

Evaluation of *in vitro* anti-diabetic activity of methanolic extract of *Ixora coccinea* flowers

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Abstract

The aim of the present study was to explore the phytochemical bioactive compounds of the 80% methanolic extract of *Ixora coccinea* flowers and to investigate its *in vitro* anti-diabetic activity by α -amylase (starch iodine method) inhibition, α -glucosidase (dinitrosalicylic acid (DNSA) method) inhibition. Preliminary phytochemical screening revealed that the methanolic extract of the flowers of *I. coccinea* possesses secondary metabolites like flavonoids, phenols, steroids and tannins. The total phenolic content of methanolic extract was estimated in the flowers of *Ixora coccinea* and the value was found to be 30.6mg /g dry weight. The assay results suggests that methanolic extract exhibit the dose-dependent increase (20 -100 μ g/ml) in inhibitory effect on alpha-glucosidase enzyme (upto 70%), and alpha-amylase enzyme (upto 72%). The current study proves that the methanolic extract of *Ixora coccinea* flowers exhibit *in vitro* antidiabetic activity which may be attributed to the high amount of bioactive compounds such as polyphenolics.

Keywords- Alpha-glucosidase, alpha-amylase, *Ixora coccinea*, phenolic, antidiabetic

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by a loss of glucose homeostasis with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. According to WHO, it is estimated that 3% of the world's population have diabetes and the prevalence is expected to double by the year 2025 to 6.3% [2]. With a long course and serious complications often resulting in high death-rate, the treatment of diabetes spent vast amount of resources including medicines, diets, physical training and so on in all countries. Thus searching for a new class of compounds is essential to overcome diabetic problems. There is continuous search for alternative drugs [3].

Diabetes mellitus is one of the most common chronic diseases in nearly all countries, and continues to increase in numbers and significance, as changing lifestyles lead to reduce physical activity, and increase obesity. Estimates of the current and future burden of diabetes are important in order to allocate community and health resources, and to emphasize the role of lifestyle, and encourage measures to counteract trends for increasing prevalence [4]. Furthermore, taking into account its present rate of increase, within few decades it will be one of the world's commonest diseases and one of the biggest public-health problems with an estimated minimum of half-a-billion cases [5]. People suffering from diabetes are not able to produce or properly use insulin in the body and therefore chronic hyperglycemia occurs. In addition, the diabetic individual is prone to late onset complications, such as retinopathy, neuropathy and vascular diseases, that are largely responsible for the morbidity and mortality observed in diabetic patients [6]. There are two main types of diabetes, namely type I and type II. Type I diabetes, that is called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes develops when the body's immune system destroys pancreatic β -cells, the only cells in the body that produce the hormone insulin that regulates blood

glucose. This type of diabetes usually strikes children and adults and the need for insulin administration is determinant for survival. Type I diabetes accounts for 5% to 10% of all diagnosed cases of diabetes and the risk factors may be autoimmune, genetic, or environmental. On the other hand, type II diabetes, also called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, accounts for about 90% to 95% of all diagnosed cases of diabetes. It usually begins as insulin resistance, a disorder in which the cells do not use insulin properly and as the need for insulin rises; the pancreas gradually loses its ability to produce it. This type of diabetes is associated with older age, obesity, family history of diabetes, history of gestational diabetes, impaired glucose metabolism, physical inactivity, and race/ethnicity. It must be noted thought that in the last decay type II diabetes in children and adolescents is being diagnosed more frequently [7]. In the case of the IDDM, insulin is of crucial importance for the survival of the patients. On the other hand, in the case of NIDDM the treatment includes medicines, diets and physical training. Up to now, many kinds of anti diabetic medicines have been developed for the patients and most of them are chemical or biochemical agents aiming at controlling or/and lowering blood glucose to a normal level. Despite the impressive advances in health sciences and medical care, there are many patients who are using alternative therapies alone or complementary to the prescribed medication.

Management of diabetes without any side effect is still a challenge to the medical community. The use of the drugs is restricted by their pharmacokinetic properties, secondary failure rates and accompanying side effects [8]. Thus searching for a new class of compounds is essential to overcome diabetic problems. There is continuous search for alternative drugs [9, 10]. Traditional plant remedies or herbal formulations exist from ancient times and are still widely used, despite all the controversy concerning their efficacy and safety [11, 12], to treat hypoglycemic and

hyperglycemic conditions all over the world. It must be noted that many ethno-botanical surveys on medicinal plants used by the local population have been performed in different parts of the world and there is a considerable number of plants described as anti diabetic. In addition a variety of compounds have been isolated (alkaloids, glycosides, terpenes, flavonoids, etc) but further studies need to be done so as these 'leads' to develop into clinically useful medicines. Thus, Ayurveda is considered as one of the oldest systems of health care dealing with both the preventive and curative aspects of life in a most comprehensive way and presents a close similarity to the WHO's concept of health propounded in the modern era. To date, metformin (a biguanide) is the only drug approved for treatment of type II diabetes mellitus. It is a derivative of an active natural product, galegine, isolated from the plant *Galega officinalis* L. [13].

Many ethnobotanical surveys on medicinal plants used by the local population have been performed in different parts of the world including Morocco, Saudi Arabia, and Taiwan etc. Several species have been described as hypoglycemic. Several medicinal plants have been used as dietary adjunct and in the treatment of numerous diseases without proper knowledge of their function. Although phytotherapy continues to be used in several countries, few plants have received scientific or medical scrutiny. Moreover, a large number of medicinal plants possess some degree of toxicity. Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. India has about 45000 plant species and among them, several thousands have been claimed to possess medicinal properties. Research conducted in last few decades on plants mentioned in ancient literature or used traditionally for diabetes, have shown anti-diabetic property [14]. However, majority of plants have not yet been screened for such activity. So, in order to contribute further to the knowledge of Indian traditional plants, our present study is focused to evaluate the antidiabetic property of the methanolic extract of *Ixora coccinea* Linn. flower using some *in vitro* studies.

PLANT DESCRIPTION



Picture 1: *Ixora coccinea* Linn (Family: Rubiaceae; Malayalam-Thetti)

Ixora coccinea (or jungle geranium, flame of the woods, and jungle flame) is a species of flowering plant in the Rubiaceae family, a small to medium sized hardy shrub and is cultivated for ornamental purpose and also it finds place in traditional Indian medicine. It is a common flowering shrub native to Southern India and Sri Lanka. It has become one of the most popular flowering shrubs in South Florida gardens and landscapes. Its name derives from an Indian deity. *I. coccinea* is a dense, multi-branched evergreen shrub, commonly 4–6 ft (1.2–2 m) in height, but capable of reaching up to 12 ft (3.6 m) high. It has a rounded form, with a spread that may exceed its height. The glossy, leathery, oblong leaves are about 4 in (10 cm) long, with entire margins, and are carried in opposite pairs or whorled on the stems. Small tubular, scarlet flowers in dense rounded clusters 2-5 in (5–13 cm) across are produced almost all year long.

Although there are around 500 species in the genus *Ixora*, only a handful are commonly cultivated, and the common name, *Ixora*, is usually used for *I. coccinea*. It is used in warm climates for hedges and screens, foundation plantings, massed in flowering beds, or grown as a specimen shrub or small tree. In cooler climates, it is grown in a greenhouse or as a potted house plant requiring bright light. *I. coccinea* is also grown in containers, looking very distinguished as a patio or poolside plant. This tight, compact shrub is much branched and tolerates hard pruning, making it ideal for formal hedges, although it is at its best when not sheared [15].

The flowers, leaves, roots, and the stem are used to treat various ailments in the Indian traditional system of medicine, the Ayurveda, and in various folk medicines. The fruits, when fully ripe, are used as a dietary source. Phytochemical studies indicate that the plant contains the phytochemicals lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, leucocyanadin, anthocyanins, proanthocyanidins, and glycosides of kaempferol and quercetin [16].

MEDICINAL PROPERTIES

Roots contain aromatic acrid oil, tannin, fatty acids. Leaves yield flavonols, kaempferol, quercetin, proanthocyanidins, phenolic acids, and ferulic acids. Flowers yield cyanidins, flavonoids, and cooling material related to quercetin [17]. The plant is reported for diverse pharmacological properties including anti-inflammatory and antimutagenic activities [18]. The aqueous extract of the leaves of *Ixora coccinea* was effective in controlling the blood glucose levels and in improving the lipid profile in diabetic rats [19]. Leaves and flower extracts were reported to possess antimicrobial activities [20]. Flower extract showed protective effect against cyclophosphamide and cisplatin induced systemic toxicity [21]. The roots of the plant *Ixora coccinea* are mostly used as an astringent, antiseptic, stomachic, sedative etc. Traditionally roots are also used in diarrhoea, dysentery, gonorrhoea, in loss of appetite, hiccups, fever, sores and chronic ulcers. The leaf and stem often used as an abluion for infantile, sedative [22]. The present study deals with phytochemical analysis and *in vitro* anti-

diabetic activity of methanolic extract of *Ixora coccinea* flowers.

MATERIALS AND METHODS.

Plant material and preparation of extracts

Fresh, whole plant of *Ixora coccinea* Linn. were collected from local area, authenticated, washed and allowed to dry at room temperature. The dried plants were then ground to fine powder. 30 g of the dry powder was weighed and was used for extract preparation. Extracts for the plant were prepared using 80% methanol [23]. 30 g of the dry powder was ground to a paste in pestle and mortar using 150 ml of the solvent and kept overnight at 20°C. It was filtered twice through Whatman filter paper. The resulting filtrate was collected in a beaker and was subjected to evaporation in a water bath for 2-3 hours at 80°C. The extract was suitably diluted in 10% DMSO for various estimations.

Estimation of total phenol content (TPC):

The total phenol content was determined by Folin-Ciocalteu reagent method which was adapted from Swain and Hillis [24] & McDonald *et al.*, [25]. 0.5 ml of extract (1:5 dilution) and 0.1 ml of Folin-Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 15 min. 2.5 ml saturated sodium carbonate was added, incubated for 30 min at room temperature and absorbance was measured at 760 nm. The total phenol content was expressed in terms of Gallic acid equivalent (mg/g) [26].

Phytochemical Screening

For preliminary phytochemical analysis the freshly prepared crude methanolic extracts of leaves were tested for the presence or absence of phytoconstituents such as reducing sugar, tannins, flavonoids, steroids and alkaloids by using standard phytochemical procedures [27].

Qualitative Analysis

ALKALOIDS:

Mayer's Test: A small quantity of the extract was treated with few drops of dil.HCl and filtered. The filtrate was tested with alkaloid Mayer's reagent. Formation of cream precipitate indicated the presence of alkaloids.

Wagner's Test: To 2-3 ml of extract, add few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

FLAVONOIDS:

NaOH Test: To 2-3 ml of extract ,few drops of NaOH solution were added .Formation of intense yellow colour that became colourless on addition of few drops of diluted HCl indicated the presence of flavonoids.

PHENOLS:

Phenol Test: When 0.5 ml of FeCl₃ (w/v) solution was added to 2 ml of test solution, formation of an intense colour indicated the presence of phenols

PHYTOSTEROLS:

Salkowski Test: To 2 ml of extract, add 2 ml conc.H₂SO₄ and was shaken well. Chloroform layer appeared red and acid layer showed greenish yellow fluorescence indicated the presence of sterols.

Libermann Burchard's test: Mix 2 ml of extract with chloroform. Add 1-2 ml acetic anhydride and 2 ml of conc. Sulfuric acid from the sides of the test tube. First red

and then blue and finally blue colour is obtained in the presence of sterols.

SAPONINS:

Foam test: The extract was diluted with 20 ml of distilled water and it was shaken in a graduated cylinder for 15 min. A 1 cm layer of foam indicated the presence of saponins.

TANNINS:

Ferric Chloride Test: Small quantity of extract was boiled in 20 ml water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or blue-black colouration which indicate the presence of tannins.

CARBOHYDRATES:

Molisch's Test: To 1 ml of extract, 2 drops of molisch's reagent is added in a test tube and 2 ml of conc. Sulphuric acid was added carefully. The test tube slightly curved. The formation of a violet ring at the junction indicated the presence of Glycosides.

AMINO ACIDS:

Ninhydrin Test: To 5 ml of extract, 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. The appearance of blue colour indicates the presence of amino acids.

ANTHROQUINONES:

0.5 g of the extract was boiled with 10 ml of sulfuric acid and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette out into another test tube and 1 ml dil. ammonia was added. The resulting solution was observed for colour changes.

IN VITRO METHODS EMPLOYED IN ANTIDIABETIC STUDIES [28]

In Vitro Alpha Amylase Inhibition Assay

3, 5-Dinitrosalicylic acid method (DNSA): The inhibition assay was performed according to Miller [29] using DNSA method. Methanolic extract of *Ixora coccinea* flowers of varied concentrations (20 to 100 µg/ml) were added to 500 µL of 0.02 M sodium phosphate buffer (pH6.9 containing 6 mM sodium chloride) containing 0.04 units of α-amylase solution and were incubated at 37°C for 10 min, followed by addition of 500 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH6.9) all the test tubes. The enzyme solution was prepared by mixing 27.5 mg of alpha-amylase in 100 ml of distilled water. The colorimetric reagent is prepared by mixing sodium potassium tartarate solution and 3, 5 di nitro salicylic acid solution 96 mM. The reaction was stopped with 1.0 mL of 3, 5 DNSA reagent [30]. The test tubes were then incubated in a boiling bath water for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 mL distilled water and absorbance was measured at 540 nm. The control samples were also prepared accordingly without any plant extracts and were compared with the test samples containing various concentrations of the plant extracts prepared with different solvents. The results were expressed as % inhibition calculated using the formula:

Inhibition of enzyme activity (%) = $(C-S)/C \times 100$

Where S is the absorbance of the sample and C is the absorbance of blank (no extract) [31].

Inhibition of alpha-glucosidase enzyme

Starch-iodine Colour Assay: The inhibitory activity was determined by incubating 1 ml of 1% w/v starch in 0.2 M Tris buffer pH 8.0 and various concentration of plant extract (20 to 100 µg/ml) for 5 min at 37°C. The reaction was initiated by adding 1 ml of alpha-glucosidase enzyme (1U/ml) to it followed by incubation for 40 min at 35°C. Then the reaction was terminated by the addition of 2 ml of 6N HCl, followed by the addition of 500 µL of iodine reagent (5 mM I₂ and 5 mM KI). The colour change was noted and the absorbance was read at 620 nm [32]. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, appropriate extract controls without the enzyme were also included. Inhibition of enzyme activity was calculated as:

Inhibition of enzyme activity (%) = $(C-S) / C \times 100$,

where S is the absorbance of the sample and C is the absorbance of blank (no extract).

STATISTICAL ANALYSIS

Statistical significance was determined by One-way Analysis of Variance (ANOVA) in SPSS 10.0 package. Paired comparison between samples was made by Duncan's multiple range test. 'p' value of 0.05 or less was considered as significant.

RESULTS

1. Yield & total phenolic content (TPC) from methanolic extract of *Ixora coccinea*

Determination of total phenolic content of the methanolic crude extracts of *Ixora coccinea* were done by using Folin-Ciocalteu colorimetric method. This reagent is a mixture of phosphotungstic (H₃PW₁₂O₄₀) and phosphomolybdic acids (H₃PMo₁₂O₄₀). It is reduced during the oxidation of phenols in a mixture of blue oxides of tungsten and molybdenum. The color produced, whose absorption maximum is between 700 and 750 nm, is proportional to the amount of polyphenols present in plant extracts. The total phenolic contents were reported as mg gallic acid equivalent per gram of dry extract. On this plant, there are very few publications that are made regarding the levels of polyphenols and flavonoids. The results showed remarkably high total phenols content in the flowers of *Ixora coccinea* at a value of 30.6mg /g dry weight. Table 1 shows the yield and total phenolic content of methanolic residue obtained from *Ixora coccinea*.

Phytochemical screening

The preliminary phytochemical screening tests for the methanol extract of *Ixora coccinea* flowers (Table 2) revealed the presence of secondary metabolites like flavonoids, tannins and phenols in the extract.

In Vitro α- amylase & α- glucosidase Inhibition Assay

There was a dose-dependent increase in percentage inhibitory activity against α- amylase enzyme. At a concentration of 20µg/ml of plant extract showed a

percentage inhibition 23% and for 100µg/ml plant extract showed inhibition of 72% (Table 3). The methanolic extract of *Ixora coccinea* flowers revealed a significant inhibitory action of α- glucosidase enzyme. The percentage inhibition at 20-100µg/ml concentrations of *Ixora coccinea* flower extract showed a dose dependent increase in percentage inhibition. The percentage inhibition varied from 70% - 17% for highest concentration to the lowest concentration (Table 4).

Table 1: Yield & total phenolic content (TPC) from methanolic extract of *Ixora coccinea* Linn:

Solvent	Yield (g/kg dry weight)*	Total Phenolic content (mg/g dry weight)
Methanol	45	30.58

* Average of three extractions

Table 2: Phytochemical analysis of methanolic extract of *Ixora coccinea* flowers

Sl. No.	Phytochemical Constituents	Name of the Test	Methanolic Extract
1	Alkaloids	Mayer's Test Wagner Test	-
2	Flavonoids	NaOH test	++
3	Phenols	Ferric chloride	+++
4	Phyto sterols	Salkowaski Liebermann's Burchard Test	-
5	Saponins	Foam test	-
6	Tannins	Ferric chloride test	++
7	Carbohydrates	Molish Test Benedicts Test	+
8	Amino acids	Ninhydrin test	-
9	Anthraquinones	H ₂ SO ₄ /CHCl ₃ /Ammonia	-

+++ = High; ++ = Moderate; + = Present; - = Absent

Table 3: In vitro antidiabetic activity of methanolic extract of *Ixora coccinea* flowers by α-amylase method

Sl. No	Concentration of Sample (µg/ml)	% of Inhibition
1	20	23.08 ± 0.28
2	40	46.15 ± 0.85
3	60	53.85 ± 0.32
4	80	66.15 ± 0.62
5	100	72.31 ± 0.35

Values expressed as mean ± SEM.

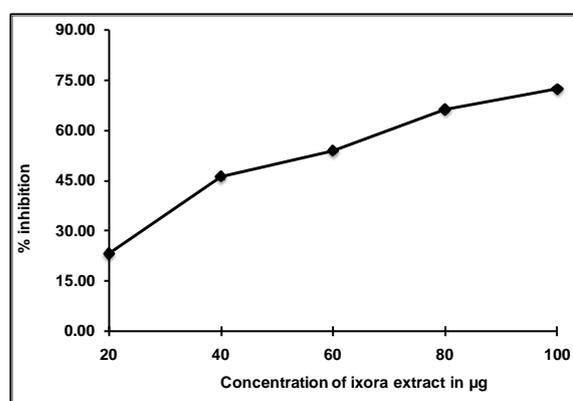
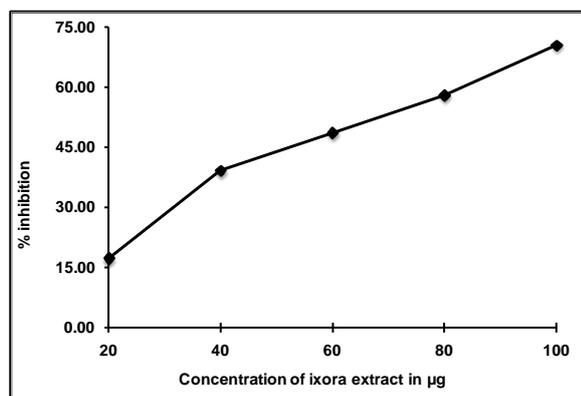


Figure 1: In vitro antidiabetic activity of methanolic extract of *Ixora coccinea* flowers by α-amylase method

Table 4: In vitro antidiabetic activity of methanolic extract of *Ixora coccinea* flowers by α -glucosidase method

Sl. No	Concentration of Sample ($\mu\text{g/ml}$)	% of Inhibition
1	20	17.19 \pm 0.65
2	40	39.06 \pm 0.35
3	60	48.44 \pm 0.62
4	80	57.81 \pm 0.54
5	100	70.31 \pm 0.42

Values expressed as mean \pm SEM.

**Figure 2: In vitro antidiabetic activity of methanolic extract of *Ixora coccinea* flowers by α -glucosidase method**

DISCUSSION

Diabetes mellitus is a metabolic disorder with increasing incidence throughout the world. Insulin is a key player in the control of glucose homeostasis. Lack of insulin affects carbohydrate, fat and protein metabolism [33]. Management of diabetes without side effects is still challenge to the medical community. It was proposed that inhibition of the activity of such alpha-amylase and alpha-glucosidase would delay the degradation of carbohydrate, which would in turn cause a decrease in the absorption of glucose, as a result the reduction of postprandial blood glucose level elevation [34]. In the present study, research has been carried out to evaluate the preliminary phytochemical investigation and the potential of methanol extract of *Ixora coccinea* flowers in inhibiting alpha-glucosidase and alpha-amylase.

The present finding of phytochemical screening of the plant extract confirmed the presence of several bioactive compounds like flavonoids, tannins and phenols which could be responsible for the versatile medicinal properties of this plant. Any of these secondary metabolites, singly or in combination with others could be responsible for the anti-diabetic activity of the plant. The present finding reveals that *Ixora coccinea* efficiently inhibits both alpha amylase and alpha-glucosidase enzymes *in vitro* in a dose dependent manner. The aqueous extracts from *Syzygium cumini* seeds and *Psidium guajava* leaves both showed a dose dependent inhibitory effect on alpha-amylase activity [35]. The antidiabetic action of *Psidium guajava* can also be attributed to the intestinal alpha-amylase and alpha glucosidase inhibitory activity.

In this present study we evaluated *in vitro* alpha amylase and alpha glucosidase activity of crude methanol extract of

Ixora coccinea flowers. The plant showed significant inhibition activity, so further the compound isolation, purification and characterization which is responsible for inhibiting activity, has to be done for the usage of antidiabetic agent.

CONCLUSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times.

In accordance with the results obtained from present study, it may be confirmed that the presence of phytoconstituents such as flavonoids, tannins and phenolics in the methanol extract could be responsible for the observed significant in antidiabetic activity. It is an easily available plant for natural remedies. Efforts are in progress to isolate and characterize the active principle, which is responsible for the antidiabetic activity of this valuable medicinal plant and further studies are required to establish the efficacy of the *I. coccinea* as an antidiabetic drug.

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