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Pharmacological activities of aqueous flower extract of *Ixora coccinea* L.

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ABSTRACT

Nature has blessed the universe with plenty of medicinal plants, so often referred as the Medicinal Garden of the world. In Indian Vedas, the medicinal plants including every part of the plant from roots to flowers were used for curing several diseases. Therefore, it is necessary to enrich the dietary system with antioxidant molecules to protect the human system against various diseases. The aim of the study was to evaluate antioxidant, anti-inflammatory, antibacterial and antidiabetic activities of aqueous flowers extract of *Ixora coccinea* belongs to the family Rubiaceae. Antioxidant activities such as DPPH· radical, superoxide ($O_2^{\cdot-}$) radical scavenging activities, phosphomolybdenum reduction and Fe^{3+} reducing power activities were carried out. The IC_{50} of DPPH· radical and superoxide ($O_2^{\cdot-}$) radical were 13.58 and 23.51 µg/mL concentrations. The RC_{50} of phosphomolybednum reduction and Fe^{3+} reduction were 20.57 and 14.57 µg/mL concentrations. The IC_{50} of haemolytic inhibition was 99.72 µg/mL concentration. Total phenolic and flavonoid content were 369.1 µg/mg Gallic acid equivalents and 55.14 µg/mg Quercetin equivalents. The IC_{50} of alpha amylase enzyme inhibition was 655.02 µg/mL concentration. The antibacterial activity showed maximum zone of inhibition of 15mm for *B. subtilis* at 500 µg/mL concentration.

Keywords: Antioxidant, DPPH· radical, Superoxide ($O_2^{\cdot-}$) radical, alpha-amylase, haemolysis.

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INTRODUCTION

Ixora coccinea Linn (Rubiaceae) is known as Jungle of Geranium (or) Flame of the woods or vetchi in Ayurveda. It is a common flowering shrub native to Asia. It is name derived from an Indian deity. Although there are some 400 species in the genus *Ixora coccinea* is a dense, multi – branched ever green shrub commonly 4-6 ft (1-2-2m) in height, but capable of reaching up to 12ft (3.6m) height (Figure 1). It is traditionally used as hepato-protective, Chemo-protective, antimicrobial, anti-oxidant, anti-nociceptive, anti–mitotic and anti-inflammatory activities. Decoction of roots used for nausea, hiccups and anorexia. Powered roots used for sores and chronic ulcers. In indo – china, root decoction used to clarify the urine, poultice fresh leaves and stems for sprains, eczema, boils and contusions. The genus name " *Ixora* " is supposed to be derived from the Sanskrit word " ikvana ", after a Malaysian deity, or possibly from the name " Iswara ", the other name of Lord Shiva to whom the flowers are offered during worship, while the species name " *coccinea* " means scarlet[1,2]. In the Ayurvedic system of medicine, the flowers are used to treat luecorrhoea, dysentery, dysmenorrhoea, haemoptysis, hypertension, menstrual irregularities, sprains, bronchitis fever, sores, chronic ulcers, scabies, and skin diseases[3-9].

Figure.1: Habit of *Ixora coccinea* L.

MATERIALS AND METHODS

Collection of *Ixora coccinea* flowers and preparation of extracts

The flowers of *Ixora coccinea* were collected from normal garden at Porur, Chennai. The collected flowers were washed with distilled water. The cleaned flowers were subjected to hot decoction method. Then the supernatant was filtered by filter paper and condensed by using rotary evaporator 50°C, which yields gummy extract [10,11].

Antioxidant activities

DPPH$^{\cdot}$ radical scavenging activity

The antioxidant activity of aqueous extract of flowers of *Ixora coccinea* was measured on the basis of scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical [12]. One mL of 0.1 mM DPPH solution in methanol was mixed with 1 mL of various concentrations of aqueous extract (20-120 µg/mL). The mixture was then allowed to stand for 30 min incubation in dark. Ascorbic acid was used as the reference standard. One mL ethanol and 1mL of DPPH solution was used as the control. The decrease in absorbance was measured using UV-Vis spectrophotometer at 517 nm. The percentage of inhibition was calculated as:

$$\% \text{ of DPPH}^{\cdot} \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] * 100$$

Superoxide ($O_2^{\cdot-}$) radical scavenging activity

The superoxide ($O_2^{\cdot-}$) radical scavenging activity of aqueous extract of flowers of *Ixora coccinea* was measured on the basis of superoxide radical inhibition[13]. Various concentrations (20-120 µg/mL) of aqueous extract were mixed with 50 mM phosphate buffer (pH-7.6). The mixtures were combined with 200 µL of 1.5 mM Riboflavin, 200 µL of 12 mM EDTA followed by 100 µL of 50 mM NBT. The reaction was started by illuminating the test tubes in UV-lamp for 15 min. The superoxide ($O_2^{\cdot-}$) radical reduces NBT to a blue colored formazon can be measured at 590 nm. Ascorbic acid was used as the reference standard. The percentage of superoxide ($O_2^{\cdot-}$) radical inhibition can be calculated as:

$$\% \text{ of superoxide } (O_2^{\cdot-}) \text{ radical inhibition} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] * 100$$

Phosphomolybdenum reduction activity

The antioxidant capacity of the aqueous extract of flowers of *Ixora coccinea* was assessed by the method[14]. The aqueous extract of different concentrations (20-120 µg/mL) was combined with 1 mL of reagent solution containing 4 mM Ammonium molybdate, 28 mM Sodium phosphate and 0.6 M Sulphuric acid. The reaction mixture was incubated in water bath at 95°C for 90 min. The absorbance of the coloured complex was measured at 695 nm. Ascorbic acid was used as standard reference.

$$\% \text{ of phosphomolybdenum reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] * 100$$

Ferric (Fe^{3+}) reducing power activity

The reducing power of aqueous extract of flowers of *Ixora coccinea* was determined by slightly modified method[15]. One mL of aqueous extract of different concentrations (20-120 µg/mL) was mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1 % w/v). The mixture was then incubated in water bath at 50°C for 30 min. 500 µL of trichloroacetic acid (10 % w/v) was added to each mixture, mixed well and 100 µL of freshly prepared $FeCl_3$ (0.1% w/v) solution and shaken well. The absorbance was measured at 700 nm using spectrophotometer. Ascorbic acid was used as the standard reference.

$$\% \text{ of } Fe^{3+} \text{ reduction} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] * 100$$

Anti-inflammatory activity by Heat induced haemolysis

Varying concentrations (20-120 µg/mL) of aqueous extract of flowers of *Ixora coccinea* and made upto 1 mL with normal saline, followed by 200 µL of freshly prepared 10% RBCs suspension[16]. All the centrifuge tubes containing reaction mixture were incubated in water bath at 50°C for 30min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 3000 rpm for 5 minutes, if precipitate appears and the absorbance was taken at 560 nm. Diclofenac was used as a standard reference.

$$\% \text{ inhibition of Haemolysis} = \left[\frac{\text{Control} - \text{Sample}}{\text{Control}}\right] * 100$$

Quantitative estimations of total phenol and flavonoids

Determination of total phenols

Folin-Ciocalteau reagent method was used to determine the total phenolic compounds[17] with slight modifications. One hundred µL (1 mg/mL) of aqueous extract of flowers of *Ixora coccinea* were mixed with 900 µL of methanol and 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water). After 5 min, 1 mL of Na_2CO_3 (20% w/v) solution was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured by UV-Vis spectrophotometer at 765 nm. The total phenolic content was expressed in terms of gallic acid equivalent (µg/mg of extract), which is a common reference compound.

Determination of total flavonoids

The total flavonoid content of aqueous extract (1 mg/mL) of flowers of *Ixora coccinea* were determined by aluminium chloride reagent method with slight modification[18]. Five hundred µL of aqueous extract were mixed with 0.5 mL of methanol and 0.5 mL of (5% w/v) sodium nitrite solution. Then, 0.5 mL (10% w/v) aluminium chloride solution was added followed by 1 mL of 1 M NaOH solution was added. The mixture was incubated for 30 min at room temperature and the absorbance was measured at 510 nm. The result was expressed as (µg/mg of extract) quercetin equivalent.

Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis was carried out for aqueous extract of flowers of *Ixora coccinea* on silica gel aluminium sheet[19] (Merck Silica gel 60 F254). The ethanol extracts were spotted at 0.5 mm above from the bottom of the TLC plate. The spotted TLC plate was placed in a 100 mL beaker containing solvent mixture Ethyl acetate (1.8 mL):Methanol (0.1mL):Distilled water-(0.1mL). The

chromatogram was developed and the spots were visualized under UV light at 254 nm as well as in iodine vapour. The ratio in which distinct coloured bands appeared was optimized and R_f values were calculated.

$$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Antidiabetic activity by Alpha-amylase inhibition method

The antidiabetic activity of aqueous extract of flowers of *Ixora coccinea* was determined by slightly modified method[20]. Different concentration of aqueous extract (100-600 µg/mL) was mixed with 1 mL of methanol, followed by 10 µL of alpha amylase solution (1 % w/v). The mixture was then incubated in room temperature at 37°C for 10 min. 500 µL of starch (1% w/v) was added to each mixture, mixed well and incubated in room temperature at 37°C for 60 min. 100 µL of freshly prepared 1N Hydrochloric acid was added to terminate the enzymatic reaction and 200 µL of freshly prepared iodine solution (1 % w/v) was added and shaken well. The absorbance was measured at 565 nm using spectrophotometer. Acarbose was used as the standard reference.

$$\% \text{ of alpha amylase inhibition} = \left[\frac{\text{Sample} - \text{Control}}{\text{Sample}}\right] * 100$$

Antibacterial activity by Agar Well diffusion assay

Nutrient agar was prepared and poured in the sterile Petri dishes and allowed to solidify. 24 hours grown bacterial pathogens were swabbed on nutrient agar plates[21]. Then, the stock crude aqueous extract individually (10 mg/mL) was prepared. Varying concentration (250 µg/mL, 375 µg/mL and 500 µg/mL) of aqueous extract of flowers of *Ixora coccinea* was loaded in the wells made using cork borer. Tetracycline was used as standard. The plates were then incubated at 37ºC for 24 hours. After incubation the inhibition diameter was measured.

RESULTS AND DISCUSSION

Antioxidant activities

DPPH˙ radical scavenging activity

Evaluation of antioxidant activity by DPPH method is the best screening option for herbal based drugs. DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl) is a stable nitrogen centered free radical which has an unpaired valence electron at one atom of nitrogen bridge. The ability of the aqueous extract of flowers of *Ixora coccinea* to scavenge free radicals formed was assessed using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH).The maximum DPPH˙ radical scavenging activity was 97.27±0.10 at 120 µg/mL concentration for aqueous extract of flowers of *Ixora coccinea.* The aqueous extract of flowers of *Ixora coccinea* demonstrated high capacity for scavenging free radicals by reducing the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radical to the yellow coloured 1,1-diphenyl-2-picrylhydrazine and the reducing capacity increased with increasing concentration of the extract (Table 1).The IC_{50} value for aqueous extract of flowers of *Ixora coccinea* was found to be 13.58 µg/mL concentration (Graph 1) and was compared with standard (Ascorbic acid, IC_{50} = 10.76 µg/mL concentration).

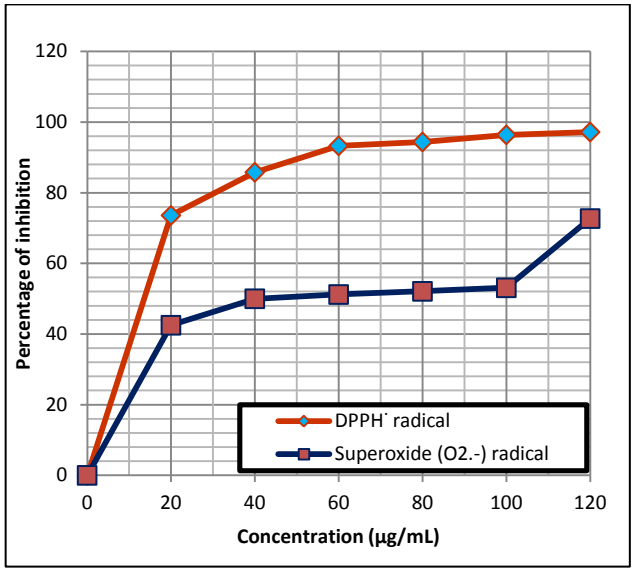
Superoxide (O_2˙-) radical scavenging activity

Superoxide anion is also very harmful to cellular components and their effects can be magnified because it produces other kinds of free radicals and oxidizing agents[22]. Reduction of NBT is the most popular method. The method is based on generation of super oxide radical by auto oxidation of riboflavin in presence of light. The maximum superoxide (O_2˙-) radical scavenging activity for aqueous extract of flowers of *Ixora coccinea* was 72.74±0.46 at 120 µg/mL concentration (Table 1). In-vitro superoxide radical scavenging activity is measured by riboflavin/light/NBT (Nitro blue tetrazolium) reduction. The experiment demonstrated higher antioxidant activity the IC_{50} of 23.51 µg/mL concentration for aqueous extract of flowers of *Ixora coccinea* (Graph 1) and was compared with standard Ascorbic acid (IC_{50} = 4.46 µg/mL concentration).

Table 1: DPPH˙ radical and Superoxide (O_2˙-) radical scavenging activity of aqueous extract of flowers of *Ixora coccinea*

S.No	Concentration (µg/mL)	% of inhibition*	
		DPPH˙ radical	Superoxide (O_2˙-) radical
1	20	73.62±0.23	42.53±0.34
2	40	85.81±0.17	50.05±0.41
3	60	93.30±0.44	51.28±0.16
4	80	94.42±0.39	52.11±0.39
5	100	96.45±0.28	53.19±0.13
6	120	97.27±0.10	72.74±0.46

(*Average value of 3 replicates)



Graph 1: DPPH˙ radical and Superoxide (O_2-) radical scavenging activity of aqueous extract of flowers of *Ixora coccinea*

Phosphomolybdenum reduction activity

The total antioxidant activity of aqueous extract of flowers of *Ixora coccinea* was measured spectrophotometrically by phophomolybdenum reduction method which is based on the reduction of Mo (VI) to Mo (V) by the formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption at 695 nm. The maximum reducing ability for aqueous extract of flowers of *Ixora coccinea* was 78.08±0.29 at 120 µg/mL concentration. (Table 2 and Graph 2). The experiment demonstrated higher antioxidant activity the RC_{50} of 20.57 µg/mL concentration for aqueous extract of flowers of *Ixora coccinea* and was compared with standard Ascorbic acid (RC_{50} = 18.28 µg/mL concentration). Metal-Catalyzed Oxidation (MCO) systems catalyze the reduction reaction, which alters the nature of proteins at the metal-binding site and cause DNA and protein damage[23].

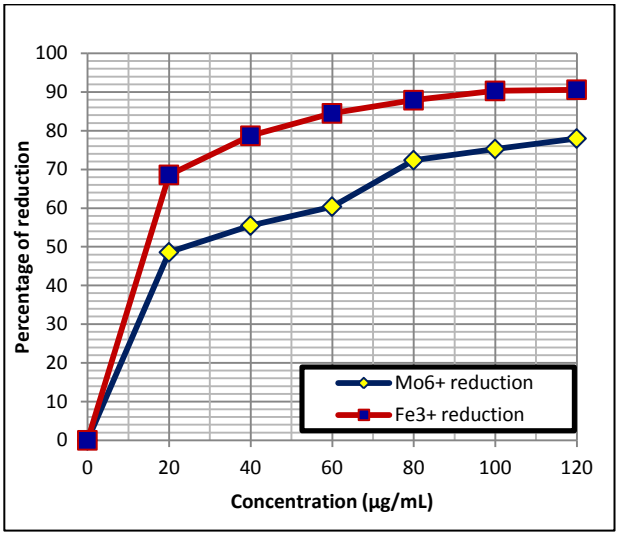
Ferric (Fe^{3+}) reducing power activity

The reducing power of Fe^{3+} to Fe^{2+} by the aqueous extract of flowers of *Ixora coccinea* was studied and showed reduction ability in a dose dependent manner. The maximum reduction for aqueous extract of flowers of *Ixora coccinea* was 90.66±0.43 at 120 µg/mL concentration. (Table 2 and Graph 2). Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action[24]. The RC_{50} value for aqueous extract of flowers of *Ixora coccinea* as found to be 14.57 µg/mL concentration and was compared with the standard (23.74 µg/mL concentration) Ascorbic acid.

Table 2: Phosphomolybdenum reduction and Fe^{3+} reduction activity of aqueous extract of flowers of *Ixora coccinea*

S.No	Concentration (µg/mL)	% of reduction*	
		Mo^{6+} reduction	Fe^{3+} reduction
1	20	48.61±0.25	68.62±0.11
2	40	55.54±0.16	78.71±0.28
3	60	60.46±0.33	84.57±0.20
4	80	72.42±0.41	87.95±0.35
5	100	75.37±0.19	90.32±0,39
6	120	78.08±0.29	90.66±0.43

(*Average value of 3 replicates)



Graph 2: Phosphomolybdenum reduction and Fe^{3+} reduction activity of aqueous extract of flowers of *Ixora coccinea*

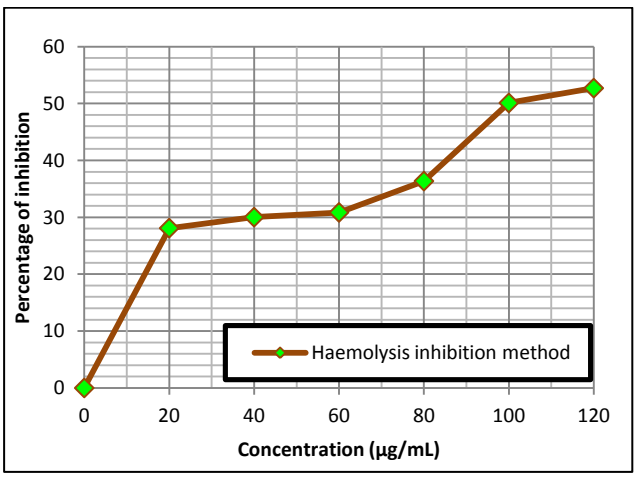
Anti-inflammatory activity by Heat induced haemolysis

Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer. Erythrocytes are considered as major target for the free radicals owing to the presence of both high membrane concentration of poly unsaturated fatty acids (PUFA) and the oxygen transport associated with redox active haemoglobin molecules, which potent promoters of activated oxygen species. The erythrocyte model has been widely used as the direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity[25]. The maximum haemolytic inhibition for aqueous extract of flowers of *Ixora coccinea* was 52.77±0.19 at 120 µg/mL concentration. (Table 3 and Graph 3).

Table 3: Heat induced haemolysis of aqueous extract of flowers of *Ixora coccinea*

S.No	Concentration (µg/mL)	% of inhibition*
		Heat induced haemolysis
1	20	28.09±0.28
2	40	30.02±0.16
3	60	30.85±0.34
4	80	36.36±0.39
5	100	50.14±0.42
6	120	52.77±0.19

(*Average value of 3 replicates)



Graph 3: Heat induced haemolysis of aqueous extract of flowers of *Ixora coccinea*

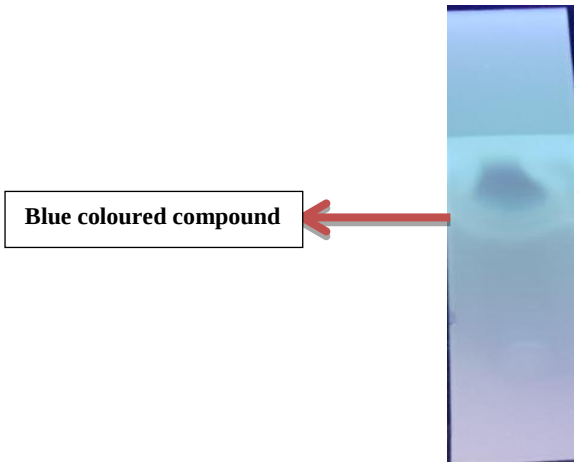
Excess production of reactive oxygen and nitrogen species generated from activated inflammatory leukocytes, especially under conditions of chronic inflammation, play an important role in various pathogenesis. Some of the conventionally used anti-inflammatory drugs, particularly the steroids and cyclooxygenase inhibitors, are observed to possess adverse side effects such as gastrointestinal irritation, ulcers, hypertension, and cardiac abnormalities[26,27]. The IC_{50} value for aqueous extract of flowers of *Ixora coccinea* as found to be 99.72 µg/mL concentration (Table 3) and was compared with the standard (17.83 µg/mL concentration) Diclofenac.

Determination of total phenols and flavonoids

Total phenol content for aqueous extract of flowers of *Ixora coccinea* was 369.1±0.19 µg/mg of GAE and flavonoid content was 55.14±0.38 µg/mg of QE in the aqueous extract. Phenolic compounds were found to be higher when compared to flavonoid content. Phenolic compounds and flavonoids have beneficial effects, which are mainly due to proper intake of healthy fruits and vegetables. Consumption of proper and nutritious foods might be responsible for significant antioxidant activity thereby preventing the entry of free radicals causing many diseases. Free radicals are produced during normal cellular function in the body, these molecules are missing an electron, giving them an electric charge. To neutralize this charge, free radicals try to withdraw an electron from or donate an electron to a neighbouring molecule[28].

Thin Layer Chromatography analysis

Thin layer chromatography analysis was carried out in the solvent system of Ethyl acetate (1.8 mL):Methanol (0.1mL):Distilled water-(0.1mL). The separated compounds in TLC were showed in Figure 2. The separated active compounds were visualized in UV light at 254 nm and iodine balls. The R_f values of the separated compounds were found to be 0.52 and 0.29.



Under UV light at 254 nm

Figure 2: Separation of active Compounds by Thin Layer Chromatography

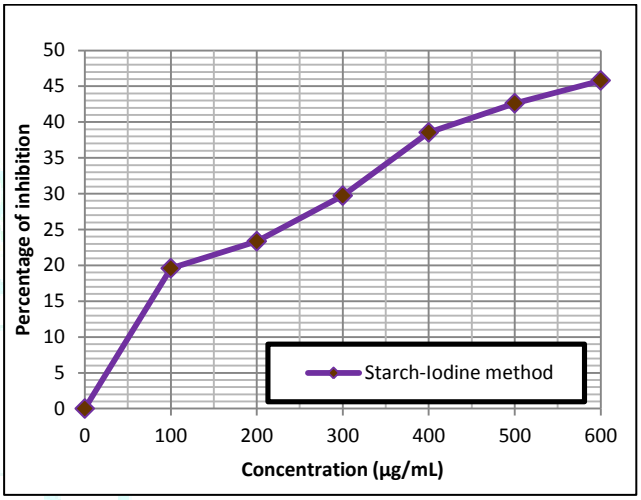
Antidiabetic activity by Alpha-amylase inhibition method

Diabetes mellitus is a chronic metabolic disorder identified by hyperglycemia due to insulin insufficiency and/or insulin resistance contributing to excess blood glucose[29]. The percentage of inhibition is 45.80±0.31 (Table 4) at 600 µg/mL concentration of the aqueous extract (Graph 4). The IC_{50} of alpha amylase enzyme inhibition was 655.02 µg/mL concentration and was compared with standard (Acarbose, IC_{50} value as 19.85 µg/mL concentration).

Table 4: Antidiabetic activity of aqueous extract of flowers of *Ixora coccinea*

S.No	Concentration (µg/mL)	% of inhibition*
		Starch-Iodine method
1	100	19.58±0.45
2	200	23.35±0.32
3	300	29.72±0.11
4	400	38.54±0.27
5	500	42.61±0.19
6	600	45.80±0.31

(*Average value of 3 replicates)



Graph 4: Antidiabetic activity of aqueous extract of flowers of *Ixora coccinea*

Antibacterial activity by Agar Well diffusion assay

The antibacterial activity of the aqueous extract (Table 5 and figure 3) could be correlated as due to the presence of secondary metabolites such as flavonoids, phenolic compounds, terpenoids, tannins and alkaloids that adversely affect the growth and metabolism of microbes. The antibacterial effect of plant extract against the testing bacterial pathogens occurs mainly due to cytoplasmic membrane disturbance, disrupting the proton motive force, electron flow, active transport mechanisms, and coagulation of cell composition. Phenolic acids are mainly involved in the reduction of specific adherence of organisms to the cells lining the bladder and the teeth, ultimately lowering the occurrence of urinary tract infections and dental caries[30].

Table 5: Antibacterial activity of aqueous extract of flowers of *Ixora coccinea*

Test organisms	Zone of inhibition (mm)			
	250 µg/mL	375 µg/mL	500 µg/mL	Standard (30 µg/mL)
S. aureus	12	12	13	17
M. luteus	13	13	14	24
B. subtilis	13	14	15	24
E. coli	14	14	14	24
S. flexneri	13	14	14	24
P.vulgaris	13	14	14	17

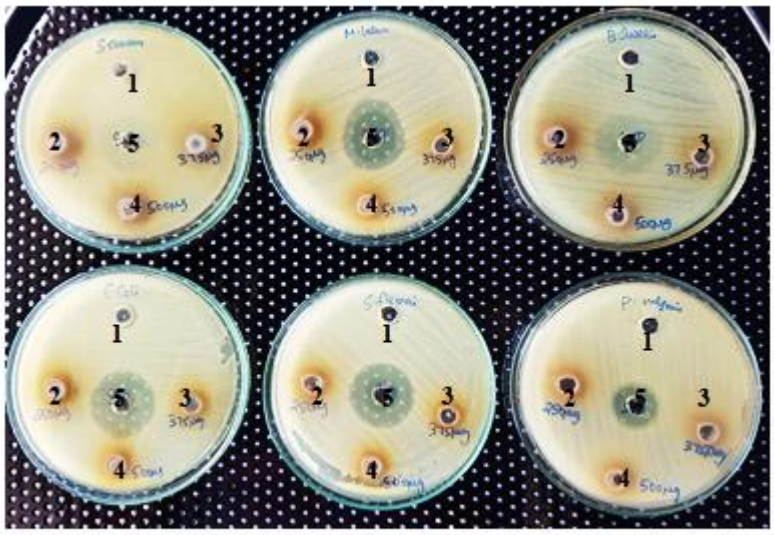


Figure 3: Antibacterial activity of aqueous extract of flowers of *Ixora coccinea*

CONCLUSION

The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases. Antioxidants derived from plants are more effective than the synthetic ones because they are non-toxic and have more than one mode of action. These lead to the screening of plants for their antioxidant properties and other medicinal properties. Based on the antioxidant, antibacterial and anti-inflammatory activities, *Ixora coccinea* could be used as a source of therapeutic agents. Natural products in the pharmaceutical industry have reduced, owing to issues such as the lack of compatibility of traditional natural product extract libraries with high-throughput screening.

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