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Evaluation of *in vitro* antioxidant and Antityrosinase activities of *Ixora coccinea* Linn. Roots

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Abstract
Ixora coccinea Linn. Commonly known as 'vetchi' is a flowering shrub of the Rubiaceae family used in the treatment of various ailments in the Ayurvedic system of medicines. This research aimed to study the qualitative phytochemistry and to determine the antioxidant and antityrosinase activities of *Ixora coccinea* roots. Phytochemical analysis indicated the presence of various plant bioactive metabolites in *Ixora coccinea* roots. Different solvent extracts were tested for their in vitro antioxidant potential using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and ferric ion-reducing power assay. The total phenolic content (TPC) of the extracts was determined by spectrophotometric methods. Methanolic and aqueous extracts of the roots were evaluated for antityrosinase activity by inhibitory potency of mushroom tyrosinase. The methanolic extracts of *Ixora coccinea* roots possessed the highest DPPH radical scavenging activity with an IC_{50} value of 26.88µg/ml compared with aqueous and ethyl acetate extracts. The estimation of total phenolic content revealed that methanolic extract has the maximum phenolic content and exhibited the highest ferric reducing power compared with the other two extracts. The methanolic extract of *Ixora coccinea* roots showed a comparable antioxidant activity with that of standard ascorbic acid and also exhibited potent tyrosinase inhibition with an IC_{50}value of 120.03µg/ml using Kojic acid as a reference standard. The current study showed that the methanolic extract of *Ixora coccinea* roots have superior skin whitening and antioxidant activities and could be used as a potential source of natural ingredient for formulating cosmetic products.

Keywords: Antioxidant activity, DPPH free radical, IC_{50} value, Antityrosinase, *Ixora coccinea* Linn. Roots, total phenolics

Introduction
Medicinal plants play an important role in human life to combat diseases since time immemorial. Nowadays in the world, there is a turn to return towards the use of herbal products and to adopt a natural way of life. According to the World Health Organization, about 65%–80% of the world's population living in developing countries depends primarily on plants for their health. Herbal medicines are rich in phenolic compounds, flavonoids, alkaloids, tannins, etc. used in the treatment of various degenerative ailments (Rex *et al.*, 2018) [1]. Plant-derived secondary metabolites have provided a variety of lead compounds, which serve as templates for developing new drugs.

Oxidative stress is the result of the imbalance between pro-oxidants and antioxidants in an organism, and it is significant in the pathogenesis of several degenerative disorders, such as arthritis, Alzheimer's, cancer, and cardiovascular diseases. Oxidative stress will be caused by either excess generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or an imbalance in antioxidant/oxidant production in the body. In case of excessive regeneration of free radicals within the body, the endogenous antioxidant system will not suffice for quenching all those ROS and RNS. This failure leads to oxidative stress which can only be prevented by taking a sufficient number of exogenous antioxidants from diet or supplements.

Human skin, the largest organ of the body forms a remarkable protective barrier against the external environment, helping to regulate temperature and fluid balance, keeping out harmful microbes and shielding against sunlight (Svobodova *et al.*, 2006) [2]. The exposure of the skin to environmental factors such as UV light, chemicals, and pollutants enhances the generation of reactive oxygen species in the cells. Oxidative stress caused by excessive reactive species (RS) is associated with skin aging and hyperpigmentation. In addition, abnormal production or distribution of melanin is the cause of various dermatological disorders such as melasma,

lentigines, age spots, and post-inflammatory hyperpigmentation (Yamaguchi *et al.,* 2007) [3]. In addition, excessive ROS results in the overexpression of collagenase, a protease that degrades collagen which can result in photoaging and wrinkling of the skin (Lopez-Camarillo *et al.*, 2012) [4]. The collagen fibers and elastic fibers present in the dermal tissue of the skin are modified or damaged, which is also characterized by wrinkles and atypical pigmentation (Masaki, 2010) [5].

Tyrosinase (EC 1.14.18.1) is the rate-limiting enzyme involved in melanin synthesis. Melanin is the main pigment that gives the skin its color. Melanin provides a beneficial effect in preventing skin damage under normal conditions by preventing UV damage and removing reactive oxygen species. Although the human body is well protected by endogenous defense systems, their effectiveness is limited in some situations (Rabiskova *et al.*, 2009) [6]. Melanin synthesis in melanocytes is accompanied by the generation of hydrogen peroxide, which if inappropriately processed, can lead to the formation of hydroxyl radicals and other ROS (Ana Sofia *et al*.,2015) [7]. ROS scavengers or inhibitors, such as antioxidants reduce hyper-pigmentation (Yamakoshi *et al.,* 2003) [8]. Many studies have revealed that herbal cosmetics are more effective inhibitors of melanogenesis which are safe, non-nontoxic, and cost-effective (Hong-Xin Cui *et al.,* 2018) [9]. A deficient anti-oxidative defense system, such as a disturbed redox balance and weak anti-oxidant enzyme activity, exacerbates skin aging and hyperpigmentation (Yokosava *et al.,* 2007) [10]. To treat hyperpigmentation-related problems, chemical cosmetics claim to have skin-whitening properties, which act by inhibiting tyrosinase in the melanin pathway. But it often poses adverse effects to the skin namely allergic contact dermatitis which has been reported with the tyrosinase inhibitor kojic acid (Cheun-Bin *et al.,* 2006) [11]. Therefore, searching for natural antioxidants that inhibit melanogenesis has been an important strategy in the development of skin medications and cosmetics. Various phenolic compounds from natural sources have been reported as melanogenic inhibitors (Bitis *et al.,* 2010) [12]. Herbal cosmetics are claimed to have efficacy and intrinsic acceptability due to routine use in daily life and circumvent the side effects which are commonly seen in synthetic products.

Ixora coccinea (or jungle geranium, the flame of the woods, and jungle flame) is a species of flowering plant in the Rubiaceae family. It is a beautiful flowering shrub native to Asia, with several medicinal properties. *Ixora coccinea Linn.* Commonly known as 'Thechi' in Malayalam or 'vetchi' in Ayurveda. It is a dense, multi-branched evergreen shrub, commonly 4–6 ft (1.2–2 m) in height, but capable of reaching up to 12 ft (3.6 m) high. The glossy, leathery, oblong leaves are about 4 in (10 cm) long, with entire margins, and are carried in opposite pairs or whorled on the stems. Fruits are globose fleshy and have 2-seeded berries. The fruits, when fully ripe, are used as a dietary source.

Ixora coccinea (I. Coccinea) showed, hepatoprotective (Baliga and Kurian 2012) [13], chemoprotective (Latha *et al.*, 2004) [14], antimicrobial (Sharma and Smita, 2010) [15], antioxidant and anti-nociceptive (Ratna sooriya *et al.*, 2005) [16], anti-mitotic, anti-inflammatory, cardioprotective, antiulcer, anthelmintic, antiasthmatic, hypolipidemic and hypoglycemic activities (Versiani *et al.,* 2012) [17]. From ancient times different plant parts have been used in the treatment of diarrhea, dysentery, leucorrhea, dysmenorrhea, hemoptysis, and catarrhal bronchitis (Banerjee *et al.,* 2011) [18]. *Ixora coccinea* showed strong reducing power and total antioxidant capacity (Moni Rani Saha *et al.,* 2009) [19]. The leaves yield flavonoids, kaempferol, quercetin, anthocyanidins, phenolic acids, and ferulic acid. Phytochemical studies have shown that the major compounds present in *I. coccinea* are lupeol, oleic acid, linoleic acid, ursolic acid, oleanolic acid, stearic acid, and sitosterol (Prabhu. and Yasmeen, 2011) [20]. Ixora coccinea is rich in phytoconstituents such as alkaloids, glycosides, flavonoids, tannins, saponins, steroids, and terpenoids (Sneha *et al.,* 2018) [21]. The present study was carried out to evaluate the antioxidant and antityrosinase activities of Ixora coccinea roots in various solvents. The screening methods are phenol estimations, antioxidant activity using 2, 2-diphenyl picryl hydroxyl (DPPH) radical scavenging, ferric reducing power, and antityrosinase activity by inhibitory potency of mushroom tyrosinase.

Materials and Methods

Plant material and preparation of extracts

The roots of *I. coccinea* Linn. Were collected from the fields of Kerala, identified, and authenticated. The roots were washed with water, shade dried, and grind into fine powder. For the extraction of active constituents of the plant material, the final uniform powder was utilized. Methanol and ethyl acetate were used for the extraction of the coarse powder of roots of *I. coccinea* (100g) in a Soxhlet apparatus. The extracts were filtered through Whatman filter paper and the filtrate obtained was evaporated to dryness on a vacuum rotary evaporator to yield the extract for further study.

Preparation of aqueous extract from roots

A cold maceration process extracted the dried powdered material with water. The solvent was removed and the resulting semisolid mass was dried in a desiccator for further use. Three extracts viz; Methanol extract (ICME), Aqueous extract (ICAQ), Ethyl acetate (ICEA) of *I. coccinea* roots were prepared. Dried extracts of *I. coccinea* roots were stored in bottles and kept in the refrigerator for further experimental analysis.

Preliminary Phytochemical screening

For preliminary phytochemical analysis, the freshly prepared crude methanolic extracts of roots were tested for the presence or absence of phytoconstituents such as alkaloids, tannins, flavonoids, phytosterols, saponins, quinones, phenols, and glycosides by using standard phytochemical procedures (Das K *et al.,*2010) [22].

Determination of total phenolics

Total phenolic contents were estimated using Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965) [23]. Gallic acid was used as the standard. The concentration of extracts solution was 10 mg/10 ml. From this solution, 1ml was taken and diluted to 10ml with the same solvent and different concentrations were taken in different test tubes. The same procedure was used for the standard gallic acid. 1 ml of Folin–Ciocalteu reagent was added, the contents were mixed thoroughly and 5 minutes later 4 ml of 20% sodium carbonate was added. The mixture was allowed to stand for 30 min with intermittent shaking. The absorbance of the blue color that developed was read at 765 nm. The total phenol content was expressed in terms of Gallic acid equivalent (mg/g).

Antioxidant assays: The following assays were performed to evaluate the plant material's antioxidant efficacy.

DPPH radical scavenging assay

The free radical scavenging capacity of the methanolic aqueous, ethyl acetate extracts of roots was determined using DPPH method. It was measured by a decrease in absorbance at 516 nm of a solution of coloured DPPH in methanol brought about by the sample (Ravishankar *et al.*, 2002) [24]. A stock solution of DPPH (1.3 mg/mL in methanol) was prepared. The concentration of extracts solution was 10 mg/10 ml. From this solution, 1ml was taken into test tubes and diluted with the same solvent to 10 ml. This is the stock solution. From the stock solution 0.10, 0.15, 0.25, 0.50, and 0.60 ml were taken in different test tubes, whose concentration was then 10, 15, 25, 50, and 60 µg/ml, respectively. Freshly prepared DPPH solution 75 µl was added to each of these test tubes containing methanolic, aqueous, and ethyl acetate extracts and kept in dark for 30 min, the absorbance was taken at 517 nm using a spectrophotometer. IC_{50} was calculated from% inhibition. Ascorbic acid was used as a reference standard. A control sample was prepared to contain the same volume without any extract and reference ascorbic acid; 95% methanol was used as blank and % scavenging of the DPPH free radical was measured using the following equation.

% inhibition = {(A control – A sample)/(A control)} ×100

A control = absorbance of DPPH alone

A sample = absorbance of DPPH along with different concentrations of extracts

Ferric Reducing power assay

The Fe3+–reducing power of the extract was determined by the method of Oyaizu (1986) [25] with a slight modification. Different concentrations (10, 25, 50, 75,100 µg/mL) of the extract were mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6). After incubation, 2.5 mL of TCA (10%) was added to terminate the reaction and centrifuged at 3000 rpm for 10 minutes. The upper portion of the solution (2.5 ml) was measured at 700nm against a blank using a UV-Visible spectrophotometer after 30minutes. Increased absorbance of the reaction mixture indicates an increase in reducing power Ascorbic acid at various concentrations (10-100 µg/ml) was used as standard. Increased absorbance of the reaction mixture indicated increased reducing power.

Antityrosinase assay or Skin whitening assay

Mushroom tyrosinase was used for the bioassay (Ye *et al.*, 2009) [26] since the mode of inhibition depends on the structure of both the substrate and inhibitor, L- DOPA was used as the substrate in this experiment. Therefore, the inhibitors discussed in this paper are inhibitors of diphenolase activity of mushroom tyrosinase, and their effect on the enzyme was determined by spectrophotometry, based on dopachrome formation at 475nm. All the samples were first dissolved in dimethyl sulfoxide (DMSO) and used for the experiment at 30 times dilution. L-DOPA solution (0.87ml, 4.5mM) was mixed with 0.9ml of 0.1M phosphate buffer (pH6.8) and incubated at 30ºC for 5minutes. Then 0.9ml of various concentrations of extracts, followed by 0.03ml of the aqueous solution of mushroom tyrosinase (4000 units) was added to the mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475nm (30 ºC), corresponding to the formation of dopachrome, for 25minutes at 1minute intervals. Controls, without inhibitor but containing 3.3% DMSO, were routinely determined. The percent inhibition of the enzyme by the active compounds was calculated as follows; inhibition (%) = [(A control- A sample)]/A control] x 100.

Statistical evaluation was done using SPSS- 20 packages one-way analysis of variance (ANOVA)). The difference was considered to be significant at $P< 0.05$. The results are an average of 6 replications and are represented as mean ± SEM.

Results and Discussions

Preliminary phytochemical screening

Methanolic extracts of *I. coccinea* roots showed the presence of alkaloids, phenols, flavonoids, glycosides, tannins, saponins, terpenoids glycosides, quinones, and steroids as shown in Table 1.

Table 1: Phytochemical analysis of various extracts of *I. coccinea*.

Compounds	Extracts		
	ICME	ICAQ	ICEA
Alkaloids	+	+	-
Glycosides	+++	++	++
Flavonoids	+++	++	+
Phytosterols	+	_	+
Phenols	+++	++	+
Saponins	+	-	-
Tannins	+++	++	_
Sterols	+	-	+
Quinones	++	+	+

+++ = large amount ++ = medium amount + = small amount - = absent.

Yield & Total phenolic content (TPC) from various extracts of *I. Coccinea*

Three extracts namely; Methanol (ICME), Aqueous (ICAQ), and Ethyl acetate (ICEA) extracts of *I. coccinea* roots were prepared. Table 2 shows the yield and total phenolic content of the root extracts obtained. Determination of total phenolic content of the root extracts of *I. Coccinea* was done by using Folin– Ciocalteu colorimetric method. This reagent is a mixture of phosphotungstic and phosphomolybdic acids. It is reduced during the oxidation of phenols into a mixture of blue oxides of tungsten and molybdenum. The color produced, whose absorption maximum is between 700 and 750 nm, is proportional to the total phenols present in plant extracts. The total phenolic contents were reported as mg gallic acid equivalent per gram of dry extract. The TPC values of methanolic, aqueous, and ethyl acetate extracts were 34.58±0.38mg/g,22.21±0.14mg/g, and 18.3±0.13 mg/g respectively. The methanolic extracts of *I. coccinea* roots possessed significantly higher levels of phenolics when compared with aqueous and ethyl acetate extracts.

Table 2: Yield and total phenolic content from various extracts of *I. coccinea.*

Extracts	Yield (g/kg dry weight)	Total Phenolic Content (mg/g dry weight)
ICME	45±0.28[a]	34.58±0.38[a]
ICAQ	28.5±0.18	22.21±0.14
ICEA	14.5±0.08	18.3±0.13

Results are expressed as mean± SEM. (n= 6). a $P< 0.05$

Antioxidant activity

DPPH radical scavenging assay

Free radical scavenging activity of methanol, aqueous, and ethyl acetate extracts of *I. coccinea* roots was evaluated by the DPPH method**.** The percentage inhibition of the test extracts increases with the increase in the concentration of the sample as shown in Table 3 & Figure 2. Ascorbic acid at a

concentration of 10 µg/ml exhibited a percentage inhibition of 52.74% and for a concentration of 60 µg/ml exhibited a percentage inhibition of 99.86%. The IC_{50} value of ascorbic acid was 7.08 µg/ml and for *I. coccinea* extracts were observed as 26.44µg/mL for methanolic extract, 45.17 µg/ml for aqueous extract, and 58.13 µg/ml for ethyl acetate extract respectively. From the results, it is observed that the methanolic extracts of *I. coccinea* roots possessed the highest DPPH radical scavenging activity compared with aqueous and ethyl acetate extracts.

Table 3: Inhibition of DPPH by various extracts of *I. coccinea*

Concentration (µg/ml)	% Inhibition			
	ICME	ICAQ	ICEA	Ascorbic acid
10	30.68±0.38	18.11±0.12	8.48±0.06	52.74±0.26
15	39.69±0.33	22.15±0.46	12.63±0.09	62.33±0.18
25	49.05±0.27	26.32±0.28	22.61±0.23	78.57±0.31
50	70.64±0.11	51.23±0.26	40.99±0.27	98.79±0.41
60	90.42±0.43	68.24±0.32	52.92±.29	99.86±0.40
IC_{50}	26.44	45.17	58.13	7.08

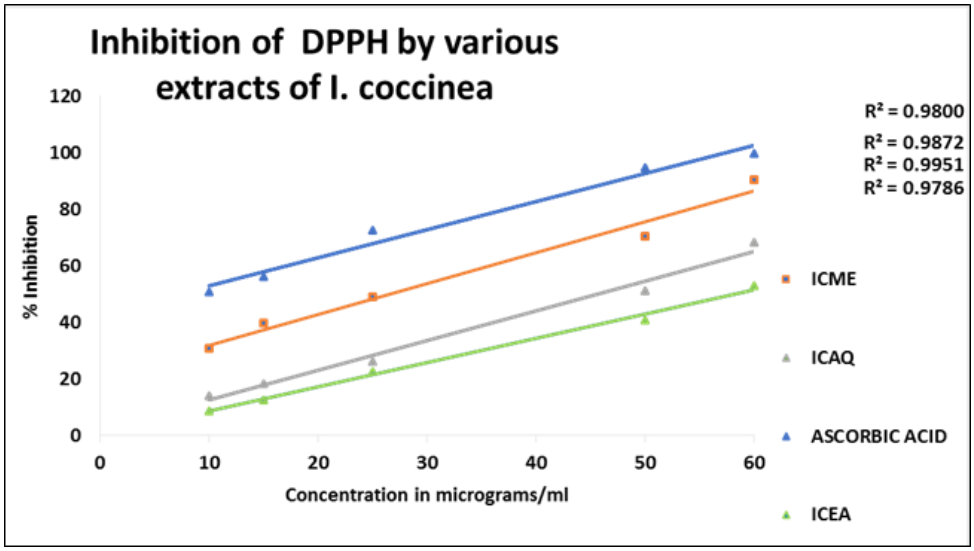

Fig 2: DPPH free radical scavenging activity (percentage inhibition vs concentration)

Graph for Ascorbic acid and extracts of *I. coccinea*

Ferric Reducing power assay

The reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form a ferric–ferrous complex that has an absorption maximum at 700 nm. The reducing power of the test extracts increases with the increase in the concentration of the sample as shown in Table 4 &Figure 3. The reducing power shows good linear relationship in both standard Ascorbic acid (R^2 = 0.9926) and ICME (0.9746), ICAQ (R^2 =9842) and ICEA (R^2 = 0.9876) extracts. From the results, it is observed that the methanolic extracts of *I. coccinea* roots. Exhibited the highest ferric reducing power compared with aqueous and ethyl acetate extracts.

Table 4: Absorbance of various concentrations of standard (ascorbic acid) and *I. coccinea* extracts in ferric reducing power

Concentration (µg/ml)	Absorbance			
	ICME	ICAQ	ICEA	Ascorbic acid
10	0.065±0.009	0.052±0.003	0.045±0.002	0.09±0.001
25	0.083±0.09	0.065±0.008	0.058±0.005	0.124±0.004
50	0.169±0.22	0.121±0.012	0.109±0.003	0.239±0.051
75	0.235±0.48	0.181±0.02	0.15±0.03	0.35±0.004
100	0.272±0.50	0.222±0.03	0.201±0.21	0.467±0.03

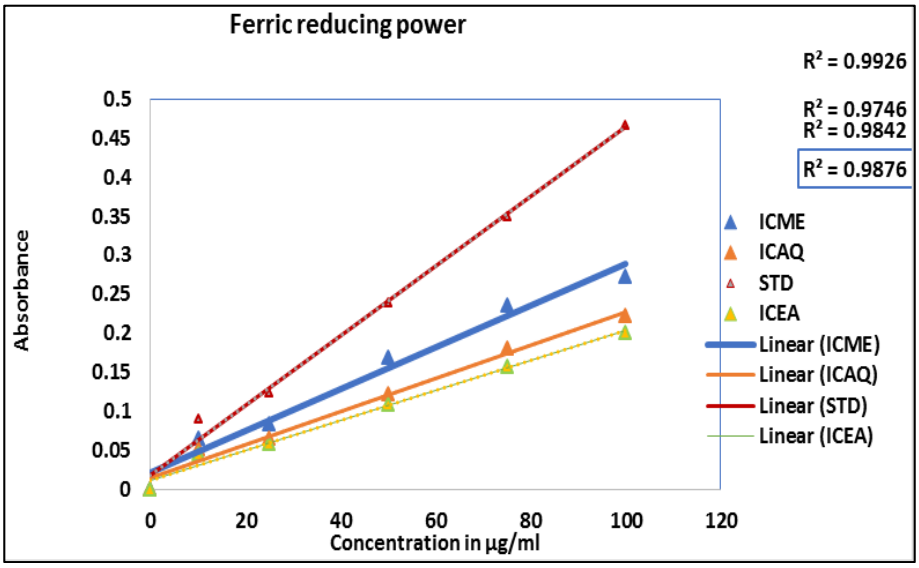

Fig 3: Effect of Methanol and aqueous extracts on reducing power

Antityrosinase assay

Anti-tyrosinase activity of Methanolic and aqueous extracts of *I. coccinea* roots was evaluated by inhibitory potency of mushroom tyrosinase. In the present study, The IC_{50} values were found to be 120.03µg/ml & 265.25µg/ml for methanolic and aqueous extracts respectively. The methanolic extract at a concentration of 250 µg/mL exhibited a percentage inhibition of 96.99±0.35% and for aqueous extract 50.48±0.28% as shown in, Table 5 & figure 4. The methanolic extracts of *I. coccinea* roots showed tyrosinase inhibition in a dose-dependent manner, compared with Kojic acid, a synthetic reference slandered. From the results, it is observed that the methanolic extracts of *I. coccinea* roots possessed the highest tyrosinase inhibitory potency as compared with aqueous extract.

Table 5: Inhibition of tyrosinase by extracts of *I. coccinea.*

Plant extracts			Kojic acid	
Concentration (µg/ml)	% Inhibition		Concentration(µg/ml)	%Inhibition
	ICME	ICAQ		
50	20.62±0.23	12.58±0.18	1.56	29.50±0.18
100	34.69±0.26	18.44±0.19	3.12	38.21±0.19
150	60.44±0.29	28.14±0.19	6.25	50.53±0.24
200	70.45±0.31	34.48±0.20	12.5	72.22±0.28
250	96.99±0.35	50.48±0.28	25	90.45±0.41
IC 50	120.03	265.25	-	7.26

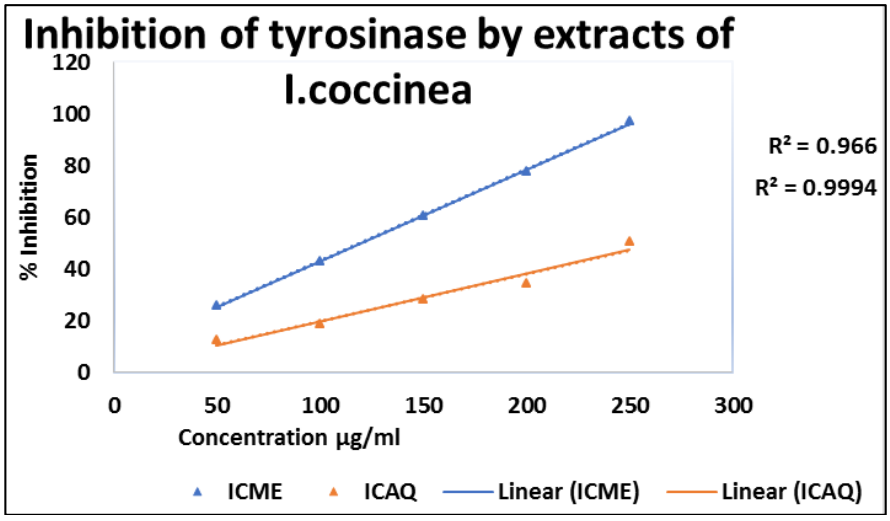

Fig 4: Tyrosinase Inhibitory activity (% Inhibition vs Concentration) graph for extracts of *I. coccinea.*

The results obtained indicate that methanolic extracts of *I. coccinea* roots have significant antioxidant and antityrosinase activities. A positive linear correlation was found between TPC and the free radical scavenging/ion-reducing activity of the extracts. It has been reported that plants with high total phenolic components which include phenols, tannins, etc. have exhibited excellent antioxidant effects, (Ramanuj Rauniyar *et al.*, 2014) [27]. The phenolic extracts protect the skin by scavenging the ROS, reducing the ROS reactivity, absorbing the UV light, inhibiting oxidation, and suppressing

the catalytic activity of tyrosinase. This reduces the risk of wrinkle formation and protects the skin from aging. (Azila Abdul Karim *et al*., 2014) [28]. Previous studies have shown that compounds with high antioxidant and radical scavenging activities exhibit significant antityrosinase activities and can be used to develop skin-whitening and cosmetic formulations (Nithya *et al*., 2011; Fatiha *et al*., 2015.) [29, 30]. Here in this study, the antioxidant and antityrosinase activities of *I. coccinea* root extracts can be endorsed due to the presence of higher amounts of phenolics. The maximum inhibition potential of methanolic extracts from *I. coccinea* roots indicated that the active principle is highly soluble in methanol and evaluation needs to be carried out with animal models to explore the active component responsible for pharmacological activity.

Conclusion

It is evident from this study that methanolic extracts from *I. coccinea* roots have significant antioxidant potential as well as strong antityrosinase activity. The high content of phenolic compounds appears to be responsible for these activities, comparable with commercial standards. This work provides new insights that will contribute to the commercial application of *I. coccinea* as an economic natural source of bioactive molecules with potent antioxidant and anti-tyrosinase activities. The potential of this easily accessible source of natural antioxidants should be explored by the pharmaceutical and cosmetic industries as an alternative to synthetic chemicals.

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