standard solution at various concentrations (5, 25, 50, 75, and 100 µg/mL) and stored in the dark at room temperature for 30 min. The absorbance was assessed at 517 nm. The % antioxidant or radical scavenging activity was calculated using the below formula,

$$\% \text{ Antioxidant activity} = \left( \frac{[Ac-As]}{Ac} \right) \times 100$$

Where Ac and As are the absorbance of control and sample, respectively. Ascorbic acid was used as standard.

### Total Antioxidant Capacity (Phosphomolybdenum Reducing Assay)

The phosphomolybdenum reduction assay was employed to assess the total antioxidant capacity of the *I. coccinea* fruit extract. 0.5 mL of ascorbic acid (5, 25, 50, 75, and 100 µg/mL) and fruit extract (10, 15, 20, 25, and 30 µg/mL) were mixed with 1.5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was read at 695 nm.<sup>[24,25]</sup>

### Purification of sPLA2

The method of modified by Vishwanath *et al.*<sup>[26]</sup> was used to purify human pleural fluid (HPF) – sPLA2. HPF was centrifuged at 10,000 rpm for 10 min, and an equal volume of 0.36 N H<sub>2</sub>SO<sub>4</sub> was added to the supernatant. The sample was stirred overnight and centrifuged for 10 min at 10,000 rpm. The supernatant was dialyzed against a phosphate buffer (10 mM, pH 4.6) using a 7–8 KD membrane. The dialyzed sample was kept in a water bath at 60–70°C for 5 min. The boiled sample was then centrifuged for 10 min at 10,000 rpm, and the supernatant will be used as a source of sPLA2, and the homogeneity was checked by SDS-PAGE.

### sPLA2 Inhibition by Egg Yolk Plate Method

The method of Gutierrez's<sup>[27]</sup> agarose egg yolk plate procedure was used to inhibit sPLA2. 1 gm of agarose was dissolved in 100 mL of 0.1M tris HCl (pH 7.4) containing 5 mM CaCl<sub>2</sub> and allowed to boil. When the mixture has reached room temperature, add 6 drops of egg yolk, stir, and

pour into Petri dishes and allow to solidify. Pierce the gel, make the proper wells, and add 20 µL of extracted PLA2 to each well. Following overnight incubation at 37°C, the plates were carefully measured with a scale to determine the zone of clearance. The zone containing only PLA2 was used as a control, while the well containing no PLA2 was used as a negative control. sPLA2 was pre-incubated with 10 g of *I. coccinea* fruit extract as a test. The percentage of sPLA2 inhibition was calculated using the following formula:

$$\text{Zone of inhibition} = \frac{\text{Control diameter} - \text{Text diameter}}{\text{Control diameter}} \times 100$$

### Anti-inflammatory Activity by Denaturation Assay

A phosphate-buffered saline (PBS) solution containing 1% w/v bovine serum albumin (BSA) was prepared, and the pH was adjusted to 7.4 with HCl. Using appropriate solvents, stock solutions of 1mg/ml of the standard (acetylsalicylic acid) and test extract (aqueous extract of *I. coccinea* fruit) were prepared. Extracted fruit samples with various concentrations of 20–100 µg were established from the stock solutions in PBS with 6 mL of 1% w/v BSA. The 400 mL of phosphate buffer saline and 1.6 mL of 1% w/v BSA solution was used as the control. Acetylsalicylic acid at 100 mg/mL in ethanol with a 5 mL 0.2% w/v BSA solution was used as the standard. The reaction mixture was heated to 60°C for 10 min and allowed to cool for 10 min. The absorbance was measured at 660 nm using a spectrophotometer. The average absorbance was recorded after each round of the experiment, which was carried out in triplicate.

### Neutralization of Indirect Hemolytic Activity

The assay was carried out in accordance with what Boman and Kaletta<sup>[28]</sup> proposed. *I. coccinea* fruit. Freshly mixed human red blood cells (1 mL) and egg yolk (1 mL) in 8 mL of PBS were used as a substrate for indirect hemolytic activity. After pre-incubating the inhibitor (extract) with sPLA2-IIA (30 g) for 30 min at 37°C, 1 mL of a substrate was added, and the reaction was allowed to run for 45 min at 37°C. A total of 9 mL of chilled PBS was added to stop the reaction. The mixture was thoroughly mixed before being centrifuged at 1500 g for 20 min. The hemolytic activity was measured in terms of released hemoglobin at 530 nm. As a positive control, the sPLA2-IIA enzyme was used alone.

### Neutralization of Edema-Inducing Activity

To study the neutralization of edema-inducing activity, the protocol established by Yamakawa *et al.*<sup>[29]</sup> and modified by Vishwanath and Gowda<sup>[30]</sup> was used. Initially, a total of 20 µL of saline and 5 g of the PLA2 enzyme either alone or with various concentrations of extracts or inhibitors were injected into the intraplantar surface of the right hind footpad

of experimental mice. Control was received 20  $\mu$ L of saline or vehicle. After 45 min of anesthesia (pentobarbitone, 30 mg/kg, i. p.), the mice were sacrificed. The hind limbs were surgically removed near the ankle joint and individually weighed. The percentage rise in the weight due to edema was determined by the ratio of the weight of the edematous limb to the weight of a standard (sham injected) limb  $\times$  100. Edema was also compared between sham injected control and uninjected limb.

## RESULTS

Inhibition of sPLA2-IIA enzyme is of intensive pharmacological interest, because sPLA2-IIA plays a significant part in a broad spectrum of conditions characterized by inflammation.<sup>[7,31,32]</sup> According to prior reports, numerous types of wild edible fruits are used to treat inflammatory diseases. Hence, the present study hypothesized to evaluate anti-inflammatory and antioxidant activity of *I. coccinea* fruit. Initially, different extracts of *I. coccinea* such as hexane, petroleum ether, chloroform, acetone, ethanol, methanol, and aqueous were prepared by Soxhlet method and subjected to phytochemicals analysis, which showed that fruit contains phytoconstituents such as alkaloids, flavonoids, terpenoids, tannins, glycosides, phenols, and steroids [Table 1].

The antioxidant activity of hexane, petroleum ether, chloroform, acetone, ethanol, methanol, and aqueous extracts of *I. coccinea* fruit was estimated by the DPPH method. All the extracts showed antioxidant activity to a considerable extent. However, aqueous extract showed greater activity [Figure 1a]. Hence, the aqueous extract ranging from 0 to 100  $\mu$ g estimated antioxidant activity which showed the antioxidant activity in a concentration-dependent manner with the IC<sub>50</sub> concentration of 48.31  $\mu$ g [Figure 1b]. As well, total antioxidant of aqueous extract of *I. coccinea* fruit was measured by the phosphomolybdenum reduction assay in the concentration range from 0 to 100  $\mu$ g, which showed

phosphomolybdenum reduction activity in dose-dependent manner. For reference, the ascorbic acid was used [Figure 2].

Further, the aqueous extract of *I. coccinea* was subjected to sPLA2-IIA inhibition in egg yolk plate method. The extract in the range of 0–100  $\mu$ g concentration inhibited sPLA2 enzyme in concentration-dependent manner. Briefly, the five wells, the well (1) added tris base buffer (10  $\mu$ L) served as negative control, the well (2) added sPLA2 enzyme (10  $\mu$ g), the wells from (3) to (5), added sPLA2 enzyme with aqueous extract of fruit of 25  $\mu$ g, 50  $\mu$ g, and 100  $\mu$ g concentrations [Figure 3]. The zone formation around the well represents sPLA2 enzyme hydrolytic activity and reducing the zones indicates sPLA2 inhibition. The crude extract of medicinal fruit of *I. coccinea* demonstrated sPLA2 inhibition to a greater extent.

The ability of the aqueous extract of *I. coccinea* evaluated for prevention of the denaturation of proteins, which is one of the aspects of the anti-inflammatory activity. It was found to be effective in preventing the heat-induced denaturation of albumin at 0–100  $\mu$ g concentrations [Figure 4]. The value of IC<sub>50</sub> was found to be 54.518  $\mu$ g. A known anti-inflammatory drug acetylsalicylic acid served as standard, its IC<sub>50</sub> concentration was found to be 16.137  $\mu$ g.

The indirect hemolytic activity is to measure the sPLA2-IIA activity in an indirect way using egg yolk phospholipids dispersed as micelles together with washed erythrocytes. Aqueous extract of *I. coccinea* tested for neutralization of sPLA2-IIA-induced hemolytic activity. Aqueous extract of fruit in the range from 0 to 100  $\mu$ g concentration reduced the sPLA2-IIA-induced indirect hemolytic activity to 24.19%  $\pm$  1.2 and the IC<sub>50</sub> value was found to be 50.22  $\pm$  1.1  $\mu$ g [Figure 5].

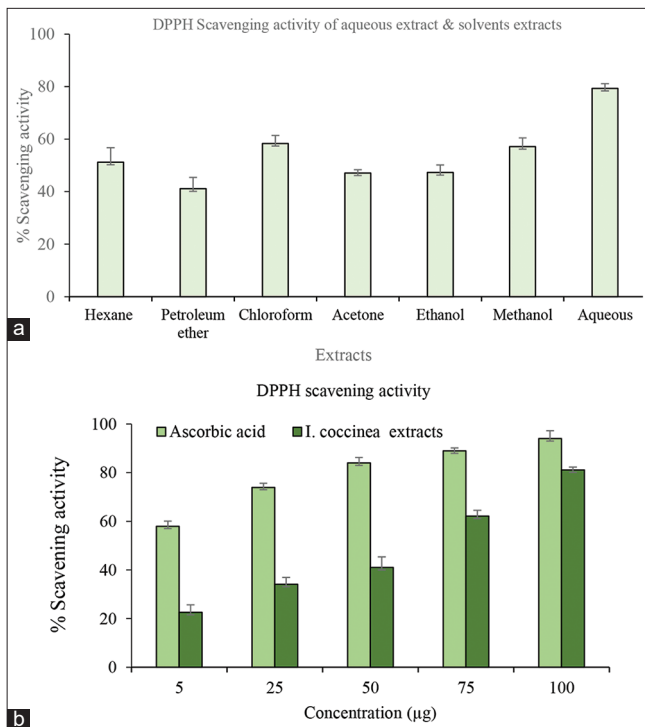
Inflammatory fluids injection into animal joints resulted in edema due to swelling of synovial cells and hyperplasia. The different doses from 0 to 100  $\mu$ g concentration of extracts of *I. coccinea* were pre-incubated with sPLA2-IIA enzyme and

**Table 1: Phytochemicals constituents of *Ixora coccinea* fruit**

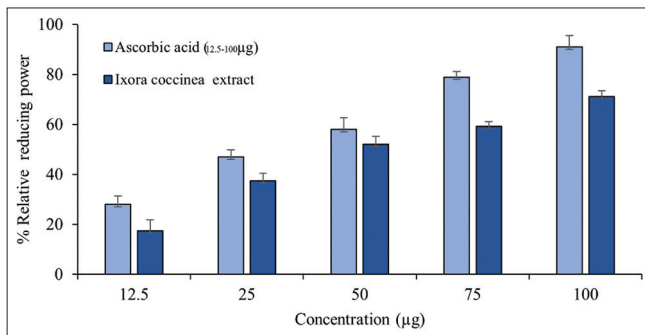
S. No.	Name of the Phytochemical	<i>Ixora coccinea</i> fruit extract with the different solvents						
		Solvents	Hexane	Petroleum ether	Chloroform	Acetone	Ethanol	Methanol
1	Flavonoids	-	+	+	-	+	+	+
2	Phenols	+	-	+	-	-	+	+
3	Terpenoids	-	+	-	+	+	-	+
4	Tannins	+	+	-	-	-	-	+
5	Alkaloids	-	-	+	+	+	-	-
6	Saponins	-	-	-	-	-	-	+
7	Glycosides	-	-	-	-	+	+	-
8	Steroids	+	+	-	-	-	-	+

(-) indicates the negative, (+) indicates the presence





**Figure 1:** (a) Estimation of antioxidant activity of extracts of hexane, petroleum ether, chloroform, acetone, methanol, ethanol, and aqueous at 100 µg concentration. (b) The antioxidant activity of aqueous extract of *Ixora coccinea* from 0 to 100 µg concentration. The  $IC_{50}$  value was found to be 48.31 µg. The data expressed as mean ± standard deviation ( $n = 3$ )

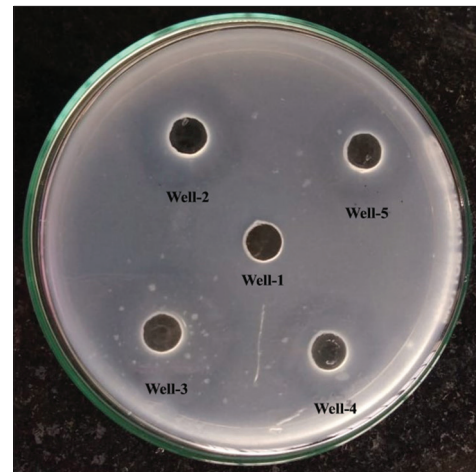


**Figure 2:** Phosphomolybdenum reduction activity of aqueous extracts of *Ixora coccinea* fruit in the range from 0 to 100 µg concentration. The data expressed as mean ± standard deviation ( $n = 3$ )

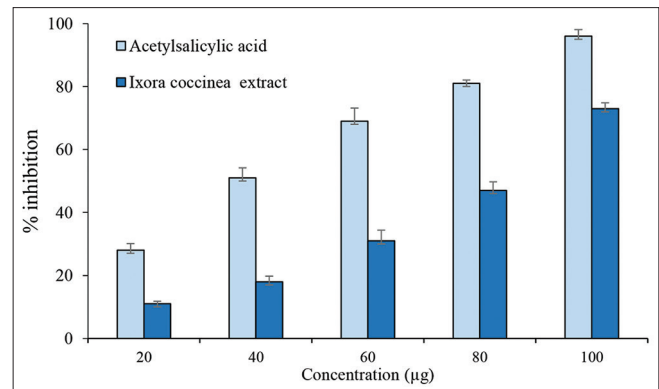
injected into the right hind paw of mice, the respective left hind paw received saline as a negative control. The *I. coccinea* extract reduced the edema in a dose-dependent pattern from  $169.51\% \pm 2.39$  to  $117.35\% \pm 2.74$  at 100 µg concentration and the  $IC_{50}$  value was found to be  $45.12 \pm 1.36$  µg [Figure 6].

## DISCUSSION

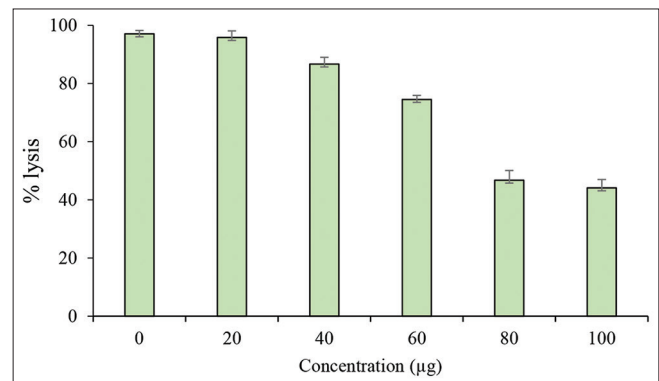
The synthesis of arachidonic acid by sPLA2-IIA regulates proinflammatory mediators concentration. The metabolism of arachidonic acid results in a lot of ROS as a by-product.



**Figure 3:** Inhibition of sPLA2 by egg yolk plate method. Well 1, negative control added only tris base buffer; well 2, positive control, added tris base buffer (10 µL) + sPLA2 enzyme (10 µg); wells 3–5, tris base buffer + sPLA2 enzyme + aqueous extract (25, 50, and 100 µg)

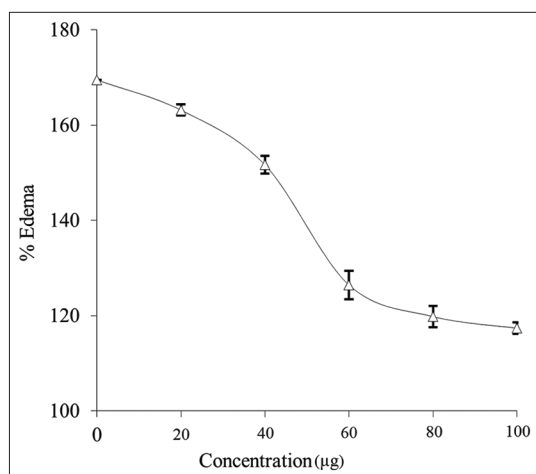


**Figure 4:** Aqueous extract of *Ixora coccinea* fruit from 0 to 100 µg range inhibited protein denaturation. The data expressed as mean ± standard deviation ( $n = 3$ )



**Figure 5:** *Ixora coccinea* fruit extract neutralized the indirect hemolytic activity of sPLA2-IIA. The data expressed as mean ± standard deviation ( $n = 3$ )

The excess of ROS concentration enhances the sPLA2-IIA activity and lipid peroxidation, both of which modulate the downstream reactions and cause an even greater production of proinflammatory mediators. Hence, bioactive molecules



**Figure 6:** Neutralization of edema-inducing activity of sPLA2 IIA enzyme by *I. coccinea* extract. The data expressed as mean  $\pm$  standard deviation ( $n = 3$ )

that possess both sPLA2-IIA inhibitory and antioxidant activity become a more effective anti-inflammatory agent.<sup>[33]</sup> The anti-inflammatory drugs act either by inhibiting proinflammatory enzymes (PLA2-IIA) or by inhibiting the release of inflammatory cytokines (interleukin-1 and tumor necrosis factor- $\alpha$ ).

In the current investigation, we evaluated the antioxidant and anti-inflammatory activities of the *I. coccinea* fruit. The phytochemical analysis of *I. coccinea* fruit showed the presence of various phytochemicals such as alkaloids, flavonoids, terpenoids, tannins, glycosides, phenols, and steroids. The solvent and aqueous extracts showed antioxidant activity to a good extent, while the aqueous extract showed greater antioxidant activity. The further estimation of antioxidant activity with aliquot concentration of fruit extracts (0–100  $\mu$ g) demonstrated the 81.12% radical scavenging activity. The phosphomolybdenum reduction assay showed the  $74.14\% \pm 2.3$  reduction power which substantiates the antioxidant activity. The findings showed a correlation between the levels of phytochemicals and their antioxidant activities.

The inhibition of sPLA2-IIA enzyme by aqueous extract of *I. coccinea* fruit demonstrated by forming the zone of inhibition in the egg yolk plate method which validated its anti-inflammatory activity. The possibility of single or synergistic action of potent bioactive molecule/s in the *I. coccinea* fruit might be the reason for the sPLA2 inhibition. Aqueous extract neutralized the sPLA2-IIA-induced indirect hemolytic activity even irrespective of the nature of substrate. The fruit extract neutralized the sPLA2-IIA-induced edema in the concentration-dependent manner. The formation of edema might be due to the combined effect of hydrolysis of membrane phospholipid that results in the loss of membrane integrity, as well as its metabolic activity due to proinflammatory products.<sup>[34–38]</sup> The prevention of denaturation of proteins by fruit extract of *I. coccinea* implies

anti-inflammatory activity. Thus, the *I. coccinea* fruit is proven to possess an anti-inflammatory property.

## CONCLUSIONS

The phytochemical screening of the *I. coccinea* fruit revealed its phytoconstituents, alkaloids, flavonoids, terpenoids, tannins, glycosides, phenols, and steroids. These compounds are thought to be responsible for the fruit's diverse range of medicinal properties. The aqueous extract of *I. coccinea* demonstrated a greater antioxidant and sPLA2-IIA inhibition potency. The fruit extract effectively neutralized the sPLA2-IIA-induced indirect hemolytic activity and mouse paw edema. The fruit extract's potency to prevent the denaturation of proteins implies anti-inflammatory activity. However, a number of significant questions have not been satisfactorily answered, and further, research is required if one is to comprehend the mechanism that lies behind the effects of the extract and the active compound or compounds that are responsible for the anti-inflammatory activities.

## ACKNOWLEDGMENTS

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