APPLICATION OF NATURAL PRODUCTS IN COSMETICS: A STUDY OF IXORA COCCINEA EXTRACTS FOR THEIR ANTITYROSINASE AND ANTIOXIDANT ACTIVITIES

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Abstract
Non-toxic natural products employed in the formulation of cosmetics are of considerable interest. Recent efforts have focused on the identification of substances that inhibit tyrosinase activity (skin whitening properties) or suppress the formation of reactive oxygen species in skin cells (antiaging). In the present study, different parts of Ixora coccinea (IC) in various solvents were examined for antityrosinase and antioxidant activities. The methods for screening are based on tyrosinase inhibition potency using mushroom tyrosinase and antioxidant activity using 2, 2-diphenyl picryl hydrazyl (DPPH) radical scavenging, inhibition of lipid peroxidation, ferric reducing power and phenol estimation. Among the parts investigated for skin whitening activity, the flowers were found to possess the superior activity. The methanolic extract of bark had the highest inhibition of DPPH when compared to flowers and leaves. However, the antioxidant potential of leaves and flowers were also comparable with bark. The inhibition of lipid peroxidation & the phenolic content was maximum in the leaves when compared to bark and flowers. Bark exhibited the best ferric reducing power when compared to other parts. Thus, Ixora coccinea serves as a potential source of ingredient for formulating the cosmetic products.

Key words: Cosmetics, Ixora coccinea, antityrosinase, 2, 2-diphenyl picryl hydrazyl (DPPH) scavenging, ferric reducing power and lipid peroxidation

Introduction
Herbal therapy is becoming increasingly popular among the patients and physicians. Many herbal preparations are used for treating various ailments including those of the skin (Jurga et al., 2011). Plenty of aromatic, medicinal and other herbs contain chemical compounds demonstrating antityrosinase, antioxidant, antimicrobial and other functional properties. The herbs with such functional properties have been reported in the literature and the topical application of these functional ingredients has wide scope in cosmetic industry (Chih-chien et al., 2011).

Skin, the outermost organ of the body is being exposed to the external environment. The exposure of the skin to UV radiation results in the generation of free radicals via lipid peroxidation. These reactive oxygen species generated plays a causative role in aging and pigmentation of the skin (Masaki, 2010). Although, the human body is well safeguarded with endogenous defense systems, their efficiency is insufficient in some circumstances (Rabiskova et al., 2009). Hence, the supplementation of antioxidants for diminishing the oxidative damage is necessary (Barja, 2002). Antioxidants of natural origin are gaining increasing importance of treating such disorders associated with oxidative stress.

Melanin formation is the most important determinant of the color of the mammalian skin. It is the important pigment protecting our skin from UV damage by absorbing sunlight and removing reactive oxygen species. Upon exposure to UV radiation, melanogenesis is initiated by the enzyme tyrosinase resulting in skin darkening (Vamos, 1981). Tyrosinase, the rate limiting enzyme, catalyses the conversion of tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA) and further oxidizes it to dopaquinone, which is used for the ultimate formation of melanin (Xiang Lan et al., 2004). The formation of melanin in the human body is reduced by several mechanisms including antioxidant, direct...
Tyrosinase inhibitors of synthetic origin are used currently in blocking the pathway for melanin biosynthesis (Jin et al., 2009). But it often poses adverse effects to the skin namely allergic contact dermatitis which have been reported with the tyrosinase inhibitor kojic acid (Cheun-Bin et al., 2006). Thus, the search for non-toxic natural sources, with antityrosinase and antioxidant properties is highly desirable in the context of formulating cosmetic products.

Ixora coccinea Linn (Rubiaceae) is known as Jungle Geranium or vetchi in ayurveda (Neelamegam, 2011). It is an evergreen flowering shrub, native to the tropical regions of Asia. It has several medicinal properties-chemoprotective, cytotoxic anti-tumor, antimicrobial and antinociceptive activity. From ancient times different plant parts have been used in the treatment of diarrhea, dysentery, leucorrhoea, dysmenorrhoea, hemoptysis and catarrhal bronchitis (Banerjee et al., 2011). The leaves have shown antimicrobial, antinociceptive anti-inflammatory and antioxidant property (Nripendra et al., 2011). The leaves yield flavonoids, kaemferol, quercetin, anthrocyanidins, phenolic acids and ferulic acids (Yasmeen & Prabhu, 2011). The flower extract has been found to contain triterpenoids, tannins and flavonoids which infer certain properties upon them. The flowers are used externally to sores, chronic ulcers, scabies and some type of dermatitis (Mukesh & Smita, 2010). The present investigation was carried out to evaluate the skin whitening and antiaging properties of different parts of the plant.

Materials and Methods

Plant parts used
Leaves, flowers and bark of Ixora coccinea (IC) were obtained from the fields of Kerala.

Plant extract preparation
Various parts of Ixora coccinea were thoroughly washed with distilled water and dried under shade. The dried materials were ground separately into powder and used for experimentation. One gram of herbal powder was taken and ground with 4 ml of 70% ethanolic solution of the plant material in a vial with screw cap and then placed in a boiling water bath at 60°C for 60 minutes. The mixture was cooled to room temperature and centrifuged at 6000 rpm for 10 minutes. The supernatant was filtered and the filtrate was collected and used for the analysis.

Skin whitening assay

Antityrosinase assay (Lee et al., 2003)

Tyrosinase (Phenoloxidase activity) which catalyzes the transformation of L-tyrosine into L-DOPA by hydroxylation and further into O-dopaquinone by oxidation. Then, through a series of non-enzymatic reactions, O-dopaquinone is rapidly transformed into melanin, which is measured at 492 nm in a spectrophotometer. Each plant extract was assayed for tyrosinase inhibition by measuring its effect on tyrosinase activity using a 96-well reader. The reaction was carried out in a 50 mM potassium phosphate buffer (pH 6.8) containing 20 mM L-tyrosine and 312 U/mL mushroom tyrosinase at 30°C. The reaction mixture was pre-incubated for 10 min before adding the enzyme. The reaction mixture without the enzyme serves as blank. The reaction mixture with the corresponding solvents (without plant material) serves as control. The change of the absorbance at 492 nm was measured. The percent inhibition of tyrosinase was calculated as follows:

\[
\% \text{ inhibition of tyrosinase} = \left( \frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control}} \right) \times 100
\]

Antioxidant assays
The following assays were performed for evaluating the antioxidant efficacy of the plant material.

DPPH radical scavenging assay (Mensor et al., 2001)

DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available, commonly used, stable free radical, which is purple in colour. Antioxidant molecules when incubated, reacts with DPPH and converts it into di-phenyl hydrazine, which is yellow in colour. The degree of discoloration of purple to yellow was measured at 520 nm, which is a measure of scavenging potential of plant extracts. 5 µl of plant extract was added to 195 µl of DPPH solution (0.1mM DPPH in methanol) in a microtitre plate. The reaction mixture was incubated at 25°C for 10 minutes, after that the absorbance was measured at 520 nm. The DPPH with corresponding solvents (without plant material) serves as control. The methanol with respective plant extracts serves as blank. The DPPH radical scavenging activity of the plant extract was calculated as the percentage inhibition.

\[
\% \text{ Inhibition of DPPH radical} = \left( \frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control}} \right) \times 100
\]

Lipid peroxidation by Ferric thiocyanate method (Mistuda et al., 1996)

Cell membrane is more susceptible to free radicals that reacts rapidly with the unsaturated fatty acids (like linoleic acid and arachidonic acid) embedded in the membrane resulting in lipid peroxidation. In this assay, linoleic acid is used as the model system for measuring the levels of lipid peroxidation. This was used to determine the amount of peroxide formed during the lipid peroxidation, in which peroxide will react with ferrous chloride and form ferric ions. Ferric ions will then unite with ammonium thiocyanate and produce a ferric thiocyanate complex whose colour is measured at 500nm. A mixture containing 10 ml of 0.05 M-phosphate buffer (pH 7.0), 5.9ml of water, 0.1 ml of plant extract and 4 ml of 2.5% linoleic acid in absolute ethanol was placed in a vial with a screw cap and then placed
in a dark oven at 40 degree centigrade overnight. To 0.1ml of this incubation mixture, added 9.7ml of 75% ethanol and 0.1 ml of 0.02M ferrous chloride in 3.5% HCl. Add 0.1ml of 30% ammonium thiocyanate, precisely 3 minutes after the addition of ferrous chloride. The absorbance of the red colour was measured at 500nm. A mixture without the plant sample was used as the negative control. (Note: Instead of plant extract, use 0.1 ml of water/ethanol/ petroleum ether as control).

**Ferric reducing power assay**

The reducing antioxidant power of the plant extracts were determined by the method of Oyaizu (1986). Different concentrations of plant extracts in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of trichloroacetic acid (10%) was added to mixture, which was then centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%). The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer after 30 mins. Increased absorbance of the reaction mixture indicates increase in reducing power. The reducing power of the plant material was expressed in terms mg of gallic acid equivalents/g of plant material.

**Determination of total phenolics (Mallick and Singh, 1980)**

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650 nm. Pipetted out different aliquots (0.2 to 2 ml) into test tubes. Made up the volume in each tube to 3.0 ml with water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 minutes, added 2.0 ml of 20% sodium carbonate solution to each tube. Mixed thoroughly, placed the tubes in a boiling water bath for exactly 1 minute, cooled and measured the absorbance at 650nm against reagent blank. The amount of phenols present is expressed as mg catechol equivalents/g of plant material.

**Statistical analysis:**

Samples were analyzed in triplicate and the results were given as Mean ± S.D.

**Results and discussion**

**Tyrosinase inhibition assay**

The various parts of *IC* in different solvents were studied for their skin whitening property through tyrosinase inhibition (Fig 1). Methanolic extracts exhibited the highest inhibition of tyrosinase followed by aqueous and petroleum ether extracts in comparison to the solvents. Among the methanolic extracts of various parts of *IC*, flowers ranked first followed by bark and leaves. The aqueous extract of bark (49.35%) exhibited the greatest antityrosinase activity in comparison to the aqueous extract of various parts. Among the petroleum ether extracts of various parts studied, leaves exhibited the highest inhibition whereas flowers and bark possessed almost similar tyrosinase inhibiting potential. Among the parts tested, the methanolic extract of flowers (71.17%) has shown the superior skin whitening activity followed by methanolic extract of bark (67.53%).

The methanolic extract of leaves were further investigated for their concentration dependant tyrosinase inhibition (Fig 1.1). Various concentrations ranging from 1 µg to 1500 µg were studied for their antityrosinase activity. The activity was in a dose dependant manner with the IC₅₀ value of 501 µg.

**Figure 1.1** illustrates the concentration dependant inhibition of tyrosinase by *IC* leaves.

The methanolic extract of *IC* flowers were further studied for their dose dependant antityrosinase activity since it exhibits the greatest tyrosinase inhibition (Fig 1.2). The IC₅₀ value was found to be 264.52 µg.
Figure 1.2 depicts the concentration dependant inhibition of tyrosinase by IC flowers

The concentration required to produce 50% inhibition of tyrosinase for the methanolic extract of IC bark was found to be 490 µg (Fig 1.3).

Figure 1.3 represents the concentration dependant inhibition of tyrosinase by IC bark

DPPH scavenging assay

The DPPH scavenging potential of various parts of IC in various solvents was investigated. Figure 2 illustrates the DPPH scavenging ability of various solvent extracts of IC leaves. The methanolic extract of IC leaves (95.88%) showed the maximum inhibition of DPPH radical followed by aqueous extract (89.00%). The least inhibition of DPPH radical was observed in petroleum ether extract.

The methanolic extract of leaves was further studied for their concentration dependant inhibition of DPPH radical (Fig 2.1). The dose dependant pattern was observed for the methanolic extract of IC leaves with the IC$_{50}$ of 117.23 µg.

The DPPH scavenging ability of various solvent extracts of IC flowers was given in figure 3. Among the various solvent extracts tested for DPPH inhibition, methanolic extract (97.89%) showed the highest inhibition followed by aqueous (90.92%) and petroleum ether extracts.
The concentration dependant inhibition of DPPH radical was also observed in methanolic extract of flowers with the IC$_{50}$ value of 15.62 µg (Figure 3.1).

The DPPH scavenging activity of various solvent extracts of IC bark was shown in figure 4. The methanolic extract of bark (98.39%) possessed the maximum DPPH scavenging potential in comparison to other solvents. The dose dependant DPPH scavenging ability of methanolic extract of IC bark was studied. The 50% inhibition for the methanolic extract of bark was found to be 15.8 µg.

From the results it was found that DPPH scavenging activity was maximum and almost similar in the methanolic extract of flowers and bark in comparison to leaves. The second highest and almost similar activity was observed in aqueous extract of flowers and leaves. The least activity was noticed in the petroleum ether extracts of all the parts.

**Lipid peroxidation inhibition assay**

The various parts of IC in three different solvents were studied for their inhibition on linoleic acid oxidation (Fig 5). The aqueous and methanolic extract of leaves and flowers exhibited good inhibition on lipid peroxidation. The maximum inhibition was observed by the methanolic extract of leaves (70.88%) followed by methanolic extract of flowers (64.42%) and methanolic extract of bark (62.94%). The aqueous extract of leaves (62.48%) and flowers (62.64%) possessed almost similar inhibiting potential on lipid peroxidation. The petroleum ether extracts of IC exhibited the least inhibiting potential on lipid peroxidation.

**Ferric reducing power**

The ferric reducing ability of various parts of IC in different solvents is given in table 1. Among the parts investigated for ferric reducing power, methanolic extract of IC bark showed maximum reducing ability followed by methanolic extract of flowers and leaves. The order of
Table 1 represents the ferric reducing ability of various parts of *Ixora coccinea*.

<table>
<thead>
<tr>
<th><em>Ixora coccinea</em> parts</th>
<th>Solvents</th>
<th>Amount (mg of gallic acid equivalents/g of plant material)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>Aqueous</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>0.9</td>
</tr>
<tr>
<td>Flowers</td>
<td>Aqueous</td>
<td>11.8</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>0.3</td>
</tr>
<tr>
<td>Bark</td>
<td>Aqueous</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>Petroleum ether</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Reducing power in terms of solvents used for various parts of *IC* was methanol > aqueous > petroleum ether.

**Estimation of phenols**

The aqueous and methanolic extract of leaves possessed the maximum amounts of phenols when compared to flowers and bark. The methanolic extract of leaves and bark has shown the similar phenolic content when compared to their corresponding aqueous extracts. The methanolic extract of flowers exhibited the greatest phenolic content in comparison with their corresponding aqueous extract. The least amount of phenols was present in petroleum ether extracts (Fig 6). Positive correlations were observed between phenolic content and DPPH scavenging of *Ixora coccinea*.

**Conclusion**

From the findings, it was observed that the flowers of *Ixora coccinea* possessed the highest skin whitening potential in comparison to other parts. Bark exerted the superior DPPH scavenging ability and reducing power as compared to flowers and leaves. However, the antioxidant potential of leaves and flowers were comparable with that of the bark. The inhibition of lipid peroxidation and phenolic content of leaves were high in comparison with other parts.

All the parts of *Ixora coccinea* exerted good antityrosinase and antioxidant activities in methanol when compared to other solvent extracts of respective parts. This indicates that the compounds responsible for the above said activities are highly extractable in methanol. It can be inferred from the results of this study that *Ixora coccinea* is a potential candidate as the active ingredient of products for topical application as skin whitening and antiaging in cosmetic formulations.

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