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Phytochemical, Antimicrobial and Antioxidant Analysis of Indigenously used Folk Medicinal Plant *Ixora notoniana* Wall.

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Abstract

Ixora notoniana plant parts are traditionally used by Paliyars, who are indigenous tribal group of Tamil Nadu state of India, to treat various disorders such as tooth ache, skin and stomach problems. The present investigation aimed to search for an alternative antibiotic and antioxidant from traditionally used medicinal plant and to give ethnopharmacological evidence for its traditional usage. Phytochemical screening, antioxidant and antimicrobial studies were conducted with leaf, stem and root extracts. Methanol was used as solvent for the extraction process owing to its high extraction power and polarity. Preliminary phytochemical analysis with the extracts confirmed the presence of alkaloid, phenol, flavonoid and saponin in the extracts. Four bacterial species (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis)* and a fungal species *(Trychopyton simii)* were used for the evaluation of antimicrobial activities of the selected plant extracts using disc diffusion method. In antimicrobial studies, leaf extract exhibited antimicrobial activities against all the tested microorganisms in the range between 9.33 mm and 13 mm of inhibition zone, whereas the stem extract showed activities against *E. coli, S.aureus* and *T. simii* only. All the plant extracts were exhibited noticeable and dose dependent antioxidant activities in both DPPH and FRAP assays. Highest antioxidant activities were observed in leaf extract (43.91%) at 200 µg/ml concentration, root extract showed lowest activity (5.45%) at 25 µg/ml concentration. Oxidative stress is one of the instrumental factor in the stomach disorder and tooth and skin problems are associated with the bacteria and fungi.The present study observe the both antioxidant and antimicrobial activities in the extracts, hence the usage of plant as a remedy for stomach, tooth and skin problems could be justified.

Keywords: Paliyar Tribal, *Ixora notoniana*, antioxidant , antimicrobial activities, methanol.

Introduction

Medicines obtained from natural plant resources were used to treat various diseases since the beginning of human civilization. Medicinal plants are still used as source of medicines all over the world for the prevention and treatment of various diseases especially in third world countries, where many diseases are endemic and modern health care facilities are insufficient (1). Plant based medicines are emerging as paramount source of drugs because of their therapeutic properties and chemical and structural diversity (2). Medicinal plants possess various natural compounds such as peptides, alkaloids, essential oils, saponins, sterols, tannins, flavonoids and phenols. These

naturally occurring compounds are significant in therapeutic applications against human and animal pathogens like bacteria, fungi and viruses (3, 4). Natural compounds from plants possess wide range of biological effects such as antioxidants, antimicrobials, anti inflammatory and anti diabetic activities (5).

The indiscriminate use of commercial antimicrobial drugs for the treatment of infectious diseases leads to the development of drug resistant human and animal pathogens (6-8). Swift adaptation of microorganisms towards conventional antibiotics is said to be the reason for the emergence of multiple drug resistant microorganisms (9). Now the drug resistant microorganisms pose a biggest threat and challenge to the human beings. On the other hand, highly reactive free radicals know to be major cause of many diseases like chronic and degenerative diseases. Free radicals are generated throughout the metabolic processes, but our body have specialized mechanism to neutralize these highly reactive species. Antioxidants present in the nutritional source also play a role in the neutralization of free radicals (10-12). However, when the balance between free radical generation and neutralization decline, the excess radicals cause many life threatening diseases like cancer (13), atheroscelrosis (14) heart disease (15), diabetes (16), preeclampsia (17), Alzheimer's disease (18), celiac disease (19), aging (20) and Parkinson's disease (21).

Evolution of multiple drug resistant microorganisms and undesirable side effects caused by certain antimicrobial drugs and persistent trouble with free radicals prompted the continuous search for the alternate source of drugs from plants (22). Medicinal plants are used as conventional and alternative medicines in rural and tribal belt of India. These plants are serving as blue print for the development of many drugs. These complementary medicines are considered as effective with no adverse side effects. In the last few decades, many investigations are carried out with the folk medicinal plants to find out suitable source of medicines (23).

The presence of C6-C3-C6 skeleton in flavonoids is said to be the reason for its antimicrobial activities against wide range of pathogenic microorganisms (24). The alkaloids such as indole alkaloids undergo dimerization to exhibit antibacterial activity, which is possibly due to larger molecules of indole that are less prone to the bacterial efflux (25). Essential oils obtained from the *Elaeagnus umbellate* fruit exhibit noticeable antioxidant and antidiabetic activities (26). The presence of bioactive compounds such as phenolic, flavonoid and tannin in *Salvia officinalis* leaves are the reason for its antioxidant activities (27).

Approximately 120 life saving drugs, used for various diseases, are bioactive compounds, isolated from medicinal plants. These life saving drugs are reported to be present in about 6% of the total available herbal plants. The wealth of global flora and fauna are to be explored for the identification of core molecules, which can be used for the treatment of many fatal diseases like cancer, HIV and diabetes (2).

Ixora notoniana is a tropical plant belongs to the family Rubiaceae and indigenous to Western Ghats of India. The leaf, stem and root of the plants are used by the native Paliyar Tribal group, for the treatment of various disorders associated with skin, tooth and stomach (28). However, till date no research is documented on the phytochemical screening, antioxidant and antimicrobial properties of *I. notoniana*. Hence the present investigation is undertaken to examine the comparative phytochemical composition and the antibacterial and antioxidant activities of methanolic extracts of different parts (Stem, leaf and root) of *I.notoniana*.

Materials and Methods

Collection of plant material and authentication

I. notoniana is a large shrub and grows up to 4-5 meter. The trunk is covered with rough brown bark with short branches. Leaves are simple, opposite, decussate and broadly ovate. Inflorescence cymose and terminal, flowers sub sessile. Fruits are berry with two seeds.

The fresh samples from *I. notoniana* plants were collected from the Sathuragiri Hills, Virudhunagar District, Tamil Nadu, India during November – December 2018. The samples were properly identified by professional taxonomist. The properly prepared herbarium specimens were duly numbered and deposited (BOT-AAGAC – 06/2018) at Botany Department of Arignar Anna Government Arts College, Namakkal, Tamil Nadu State, India.

The collected samples were properly evaluated to ensure the quality according to the WHO guidelines (29) on herbal quality control. The samples were washed with clean water to remove dirt and foreign materials were manually separated with care. The stem and root samples were chopped into small pieces, approximately to the size of half centimeter, prior to the drying. Further, the samples were shade dried for 20-25 days. The well dried plant materials were ground with mixer grinder and sieved with 40 mesh size filter. The sample powders were properly labeled and refrigerated till further use.

Chemicals

All the used chemicals were of analytical grade and purchased from Himedia India Ltd.

Plant extraction

I. notoniana leaves, stem and root extracts were prepared according to scientifically accepted procedures using Soxhlet apparatus (30, 31). Methanol was used as solvent for the extract preparation, owing to its highest extraction power and polarity [32). A total of 50 g plant samples were extracted with methanol. The extracts were then filtered and dried using rotary evaporator. The dried extracts were properly labeled and refrigerated at 4°C.

Phytochemical screening

Crude extracts obtained with solvent methanol were screened to identify the presence of secondary metabolites such as alkaloid, phenol, flavonoid, glycosides and saponin by using standard phytochemical screening methods and procedures (33-37).

Antimicrobial studies

The antimicrobial activities of the extracts were evaluated with disk diffusion method. Four bacterial strains and one fungal strain were used. Among the four bacterial strains two were gram positive (*Bacillus subtilis*- MTTC 441, *Staphylococcus aureus*-MTTC 3160) and two were gram negative (*Escherichia coli*-MTTC 46*, Pseudomonas aeruginosa* - MTTC 1688*)* the sole fungal microorganism was *Trychopyton simii*. Loop full of stock cultures were taken and mixed with nutrient broth in sterile condition. The cultures were allowed to grow for 24 hours in shaker at the speed of 110 rpm. From the nutrient broth culture, strains were taken and spread on the prefilled petriplate, which contained solid Muller Hinton Agar medium. The dried test extracts at the concentration of 50mg were loaded on the sterile disc.

The sterile discs were placed on surface the petriplate and incubated for 24 hours at 37°C. Ciproflaxin 5µg per disc was used as control. For fungal organism potato agar medium was used for culturing. Fluconazole 25 µg/disc was used as control. The inhibition zone was measured at the end of experiment. The potential of the extracts against the pathogen is assessed by the presence or absence of inhibition zone in the culture medium. The diameter of the inhibition zone is considered as parameter for the evaluation antimicrobial activity (38). All the antimicrobial experiments were performed in triplicates and the results were averaged.

Antioxidant Studies

DPPH assay

The free radical scavenging activities of *I. notoniana* leaves, stem and root extracts on DPPH were determined using the method formulated by Blois [39]. In this method, the purple coloured DPPH get reduced by a hydrogen or electron donor and its colour changes into yellow. The plant extracts were serving as hydrogen donor and reduce the DPPH. The discolouration rate of DPPH is directly proportional to the ability of extract to donate hydrogen atom or electron (22). The bleached DPPH show maximum light absorption at 517 nm and could be measured with spectrophotometer. In this investigation, an aliquot of 0.5 ml of different concentration of sample solution was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The mixture was shaken vigorously and incubated for 37 min in the dark at room temperature. The absorbance was measured at 517 nm using UV spectrophotometer. Ascorbic acid was used as a positive control. All the experiments were performed in triplicates and results were averaged.

Iron chelating activity (FRAP)

FRAP assay (Ferric reducing antioxidant power) is widely used method to evaluate the antioxidant activity of plant based samples (40, 41). FRAP assay is ease to perform, fast and results generated from this method are linearly related to molar concentration of antioxidants. Antioxidants donate a single electron or hydrogen atom in the reaction for the reduction of Fe^{3+} to Fe^{2+}. Substance with higher antioxidant capacity gives higher FRAP values (42).

The method of Benzie and Strain (43) was adopted for the assay in this study. The principle is based on the formation of *O*-Phenanthroline-Fe2+ complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 25 to 500µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates and results were averaged.

Statistical Analysis

All the experiments were performed in triplicates and the data presented as means of $\pm$ SE. Two way ANOVA test was performed with P value <0.05 for DPPH and FRAP assay using Excel Stat (38, 44].

Results and Discussion

Phytochemical screening

The phytochemicals are considered as pillar of all traditional medicinal systems. Phytochemicals from plants possess wide range of biological effects such as antioxidants, antimicrobials anti inflammatory and anti diabetic activities (5). Phytochemical screening conducted with the extracts of *I. notoniana* indicated the presence of alkaloid, phenol, flavonoids and saponin (Table 1). The leaf extract showed the presence of four phytochemicals, whereas stem and root extracts exhibited the presence of only 3 and 2 phytochemicals respectively. Saponin was detected in all the three extracts. Phenols and flavonoids were detected only in leaf and stem extracts, whereas the presence of alkaloid was recorded only in leaf and root extracts. Glycoside was not recorded in any one of the three extracts.

Antimicrobial studies

Continuous evolution of multiple drug resistant microorganisms and undesirable side effects caused by certain antimicrobial drugs necessitated the search for the drugs from alternative source. Many investigations are carried out worldwide with plants to identify suitable alternative drugs (44). Medicinal plants used by rural and tribal traditional medicinal practitioners possess variety of compounds with known therapeutic properties (45, 46). The plant extracts or crude drugs are complex mixtures,

Table 1. Phytochemical composition of the stem, leaf and root of *I.notoniana* Wall.

S. No	Phytochemicals	Name of the test	Plant Parts		
			Stem	Leaf	Root
1.	Alkaloid	Mayer's	-	+	+
2.	Flavonoid	Alkaline Reagent	+	+	-
3.	Phenol	Ferric Chloride	+	+	-
4.	Glycoside	Legal's	-	-	-
5.	Saponin	Foam	+	+	+

(+) : Present (-) : Not detected

Table 2. Antibacterial activity of *I. notoniana* Wall**.**

S.No	Sample & Concetration / Disk	Name of microorganisms & Diameter of inhibition zone (mm)			
		E. coli	***P. aeruginosa***	***S. aureus***	***B. subtilis***
1.	Stem - 50 mg/ml	08.67 ± 1.20	-	08.33 ± 0.33	-
2.	Leaf - 50 mg/ml	12.67 ± 1.20	10.67 ± 0.88	12.00 ± 1.15	09.33 ± 0.88
3.	Root - 50 mg/ml	-	-	-	-
4.	Control - 5µg/ml	24.33 ± 1.20	18.33 ± 1.45	27.33 ± 0.33	27.00 ± 1.15

(-) : Not detected ; Results were expressed as mean ± SE ; Control (Positive) - Ciprofloxacin

Table 3. Antifungal studies of *I.notoniana* Wall.

S.No	Sample & Concetration / Disk	Name of microorganism & Diameter of inhibition zone (mm)
		T.simii
1.	Stem - 50 mg/ml	09.33 ± 0.88
2.	Leaf - 50 mg/ml	13.00 ± 0.57
3.	Root - 50 mg/ml	-
4.	Positive Control Flucanozole - 25µg/ml	18.67 ± 1.20

(-) : Not detected ; Results were expressed as mean ± SE

Table 4. DPPH antioxidant assay in leaf, stem and root of *I. notoniana* Wall.

S.No	Concentration of Sample (µg/ml)	Free radical scavenging activity (%)			
		Stem Extract	Leaf Extract	Root Extract	Ascorbic Acid
1.	25	07.91 ± 0.98	15.12 ± 0.51	06.81 ± 0.22	45.12 ± 0.86
2.	50	13.29 ± 1.21	22.63 ± 0.44	13.47 ± 0.66	55.64 ± 0.40
3.	100	21.09 ± 1.12	33.29 ± 0.77	19.41 ± 0.73	66.59 ± 0.44
4.	200	28.16 ± 0.66	43.91 ± 0.70	23.80 ± 0.73	79.37 ± 0.76

± - Standard Error of triplicates.
Difference between values are significant at $p<0.05$ with two way ANOVA test.

Table 5. FRAP assay of leaf, stem and root of *I.notoniana* Wall.

S.No	Concentration of Sample (µg/ml)	Iron chelating activity (%)			
		Stem Extract	Leaf Extract	Root Extract	EDTA
1.	25	06.74 ± 0.38	16.35 ± 0.67	05.45 ± 0.43	42.15 ± 0.98
2.	50	11.64 ± 0.67	25.13 ± 0.51	10.11 ± 0.82	60.43 ± 1.17
3.	100	17.52 ± 0.67	31.96 ± 0.77	14.23 ± 0.47	72.17 ± 1.17
4.	200	25.72 ± 0.68	42.15 ± 0.64	21.88 ± 0.82	77.88 ± 0.88

± - Standard Error of triplicates. Difference between values are significant at $p<0.05$ with two way ANOVA test.

containing large number of biologically active substances. Each of such substances play a specific role and they collectively function as single agent for the control of diseases (47). Plant based compounds play a significant role in the development of new medicines. It is estimated that 87% of currently used drugs are either directly or indirectly developed from the natural sources (48).

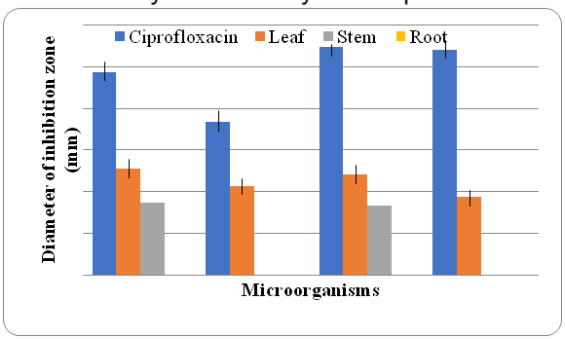

Figure 1. Antibacterial studies on the leaf, stem and root of *I.notoniana* Wall.

Disk diffusion method was used in the present study to evaluate the antimicrobial activities of extracts obtained from stem, leaf and root of *I. notoniana* plant. Disk diffusion method is cheap, flexible, visible and has shortened turnaround time. It is widely practiced in many laboratories all around the world and reported in multiple papers (49-52).

The results of antimicrobial activities of all extracts and standard antibiotics are furnished in Table 2 & 3. The leaf extract was more effective against all the four bacterial strains with mean inhibition zones of 12.67 mm, 10.67 mm, 12.00 mm and 9.33 mm in *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis* respectively (Figure 1). Stem extract was effective against *Escherichia coli* and *Staphylococcus aureus* with mean inhibition zones of 8.67 mm and 8.33 mm respectively. No inhibition zone is recorded with the root extracts in all the tested four bacterial and fungal strains.

The positive control Ciprofloxacin exhibited strong inhibition zone in all the tested microorganisms in the range of 18.33 mm (*P. aeruginosa*) to 27.33 mm (*S. aureus*). Results of antifungal studies with *Trychopytonsimii* also revealed similar effects, the diameter of inhibition zone was 13mm, 9.33 mm and 18.67 mm in leaf, stem and control Flucanozole respectively (Figure 2). Statistical analysis

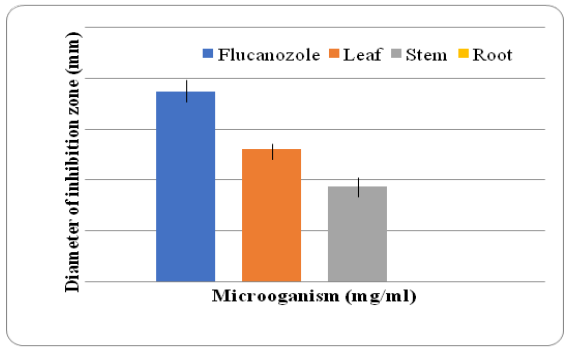

Figure 2. Antifungal studies on the leaf, stem and root of *I.notoniana* Wall.

with the antimicrobial studies indicated the significance difference between control, stem and leaf extracts, however, the activities of each extract with different microorganisms were not significantly different.

Phenols and flavonoids are proved to be effective antimicrobial, antioxidant and antimutagenic activities (53, 54). Saponin from plant source exerted diverse pharmacological properties including antimicrobial activities (55, 56). Priliminary phytochemical analysis with *I. notoniana* extracts indicated the presence phenols, flavonoids and saponins. The presence of such natural products in the extracts might be associated the exhibition of antimicrobial activities of extracts. The presence of phytochemicals in the plant extracts and their ability to inhibit bacterial and fungal strain could be interpreted with the usage of the *I. notoniana* as medicine for skin and tooth problem in the Paliyar medicinal system.

Antioxidant Studies

Oxidative stress caused by the constant generation of free radicals in the metabolic processes and insufficient intake of food based antioxidants necessitated the search for the drugs from alternative source. Plants are prominent source of natural antioxidants, because of the presence of various phytochemicals like phenolic acids, saponins, flavonoids and tocopherols (57). Plant based polyphenols, flavonoids are reported to be very effective in scavenging most oxidant molecules, including singlet oxygen and other free radicals (58- 60).

Antioxidant potencies of extracts were evaluated with DPPH and FRAP assays in the present study. Both assays are widely used, reliable, easy to perform, less time consuming and reproducible (41, 61-63).

All the three extracts (leaf, stem and root) scavenged the free radicals of DPPH in dose dependent manner (Table 4). The free radical scavenging of leaf extracts were statistically greater than the stem and root extracts. Among the three extracts, the highest scavenging activity 43.91% was observed in leaf extract at 200 µg/ml concentration, which was only half of the value of control ascorbic acid, the scavenging activity of control was 79.37% at the same concentration (Figure 3). Statistical analysis indicated that the difference between the types of extracts (leaf, stem and root) and extract concentrations were statistically significant.

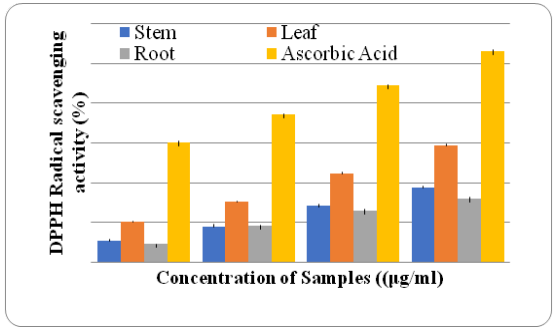

Figure 3. DPPH antioxidant assay in leaf, stem and root of *I. notoniana* Wall.

The results of FRAP analysis with different extracts is presented in table 5. All the extracts exhibited dose dependent reducing activity at all concentration ranging from 25µg/ml to 200 µg/ml. The ranking order for the reducing power was EDTA, leaf, stem, and root extracts (Figure 4). The highest reducing activity (42.15% at 200µg/ml) observed that in leaf extract whereas the chelating activity was 77.88% in the case of control EDTA at the same

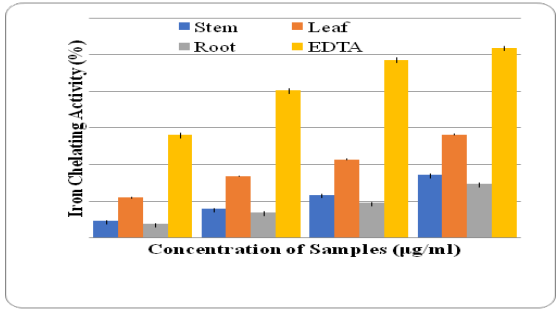

Figure 4. FRAP assay of leaf, stem and root of *I. notoniana* Wall.

concentration. The difference between the types of extracts (leaf, stem and root) and extract concentrations were statistically significant.

Lack of previous reports on antimicrobial and antioxidant studies of *I. notoniana* made the comparison of our results with those of previous studies difficult. The evaluation of antioxidant activities with DPPH and FRAP assays proved the ability of plant extracts to act as a natural antioxidants. In consistent with many earlier studies, all the extracts exhibited antioxidant activities in dose dependent manner (64-66]. However the intensity of activities of plant extracts remained below the positive controls (Ascorbic acid and EDTA). These results are similar to many previous studies (67, 68). In both antimicrobial and antioxidant studies, the leaf extracts were found to be more effective. The activities of leaf extracts were significantly higher than those of stem and root extracts. It is consistent with many previous studies (69, 70).

Oxidative stress is said to be the reason for many chronic and degenerative disorders and it is proved to be one of the instrumental factor in stomach disorder(71). Screening of *I. notoniana* extracts showed the presence of phenol and flavonoids, hence the capability of the plant to act as a natural antioxidant and the plants usage as remedy for stomach disorder by Paliyar Tribal could be understood with the present study.

Conclusion

Ixora notoniana is used by indigenous Paliyar tribal group for the treatment of problems associated with tooth, skin and stomach. The present investigation conducted to pharmacologically evaluate the extracts obtained from stem, leaf and root of the plant. Preliminary phytochemical analysis with the extracts confirmed the presence of alkaloid, phenol, flavonoid and saponin. Antioxidant and antimicrobial activities were conducted with the extracts. All the plant extracts exhibited noticeable antioxidant and antimicrobial activities. The presence of certain phytochemicals and pharmacological activities of the extracts provide some insight on the scientific evidence of the effectiveness of its traditional use. Further studies involving purification and isolation of the plant extract could provide an alternative medicine for many disorders.

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Conflicts of Interest

There are no conflicts of interest

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