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Phytochemical Evaluation and Screening of *in vitro* Antioxidant Potentiality of Extracts of *Ixora chinensis* Lam Leaves

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From *Ixora chinensis* Lam leaves, we obtained petroleum ether (60-80°), ethyl acetate and *n*-butanol extracts by soxhilation. The crude extracts obtained were tested for chemical identification of groups that may be the basis of the expected therapeutic effects. The preliminary phytochemical investigation led to the presence of flavonoids, triterpenoids, sterols, glycosides, carbohydrates, *etc*. *in vitro* antioxidant activity was evaluated using DPPH free radical scavenging assay, superoxide radical scavenging and nitric oxide methods and % inhibitions were calculated for all the methods.

Keywords: *Ixora chinensis* Lam leaves, Flavonoids, Antioxidant, DPPH, Super oxide ion scavenging.

INTRODUCTION

In humans, antioxidants exert important roles in preventing several degenerative diseases and stress related pathologies. The intake of antioxidant molecules can scavenge free radical species to defend the cells from damages. The antioxidant phytochemicals are also proved as protective against cardiovascular diseases and cancer, associated with overproduction of reactive oxygen species. Natural antioxidants are more safe and healthy than synthetic antioxidants [1].

The plants belonging to Rubiaceae family are generally a rich source of substances of phytochemical interest. Numbers of plants from this family were used in traditional system of medicine [2,3].

Ixora chinensis [4] is an ornamental flowering shrub that is found in southern China. It is widespread in Southeast Asian gardens and used to treat various ailments like rheumatism and wounds. Chinese *Ixora*, santan-pula, siantan are the common names in English. It is about 1 m tall and leaves are subsessile, 6-10 × 3-6 cm, oblong, elliptic, elliptic-obovate, entire and glabrous.

Phytochemical studies of some other species like *I. coccinea* [5-7], *I. finalaysonia* [8,9], *I. arborea* [10], *etc*., indicated the presence of important phytochemicals [11-13] such as lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, anthocyanins, proanthocyanidins, glycosides of kaempferol and quercetin. The great antioxidant activity indicates the potential of the extracts as a source of natural antioxidants or nutraceuticals with possible application to reduce oxidative stress with consequent health benefits. Therefore, the objectives of the present study were to investigate the phytochemicals and screening of *in vitro* antioxidant activity of *I. chinensis* leaves through the DPPH free radical scavenging, superoxide anion radical scavenging and nitric oxide scavenging methods.

EXPERIMENTAL

Silica gel (ACME Chemical works, Mumbai) was used for TLC. Silica gel of mesh size 60-120 (Merck, Mumbai) was used for column chromatography. All the solvents *e.g.*, pet. ether (60-80 °C), ethyl acetate, *n*-butanol, chloroform, methanol and ethanol (70 %) L.R. grade used for isolation. All the melting points were recorded in a Toshiwal electrically heated melting point apparatus and uncorrected. IR spectra of the compounds were recorded using Thermo Nicolet Nexus 670 I.R spectrophotometer. ^{1}H NMR spectra were taken on varian EM-360 (300 MHz) NMR spectrometer using $CDCl_3$ as solvent. ^{13}C NMR was recorded on Bruker instrument with $CDCl_3$ as solvent at 300 MHz. Mass spectra were recorded on a GC-MS, data on E:/ISO/21184-1.QGD.

The fresh leaves of *I. chinensis* Lam were collected from the local areas of Hyderabad, India and authenticated by P.

Venu, Additional Director, Office-in-charge, Botanical Survey India, Deccan Regional Center, Hyderabad, India. Voucher specimen (ICL/2013/22) was deposited at Malla Reddy College of Pharmacy, Dhullapally, Hyderabad, India.

Extraction of plant material: The shade dried leaves, pulverized and 200 g powder was extracted with petroleum ether (60-80 °C), ethyl acetate and *n*-butanol into 15 batches in Soxhlet extractor. After complete extraction, the different solvents were concentrated and finally dried under reduced pressure in a rotary flash evaporator. The residue (semi-solid) was dried in a dessicator and preserved. After drying the respective extracts were weighed and percentage yield were calculated (Table-1).

Preliminary qualitative chemical investigation: All the extracts were subjected to preliminary qualitative chemical investigation [14-16].

Salkowaski test: Dry extract of leaves (2 mg) was shaken with chloroform. To the chloroform layer, sulphuric acid was added slowly on the sides of test tube. Formation of red colour indicated the presence of sterols.

Liberman-Burchard's test: Dry extract of leaves (2 mg) was dissolved in acetic anhydride, heated to boiling, cooled and then 1 mL of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicated the presence of steroids.

Shinodas's test: In a test tube containing 0.5 mL of the extract of leaves, 10 drops of dilute hydrochloric acid was added, followed by a piece of magnesium. Formation of reddish brown coloured precipitate indicated the presence of flavonoids.

Liberman-Burchard's test: 2 mg of dry extract of leaves was dissolved in acetic anhydride, heated to boiling, cooled and then 1 mL of concentrated sulphuric acid was added along the sides of the test tube. Formation of violet coloured ring indicated the presence of triterpenoids.

Foam test: In a test tube containing about 5 mL of extract, a drop of sodium bicarbonate solution was added. The test tube was shaken vigorously and left for 3 min. Formation of honeycomb like foam/froth indicated the presence of saponins.

Molish's test: In a test tube containing 2 mL of the extract of leaves, 2 drops of freshly prepared 20 % alcoholic solution of α-naphthol was added. 2 mL of concentrated sulphuric acid was added, so as to form a layer below the mixture. Red-violet ring appeared indicating the presence of carbohydrates, which disappeared on the addition of excess of alkali with *n*-butanolic extract.

Benedict's test: To 2 mL of the extract of leaves, 5 mL of Benedict's solution was added and boiled for 5 min. Formation of a brick red coloured precipitate indicated the presence of carbohydrates.

Fehling's test: To 2 mL of extract of leaves, 1 mL of mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes. Formation of brick red coloured precipitate indicated the presence of reducing sugar.

Mayer's test: To a few drops of the Mayer's reagent, 2 mg of extract of leaves was added. Formation of no precipitate indicated the absence of alkaloids.

Wagner's test: 2 mg of leaf extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added. No precipitate was seen, which indicated absence of alkaloids.

Test for tannins: To 2 mL of the extract of leaves, few drops of 5 % w/v $FeCl_3$ solution was added. A green coloured solution indicated the presence of gallo tannins, while brown coloured solution indicated the presence of pseudo tannins.

Millon's test: 1 mL of ethanolic extract of leaves was dissolved in 1 mL of distilled water and 5-6 drops of Millon's reagent were added. Formation of white coloured precipitate, which turned to red on heating indicated the presence of proteins.

Biuret test: To 1 mL of hot extract of leaves, 5-8 drops of 10 % w/v sodium hydroxide solution was added, followed by 1 or 2 drops 3 % w/v copper sulphate solution. Formation of a violet red coloured solution indicated the presence of proteins.

Test for resins: 1 mL of extract of leaves was dissolved in acetone and the solution was poured in distilled water. No turbidity was seen which indicated the absence of resins.

Legal test: Leaf extract was dissolved in pyridine, sodium nitroprusside solution was added to it and made alkaline. Pink red colour was produced.

Baljet test: To the extract, sodium picrate solution was added. Formation of yellowish orange coloured solution indicated the presence of glycosides.

Borntrager test: Few mililitter of dil. sulphuric acid was added to the extract, boiled, filtered and then extracted the filtrate with ether or chloroform. To the separated organic layer, ammonia was added; violet colour was produced in organic layer, which indicated the presence of glycosides.

Keller Killani test: Sample was dissolved in acetic acid containing traces of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of the liquid, reddish brown colour was produced which gradually turned to blue, indicated the presence of glycosides.

The preliminary qualitative chemical investigation of petroleum ether, ethyl acetate and *n*-butanol extracts of leaves of *I. chinensis* Lam, was given in Table-2.

DPPH radical scavenging assay [17]: It is the most accepted model for evaluating the free radical scavenging activity of any new drug. 1,1-Diphenyl-2-picryl hydrazyl (DPPH) is a stable free radical which gives a maximum absorption at 517 nm (purple colour). When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour. More is the decolourization more is the reducing ability.

TABLE-1
NATURE, COLOUR, WEIGHT AND PERCENTAGE YIELD OF RESPECTIVE SOLVENTS EXTRACTS

S. No.	Extract	Nature of the extract	Colour of the extract	Weight (g)	Yield (%)
1	Petroleum ether (60-80°) extract	Sticky mass	Light green colour	44	0.87
2	Ethyl acetate extract	Sticky mass	Light green colour	39	0.78
3	*n*-Butanol extract	Sticky mass	Dark green colour	50	1.00

TABLE-2
PRELIMINARY QUALITATIVE CHEMICAL INVESTIGATION OF DIFFERENT SOLVENT EXTRACTS OF *Ixora chinensis* Lam LEAVES

S. No.	Chemical test	Petroleum ether	Ethyl acetate	*n*-Butanol
1	Sterols	+	–	+
2	Flavonoids	–	+	+
3	Triterpinoids	+	+	+
4	Saponins	–	–	+
5	Carbohydrates	–	+	+
6	Alkaloids	–	–	–
7	Proteins	–	–	–
8	Tannins	–	+	+
9	Resins	–	–	–
10	Glycosides	–	+	+

Note: '+' Present, '–' Absent

$$\text{Inhibition } (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 - absorbance of the control (blank, without extract) and A_1 - absorbance of the extract.

The effective concentration of sample required to scavenge DPPH radical by 50 % (IC_{50} value) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations [18].

Superoxide anion scavenging activity assay [19]: Superoxide anion was generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium and its absorbance was measured at 560 nm. The scavenging activity of various leaf extracts towards superoxide anion radicals was measured by colourimetric method [20]. The reaction mixture (200-1000 µg/mL) dilutions was made 1 mL with solvent, then 1 mL of PMS in phosphate buffer (0.1 M pH 7.4) and 1 mL of NBT(150 µM) in phosphate buffer. The reaction mixture was incubated (25 °C for 5 min) and absorbance was measured. The abilities to scavenge the superoxide radical and % inhibitions were calculated using the above formula.

Nitric oxide scavenging activity assay [21]: Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions [22]. 1 mL of 10 nM sodium nitroprusside solution was mixed with 1 mL of different extracts 200-1000 µg/mL in phosphate buffer (0.2 M pH 7.4). The mixture was incubated and then mixed with 1.0 mL of pre-prepared Griess reagent (1 % sulphanilamide, 0.1 % napthyl ethylenediamine dichloride and 2 % phosphoric acid). The absorbance was measured at 540 nm and the % inhibition was calculated using the same formula.

The nitric oxide radical scavenging activity was calculated according to the following equation:

$$\text{Inhibition } (\%) = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance of the extract.

RESULTS AND DISCUSSION

The results of qualitative chemical investigation of petroleum ether, ethyl acetate and *n*-butanol extracts of leaves of *I. chinensis* Lam, indicated the presence of following compounds listed in Table-3.

TABLE-3
PRELIMINARY PHYTOCHEMICAL ANALYSIS OF DIFFERENT EXTRACTS OF *I. chinensis* Lam LEAVES

Extracts	Chemical constituents
Petroleum ether (60-80 °C) extract	Sterols, triterpenoids
Ethyl acetate extract	Flavonoids, glycosides, sterols and carbohydrates
n-Butanolic extract	Glycosides, flavonoids, carbohydrates, triterpenoids, saponins and tannins

Antioxidant activity: The different concentrations of leaf extracts were tested for the antioxidant activity by DPPH, super oxide radical scavenging and nitric oxide methods and their IC_{50} values were calculated (Table-4).

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolourize in the presence of antioxidants. The DPPH radical contains as odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple colour. When DPPH accepts a Hydrogen atom donated by an antioxidant compound, the DPPH is decolourized which can be quantitatively measured from the changes in absorbance.

Superoxide anion scavenging activity is due to the reduction of the yellow dye (NBT^{2+}) to produce the blue formazon, which is measured at 560 nm. The decrease of absorbance indicates the high antioxidant power.

Nitric oxide scavenging activity is due to the ability of the extracts which scavenges the nitric oxide formed from the sodium nitrofrusside by inhibiting the chromophore formation, hence absorbance decreases as the concentration of the extracts increased [23].

The *n*-butanolic and ethyl acetate extracts showed significant antioxidant activity in all the methods compared with standard, whereas petroleum ether extract showed the moderate activity.

Conclusion

The results showed that *n*-butanolic and ethyl acetate extracts showed significant antioxidant activity in all the methods compared with standard, whereas the petroleum ether extract showed moderate activity. It can be concluded that *Ixora chinensis* Lam leaves possesses significant antioxidant activity and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants, which might be due to the presence of tannins, flavonoids and triterpenoids. These finding suggest that this plant is a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases such as cancer and various other human ailments. Further studies are required for the isolation and characterization of antioxidant components and also *in vivo* studies are needed for understanding their mechanism of action as a better antioxidant.

TABLE-4
FREE RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS FROM *Ixora chinensis* Lam LEAVES

Drugs	Concentration (µg/mL)	DPPH radical inhibition (%)	Super oxide anion inhibition (%)	Nitric oxide inhibition (%)
Pet. ether extract	50	11.97 ± 1.67	10.61 ± 1.26	13.06 ± 1.46
	100	22.24 ± 0.05	17.81 ± 0.58	24.41 ± 2.10
	200	44.90 ± 1.12	25.26 ± 2.11	52.45 ± 1.60**
	300	68.15 ± 0.93**	43.61 ± 1.48**	79.08 ± 2.20**
	400	85.36 ± 3.17**	62.19 ± 2.61**	87.32 ± 3.28**
	IC_{50}	230 µg/mL	348 µg/mL	194 µg/mL
Ethyl acetate extract	50	12.38 ± 1.06	10.22 ± 1.10	11.15 ± 1.94
	100	29.22 ± 1.41	17.45 ± 1.27	31.32 ± 1.04
	200	55.28 ± 1.61**	38.19 ± 1.25	59.10 ± 1.12**
	300	79.13 ± 2.42**	52.84 ± 0.56**	79.21 ± 1.91**
	400	93.16 ± 1.90**	80.13 ± 1.45**	92.05 ± 1.52**
	IC_{50}	186 µg/mL	261 µg/mL	126 µg/mL
Methanolic extract	50	13.36 ± 1.36	11.24 ± 1.14	14.14 ± 1.29
	100	28.26 ± 2.14	18.45 ± 1.72	30.36 ± 1.40
	200	56.25 ± 1.46**	39.13 ± 1.32	61.02 ± 1.72**
	300	80.14 ± 2.52**	54.24 ± 0.66**	82.31 ± 1.79**
	400	97.17 ± 2.40**	83.24 ± 1.36**	94.15 ± 1.50**
	IC_{50}	190 µg/mL	270 µg/mL	130 µg/mL
Ascorbic acid (Standard)	5	14.31 ± 1.20	12.38 ± 1.80	18.81 ± 1.72
	10	30.46 ± 3.09	20.68 ± 1.28	46.68 ± 0.62
	20	59.68 ± 1.38**	41.36 ± 1.82	64.26 ± 2.31**
	30	88.25 ± 1.49**	62.84 ± 1.82**	83.36 ± 1.28
	40	94.60 ± 2.30**	80.82 ± 2.18*	95.10 ± 2.16**
	IC_{50}	18 µg/mL	24 µg/mL	11 µg/mL

Values are mean ± SEM of 6 parallel measurements. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's test (n = 6). All the values are statistically significant at **P < 0.01.

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