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RESEARCH ARTICLE

Phytochemical and antioxidant potential of *Indigofera colutea* (Burm.f.) Merr. (Fabaceae)

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Abstract

The total phenolic content, flavonoid content and antioxidant activity of *Indigofera colutea* were evaluated using three assays: ferric reducing antioxidant power (FRAP), phosphomolybdenum reducing power and hydrogen peroxide scavenging activity. Among the tested extracts, the ethanolic extract exhibited the highest total phenolic content (0.45 mg TAE/g dw) and total flavonoid content (1.527 mg QE/g dw), while the petroleum ether extract showed the lowest levels (0.039 mg TAE/g dw and 0.204 mg QE/g dw, respectively). In the antioxidant assays, the methanolic extract demonstrated the strongest ferric reducing power, whereas the ethanolic extract exhibited the highest activity in the phosphomolybdenum reducing power assay. The hydrogen peroxide scavenging activity was most effective in the ethanolic extract, followed by methanol, aqueous and acetone extracts, with petroleum ether and chloroform extracts showing the weakest activity. These findings suggest that *I. colutea*, particularly its ethanolic and methanolic extracts, is a promising source of natural antioxidants for potential therapeutic applications.

Keywords: antioxidant; Fabaceae; *Indigofera*; phytochemical

Introduction

The genus *Indigofera* Linnaeus (1753: 75), belonging to the tribe Indigofereae, is the third largest genus within the family Fabaceae. Species of this genus are predominantly distributed across tropical and subtropical regions worldwide. In India, approximately 60 species and 11 varieties are reported, among which 16 species and 7 varieties are endemic (1). *Indigofera* species are valued for various ecological and commercial uses, including animal fodder, ornamental landscaping, traditional medicine, natural dye production and treatment of diseases. Numerous studies have explored the pharmacological activities of different *Indigofera* species (2-7).

The systematic literature review for this inquiry indicated that consuming plant products having antioxidant chemicals could lessen the chance of being attacked by many diseases (8). Phytochemicals have received increased attention in recent years due to their numerous bioactive properties, including antioxidant activity (9). Furthermore, it is well known that antioxidant activity and phytochemical composition of plant extracts are affected by plant species, phenological stage, biotic stresses, genetic composition, geographical location and/or changes in climatic circumstances (10). The whole plant and roots of *Indigofera colutea* (Burm.f.) Merr., have been used against diarrhea, multiple boil cleaning, scabies (11-13) and in stomach ache (14). With this background, the current investigation deals with quantitative analysis of total phenolic content, flavonoid content and antioxidant activities of different solvent extracts of the species.

Materials and Methods

Plant material collection

The species was collected from Kadabagatti forest area, Gokak hills, Belagavi district of Karnataka and identified with the aid of flora (15) (Fig. 1). The herbarium was prepared and deposited in the Department of Botany, J.S.S. Arts, Science and Commerce College, Gokak, Belagavi.

Preparation of extracts

To evaluate the phytochemical and antioxidant potential, extracts of *I. colutea* were prepared using six solvents of varying polarity: petroleum ether (non-polar), chloroform (slightly polar), acetone (moderately polar), methanol, ethanol (polar protic) and distilled water (highly polar). This polarity gradient was selected to ensure the extraction of a broad spectrum of phytochemicals with varying solubility properties.

Fig. 1. *Indigofera colutea* (Burm.f.) Merr. (Fabaceae): (A) Habit, (B) Close up view of flowers, (C) Close up view of fruiting branch.

Fresh plant materials were washed thoroughly, shade-dried and ground into fine powder. For each solvent, 5 g of powdered plant material was extracted in 50 mL of the respective solvent in triplicate (n = 3). The mixture was first vortexed for 10-15 min and then placed in a rotary shaker at room temperature (25 °C) for 24 hr. After incubation, the samples were centrifuged at 10000 rpm for 20 min and the supernatant was collected. The supernatants were then pooled and diluted with the respective solvent to a final volume of 50 mL. Extracts were stored at 4 °C until further use.

For all antioxidant assays, 1 % (v/v) solutions of each extract were prepared using double-distilled water or the corresponding solvent.

Methods for total phenolic content

The Folin-Ciocalteu technique was used to quantify the total phenolic content (TPC) in the plant extracts (16). An aliquot of the extracts (0.125 mL), 0.125 mL of Folin-Ciocalteu reagent and 1.25 mL of saturated sodium carbonate solution were combined to create the reaction mixture. After 90 min of room temperature incubation, the reaction mixture was tested for absorbance at 760 nm. After the samples were made in triplicates, the absorbance mean value was found. The results were represented in mg of tannic acid equivalents (TAE)/g of sample. A calibration curve was generated using a reference solution of tannic acid (10-100 µg/mL, R_2 = 0.992).

Methods for total flavonoid content

The colorimetric method developed by Luximon-Ramma et al. (17) was used to measure the total flavonoid contents (TFC) of the plant extracts. After combining 1.5 mL of the extract with 1.5 mL of 2 % methanolic $AlCl_3$, the reaction mixture was created and allowed to sit at room temperature for 10 min. Measuring the absorbance at 420 nm. Three duplicates of the samples were made and the mean absorbance was calculated. Using a standard solution of quercetin (10-100 µg/mL, R_2 = 0.920), the calibration curve was created. The findings were given as mg of quercetin equivalents (QE) per gram of material.

Ferric reducing antioxidant power assay (FRAP assay)

A modified version of the Pulido et al. (18) approach was used to measure the capacity to remove ferric ions. Three milliliters of FRAP reagent (10 parts 300 mM sodium acetate buffer at pH 3.6, 1 part 10 mM TPTZ solution (2,4,6-Tri(2-pyridyl)-1,3,5-triazine) and 1 part 20 mM $FeCl_3 \cdot 6H_2O$ solution) were combined with an aliquot (100 µL) of extract and the reaction mixture was incubated at 37 °C for 15 min. The absorbance was then calculated at 595 nm. An aqueous solution of ascorbic acid (10-100 µg/mL, R_2 = 0.949) was used to create a calibration curve. FRAP results were given as milligrams of ascorbic acid equivalent per gram of material, expressed on a dry weight (dw) basis.

Phosphomolybdenum reducing power assay

The phosphomolybdenum reduction assay was used to evaluate the extracts' reducing power in accordance with Prieto et al. (19). A 90-minute incubation period was conducted at 95 °C for an aliquot containing 0.3 mL of extract and 3 mL of the reagent (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). At 695 nm, the absorbance of the watery solution was measured in comparison to a blank. The ascorbic acid equivalents (AAE) per gramme of sample were calculated using a calibration curve that was generated with ascorbic acid as the reference (10-100 µg/mL, R_2 = 0.953).

Hydrogen peroxide scavenging activity

Replacement titration was used to measure the amount of hydrogen peroxide scavenging activity (20). The test compounds and 40 mM H_2O_2 were mixed to create a solution, which was then titrated with 5.09 mM NaS_2O_3 until the yellow colour disappeared. The relative activities of the test compounds to scavenge hydrogen peroxide were expressed as percentage (%) of the titer volume change $[(V_{control}-V_{sample})/V_{control}] \times 100$.

Statistical analysis

Every experiment was measured in triplicate and the data were reported as mean ± standard deviation. GraphPad InStat and Microsoft Excel were used to calculate the Pearson's correlation coefficient of flavonoids and phenolics using several antioxidant assays.

Results and Discussion

Phenolics and flavonoid content

Based on the comprehensive literature review associated with this investigation, plant phenolics are among the principal classes of compounds that act as primary antioxidants or free radical terminators. Phenolic antioxidants "stop" free radicals produced by the substrate and free oxygen radicals by offering hydrogen atoms or electrons. As such, it is valuable to ascertain their total content in the selected plant for the investigation. The most significant natural phenolics are probably flavonoids, which are among the most varied and abundant classes of natural

chemicals (21). These substances have a wide range of biological and chemical functions, including the ability to scavenge radicals. For flavanols, these characteristics are unique. Consequently, the extracts' contents for each of the two phenolic groups are likewise ascertained (Table 1).

The content of total phenolics in the extracts of *I. colutea* (Burm.f.) Merr. is expressed as tannic acid equivalents (TAE). It can be observed that the content of phenolics in the different solvent extracts is highest in ethanolic extract (0.45 mg TAE/g dw) and lowest in petroleum ether extract (0.039 mg TAE/g dw). The phenolic content followed the order: ethanol > acetone > methanol > aqueous > chloroform > petroleum ether. This correlates with solvent polarity and suggests ethanol is most effective in extracting phenolic compounds. Table 2 also represents the total flavonoid content in different solvent extracts of *I. colutea* (Burm.f.) Merr. The results revealed that ethanolic extract also have the highest total flavonoid content (1.527 mg QE/g dw) and petroleum ether extract have the lowest flavonoid content (0.204 mg QE/g dw) in comparison with other fractions of plant extract. The flavonoid content is in descending order of ethanol > acetone > aqueous > methanol > chloroform > petroleum ether.

Table 1. Total phenolics and flavonoid content in *Indigofera colutea* (Burm.f.) Merr.

Solvents	Total phenolics (mg TAE/dw of plant material)	Total flavonoids (mg TAE/dw of plant material)
Aqueous	0.318	0.47
Methanol	0.32	0.387
Ethanol	0.45	1.527
Acetone	0.401	1.07
Chloroform	0.188	0.303
Petroleum ether	0.039	0.204

The results of total phenolic content, flavonoid content, were evaluated by analyzing ten concentration levels of standard solutions. Each concentration was repeated three times. The calibration curves of the standards were constructed with the correlation coefficients and regression equations (Table 2). The calibration graphs of different standards were shown in Fig. 2(A & B). The phenolic content discrepancy observed within the species may be influenced by the polarity of the extraction solvents. Depending on the type of plant material, the solvents' capacity to extract the most phenolic chemicals also changed (22). The yield of total phenolics and flavonoid content was greatly impacted by the characteristics of the extraction solvents. Due to their antioxidative and potential anticarcinogenic properties, the effects of current dietary phenolics are highly interesting (23). Flavonoids, which make up a significant portion of polyphenolic compounds, may also be useful in the treatment of inflammatory illnesses, cancer and cardiovascular dysfunction (24). The determination of total phenolics and flavonoid contents in the studied species indicated a significant amount of phenolics and flavonoids.

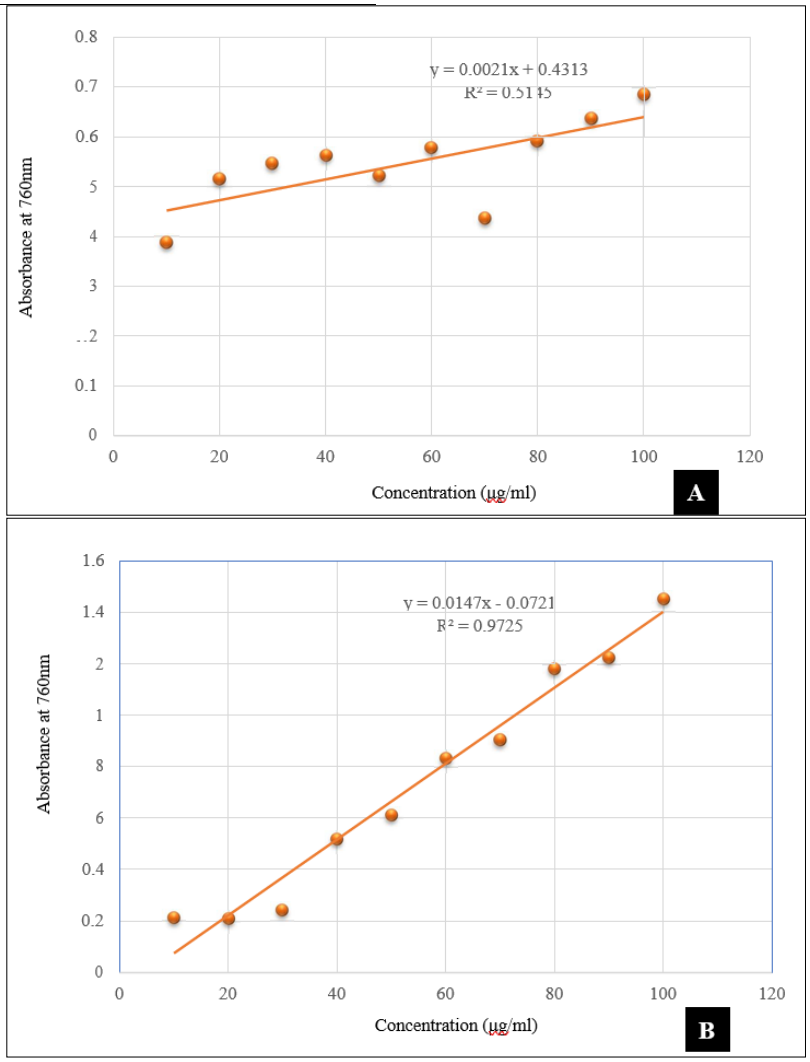

Fig. 2. (A) Standard plot of phenolics; (B) Standard plot of flavonoid.

Table 2. Values of standard calibration graph

Name of standard	Name of quantified compounds	*y* Value	R^2
Tannic acid	Phenols	*y* = 0.0021x + 0.4313	0.5145
Quercetin	Flavonoids	*y* = 0.0147x - 0.0721	0.9725

Ferric reducing antioxidant power (FRAP)

The FRAP assay is based on the ability of phenolics to reduce yellow ferric tripyridyltriazine complex (Fe (III)-TPTZ) to blue ferrous complex (Fe (II)-TPTZ) by the action of electron donating antioxidants (25). The result of blue colour measured spectrophotometrically at 595 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. As shown in Fig. 3(A & B), methanolic extract exhibited the highest ferric reducing power (2.030 mg AAE/g), followed by ethanol and distilled water. Petroleum ether showed the lowest (0.651 mg AAE/g). These trends align with previous reports indicating a strong correlation between phenolic content and reducing capacity. The plant extract with ethanol showed the highest antioxidant activity in FRAP assay exhibited the significant activity different from all solvents used. The ferric reducing ability of the extracts revealed that all of them gave well FRAP activity and the activity increased with the increase in concentration of the plant extract. This is consistent with the findings published in the previous study (25, 26). The examined species had varying but significant antiradical and antioxidant properties. Reductanes, which have been shown to exhibit antioxidant activity by disrupting the free radical chain by donating a hydrogen atom, are typically linked to the reducing characteristics of plant extracts (27).

Phosphomolybdenum reducing power

The phosphomolybdenum assay was based on the reduction of Mo (VI) to Mo (V) by antioxidant and subsequent formation of a green phosphate/Mo (V) complex under acidic conditions. Herein, the total antioxidant activities of various solvent extracts were measured and compared with that of ascorbic acid. According to the results, each extract exhibited a better degree of total antioxidant activity and were exhibited in a concentration- dependent manner. In this assay, ethanol extract exhibited the highest activity (2.829 mg AAE/g plant material) followed by methanol extract (1.085 mg AAE/g plant material), acetone extract (0.800 mg AAE/g plant material) and petroleum ether extract (0.325 mg AAE/g plant material). Distilled water and petroleum ether extracts exhibited the lowest antioxidant activity, likely due to their

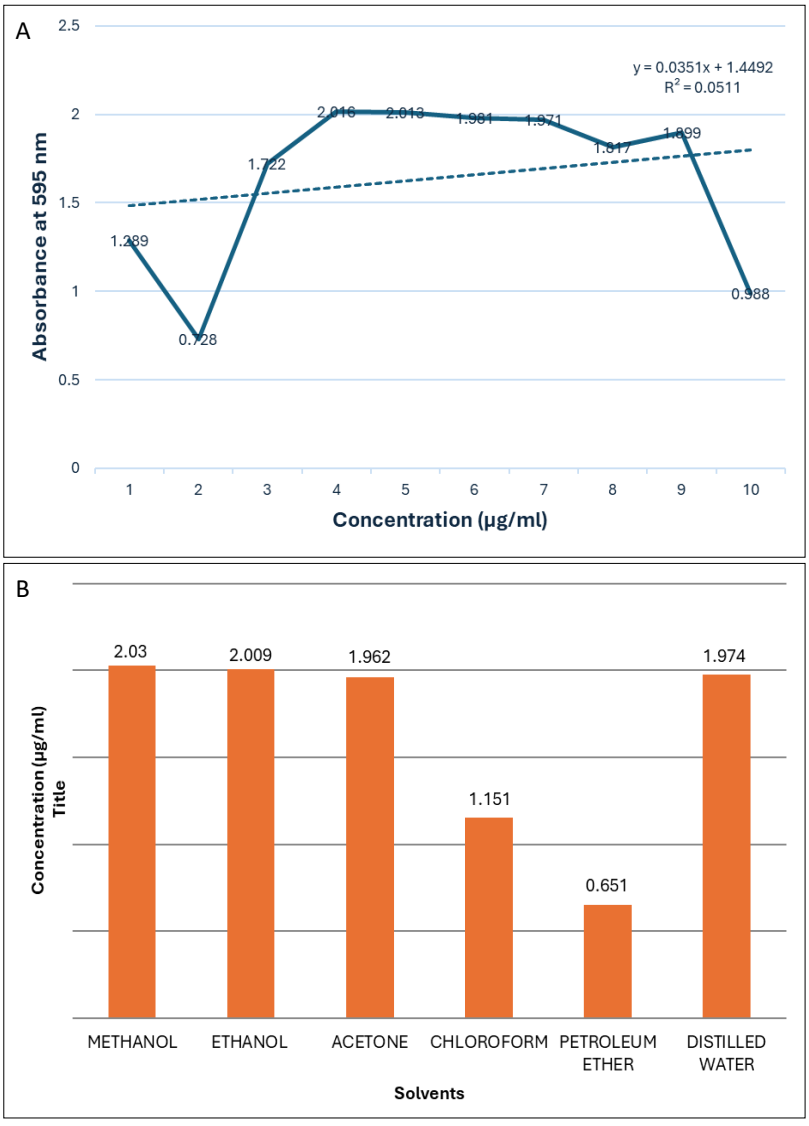

Fig. 3. (A) Standard plot (µg/mL) of antioxidants in various solvents; (B) Concentration (µg/mL) of antioxidants in various solvents.

limited efficiency in extracting phenolic and other electron-donating compounds. Water, being highly polar and petroleum ether, being non-polar, may not be effective in dissolving the moderately polar phytochemicals primarily responsible for antioxidant activity. Numerous studies have demonstrated a correlation between antioxidant activity and the ability of bioactive substances to donate electrons (28, 31). The chemical components with reducing power demonstrated that they are electron donors and can lower the oxidised intermediates of lipid peroxidation processes (32). This allows them to function as both primary and secondary antioxidants.

Hydrogen peroxide scavenging activity

The H_2O_2-scavenging activity of the extract may be attributed to its phenolic contents as well as other active components such as anthocyanin, tannins and flavonoids which can donate electrons to H_2O_2, thus neutralizing it to water (32). Although hydrogen peroxide is relatively stable, it can become toxic in cells by generating highly reactive hydroxyl radicals. Therefore, its removal is essential for maintaining antioxidant defense in biological and food systems (33). The scavenging reaction included 40 mM H_2O_2 and test compounds. After a period of incubation, the remaining H_2O_2 was measured by titration. The extract of *I. colutea* exhibited some extent of hydrogen peroxide scavenging capacity. The ethanol extracts possessed stronger scavenging activity (31.7 %) followed by methanol (20.7 aqueous (16 %) extracts and acetone (8 %). Whereas petroleum ether and chloroform were found to be rather similar in action and petroleum ether extracts exhibited a weaker scavenging activity against hydrogen peroxide as compared to other plant extracts.

Although H_2O_2 is mildly reactive, its decomposition into hydroxyl radicals can be harmful. Ethanol extracts exhibited the highest scavenging activity (31.7 %), likely due to the presence of ortho-dihydroxylated phenolic compounds. Additionally it has been demonstrated that polyphenols, particularly those containing the orthohydroxy phenolic compounds like quercetin, gallic acid, caffeic acid and catechin, can shield human cells against harm caused by hydrogen peroxide. Consequently, it is likely that the phenolic chemicals in the species extract are responsible for scavenging hydrogen peroxide (33).

Conclusion

This study revealed significant variation in the antioxidant potential and phytochemical composition among the tested extracts. Species rich in phytochemicals were found to exhibit strong antioxidant properties, indicating their potential benefits for human health. Notably, the ethanolic and methanolic extracts of *I. colutea* demonstrated the highest levels of antioxidant activity and phytochemical content. These results highlight the promising role of these extracts in the formulation of antioxidant-based therapeutics. To further elucidate the mechanisms involved and confirm their pharmaceutical relevance, future research should focus on isolating the active compounds and conducting *in vivo* studies.

Authors' contributions

GTC conceptualized the study, collected the specimens and wrote the manuscript. SVK assisted in the collection of specimens, provided critical input in the manuscript preparation and reviewed the final draft. MRB provided guidance in the manuscript preparation and contributed to the analysis of the results. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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