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PHYTOCHEMICAL STUDIES OF *Ixora coccinea Linn*- AN ETHNOBOTANICAL PLANT FROM KARWAR DISTRICT

DAMLE S* AND SHARON K

Department of Life Sciences, Kishinchand Chellaram College, Dinshaw Waccha Road, Churchgate, Mumbai-400020, Maharashtra

***Corresponding Author Email ID: visaanika@gmail.com**

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ABSTRACT

Western Ghats, being one of the global hotspots of biodiversity, supports an enormous vegetal wealth [1]. It has nearly 5800 species of flowering plants which includes *Poaceae, Leguminasae, Orchidaceae, Acanthaceae, Euphorbiaceae, Asteraceae, Lamiaceae and Rubiaceae* [2]. Tribal people are the major stakeholders of the indigenous knowledge of uses of plants. Conserving their knowledge by documentation protects them from exploitation. In the survey conducted- Karwar in Karnataka was one of the study sites. *Ixora coccinea* Linn was one of the plants used by the village doctor (Vaidya) of the tribe. Qualitative analysis to detect the antimicrobial class of compounds in stem and leaf, were carried out followed by TLC and Quantification of Total flavonoids and Phenolics. The study reveals presence of certain antimicrobial class of compounds. The total phenolic content of stem and leaf was found to be 125.37mg/g and 124.22mg/g GAE phenolics respectively, whereas total flavonoid content was found to be 0.721mg/g and 5.981mg/ g QE respectively. The TLC results revealed a number of bands on the plate which was followed by Bioautography which showed zone of inhibition at 0.51 Rf value. This study therefore shows *Ixora coccinea* Linn to be a plant with potential bioactive components which can be of medicinal use.

Keywords: Antimicrobial activity, Bioautography, *Ixora* coccinea Linn, Karwar, TLC

INTRODUCTION

Western ghats is one of the eight world's "Hotspots" of biological diversity [1]. Plants have been used by man since long, not only for medicinal use, but also for food, shelter, clothing etc., A study of the relationship between man and plants is broadly termed as Ethnobotany. The major stakeholders of this knowledge are the Tribes, who live in the forests and make a living dependent on the trees and plants that surround them. Lessening of interaction between modern man and plants is due to the development of industries and deforestation. Therefore, there is a worry that the children and youth are not learning the knowledge, skills of handling and using indigenous plants [3]. A report of ICMR has generated a database on Ethnomedicinal plants of Western Ghats which reports 500 plants that can be used for medicinal purposes, belonged majorly to the families *Fabaceae, Euphorbiaceae, Lamiaceae, Asteraceae, Piperaceae* etc. [4]

Ixora coccinea Linn belongs to the family *Rubiaceae* and Order Gentianales. It is said to have antioxidant, antimicrobial and anti-inflammatory activities [5]. Every part of this plant was found to have a medicinal property, according to the literature. The ripe fruit of this plant was used as dietary source, according to a study conducted by Baliga & Kurian [6]. Chemical structures of some phytochemicals from it's leaves, roots and flower have been elucidated. The leaves of this plant shows the presence of Flavonoids (rutin), Lupeol and A- type proanthocyanidin trimer [7]. The flowers on the other hand shows the presence of cycloartenol esters, cyanidin, delphidine, quercetin, astringent principle, flavonoids, cyanidine- 3- rutinoside, leucocyanidine glycoside [8].The plant phenolics display a wide ranges of action from protection against pathogen attack, protection against UV rays to giving plant it's pigments [9]. Polyphenols have antioxidant activity which helps in preventing many oxidative stress related ailments [10]. Therefore, assessing the total polyphenol and flavonoid content can give us an insight to this plant's antioxidant properties. *Ixora coccinea* Linn is known to have anti-microbial and other pharmocological properties. The antimicrobial activity of the leaves was reported against Gram positive and Gram negaitve bacteria like *Escherichia coli* and *Staphylococcus aureus* etc., in different studies [11-13]. The current study was aimed at collection of *Ixora coccinea* Linn from Karwar district since it is one of the plants used by the tribe for medicinal purpose. The objective was to authenticate it and analyse the Phyotchemical constiuents and its Bioactivity which would give insights for its application in biopharmaceutical.

MATERIALS AND METHODS

1. Ethnobotanical Survey

In Karwar, a place called "Phaate" was targeted where there was a tribal settlement.

A Tribal doctor, lady Vaidya was contacted. She specializes in treating women with white discharge problems. She also possessed knowledge about plants that treated other ailments.

2. Sample preparation

The plant samples were collected on field wrapped in newspaper and were transported to the lab. The leaves, stem and flowers were separated and shade dried, after which they were oven dried at not more than 50^{o}C, till a constant weight was obtained. The different parts of the plant were ground and stored in dark bottles at a cool, dry place. For phytochemical test, dry powdered sample was used. For Thin Layer Chromatography analysis, 0.5g of the powdered sample was mixed with 5ml of AR grade Methanol and subjected to ultrasonication 3 times, for 15 minutes each. Following this, the sample was filtered using Whattman filter paper (pore size: 0.45micron) and the filtrate was collected in a glass stoppered test tube.

3. Phytochemical analysis- Qualitative tests

The dried powder was subjected to qualitative analysis to check for the presence of antimicrobial class of compounds. The tests were as follows [14]:

A. **Test for flavonoid (Alkaline reagent test)**

To the powder, add a few drops of NaOH. Formation of intense yellow color which turns colourless by addition of few drops of acetic acid indicates presence of flavonoids.

B. **Test for Phenolic compounds:**

To the powder, add few drops of 10% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compounds.

C. **Test for tannins: (Ferric chloride test)**

To the test solution, add a few drops of ferric chloride solution. An intense green, purple, blue/ black colour indicates presence of tannin.

D. **Test of terpenoids and steroids (Salkowski's test)**

Powder was treated with chloroform, followed by addition of few drops of concentrated sulphuric acid. Shake well and allow to stand. Appearance of red colour in the lower layer indicates presence of steroids. Formation of reddish brown interface after addition of concentrated sulphuric acid indicates presence of terpenoids.

E. **Test for alkaloids (Mayer's test)**

To the powder, add 1ml Mayer's reagent. Whitish- cream colored precipitate indicates the presence of alkaloids.

F. **Test for glycosides (Killer Killiani test)**

Powder is dissolved in 1% ferric sulphate made in 5% glacial acetic acid. Add one or two drops of concentrated Sulfuric acid. A blue colour indicates presence of glycosides.

4. Quantification Studies

A. Total Phenolics Content by Folin-Ciocalteau Method:

To determine the total phenolics content in the dried powdered sample, 0.5g of the dried powdered sample was macerated with 5ml of Methanol for 2 hours at RT on shaker after which the supernatant was collected for estimation. For standard Gallic acid calibration curve, a stock of 1mg/ml of Gallic acid was prepared in methanol which was further diluted to obtain different concentrations of gallic acid (50, 80,100,150,200,250,300 & 400 ug/ml). A volume of 0.5ml of test and standard was mixed with 2 ml of 1:10 diluted Folin ciocalteau reagent respectively, followed by 4ml of 7.5% sodium carbonate solution. It was mixed and incubated at RT for 30 minutes, following which its absorbance was measured at 765nm using Double beam UV Visible spectrophotometer [15-16].

B. Total Flavonoid content by $AlCl_3$ method:

To determine the total flavonoid content, Alumunium chloride method of estimation was followed. Different concentrations of Quercetin were used to obtain the standard calibration curve. 1mg/ml of Quercetin was made in Methanol which was further diluted to obtain the following concentrations- 6.25, 12.5, 25, 50, 80 & 100ug/ml. For experimental set (test), 0.5g of plant dried powder was subjected to cold maceration at 4°C for 16 hours in 70% Ethanol, following which, the supernatant was used for quantification. A volume of 0.5ml of test and control sample was mixed with 1.5ml of Methanol, 0.1ml of 10% Aluminium Chloride, 0.1ml of Potassium acetate and 2.8ml of Distilled water. The blanks for each tube was also prepared, in which 0.1ml 10%aluminium chloride was eliminated and replaced with 2.9ml of Distilled water. The reaction mixture was vortexed and absorbance was measured at 415nm using Double beam UV Visible Spectrophotometer [17-19].

5. Thin layer Chromatography

TLC plates Silica Gel 60F 254 were used to run Thin layer chromatography. 2µl and 5µl of 100mg/ml methanolic extract of stem and leaf was applied on the plates using Linomat- 5 applicator. The plates were then developed in Toluene: Chloroform: Ethanol (4:4:1) mobile phase till the solvent reached 70mm of the plate. The plates were dried using hair dryer, observed and scanned under white light and UV (254nm). The plate was derivatized using Natural Product reagent (NP reagent) and scanned under 356nm. This was followed by derivatization with

Anisaldehyde sulphuric acid reagent. The plates were scanned at 354nm and white light. The compounds are recorded as peaks on the chromatogram.

6. Bioautography

For this purpose, two Gram positive (*Staphylococcus aureus, Corynebacterium diptherea e*) and two Gram negative (*Escherichia coli, Salmonella typhi*) cultures were selected. The stem and leaf methanolic extracts (100mg/ml) were applied on four TLC silica gel 60F 254 plates and developed in Toluene: Chloroform: Ethanol (4:4:1) mobile phase. For positive control, 10ppm Ciprofloxacin antibiotic was applied at the application spot. The TLC plates were placed aseptically in sterile 6 inch petri plates. A 0.3ml of 0.1OD adjusted 18 hour old culture was added to 20ml sterile Mueller-Hinton molten butt and poured on the TLC plates for the respective cultures. The plates were incubated at 37^{o}C for 24 hours. Few drops of INT indicator (0.2mg/ml) was added to observe the actively growing culture and for any zone of inhibition on the plate. The zone on the plate which is seen as a white patch against a background of pink colour (growth) is the region where the antibacterial compound is present.

RESULTS:

1. Ethnobotanical survey

According to the information provided by the village doctor (vaidya Sita Gaonkar), of the plants surveyed, *Ixora coccinea* Linn was selected for further phytochemical investigation. The data obtained from the survey is shown in **Table I.**

2. Qualitative analysis: The results obtained from the different qualitative tests are represented in **Table II.**

3. Quantification Studies

A standard calibration graph of Gallic acid **(Figure 1)** was obtained, from which the concentration of phenolics from Stem and leaf sample of *Ixora coccinea* Linn was measured. The experiment was performed in triplicates and is reported as mg GAE/gram of plant extract. Standard calibration graph of Quercetin **(Figure 2)** was obtained from which the concentration of Flavonoids from the stem and leaf sample of *Ixora coccinea* Linn was measured.

The total phenolics and total flavonoid content of *Ixora coccinea* Linn from the standard graphs are tabulated in **Table III.**

4. Thin Layer Chromatography

A. Images of Leaf and Stem samples under different wavelengths **(Figure 3)**

B. Scan at different wavelengths

1. Post development under 254nm, without derivatization **(Figure 4)**. The respective chromatogram of Leaf and Stem sample, obtained when scanned under 254nm **(Figure 5).**

The Rf value and Area under peak for Stem sample and Leaf sample scan under 254nm was recorded. As observed from the graph, 9 peaks corresponding to 9 compounds and 8 peaks corresponding to 8 compounds in Stem and Leaf sample respectively were present. Of these compounds, some peaks were observed which had the same Rf in both the samples, that is **Rf= 0.51, 0.44, 0.81 and 0.97**. One compound present in **Stem at Rf= 0.21** was not present in leaf, hence the difference in the number of compounds present.

2. Derivatization with Natural Product reagent followed by anisaldehyde, under 366nm **(Figure 6).** The respective chromatogram of Leaf and Stem sample, obtained when scanned under 366nm **(Figure 7)**.

The Rf value and Area under peak for Stem sample and Leaf sample scan under 366nm, post derivatization was recorded. A total of 10 peaks corresponding to 10 compounds were seen to be present in both Stem and Leaf samples. Except two compounds, whose Rf values were **0.45 and 0.61** which seemed to be common in Stem & Leaf, all other compounds had differences in Rf values.

5. Bioautography Studies: Figure 8 shows developed plates after incubation with the respective organisms (*Corynebacterium diphthereae, Styphalococcus aureus*, *Escherichia coli & Salmonella typhi)* for 24hrs at 37^{o}C, followed by treating the plates with 0.2mg/ml INT indicator.

Zone of inhibition was observed on the plate incubated with *Corynebacterium dipthereae* only and not with other organisms. The Rf value of the Zone of inhibition observed was **0.51.**

Table I- Details of Ethnobotanical survey at Karwar, Karnataka

	Plant Local name	Medicinal Use	Botanical Name after Authentication	Common name
1	Pitkule	Roots of red and white variety is mixed with rice and water and made to drink and apply on boils	*Ixora coccinea* Linn	Flame of the woods

Table II- Qualitative Phytochemical test results of *Ixora coccinea* Linn

	Class of Compounds	Result- Leaf	Result- Stem
1	Flavonoids	+	+
2	Phenolic compounds	+	+
3	Tannins	+	-
4	Terpenoids	+	+
5	Steroids	+	+
6	Alkaloids	+	+
7	Glycosides	-	-

Key for Table II: +- Positive, -Negative

Table III- Results of the total phenolics and total flavonoid content in Stem and Leaf of *Ixora coccinea* Linn		
Sample	**Total phenolics content in mg/g of extract (in GAE)**	**Total flavonoids content in mg/ gram of extract (in QE)**
Stem	**125.377± 3.744781**	**0.721486±0.661**
Leaf	**124.2232± 1.575961**	**5.9816±0.0661**

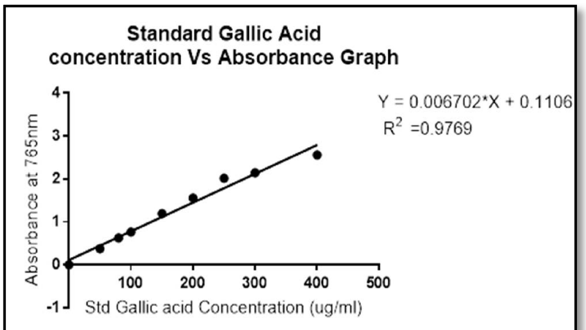


Figure 1: Standard Calibration Curve for Gallic acid

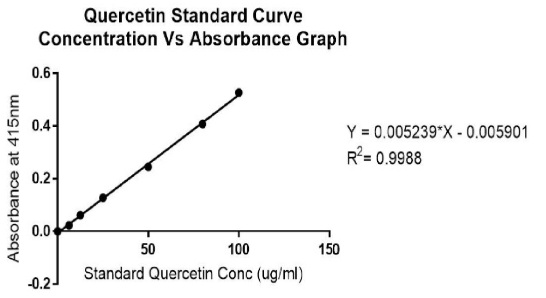
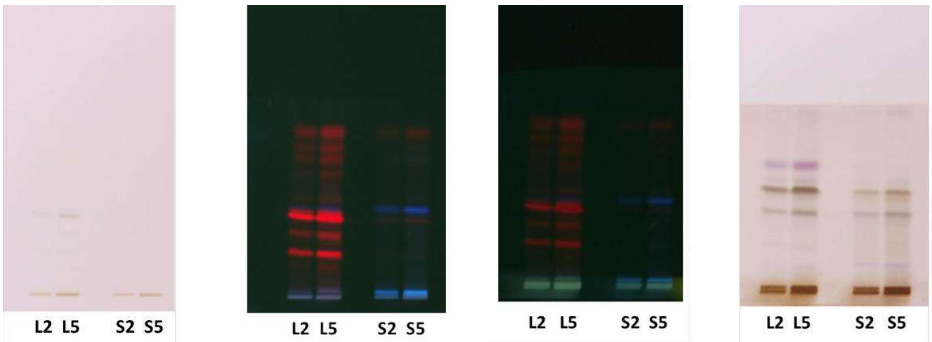


Figure2: Standard Calibration Curve for Quercitin



Post development, 540nm, no derivatization

Post development, 366nm, no derivatization

Post development, 366nm, Derivatization with Natural Product reagent

Post development, 540nm, Derivatization with Anisaldehyde reagent

Figure 3: Images of Leaf (L2- 2Microlt; L5- 5 Microlt) and Stem (S2- 2 Microlt; S5- 5 Microlt) samples under different wavelengths

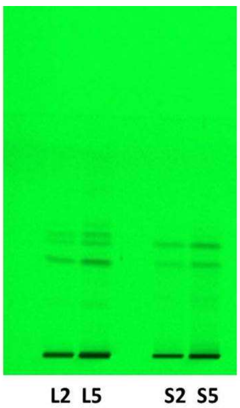


Figure 4: TLC Plate observed under 254nm after developing in Mobile phase

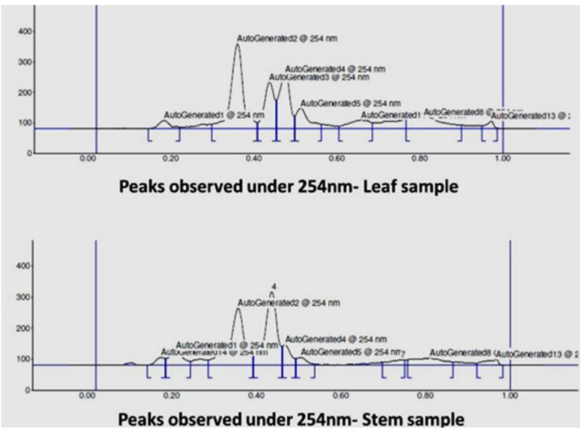


Figure 5: The respective chromatogram of Leaf and Stem sample, obtained when scanned under 254nm

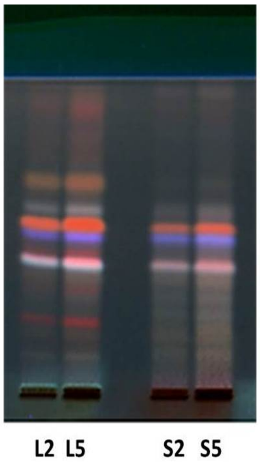


Figure 6: Derivatization with Natural Product reagent followed by anisaldehyde, under 366nm

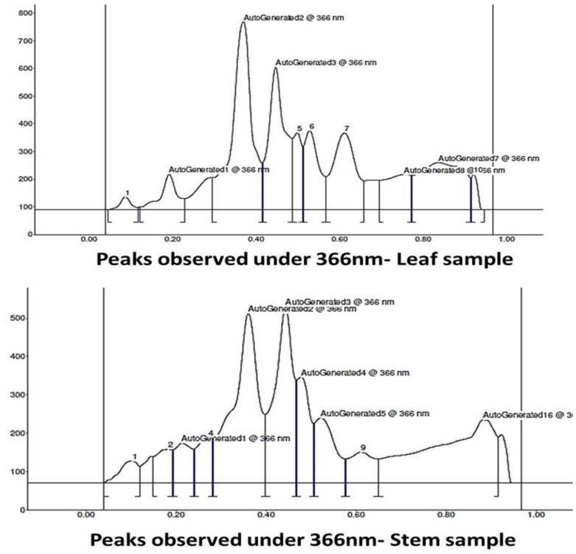


Figure 7: The respective chromatogram of Leaf and Stem sample, obtained when scanned under 366nm

*Corynebacterium diptherea*e *Styphalococcus aureus* *Escherichia coli* *Salmonella typhi*

Key to Figure 8: 1-*Ixora coccinea* LinnLeaf; 2- *Ixora coccinea* Linn Stem; +ve control- Ciprofloxacin (5ppm). 5Microlt of each was applied on TLC plates.

DISCUSSION:

The ethnobotanical survey revealed few plants with potential medicinal value. *Ixora coccinea* Linn was authenticated by St. Xavier's Blatter Herberium. Ethnobotanical surveys have been conducted in Karwar in the past. A study conducted by Bhandary and Chandrashekar in 2014 [20], amongst the Koraga, Malekudya and Halakki vokkaliga tribes, documented ethnomedicinal uses of 342 species of plants belonging to 34 families, which included herbs, climers, trees etc. Another study conducted by Harsha et al., in 2003[21] in Uttara Kannada district, documented the 52 herbal preparations from 31 plants belonging to 21 families. There is a scope to conduct more ethnobotanical surveys in Karwar, as there are many tribal settlements yet to be explored.

Ixora coccinea Linn have been studied extensively with respect to it's phytochemical composition, antioxidant activity, antimicrobial activity etc., The purpose of this study was to check if the plant from a different geographical location shows similar properties and bioactivity. The qualitative phytochemical analysis results reveal the presence of class of compounds which supposedly shows anti-microbial activity, except Glycosides. The results are in agreement with the previous literature regarding *Ixora coccinea* Linn.

The quantitative estimation studies reveal that the stem and leaf of this plant has 125.37 and 124.22mg/g GAE phenolics respectively. The amount of phenolics present in either part of the plant are almost same. According to literature, the leaves of *Ixora coccinea* Linn supposedly shows more phenolic content than the stem [22]. This maybe attributed to the difference in the geographical location of the plant. The total flavonoid content in stem and leaf was found to be 0.721 and 5.981mg/ g QE respectively. The leaf was found to contain more flavonoids as compared to the stem. This phenolic and flavonoid content may contribute towards the plant's antioxidant activity.

TLC fingerprint at different wavelengths and derivatizing agents reveals the presence of a number of compounds. Post development with mobile phase, the plates when exposed and scanned under 254nm UV light, showed the presence of nine fluorescent compounds in leaf and eight in stem as represented by the graphs. The compound present at Rf value 0.21 in leaf sample was not present in stem; whereas most of the compounds in stem and leaf match with respect to their Rf values, inferring that similar fluorescing compounds might be present in leaf and stem. Post derivitzation with Natural Product reagent and Anisaldehyde, ten compounds each was observed at 354nm as represented in the graphs. Most of the Rf values of the compounds observed in leaf does not match those in stem sample, inferring that different compounds were detected by anisaldehyde in Leaf and stem, unlike detection of UV fluorescence without derivatization. Anisaldehyde derivatizing agent is usually used for the detection of Lichen constituents, phenols, terpenes, sugars and steroids. This can be visualized as colored spots which are- Violet, blue, red, grey and green respectively. From the image after anisaldehyde treatment, Bands of Blue, red, grey and green were observed at 366nm and 545nm, confirming the presence of Phenols, Terpenes, Sugars and Steroids in both, leaf and stem sample[23]. The derivitized fingerprint with Natural Product reagent shows presence of flavonoids which is in agreement with the quantitative study performed. Therefore, TLC helps in initial detection of compounds in plant extract. This fingerprint can be used to authenticate herbal samples, which claim to have *Ixora coccinea* Linn extract.

Bioautography studies reveal the presence of bioactive compounds in Leaf as well as stem of *Ixora coccinea* Linn. The Rf value of the Zone of inhibition obtained was 0.51. This maybe the compound that was detected under 254nm, at 0.51 Rf. Compounds that fluorescence under UV light usually belong to Flavonoid class of compounds. Derivatization of the fingerprint with class of compounds derivatizing agents may reveal the identity of the class of compound. Further, this compound maybe isolated using preparative TLC and it's structure can be identified using NMR/ UV spectroscopy. Bioautography studies with *Ixora coccinea* Linn previously performed by Marimuthu et al., [24] showed Zone of inhibition with different organisms of different class of compound extracts of *Ixora coccinea* Linn. This study suggests that more than one compound may be responsible to show that zone of inhibition. Therefore, the leaf and stem samples can be developed in mobile phase specific for each class of compounds to confirm the previous studies.

CONCLUSION AND FUTURE PROSPECTS

The present study exhibits the phytochemical constituents and bioactivity of *Ixora coccinea Linn*. It shows the presence of class of compounds which is known to possess anti- microbial activity. TLC fingerprint reveals a number of compounds, as observed under 254nm and post derivatization with anisalydehyde at 366nm. Bioautography studies suggests that the plant has potential bioactive compounds which needs further investigation. *Ixora coccinea* Linn was selected as a study plant from the Ethnobotanical survey conducted in Karwar. A comparative study of *Ixora coccinea* Linn, sampled from a city/ metropolitan area can be conducted and any differences in the above property maybe noted. The TLC fingerprint can be used to authenticate herbal samples containing *Ixora coccinea* Linn. The study also further aims at proposing a sustainable value chain model in collaboration with pharmaceutical companies to ameliorate the poverty level of the tribes, who are seldom rewarded for their traditional knowledge.

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