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Research Article

SCREENING OF PHYTOCHEMICALS AND *IN VITRO* ANTIOXIDANT ANALYSIS OF *IXORA MACROTHYRSA* (TEJISM. AND BINN.)

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ABSTRACT

Objective: The aim of the study was to investigate the phytochemical screening and quantitative and *in vitro* antioxidant analysis of the hydroethanolic leaf extract of *Ixora macrothyrsa* (Tejism. and Binn.)

Methods: The powdered plant material was extracted using different solvents for phytochemical screening. For the quantitative analysis, proteins, carbohydrates, tannins, free amino acids, and proline were estimated. *In vitro* antioxidants such as ascorbic acid, total phenols, chlorophyll, and carotenoids were also analyzed using standard methods.

Results: The preliminary qualitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* showed the presence of carbohydrates, proteins, alkaloids, flavonoids, phenols, saponins, glycosides, and tannins. The secondary metabolites such as proteins, carbohydrates, tannin, free amino acid, and proline were quantified as 24.57±0.61 mg/g, 11.07±0.09 mg/g, 6.17±0.20 mg/g, 9.20±0.17 mg/g, and 34.47±0.44 mg/g, respectively. *In vitro* antioxidants such as ascorbic acid, total phenols, chlorophyll, and carotenoid were estimated to be 35.73±0.28 mg/g, 15.9±0. mg/g, 9.33±0.09 mg/g, and 5.9±0.58 mg/g, respectively.

Conclusion: It can be concluded that the hydroethanolic extract of the leaves of *I. macrothyrsa* showed the presence of major secondary metabolites and has potent antioxidant activity.

Keywords: *Ixora macrothyrsa*, Hydroethanolic leaf extract, Phytochemicals, *In vitro* antioxidant, Secondary metabolites.

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INTRODUCTION

Phytochemicals are biologically active compounds present in plants which contribute for its color, aroma, and flavor. Phytochemicals protect the plant from various factors such as stress, pollution, ultraviolet (UV) exposure, and insects [1]. Phytochemicals assemble in various parts of the plants, and the pigment molecule gets accumulated in the outer layer of plant tissues [2]. These secondary metabolites defend humans against various diseases by stimulating the immune system [3]. The phytochemical constituents include alkaloids, flavonoids, tannins, phenolics, saponins, and several essential oils [4]. The bioactive substances from the plant are excellent sources of medicine that play a major role in human health against a large number of diseases [5].

Antioxidants protect humans against infectious diseases by scavenging the free radicals [6]. Antioxidants scavenge the free radicals by inhibiting the oxidation process [7]. Medicinal plants constitute a large variety of free radical scavenging molecules including phenols, flavonoids, vitamins, and terpenoids which are potent antioxidants [8]. Natural antioxidants from plant constituents are more effective in the prevention of oxidative stress [9]. Among the naturally occurring antioxidants, ascorbic acid, carotenoid, and phenolic compounds are much more effective than others [10]. Natural antioxidants are risk free and they are protective against viruses, cancer, and tumor [11].

Ixora is a genus of shrubs and short trees which comprises about 400 taxa. It belongs to the family Rubiaceae and subfamily Ixoroideae [12]. *Ixora* are grown in Indian garden which comprises of colored, beautiful flowers, and leaves [13]. The roots and flowers of the plant are involved in the treatment of dysentery, loss of appetite, nausea, and chronic ulcers [14]. Furthermore, the leaf of *Ixora macrothyrsa* has a potent antidiabetic property by lowering the blood glucose level [15].

Plant collection and authentication

I. macrothyrsa (Tejism. and Binn.) was collected from Coimbatore, Tamil Nadu. The plant was identified and authenticated by the Taxonomist, Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India, and the authentication number for the plant is BSI/SRC/5/23/2013-14/Tech/1417.

METHODS

Plant extraction and qualitative phytochemical analysis

The leaves of *I. macrothyrsa* were shade dried and grounded to a coarse powder by a mechanical device. The extract was prepared using different solvents, namely water, 50% ethanol, ethanol, acetone, benzene, petroleum ether, and chloroform, by cold maceration process. The filtrate was then used for the preliminary phytochemical analysis. The phytochemical analysis was carried out for the presence of phytoconstituents using standard procedures [16].

Preparation of hydroethanolic extract

The leaves of *I. macrothyrsa* were soaked in 50% alcohol and macerated in cold for 3 days. Occasional stirring was done during maceration. The suspension was then filtered using a fine muslin cloth, and the residue was removed. The filtrate was taken in a round-bottomed glass flask, and the sample was evaporated to dryness at a low temperature in a rotary evaporator. Finally, dark brown-colored crystals obtained were then stored in an airtight desiccator. The crystals are then used to carry out quantitative analysis and *in vitro* antioxidant study.

Quantitative analysis of phytochemicals

Determination of protein

Weighed 100 mg of crude plant extract and homogenized with 5.0 ml of ice-cold phosphate buffer. The sample was centrifuged at 2000 rpm for 5 min. Supernatant was separated and added equal volume of

10% ice-cold trichloroacetic acid to precipitate protein. The tube was incubated for 10 min at 4°C for 1 h. The precipitated protein was centrifuged and the supernatant was discarded. The pellet was then dissolved in 1.0 ml of 0.1 N NaOH. 0.5 ml of the protein solution was taken and mixed with 5.0 ml of alkaline copper reagent. The tube was shaken well and allowed to stand at room temperature for 10 min. Protein standard was taken in different aliquots and added 5.0 ml of alkaline copper reagent. A separate tube with distilled water and 5.0 ml of alkaline copper reagent served as a blank. Then, added 0.5 ml of Folin–Ciocalteu reagent to all the tubes and the final volume was made up to a known quantity using distilled water. The tubes were then incubated for 30 min in the dark, and the optical density of the solution was read at 660 nm [17].

Determination of carbohydrate

100 mg of plant sample was taken in a boiling tube. The sample was then hydrolyzed by adding 5.0 ml of 2.5 N HCl in boiling water bath and cooled to room temperature. The reaction mixture was neutralized by adding sodium carbonate and made up to 100 ml with distilled water and centrifuged. Supernatant was collected and 1.0 ml of sample was taken for analysis. Standards were prepared and 0.2–1.0 ml of the working standard was taken. 1.0 ml of water serves as a blank. To all the tubes, the volume was made up to 1.0 ml with distilled water. 4.0 ml of anthrone reagent was added. Tubes were heated for 8 min in a boiling water bath. Cooled rapidly and green color obtained was read spectrophotometrically at 630 nm [18].

Determination of tannin

About 500 mg of crude plant crystals was taken in a 250 ml conical flask containing 75 ml of distilled water. The flask was boiled in a boiling water bath for 30 min and transferred to separate tube. The tube was centrifuged at 2000 rpm for 20 min. The supernatant was collected in 100 ml volumetric flask and made up to a known volume. 1.0 ml of the plant extract was transferred to a 100 ml volumetric flask containing 75 ml of distilled water. To this, 5.0 ml of Folin-Denis reagent and 10 ml of sodium carbonate solution are added and made up to 100 ml with distilled water. The flask was shaken well and incubated for 30 min, and the absorbance was read at 700 nm against a reagent blank (water) [19].

Determination of proline

About 100 mg of crude plant sample was taken and homogenized with 0.5 ml of 3% aqueous sulfosalicylic acid. The tube was then centrifuged at 12,000 g for 10 min. To 1.0 ml of the homogenized tissue, added 1.0 ml acid-ninhydrin and 1.0 ml of glacial acetic acid and incubated for 1 h at 100°C, and the reaction was terminated in an ice bath. The reaction mixture was extracted by the addition of 2.0 ml of toluene. The contents were mixed vigorously and incubated at room temperature for 30 min. The chromophore containing toluene was warmed and its optical density was measured at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve using D-proline [20].

Determination of total free amino acid

Nearly 100 mg of dried plant crystals was taken in a tube. The sample was homogenized with 5.0 ml of 80% ethanol and centrifuged at 2000 rpm for 10 min. The supernatant was discarded, and the pellet was reextracted with the same solvent and centrifuged again. The supernatant were pooled. To the supernatant, an equal volume of petroleum ether was added to remove the chlorophyll pigments using separation funnel. The lower layer was taken as sample. Added 0.5 ml of acetate buffer to the 1.0 ml of alcoholic extract, followed by the addition of 1.0 ml of 1% ninhydrin solution. The reaction mixture was heated in a boiling water bath at 100°C for 15 min. The color developed on the tube was then cooled. The volume was made up to 10 ml with distilled water. 0.5 ml distilled water with all the reagents was served as a blank. The color intensity was measured at 570 nm [21].

***In vitro* antioxidant analysis**

Determination of ascorbic acid

Extraction

About 3 g of air-dried powdered sample was ground with 25 ml of 4% oxalic acid, and the extract was filtered. 10 ml of the filtrate was taken in a conical flask. To remove the ethanolic hydrogen atoms, bromine water was added drop by drop with constant stirring until the filtrate turned orange–yellow. The excess of bromine was removed by blowing in the air. Using 4% oxalic acid solution, the final volume was made up to 25 ml and it was used as the source material.

Estimation

2.0 ml aliquots of the above extract were pipetted into each of the different test tubes and the volume was made up to 3 ml with distilled water. 1.0 ml of 2% 2, 4-dinitrophenylhydrazine reagent was added followed by 1 or 2 drops of 10% thiourea to each test tube. The contents were mixed and kept at 37°C for 3 h. Added 7.0 ml of 80% sulfuric acid to dissolve the orange–red osazone crystals, and the absorbance was measured at 540 nm in UV-visible spectrophotometer against a reagent blank. The ascorbic acid content present in the sample was calculated using ascorbic acid as standard with the help of standard graph [22].

Determination of total phenol

1.0 mg of plant extract was taken in a dry test tube. Added 0.5 ml of Folin–Ciocalteu reagent and allowed to stand for 15 min followed by the addition of 1.0 ml of 10% sodium carbonate solution. Finally, the mixtures were made up to 10 ml with distilled water and allowed to stand for 30 min at room temperature. Total phenols present in the plant extract were determined spectrophotometrically at 760 nm. The reaction mixture without sample was used as blank. Total phenol content of leaf extract was determined using gallic acid as a standard with the help of standard graph [23].

Determination of chlorophyll

0.5 g of sample was taken in a clean mortar and was macerated with the addition of 5.0–15 ml of 80% acetone. The contents were centrifuged at 5000 rpm for 5–8 min. The supernatant was taken in a 100 ml volumetric flask. The residue was again ground with 5.0–15 ml of 80% acetone and again centrifuged. The supernatant was then transferred to the same volumetric flask. The procedure was repeated until the residue became colorless. The mortar and the pestle were washed thoroughly with 80% acetone, and the supernatant was taken to the same volumetric flask. Finally, the volume was made up to 100 ml by adding 80% acetone. The absorbance of the solution was measured spectrophotometrically at 645 nm and 663 nm against reagent blank (80% acetone) [24].

Calculation

The amount of chlorophyll of the extract as mg chlorophyll gram tissue will be:

$$\text{Total Chlorophyll}\left(\text{mg/g}\right)=20.2\left(A_{645}\right)+8.02\left(A_{663}\right)\times\frac{V}{1000\times W}$$

Where

A=Absorbance of specific wavelengths

W=Fresh weight of the tissue extracted.

Determination of carotenoids

About 1 g of sample was taken and extracted with 100 ml of 80% methanol solution. The reaction mixture was centrifuged at 4000 rpm for 30 min. The supernatant was concentrated to dryness. The residue was then dissolved in 15 ml of diethyl ether. Added 15 ml of 10% methanolic Potassium hydroxide solution, and the contents were washed with 5% ice-cold saline water to remove alkali. The free ether extract was dried over anhydrous sodium sulfate for 2 h. The ether extracts were filtered, and its absorbance was measured at 450 nm using ether as blank [25].

RESULTS AND DISCUSSION

Phytochemical analysis

The preliminary phytochemical analysis of the hydroethanolic leaf extract of *I. macrothyrsa* revealed the presence of carbohydrates, proteins, alkaloids, flavonoids, phenols, steroids, saponins, tannins, and glycosides as shown in Table 1.

The extraction of various phytochemicals was more efficaciously done in polar solvents such as acetone, ethanol, and water than the non-polar solvents. Furthermore, the hydroethanolic leaf extract of *I. macrothyrsa* under the study showed the presence of a maximum number of the tested phytochemicals. Hence, it can be reported that the hydroethanolic leaf extract of *I. macrothyrsa* was the best one for extracting the active principle when compared to other solvents.

Quantitative phytochemical analysis

The quantitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 24.57±0.61 mg/g of protein (Fig. 1). Protein is the most important primary compound with regard to the human body. Since they act as building blocks, it involves in repairing the tissues in our body. Hence, for building body or muscles, a high protein diet is recommended. The body makes use of proteins for energy production, when it lacks the presence of carbohydrates and fats [26].

The quantitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 11.07±0.09 mg/g of carbohydrate (Fig. 1). Carbohydrates are important for controlling immunological recognition, in defense against pathogens, protein folding and placement. Since carbohydrate in plants is considered as a third bioinformative macromolecule, it is used in determining the quality of food, fruits, and its production [27].

The quantitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 6.17±0.20 mg/g of tannin (Fig. 1). Tannin helps in the inhibition of fungi, yeasts, bacteria, and viruses. Tannins are rich in antioxidants and help in free radical scavenging activity, antimicrobial, gastroprotective, and antiulcerogenic activities. Moreover, tannin helps in the inhibition of lipid peroxidation in heart mitochondria and tends to possess anti-fibrotic effects [28]. Tannin helps to improve the digestive utilization of feed due to a reduced degradation of protein in the rumen and a subsequent increase in amino acid flow to the small intestine [29].

The quantitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 9.20±0.17 mg/g of proline (Fig. 1). Proline acts as an osmolyte and antioxidant and helps the plants to maintain the survival of cell turgor [30]. Proline is a proteinogenic amino acid with an exceptional conformational rigidity and it is important for primary metabolism [31]. Proline has been reported to enhance growth, physiological, biochemical, and anatomical characteristics and helps to improve antioxidant defense system in plants during salinity stress [32].

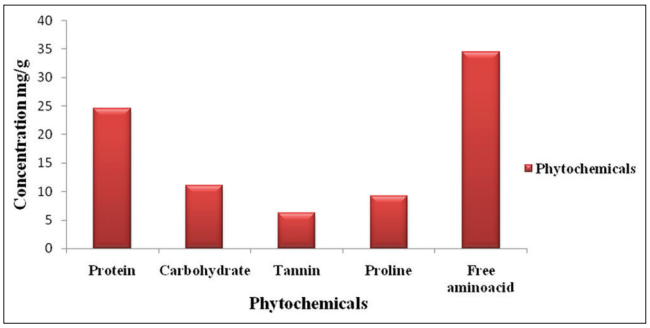

Fig. 1: Quantitative phytochemical analysis

Table 1: Qualitative estimation of phytochemicals

Test	Water	50% ethanol	Ethanol	Acetone	Benzene	Petroleum ether	Chloroform
Carbohydrates							
Fehling's test	+	+	+	+	–	–	+
Benedict's test	+	+	+	–	–	–	–
Molisch's test	–	–	–	–	+	+	+
Proteins							
Millon's test	+	–	+	–	–	––	–
Biuret test	+	+	–	–	+	+	–
Ninhydrin test	–	+	–	+	–	–	–
Steroids							
Salkowski's test	–	+	–	–	–	–	+
Thiols	–	–	–	–	+	–	+
Alkaloids							
Dragendorff's test	–	–	–	–	–	–	–
Wagner's test	–	+	+	–	–	–	–
Mayer's test	–	–	–	–	–	–	–
Flavonoids							
Alkaline reagent test	–	–	–	–	–	–	–
Zinc hydrochloride test	+	+	+	+	–	–	–
Shinoda test	+	+	+	–	+	+	–
Phenols							
Ferric chloride test	+	+	+	+	+	–	–
Lead acetate test	+	+	+	+	–	–	–
Saponins							
Foam test	–	+	+	–	–	–	–
Glycosides							
Legal's test	–	–	–	–	+	+	+
Keller–Kiliani test	+	+	–	–	–	–	–
Tannins							
Ferric chloride test	+	–	+	–	+	+	+
Lead acetate test	+	+	–	–	+	–	+

+indicates the presence of the compound and–indicates the absence of the compound

The quantitative analysis of the hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 34.47±0.44 mg/g of free amino acid (Fig. 1). Amino acid plays a major role in protein synthesis and in the formation of secondary metabolites that participate in cell signaling, gene expression, regulation of homeostasis, phosphorylation of proteins, synthesis of hormones, and antioxidant activity. Furthermore, amino acids involve in various physiological processes, namely formation of skeletal muscle, atrophic conditions, sarcopenia, and cancer [33].

***In vitro* antioxidant analysis**

Quite a high percentage of ascorbic acid has been detected in the leaves of the plant (35.73±0.28 mg/g) (Fig. 2). Ascorbic acid is a potent antioxidant involving in the flowering time of the plant, senescence, apoptosis, and responses to pathogens against diseases [34]. Due to its antioxidant activity, it impairs the production of free radicals involving the formation of intracellular substances including collagen, bone matrix, and tooth dentine [35]. Therefore, a clinical manifestation of scurvy hemorrhage from mucous membrane of the mouth and gastrointestinal tract, anemia, and pains in the joints can be related to the association of ascorbic acid and normal connective tissue metabolism [36].

The amount of phenol content in the hydroethanolic leaf extract of *I. macrothyrsa* was found to be 15.9±0.17 mg/g (Fig. 2). Phenols are important plant constituents because it has the ability to scavenge hydroxyl groups and may contribute directly to antioxidative action [37]. Phenols increases bile secretion, reduces blood cholesterol, and antimicrobial activity against some strains of bacteria such as *Staphylococcus aureus*. In addition to this, phenols have antiulcer, anti-inflammatory, cytotoxic, antitumor, antispasmodic, and antidepressant properties [38].

The hydroethanolic leaf extract of *I. macrothyrsa* was found to contain 9.33±0.09 mg/g (Fig. 2) of chlorophyll and 5.9±0.58 mg/g (Fig. 2) of carotene. Chlorophyll belongs to the class of primary compounds that capture sunlight and makes it available to plant system for its cultivation on photosynthesis [39]. Chlorophyll acts as an oxygenator. Deposition of metals and drugs is eliminated from the body by the purifying action of chlorophyll. Chlorophyll also helps in the prevention of constipation and attenuates the discomfort caused by gastritis [40]. Carotenoids act as photoprotective agent and may reduce the risk of sunburns, photoallergy, and even some types of skin cancer. It was proved that carotenoids have a positive role on the epithelialization process and influence the cell cycle progression of the fibroblasts [41].

CONCLUSION

The results obtained from the present study indicate that the leaves of *I. macrothyrsa* have a higher amount of phytochemicals and strong antioxidant activity. Therefore, the leaves may have the capability to scavenge the free radicals and reduce the risk caused by the free radicals. However, further studies are required to prove the free radical scavenging activity and isolation of other phytoconstituents in the plants.

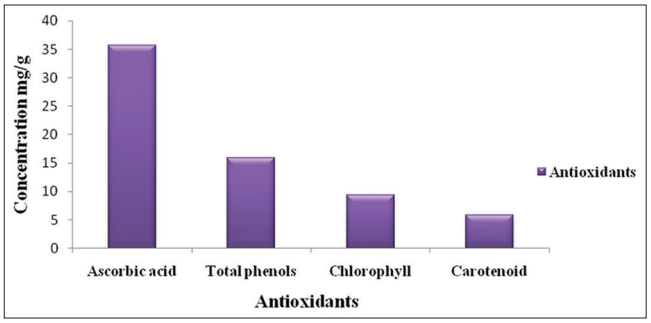

Fig. 2: Quantification of antioxidants

AUTHORS' CONTRIBUTIONS

The authors have contributed equally to this work.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this article.

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