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Research Article

ESTIMATION OF TOTAL PHENOLIC, TOTAL FLAVONOID CONTENT AND EVALUATION OF ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF *IXORA COCCINEA* LINN. STEMS

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ABSTRACT

Ixora coccinea Linn. *(Rubiaceae)* has mentioned in Ayurveda as Paranti and traditionally stems used in inflammatory diseases like sprains, eczema, contusions and boils. Present study deals with evaluation of anti-inflammatory and antioxidant activity of extracts of *I.coccinea* stem. Anti-inflammatory activity was studied *in vivo* by carrageenan-induced paw edema in rat and *in vitro* by human red blood cell membrane stabilization method. Total tannin and flavonoid content of extracts was determined by using the Folin- Ciocalteu method and aluminum chloride method, respectively. Antioxidant activity was evaluated by *in vitro* assay involving nitric oxide scavenging, hydrogen peroxide scavenging, 2,2-diphenylpicrylhydrazyl (DPPH) radical scavenging, and ion chelating activity. Chloroform extract showed significant reduction in carrageenan induced rat paw edema ($p<0.05$) and protection of HRBC in hypotonic solution. Methanol extract contain more total tannin and flavonoid content as compared with petroleum ether and chloroform extract. All extracts showed concentration dependant free radical scavenging activity. Methanol extract and chloroform extract have shown better antioxidant activity and due to this antioxidant nature might be responsible for its anti-inflammatory activity. These activity supports to use of *I.coccinea* extract in traditional used in treatment of various inflammatory disaeses.

Key words: Anti-inflammatory activity; Antioxidant activity; Carrageenan-induced paw edema; HRBC; *Ixora coccinea*

INTRODUCTION

Despite the development in medical field during the past years, the treatment of severe diseases is still challenging. Inflammatory diseases remain with others one of the world's major health problems (Martínez-Vázquez *et al*., 2012). Inflammation or phlogosis is an immunlogical reaction of living tissues to injuries that leads to accumulation of blood fluids and cells. Even though it is an immunological defense, the complex reaction but mediators involved in the inflammation can induces many diseases. Hence, the use of anti-inflammatory substances is helpful to treat therapeutically this pathological condition (Sosa *et al*., 2002). An anti-inflammatory drug like NSAID's used to decrease the swelling and pain of inflammation of tissue. But prolonged use of anti-inflammatory agents may produce gastro-intestinal toxicity, cardiovascular and other toxicity. Therefore, there is a need for anti-inflammatory drugs having less severe side effects to use for chronic inflammatory diseases. Therefore, in recent time, more interest is shown in traditional drugs for treatment of various diseases, but there is no or less scientific evidences (Madhavi *et al*., 2012).

The oxidative condition presents a range of free radicals including nitric oxide, hydroxyl radical, superoxide and peroxynitrite in living organisms. There are a number of evidences about the role of free radicals in the development of various diseases including cancer, neurodegeneration and inflammatory diseases (Ferguson, 2010; Halliwell, 2006). Antioxidants therefore gained importance for their capacity to reduce these diseases produce from free radicals. In this context, antioxidant properties of various medicinal plants are being investigated throughout the world because of

the toxicological concerns associated with the synthetic antioxidants (Peschel *et al*., 2006).

Ixora coccinea Linn. Family-Rubiaceae is medicinal plant widely found in tropical region of East Indies and subtropical region of India and Shrilanka. (Basu *et al*., 1999). Flowers contain cycloartenol esters (Ragasa *et al*., 2004) and leaves contain Ixorene dammarane triterpene (Ikram *et al*., 2013). Leaves show anti-inflammatory (Muke *et al*., 2013), cardioprotective effect (Momin *et al*., 2012), antinociceptive (Ratnasooriya *et al*., 2005), antioxidant (Babu *et al*., 2014; Saha *et al*., 2008), antidiarrhoeal (Yasmeen *et al*., 2010), antiasthmatic (Missebukpo *et al*., 2011), hypoglycaemic and hypolipidaemic activity (Maniyar and Bhixavatimath, 2011).The roots and flowers shows anthelmintic (Surana *et al*., 2012) and antioxidant activity (Saha *et al*., 2008; Surana *et al*., 2013).

From literature review it reveals that stems of *I. coccinea* has traditionally used in inflammatory conditions like sprains, eczema, contusions and boils (Mhaskar *et al*., 2000) and no scientific work was done on stems of *I. coccinea* for anti-inflammatory activity and antioxidant activity. This study was designed to assessment of anti-inflammatory, antioxidant potential and total tannin and flavonoid contents of extracts of *I. coccinea* stems.

MATERIALS AND METHODS

Drugs and chemicals

DPPH used in this study was obtained from Sigma-Aldrich, India, Diclofenac sodium and carrageenan was purchased from TCI Chemical (India) Pvt. Ltd., sulfanilamide and NADH obtained from Hi Media Laboratories Pvt., India. Rutin, Folin-Ciocalteu Reagent ascorbic acid, tocopherol, ferrozine obtained from Qualigens Fine Chemicals and sodium nitroprusside, N-(1-naphthyl)-ethylenediamine hydrochloride, sodium nitrite, ferrous chloride purchased from S.D. Fine Chemicals Ltd., India. Other reagents were purchased locally. All chemicals used in the study were of analytical grade.

Authentication of plant material and Preparation of extract

The stems of *I. coccinea* were collected from Dhule District. Dr. P. S. N. Rao, Scientist, Botanical survey of India (BSI), Pune did the identification and authentication of plant. The herbarium of the plant has been deposited at BSI, Pune have voucher specimen Number, ARS-1.

The air-dried stems of *I. coccinea* were pulverized in grinder (# 60-80) and successively extracted with petroleum ether (60-80°C), chloroform and methanol by Soxhlet apparatus. After completion of extraction, solvent was distilled out and dried the extract in vacuum dryer.

Animals

The tests with animals (Male Wistar rats weighing 150-200g) experimental were performed according to guidelines for the care and use of laboratory animals of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCESEA) and approved by the Institutional Animal ethical committee.

Determination of total Tannins

The extracts of *I. coccinea* were analyzed for its tannins content according to the Folin-Ciocalteu colorimetric method described by Spanos with slight modifications (Spanos and Wrolstad, 1990). The assay was performed by taking stock solution of each extract (1mL, 1mg/mL) and 1mL of different dilutions of standard gallic acid (25 to 250µg/mL) in volumetric flask (25mL). Followed by addition of water (10mL) and Folin-Ciocalteu Reagent (1.5mL) and allowed to stand for 10min. Then sodium carbonate solution (4mL) was added in each volumetric flask and made volume up to the mark with distilled water. Absorbance was taken after 1h at 765nm by UV visible spectrophotometer (Shimatzu) against blank. The calibration curve of gallic acid was plotted and tannin content determined from calibration curve.

Total flavonoid content

The flavonoids content of *I. coccinea* was determined according to the Aluminium chloride method performed by Kumaran and Karunakaran (Kumaran and Joel Karunakaran, 2007). Total flavonoid content was analysed by taking 0.5mL stock solution of each extract (1mg/mL) and different dilution of standard

rutin solution (10 to 100µg/mL) in separate test tubes. In each test tube methanol (1.5mL), aluminium chloride solution (0.1mL), potassium acetate solution (each 0.1mL) and distilled water (2.8mL) were added and mixed well. Blank solutions for all the extract and for different dilution of standard rutin were prepared by addition of distilled water in place of aluminium chloride solution. Filtered all prepared solutions were through Whatmann filter paper before determining absorbance. Absorbance was taken at 415nm against the suitable blank. The amount of flavonoid in the extract was determined from calibration curve of rutin.

Anti-inflammatory activity

***In vitro* Human Red Blood Cell (HRBC) membrane stabilizing activity**

This method is useful to evaluate membrane Stabilizing activity of HRBC when in inflammation blood capillaries are breaks. The 10% Human red blood cell suspension prepared in normal saline solution (Khouya *et al.*, 2015).

HRBC Membrane Stabilizing activity was performed according to method described by Kumar *et al.*, 2011. Various dilutions of extracts (25to150µg/mL) were prepared in distilled water. In each extract, 1mL phosphate buffer (0.15M pH 7.4), 2mL hyposaline and 0.5mL HRBC suspension were added. The resultant mixture was incubated at 37^0C for 30min. After, it centrifuged at 3000rpm for 10min. The hemoglobin content of the supernatant solution was determined by taking absorbance at 560nm using UV-visible spectrophotometer (Shimatzu). Diclofenac sodium (10 to 50µg/mL) were used as standard and a control was prepared excluding the test component (Kumar *et al.*, 2011). The % inhibition of haemolysis or membrane stabilization of HRBC was detrmined by using following formula-

$$\% \text{ inhibition of haemolysis} = \frac{A_{control} - A_{test}}{A_{control}} X\ 100$$

Where; $A_{control}$ = Optical density of control i.e. HRBC in hypotonic-buffered saline solution alone; A_{test} = Optical density of test component in HRBC in hypotonic-buffered saline solution

***In Vivo* Carrageenan-induced paw edema**

The Wister albino rats were divided into 5 groups, each group consists of 6 animals. The edema was produced by injection of 0.1mL of carrageenan (1%w/v suspension in normal saline solution) locally into subplantar region of the left hind paw of rats. Extracts of *I. coccinea* (100mg/kg body weight) and diclofenac (10mg/kg, body weight) were given orally 1h prior of carrageenan injection. Control group only received vehicle. The paw volume of rat up to the ankle joint was measured at 0h, 1h, 2h and 3h by using Plethysmometer PLM-01(Ferreira *et al.*, 2013; Nagulsamy *et al.*, 2015).

Antioxidant activity

Nitric Oxide Radical Scavenging Assay

Nitric oxide (NO) scavenging activity of *I. coccinea* extracts was performed by sodium Nitroprusside-Griess reagent method (Gülçin *et al.*, 2012; Kumbhare *et al.*, 2012). The different concentration (25to125µg/mL) of *I. coccinea* extracts in methanol were mixed with 1mM sodium nitroprusside solution in phosphate buffer saline solution and incubated at 37°C for 2.5h. Blank solution was also prepared excluding test component. After incubation, and 0.5mL of Griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine hydrochloride) and absorbance was taken at 546 nm immediately. Ascorbic acid was used as standard. Percent inhibition was calculated as per following formula. IC_{50} was calculated for each extract.

$$\% \text{ inhibition} = \frac{A_{control} - A_{test}}{A_{control}} X\ 100$$

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity was measured by the method described earlier. (Amzad Hossain and Shah, 2015; Xiao *et al.*, 2012). A hydrogen peroxide solution (40mM) was prepared in phosphate buffer pH 7.4. Different dilution (25to125µg/mL) of *I. coccinea* extracts was prepared in methanol. In that hydrogen peroxide solution (0.6mL) was added. The absorbance of hydrogen peroxide was determined after 10min. at 230nm. Percent inhibition calculated as per above formula & IC_{50} calculated

DPPH Free radical scavenging assay

DPPH Free Radical Scavenging inhibiting Activity was performed as described by Nwaehujor *et al.*, 2014. Various concentrations of each *I. coccinea* extract were prepared in methanol. In each different dilution (25to125µg/mL) of extract solution (4mL), 100µL of DPPH solution (13mg/Lit.) was added. 100 µl of DPPH solution in methanol (4 mL) was treated as control. Ascorbic acid was used as standard. Absorbance of each solution was taken after 15min. at 517nm. Percent inhibition calculated as per above formula & IC_{50} calculated.

Ferrous chelating activity

The chelating activity of the *I. coccinea* extracts toward ferrous ions was studied by the previously describe method (Dinis *et al.*, 1994). In 1mL of each dilution (25to125 µg/mL) of *I. coccinea* extracts, 2 mM $FeCl_2$ (0.1mL) was added and the chelating reaction was started by the addition of 5mM ferrozine (0.2mL) and the reaction mixture was vigorously shaken for 10s and stand for 10min. at room temperature and the absorbance was determined at 562nm. Reaction mixture without test sample was used as a control and without ferrozine mixture was used as a blank.

Statistical analysis

All animal experimental results are given as the mean ± standard error of the mean (SEM).To compare experimental and control groups, we used two-way analysis of variance (ANOVA), followed by by Bonferroni's test. A value of $P<0.05$ was considered to be significant. All *in vitro* experimental results are given as the mean± standard deviation (SD)

RESULT AND DISCUSSION

Determination of total phenolics

The Folin-Ciocalteu method is responsive to reducing compounds like polyphenols and tannins there by forming blue colored complex. Table I shows the total tannin content of the *I. coccinea* extracts measured using the Folin-Ciocalteu method. The linear regression equations and linear regression coefficient of calibration curve was $y = 0.004x$ and $R^2 = 0.992$ respectively, where x is the absorbance and y is the concentration of gallic acid solution (µg/mL). The total tannin content of the extracts were 19.17±1.01, 34.25±1.28 and 121.75±1.14 mg GAE/g for petroleum ether, chloroform and methanol extract respectively (Table I).

Table I. Total phenolics and flavonoid content of *I. coccinea* stems extracts

Sample	Total phenolics	Total flavonoid
PIC	19.17±1.01	6.76±0.64
CIC	34.25±1.28	17.38±1.36
MIC	121.75±1.14	38.71±0.85

Values represent mean ± standard deviation (n=3)

Plant tannin components are secondary metabolites with beneficial role in animals as well as human health. The valuable effects of those tannins are due to their antioxidant activity involving their capability to scavenge free radicals, accept hydrogen atoms, donate electrons or to chelate metal ions (Kasote *et al.*, 2015). Tannin contents of all the *I. coccinea* extracts may appear to function as good antioxidant.

Determination of total flavonoid

The total flavonoid content of the *I. coccinea* extract was determined using the aluminium chloride colorimetric method. The total flavonoid content values were obtained from the calibration curve. The linear regression equations and linear regression coefficient of calibration curve was $y = 0.007x$ $R^2 = 0.996$ respectively, where x is the absorbance and y is the concentration of rutin solution (µg/mL) expressed as mg of rutin equivalent /g of extract. Results of total flavonoids content of *I. coccinea* extracts (Table I).

Anti-inflammatory activity

***In vitro* human red blood cell membrane stabilizing activity**

HRBC membrane is similar to lysosomal membrane. In inflammatory process histamine released from injured tissue causes blood capillaries more permeable and damaged tissue releases their enzymes, which break down the damaged tissue but may also destruct nearly

healthy tissue cells (Nagaharika *et al*., 2013). *I. coccinea* extracts stabilizes HRBC and may stabilizes lysosomes in injured tissue cells and thereby prevent release of lysosomal enzymes into cells, thus preventing deterioration of tissue. Petroleum ether, chloroform, methanol extracts of *I. coccinea* and diclofenac sodium showed HRBC membrane stabilizing activity, stabilizing HRBC membrane upto 21.207%, 30.865%, 26.686% and 35.173% respectively at conc. 250µg/mL in hypotonic solution (Figure 1). By comparing HRBC membrane stabilizing activity of all the extracts it was found that chloroform extract has better *in vitro* anti-inflammatory activity.

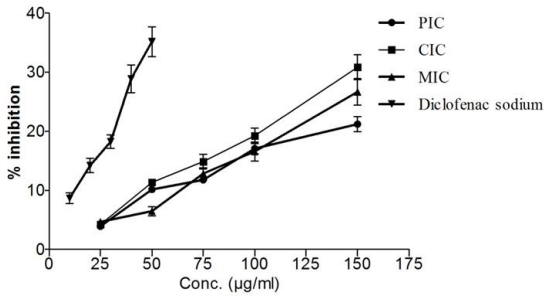

Figure 1. *In vitro* HRBC Membrane Stabilizing activity of extracts of *Ixora coccinea* stems. Each value represents mean ± SD (n=3).

In vivo anti-inflammatory activity carrageenan-induced paw edema

Carrageenan-induced rat paw edema is a widely used to evaluate anti-inflammatory activity and constitutes a simple and regular animal model without any injury to the paw of animal. Animal paw edema used to investigate new anti-inflammatory drugs along with mechanisms involved in inflammatory process (Udavant *et al*., 2012). As a carrageenan- induced rat paw edema shows biphasic mechanism in which mediators activate in sequence to induce the inflammation. Histamine, bradykinin and serotonin are the mediators induce inflammation in the early phase. Prostaglandins (PGs) are detectable in the later phase of inflammation and PGs increase vascular permeability (Huang *et al*., 2011; Pinheiro *et al*., 2013).

The anti-inflammatory activity of petroleum ether, chloroform, methanol extract of *I. coccinea* and diclofenac sodium against paw edema induced by carrageenan (Figure 2). Chloroform extract showed significant reduction of inflammation in carrageenan induced rat paw edema ($p<0.05$). The chloroform extracts of *I. coccinea* at the doses of 100mg/kg moderately inhibited rat paw edema 28.42, 36.07 and 38.36 % at 1h, 2h and 3h. respectively. Result revels that chloroform extract may inhibit production of histamine, serotonin and bradykinin in starting phase (Figure 2A) and also inhibit PGs in later phase (Figure 2B).

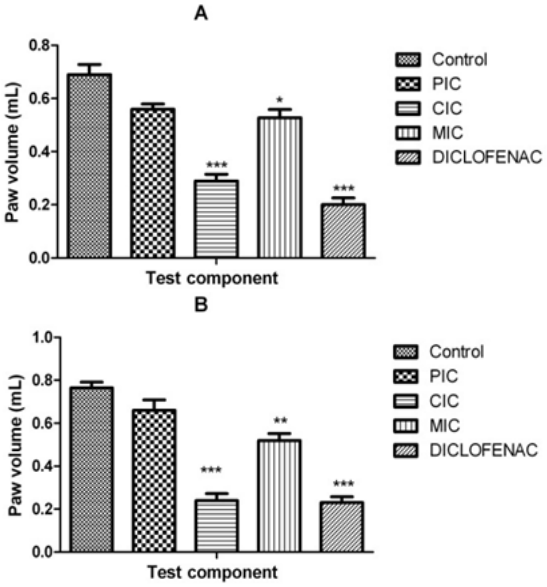

Figure 2. The effect of the oral administration of the crude extracts (PIC-Petroleum ether extract, CIC- Chloroform extract and MIC methanol extracts of *Ixora coccinea*) In early phase of carrageenan-induced paw edema (A) and in late phase of carrageenan-induced paw edema in rats(B). Each column represents the mean ± standard errors of the mean. *P<0.05, **P<0.01 and **P<0.001 represent significant differences compared to the control group. The statistical analysis was performed two-way ANOVA followed by Bonferroni's test.

Antioxidant activity
Nitric Oxide Radical Scavenging Assay

Nitric oxide (NO) is an important pleiotropic mediator produced by endothelial cells, macrophages, neurons, etc. NO is involved in regulation of many physiological processes like vasodilatation, smooth muscle

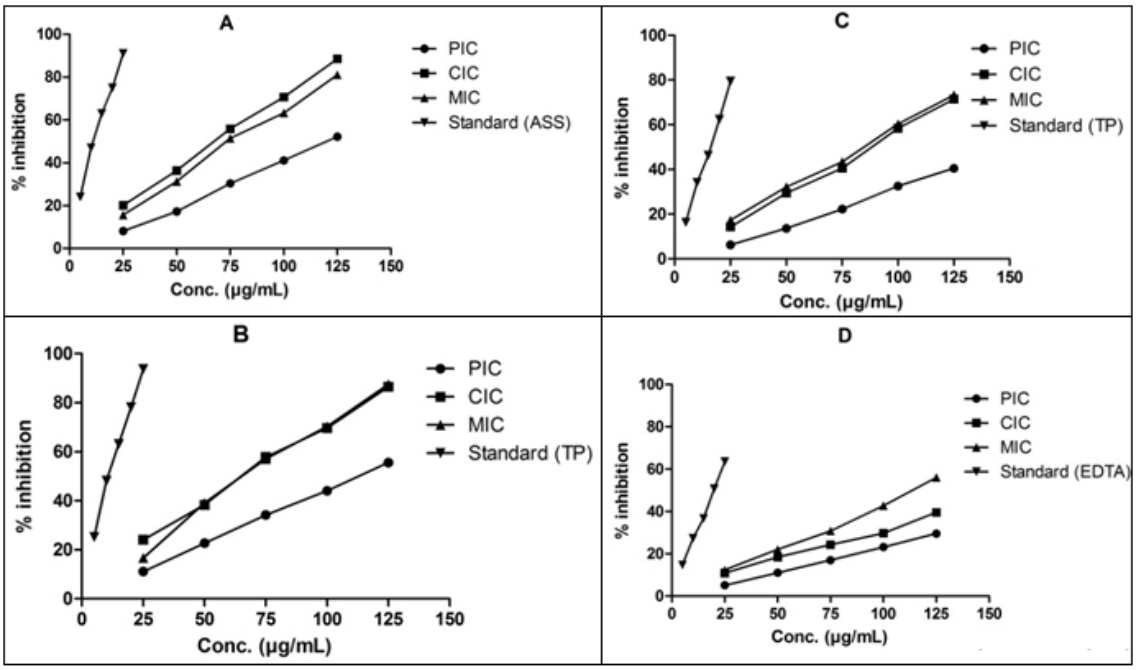

Figure 3. Antioxidant activity of *Ixora coccinea* extracts at different concentrations. Each value represents mean (n=3). A=Nitric oxide free radical scavenging activity, B=Hydrogen peroxide scavenging activity, C= DPPH free radical scavenging and D=Ferrous ion chelating activity

relaxation, neuronal messenger, inhibition of platelet aggregation and regulation of cell mediated toxicity. (Hagerman *et al*., 1998). Nitric oxide scavengers have been shown to have good effects in some phases of inflammation and also in tissue damage observe in inflammation (Kelml *et al*., 2000).

Petroleum ether, chloroform, methanol extracts of *I. coccinea* stem and ascorbic acid were found to be scavenger of nitric oxide free radical with an IC_{50} 125.95µg/mL, 69.06µg/mL, 77.28µg/mL and 12.88 µg/mL respectively (Figure 3a).

Hydrogen peroxide scavenging activity

H_2O_2 radical scavenging activity is a useful method for assessing the antioxidant properties of samples. H_2O_2 is usually nonreactive to cells but may give rise to hydroxyl radicals, which is toxic to cell. Therefore removal of H_2O_2 is important and it can be carry by antioxidants that donate an electron to H_2O_2 and reduce in to H_2O, thus blocking the production of hydroxyl radicals and protecting from cell injury. The electron donation potential of a sample is directly proportional to its antioxidant potential (Verma *et al*., 2015).

Petroleum ether, chloroform, methanol extracts of *I. coccinea* stem and tocopherol were found to be scavenger of H_2O_2 with an IC_{50} 111.85µg/mL, 67.84µg/mL, 68.49µg/mL and 11.58µg/mL respectively (Figure 2b). Scavenging of H_2O_2 by extracts is due to their polyphenolics, which may donate electrons to H_2O_2. The extracts of *I. coccinea* might be used to provide a good H_2O_2 scavenger for humans.

DPPH Free Radical Scavenging Assay

DPPH is nitrogen centered free radical having an odd electron which gives a strong absorption at 517nm. DPPH purple color changes to yellow when its odd electron paired with the radical scavenger to form the reduced DPPH-H(Cai *et al*., 2003). The decrease in absorbance of DPPH free radical at 517 nm was due to radical scavenger by donating hydrogen (Dehpour *et al*., 2009). Various concentrations petroleum ether, chloroform,

methanol extracts of *I. coccinea* and ascorbic acid were found to be good scavenger of DPPH radical with an IC_{50} of 162.87µg/mL, 87.72µg/mL, 82.92µg/mL and 16.07µg/mL, respectively (Figure 3c).

Ferrous ion chelating activity

Ferrous ion is one of the most effective pro-oxidants. In biological systems ferrous ion is interacting with hydrogen peroxide can lead to formation of very reactive hydroxyl radicals. Ferrozine forms complex with ferrous ions due to it is ferroin compound. The ferrozine-ferrous complex (magenta colored) formation is inhibited by chelating agent and intensity of magenta colour of complex is decrease. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction(Sudan *et al*., 2014). The formation of the ferrozine- ferrous complex is inhibited by the *I. coccinea* extract, indicating ferrous chelating activity with concentration dependent manner (Figure 3d). *I. coccinea* extract as chelating agents that may forms bonds with a metal and act as effective secondary antioxidants because it may reduce the redox potential of metal, and thereby stabilize the oxidized form of metal ion. Petroleum ether, chloroform, methanol extracts of *I. coccinea* stem and EDTA were found to be ferrous chelating activity with an IC_{50} of 216.45µg/mL, 157.73µg/mL, 114.68 µg/mL and 19.63 µg/mL respectively.

CONCLUSIONS

The chloroform and methanol extracts of *I. coccinea* stems showed anti-inflammatory and antioxidant activities supporting use in traditional medicine to treat in inflammatory condition. This study also affirmed that *I. coccinea* extracts is a potential source of antioxidant and could be used to prevent the diseases associated due to free radical. Due to antioxidant activity, extract might show anti-inflammatory activity. However, future work is required for the isolation and characterization of the active constituents responsible for above activities.

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