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IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACTS OF LEAVES OF *INDIGOFERA INDICA* AND STEMS OF *STEREOSPERMUM SUAVEOLENS* GROWN IN SRI LANKA

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Keywords:

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ABSTRACT

Oxidative stress due to free radicals plays a fundamental role in pathogenesis of several human diseases. Accordingly, wide search is operative to discover novel and potent antioxidants to be used as prophylactics and therapeutics, and also in food and cosmeceutical industries. Thus, the aim of this study was to evaluate the antioxidant capacity of leaves of *Indigofera indica* (Family: Leguminosae) and stems of *Stereospermum suaveolens* (Family: Bignoniaceae) grown in Sri Lanka. Oven dried (at 40°C), powdered leaves and air dried stems were extracted to methanol by cold extraction technique and their antioxidant activities were evaluated *in vitro* using four direct antioxidant assays [1,1-diphenyl-2-picrylhydrazyl (DPPH)], radical scavenging activity: 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity: ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) and two indirect antioxidant assays [total polyphenolic content (TPC) and total flavonoid content (TFC)]. The results showed that both extracts exhibit moderate antioxidant activity [DPPH assay, leaves Vs stem: 11.53 ± 0.50 Vs 13.46 ± 0.22 mg Trolox equivalent /g; ABTS assay, leaves Vs stem 156.66 ± 12.87 Vs 146.14 ± 3.15 mg Trolox equivalent /g, FRAP assay, leaves Vs stem 11.76 ± 3.93 Vs 13.59 ± 4.36 mg Trolox equivalent /g; ORAC assay, leaves Vs stem 198.83 ± 6.09 Vs 209.61 ± 16.10 mg Trolox equivalents /g; TPC assay, leaves Vs stem: 193.45 ± 9.60 Vs 102.26 ± 6.04 mg Gallic acid equivalents /g, and TFC assay, leaves Vs stem: 22.56 ± 0.42 Vs 0.12 ± 0.04 mg Quercetin equivalents /g]. Further, the radical scavenging activities determined by DPPH and ABTS assays were dose dependent. These results establish, for the first time, that leaves of *I.indica* and stem of *S.suaveolens* grown in Sri Lanka are good source of natural antioxidants that may be effective against oxidative stress induced pathophysiology of several diseases.

1.0 INTRODUCTION

It is now well recognized that over production of reactive oxygen species causes oxidative stress¹. Oxidative stress plays a fundamental role in the pathogenesis of various human diseases such as coronary heart diseases, atherosclerosis, stroke, cancer, Alzheimer's and Parkinson's disease, renal disorders, dengue, malaria, leptospirosis or chickungunya^{2,3,4}. Reactive oxygen species contribute to normal ageing process and also known to play a role in premature ageing⁵. They are also involved in oxidative deterioration, nutritional losses, undesired flavour development and discolouration of food items, especially, those rich in polyunsaturated fats⁶.

Now, there is strong evidence that antioxidants, substances which quench free radicals (reactive oxygen and nitrogen species), offer health promoting, prophylactic and therapeutic actions against several diseases / disorders^{7,8}. Further, synthetic antioxidants such as 2,3-tert-butyl-4-methoxy phenol (BHA), 2,6-ditert-butyl-4-methyl phenol (BHT) and propyl gallate (PG) are widely used in the food industry, even though these have some serious concerns about their toxicity and carcinogenicity⁹. Accordingly, there is an imperative need and demand to search for new sources of safe and inexpensive antioxidants, preferably from plant sources.

Thus, the aim of this study was to evaluate the *in vitro* antioxidant potential of leaves of *Indigofera indica* L (Family: Leguminosae, Nilawari in Sinhala, Asidii in Tamil) and stems of *Stereospermum suaveolens* (Roxb) DC (Family: Bignoniaceae, Palol in Sinhala, Ambu in Tamil) which are either used singly or in combination with other herbs in Sri Lankan traditional and folk medicine to treat several diseases^{10,11} which are thought to be mediated, at least partly, by oxygen free radicals^{12,13,14,15}. What is more, the antioxidant potential of Sri Lankan variety of these two botanicals has not been investigated *hitherto* and it is worth examining since plant metabolites can vary significantly and hence their therapeutic potentials vary significantly, both quantitatively and qualitatively within a species due to genetic, geographic, environmental and climate difference³, conditions of drying and storage and the type of solvent used for extraction³.

2.0 MATERIALS AND METHODS

2.1 Collection and Authentication

Few branches from a mature shrub of *I.indica* were plucked from a home garden at Beliatta (GPS coordinates: 6° 2' 0" North, 80° 45' 0" East) southern province Sri Lanka, in October

2014. Air dried pieces of mature stems of *S.suaveolens* were purchased from the drug outlet of Wickramarchci Ayurveda Institute in Gampaha district Sri Lanka, in October 2014. The leaves of *I.indica* was identified by Emeritus Prof. (Mrs) A. Senaviratne , Department of Plant Sciences , University of Colombo , Sri Lanka . The stem has been identified by the Pharmacognosist / Purchasing officer of the drug outlet. Voucher specimens of leaves of *I.indica* (CS / 04 / 2014) and pieces of stem of *S.suaveolens* (CS/03/2014) were deposited at the Department of Medical Laboratory Sciences at General Sir John Kotelawala Defence University, Sri Lanka.

2.2 Chemicals and Equipments

Folin-Ciocalteu reagent , gallic acid , quercetin, 6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPHH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABST), potassium persulphate, 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), sodium fluorescein and 2,4,6-tripyridyl-s-triazine(TPTZ) were purchased from Sigma Aldrich (USA) . All the other chemicals used in preparation of buffers and solvents were of analytical grade . All the analyses were carried out using High-throughput-96-well microplate readers (SpectraMax Plus384, Molecular Devices, USA and SPECTRAMax-Gemini EM, Molecular Devices Inc , USA).

2.3 Preparation of methanolic extracts of leaves of *I.Indica* and stems of *S.suaveolens*

The leaves of *I.indica* were washed thoroughly in distilled water and were oven dried at 40°C for 2 days. The dried leaves and dried pieces of stem were crushed and powdered and converted into a powder using an electrical grinder. Two point five grams (2.5 g) of powdered leaves and stem bark were dipped separately in methanol (250 ml) and left overnight at room temperature (30°C). The resulting extracts were filtered and centrifuged at 500 rpm for 5 minutes. Filtrates were separately concentrated in vacuum and freeze dried. Freeze dried stem and leaf extracts were used for evaluation of *in vitro* antioxidant activity.

2.4 Total polyphenolic content (TPC) of stem and leaf extracts

The TPC of bark and leaf extracts were determined (n=6) with Folin-Ciocalteu reagent using 96 well microplates as described by Singleton *et al.* 1999¹⁶. Twenty microliters (20 µl) of 1 and 0.5 mg/ml of stem and leaf extracts were added to 110 µl of ten times diluted freshly prepared Folin-Ciocalteu reagent. Seventy microliters (70 µl) of sodium carbonate solution was added to the mixture and incubated at room temperature (30 ± 2°C) for 30 minutes and absorbance was recorded at 765 nm . Five different concentrations of gallic acid (1,0.5, 0.25,

0.12 and 0.06 mg/ml) were used to construct the standard curve. TPC of stem and leaf extracts were expressed as mg of gallic acid equivalents per gram of extract of stem and leaf.

2.5 Total flavonoid content (TFC) of stem and leaf extract

Total flavonoid content of stem and leaf extracts (n=3) were determined by aluminium chloride method using 96 well micro plates as described by Siddhuraju and Becker¹⁷ . One hundred microlitres of 2 % aluminium chloride in methanol solution was added to 100 µl of 2 mg / ml stem and leaf extracts in methanol. The mixture was incubated at room temperature (30 ± 2°C) for 10 minutes and absorbance was recorded at 367 nm. Pre –plate reading was recorded before adding the aluminium chloride solution. Five different concentrations of quercetin (125, 62.5 , 31.25, 15.62 and 7.81 mg/ml) were used to construct the calibration curve . TFC of stem and leaf extracts were expressed as mg quercetin equivalents per gram of extract of stem and leaf.

2.6 Ferric reducing antioxidant power (FRAP) of stem and leaf extracts

The assay was carried out according to the method of Benzie and Szeto¹⁸ with some modifications in 96 well micro titre plates (n=6). The working FRAP reagent prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM FeCl₃.6H₂O in a ratio of 10:1:1 just before use and heated to 37°C .The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. Reaction volume of 200 µl containing 150 µl working FRAP reagent , 30 µl acetate buffer and 20 µl of 2 and 1 mg/ml of stem and leaf extracts were incubated at room temperature (30 ± 2°C) for 8 minutes and absorbance was recorded at 600 nm . Five different concentrations of Trolox (1, 0.5, 0.25, 0.12 and 0.06 mg/ml) were used to construct the standard curve. The results were expressed as mg of Trolox equivalents per gram of extract of stem and leaf.

2.7 DPPH radical scavenging activity of stem and leaf extracts

The DPPH radical scavenging assay was performed according to the method described by Blois¹⁹ using 96 well micro titre plates. Reaction volumes of 200 µl , containing 125 µM of DPPH radical and 50 µl of different concentrations of stem and leaf extracts (stem and leaf extracts:5, 2.5, 1.25, 0.625, 0.3125 mg/ml) were incubated at 30 ± 2°C for 15 minutes and absorbance was recorded at 517 nm. Five different concentrations of Trolox (20 , 10, 5, 2.5 and 1.25 µg/ml, n=3) were used to construct the standard curve. Results were expressed as Trolox equivalents antioxidant capacity in mg of Trolox equivalents per gram of extract of stem and leaf.

2.8 ABTS⁺ radical scavenging activity of stem and leaf extracts

The ABTS⁺ radical scavenging assay was performed according to the method described by Re *et al*²⁰ in 96 well micro titre plates. A stable stock solution of ABTS radical cation was prepared by reacting 10 mM of ABTS in potassium persulfate at 37°C for 16 hrs in dark. Reaction volume of 200 µl, containing 40µM of ABTS⁺ radical and 50 µl of 200 and 100 µg/ml of stem and extracts were incubated at 30 ± 2°C for 10 minutes. The absorbance was recorded at 734 nm. Five different concentrations of Trolox (50, 25, 12.5, 6.25, and 3.12 µg/ml , n= 3) were used to construct the standard curve. The results were expressed as Trolox equivalents antioxidant capacity in mg of Trolox equivalents per gram of extract of stem and leaf.

2.9 Oxygen radical absorbance capacity (ORAC) of stem and leaf extracts

The ORAC radical scavenging assay was performed according to the method described by Ou *et al*²¹ with some modifications in 96 well micro titre plates (n=3). The assay was conducted at 37°C and pH 7.4, with a blank sample in parallel. Trolox standards (1.5, 0.75 µg/ml), fluorescein (4.8 µM) and AAPH (40 µg/ml) solutions were prepared prior use in phosphate buffer (75 mM, pH 7.4). Stem and leaf samples were initially dissolved in DMSO and the DMSO concentration of the assay in blank and samples was 0.125 µg/ml. Reaction volume of 200 µl , containing 100 µl of 4.8 µM fluorescein and 50 µl of 50 µg/ml stem and leaf extracts were pre incubated at 37°C for 10 minutes followed by the addition of 50 µl of AAPH (40 mg/ml) to each well to initiate the reaction. The plate was placed on the fluorescent microplate reader (SpectraMax Plus384, Molecular Devices USA and SPECTRAMax-Gemini EM, Molecular Devices Inc , USA) set with excitation and emission at 494 nm and 535 nm and decay of fluorescein was recorded in 1 minute intervals for 35 minutes. Trolox was used as a standard antioxidant. ORAC activities of the samples were calculated by comparing net area under curve of fluorescein decay between blank and the samples. The results were expressed as ORAC values in mg of Trolox equivalents per gram of extract of stem and leaf.

2.10 Phytochemical analysis

The methanolic extracts of leaves of *Lindica* and stem of *S.suaveolens* were subjected to qualitative tests for flavonoids, polyphenols, tannins, alkaloids, terpenoids, 2-deoxy sugars and steroids²².

2.11 Statistical Analysis

Data is given as Mean \pm Standard Deviation of mean (SD). The extract concentrations for 50% inhibition (IC₅₀ value) and dose dependencies (by Pearson's correlation test) were determined. Statistical comparisons (by ANOVA followed by Tukey's Post Hoc test) were made using Statistical Analysis System (SAS) package. Significance was set at $p < 0.05$.

3.0 RESULTS

The results accrued with different *in vitro* antioxidant assays are summarized in Tables 1, 2 and 3.

Table 1: *In vitro* antioxidant activities of methanolic leaf extract of *Indigofera indica* and stem extract of *Stereospermum suaveolens* in different assays. (mean \pm SD)

Sample	Anti-oxidant Capacity					
	TPC (mg gallic acid equivalents/ g of extracts of leaf /bark)	TFC (mg quercetin equivalents/ g of extracts of leaf /bark)	FRAP (mg Trolox equivalents/ g of extracts of leaf /bark)	ORAC (mg Trolox equivalents/ g of extracts of leaf /bark)	DPHH (mg Trolox equivalents/ g of extracts of leaf /bark)	ABTS (mg Trolox equivalents/ g of extracts of leaf /bark)
Leaf	193.45 \pm 9.60 ^a	22.56 \pm 0.42 ^a	11.76 \pm 3.93 ^a	198.83 \pm 6.09 ^a	11.53 \pm 0.50 ^a	156.37 \pm 13.32 ^a
Bark	102.26 \pm 6.04 ^b	0.12 \pm 0.04 ^b	13.59 \pm 4.36 ^a	209.61 \pm 16.10 ^a	13.46 \pm 0.22 ^a	126.79 \pm 13.87 ^a

Data represented as mean \pm SD. TPC: Total polyphenolic content (n=6), TFC: Total flavonoid content (n=3), FRAP: Ferric reducing antioxidant power (n=6), DPHH, ABTS, ORAC (n=3). Values in column superscripted by different letters within a column are significantly different at $p < 0.05$.

Table 2: DPHH radical scavenging activities of different concentrations of methanolic leaf extract of *Indigofera indica* and stem extract of *Stereospermum suaveolens*.

% Radical scavenging activity.		
Concentration (μ g/ml)	Leaf	Bark
1250	98.60 \pm 0.61	98.10 \pm 1.00
625	67.47 \pm 4.43	73.67 \pm 0.81
312.5	36.70 \pm 2.63	46.54 \pm 2.36
156.25	18.47 \pm 1.75	24.81 \pm 0.41
78.125	4.73 \pm 2.13	11.57 \pm 0.64

Data represented as mean \pm SD. Leaf and stem extracts (n=3).

Table 3: ABTS radical scavenging activities of different concentrations of methanolic leaf extract of *Indigofera indica* and stem extract of *Stereospermum suaveolens* in different assays.

% Radical scavenging activity.		
Concentration (μ g/ml)	Leaf	Bark
125	95.26 \pm 0.95	93.99 \pm 0.83
62.5	68.9 \pm 2.18	66.35 \pm 6.31
31.25	42.47 \pm 3.12	36.08 \pm 4.04
15.62	23.76 \pm 0.81	25.38 \pm 3.20
7.81	11.00 \pm 0.23	12.37 \pm 1.28

Data represented as mean \pm SD. Leaf and stem extracts (n=3).

As shown in Table 1, both methanolic extracts of leaves of *I.indica* and stem extract of *S.suaveolens* exhibited moderate antioxidant activity in five antioxidant capacity determination assays (TPC, FRAP, ORAC, DPHH and ABTS) whilst only the leaf extract of *I.indica* showed moderate antioxidant activity in TFC assay. Further, the antioxidant activity

of leaf extract of *L.indica* was significantly ($p < 0.05$) higher than the stem extract of *S.suaveolens* in TPC (by 89.2%), and TFC (by 187%) assays. On the other hand, no significant ($p > 0.05$) difference in antioxidant activities between leaf extract of *L.indica* and stem extract of *S.suaveolens* was evident in FRAP, ORAC, and ABTS assays.

As shown in Tables 2 & 3, the antioxidant activity of both leaf extract of *L.indica* and stem extract of *S.suaveolens* were dose dependent in DPHH assay (leaf: $r^2= 0.99$, $p < 0.05$ and stem : $r^2= 0.98$, $p < 0.05$) and ABTS radical assays (leaf: $r^2= 0.99$, $p < 0.05$ and stem : $r^2= 0.98$, $p < 0.05$). In the DPPH assay, IC_{50} value for leaf extract of *L.indica* was 456.85 ± 20.26 $\mu\text{g/ml}$ whilst the IC_{50} value for stem extract of *S.suaveolens* was 390.93 ± 6.52 $\mu\text{g/ml}$. In this assay, IC_{50} for Trolox , reference agent, was 5.04 ± 0.14 $\mu\text{g/ml}$. In contrast, IC_{50} values for leaf extract of *L.indica* and stem extract of *S.suaveolens* in ABTS radical scavenging assay were respectively 36.81 ± 3.00 $\mu\text{g/ml}$ and 45.56 ± 5.13 $\mu\text{g/ml}$. Further, in this assay IC_{50} for Trolox was 5.04 ± 0.14 $\mu\text{g/ml}$.

Phytoconstituent analysis revealed the presence of alkaloids (Primary, Secondary, Tertiary N function and quaternary amine base) flavonoids, tannins, terpenoids and 2-deoxy sugars in both leaf extract of *L.indica* and stem extract of *S.suaveolens*. Moreover steroids and saponins were found to be absent in both leaf and stem bark extracts.

4.0 DISCUSSION

Generation of free radicals is a continuous process in living systems^{1,2}. This study examined the *in vitro* antioxidant capacity of leaves of Sri Lankan variety of leaves of *L.indica* and stem of *S.suaveolen* using methanolic extracts and four direct antioxidant assay models: DPHH radical scavenging , ABTS radical scavenging, ORAC (Oxygen Radical Absorption Capacity) and FRAP (Ferric Reducing Antioxidant Potency) . In addition, TPC (Folin–Ciocalteu assay) and TFC (Total Flavonoid Content) assays were also used to determine antioxidant activity; these are now considered as indirect antioxidant assays since significant association between TPC and TFC and antioxidant capacity of plant extracts have been shown^{3,23}. Interestingly, antioxidant activities of both leaf and stem extracts were dose dependent in ABTS and DPHH radical scavenging assays (dose response studies were performed only in these two assays).

Methanol was selected for extraction since most of the antioxidant phytoconstituents of medical herbs have been extracted either to methanol or to aqueous methanol^{3,13,15}, even though the ideal way is to use sequential extraction procedure using solvents of different

polarity³. We employed six assay models to evaluate the antioxidant capacities of the two extracts: it is recommended to use multiple assays in the evaluation of antioxidant capacities of foods, dietary supplements and medicinal herbs because using one assay is considered as an over simplification and inappropriate since different reactive oxygen species have different reactive mechanisms³. Further, assay methods measure different characteristics of the putative antioxidant^{3,16,17,18,19,20,21}.

All the assay techniques selected are accurate, validated, sensitive, reproducible assay models which are now widely being used to determine antioxidant potential of herbs^{6,16,17,18,19,20,21}. Further, these assays are primarily based on redox reactions which falls into two broad categories^{23,24,25}: hydrogen atom transfers based assays / HAT assays and single electron transfer reaction based assays / SET assays^{24,25}. ORAC model belongs to the former category and ABTS, DPPH, FRAP, TBC, and TFC belong to the latter category^{24,25}. Hence, the results reported are valid and meaningful and likely to be widely accepted. In contrast, other studies on antioxidant activities of *I.indica* and stem extract of *S.suaveolens*, have used only one or two antioxidant assay models^{13,14,15}.

The results of this study, convincingly show, that the both methanolic leaf extract of *I.indica* and stem extract of *S.suaveolens* have moderate *in vitro* antioxidant activity in comparisons to known natural antioxidants used in experimental studies; namely gallic acid (in TPC assay), quercetin (in TFC assay) and Trolox, water soluble form of vitamin E (in FRAP, ORAC, DPPH and ABTS assays)^{18,19,20,21}. This is a novel finding for the Sri Lankan variety of *I.Indica* leaves and *S.suaveolens* stems which indicates their potential as prophylactic and therapeutic agents against several diseases. However, the overall potency of leaf extract of *I.Indica* appears to be higher than that of stem of *S.suaveolens*. The presence of moderate antioxidant activity in *I.Indica* leaves and *S.suaveolens* stem is a desirable feature of therapeutic importance since strong antioxidants are reported to act as pro-oxidants under certain conditions^{26,27}.

The antioxidant activity of both extracts in ABTS assay was found to be substantially higher than in DPPH assay. Such differences are also reported with other natural products as well. For example, with sap and treacle of Sri Lankan variety of *Caryota urens*²⁸ and leaf and stem extracts of *Cinnamomum zeylanicum*²⁹ Steric inaccessibility³⁰ and solubility differences³⁰ of different phytoconstituents could account for the observed difference in antioxidant activity in these two assays.

Polyphenols including flavonoids and tannins and phenolic diterpenes are known to possess potent antioxidant activities³¹. Both extracts had high values for TPC and TFC assays and phytochemical profile revealed the presence of flavonoids, phenolics and terpenoids. Obviously, these phytoconstituents could precipitate the antioxidant activity observed in this study, possibly via synergistic interactions and multiple mechanisms. Supportive evidence for this notion is that antioxidant activity was evident in both extracts when determined by several assays which have different reaction mechanisms^{23,24,25}.

5.0 CONCLUSION

In conclusion, this study shows for the first time, that the leaves of *I.Indica* and stem bark of *S.suaveolens* (two medicinal plants used in Sri Lankan traditional medicine) exhibits moderate antioxidant activities *in vitro*, mediated via multiple mechanisms. Additionally, the findings may rationalize their use in Sri Lankan traditional medicine for several diseases and also their ability as potential antioxidants in food and cosmeceutical industry.

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