



Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading

Evaluation of phytochemical constituents and antioxidant activities of successive solvent extracts of leaves of *Indigofera caerulea* Roxb using various *in vitro* antioxidant assay systems

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ARTICLE INFO

Article history:

Received 15 June 2012

Received in revised form 27 June 2012

Accepted 18 October 2012

Available online 28 October 2012

Keywords:

Indigofera caerulea Roxb

Free radicals

Phytochemicals

Flavonoids

Reducing power

Scavenging activity

ABSTRACT

Objective: To evaluate the phytochemical constituents and antioxidant activities of successive solvent extracts of *Indigofera caerulea* Roxb using various *in vitro* antioxidant assay systems.

Methods: Total phenol and antioxidant activity of different solvent extracts of *Indigofera caerulea* Roxb leaves were investigated. Extraction was done sequentially in soxhlet apparatus using various solvents (Petroleum ether, Ethyl acetate and Methanol). Antioxidant activity was evaluated by 2, 2-diphenyl-1-picryl hydrazyl free radical scavenging assay, hydroxyl radical scavenging assay, superoxide anion radical scavenging assay and Total ion reducing power assay. Total phenol and flavonoid contents were also measured. **Results:** Methanolic extract had more total phenol content and more antioxidant activities, confirming to the hypothesis that phenol content and antioxidant activity has a direct correlation. **Conclusions:** All the results of the *in vitro* antioxidant assays revealed that the methanolic extract of *Indigofera caerulea* Roxb leaves had notable antioxidant and free radical scavenging activity. The results obtained appeared to confirm the antioxidant and free radical scavenging potential of *Indigofera caerulea* Roxb.

1. Introduction

Free radicals are chemical species which contains one or more unpaired electrons. They are highly reactive and unstable compounds produced in the body during normal metabolic functions or introduced from an external source such as pollution, pesticides, UV radiation, cigarette smoking etc. or a failure in antioxidant defense mechanism. Human bodies are protected from oxidative damage of free radicals through some complex defense systems which are called antioxidants [1,2]. Antioxidant work to maintain the oxidant at optimum level and reduce free radicals. Examples of these radicals include hydroxyl, superoxide hydrogen peroxide and superoxide anion radicals. Excessive generation of these free radicals results in cellular stress that damages the lipid cells, proteins, DNA, cell membrane

structure and function [3]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced due to oxidative stress within the human body. Oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems [4]. Excessive amount of ROS is harmful because it can initiate bimolecular oxidation, which leads to various diseases such as cardiovascular diseases, liver injury, Alzheimer's diseases, diabetes, rheumatoid arthritis, aging and inflammation [5,6].

Antioxidants protect the human body from ROS effects. Antioxidants are considered as possible protective agents reducing oxidative damage by directly reacting with free radicals. Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intra cellular redox potential. Although, many antioxidants such as ascorbic acid, β carotene, Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) are in use but gradually invites concern because of many side effects and carcinogenicity

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[7,8]. Therefore, the interest has been increased worldwide considerably in searching for naturally occurring antioxidants to replace synthetic antioxidants because of their presumed safety, nutritional and therapeutic value [9]. Natural antioxidants and their radical scavenging potential have been reported in several ethnomedicinal plants. [10–12]

Indigofera caerulea Roxb belonging to the family Fabaceae is a shrub, distributed along the Eastern Ghats of Tamil Nadu, India. The leaf juice is administered orally to cure night blindness and Root juice is used as a cure for jaundice and epilepsy. The root powder and leaf paste are reported to treat jaundice. The leaves when taken internally along with *Leucus aspera* and pepper can treat snakebite and are used by the native medical practitioners and tribal people to treat various diseases such as jaundice, epilepsy, night blindness and liver diseases [13–15]. The aqueous and organic solvent extracts are reported to have antibacterial and pharmacognostical properties [16]. Although information pertaining to the pharmacological activities is revealed, investigations in sighting the presence of the compounds associated with these properties are unveiled. Hence, based on these supportive evidences, the present study was aimed at screening and quantifying the phytochemical involved the scavenging the free radicals in *Indigofera caerulea* Roxb.

2. Materials and methods

2.1. Plant collection and extraction

Fresh leaves of *Indigofera caerulea* Roxb belonging to family Fabaceae were collected during the morning hours of its flowering season, from the foothills of Pacchaimalai Hills, a part of Eastern Ghats of Tamil Nadu, India. The species identification was done by Botanical Survey of India, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India (Ref No. BSI/SRC/5/23/2010– 11/Tech.–1729). The leaves were separated, washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in airtight bottles.

2.2. Preparation of extract

The dried powder of the leaves was extracted sequentially [17] by Soxhlet apparatus [18], using different solvents depending upon their polarities which included petroleum ether, ethyl acetate and methanol. The extracts were concentrated and freed of solvent under reduced pressure, using a rotary evaporator. The dried crude concentrated extracts were weighed to calculate the extractive yield and stored in a refrigerator at 40 C in airtight bottles.

2.3. Chemicals

1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma–Aldrich

Co., St. Louis, USA) Rutin (Acros Organics, Newjersey, USA) Ascorbic acid, trichloroacetic acid, ethylenediamine tetra acetic acid, phosphoric acid, thiobarbituric acid (S.D. Fine Chem, Ltd., Biosar) India. 2-Deoxy-D-ribose, nitro blue tetrazolium (NBT), Phenazine methosulphate and NADH (Himedia, Laboratories Pvt. Ltd., India) Folin–Ciocalteu reagent and potassium ferricyanide (Qualigens Fine Chemicals, Glaxo Smithkline Pharmaceutical Ltd., India) were all used in the study.

2.4. Quantitative phytochemical analysis

The preliminary phytochemical screening of various successive solvent extracts of *Indigofera caerulea* Roxb was done to determine the presence of bioactive components. The presence of alkaloids, saponins, tannins, flavonoids, phytosterols, terpenoids, resins, steroids, proteins, cardiac glycosides were determined. The various solvent extract of *Indigofera caerulea* Roxb were tested for its *in vitro* antioxidant activity using the existing standard methods. In all these methods, a particular concentration of the extract or standard was used to give the final concentration of 20–160 μ g/ml by adding all the reagents. Absorbance was measured both for the control and the plant sample against the blank solution. Free radical scavenging activity was calculated in percentage; total phenolic content was calculated using the standard curve of gallic acid and total flavonoid content was calculated using the standard curve of quercetin.

2.5.1 Estimation of total phenol content

About 0.1 mL of the extract (10 μ g /mL) was mixed with 0.5 mL of Folin–Ciocalteu reagent (diluted 1:10 ratio with distilled water) and 1.5 mL of sodium carbonate. The resulting mixture was vortexed for 15 sec and incubated at 40^o C for 30 min for colour development. The absorbance of total phenolics was measured at 765 nm using Hewlett Packard, UV/visible light. Total phenolic content was expressed as mg/g gallic acid equivalent using the expression from the calibration curve $Y=0.0183x+0.101$, where $R^2 =0.9338$ where x is the absorbance and Y is the tannic acid equivalent in mg/g. The experiment was conducted in triplicates and the results were expressed as mean \pm SD values.

2.5.2 Estimation of total flavonoids

The amount of flavonoid content in different solvent extracts was determined by aluminium chloride colorimetric method [19]. The reaction mixture of 3.0 mL consisted of 1.0 mL of sample (1 mg/mL), 1.0 mL methanol, 0.5 mL of (1.2%) aluminium chloride and 0.5 mL (120 mM) potassium acetate and was incubated at room temperature for 30 min. The absorbance of all samples was measured at 415 nm. Quercetin was used as positive control. The flavonoid content was expressed in terms of quercetin equivalent (mg/g of extracted compound). The assay was carried out in triplicate and the mean values with \pm SEM are presented.

2.6 *In vitro* antioxidant activity

2.6.1 DPPH Free Radical Scavenging Activity

The free radical scavenging activity of different solvent extracts was measured by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) using the modified method of Mc Cune et al [20]. The reaction mixture of 3.0 mL, which consisted of 1.0 mL of DPPH (0.3 mM), 1.0 mL of extract (different concentrations) and 1.0 mL of methanol, was incubated for 10 minutes under dark condition, after which the absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The assay was carried out in triplicate and the mean values with \pm SEM were presented. The percentage inhibition was determined by comparing the results of the test and the control. Percentage of inhibition was calculated using the formula

$$\% \text{ Inhibition} = \frac{(B - A)}{B} \times 100$$

Where, B is the absorbance of blank (DPPH and methanol); A is the absorbance of sample (DPPH, methanol and sample).

2.6.2 Superoxide Anion Radical Scavenging Activity

The superoxide anion radical scavenging activity of different solvent extracts was measured by the method of Robak et al [21]. Superoxide radicals generated by oxidation of NADH was assayed by the reduction of NBT. The reaction mixture of 3.0 mL consisted of 0.5 mL of NBT (0.3 mM), 0.5 mL of Tris-HCl buffer (16 mM, pH 8), 0.5 mL NADH (0.936 mM), 0.5 mL PMS (0.12 mM) and 1.0 mL of different concentrations of different solvent extracts. The superoxide radical generating reaction was started by the addition of 0.5 mL of PMS solution to the mixture. The reaction mixture was incubated at 25 °C for 5 min and then the absorbance was measured at 560 nm against a blank sample. Gallic acid was used as a positive control. The assay was carried out in triplicates and the mean values with \pm SEM were presented. The percentage inhibition was determined by comparing the results of the test and the control.

2.6.3 Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of different solvent extracts was measured by studying the competition between deoxyribose and test compound for hydroxyl radicals generated by Fe²⁺-ascorbic acid-EDTA-H₂O₂ system (Fenton reaction). The reaction mixture of 1.0 mL, which consisted of 100 mL of 2-deoxy-D-ribose (28 mM in 20 mM KH₂PO₄-KOH buffer, pH 7.4), 500 mL of the various solvent extracts, 200 mL EDTA (1.04 mM) and 200 mL FeCl₃ (1:1 v/v), 100 mL H₂O₂ (1.0 mM) and 100 mL ascorbic acid (1.0 mM), was incubated at 37°C for 1 h. One milliliter of thiobarbituric acid (1%) and 1.0 mL of trichloroacetic acid (2.8%) was added and incubated at 100 °C for 20 min. After cooling, the absorbance of pink color was measured at 532 nm against a blank sample. Gallic acid was used as a positive control. The assay was carried out in triplicates and the mean values \pm SEM were presented. The percentage inhibition was determined

by comparing the results of the test and the control [22].

2.6.4 Reducing Capacity Assessment

The reducing capacity assessment of different solvent extracts were determined by using [23] 1.0 mL of each solvent extracts that was mixed with 2.5 mL of potassium phosphate buffer (200 mM, pH 6.6) and potassium ferricyanide (2.5 mL, 30 mM). The mixture was then incubated at 50°C for 20 min. Thereafter 2.5 mL of trichloroacetic acid (600 mM) was added to the reaction mixture and then centrifuged for 10 min at 3,000 rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of FeCl₃ (6 mM) and the absorbance was measured at 700 nm. Ascorbic acid was used as a positive control. The assay was carried out in triplicate and the mean values with \pm SEM were presented.

2.7. Statistical analysis

Graph pad PRISM software (version 4.03) was used for the calculating IC₅₀ values. The results were expressed as Mean \pm SEM and all the experiments were performed in triplicates.

3. Results

3.1. Phytochemical analysis

The three different crude solvent extracts of *I. caerulea* Roxb were subjected to preliminary phytochemical analysis (Table 1) which revealed the presence of phenols, flavonoids, saponins and phytosterols to a greater extent compared to the other constituents like alkaloids, tannins, triterpenes, terpenoids and carbohydrates respectively in the polar solvents. Similar analysis performed with either petroleum ether or ethyl acetate revealed the negligible quantities of these phytoconstituents. However in any of the highly polar solvent extract did steroids showed its presence.

3.2. Total phenol and Flavonoid contents

In general the extractive yield of various solvent extracts of *I. caerulea* Roxb (Table 2) varied among different solvents. The percentage of extractive yield can be ranked from low to high, wherein petroleum ether extract was 1.37 \pm 0.07 mg/g, while ethyl acetate extracts yielded 3.65 \pm 0.12 mg/g. However, methanolic extract revealed to be highest (8.22 \pm 0.74 mg/g). Total phenolic content was more than the flavonoid content in methanolic and ethyl acetate extracts, while it was vice-versa for flavonoid content which was more than phenols in the same extract (Table 1). The calculation of total phenolic content of *I. caerulea* Roxb extracts carried out using gallic acid standard and presented as gallic acid equivalents (GAE) per gram and flavonoid calculated and presented as quercetin equivalents, varied widely in plant materials with usage of different solvents, which ranged from 73.10 \pm 3.10 to 97.10 \pm 2.50 mg of quercetin equivalent /g of extract respectively.

3.3. DPPH radical scavenging activity

The three extracts investigated showed varied levels of DPPH free radical scavenging activity. Half maximal inhibitory concentration (IC₅₀) values ranged from 13.23 μ g/mL to 140 μ g/mL (Table 2) The IC₅₀ value of ethyl acetate showed more than 1,000 μ g/mL The IC₅₀ value of methanolic extract was 52.5 μ g/mL (Table 2) while that of standard ascorbic acid value was 13.23 μ g/mL The IC₅₀ value of petroleum ether extract was 140 μ g/mL Low IC₅₀ value which indicates high antioxidant activity. In this study the DPPH radical significantly reduced by various solvent extracts and standard with increasing concentrations [4,24].

3.4. Hydroxyl Radical Scavenging Activity

The hydroxyl radical is an extremely reactive in biological systems and has been implicated as highly damaging species in free radical pathology. Out of these three extracts investigated petroleum ether extract showed IC₅₀ value more than 1000 μ g/mL (Table 2). The best activity was shown by methanolic extract, and its IC₅₀ value was 180 μ g/mL (Table 2). Gallic acid was used as a standard with IC₅₀ value of 120 μ g/mL (Table 2). In this method the plant extracts showed potent hydroxyl radical scavenging activity which may be due to the presence of various phytochemicals including polyphenols and flavonoids in *Indigofera caerulea* Roxb.

3.5. Superoxide Anion Radical Scavenging Activity

Superoxide (O₂⁻) radical is known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributing to the tissue damage and various diseases. Out of the three extracts investigated the IC₅₀ values of methanolic extract was 225 μ g/mL while that of ethyl acetate extract was 260 μ g/mL (Table 2). The IC₅₀

value of petroleum ether extract was 420 μ g/mL (Table 2) which was comparatively lower than that of the standard gallic acid 120 μ g/mL (Table 2). These data showed that the superoxide anion radical scavenging activity of various successive solvent extracts of *I. caerulea* Roxb increases respectively with increasing the polarity of the solvents used for extraction [25].

3.6. Reducing Capacity Assessment

The potassium ferricyanide reduction method was used to measure the ability of phenolic compounds to quench radicals through electron donation. Activity was monitored by measuring its absorbance at 700 nm, as antioxidants reduce ferric ions to ferrous form. A linear increase in reducing power was observed over the concentration range of 20–160 μ g/mL in sample, equivalent to 5–40 μ g/mL for ascorbic acid equivalents. Significant differences were observed between different solvent extracts in terms of reducing power. This may be a consequence of the variability of phenolic compounds present, as affected by polarity of solvents used for extraction. Out of three studied extracts, petroleum ether extract showed poor reducing capacity in *Indigofera caerulea* Roxb (Table 3). There was a concentration-dependent increase in the absorbance of reaction mixture for all the three extracts and the standard ascorbic acid. According to these results, there is good relationship between total phenols and antioxidant activity.

4. Discussion

Plant phenolics constitute one of the major groups of compounds acting as a primary antioxidant free radical terminators. These compounds possess a wide spectrum of chemical and biological activities including radical scavenging properties. Natural extracts with proven

Table 1

Preliminary phytochemical analysis of successive solvent extract of *Indigofera caerulea* Roxb

Extract	Parts used	Alk		Fla	Tan	Ph	Tri	Ste	Sap	Ter	Phy	Car
		Dr	Ma									
P. ether	Leaves	-	-	-	+	+	+	-	+	++	++	++
E. acetate	Leaves	++	-	++	+	++	+	-	-	+	++	++
Methanol	Leaves	++	-	+++	++	+++	++	-	+++	++	+++	++

-: No presence; +: Less presence; ++: Moderate presence; +++: High presence; Alk: Alkaloids; Dr: Dragendroff; Ma: Mayer; Fla: Flavonoids; Ta: Tannins; Ph: phenols; Tri: Triterpens; Ste: Steroids; Sap: Saponins; Ter: Terpenoids; Phy: Phytosterols; Car: Carbohydrates

Table 2

Evaluation of phytochemicals and antioxidants of successive solvent extracts in leaves of *Indigofera caerulea* Roxb

Solvent	% Yield (w/w)	TPC (mg/g) GAE	Flavonoid content (mg/g) quercetin eq	IC ₅₀ Values (μ g/mL) Antioxidant assays		
				DPPH	OH	SO
Petroleum ether	1.37±0.07	ND	ND	140	-	420
Ethyl acetate	3.65±0.12	69.48±2.26	97.10±2.50	-	310	260
Methanol	8.22±0.74	112.94±1.11	73.10±3.10	52.5	180	225

Values are expressed in mean± standard error of the mean (n=3).

GAE- Gallic acid equivalents, OH -Hydroxyl, SO - Superoxide anion, ND- not detected, TPC-Total Phenol content, - indicates >1,000 μ g/mL

Table 3Reducing capacity assessment of the different solvent extracts of leaves of *Indigofera caerulea* Roxb

Standard Ascorbic acid		Petroleum ether extract		Ethyl acetate extracts		Methanol Extract	
Conc. (μ g/mL)	Reducing power (Absorbance At 700nm)*	Conc. (μ g/mL)	Reducing power (Absorbance At 700nm)*	Conc. (μ g/mL)	Reducing power (Absorbance At 700nm)*	Conc. μ g/mL)	Reducing power (Absorbance At 700nm)*
5	0.033±0.000	20	0.022±0.007	20	0.038±0.004	20	0.064±0.000
10	0.110±0.009	40	0.025±0.002	40	0.040±0.003	40	0.082±0.002
15	0.173±0.024	60	0.028±0.000	60	0.042±0.001	60	0.089±0.000
20	0.189±0.047	80	0.033±0.004	80	0.044± 0.000	80	0.099±0.000
25	0.223±0.003	100	0.039±0.002	100	0.047±0.001	100	0.135±0.005
30	0.231±0.05	120	0.054±0.002	120	0.060±0.005	120	0.145±0.001
35	0.234±0.078	140	0.066±0.003	140	0.075±0.003	140	0.165±0.002
40	0.237±0.005	160	0.079±0.000	160	0.110±0.012	160	0.209±0.008

*Values are expressed in mean±SEM (n=3).

antioxidant activity are usually present with their phenolic moiety, for example flavonoids, coumarins, proanthocyanidins and tocopherols [26].

Different free radical generating systems were used in this study to assess the free radical scavenging and reducing properties of the crude polar and non polar extracts of *Indigofera caerulea* Roxb, along with evaluation of the total phenol content. Quantitative estimation proved that the methanolic extract of *Indigofera caerulea* Roxb possesses the highest concentration of phenolic compounds. Total phenolic content was more than the flavonoid content in polar solvent extracts, while in non polar solvent extracts, flavonoid content was more than phenols. Highest total flavonoid content was present in ethyl acetate extract. Thus, it indicates the fact that the technique used in the present work was better for extracting total phenol and flavonoid content when compared with that of cold percolation method [27].

DPPH being a stable free radical accepts electrons or hydrogen radicals to become a stable diamagnetic molecule. Antioxidants on interaction with DPPH, transfer electrons or hydrogen atoms to DPPH and thus neutralize its free radical character converting to 1–1diphenyl–2–picryl hydrazine as represented by the degree of discoloration [28, 29]. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radicals in progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant activity [30, 31]. The methanolic extract had significant scavenging effect on the DPPH radical, which was generally significantly increasing with the increase in the concentration from 20–160 μ g/ mL.

The present study shows the ability of the leaf extract to inhibit hydroxyl radical mediated deoxyribose degradation in a concentration dependent manner. The extract had significant scavenging effects on the hydroxyl radical, which increased with the increase in the concentrations. On the other hand, superoxide is known to be very harmful species to cellular components as a precursor of more reactive

species. The results clearly indicate that methanolic extracts have a noticeable effect as scavenging superoxide radical when compared to that of other other two extracts.

The reducing power of the extract was evaluated by the transformation of Fe^{3+} to Fe^{2+} through electron transfer ability which serves as a significant indicator of its antioxidant activity. The reducing properties of the plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by donating a hydrogen atom by breaking the free radical chain. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The data obtained the present study suggest that it is likely to contribute significantly towards the observed antioxidant effects [1].

Recent investigations have shown that many flavonoids and related polyphenols contribute significantly to the antioxidant activity of medicinal plants. The diversity in radical scavenging shown in these assays may also be due to factors like stereo selectivity of the radicals or the differential solubility that may be justified in case of crude extracts, which contain a variety of antioxidants. Therefore, it is suggested that further work be performed on the isolation and identification of the bioactive compounds, which may be useful for therapeutic purpose.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

One of the authors, Mr. Ponmari. G is thankful to University Grants Commission, New Delhi, India for providing financial support as Rajiv Gandhi National Fellowship–Junior Research fellowship and the UGC grant number: F.14–2(SC)/2010(SA–III).

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