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Ichnocarpus frutescens Stem Extract: Effect of Different Concentrations of Solvent on Total Phenol Content, Total Flavonoid Content and on Its Antioxidant Activity

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Abstract Free radicals formed from reactive oxygen species can oxidize biomolecules leading to tissue injury and cell death. Oxidative stress is generated by imbalance between reactive oxygen species and antioxidants. Hence, to overcome this problem, an attempt has been drawn by studying *Ichnocarpus frutescens* stem extract. In order to study it we have gone through total phenolic content, total flavonoid content as quantification of secondary metabolite. As far as antioxidant activity concerns, DPPH radical scavenging, reducing power assay and total antioxidant activity were performed and analyzed. During this analysis, we have found that 80% ethanol showed maximum activity as compared to 50% and 95% ethanolic extract of *Ichnocarpus frutescens* stem.

Keywords: *total phenolic content, total flavonoid content, free radical, oxidative stress, reducing power, radical scavenging*

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1. Introduction

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as byproducts of biological reaction or from exogenous factors such as sunlight, ultraviolet light, ionizing radiation, chemical reactions. [1] Free radicals thus formed, being highly reactive, can oxidize biomolecules leading to tissue injury and cell death. [2] Oxidative stress is generated by imbalance between reactive oxygen species (ROS) and antioxidants. Oxidative stress occurs when there is excessive free radical production and/or low antioxidant defense, which leads to chemical alterations of biomolecules causing structural and functional modifications. [3] Oxidative damage plays a significant pathological role in human diseases such as cancer, inflammation, arthritis, diabetes and atherosclerosis. [4] It has already been reported that phenolic compounds contribute to the antioxidant activity of the plants and there is a great demand to find more effective antioxidants from plant species. [5]

Ichnocarpus frutescens R. Br. (Apocyanaceae), commonly known as Black Sariva, is a climbing shrub found almost in all parts of India, ascending to an altitude of 1200m. [6] Considered as a substitute for *Hemidesmus indicus* (Indian Sarsaparilla), this plant is used by tribes in atrophy, convulsions, cough, delirium, dysentery, measles, splenomegaly and tuberculosis. It is also used in abdominal and glandular tumors and its roots are used as alterative, anti-dysentric, anti-pyretic, demulcent, diaphoretic, hypoglycemic. [7,8,9]

2. Materials and Methods

2.1. Plant Material, Procurement and Preparation

The stems of *Ichnocarpus frutescens* R. Br. were procured from Dehradun, Uttrakhand and were authenticated by taxonomist Dr. Anjula Pandey, Dept. of National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi. A voucher specimen (Specimen No: NHCP/NBPGR/2009-13/889) is preserved in the Herbarium Section of the Taxonomy Department NBPGR, New Delhi. Stems were carefully collected, air-dried, and reduced to coarse powder, which was subjected to exhaustive extraction with 95% ethanol, 80% ethanol and 50% ethanol. The extracts were filtered through Whatman's filter paper number 41 and

concentrated under reduced pressure to obtain dried dark brown residue.

2.2. Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) in extracts was determined by the Folin–Ciocalteu method [10] and gallic acid as standard. Briefly, 0.5mL of crude extract (1mg/mL) were made up to 10 mL with distilled water, mixed thoroughly with 1.5 mL of Folin–Ciocalteu reagent for 5 min, followed by the addition 4 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand for further 30 minutes at room temperature. The absorbance was measured at 765nm using a UV-VIS spectrophotometer (UV-2450PC, Shimadzu, Kyoto, Japan). The total phenolic content is expressed as milligrams of gallic acid equivalent (GAE) to per gram of dry extract.

2.3. Determination of Total Flavonoid Content (TFC)

Flavonoid content in the examined plant extracts was determined using spectrophotometric method using Quercetin as standard.[11] The sample contained 0.5 mL of methanolic solution of the extracts in the concentration of 1 mg/mL and 0.5 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λmax = 415 nm. The same procedure was repeated for the standard solution of quercetin and the calibration line was constructed. The content of flavonoids in extracts was expressed in terms of milligrams of quercetin equivalent (QUE) to per gram of dry extract.

2.4. DPPH Radical Scavenging Activity

The ability of the plant extract to scavenge 1, 1-diphenyl-2-picryhydrazyl (DPPH) free radicals was assessed by the standard method. [12,13] The stock solution of extracts were prepared in ethanol to achieve the concentration of 1 mg/ml. Different concentrations of extracts were mixed with 3 ml of ethanolic solution of DPPH (DPPH, 0.004%). After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517nm using UV-Visible Spectrophotometer. Initially, absorption of blank sample containing the same amount of ethanol and DPPH solution was prepared and measured as control. Gallic acid was used as standard. The experiment was carried out in triplicate. Percentage inhibition was calculated using equation (1), whilst IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\%\ inhibition = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \right) X100. \quad (1)$$

2.5. Reducing Power Assay (RP)

The reducing powers of the extracts were determined by the method. [14] Various concentration of extracts were prepared in 1 mL of distilled water was mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50◦C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 RPM for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Gallic acid was used as the reference material. All the tests were performed in triplicate and the graph was plotted with the average of three observations.

2.6. Total Antioxidant Activity

The total antioxidant capacity of the extracts were evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto et al. 1999. [15] 1mL of various concentrations of extract was combined with 1 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm against reagent blank using a spectrophotometer. The antioxidant activity was expressed as the number of equivalent of ascorbic acid.

3. Result and Discussion

3.1. Total Phenolic and Flavonoid Content

Total phenolic content (TPC) and total flavanoid content (TFC) was determined for 95% ethanol extract, 80% ethanol and 50% ethanol extracts. Results are summarized in Table 1. The TPC and TFC were found to be in the order of 80% ethanol > 50% ethanol > 95% ethanol which indicates that solvent has a direct effect on the extraction of active metabolites. Further the anti-oxidant activity can also be correlated with the total phenolic content. Greater anti-oxidant activity of many plants is attributed to the high phenolic content. TPC is found to be maximum in 80% ethanol (3.58±0.35 mg QUE g^{-1}) and least in 95% ethanol (1.16±0.011mg QUE g^{-1}). The low solubility of phenolic compounds in absolute solvents may be attributable to the strong hydrogen bonding between protein and polyphenols. However, the solubility increases upon addition of water to organic solvents that weakens the hydrogen bonds. [16] (Table 1).

Table 1. Total phenolic and flavanoid content of endocarp extract

Name of the Extract	Total Phenolic Content (Gallic acid equivalent) mg/g extract	Total Flavonoid Content (Quercetin equivalent) mg/g extract
50% Ethanol	1.97±0.121	0.51±0.112
80% Ethanol	3.58±0.35	1.01±0.113
95% Ethanol	1.16±0.011	0.21±0.021

Each value represents the mean±SD, n=3.

3.2. Total Phenolic and Flavonoid Content

Free radical scavenging activity of different plant extracts can be assessed by DPPH assay. DPPH radicals reacts with suitable reducing agent and on reduction of free DDPH radicals solution changes from purple to light yellow which can also be measured spectrophotometrically at 517nm. The scavenging potential of plant extract antioxidants corresponds to the degree of the discoloration. [17] The effect of different solvents and their concentration levels on DPPH radical scavenging activity of *Ichnocarpus frutescens* extracts (Table 2) reveals high antioxidant activity of all sample extracts (Figure 1). However, 80% ethanol extract strongly scavenged DPPH radicals with the IC_{50} being 27 μg/mL followed by 50% ethanol extract (IC_{50}=38 μg/mL). The minimum inhibitory action was shown by 95% ethanol extract (IC_{50}=53 μg/mL). Higher inhibitory activity of aqueous solvent extracts against the DPPH radical as compared to corresponding absolute solvents, which may be attributed to the higher polyphenol content in these extracts.

Table 2. Antioxidant properties of *Ichnocarpus frutescens*

Name of Extract	DPPH Radical Scavenging ability (IC_{50}, µg/ml)	Ferric Reducing Power Conc., mg/ml (Abs. 0.5)	Total Antioxidant Capacity (mmol AA/mg extract)
50% Ethanol	38	361	2.62
80% Ethanol	27	246	3.93
95% Ethanol	53	478	1.64
Standard	1.98	57	-

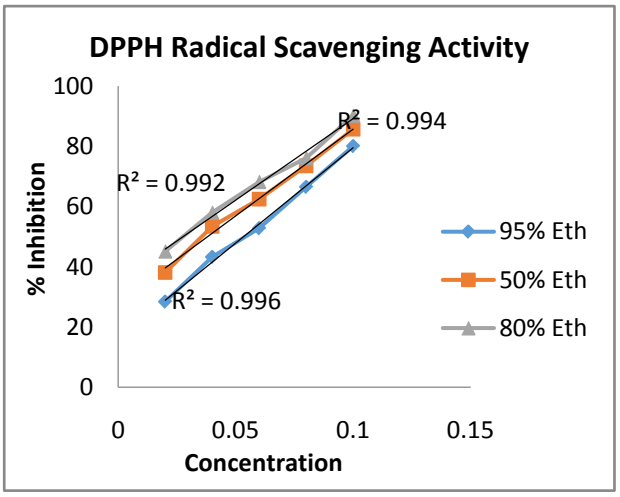

Figure 1. DPPH radical scavenging activity

3.3. Reducing Power

Presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). Strong reducing agents, however, formed Perl's Prussian blue color and absorbed at 700 nm. The higher the absorbance of the reaction mixture, the higher would be the reducing power. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts. [18] Reducing power for the various extracts has been tabulated in Table 2 which revealed the 80% ethanol extract has maximum ferric reducing activity followed by 50% ethanol and 95% ethanol extract (Figure 2).

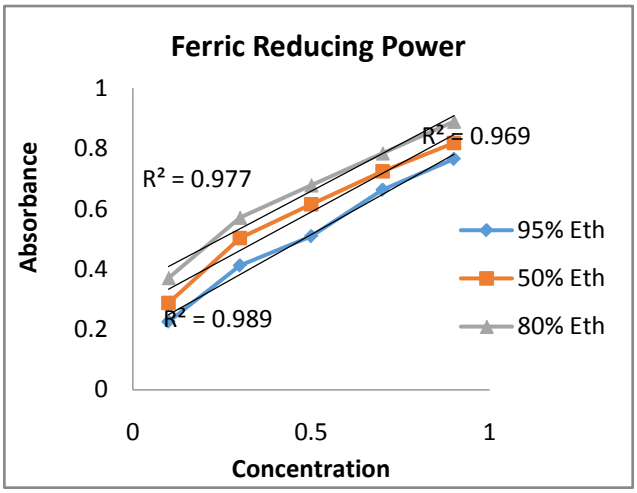

Figure 2. Ferric reducing power

3.4. Total Antioxidant Activity

The total antioxidant capacity of the extract was calculated based on the formation of the phosphomolybdenum complex which was measured spectrophotometrically at 695nm. A direct correlation was found to exist between the concentration of the extract used and the spectrophotometrically measured phosphomolybdenum complex. The total antioxidant activity of various extracts was vary with the solvent composition and it was observed that aqueous solvent extracts showed greater activity as compared to absolute solvent. The antioxidant activity was found to be maximum in 80% ethanol extract (3.93 mmol ascorbic acid equivalent/mg extract) and minimum in 95% ethanol (1.64 mmol ascorbic acid equivalent/mg extract). (Table 2)

4. Conclusion

In the present investigation anti-oxidant properties, phenolic content and flavonoid content was studied for the stem of *Ichnocarpus frutescens*, which is commonly known as *Black Sariva* and used as a substitute for Indian Sarsaparilla (*Hemidesmus indicus*). The drug was found to posses antioxidant activity and may be useful for reducing the oxidative stress, which may further contribute for prolongation of many disease. The main purpose of the study was to determine the effect of extraction solvent on the extraction of phenols from the drug as well as anti-oxidant property of the drug. It was observed that 80% ethanol showed maximum activity when compared to 50% ethanol and 95% ethanol extract.

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