Evaluation of anti-bacterial, anti-fungal along with in-vitro anti-inflammatory activity of highly anti-oxidant rich flower of *Ixora Coccinea*

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Abstract:
Ixora coccinea flower also known as rongon, a perennial plant belonging to the family Rubiaceae, is used as phytomedicine to treat a number of chronic diseases including ulcer, inflammation, bronchitis, anemia, diarrhea, microbial infections, and skin infections. For exploration of pharmacological activities, the preliminary phytochemicals screening of the flowers are needed to reveal the presence of tannins, saponins, flavonoids, alkaloids, and glycosides. In vitro experiments on the methanolic flower extract of Ixora coccinea have likely revealed several bioactive chemicals with potential health benefits. Phytochemical screening identified compounds such as alkaloids, flavonoids, and phenols, known for their antibacterial, anti-inflammatory, and antioxidant properties. The extract's Total Phenolic content was 20.18 µg GAE/mg, and Total Flavonoid content was 21.49 µg QAE/mg, indicating a significant presence of these compounds, which are effective against free radical damage. The extract showed antimicrobial activity against Staphylococcus aureus and Pseudomonas aeruginosa with an MIC of 25 mg/ml and a zone of inhibition of 0.7 cm, and antifungal activity against Aspergillus niger with an MIC of 50 mg/ml. These dual antibacterial and antifungal properties suggest broad-spectrum antimicrobial potential, useful for treating infections like throat, skin, and urinary tract infections. Anti-inflammatory studies using heat-induced hemolysis, hypotonicity-induced hemolysis, protein denaturation assay, and proteinase inhibitory effect showed IC50 values of 357, 288, 242, and 259 µg/ml, respectively, comparable to aspirin. This strong anti-inflammatory potential indicates the extract could be tested for treating degenerative disorders like arthritis, dementia, Alzheimer's disease, and diabetes.
In conclusion, Ixora coccinea flower extract shows promise as an antioxidant, antimicrobial, and anti-inflammatory agent, warranting further in vivo studies to explore its full potential.

Keywords: *Ixora coccinea*, anti-inflammatory, antibacterial, anti-fungal, protein denaturation, antioxidant etc.

Introduction:

For centuries, mankind has turned to Mother Nature's botanical bouquet for healing remedies, relying on extracts crafted from vegetation's versatile vocabulary. Ranging from common calamus to curious calendula, countless plants proudly proffer preventative properties. Though contemporary cures clutch captivating careers, curious customers continually consider countless configurations carefully crafted from nature's nourishing nutrients [1]. Recently, renewed rapture regarding recognized remedies revived research into rich reservoirs residing within roots, rhizomes, leaves, and blooms. This unveils a universe of unpredictable utilities uniquely unleashed by assorted aromatic arrangements. While archaic applications anchored ancestral alleviation approaches, analyzing active accoutrements under an analytic argues eye advances our understanding from unexplained experiences to established evidence. Herbal medicines can be used for a wide range of ailments; from plants that contain extensive bioactive compounds that can be used to treat specific health disorders to antioxidant-rich combinations that support general well-being[2].
Flame of the woods flaunts its fiery flowers, a fantastic flash of colors casting light across the verdant vista. *Ixora coccinea*, a stalwart shrub of South Asian ancestry, emanates effusive efflorescence's in vivid reds, oranges, pinks, and yellows to liven landscapes with its luminous blooms. Revered for centuries in venerable Ayurvedic and antique Chinese medical arts, prudent practitioners prize particular plant parts for purported healing properties—soothing skin, speeding wound repair, regulating rises in body heat. Beyond mere decorative duties, this resilient evergreen renders valuable services, its versatile constitution contributing components that traditions have tailored to address afflictions. Though indigenous to India and Sri Lanka, it spreads spectacular splendor universally as a picturesque presence profuse with patterns of rainbow radiance [3,4]. Culturally, the species Ixora coccinea is often cultivated in gardens, parks, and as hedge plants due to its hardiness and adaptability to diverse climatic conditions. Its propensity to attract butterflies and birds adds to its popularity in landscaping. This adaptable shrub has also found use in landscaping, traditional medicine, and cultural customs, making it a plant with both aesthetic and utilitarian significance.

Inflammation is the body's typical response to harmful stimuli like infections, cell damage, or allergens. It refers to an intricate biological process involving several cells, proteins, and chemical reactions. The immune system causes inflammation when tissue is damaged or infected in order to defend and repair the body. Blood arteries widen when there is inflammation, increasing the amount of blood that reaches the injured area. This causes swelling, warmth, and redness. White blood cells in particular, which are immune cells, go to the site of infection or injury to eliminate pathogens, remove damaged tissue, and start the healing process [5,6].

Fig. 1: Flowers of *Ixora coccinea*

Materials & Methods:

Collection and Authentication of Plant:

Select the spring season stem cuttings, make a cut slightly below a leaf, and cover the cut end with hormone rooting powder to propagate Ixora coccinea. The cuttings should be planted in a container with coarse sand, perlite, and peat moss. The container should be placed in a brilliantly lighted location with filtered light, between 21 and 27 °C. After the cuttings have taken root, which should take four to six weeks, expose them over a period of two to three weeks. Every two weeks, apply a normal dose of liquid fertilizer, let the soil dry, and then give it a little watering. Three months after propagation, move the young plant into a larger container and label it as mature. Botanical Survey of India observed and confirmed the plant, granting it the authentication number JIS/PG-01.

Extraction procedure:

The study involved collecting 2 kg of Ixora coccinea flowers from a garden, drying them in a shaded area for at least 15 days, and then grinding them into a powder. The powder was further dried and crushed. The flower sample was weighed and a 30% methanol extract was created. The dry powder was mashed into a paste and stored at 20°C for three to four days. Whatman filter paper was used twice to filter the extract. The filtrate was then evaporated in a water bath and 10% DMSO was used to dilute the extract for various estimation purposes [7].

Phytochemical Screening: [8]

The study utilized reagents and chemicals to qualitatively test Ixora coccinea flower extracts for chemical constituents, followed by phytochemical screening to identify alkaloids, carbohydrates, steroids, cardiac glycosides, flavonoids, amino acids, phenolics, and tannins.

Determination of Alkaloids:

Mayer’s test: Mayer's reagent was distributed over the sample in tiny drops (two to three milliliters). The formation of white precipitate indicated the existence of alkaloids.

Wagner’s test: Wagner's reagent was introduced to just a couple drops of the sample (two to three milliliters). The reddish-brown precipitate's appearance suggested an existence of alkaloids.

Determination of Carbohydrates:

Fehling’s test: The extract had been incorporated with 5 milliliters of Fehling's solution (A and B), and the mixture had been brought to a boiling temperature for ten to fifteen minutes. A yellow or red precipitate's formation suggests a lower sugar concentration.

Determination of Glycosides:

Modified Bontrager’s test: Take 1 g of the sample medication and mix in 5% ferric chloride and dilute HCL. For a 10 minute period, boil it. Pass the solution through a filter. Carbon tetrachloride (CCl4) or benzene is used to extract the filtrate.

Shake after adding the same amount of ammonia solution to the filter. Look for any shade of pink or red shows that the C-type anthraquinone glycosides moiety is present in the ammoniacal layer.

Determination of Steroids:
Lieberman-Buchard reaction: Chloroform was combined with the sample (2 ml). Two drops of concentrated sulfuric acid were introduced from the tube's edges, along with one to two millilitres of acetic anhydride. The colors came in shades of red, blue, and eventually green.

Determination of Proteins:
Millon's test: Millon's reagent (5 ml) was combined with 3 ml of the test sample. Precipitate that is white forms. Precipitate turns brick red when it warms up or dissolves to produce a red solution.

Determination of Amino Acids:
Ninhydrin test: Boiling water was used to heat the test sample (3 ml) and three drops of 5% ninhydrin solution for ten minutes. The color purple emerged.

Determination of Flavonoids:
Alkaline Test: The presence of flavonoids is shown by the test solution taking on a vivid yellow hue when just a couple drops of sodium hydroxide solution is included, which goes colorless when a few drops of di acid is introduced.

Determination of Sterols:
The sample was treated with 5% potassium hydroxide solution, The pink tone of the sample indicated the presence of sterols.

Determination of Tannins:
Lead acetate test: Tannins were discovered in the sample following treatment with a 10% lead acetate solution, as shown by the development of a white precipitate. When the extract was treated with an aqueous bromine solution, the presence of tannins was shown by the production of a white precipitate.

Determination of Saponins:
Foam test: 1 milliliter of the extraction was combined with 5 milliliters of distilled water and vigorously stirred. Foam creation indicated the presence of saponins.

Total phenolic content
The concentrations of phenolic compounds in the sample will be measured according to the Folin-Ciocalteu method. Briefly, the samples solution (0.5 ml) at different concentrations (ranging from 500, 500, 1000 µg/ml) was mixed with 2.58 ml of Folin–Ciocalteu's phenol reagent. After 3 min, 0.3 ml of saturated sodium carbonate solution was added to the mixture.The reaction mixtures were incubated at room temperature (25°C) for 20 min. The absorbance was measured at 760 nm with a spectrophotometer. Gallic acid solutions with concentrations ranging from 400, 600, 800 µg/ml were used for calibration. A dose response linear regression was generated by using the gallic acid standard absorbance and the levels in the samples were expressed as gallic acid equivalent (mg of GAE/gm of extract). The blank was taken as a mixture of 0.5 ml 200ug/ml aspirin sol and 2.58 ml of Folin, and 0.3 ml of saturated Na2CO3 solution [9].

Total Flavonoid Content
The total flavonoid content was estimated by aluminium chloride method. Plant samples (0.5 ml) ranging from 100, 200, 300 µg/ml were mixed with 2.5 ml of distilled water and 150 µl NaNO2 solution (5 %). The contents were vortexed for 10 s and left at room temperature for 5 min. Then, 300 µl AlCl3 (10 %), 1 ml NaOH (1 mM) and 550 µl of distilled water were added. The solution was mixed well and kept for 15 min. The absorbance for each sample was measured at 510 nm. Quercetin concentrations ranging from 400, 600, 800 µg/ml were prepared and the standard calibration curve was obtained. The total flavonoid content was calculated using standard quercetin calibration curve. The results were expressed as mg of quercetin equivalent (QE) per gram of extract. The blank was taken as 200 ug/ml of aspirin sol plus 2.5 ml dH2O plus 150ul of NaNO2 sol plus AlCl3 sol plus NaOH sol plus 150 ul dH_2O[9]

Anti-Microbial Study:
The Minimum Inhibitory Concentration Analysis:
To use the autoclaved equipment in the antimicrobial research, it must first be obtained and then properly autoclaved. The same procedure is done to the distillation with the distilled water to sterilize the distilled water. The nutrient broth is then made. Different concentrations, which are 25, 50, 75, 100 mg/ml, were prepared from the extract sample using 1%DMSO (Dimethyl sulfoxide) and then 3ml of broth is poured and dissolved into different tubes. Then one loop full of bacterium *Streptococcus aureus* and *Pseudomonas aeruginosa* and the fungus *Aspergillus niger* were added separately to the tubes. After that, the tubs are placed in a BOD incubator and left for 24 hours at 37 °C. Each test tube

is checked for turbidity after 1 day of incubation. The concentrations that appear to be inhibitory are those at which the solutions seem clear. The minimum inhibitory concentration of the sample for demonstrating antimicrobial activity is the concentration at which antimicrobial activity is observed, i.e., the solution appears to be clear. The aforementioned experiment is carried out in an aseptic environment [10].

Identifying the Inhibition Zone:
For Streptococcus aureus, potato dextrose agar was created, while Celrimide agar was made for Pseudomonas aeruginosa and Mannitol Salt Agar for Streptococcus aureus. Zone of inhibition is established using conventional protocol.

Anti-Inflammatory study:
In order to perform membrane stabilization experiments, phosphate buffer (pH 7.4) and a human blood sample are prepared.

Hypotonicity-induced haemolysis:
Distilled water was used to create extracts from samples at levels of 100, 500, and 1000 µg/ml, respectively. To each concentration, one milliliter of phosphate buffer, two milliliters of hyposaline, and half a milliliter of the human red blood cell suspension have been applied. It was centrifuged for 20 minutes at 3000 rpm and incubated for 30 minutes at 37 °C. The hemoglobin level of the residual solution was determined by spectrophotometry at 560 nm. The extracts were excluded to provide a control, and aspirin (100 µg/ml) was selected as the benchmark. The quantity of hemolysis or membrane stabilization that was suppressed was measured.
% inhibition of haemolysis = [(A1 – A2)/ A1] x 100 Where A1 = absorbance related to control, and A2 = absorbance of the mixture of test samples

Heat-Induced Hemolysis:
0.05 milliliters of blood cell suspension and 0.05 milliliters of hydromethanolic leaf extracts were mixed with 2.95 milliliters of phosphate buffer (pH 7.4). For twenty minutes, the mixture had been incubated at 54 °C in a shaking water bath. After the incubation period, the resulting mixture had been centrifuged for three minutes at 2500 rpm, and a UV/VIS spectrophotometer (Optima, SP-3000, Tokyo, Japan) was used to measure the absorbance of the supernatant at 540 nm. Phosphate buffer solution was used as the experiment's control. The amount of hemolysis or membrane stabilisation that was suppressed was measured.
% inhibition of haemolysis = [(A1 – A2)/ A1] x 100 Where A1 = absorbance related to control, and A2 = absorbance of the mixture of test samples

Effect on Protein Denaturation:
The reaction mixture (5 ml) consisted of 5.78 ml of phosphate buffered saline (PBS, pH 6.4), 0.02 ml of extract, and 0.2 ml of 1% bovine albumin. After mixing, the mixture was heated to 70 °C for five minutes, and it continued to incubate for 15 minutes at 37 °C in a water bath. After cooling, the turbidity has been determined at 660 nm using a UV/VIS spectrometer (Optima, SP-3000, Tokyo, Japan). Solution of phosphate buffer served as the control. To determine the percentage of protein denaturation inhibition, we performed this calculation:

% Inhibition of haemolysis = [(A1 – A2)/ A1] x 100 Where A1 = absorbance related to control, and A2 = absorbance of the mixture of test samples

Proteinase Inhibitory Activity
The reaction solution (2 mL) consisted of 0.06 mg trypsin, 1 mL of 20 mM Tris-HCl buffer (pH 7.4), and 1 mL test sample (0.02 mL extract 0.980 mL methanol). The solution was incubated (37 °C for 5 min), and then 1 mL of 0.8% (w/v) casein was added, and the mixture was further incubated for an additional 20 min. At the end of incubation, 2 mL of 70% perchloric acid was added to terminate the reaction. The mixture was centrifuged, and the absorbance of the supernatant was measured at 210 nm against buffer as the blank. Phosphate buffer solution was used as the control. The percentage inhibition of protein denaturation was calculated by using the following formula:
% inhibition of denaturation = 100 × (1 − A2/A1), where A1 = absorption of the control sample, and A2 = absorption of the test sample.

Result:
Phytochemical screening:
Ixora coccinea peels were extracted in methanol, and the presence of several phytochemicals such as alkaloids, glycosides, tannins, flavonoids, saponins, steroids, and carbohydrates was assessed. The analysis showed that the floral extract included considerable amounts of secondary metabolites, including methanolic chemicals, alkaloids, carbohydrates, tannins, and reducing sugars. [Table 1]

Table 1: secondary metabolites found in Ixora coccinea flowers

Phytochemicals / secondary metabolites	Result
Alkaloids	+
Glycosides	+
Tannins	+
Flavonoids	+
Saponins	+
Steroids	+
Carbohydrates	+

Antioxidant Study:

Total Phenolic Content:

In terms of gallic acid comparable, the total phenolic content (mg/gm) concentration was determined using the gallic acid curve standard equation, y= 0.0069x+0.0673. The overall phenolic content, expressed in terms of gallic acid, is 20.18 µg GAE (Gallic Acid Equivalent) /mg of extract.

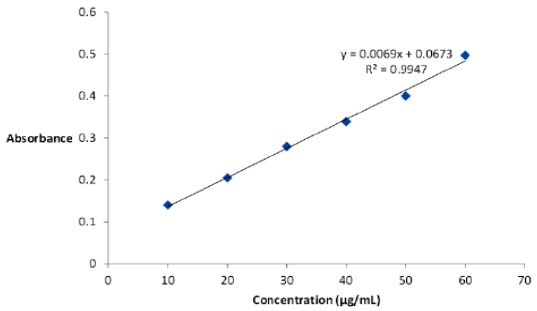

Fig 2: Gallic acid standard curve for the study of total phenolic content

Total flavonoid content:

In terms of quercetin acid equivalent, the total flavonoid content (mg/gm) concentration was determined using the quercetin acid standard deviation equation, Y= 0.031x+0.0159.

In terms of quercetin acid, the total flavonoid concentration is 21.49 µg QAE (Quercetin Equivalent)/mg of extract.

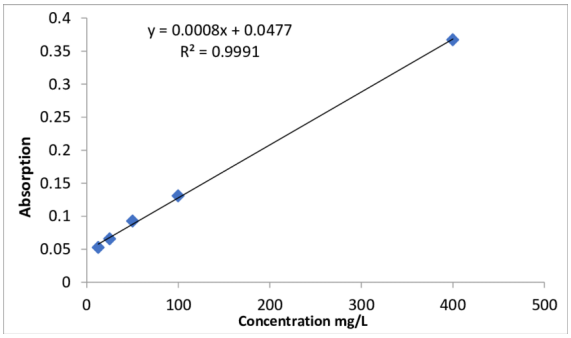

Fig 3: Quercetin standard curve for study of total flavonoid content

Anti-microbial study:

Minimum Inhibitory Concentration (MIC) Study:

The study discovered the minimum quantity of extract required to halt the microorganism's growth. [Tables 2 and 3].

Table 2: Microbial growth at various concentration of flower extract

Concentration (mg/ml)	15	25 (Minimum Inhibitory Concentration)	50	75	100
Staphylococcus aureus	+	-	-	-	-
Pseudomonas aeruginosa	+	-	-	-	-
Zone of inhibition	**0**	**0.7**	**1.2**	**1.8**	**2**

Table 3: Microbial growth at various concentration of flower extract

Concentration (mg/ml)	**25**	**50 (Minimum Inhibitory Concentration)**	**75**	**100**
Aspergillus niger	+	-	-	-
Zone of inhibition	**0**	**0.3**	**0.9**	**1.1**

(+): Bacterial Growth Visible **(-): Bacterial Growth inhibited**

Anti- Inflammatory study:
Effect on protein denaturation:

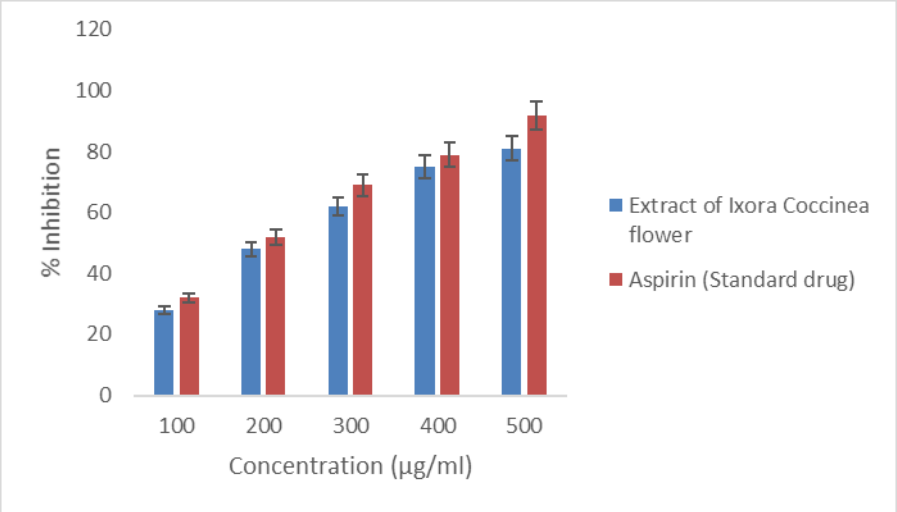

Fig 4: Comparison of inhibition of protein denaturation between extract of flower of *Ixora coccinea* and standard drug aspirin

The study of anti-inflammatory activity by protein denaturation method of the extract of *Ixora coccinea* has compared with the standard drug aspirin has shown IC_{50} value is 242 µg/ml where aspirin has shown 192 µg/ml

Proteinase Inhibitory Activity:

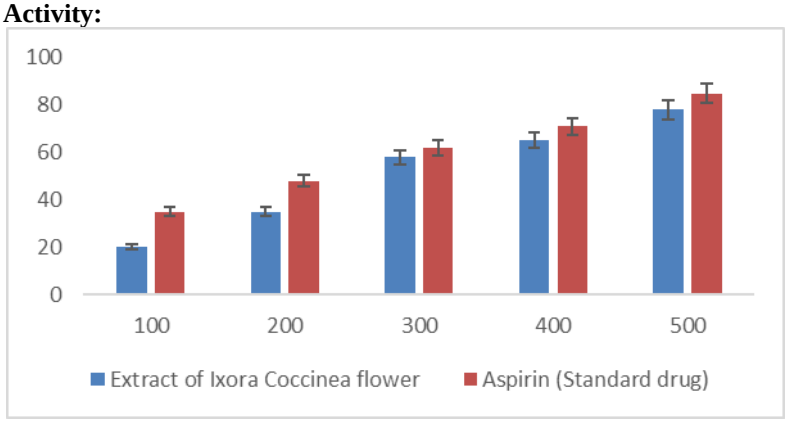

Fig 5: Comparison of inhibition of Proteinase Inhibitory Activity between extract of flower of *Ixora coccinea* and standard drug aspirin

The study of anti-inflammatory activity by proteinase inhibitory method of the extract of *Ixora coccinea* has compared with the standard drug aspirin has shown IC_{50} value is 259 µg/ml where aspirin has shown 241 µg/ml

Hypotonicity-induced hemolysis:

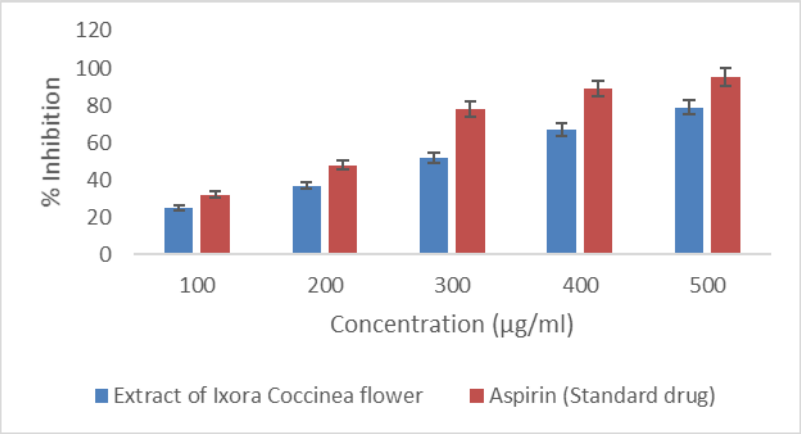

Fig 6: Comparison of inhibition hypotonicity-induced hemolysis between extract of flower of *Ixora coccinea* and standard drug aspirin

The study of anti-inflammatory activity by proteinase inhibitory method of the extract of *Ixora coccinea* has compared with the standard drug aspirin has shown IC_{50} value is 288 µg/ml where aspirin has shown 192 µg/ml

Heat-Induced Hemolysis:

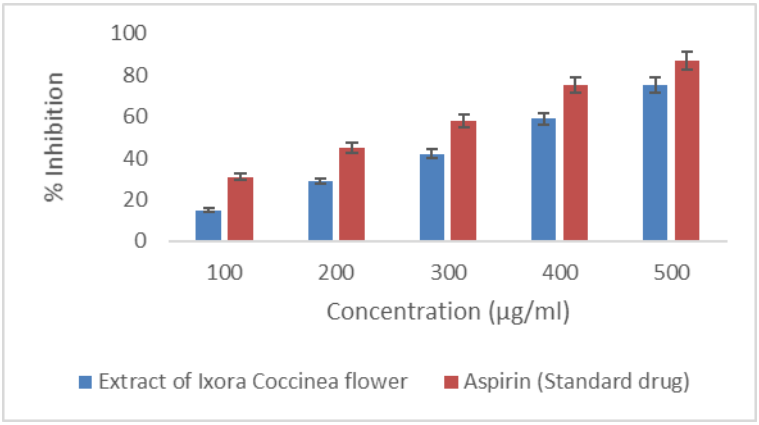

Fig 7: Comparison of inhibition heat-induced hemolysis between extract of flower of *Ixora coccinea* and standard drug aspirin

The study of anti-inflammatory activity by proteinase inhibitory method of the extract of *Ixora coccinea* has compared with the standard drug aspirin has shown IC_{50} value is 357 µg/ml where aspirin has shown 258 µg/ml

Discussion and conclusion:

The existence of several bioactive chemicals and their possible health advantages were probably discovered by the in vitro experiments carried out on the methanolic flower extract of *Ixora coccinea.* The phytochemical screening would have identified the classes of compounds present, such as alkaloids, flavonoids, phenols, etc. [Table 1], which are well-known for having antibacterial, anti-inflammatory, and antioxidant qualities. The phytochemical screening such as Total Phenolic was 20.18 µg GAE (Gallic Acid Equivalent) /mg of extract and Total flavonoid content was 21.49 µg QAE (Quercetin Equivalent)/mg of extract [Figure 2 & 3]. The presence of such good amount phenolic and flavonoid compounds is always a promising outcome to combat the free radicle damage. The anti-microbial activity of the extract on *Staphylococcus aureus* and *Pseudomonas aeruginosa* have shown the MIC (Minimum Inhibitory concentration) at the concentration 25 mg/ml with a zone of inhibition 0.7 cm and the anti-fungal activity is showing the MIC at 50 mg/ml on *Aspergillus niger* [Table 2 & 3]. Having anti-bacterial along with anti-fungal activity both together can promise to have a broad spectrum anti-microbial activity which can also suggest that the extract can give the protection against various infectious disease like throat infection, skin infection, urinary tract infection etc. There were various anti-inflammatory studies performed to see the anti-inflammatory potential of the extract. The methods which have been utilized here were heat-induced haemolysis, hypotonicity induced haemolysis, protein denaturation assay, proteinase inhibitory effect. Each of the method have shown the inhibitory concentration 50% (IC50) at 357, 288, 242, 259 µg/ml respectively [Figure 4,5,6 & 7]. The result was compared with the aspirin which is proven marketed anti-inflammatory drug. It has shown the dose dependent increase of % inhibition of inflammation similar to aspirin. Inflammation is the one of the main causes of various degenerative disorders such as arthritis, dementia, alzheimer's disease, diabetes mellitus and so on. An extract having such a strong anti-inflammatory potential is a proof to future research that this drug can be tested for multiple degenerative disorders.

To conclude, it can easily be said that the extract of flower of *Ixora coccinea* has it's potential as an anti-oxidant, anti-microbial and anti-inflammatory agent. In future, the flower of *Ixora coccinea* can be utilized for various in-vivo studies to accurately identify and estimate the various effects.

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