PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTIOXIDANT, ANTIMICROBIAL ACTIVITIES OF *IXORA COCCINEA* L.

By

Akshaya. S

(Reg. No: 19PBO003)

The Thesis Submitted to

Department of Botany

Avinashilingam Institute for Home Science and Higher Education For Women

Coimbatore-641043

In partial fulfilment of the degree of

MASTER OF SCIENCE

MAY 2021

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Signature of the Head

Dr. M.K.Nisha

Signature of the Supervisor

Dr. S. Amutha

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INTRODUCTION

Plants play a key role in life; it is impossible without the use of plant and plant products. The plants are mainly used as food and medicine. There are a wide range of medicinal plants available across the world, which have capacity to cure diseases (Pooja *et al.*, 2019).

The use of Allopathy medicines gradually decreasing because of its side effects. There are about 80% people all over the world prefers herbal and traditional medicines (Ancilla *et al.,* 2016). The modern medicines are mostly derived from plants. The herbal medicine has no side effects and it has ability to decrease the harmful effects of modern medicine (Ratty and Das, 1988) so it is also taken as supplementary treatment to reduce the side effects of synthetic medicine (Haji *et al*., 2020).

The increasing number of diseases in present world urges the researches and scientists to search for new drugs to compete with the diseases. It is the main reason for development and conservation of the medicinal plants (Lachimanan *et al.,* 2012).

Major ancient system of medicines are Ayurveda, Siddha, Unani and folk medicines. In India Ayurveda is generally following system of medicine from ancient time. In Ayurveda, the plants used have biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and less toxicity (Riddhi *et al*., 2017).

India has a rich source of medicinal plants. Different parts of plants such as root, leaves, stem fruits, seeds are used for treatment of several diseases (Philomina *et al.,* 2011). The Western ghats of India is one of the hotspots of biological diversity. The tribal people live in the forests of western ghats have the knowledge of traditional medicinal plants and their using methods. Arises of industrialization and deforestation shortens the interaction between man and plants, so the new generation has no knowledge of handling and using of indigenous plants. The ICMR (Indian Council of Medical Research) generated a report of database shows that there are 500 ethnomedicinal plants used for medicinal purposes (Damle and Sharon, 2017).

From ancient period the medicinal plants are used as anti-microbial agents. The chemical constituents present in the different species of *Ixora* of Rubiaceae family is used as ethnobotanical plant in many countries of the world. The leaves of this plants are reported to have anticancer, anti-inflammatory, anti-diarrheal, anti-asthmatic, anti-ulcer, hypotensive, anti-

viral and antinociceptive activities. Traditionally the leaves, flowers, root and frits are used for various health issues (Saleha *et al*., 2015).

Traditionally the flowers of *Ixora* are used as anti-inflammatory, aromatic, anti-pyretic drug and use in extensive thirst and fatigue in Siddha medicine. All these properties are due to the polyphenolic compound which fights against free radicals and cure diseases (Sankhadip *et al*., 2013).

Ixora coccinea L. is the plant species of rubiaceae family. Rubiaceae is a family which consists of 630 genera and about 1300 species are found tropical and warm regions of the world. Many plants species of Rubiaceae exhibits antimalarial, antimicrobial, antihypertension, antidiabetic, antioxidant and anti-inflammatory activities (Sirigiri *et al*., 2015).

Ixora coccinea is also known as jungle of geranium, jungle flame, flame of the woods. It is a flowering shrub native to southern India, Bangladesh and Sri Lanka. It is a dense glabrous, multi branched ever green shrub, which commonly attains the height about 4-6 feet, but there is chance to reaching up to 12 feet height. It is grown in rounded form. The leaves are glossy, leathery, and oblong and the length about 10cm have entire margins, the leaves are arranged opposite whorled arrangement on the stem. The flowers are tubulous brilliant red color in dense rounded clusters (Mani Maran *et al*., 2011).

The roots are traditionally used as an astringent, antiseptic, stomachic, sedative etc., and also used for treat diarrhea, dysentery, gonorrhea, loss of appetite, hiccups, fever, sores and chronic ulcers. The flowers are used in hemoptysis, leucorrhea, dysentery, dysmenorrhea and catarrhal bronchitis. The decoction of flowers or bark is used as lotion for dilemmas of eye (Khare, 2007). The presence of various phytochemicals such as vitamins, minerals, phenolic acids, tannins, lignins, flavonoids, terpenoids, alkaloids, amines, coumarins, betalins and secondary metabolites (Akanji *et al*., 2018).

The biomolecules present in the body such as proteins, lipids, lipoproteins and DNA are reacting with the Reactive Oxygen Species (ROS) causes various disorders such as diabetes, cancer, genotoxicity and inflammation. The antioxidants help to regulate those oxidation reactions. There are several studies are done in different parts of *Ixora coccinea.* The flower contains anthocyanins, lupeol, cycloartenol esters, ursolic acid and oleanolic acid. The root contains octadecadienoic acid, myristic acid, quercetrin and the leaves contain plastaquinones (Surana *et al*., 2013).

Free radicals and active oxygen species are the main reason for numerous diseases. Antioxidants plays major role in fight against the free radicals and prevent diseases such as diabetes, cardio vascular disorders, brain dysfunction, aging and pre mature greying of hair (Ravikumar *et al.*, 2013).

In developing countries, the microbial infections are most common especially wound infections due to the poor sanitation. Commonly the wound infection causing microbes are *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* and *Klebsiella pneumoniae.* Commercially there are plenty of anti-microbials are available, but the side effects and drug resistance are caused due to aimless use of antimicrobial drugs. To conquer this problem, the recent researches are going on in herbal field for discovery of plant derived anti microbials (Nagaraj *et al*., 2011).

1.1 AIM AND OBJECTIVES

Ixora coccinea is a medicinal plant, which is used as medicine for a wide range of health problems. So, this plant is selected for study the phytochemicals present in it and the *In vitro* antioxidant and antimicrobial properties.

- To analyse the secondary metabolites by preliminary phytochemical screening of various leaf extracts of *Ixora coccinea* L.
- To analyse the secondary metabolites by Thin Layer Chromatography of various leaf extracts of *Ixora coccinea* L.
- To investigate the antioxidant property of methanolic leaf extract of *Ixora coccinea* L. by DPPH radical scavenging method.
- To investigate the antimicrobial property of methanolic leaf extract of *Ixora coccinea* L. by agar well-diffusion method.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 INTRODUCTION

Ixora coccinea L. is commonly called jungle of geranium is a well-known ornamental plant. It is used in traditional medicine as an astringent and to treat dysentery, sores, fever and chronic ulcers. The leaves, flowers and root and fruit are used for curing various diseases.

2.2 TAXONOMICAL POSITION

Kingdom : Plantae
Division :Angiospermae
Class :Dicotyledonae
Sub class : Gamopetalae
Series : Inferae
Order : Rubiales
Family : Rubiaceae
Genus : *Ixora*
Species : *coccinea*

2.3. VERNACULAR NAME

English : Jungle geranium, Burning love, Scarlet Ixora, Red Ixora

Hindi : Rugmini, Rangan,

Tamil : Idly poo, Vedchi

Malayalam : Thechi, Chethi

Kannada : Gurugudu, Kepala, Holedaasala

Marathi : Bakora, Padkali

Sanskrit : Bandhujeevaka, Parali, Raktaka

Telugu : Bandhuca, Koranam



2.4 BOTANICAL DESCRIPTION

Ixora coccinea is a dense, multi branched evergreen shrub with branched tap root system. The stem is branched, cylindrical and woody in nature. The leaves are simple, opposite decussate, leathery oblong in shape, inter petiolar stipules are present and the veins are unicostate reticulate. The inflorescence is axillary dense corymbose cyme. The flowers are bracteate, bracteolate, dichlamydeous, bisexual, complete, tetramerous, actinomorphic and epigynous. There are four united sepals which are green coloured, regular and persistent, it is arranged in valvate aestivation. The petals are also four, bright red coloured, united with showy twisted aestivation. The corolla tube is long and slender. There are four stamens which are epipetalous attached to the throat of corolla tube. The filaments are short which alternate the petals. Anthers are dithecous, basifixed and introse. It dehisces longitudinally. The ovary is inferior with two carpels and syncarpous. The ovules are present inside the locules, two locules having one ovule each showing axile placentation. The style is simple, filiform with bifid stigma. The fruits are berry with endospermous seed.

2.5. MEDICINAL USES

The leaves, barks, roots and flowers are used for curing various diseases. The roots are said to be analgesic, antiseptic, astringent, diuretic, sedative and stomachic. They are used in the treatment of hiccups, fever, gonorrhea, loss of appetite, nausea, sores, chronic ulcer, diarrhoea and dysentery. The flower decoction is used for treat haemoptysis, dysmenorrhea, catarrhal bronchitis and lotion against eye troubles. The flower and leaves decoction inhibit all kinds of tumors.

Moni Rani *et al*., (2008) investigated in methanolic flower extract of *Ixora coccinea* for its free radical scavenging activity. The antioxidant activity was carried out by DPPH free radical scavenging method, reducing power and total antioxidant capacity by phospho-molybdenum method. The phytochemical analysis resulted that the presence of flavonoids, steroids and tannin compounds. In all assays the extract shows potent antioxidant activity. The DPPH assay resulted the IC_{50} value of the extract as 100.5µg/mL, the ascorbic acid had the value of 58.9µg/mL.

Angeline *et al*., (2010) studied in methanolic extract of *Ixora coccinea* flower, leaves and stem for its antioxidant activity and total phenolic content. The antioxidant activity study is carried out by DPPH radical scavenging activity, Total antioxidant capacity and Xanthine oxidase inhibition assay. The highest total phenolic content was found in flower extract, and comparatively low in leaves than flower extract so, the leaves and flower extract are used as antioxidants.

Nagaraj *et al*., (2011) evaluated wound healing and antimicrobial potential of *Ixora coccinea* root extract. The root extracts combined with ethanol, aqueous, petroleum ether, benzene, chloroform and ethyl acetate were studied for its anti-microbial activity against the bacterial strains *staphylococcus aureus, enterococcus faecalis, Escherichia coli, Pseudomonas*

Philomina and Pradeep (2011) analysed in *Ixora coccinea* flower extract for the antimicrobial activity using well diffusion method and filter paper method against the bacterial strains including *Escherichia coli, Enterobacterium aerogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis* and *Corynebacterium glutamicum.* The result shows the zone of inhibition increase with the increase in concentration of extract.

Sankhadip *et al*., (2011) compared the *in vitro* and *in vivo* anti-oxidant properties of *Ixora coccinea* and Ixora parviflora. Both the plants showed effective result in *in vitro* and *in vivo* antioxidant activity. The aqueous extract and ethyl acetate extract of *Ixora coccinea* exhibit more activity than Ixora parviflora extracts.

Shiny *et al*., (2012) examined the phytochemical and pharmacological activities of *Ixora coccinea*. The extracts were prepared by using petroleum ether, chloroform, methanol and water. The anti-microbial activity is assessed using cup plate method against the strains of bacteria and fungi, the extract showed better anti-bacterial activity than antifungal activity. The extract shows potent antioxidant activity comparable to ascorbic acid.

Lachimanan *et al.,* (2012) investigated in plant extracts of some species of *Ixora* to compare its antimicrobial activity. The methanolic plant extracts were tested using disc diffusion method against about 14 strains of bacteria, 5 stains of yeasts and 11 strains of fungi. The study resulted that all species of *Ixora* shows antimicrobial properties against at least one strain of bacteria or yeast or fungi.

Ravi kumar *et al*., (2013) evaluated the *in vitro* antioxidant activity of ethanolic leaf extract of *Ixora coccinea* leaf extract were carried out by DPPH, reducing power and nitric oxide assay methods are compared with standard gallic acid. The presence of various phytoconstituents is responsible for this activity.

Surana *et al.,* (2013) analyzed in *Ixora coccinea* root extract for *in vitro* and *in vivo* antioxidant activities. The powdered *Ixora coccinea* root is extracted with petroleum ether, chloroform, ethyl acetate and methanol. The *in vitro* anti-oxidant assay is carried out by DPPH free radical scavenging activity, nitric oxide scavenging activity and hydrogen peroxide scavenging activity, the *in vivo* anti-oxidant method is carried out by prolongation of haloperidol- induced catalepsy in mice. The free radical scavenging effect increase with increasing concentration.

Sankhadip *et al*., (2013) investigated in *Ixora coccinea* leaf extract for the isolation of quercitrin, which depends on fractionation of defatted hydro alcoholic extract by petroleum ether, ethanol, benzene and ethyl acetate followed by purification through column chromatography. The isolated quercitrin has characterized by using UV, IR, mass spectral data, NMR data and conformed by HPTLC analysis. Later the antioxidant activity of quercitrin was carried out by DPPH radical scavenging method. By DPPH method 80µg/mL of quercitrin exhibit 90.46% and ascorbic acid shows 96.30% of inhibition. The IC_{50} value of quercitrin is 6.167 and ascorbic acid is 4.321µg/mL. the isolated quercitrin shows a great anti-oxidant activity in both the models.

Fawole *et al*., (2013) investigated in ten different medicinal plants of India. The antioxidant study using DPPH, total phenolic content (TPC) and ferric reducing antioxidant power assay (FRAP). Out of the plants extract of ten plants, only five were showed highest radical scavenging activity. The ethanolic leaf extract of *Psidium guajava, Ixora coccinea, Vinca rosea, Physalis angulate* and *Mangifera indica* shows highest antioxidant activity were selected for antibacterial study. The bacterial strains used are *Aeromonas hydrophila,*

Micrococcus luteus and *vibrio vulnificus.* The inhibition zone is greater than 8 in all tested pathogenic bacteria.

Kawade *et al*., (2013) analysed the anti-inflammatory and antioxidant activity of ethanolic root extract of *Ixora coccinea*. The anti-inflammatory test was carried out by using cotton pellet granuloma test and carrageenan induced paw edema, the result shows effective anti-inflammatory activity against acute and chronic inflammation. The antioxidant activity was examined using thiobarbituric acid reactive substances assay. It shows effective and dose dependent antioxidant activity.

Mani maran *et al*., (2014) studied the phytochemical screening and antimicrobial activity in methanolic extract of leaf, stem and flower of *Ixora coccinea*. The phytochemical screening revealed the presence of terpenoids, flavonoids, coumarin, alkaloid and phenolic groups. The antimicrobial assay was carried out by using agar disc diffusion, microdilution and thin layer chromatography bioautography assay. The leaf and stem extract showed more activity than flower extract against the pathogenic microorganisms. It particularly exhibits strong antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus flexneri.*

Poornima *et al*., (2014) compared the antibacterial activity of the leaf extract of different colored varieties of *Ixora coccinea* such as dwarf, red, white and yellow. The antibacterial assay was done using agar well diffusion method and broth dilution method against the bacterial strains such as *Pseudomonas aeruginosa, Salmonella typhi, Micrococcus luteus* and *Vibrio cholerae*. The red variety showed the maximum inhibition compared to others. The preliminary phytochemical screening of the *Ixora coccinea* red exhibit the presence of alkaloids, phenol, flavonoids, steroid and saponins.

Aadesh *et al*., (2014) evaluated *Ixora coccinea* extract for its enhancing cutaneous wound healing by upregulating the expression of collagen and basic fibroblast growth factor. The petroleum ether, chloroform, methanol and water sequential extracts were studied for its in vitro antioxidant, anti-microbial and fibroblast proliferation activities. The study revealed that the methanolic extract shows the wound healing, antimicrobial and antioxidant activities.

Soma *et al*., (2014) investigated in flower and leaf extracts of *Ixora coccinea* for the evaluation of its antibacterial activity against some pathogenic clinical samples of bacteria. This is carried out by agar- well diffusion method. Both the methanolic and ethanolic extract

of leaf and flower shows potent antibacterial activity. The phytochemical analysis resulted the presence of alkaloids, steroids, flavonoids and tannins.

Saleha *et al*., (2015) studied in different species of Ixora for its comparative antimicrobial activities. For this study the leaf extracts of four different species of Ixora are used such as *Ixora chinensis, Ixora lutea, Ixora coccinea* and *Ixora parviflora* they were tested against nine bacterial strains by using agar-well diffusion method. The gram-positive bacterial strains used were *Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* and gram$^-$ strains were *Escherichia coli, Acinetobacter, Salmonella paratyphi, Salmonella typhi, Klebsiella proteus* and *Pseudomonas.* 10µg/disc of Gentamicin was used as standard. The leaf extract of *Ixora parviflora* showed antimicrobial activity against *Acinetobacter, Bacillus subtilis, Salmonella typhi* and *Salmonella paratyphi* even in low concentration.

Sunitha *et al*., (2016) examined the phytochemicals and anti-oxidant activity of flower extract of *Ixora javanica*. The phytochemical screening revealed that the presence of flavonoids, polyphenols, anthocyanins etc., The ethanolic extract showed highest antioxidant activity, ethyl acetate extract showed intermediate and petroleum ether extract showed comparatively low activity.

Ancilla and Judia (2016) studied antioxidant activity of crude extract and carotenoid pigment from the flower of *Ixora coccinea, Tecoma stans, Peltophorum ptelocarpum* and *Hibiscus rosasinensis.* The anti-oxidant activity of the crude extract and carotenoid extract were determined by reducing power assay and phosphomolybdenum method. The extract of *Tecoma stans* and *Peltophorum pterocarpum* showed higher antioxidant activities than other.

Riddhi *et al*., (2017) compared the Phyto-pharmacognosy activity of stem of *Ixora coccinea* and *Ixora arborea*. The phytochemical screening of water and methanolic extract showed the presence of carbohydrates, alkaloids, starch, saponins and tannin in both the plants. The flavonoids present only in *Ixora coccinea*.

Damle and Sharon (2017) investigated in *Ixora coccinea* of Karwar district for determination of phytochemicals present in it. The qualitative analysis resulted that the presence of flavonoids, phenolic compounds, terpenoids, steroids and alkaloids in leaf and stem extract which is responsible for the antimicrobial activity against *Corynebacterium diphtheriae, Salmonella typhi, Staphylococcus aureus, Escherichia coli.* The R_f value of leaf and stem extracts showed a total 10 peaks corresponding to 10 compounds.

Akanji *et al*., (2018) examined on *Ixora coccinea* leaf extract for the determination of its chemical composition, antimicrobial activity and proximity analysis on some clinical pathogens. The phytochemical present in the ethanolic leaf extract were alkaloids, cardiac glycosides, anthraquinone, phenol, tannin, saponin and flavonoids. The elemental composition present in the extract were sodium, potassium, calcium, magnesium, zinc, iron, copper, manganese and phosphorus. The proximal study revealed the presence of ash, moisture, crude protein, crude fat, crude fiber and carbohydrates. The antimicrobial study reveals that the plant extract has activity against *staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Escherichia coli* and a strong activity against *Pseudomonas aeruginosa.* The zone of inhibition increases with the increase in concentration of plant extract.

Khatoon *et al*., (2018) analysed the antioxidant properties and phytochemical studies on *Adenium obesum, Ixora coccinea* and *Aegle marmelos.* The phytochemical screening resulted the presence of about 26 phytoconstituents. The antioxidant assay carried out by DPPH free radical scavenging activity resulted that the methanolic extract of *Ixora coccinea* leaves shows significant antioxidant property.

Sivaraj *et al*., (2019) investigated the antioxidant, anti-inflammatory, antibacterial and antidiabetic activities of aqueous flower extract of *Ixora coccinea.* The antioxidant study was carried out by DPPH radical scavenging activity, superoxide radical scavenging activity, phosphomolybdenum reduction and ferric reducing power activity. The antidiabetic activity was carried out by alpha amylase inhibition method and antibacterial activity by agar-well diffusion method, it resulted maximum zone of inhibition against *Bacillus subtilis* at 500µg/mL concentration. The total phenolic and flavonoid content were 20.57 and 369.1µg/mL concentration gallic acid equivalents and 55.14µg/mg quercetin equivalents were observed.

Haji *et al*., (2020) studied in *Ixora coccinea* for the determination of antioxidant and antimicrobial activities and quantification of phenolic compounds using HPTLC. The methanolic extract were suitable for extracting polyphenol. The antioxidant activity is determined through DPPH and FRAP method. The presence of gallic acid, pyrocatechol, catechin, chlorogenic acid, p-coumaric acid, coumarin and quercetin were responsible for the antioxidant activity. It also shows antimicrobial activity against *Bacillus cereus, bacillus subtilis* and *Shigella dysenteriae*. There is no notable activity against *Escherichia coli.*

MATERIALS AND METHODS

CHAPTER III
MATERIALS AND METHODS

3.1 CLEANING OF GLASSWARES

All the glassware was immersed in cleaning solution for 3 hrs. Then it is washed thoroughly with tap water, followed by detergent solution and finally rinsed with distilled water. The washed glassware was dried in hot air oven and stored.

3.2 COLLECTION OF PLANT MATERIAL

The fresh leaves were collected from the campus surrounding of Avinashilingam university and is washed and shade dried for about ten days. The dried leaves were made in to coarse powder.

3.3 PREPARATION OF PLANT EXTRACT

For 100g of leaf powder 500ml of solvents were used. The extraction was prepared by using hexane, chloroform, ethyl acetate and methanol. The extracts were kept in orbital shaker for 24hrs. After 24hrs the extract were filtered using a filter paper and the supernatant is collected. Then the supernatant was allowed to evaporate. A greasy final material is collected in an Eppendorf tube, labelled and stored.

3.4 QUALITATIVE PHYTOCHEMICAL ANALYSIS

The different qualitative chemical tests were performed for establishing the profile of given extract of its chemical composition. The crude drug was dissolved in methanol and subjected to various phytochemical analyses. The following tests were performed on the extracts to detect various phytoconstituents present in them.

3.4.1 DETECTION OF ALKALOIDS (EVANS, 1997)

Solvent free extract (50 mg) was stirred with few mL of dilute hydrochloric acid and filtrate. The filtrate was tested carefully with various alkaloid reagents as follows:

A) MAYERS TEST (Evans,1997)

To a few mL of filtrate, a drop or two of mayers reagents was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

MAYERS REAGENT:

Mercuric chloride (1.358g) was dissolved in 60 mL of water and potassium chloride (5g) was dissolved in 10 mL of water. The two solutions were mixed and made up to 100 mL distilled water.

B) WAGNERS TEST (Wagner, 1993)

To a few drops of filtrate, few drops of wagners reagent was added by the side of the test tube. A reddish-brown precipitate confirmed the test as positive.

WAGNERS REAGENT

Iodine (1.27g) and potassium iodide (2g) were dissolved in 5 mL of water 44and made up to 100mL with distilled water.

C) DRAGENDORFF'S TEST (Waldi, 1965)

To a few mL of filtrate, 1 or 2 mL of dragendorff''s reagent was added. A prominent yellow precipitate indicated the test as positive.

DRAGENDORFF'S REAGENT

STOCK SOLUTION

Bismuth carbonate (5.2g) and sodium acetate (4g) were boiled for a few min with 50 mL glacial acetic acid. After 12 hrs, the precipitated sodium acetate crystals were filtered off using sintered glass funnel. Clear and red-brown filtrate (40 mL) was mixed with 160 mL of ethyl acetate and 1 mL of water stored in amber-coloured bottle.

WORKING SOLUTION

10mL of stock solution was mixed with 20 mL of acetic acid and made up to 100mL with water.

3.4.2 DETECTION OF CARBOHYDRATES AND GLYCOSIDES (Ramakrishnan *et al*., 1994)

The extract (100 mg) was dissolved in 5 mL of water and filtrate. The filtrate was subjected to the following tests.

A) FEHLING'S TEST

One mL of filtrate was boiled on water bath with 1mL each of fehling solutions I and II. A red precipitated indicated the presence of sugar.

FEHLING'S SOLUTION

FEHLINGS SOLUTION I: copper sulphate (34.66g) was dissolved in distilled water and made up to 500 mL with distilled water.

FEHLINGS SOLUTION II: potassium sodium tartarate (173g) and sodium hydroxide (50 g) were dissolved in water and made up to 500 mL.

B) BENDICT TEST

To 0.5 mL of filtrate, 0.5 mL of benedict's reagent was added. The mixture was heated on a boiling water bath for 2 min. A characteristic-coloured precipitate indicated the presence of sugar.

BENDICT'S REAGENT

Sodium citrate (1739) and sodium carbonate (100g) were dissolved in 800 mL of distilled water and boiled to make it clear. Copper sulphate (17.3g) dissolved I 10mL distilled water was added to it.

For detection of glycosides, 50mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hrs on a water bath, filtrate and the hydrolysate were subjected to the following tests.

3.4.3 DETECTION OF SAPONINS BY FOAM TEST (Kolkate, 1999)

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 10 min. A two cm layer of foam indicated the presence of saponins.

3.4.4 DETECTION OF PHYTOSTEROLS (Finar, 1986)

LIBERMANN-BURCHARD'S TEST

The extract (50 mg) was dissolved in 2 mL of acetic anhydrate. To this, one or two drops of concentrated H_2SO_4 was added slowly along the sides of test tube. An array of colour changes showed the presence of phytosterols.

3.4.5 DETECTION OF FIXED OILS AND FATS (Kolkate, 1999)

A) SPOT TEST

A small quantity of extract was pressed between two filter paper. Oil stain on the paper indicated the presence of fixed oil.

3.4.6 DETECTION OF PHENOLIC COMPOUNDS AND TANNINS

A) FERRIC CHLORIDE (Mace, 1963)

The extract (50 mg) was dissolved in 5mL of distilled water. To this a ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

B) GELATIN TEST (Evans, 1997)

The extract (50 mg) was dissolved in 5 mL of distilled water and 2 mL of 1 % way out of gelatin containing 10 % sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

C) LEAD ACETATE TEST

The extract (50 mg) was dissolved in distilled water and to this; 3 mL of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

D) ALKALINE REAGENT TEST

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. Yellow fluorescence indicated the presence of flavonoids.

3.4.7 TEST FOR QUINONES

To 1ml of extract, 1ml of conc. H_2SO_4 was added. Formation of pink colour indicates presence of quinones.

3.4.8 TEST FOR TERPENOIDS

To 0.5ml of extract, 2ml of chloroform was added and conc. H_2SO_4 was added carefully. Formation of red-brown colour indicates the presence of terpenoids.

3.4.9 TEST FOR TRITERPENOIDS

To 1.5ml of extract, 1ml of acetic anhydride and conc. H_2SO_4 was added. Formation of blue-green colour indicates the presence of triterpenoids.

3.4.10 TEST FOR COUMARINS

To 1ml of extract, 1ml of 10% NaOH was added. Formation of yellow colour indicates the presence of coumarins.

3.4.11 TEST FOR ANTHRAQUINONES

To 1ml of plant extract, a few drops of 10% ammonia solution were added. Appearance of pink colour precipitate indicates the presence of anthraquinones.

3.5 THIN LAYER CHROMATOGRAPHY (TLC)

Thin Layer Chromatography can be used to identify compound present in a given substance and is less time consuming, low cost and can be performed with less complicated technique. It has a wide application and significantly used in pharmaceutical purposes. The mobile phase should be prepared freshly at the time of experiment. The purity of the solvents and quality of the solvent mixture should be strictly followed. The mobile phase contains a solvent mixture of chloroform and ethanol in the form of different concentrations (3.5:1.5,3:2,2.5:2.5). TLC plate should be 8cm long in which spots were kept at above 1cm. Silica gel plate is used to separate the compounds.

Retardation factors (Rf) values were calculated by the formula,

$$R_f = Distance\ travelled\ by\ the\ compound\ /Distance\ travelled\ by\ the\ solvent$$

3.6 ANTIOXIDANT ASSAY

The discovery of the implication of free radicals is used to identifying diseases has led to increased interest in functional food that contain many different dietary phytonutrients, including antioxidants. One of the most important issues in the natural antioxidants analysis is determination of their antioxidant activity. The total antioxidant activity of plant extract has been evaluated using a new TLC spot method that involves reaction between DPPH (2,2-dipheny1-1-picrylhydrazyl) and antioxidants from the extract.

3.6.1 DPPH RADICAL SCAVENGING ACTIVITY

A. DPPH SOLUTION PREPARATION

DPPH standard was prepared by adding 4.3mg of DPPH dissolve in 3.3ml of methanol (DPPH is light sensitive so, the bottle is covered by aluminium foil).

B. PREPARATION OF STANDARD SOLUTION

Required quantity of ascorbic acid was dissolved in methanol to give the concentration of 5, 10, 20, 30, 40 and 50µg/mL.

C. PREPARATION OF TEST SAMPLE:

Stock solution of samples were prepared by dissolving 10mg of dried methanolic extract in 10mL of methanol to give concentration of 1mg/mL

PROTOCOL FOR ESTIMATION OF DPPH SCAVENGING ACTIVITY

150µl of DPPH solution was added to 3ml methanol and absorbance was taken immediately at 517nm for control reading. Different volume levels of test sample (100, 120, 140, 160, 180 and 200µl) were screened and made to 200µl of each dose level by dilution with methanol. 150µl DPPH solution was added to each test tube. Diluted with methanol up to 3ml. incubate at room temperature for 30 minutes at dark. Absorbance was taken at 517nm in UV spectrometer after 15 minutes using methanol as a blank. The percentage reduction and IC_{50} were calculated as follows

The free radical scavenging activity (FRSA) (% antiradical activity) was calculated using the equation:

$$\%\ \textbf{antiradical activity} = \frac{\textbf{control absorbance} - \textbf{sample absorbance}}{\textbf{control absorbance}} \times 100$$

3.7 ANTIMICROBIAL ASSAY

The antimicrobial assay was used to determine the growth inhibition of microorganism by plant extract. For the present study agar well diffusion method and broth dilution method are used.

3.7.1 MICROBIAL STRAINS USED

- *Escherichia coli* (gram- negative bacteria)
- *Staphylococcus aureus* (gram-positive bacteria)
- *Enterococcus faecalis* (gram – positive bacteria)
- *Candida albicans* (fungi)

3.7.2 PREPARATION OF MOTHER CULTURE

Prepare sterile test tubes for culturing the microbial strains. Take a conical flask and add 1.3g of nutrient broth powder in 100ml of distilled water. Mix and dissolve them completely and sterilize by autoclaving for 15 minutes. In a laminar air flow chamber, pour 5ml of moderately hot nutrient broth in to the test tubes. The test tube is labelled with the name of the microbe and date when the culture is prepared for each microbe separately. Transfer a small inoculum of microbes from the pure culture using an inoculation loop (the inoculation loop is heat until red hot before and after use) to the test tube containing nutrient broth. Seal the mouth of the test tube using a sterile cotton plug and stick parafilm around the mouth. Incubate over night at 37ºC with continuous shaking.

3.7.3 PREPARATION OF ANTIMICROBIAL CULTURE

Prepare sterile petri dishes for culturing. Take a conical flask and add 28g of nutrient agar powder in 1000ml of distilled water. Mix and dissolve completely and sterilize by autoclaving for 20 minutes. Let the agar cool moderately. In a laminar air flow chamber, pour agar 2/3 of the bottom of petri dishes and close the lid immediately. Leave the petri dishes for few minutes to solidify the medium. Once it gets solidify, the lid of the Petri dishes is labelled with the name of the microorganism, name of the plant extract, date of inoculum. And at the bottom label the concentration levels, control at the centre, around it at equal space mark 25, 50, 100, and positive control. Dip a sterile cotton swab in to the mother culture and swab on the agar plate evenly. Make a well using a sterile cork borer of 8mm at the marked space. Add the plant extract diluted with dimethyl sulphoxide (DMSO) to the well as the marked concentration using a micro pipette, DMSO was used as the control and the tetracycline is used as the positive control. Close the lid of the culture dish and cover the edges using parafilm. Incubate the culture dish at 37ºC for 24hrs After 24hrs of incubation the culture plates are taken out and measure the diameter of the inhibition zone for result.

3.7.4. DETERMINATION OF MIC (Eloff, 1998)

The minimum inhibitory concentration (MIC) was determined by using the broth dilution method. The two-fold dilution was used. For preparing 100mL of nutrient broth, weigh 1.3g of nutrient broth powder in a sterile conical flask and dissolve with 100mL of distilled water. Autoclave the medium for 20 minutes and allow it for cool moderately. In a laminar air flow chamber, mark the sterilised test tubes (1,2,3,4,5,6,7,8,9 and control) and pour 3.6mL of

nutrient broth in to all test tubes. The stock solution is prepared by dissolving 2mg of methanolic extract per mL of DMSO. 0.4mL of this solution were added into the test tube 1 to make the concentration 200µg/mL. From the test tube 1, 2ml is pipetted out and add into tube 2 and mix well. This is repeated for all test tubes except the control. From the last test tube 2ml was discarded. Add 0.1ml of *E. coli* culture to all test tubes. Cover the mouth of the test tubes using sterile cotton plug and incubate the tubes at 37º C for 18hrs. After 18hrs the turbidity of the test tubes were observed and the MIC is recorded.

RESULT AND DISCUSSION

CHAPTER IV

RESULT AND DISCUSSION

4.1 QUALITATIVE PHYTOCHEMICAL ANALYSIS

Sequential extraction was taken from leaf of *Ixora coccinea* for analysis of preliminary qualitative phytochemicals. Alkaloids, carbohydrates, phenolic compounds, tannins, quinone, terpenoids, anthraquinones and phytosterols were present in all the extracts such as Methanol, Ethyl acetate, Chloroform and Hexane as shown in (Table No:4.1). The methanolic extract showed positive results for all except triterpenoids and phytosterols, but these were present in hexane extract.

According to Poornima *et al*., (2014) the methanolic leaf extract showed positive results for alkaloids, phenols, flavonoids, steroids, saponins and negative result for terpenoids. Interestingly in the present study the methanolic leaf extract showed positive results for terpenoids.

4.2 THIN LAYER CHROMATOGRAPHY

The thin layer chromatography (TLC) is used to separate mixture of compounds present in the extract. The mobile phase is the mixture of chloroform and ethanol in a series of concentration (3.5:1.5, 3:2, 2.5:2.5). The extract combined with different solvents is placed in separate plates, at 1cm above from the end of the TLC plate. Various secondary metabolites were present in the various extracts. The retention factor (R_f) is calculated, it is range between 0-1 as shown in (Table No: 4.2; Figure: 4.1 a-f).

4.4 ANTIOXIDANT ASSAY

DPPH RADICAL SCAVENGING ACTIVITY

The total free radical scavenging activity of the methanolic leaf extract of *Ixora coccinea* was determined with the DPPH method. The percentage of inhibition of free radical scavenging activity and IC_{50} were calculated.

Percentage of antiradical activity =

Control absorbance−sample absorbance/ control absorbance x100

The inhibition percentage of methanolic leaf extract and standard ascorbic acid are ranging from 6.38% – 61.55% and 67.94% – 80.47% respectively. The results are shown in (Table No: 4.3). A graph is plotted with % of absorbance versus concentration of the antioxidant for both methanolic leaf extract and the standard Ascorbic acid (Fig No: 4.2). The percentage of absorbance increases with increasing in concentration.

According to Angeline *et al*., (2010) the methanolic flower, leaves and stem extract showed the 50% inhibition at the concentration of 6.6mg/mL, 109.95mg/mL and 272.42mg/mL respectively. According to Aadesh *et al*., (2014) the methanolic leaf extract showed the higher DPPH scavenging property, the 50% inhibition at the concentration 9.63µg/mL.

4.5 ANTIMICROBIAL ASSAY

4.5.1 ANTIMICROBIAL CULTURE

The antimicrobial activities of the methanolic leaf extract of *Ixora coccinea* were studied and the result was shown in the (Table No: 4.4). The extract is tested against three bacterial strains and a fungal strain using agar well-diffusion method. Interestingly the extract showed antimicrobial activity against all the tested microbes. The extract in which three concentrations (25µg/mL, 50µg/mL and 100µg/mL) were tested against the bacterial strains; *Staphylococcus aureus, Escherichia coli* and *Enterococcus faecalis* the tetracycline is used as standard. The fungal strain *Candida albicans* is tested against four concentrations (25µg/mL, 50µg/mL, 75µg/mL and 100µg/mL). The zone of inhibition increases with increasing concentration. The highest antimicrobial activity is observed against *Enterococcus faecalis*. The standard also showed a potent inhibition zone against all the tested bacteria (Fig No: 4.3(i)).

According to Mani maran *et al*., (2014) the methanolic extract of *I. coccinea* subjected for antimicrobial activity by using agar disc diffusion method. The extract showed potent activity against the bacteria and fungi. The zone of inhibition ranges between 6.7 to 11.3 mm.

According to Saleha *et al*., (2015) the leaf extract of different species of *Ixora* are subjected to antimicrobial assay against nine strains of bacteria. The extracts including *I. chinensis, I. lutea* and *I. coccinea* were not shown any antimicrobial activity against any of the bacteria tested. But interestingly the present study the leaf extract of *I. coccinea* showed a potent antimicrobial activity against *S. aureus, E. faecalis, E. coli* and *C. albicans.*

4.5.2 MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration was determined against *E. coli* by using two-fold broth dilution method. The methanolic leaf extract of *I. coccinea* diluted with DMSO and the nutrient agar broth is used as growth medium. The concentration is taken as 200µg/mL, 100µg/mL, 50µg/mL, 25µg/mL, 12.5µg/mL, 6.25µg/mL, 3.125µg/mL, 1.56µg/mL and 0.78µg/mL. The turbidity of the solutions is observed shown in (Fig No: 4.2). The minimum growth is observed in the concentration 3.125µg/mL.

According to Aadesh *et al*., (2014) the methanolic extract of *I. coccinea* was showed potent activity of MIC ranging from 0.125-2mg/mL.

4.1. Qualitative preliminary phytochemical analysis of various leaf extract of *Ixora coccinea* L.

S.no	Tests	Hexane crude extracts	Chloroform crude extracts	Ethyl acetate crude extracts	Methanol crude extracts
1 A	**ALKALOIDS** Mayer's Test	-	-	-	-
B	Wagner's Test	+	+	+	+
C	Dragendroff's Test	-	-	+	+
2 A	**CARBOHYDRATES AND GLYCOSIDES** Fehling's Test	-	++	+	++
B	Benedict's Test	-	-	-	++
3	**SAPONINS**	-	-	-	++
A	Foam Test				
4 A	**PHYTOSTEROLS** Libermann-Buchard's Test	++	-	-	-
5 A	**FIXED OILS AND FATS** Spot Test	-	-	-	+

6	**PHENOLIC COM-POUNDS AND TANNINS**				
A	Ferric chloride Test	-	-	+	++
B	Gelatin Test	-	-	-	++
C	Lead Acetate	++	-	+	++
D	Alkaline Reagent	+	-	-	++
7	**QUINONES**	-	+	-	+
8	**TERPENOIDS**	+	+	-	++
9	**TRITERPENOIDS**	+	+	+	-
10	**COUMARINS**	+	-	-	+
11	**ANTHRAQUINONE**	-	-	+	+

++ - Strongly present, + - Present, - -absent

Table 4.2. Thin Layer Chromatographic profile of various crude leaf extract of *Ixora coccinea* L.

s.no	*Ixora coccinea* (leaves)	Chloroform :methanol 3.5:1.5	Chloroform :methanol 3:2	Chloroform :methanol 2.5:2.5
1	Hexane crude extract	0.4333, 0.5, 0.5666, 0.65, 0.73	0.5666, 0.6333, 0.7166, 0.7666, 0.8	0.5166, 0.666, 0.7333, 0.8333
2	Chloroform crude extract	0.4333, 0.5, 0.5833, 0.6666	0.6666, 0.7166, 0.75, 0.8	0.5833, 0.6333, 0.6666, 0.7, 0.75, 0.85
3	Ethyl acetate crude extract	0.7166, 0.7833, 0.8333, 0.8833	0.7333, 0.8, 0.8666, 0.9166	0.7833, 0.8333, 0.8833, 0.9333
4	Methanol crude extract	0.7833, 0.8333, 0.8833, 0.9166	0.8166, 0.8666, 0.9166, 0.9666	0.8166, 0.8833, 0.9333, 0.9666

Table 4.3. Antioxidant activity of methanolic leaf extract of *Ixora coccinea* L. by DPPH radical scavenging method.

Concentration (µg/ml)	Standard Ascorbic acid	Methanolic extract (% inhibition)
20	67.94	6.38
40	69.9	9.99
60	71.33	14.41
80	72.31	21.19
100	73.63	27.85
120	74.84	32.38
40	77.13	38.33
160	78.92	44.4
180	79.83	52.21
200	80.47	61.55

Table 4.4. Antimicrobial activity of methanolic leaf extract of *Ixora coccinea* L.

S. no	Micro organisms	Inhibitory zone diameter (mm)			Positive control (Tetracycline)
	Bacteria	**25µl**	**50µl**	**100µl**	**50µl**
1	*Staphylococcus aureus*	4	7	9	27
2	*Escherichia coli*	-	4	9	19
3	*Enterococcus faecalis*	2	7	12	25

S. no	Micro organism	nhibitory zone diameter (mm)			
	Fungi	**25µl**	**50µl**	**75µl**	**100µl**
1	*Candida albicans*	5	6	7	9

Fig No: 4.1 (a) TLC profile of *Ixora coccinea* (leaf) on Hexane crude extract solvent system Chloroform: Methanol 3.5:1.5

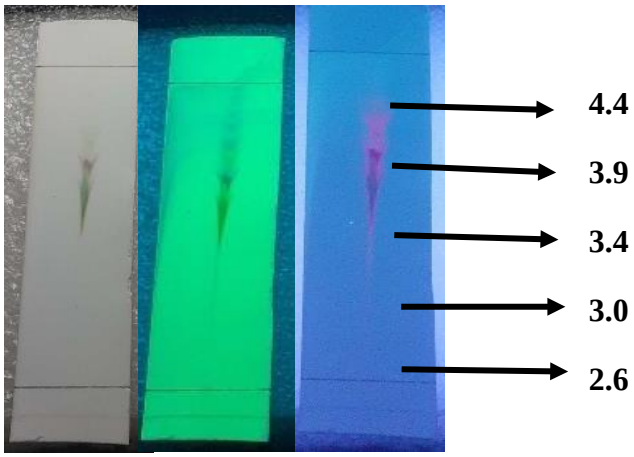


Fig No: 4.1 (b) TLC profile of *Ixora coccinea* (leaf) on Hexane crude extract solvent system Chloroform: Methanol 3:2

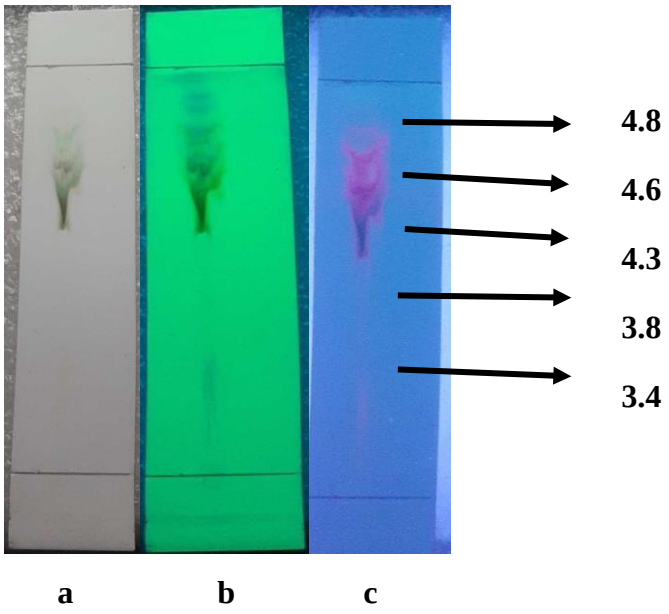


Fig No: 4.1 (c) TLC profile of *Ixora coccinea* (leaf) on Hexane crude extract solvent system Chloroform: Methanol 2.5:2.5

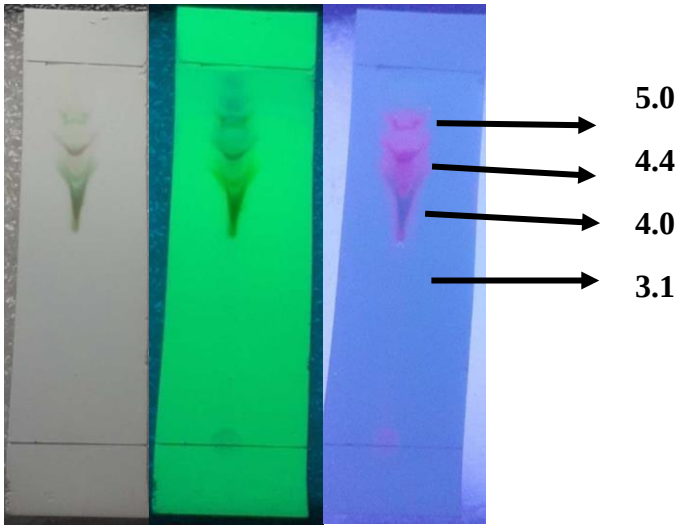


Fig No: 4.1 (d) TLC profile of *Ixora coccinea* (leaf) on Chloroform crude extract solvent system Chloroform: Methanol 3.5:1.5

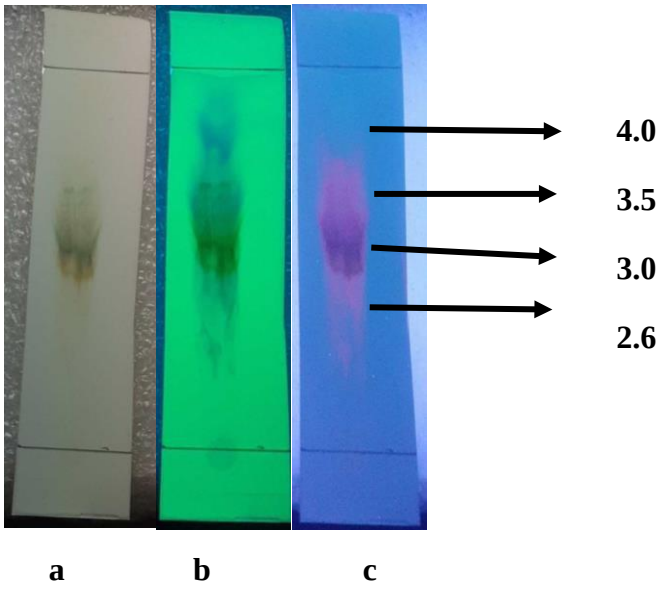


Fig No: 4.1 (e) TLC profile of *Ixora coccinea* (leaf) on Chloroform crude extract solvent system Chloroform: Methanol 3:2

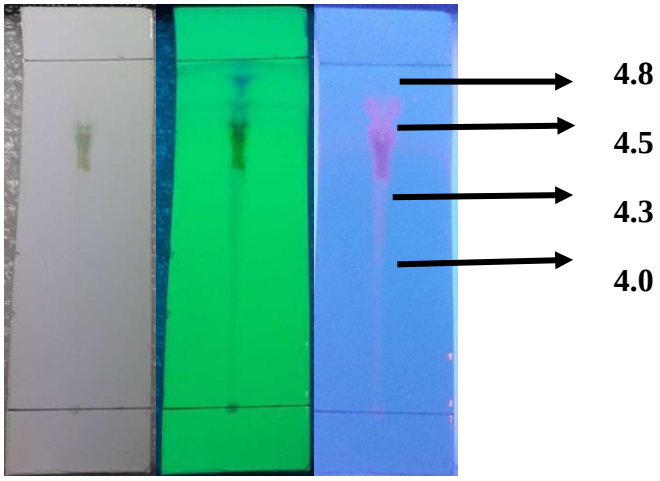


Fig No: 4.1 (f) TLC profile of *Ixora coccinea* (leaf) on Chloroform crude extract solvent system Chloroform: Methanol 2.5:2.5

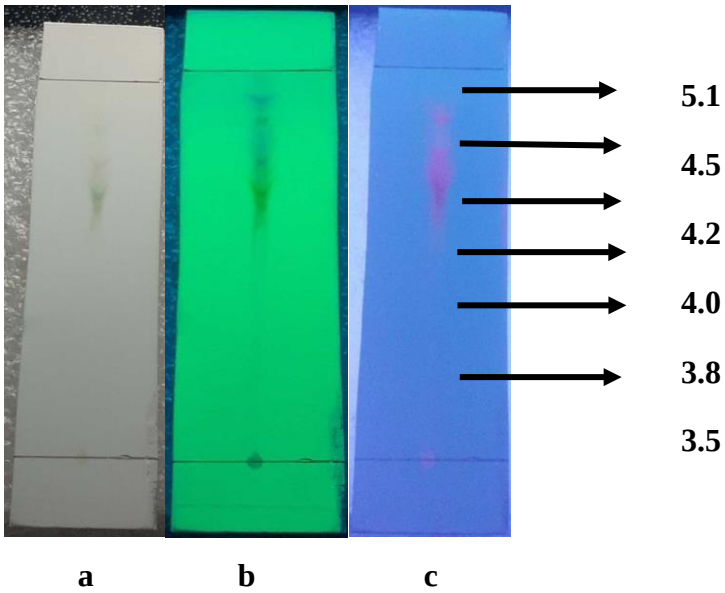
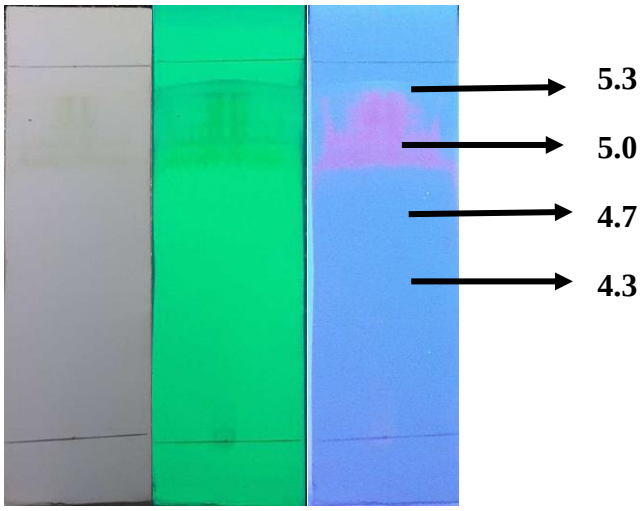


Fig No: 4.1 (g) TLC profile of *Ixora coccinea* (leaf) on Ethyl acetate crude extract solvent system Chloroform: Methanol 3.5:1.5



a. Visible light

b. Short UV

c. Long UV

Fig No: 4.1(h) TLC profile of *Ixora coccinea* (leaf) on Ethyl acetate crude extract solvent system Chloroform: Methanol 3:2

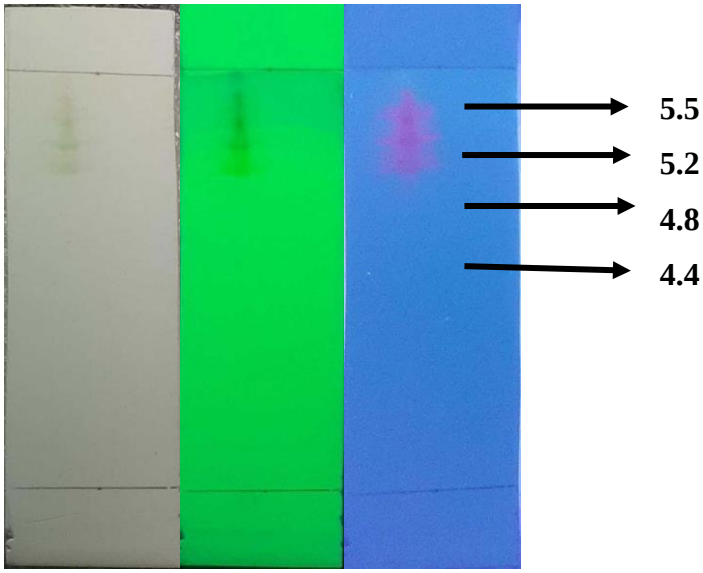


Fig No: 4.1 (i) TLC profile of *Ixora coccinea* (leaf) on Ethyl acetate crude extract solvent system Chloroform: Methanol 2.5:2.5

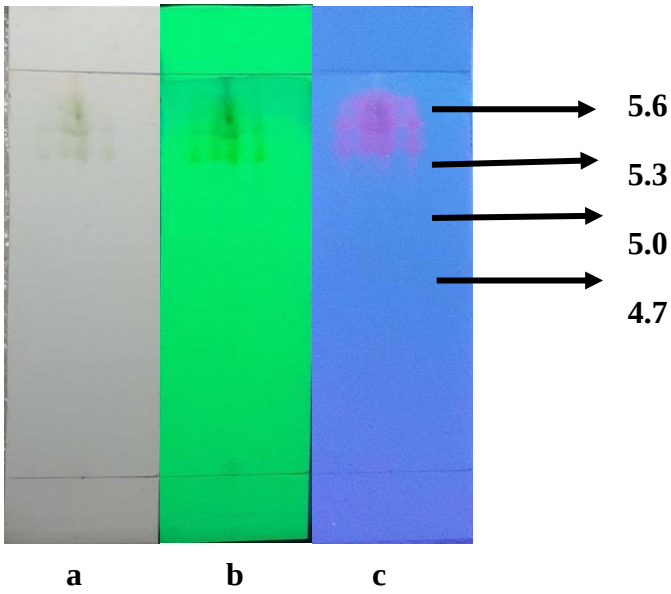


Fig No: 4.1(j) TLC profile of *Ixora coccinea* (leaf) on Methanol crude extract solvent system Chloroform: Methanol 3.5:1.5

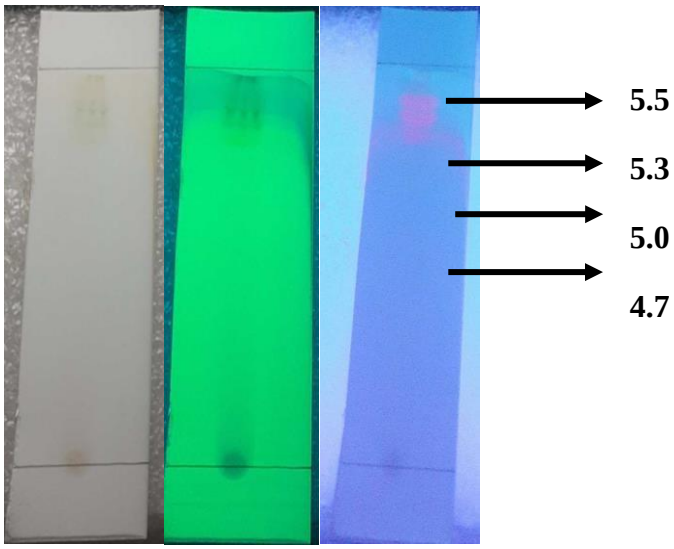


Fig No: 4.1(k) TLC profile of *Ixora coccinea* (leaf) on Methanol crude extract solvent system Chloroform: Methanol 3:2

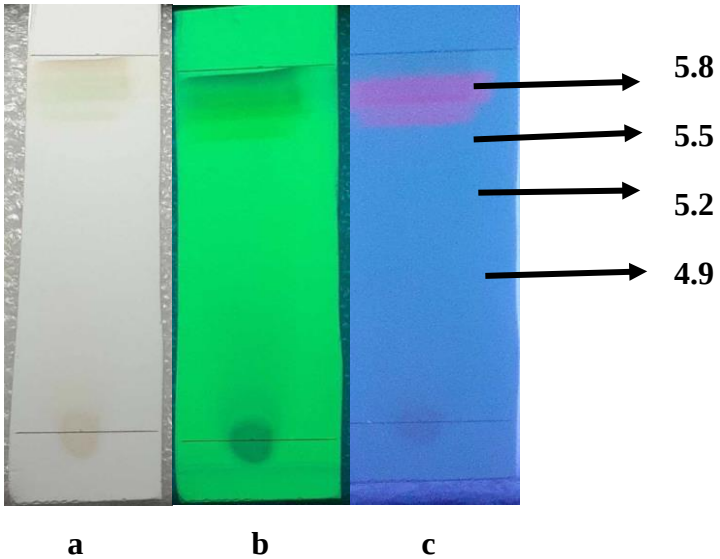


Fig No: 4.1(l) TLC profile of *Ixora coccinea* (leaf) on Methanol crude extract solvent system Chloroform: Methanol 2.5:2.5

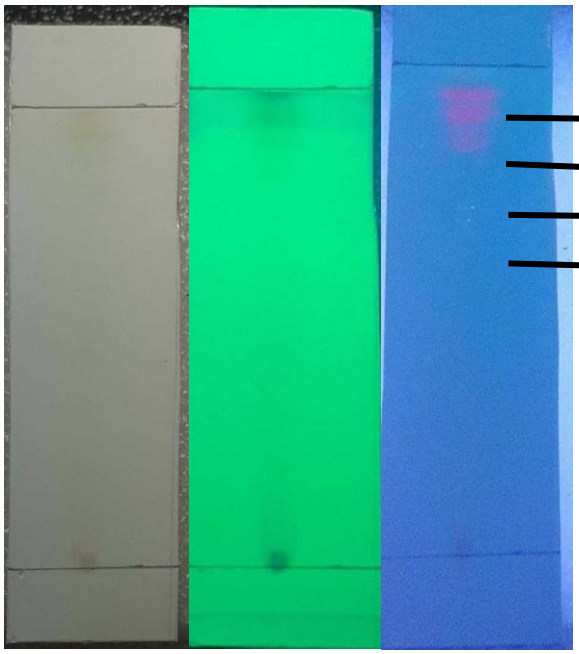


Fig No: 4.2 Antioxidant activity of methanolic leaf extract of *Ixora coccinea* L. by DPPH radical scavenging method.

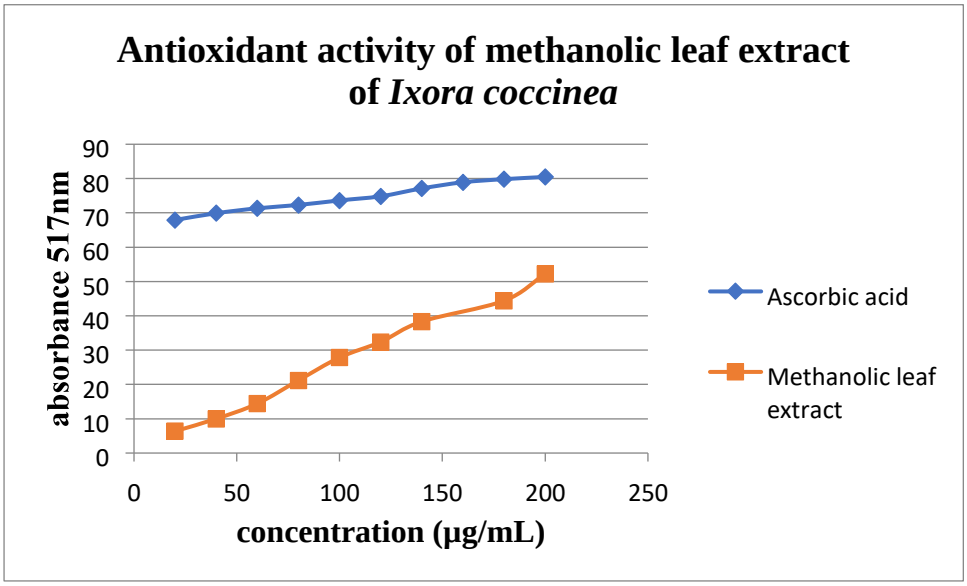
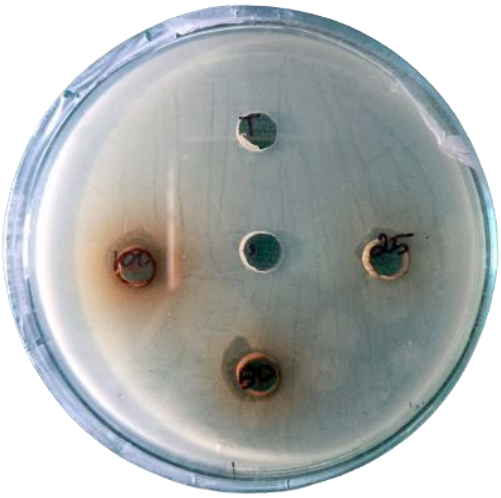
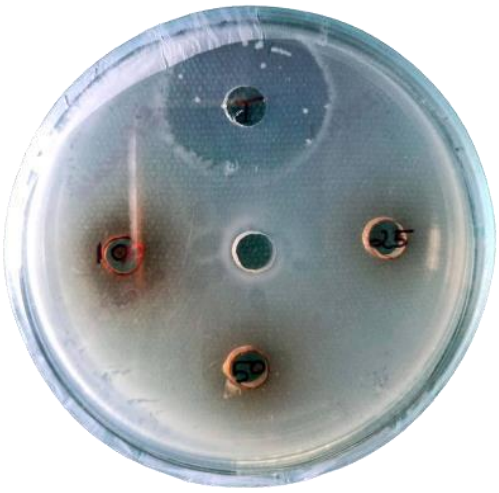


Fig No: 4.3 (i) Antimicrobial activity of methanolic leaf extract of *Ixora coccinea* L. by Agar well-diffusion method.



a

b



c

d

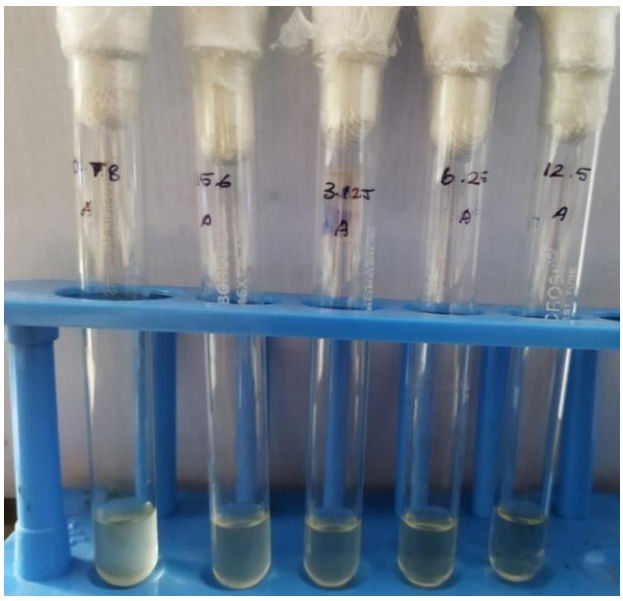
a. ***Staphylococcus aureus***

b. ***Escherichia coli***

c. ***Enterococcus faecalis***

d. ***Candida albicans***

Fig. No: 4.3 (ii) Determination of Minimum Inhibitory Concentration



e d c b a

Showing the turbidity increasing and concentration decreasing from a-e.

a. 12.5µg/mL

b. 6.25µg/mL

c. 3.125µg/mL

d. 1.56µg/mL

e. 0.78µg/mL

SUMMARY AND CONCLUSION

CHAPTER V
SUMMARY AND CONCLUSION

The present study concluded that the leaf extract of *Ixora coccinea* possess a wide range of medicinal properties. The leaf extracted with different solvents such as hexane, chloroform, ethyl acetate and methanol were used for phytochemical analysis. The methanolic extract was used in antioxidant and antimicrobial assay.

- The phytochemical screening of leaf extracted with hexane, chloroform, ethyl acetate and methanol revealed the presence of a wide range of phytoconstituents such as alkaloids, carbohydrates, phenolic compounds, tannins, quinone, terpenoids, anthraquinones and phytosterols.
- The TLC was performed with leaf extracted with various solvents including hexane, chloroform, ethyl acetate and methanol. The mobile phase used are chloroform and ethanol in the ratio of concentrations such as 3.5:1.5, 3:2 and 2.5:2.5. The retention factor (R_f) value is obtained different values for different extracts.
- The antioxidant assay is carried out by using DPPH free radical scavenging method in the methanolic extract showed a potent antioxidant activity. The percentage of absorbance increases with increasing in concentration. The values of methanolic leaf extract and standard ascorbic acid are ranging from 6.38% – 61.55% and 67.94% – 80.47% respectively.
- The antimicrobial study of methanolic leaf extract of *I. coccinea* against some clinical microorganisms such as *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Candida albicans* by using agar well-diffusion method. The different concentration showed antimicrobial activity against all the tested pathogens. *Enterococcus faecalis* exhibit higher zone of inhibition among the others. The zone of inhibition increasing with the increasing concentration. The *E. coli* formed no zone in lower concentration, but in higher concentration it exhibited. The zone of inhibition formed for each pathogen: *S. aureus* (4-9mm), *E. coli* (4,9mm), *E. faecalis* (2-12mm) and *C. albicans* (5-9mm).
- The serial two-fold broth dilution method is used to determine the Minimum Inhibitory Concentration of the methanolic leaf extract against *E. coli* is showed 3.125%.

CONCLUSION

The preliminary phytochemical screening carried out on the leaf extract of *I. coccinea* showed the presence of different phytoconstituents including alkaloids, flavonoids, carbohydrates, saponins, quinones, anthraquinones, terpenoids, triterpenoids, coumarins, phytosterols, phenolic compounds and tannins may responsible for its antioxidant and antimicrobial properties. The methanolic leaf extract showed potent antioxidant activity and antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Candida albicans.* Further studies are focused on the anti-quorum sensing and bio film formation by the leaf extracted with different solvents.

REFERENCE

REFERENCE

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ABBREVIATION

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µl	-	micro litre
DMSO	-	dimethyl sulphoxide
DPPH	-	2,2-diphenylpicrylhydrazyl
FRAP	-	ferric reducing ability of plasma
FRSA	-	free radical scavenging activity
H_2SO_4	-	sulphuric acid
IC_{50}	-	inhibition concentration at 50%
mg	-	milli gram
MIC	-	minimum inhibitory concentration
mm	-	milli meter
NaOH	-	sodium hydroxide
nm	-	nano meter
R_f	-	retention factor
TLC	-	thin layer chromatography
TPC	-	total phenolic content
UV	-	ultra violet