Research Paper

Physicochemical and Antioxidant Assays of Methanol and Hydromethanol Extract of Ariel Parts of *Indigofera tinctoria* Linn

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Free radicals or reactive oxygen indices give rise to oxidative injury, which is a fundamental mechanism underlying a number of disease like diabetes, cancer, neurodegenerative disorders. Deleterious effects of reactive oxygen species can be nullified by using different natural antioxidants derived from plants *Indigofera tinctoria* is such plant. This study was planned in order to trace and determine the antioxidant capability of *Indigofera tinctoria*. All the reagents and chemicals used in this study were obtained from reliable firms. The plant extracts were subjected to phytochemical screening, quantitative assays and antioxidant profiling. The results suggested that plant extracts contained all pharmacologically important phytoconstituents in appreciable amounts and having excellent antioxidant capabilities.

Key words: Antioxidants, *Indigofera tinctoria*, pharmacology, reactive oxygen species

Medicinal plants are highly conventional and one of the foremost sources of novel medicinal therapeutics due to the presence of various bioactive components or phytochemicals[1,2]. Most of these components are antioxidant in nature. The antioxidants from plants usually are secondary metabolites that have the ability to cope up oxidative stress resulted by the reactive oxygen species (ROS). These antioxidants produced in the plant as a result for their own defense mechanisms. From the past two or three decades, a number of medicinally important plants have been studied for their ability of neutralizing specific ROS, as the hydroxyl radical, the superoxide radicals, nitric oxide radicals[3].

India is a hub of such pharmacologically important known medicinal plants. *Indigofera tinctoria* belongs to the family Fabaceae and is an annual herb with a height of 4-6 feet. It is cultivated in India, China and other countries as a source of dyeing agent *i.e.* indigo. The herb is extensively used in the Indian system of medicine for neurological disorders, epilepsy, bronchitis and hepatic ailments[4].

The roots, stems and leaves of *Indigofera tinctoria* are bitter, thermogenic, laxative, trichogenous, expectorant, hepatoprotective, anticancer, antihelminthic and used in treating gastropathy, splenomegaly, cephalalgia, cardiopathy, epilepsy, neuropathy, chronic bronchitis, asthma, ulcers, skin diseases, diuretic and are useful for promoting the growth of hair[5].

Due to the less available data and reports to trace out the rich antioxidant abilities of *Indigofera tinctoria*, this study was designed in order to experimentally find out the content of various phytochemicals and antioxidant profiling of methanol and hydrometholic extracts of *Indigofera tinctoria*.

MATERIALS AND METHODS

Phenol reagent, dragendorff's reagent, methanol, gallic acid, aluminium chloride, HCl, sodium nitroprusside, hydrogen peroxide were purchased

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from Merck, USA. Reduced NADH, phenazine methosulpahte, nitroblue tetrazolium, L-ascorbic Acid were obtained from HiMedia, Mumbai. All other chemicals and reagents were high purity grade analytical reagents.

Collection and processing of plant material:
Botanically identified, experimental plant was procured from SM Heena Industries, Rajasthan. The plant was shade dried, milled to coarse powder. 40 g of plant material was placed in soxhlet thimble and was refluxed with solvents for 48-72 at 40-60º to obtain sequential methanol and nonsequential hydromethanol extracts. Extracts were collected and cooled at room temperature and poured in china dishes and then evaporated at 40º using hot air oven. Dried extracts were kept in desiccators for two days and stored at 5º in air tight containers. For the various assays, plant extracts were prepared with a concentration of 1 mg/ml.

Preliminary phytochemical screening:
The methanol and hydromethanol extracts were subject to screen the various phytoconstituents as flavonoids, glycosides, saponins, alkaloids, amino acids, proteins, carbohydrates, steroids, tannins and phenols present in them using standard protocols (Table 1)[6-8].

Total phenolic content:
Freshly prepared plant extract and gallic acid standard were treated with 1 ml of folin ciocalteu's reagent and mixed well. After 5 min, 4 ml of 7% Na_2CO_3 solution and 4 ml distilled water was added to it. This reaction mixture was incubated for 90 min in dark and then centrifuged at 10 000 rpm for 5 min. The supernatant was taken and its absorbance was recorded at 750 nm and the total phenolic content was expressed as mg gallic acid equivalent (GAE) per g of plant sample (fig. 1 and Table 2)[9].

Total saponin content:
Sample and standard (saponin) were treated with 400 µl vanillin-acetic acid reagent and 1.6 ml of perchloric acid. This reaction mixture was kept on water bath at 70-75° for several min. It was than cooled on ice bath for 2 min and 2.5 ml of glacial acetic acid was poured into it. Absorbance was taken at 550 nm after mixing it well. The total saponin content was expressed as mg sapogenin equivalent (SE) per g of plant sample (fig. 2 and Table 2)[10].

Total flavonoid content:
To 0.5 ml sample and standard (rutin), 2 ml distilled water and 0.15 ml 5% $NaNO_2$ solution were added. After 6 min, 0.15 ml 10% $AlCl_3$ solution was added and kept for another 6 min. To this reaction mixture, 2 ml 4% NaOH solution and 0.2 ml water was added to make up the final volume 5 ml. The reaction mixture was mixed well and allowed to stand for 15 min after which absorbance was recorded at 510 nm. Total flavonoid content was expressed as mg rutin equivalent (RE)/g plant sample (fig. 3 and Table 2)[11].

Total tannin content:
Sample and standard (tannic acid) were diluted with 8 ml distilled water and added 6.5 ml Folin–Ciocalteu reagent, 1.5 ml 20% Na_2CO_3 solution was to them. Absorbance was recorded at 775 nm and its content was expressed as mg tannic acid equivalents (TAE) per g of plant sample (fig. 4 and Table 2)[12].

Total proanthocynidin content:
Extract and standard (rutin) were treated by 1 ml ethanol (70%, v/v), 1.5 ml 25% HCl and 1 ml H_2O. This reaction mixture was heated at 85-90° for 80 min and cooled to add 1.5 ml n-butanol.

TABLE 1: PHYTOCHEMICAL SCREEING OF *INDIGOFERA TINCORIA*

Phytochemicals	Methanol extract	Hydromethanol extract
Alkaloids	+	−
Amino acids	−	+
Carbohydrates	+	+
Flavonoids	+	+
Glycosides	+	+
Proteins	+	+
Saponins	+	+
Steroids	+	+
Tannins and phenols	+	+

+: Presence of the phytochemical, −: absence of the phytochemical from the extracts

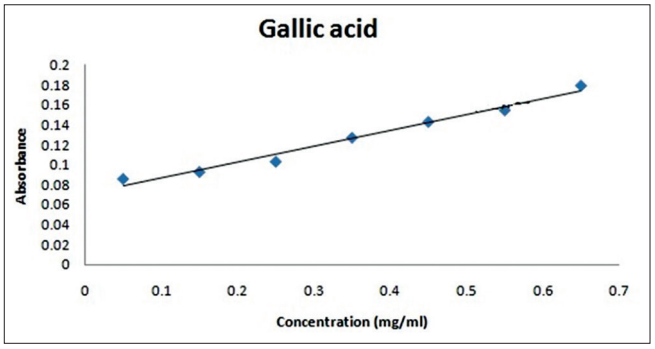

Fig. 1: Gallic acid standard curve of total phenolic content. Y=0.158x+0.070, R^2=0.979.

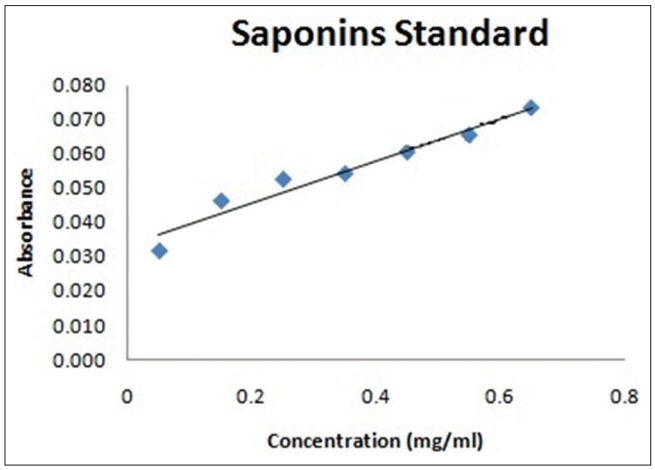

Fig. 2: Sapogenin standard curve of total saponin content. Y=0.0161x+0.033, R^2=0.950.

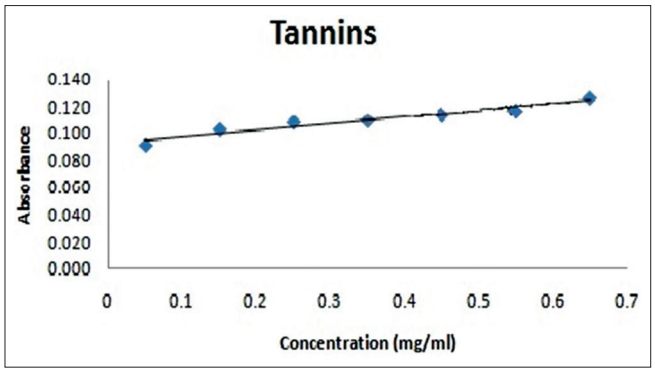

Fig. 4: Tannic acid standard curve of total tannin content. Y=0.049x+0.093, R^2=0.933.

TABLE 2: PHYSICO CHEMICAL QUANTITATIVE ASSAYS OF METHANOL AND HYDROMETHANOL EXTRACTS OF *INDIGOFERA TINCORIA*

Parameter analyzed	Met	HM
Total phenolic content (mg GAE/g)	207.67±0.002	210.62±0.005
Total saponin content (mg SE/g)	89.9±0.001	87.5±0.001
Total flavonoid content (mg RE/g)	96.4±0.003	104.16±0.008
Total tannin content (mg TAE/g)	229±0.001	230±0.001
Total proanthocynidin content (mg RE/g)	6.107±0.002	7.17±0.001

All values are expressed in mean±SD, Met: methanol, HM: hydromethanol extract, GAE: gallic acid equivalent, SE: sapogenin equivalent, RE: rutin equivalent, TAE: tannic acid equivalent, SD: standard deviation

The absorbance was than recorded at 545 nm. Total proanthocynidin content was expressed as mg rutin equivalent (RE) per g plant sample sample (fig. 5 and Table 2)[13].

DPPH scavenging assay:

Extract and standards 250 µl were treated with 2.5 ml of 0.004% DPPH solution and incubated at room temperature for 30 min in dark. The absorbance was recorded at 517 nm[14].

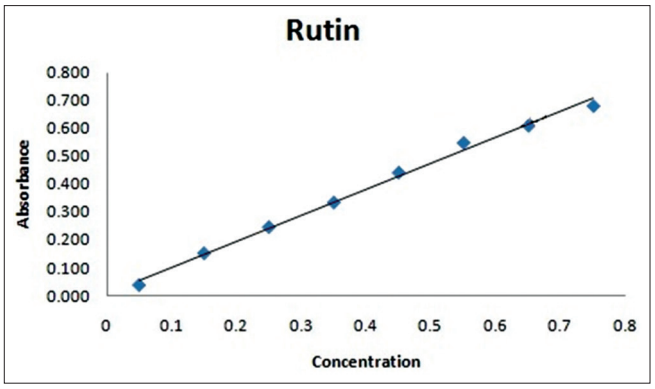

Fig. 3: Rutin standard curve of total flavonoid content. Y=0.927x+0.01, R^2=0.994.

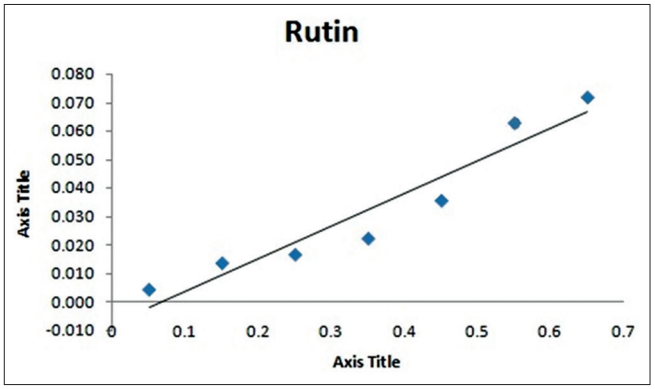

Fig. 5: Rutin standard curve of proanthocynidin content. Y=0.114x-0.007, R^2=0.917.

FRAP (feric reducing antioxidant power) assay:

The stock solutions included 300 mM acetate buffer (pH=3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solutions were prepared. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ and 2.5 ml $FeCl_3.6H_2O$. The temperature of the solution was raised to 37° before using. Plant extract (150 µl) and standard ($FeSO_4$) were allowed to react with 2850 µl working solution for 30 min in dark. The absorbance of the colored product was recorded at 593 nm[15].

Total antioxidant capacity:

Extracts and standard were treated with reagent mixture (0.6 M H_2SO_4, 28 mM $NaPO_4$, 4 mM ammonium molybdate; 1:1:1). The reaction mixture was incubated at 95° for 90 min and absorbance was taken at 695 nm[16].

Hydroxyl radical scavenging capacity:

Reaction mixture consists of 200 µl 10 mM $FeSO_4.7H_2O$, 200 µl 10 mM EDTA and 200 µl 10 mM deoxyribose was mixed with 0.5 ml sample. The

volume was made up to 1.8 ml by phosphate buffer. To it, 200 µl 10 mM H_2O_2 was also added and incubation was provided at 37° for 1 h. This reaction mixture was treated with 1 ml 0.5% TBA and 1 ml of ice cold TCA (2.8% in 25 mM NaOH) and incubated at 80° for 30 min. The absorbance was than recorded at 532 nm[17].

Superoxide radical Scavenging activity:
Samples were treated with 1 ml NBT, 1 ml NADH and 100 µl PMS solution and incubated at 25° for 3 min. The absorbance was recorded at 560 nm[18].

Metal chelating capacity:
Samples and EDTA standard (0.5 ml) were allowed to react with 1 mM $FeSO_4$, 0.5 ml Tris-HCl buffer (0.2 M; pH=7.4), 0.5 ml 0.1% bipyridyl solution, 0.4 ml 10% hydroxylamine HCl and 2 ml ethanol. The reaction mixture kept for 2 min at 25° and absorbance was recorded at 522 nm[19].

Reducing power assay:
Plant extracts and rutin standards were treated with 2.5 ml 0.2 M phosphate buffer of pH 6.6 and 2.5 ml 1% potassium ferricyanide. Incubation was given at 50° for 20 min. To it, 2.5 ml 10% TCA solution was added. 2.5 ml of the upper layer was taken of the above mixture, mixed with distilled water and 0.5 ml 0.1% $FeCl_3$. Absorbance was taken at 700 nm[20].

Nitric oxide radical scavenging activity:
Samples (0.5 ml) were mixed with 0.5 ml 0.1 M phosphate buffer of pH 7.4 and 2 ml 10 mM sodium nitroprusside solution. Incubation was provided at 25° for 2.5 h. Taken 0.5 ml of above reaction mixture and 1 ml 0.33% sulphanilic acid in 20% glacial acetic acid was added to it. Kept it at room temperature for 5 min, added 1 ml 5% N-NED-HCl, mixed and incubated at room temperature for 5 to 30 min. Absorbance was recorded at 564 nm of pink color[21].

RESULTS AND DISCUSSION

Phytochemical investigation of the methanol and hydromethanol extract of *Indigofera tinctoria* revealed the occurrence of pharmacologically important phytoconstituents as flavonoids, saponins, glycosides, steroids, tannins and phenols. Alkaloids were found to be absent in hydromethanol extract while present in low amount in methanol extract. Both extracts were also found to possess amino acids, proteins and carbohydrates. Flavonoids produced in plants due to microbial response. They are most potent and excellent antioxidants possess the capability of acting as anticancer agents[22]. Saponins, which have hemolytic and antiinflammatory ability, were also present in both extracts[23]. Plant extracts also possess glycosides, which are known to lower the blood pressure[24]. Steroids have been reported to have antibacterial activity[25] and antidiarrhoeal activity as it increase intestinal absorption sodium ions and water[26].

The total phenolic content in methanol content was 207.69 mg/g of dry weight of extract and 210.62 mg/g of dry weight for hydromethanol extract. Such amount of phenolic components is able to scavenge free radicals by donating active hydrogen and thus help reducing the detrimental effects of oxidative stress. This property of plant phenolic makes them a key group of compounds that perform as free radical scavengers or primary antioxidants. Total saponin content was 89.9 mg/g for methanol extract and 87.5 mg/g for the hydromethanol extract.

Flavonoid content was found to be 96.4 mg/g of dry weight for methanol extract and 104.16 mg/g of dry weight for hydro methanol extract. Flavonoids are chemically characterized by two benzene rings joined by a linear carbon chain. Various reports indicate that regular flavonoids may trim down the risk of several chronic diseases including neurodegenerative diseases, atherosclerosis, and cancer[27]. The flavonoids also reported to have antiviral, antiallergic, antiplatelet and antiinflammatory properties.

Tannins are phenolic compounds having antioxidant and antibacterial properties and also have ability to form complexes with macromolecules and metal ion[28]. The amount of tannins present in methanol and hydromethanol extract was 229 and 230 mg/g of dry weight of respective extracts.

Acid butanol assay is used to determine the total proanthocynidin content and it was observed this is an easy, simple and rapid test. Total proanthocynidin content was 6.107 mg/g of dry weight of extract in methanol extract and 7.17 mg/g of dry weight of extract in hydro methanol extract. Literature have indicated that proanthocynidins exhibit antimicrobial, anticarcinogenic and antimutagenic potentials[29-31].

DPPH test present information on the reactivity of the test sample with a stable free radical entity. DPPH gives a strong absorption band in visible spectrum at 517 nm wavelength. When an odd electron becomes paired off in the presence of a free radical scavenging agent, the DPPH solution is decolorized and its color changes from deep violet to light yellow as the absorption reduces. The degree of reduction in absorbance measurement is pinpointing of the antioxidant ability of the sample extract. To scavenge DPPH radicals, inhibition concentration of methanol extract was 0.709 and 0.881 mg/ml of hydromethanol extract, which was found to be much higher when compared to the IC_{50} of tocopherol standard (0.94 mg/ml) (Table 3).

FRAP is an excellent method in order to determine the free radical scavenging activity. Low pH causes reduction of ferric-TPTZ to ferrous-TPTZ, which gives an intense blue color, measured at 593 nm using spectrophometer. The FRAP value for methanol extract and hydromethanol extract were 10.807 and 18.256 mM Fe (II) ions/mg of plant extracts (Table 3).

Total antioxidant capacity or TAC is based on phosphomolybdenum model in which formation of a green phosphate-Mo (V) complex occurs after the reduction of Mo (VI) to Mo (V) at low pH. Total antioxidant capacity for methanol extract of *Indigofera tinctoria* was 622.74 mg/g of plant extract and 722.72 mg/g of hydromethanol extract (Table 3).

The ability to scavenge hydroxyl radicals is measured by the ability of extracts to prevent degradation of deoxyribose by the hydroxyl radicals generated in the reaction mixture. Highly reactive hydroxyl radicals can be generated in biological systems by fenton reaction. The IC_{50} to scavenge the hydroxyl radicals for methanol and hydromethanol extract were 0.020 and 0.028 mg/ml, respectively (Table 3).

TABLE 3: ANTIOXIDANT ASSAYS OF METHANOL AND HYDROMETHANOL EXTRACTS OF *INDIGOFERA TINCORIA*

Parameters analyzed	Met	HM
DPPH scavenging activity (IC_{50}-mg/ml)	0.709±0.018	0.881±0.027
FRAP activity (mM Fe (II) ions/mg dried plant extract)	10.807±0.016	18.256±0.036
Total antioxidant capacity (mg GAE/g)	622.74±0.002	722.72±0.007
Hydroxyl radical scavenging activity (IC_{50}-mg/ml)	0.020±0.003	0.028±0.002
Superoxide radical scavenging activity (IC_{50}-mg/ml)	0.394±0.004	0.457±0.004
Metal chelating capacity (IC_{50}-mg/ml)	0.758±0.017	0.391±0.021
Reducing power assay (mg RE/g)	2803±0.015	2494.74±0.028
Nitricoxide radical scavenging activity (IC_{50}-mg/ml)	0.709±0.002	0.709±0.004

Met: Methanol extract, HM: hydromethanol extract, GAE: gallic acid equivalent, RE: rutin equivalent, DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: ferric reducing ability of plasma

Superoxide radical scavenging activity was determined by PMS-NADH system. Superoxide radicals play an important role in living cells as they on decomposition, produced more harmful free radical entities as hydroxyl radicals and singlet oxygen. IC_{50} value to scavenge superoxide radicals was almost similar for hydromethanol extract of *Indigofera tinctoria* (0.457 mg/ml) and ascorbic acid standard (0.462 mg/ml). For methanol extract it was found to be 0.394 mg/ml (Table 3).

Metal chelating capacity is important as it reduce the concentration of transition metals as iron, which accelerate rate of lipid peroxidation by fenton reaction. According to the results, the plant extracts showed good metal chelating activity. IC_{50} values of 0.75 and 0.39 mg/ml were observed for metal chelating capacity, respectively in methanol and hydromethanol extract. The standard EDTA IC_{50} value was 0.29 mg/ml (Table 3).

Reducing power for methanol extract was found to be 2494.74 mg/g of plant extract and 2803 mg/g plant extract for hydromethanol extract of *Indigofera tinctoria*. Like the antioxidant activity, the reducing power increased with increasing amount of the extract. For the measurement of the reductive ability, the Fe^{3+}-Fe^{2+} transformation was investigated in presence of the extract. Presence of various antioxidants results in the reduction of the ferricyanide complex to the ferrous form (Table 3).

In biological systems, nitric oxide (NO) plays a potent role of physiological process such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical, which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumor activities. IC_{50} values of 0.035 mg/ml for nitric oxide radical scavenging activity was observed for both methanol and hydromethanol extract (Table 3).

This study put forwarded that methanol and hydromethanol extracts of *Indigofera tinctoria* possess antioxidant properties when compared with the ability of well exemplified and widely used antioxidant standards. The high potential of being good antioxidant and free radical scavengers of these two extracts of *Indigofera tinctoria* is due to the presence of phytoconstituents, which possess a number of therapeutic and pharmacological properties and are promising source of herbal medicinal cure of various ailments including cancers and neurogenerative disorders.

The studies are further warranted for the isolation and structural characterization of these phytoconstituents present in the plant extract. Furthermore, *in vivo* studies are also needed to be performed in order to under the mechanism of action of these antioxidant components and before the access to preceding clinical trials.

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Nil.

Conflicts of interest:
There are no conflicts of interest.

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