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PHYTOCHEMICAL EVALUATION, *IN VITRO* ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF *INDIGOFERA ASPALATHOIDES*

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ABSTRACT

***Objective**: Phytochemistry is a study and exploration of metabolic products of plants possessing high medicinal values. The metabolites produced provide protection against microbial infections and infectious diseases. The inappropriate and indiscriminate use of antimicrobials has increased the prevalence of multidrug resistant microorganisms ushering the need for an alternate antimicrobial agent. Indigofera aspalathoides, a common shrub belonging to Fabaceae family was preferred for its phytochemical constituents was enumerated have not been evaluated its antioxidant and antibacterial potential. **Methods**: The preliminary screening of I.aspalathoides extract was validated using solvent extraction to check for the presence of flavonoids, alkaloids, glycosides, polyphenolics, tannins and terpenoids using various assays specific for each compound. The radical scavenging activity of the extract was performed using DPPH assay and the percent inhibition was calculated. The human pathogenic bacteria were used for assay by well diffusion method against five MTCC strains. **Results**: The results indicated a reasonable antibacterial potential and significant antioxidant activity, thus supporting its traditional medicinal practices. In free radical scavenging assay, 63.51% inhibition was observed in methanolic extract compared to other extracts. The antibacterial assessment of different extracts was tested against Gram-positive and Gram-negative bacteria using well diffusion method. Among the various extracts, methanol extract showed maximum inhibition against Acinetobacter baumannii (21%), followed by Escherichia coli(14.94%), Staphylococcus aureus (13.63%)and Klebsiella pneumonia (12.35%). The least activity was observed in Pseudomonas aeruginosa (8%). **Conclusion**: Thecrude extract of I.aspalathoides contains severalbioactive phytochemicals that possessed medicinal properties such as antioxidant and antibacterial activities. The active ingredient should be subjected to further purification so as to responsible may further be serve as an alternative drug of choice.*

KEY WORDS

Antioxidant, Antibacterial, Ferrous ion chelation, Indigofera aspalathoides,Phytochemicals.

INTRODUCTION

Medicinal plants remain a source of inspiration for novel drug compounds as plant therapy have made large contributions to human health. Their role is twofold in the development of new drugs: either they serve as a base material for the development of new drugs or a phytomedicine for the treatment of many diseases. Over thousands of years Nature is an impressive source of medicinal properties and a number of plant based drugs were isolated from natural sources [1] (I wu, 1993). Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of diseases. More attention is being paid to the protective effects of natural antioxidants against drug-induced toxicity whenever free radicals are generated (Guptaet al.,

2004). Ayurvedha (traditional medicinal systems)which was followed in India describes many medicinal plants for curing different ailments of human because of their medicinal properties and using plant extracts in traditional medicine continues to provide health coverage for over 80% of the world's population, especially in the developing world (Geberet al., 2002, WHO 2002). Since then efficacy of many medicinal plants in the treatment of many diseases have been put to test in many laboratories (Shajahan et al., 2004).

Recently scientific interest towards medicinal plants has burgeoned due to the increased efficiency of plant derived drugs and raising concern about the side effects of modern medicine (Balandrin et al., 1985). In the traditional medicinal system, the plant *Indigofera aspalathoides* was used for the treatment of leprosy, cancer and edematous tumors (Kirtikar et al., 1975). The decoction of leaves and flowers was used for treating various types of skin rashes. The root was used in preparing medicated oil and externally applied for scabies, leprosy etc. The medicated oil prepared from its root was also given internally as powder for the treatment of leprosy, dermatitis and various forms of ulcers. The plant is mostly shrubs, though some are herbaceous and a few could grow up to 5-6 feet in height. The dye, indigo which is among the most widely used natural dye in the world is obtained mainly from the leaves through a process of fermentation (Murugesa Mudaliar).

Many efforts have been made to discover novel compounds from various sources such as microorganisms, animals, and plants; one such richest resource is folk medicines. Systematic screening of herbals may result in the discovery of novel effective compounds (Tomoko et al., 2002). The present study was undertaken to evaluate the bioactive phytochemical constituents of *Indigofera aspalathoides* and to investigate its antioxidant and antibacterial potential.

MATERIALS AND METHODS

Plant collection and authentication

The medicinal plant *Indigofera aspalathoides* was collected from Government Siddha Medical College campus, Arumbakkam, Chennai, Tamilnadu, India in a sterile polythene bag. Morphological characters of the selected plant were recorded at the time of plant collection in the field notebook by using hand lens and it was authenticated by the Chief Botanist, Tamil Nadu Aromatic and Medicinal Plants Corporation Limited (TAMPCOL) at Government Siddha Medical College, Arumbakkam, Chennai, India. The herbarium No.784 was maintained in Madras University Botany Laboratory at CAS in Botany, University of Madras, Tamil Nadu, India.

Preparation of plant extract

Coarsely pulverized plant powder of *I. aspalathoides*, 100 g was successively extracted with the various polar solvents (hexane, chloroform, ethyl acetate and methanol) in a Soxhlet apparatus until the solvent becomes colorless in the timble. Total yield of the extract was noted and the extracts were concentrated under reduced pressure 'in vacuo'and

stored at 4ºC until use. The concentrated extracts were used for assaying phytochemical constituents, antioxidant property and antibacterial activity (Akowuah et al., 2005).

Preliminary screening for phytochemicals

The preliminary screening for phytochemicals from chloroform and ethyl acetate extract of *I. aspalathoides* was carried out using standard methods of (Harborne JB, 1984) standard procedure.

DPPH Free Radical Scavenging Assay

The extracts were tested for their antioxidant property *in vitro* by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and the results were compared with Butylated hydroxyanisole (BHA) which serves as a standard. Percent inhibition was calculated by following equation (Leong and Shui et al., 2002).

Percent inhibition = O.D of blank – O.D of sample /O.D of blank) ×100

Determination of chelating effects of ferrous ion

The chelation of ferrous ions was estimated by method of (Dinis *et al*., 1994). Briefly 50µl of 2mM $FeCl_2$ was added to 1 ml of different concentrations of the extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. Absorbance was measured at 562 nm. The percentage inhibition of ferrozine Fe^{2+} complex formation was calculated and Na_2EDTA was used as positive control.

$$\text{Metal chelating \%} = \frac{A_O - As}{Ao} \times 100$$

A_0 = Absorbance of control; As = Absorbance of sample

Antibacterial activity – Agar well diffusion

Antibacterial activity of chloroform, ethyl acetate and methanol extract of *I. aspalathoides* was determined using agar well diffusion method. The most commonhuman pathogenic bacteria were obtained from IMTECH, Chandigarh.The bacterial cultures such as *Acinetobacter baumannii* (ATCC 17978), *E. coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27583) and *Staphylococcus aureus* (ATCC 25923) were maintained in Mueller Hinton Agar (MHA) slants and used prior to assay.

Preparation of inoculum

The obtained bacterial isolates were cultured on nutrient agar and incubated at 37ºC for 24h and the microorganisms were repeatedly sub cultured in order to obtain pure isolation. Morphological and biochemical reactions were carried to ascertain proper identification. They were inoculated into nutrient agar slants and stored at 4ºC. Overnight broth culture of the respective bacterial strains was adjusted to turbidity equivalent to 0.5 McFarland standards. (0.2 ml culture of organisms was dispensed into 20 ml sterile nutrient broth and incubated for 24 h and standardized at 10^5-10^7 CFU/ml adjusting the optical density to 0.1 at 600 nm.

Antibacterial activity of *I. aspalathoides* was determined using the agar well diffusion assay method .The human pathogenic bacteria namely *Acinetobacter baumannii* (ATCC 17978), *E. coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27583) and *Staphylococcus aureus* (ATCC 25923)were procured from the Microbial type culture collection (MTCC) center, IMTECH, Chandigarh, India. When the media solidified, 0.1 ml of active growth culture was poured over feeder layer and spread evenly by sterile spreader. The 6 mm diameter well was made by using a sterile cork borer. Each well received different concentration (50, 100 and 150 µg/ ml) of crude extract. They were dissolved in 0.4% DMSO (Dimethyl sulfoxide). Appropriate control was maintained. They were incubated at 37ºC for 48 h. After incubation the inhibition zone was measured by millimeter.

RESULTS

The preliminary phytochemical screening of the entire plant (*I. aspalathoides*) revealed the presence of alkaloids, anthraquinones, flavonoids, glycosides, polyphenol, tannins, triterpenes and terpenoids. Flavonoids have chemo-preventive role in cancer through the induction of enzyme affecting carcinogen

metabolism. The therapeutic potential of the plant could be considered owing to the presence of these bioactive constituents present (**Table 1a**).

Table 1a: Screening for phytochemical constituents of *I. aspalathoides*

S. No	Constituents	Dichloromethane	E. Acetate
		Inference	Inference
1.	Alkaloids	-	+
2.	Anthraquinones	+	-
3	Flavonoids	+	+
4	Glycosides	+	+
5	Phytosterol	-	-
6	Polyphenol	+	+
7.	Tannins	+	+
8.	Terpenoids	+	-
9.	Triterpenes	+	+
10.	Sterols	-	-

Table 1b: Quantitative phytochemicals present in Dichloromethane extract of *I. aspalathoides*

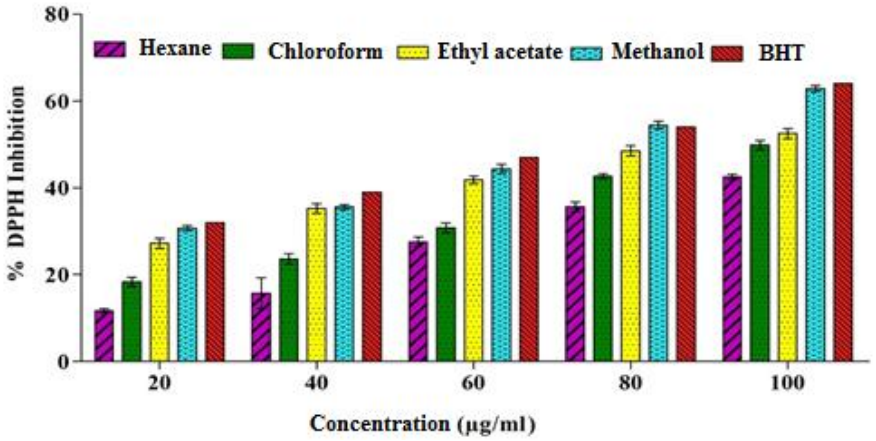
S. No	Constituents	Quantity (mg/g)
1	Alkaloids	9.75
2	Carbohydrate	250.6
3	Phenolic compounds	2.99
4	Saponins	0.993
5	Steroids	13.44
6	Tannins	8.04
7	Triterpenoids	8.33

Hence, this plant provides a significant role in the prevention and treatment of various diseases and compounds were found to be responsible for many pharmacological activities.

The remarkable contribution of plants to the drug industries was possible, because of the large number of phytochemical and biological studies all over the world. Crude preparations of whole plant parts (containing both the active and non-active components) have been suggested to have higher efficacy than semi-crude or pure plant substances (Sanaa et al., 2007). At that stage, study of the interactions between active and non-active components may throw even more light onto the differential activity of various extracts (Kafaru et al., 1994). Synergistic study of the interaction of active phytocompounds with antibiotics was required to exploit the potential of plant extracts in the combination therapy of infectious diseases caused by multi drug-resistant organisms (Iqbal Ahmad et al., 2001).

DPPH Radical Scavenging Activity

Fig 2. Free radical scavenging assay using DPPH



DPPH, a stable free radical (deep violet colour), react with the antioxidant substance and convert it to 1, 1-diphenyl, 1-2-picryl hydrazine with decolouration. The scavenging effects of hexane, dichloromethane, ethyl acetate and methanol extracts from *I. aspalathoides*on DPPH radicals was found to be increased with an increase in concentration from 20–100 µg/ml, the scavenging activity ranged from 12.15 to 43.12%**,** 19.30 to 50.90%**,** 28.35 to 53.66% and30.18 to 63.51%. The results indicated that hexane, dichloromethane, ethyl acetate and methanol extracts, showed poor, moderate and good activities. Among the extracts, methanol showed higher activity than ethyl acetate, dichloromethane and hexane. The order of DPPH radical scavenging assay was found to be methanol > ethyl acetate > dichloromethane > hexane.

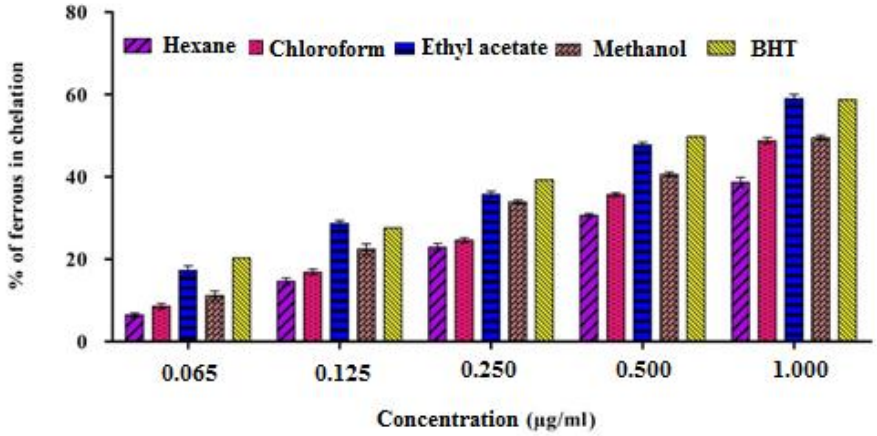
DPPH is a stable, violet colored and nitrogen centered free radical. Conversion of free radical from violet to yellow color by electron or hydrogen donating ability of the antioxidant compound found in the extract (Michalaket al., 2006). Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's diseases, mongolism, aging process and perhaps dementias. Flavonoids and Flavones are widely distributed secondary metabolites with anti-oxidant and antiradical properties (Oliveiraet al., 2009). Therefore, an approach with multiple assays for evaluating the anti-oxidant potential of extracts would be more informative and even necessary. The results should be encouraged in future *in vivo* studies, which could ultimately lead to the application of these medicinal plants in pharmaceutical formulations.

Ferrous ion chelation ability

In this assay, the chelating agents disrupt the ferrozine-Fe^{2+} complex, thus decreasing the red colour. The antioxidant compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ions before the formation of ferrozine.

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\cdot}OH$

Fig 3: Ferrous Ion Chelation of *I. aspalathoides* at different concentrations



The chelating activity of the hexane, dichloromethane, ethyl acetate and methanol extracts of *Indigofera aspalathoides* was done at five different concentrations (0.063, 0.125, 0.250, 0.500, and 1.000 µg/ml) and EDTA was used as reference standard for ferrous ions. The strongest chelating effect (72.22%) was obtained with ethyl acetate extracts at 1.0 mg/ml. At this concentration, the lowest chelating effect (49.56%) was exhibited by the dichloromethane extract. Among all the concentration, 0.065 to1000 µg/ml showed significantly higher activity. One of the mechanisms of anti-oxidative action is chelation of transition metals, thus preventing catalysis of hydroperoxide decomposition and fenton type reactions (Awadh et al., 2002, Makari et al., 2008). Alzheimer's and Parkinson's diseases are one of the lines of treatments currently under investigation is selective low affinity binding of transition metals (Augustin et al., 2005). Therefore, if the plant extract displays a mild chelating activity *in vivo* then it can be of therapeutic potential in the treatment of diseases.

Antibacterial activity (Well diffusion method)

Table 2. Antibacterial activity of *I. aspalathoides* against Clinical pathogens

Extract	Zone of inhibition (mm)														
	A. baumannii µg/ml			*E. coli*			*K. pneumonia*			*P. aeruginosa*			*S. aureus*		
	50	100	150	50	100	150	50	100	150	50	100	150	50	100	150
DCM	10	11	13	7	9	11	-	8	9	7	8	10	-	8	9
E. acetate	10	12	15	8	10	12	7	8	10	8	10	11	8	10	12
Methanol	12	15	18	7	9	13	-	9	11	-	8	13	8	11	11

Dcm**– Dichloromethane,** E.acetate **- Ethyl acetate**

The antibacterial activity (Table 2) result of dichloromethane, ethyl acetate and methanolic extract of *I. aspalathoides*revealed concentration-dependent activity against all the tested pathogens with the zone of inhibition ranged from 12-18 mm at various concentrations. Methanolic extract responded as well for the antibacterial activity against Gram-negative bacteria (12-18 mm) than the dichloromethane extract (6-9 mm). Ethyl acetate extract showed moderate activity against Gram positive bacteria *S. aureus*with zone of inhibition while Gram negative bacteria (*A. baumannii*) showed higher activity with 10-15 mm diameter.

The preliminary results of this investigation appear to indicate that number of Indian medicinal plants have high potential of antimicrobial activity. The plants demonstrating broad spectra of activity might help to discover new chemical classes of antibiotics that

could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases.

The phytochemical metabolites detected in this study, namely, alkaloid, tannin, saponin, steroidal aglycon and cardiac and cynagenetic glycosides have been associated with the antimicrobial activities of several herbs (Gordon et al., 1990). Therefore there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen local medicinal plants for possible antimicrobial properties (Leven et al., 1975). The better therapy for many microbial diseases can be found in the bark, leaves of hitherto neglected plants.

CONCLUSION

The results exposed antioxidant potential of *I. aspalathoides*, one of the commonly used medicinal plants, which help protect against lipid peroxidation and free radical damage. The bacteriostatic effect induced by the extract is found obvious with the presence of active ingredient with no side effects when compared to the commonly used synthetic chemotherapeutic agents. The investigation verified that the traditional use of *I. aspalathoides* for human ailments partly explained its use in herbal medicine as one of the rich source of bioactive phytochemicals (alkaloids, anthraquinones, flavonoids, glycosides, polyphenols, sterols, tannins, terpenoids, and triterpenes). However, further study on chemical constituents and their mechanisms in exhibiting certain biological activities are needed to understand the full phytochemical profile and the complex pharmacological effects of this plant.

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