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In vitro study on antioxidant and antibacterial effects of polyphenol rich fraction from flower of *Ixora coccinea*

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Abstract
The present work was designed to search the possible antioxidant and antibacterial effect of the polyphenol rich fraction from flower of *Ixora coccinea* (Rubiaceae). The extract were tested against pathogenic bacteria, *Escherichia coli, Pseudomonas Klebsiella pneumoniae,* and *Staphylococcus aureus* by disc difusion methods. The content of phenolic compounds, flavones, and flavonols was measured. The antioxidant activities were evaluated using four assays: total antioxidant capacity, ABTS, lipid peroxidation, superoxide and nitric oxide scavenging. Antibacterial activity was studied using the agar disk diffusion method was determined. Results obtained showed a positive correlation between the antioxidant content of polyphenol rich fraction and the antibacterial capacity, polyphenol rich fraction were the maximum efficient against Gram-positive and Gram-negative bacteria. It is concluded that pollens can be a good source of bioactive molecules that exhibit potent antioxidant effects and strong antibacterial activities.

Keywords: antioxidant; antibacterial; polyphenol rich fraction; *Ixora coccinea*

Introduction
Medicinal plants are one of the main resources of therapeutic agents. Indeed, 80% of the world's population uses plants in health care. Recently, the interest in the search for natural substances has considerably increased, because these substances are intended for use in foods or drugs to replace synthetic compounds, which are limited because of their side effects (Oliveira *et al*., 2012) [15]. There is an increasing interest in using medicinal plants and their phytoconstituents as natural sources because of their well-known ability to scavenge free radicals. Effectively, plants are sources of natural antioxidants compounds that possess various pharmacological properties with little or no side effects and protect human health from many diseases (1998). The prevention of oxidative stress related disease by medicinal plant products is delaying the oxidation of lipids or other molecules by inhibiting the propagation of oxidative chain reactions (Piao *et al*., 20009) [16].
Traditional herbal medicines have shaped the basis of human health care, and further research will improve global health. Presently, about 80% of the world population (according to WHO) uses herbal drugs for some aspects of primary health care. Globally, the use of medicinal plants predates antibiotics and other contemporary drugs (Aslam and Ahmad, 2016) [1]. In addition, many culinary herbs and spices were tested for their biological activities in Alzheimer's disease management and other chronic diseases (Jivad and Rabiei, 2014) [8]. The natural antioxidant defence mechanism, in all human and other aerobic organisms, prevents the oxidative damage. Since the natural antioxidant defence mechanism is inadequate on its own, the nutritional consumption of antioxidants is suggested (Tangvarasittichai, 2015) [21]. Currently, synthetic antioxidants are replaced by natural antioxidants as the former are reported to have carcinogenic properties. Plants are the primary source of natural antioxidant molecules capable of eliminating or neutralizing the harmful reactive oxygen species (ROS). The natural antioxidants are also free-radical scavengers, reduction agents, pro-oxidant metal complexes, singlet oxygen quenchers, etc. They can also safeguard the human body from free radicals and delay the progression of many chronic illnesses (such as cancer, heart disease, and stroke) and boost the plasma's antioxidant ability and prevent lipid oxidative rancidity in foods. Bacteria are available naturally in the adjoining environment; therefore they can easily reach food during harvesting, slaughtering, processing, and packaging. These bacteria can survive under adverse conditions used in the food preservation such as low temperature, modified atmosphere packaging, vacuum packaging, as well as resist conventional pasteurization (Sade *et al*., 2017) [18]. Thus, there is a considerable concern among consumers regarding the risk of using synthetic additives for human health, that led to decrease the use of these chemicals in food preservation (Kalem *et al*., 2017) [9]. Therefore, new eco-friendly methodologies are required to reduce the growth of pathogenic bacteria and prolong the shelf-life of food products, without using chemical preservatives. Recently, many researchers investigated the possible

utilization of some plant extracts as effective natural preservatives (Suppakul *et al*., 2016) [19]. Traditionally, the crude extracts of different parts of herbal plants, including root, stem, flower, fruit, and twigs, were widely used for treatments of some human diseases. Medicinal plants contain several phytochemicals such as flavonoids, alkaloids, tannins, and terpenoids, which possess antimicrobial and antioxidant properties (Talib and Mahasneh, 2010) [20]. *Ixora coccinea* Linn, (Rubiaceae) commonly known as jungle of geranium and red ixora, is an evergreen shrub found throughout India. Depending on the medical condition, the flowers, leaves, roots, and the stem are used to treat various ailments in the Indian traditional system of medicine, the Ayurveda, and also in various folk medicines. The fruits, when fully ripe, are used as a dietary source. Phytochemical studies indicate that the plant contains important phytochemicals such as lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, lecocyanadin, anthocyanins, proanthocyanidins, glycosides of kaempferol and quercetin. Pharmacological studies suggest that the plant possesses antioxidative, antibacterial, gastroprotective, hepatoprotective, antidiarrhoeal, antinociceptive, antimutagenic, antineoplastic and chemopreventive effects, thus lending scientific support to the plant's ethnomedicinal uses.

Materials and Methods

Collection and preparation of plant extracts

The flower of *Ixora coccinea* were collected from Government Siddha Medical College Herbal Garden, Arumbakkam, Chennai-600 106. The plants authenticated (Voucher Number. GSMC/MB-393/21) identification done by Dr. S. Sankaranarayanan, Asst. professor, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. The flower of *Ixora coccinea* (100 g) was crushed using food masher then extracted with sterile water twice at room temperature for 1 h. The extracts partition with ethyl acetate and concentrated under rotary evaporator at 55°C. After evaporation, freeze dryer was applied to remove the moisture from extracts. The dry extracts were stored at −20°C until analysis. The measurements in this study were done in triplicate and the biological activities of food samples were determined at a concentration of 25, 50, 75 and 100 from 1 mg/mL.

Phytochemical Analysis

The aqueous flower extract of *Ixora coccinea* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973) [7].

Thin Layer Chromatography Profile

The polyphenol rich fraction from flower of *Ixora coccinea* was loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of Petroleum ether, Chloroform and methanol (1:0.5:0.1, V/V/V) was used for the development of the exudates on silica gel plates silica gel 60 F_{254} (10x20 cm, 0.2mmlayer). Visible and the non-visible spot given and it is fluorescent with UV light at 360nm and 240nm.

ABTS Free Radical-Scavenging Activity

The ABTS free radical-scavenging activity of each sample was determined according to the method described by Loizzo *et al*. (2015) [11]. The ABTS radical cation was produced by reacting ABTS with potassium per sulfate. A mixture of ABTS (2 mM) and potassium persulfate (70 mM) was allowed to stand overnight at room temperature in the dark to form the radical cation ABTS, 16 h prior to use. The ABTS solution was then diluted with 80% methanol to obtain an absorbance of at 734 nm. 100 μL of appropriately diluted samples was added to 2 mL of ABTS solution and the absorbance was recorded at 734 nm after 1 min of incubation at room temperature.

Inhibition of Lipid Peroxidation

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al*. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer and different concentrations of polyphenol rich fraction from flower of *Ixora coccinea* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Superoxide Radical Scavenging Assay

This assay was based on the capacity of the polyphenol rich fraction from flower of *Ixora coccinea* to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM Ethylene diamine tetra acetic acid (EDTA), NBT (75 μM) and different concentration of extracts. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer.

Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

% Super oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of polyphenol rich fraction from flower of *Ixora coccinea*.

Nitric Oxide Radical Scavenging Activity

Nitric oxide scavenging ability of polyphenol rich fraction from flower of *Ixora coccinea* was measured according to the method described by Makhija *et al*. (2011) [12]. 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation:

% Nitric oxide radical scavenging capacity= $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of polyphenol rich fraction from flower of *Ixora coccinea*.

Culture Collection and Maintenance

The bacterial strains of *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa.* These standard strains were obtained from Microbial Type Culture Collection and gene bank (MTCC); Institute of Microbial Technology, Chandigarh, India. The stock culture was maintained on Mueller Hinton agar medium at 4 ◦C.

Antibacterial activity

The antibacterial activities of the polyphenol rich fraction were assayed using the disc diffusion method (Drago *et al.,* 1999). Bacteria were grown overnight on Mueller Hinton agar plates, five colonies were suspended in 5 ml of sterile saline (0.9%) and the bacterial population in the suspension was adjusted to ~$3x10^8$ CFU/ml. The different concentration of polyphenol rich fraction from flower of *Ixora coccinea* was introduced on to each disc and the control disc received only 7% ethanol. The plates were incubated at 37°C for 24 h and the inhibition zone was measured and calculated. The experiments were carried out in duplicate three times. The results (mean value, *n*=3) were recorded by measuring the zones of growth inhibition surrounding the discs.

Results and discussion

Phytochemical screening

The phytochemical screening from flower of *Ixora coccinea* were studied showed the presence of alkaloids, flavonoids, tannins, terpenoids, glycosides and phenols (Table -1). Recent interest in plant secondary metabolites has focused on their potential benefits to human health.

Table 1: Phytochemical screening of aqueous extract from flower of *Ixora coccinea*

Sl. No.	Phytochemical Constituents	Observation	Aqueous extract of flower of *Ixora coccinea*
1	Alkaloids		
	Dragendorff's Test	Orange / red precipitate	+
	Mayers test	Yellow or white precipitate	+
2.	Flavonoids		
	Alkalai Reagent	Intense yellow colour	+
	Lead acetate test	Precipitate formed	+
3.	Glycosides		
	Keller-Killiani test	Reddish brown colour ring formed	-
4.	Tannin -$FeCl_3$ test	Blue black coloration	-
5.	Saponins		
	Frothing test	Foam	+
6.	Terpenoids		
	Salkowski test	Dark reddish brown color in interface	-
7.	Polyphenols		
	Ferrozine test	Raddish blue	+
8.	Anthocyanin test Ammonia	Ammonia layer yellow in color	+

-- = Negative (absent); + = Positive (present)

TLC Profile

The polyphenol rich fraction from flower of *Ixora coccinea* loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of petroleum ether, chloroform and methanol in the ratio of 1:0.5:0.1 were efficient to extract the antidiabetic compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-2).

Table 2: TLC Rf Value of polyphenol rich fraction from flower of *Ixora coccinea*

S. No	UV 240 nm Rf value	UV 360 nm Rf value
1	-	0.86
2	0.67	0.67
3	0.48	0.48
4	0.27	0.27

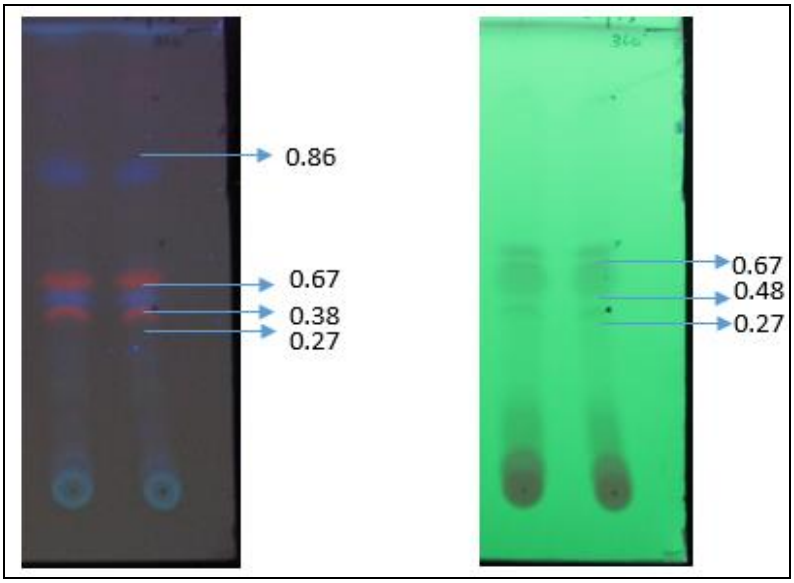

Fig 1: TLC plate viewed UV Light

ABTS Radical Assay

The antiradical activity of polyphenol rich fraction form flowers of *Ixora coccinea*, as the demonstrative of nutritional food source, were evaluated *in vitro* by ABTS assay, as well as by assessment of possible to discoloration of ABTS. Substantial changes were witnessed for the polyphenol rich fraction, as well as between the assays employed. In table-3 the outcomes of antioxidant activity gained for tested samples, as well as Vitamin-C used as standard are presented. It can be evidently understood that polyphenol rich fraction exhibited prominent antioxidant activity, expressively higher than Vitamin-C. Nevertheless, in current experimental presented that these activities were mainly due to presence of polyphenol compounds. Several reports have indicated that free radical scavenging activity is greatly influenced by the phenolic contents of the sample, flavonoid, and the presence of hydroxycinnamic acids such as caffeic acid phenyl ester (Hajji *et al*., 2010) [6].

Table 3: Free radical-scavenging ability using ABTS assay of polyphenol rich fraction of *Ixora coccinea*

Different concentration of extract	ABTS radical activity	
	Polyphenol rich fraction of *Ixora coccinea*	Standard Vitamin-C
25 µl/ml	22.34±1.46	19.34±2.36
50 µl/ml	41.32±1.89	37.32±1.46
75 µl/ml	57.32±2.36	53.32±2.36
100 µl/ml	76.32±1.89	71.32±1.46
EC_{50} value	62.34	69.32

[a]Results are expressed as percentage inhibit of ABTS ability with respect to control. Each value represents the mean+SD of three experiments

Inhibition of Lipid Peroxidation Activity

Inhibition of lipid peroxidation experimental was used as substrate egg yolk for free radical facilitated lipid peroxidation, which is a non-enzymatic method. Polyphenol rich fraction form flowers of *I. coccinea* inhibited the lipid peroxidation brought by ferrous sulfate in egg yolk homogenates. Determined inhibition was documented in polyphenol rich fraction form flowers of *I. coccinea* 67.32% with EC_{50} value 76.34 µl/ml and lowermost inhibition percentage ascorbic acid 65.34% with EC_{50} 81.23 (Table-4). Normally, the mechanism of

polyphenol compounds for neutralizing lipid free radicals and stopping breakdown of hydro peroxides into free radicals (Gulçin, 2012).

Table 4: Inhibition of lipid peroxidation activity of polyphenol rich fraction form flowers of *I. coccinea*

Different concentration of extract	Inhibition percentage of Lipid peroxidation	
	Polyphenol rich fraction form flowers of *I. coccinea*	Standard Vitamin-C
25 µl/ml	16.32±2.34	14.32±0.23
50 µl/ml	31.24±1.48	27.32±1.78
75 µl/ml	48.32±2.36	45.32±2.45
100 µl/ml	67.32±1.45	65.34±1.23
EC_{50} value	76.34	81.23

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Superoxide Scavenging Activity

Polyphenol rich fraction form flowers of *I. coccinea* displayed authoritative scavenging activity for superoxide radicals in a concentration dependent development than positive control. Polyphenol rich fraction form flowers of *I. coccinea* exhibited maximum radical activity in the percentage of 78.32% with EC_{50} value 60.32 µl/ml when related to positive control 72.34% with EC_{50} Value 67.23 µl/ml (Table-5). These superoxide radicals are enormously toxic and may be twisted either through xanthine activity or through mitochondrial reaction. Superoxide radical is recognized to be an identical damaging species to cellular components as an ancestor of additional oversensitive specie. Many studies mentioned effective free radical inhibition of the phenolic-rich extraction (Li *et al*., 2011). However, the polyphenol content was not in accordance with the hydroxyl scavenging capacity.

Table 5: Superoxide scavenging activity of polyphenol rich fraction form flowers of *I. coccinea*

Different concentration of extract	Percentage of Superoxide scavenging activity	
	Polyphenol rich fraction form flowers of *I. coccinea*	Standard Vitamin-C
25 µl/ml	21.34±1.34	19.34±1.79
50 µl/ml	44.32±2.34	41.32±2.89
75 µl/ml	59.32±1.47	56.32±1.78
100 µl/ml	78.32±1.23	72.34±0.36
EC_{50} value	60.32	67.23

[a] Results are expressed as percentage of Superoxide scavenging activity with respect to control. Each value represents the mean+SD of three

Nitric Oxide Radical Scavenging

Polyphenol rich fraction form flowers of *I. coccinea* indicated a strong nitric oxide scavenging ability which was equivalent to the standards ascorbic acid. The EC_{50} value (63.21 µl/ml) of polyphenol rich fraction form flowers of *I. coccinea* was less than ascorbic acid (68.32 µl/ml). Percentage of nitric oxide radical scavenging activity polyphenol rich fraction form flowers of *I. coccinea* and standards were presented in Table-6. In the present outcome, nitrite was formed by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by polyphenol rich fraction. Imperative scavenging activity may be due to the antioxidant property of polyphenol, compounds present in flowers of *I. coccinea*, which contest with oxygen to respond with nitric oxide, prominent to less production of nitric oxide (Tylor *et al*., 1999) [22].

Table 6: Nitric oxide radical scavenging assay of polyphenol rich fraction form flowers of *I. coccinea*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Polyphenol rich fraction form flowers of *I. coccinea*	Standard Vitamin-C
25 µl/ml	21.36±2.36	19.34±1.37
50 µl/ml	36.32±1.79	31.24±2.34
75 µl/ml	54.32±1.47	51.24±1.79
100 µl/ml	77.56±2.36	72.34±0.89
EC_{50} value	63.21	68.32

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

Antibacterial Activity

The antibacterial activity of the polyphenol rich fraction form flowers of *I. coccinea* was evaluated against *Escherichia coli, Pseudomonas Klebsiella pneumoniae,* and *Staphylococcus aureus* bacteria. The antibacterial activity of the extracts was assessed by determining disc diffusion values (Table-7). The results revealed that polyphenol rich fraction form flowers of *I. coccinea* potently inhibited *E. coli,* and *S. aureus*. The polyphenol rich fraction was more effective than against *S. aureus*. Phenolic compounds and can act as antimicrobial agents via several different mechanisms, including, which can result in cell destruction, and attenuation of pathogenicity (Dias *et al*., 2018).

Table 7: The antibacterial activity of the polyphenol rich fraction form flowers of *I. coccinea* by disc diffusion method

Pathogenic organism	Different concentrations Crude extract (µl/ml)			
	25 µl/ml	**50 µl/ml**	**75 µl/ml**	**100 µl/ ml**
Staphylococcus aureus	9.3±0.2	11.3±1.2	14.1±0.4	16.3±1.3
Escherichia coli	8.2±2.5	10.3±1.5	13.6±1.3	15.4±0.5
Pseudomonas aeruginosa	7.8±1.3	8.6±0.8	11.3±1.6	13.7±1.8
Enterococcus faecalis	6.7±0.9	7.9±2.4	10.7±1.4	12.3±2.3

*The inhibitory Zone size measured included the 6.0 mm size of the well by means of caliper. All the assays were duplicated, and the mean values were recorded.

Conclusion

We attempted to explore the diverse phytochemical efficacy of polyphenol rich fraction form flowers *Ixora coccinea* against several diseases and oxidative stress, by evaluating the antioxidant and antibacterial activities of its polyphenol rich fraction form flowers. Furthermore, to the best of our knowledge, this is the first comprehensive study of the antioxidant and antibacterial potential of the polyphenol rich fraction form flowers. In addition, the TPCs were higher in the polyphenol rich fraction, and these results were in agreement with the percentage radical inhibition results, which were higher for the polyphenol rich fraction.

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