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Activity guided separation of phytoconstituents from the flowers of *Ichnocarpus frutescens* L. and evaluation for antioxidant property

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ABSTRACT

For achieving and maintaining vibrant health tackling free radicals is very important. Each human cell receives a tremendous number of assaults per day from free radicals. Free radicals are those militant molecules that attack our cells. Therefore, the preventative measure is to combat these free radicals before they do damage. They have only one purpose and that is to invade and destroy healthy cells making the body vulnerable to disease which has negative impact on our health. Free radicals disrupt the biochemical processes within cells, and our body's natural defense simply can not keep up with the on slaught and so we become ill. Scavenging of free radical seems to play a considerable part in the antioxidant activity of flavonoidal compounds. In very recent year's flavonoids as potent radical scavengers have attracted a tremendous interest as possible therapeutic against free radical mediated diseases. Literature survey revealed that various parts of *Ichnocarpus frutescens* L.are known for its flavonoidal content hence the various extracts, fractions and isolated flavonoids of the flowers of *Ichnocarpus frutescens* L. were screened for the antioxidant property. Various extracts and fractions were prepared by using solvents of different polarity, flavonoids were isolated and antioxidant activity was studied by using DPPH assay and by measuring the scavenging capacity of the hydroxyl radicals (degradation of 2-deoxyribose with hydroxyl radicals). In the studied models, amongst the various extracts, fractions and isolated flavonoids the isolated pure flavonoid separated from ethyl acetate fraction of alcoholic extract showed the remarkable and concentration dependent antioxidant activity than the other extracts and fractions. The IC_{50} values were calculated. The results were comparable with standard ascorbic acid. Hence we conclude that isolated pure flavonoid-I (F-I) separated from ethyl acetate fraction of alcoholic extract of flowers of *Ichnocarpus frutescens* L is more bioactive than other fractions and extract studied.

Key words: *Ichnocarpus frutescens* L.**,** Antioxidants, DPPH assay,

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INTRODUCTION

Ichnocarpus frutescens L. (Fam-Apocynaceae) [1] commonly known as black creeper is a climbing shrub with slender branches; branchlets pubescent, Leaves are elliptical, obviate or oblong, entire, base attenuate, glabrous above, pubescent beneath, lateral nerves 4 to 6 pairs. Flowers are white in auxiliary and terminal peniculate cymes, 5 lobes. Found throughout India usually up to an altitude of 4000 feet's also found in hedges in deciduous forests [2].

Free radicals are responsible for many ailments including, cancer, cardiovascular diseases, stroke, arthritis, lung damage, inflammation etc. Antioxidant agents of natural origin have attracted special interest because these are the compounds that help to inhibit many oxidation reactions caused by free radicals thereby preventing and delaying damage to the cells and tissues [3]. Hence the present study was aimed at evaluating the antioxidant activity of various extract, fractions and isolated flavonoids of the flowers of *Ichnocarpus frutescens* L. Literature survey revealed that *Ichnocarpus frutescens* L. is well known for its flavonoidal content; hence this plant is selected for the present study. [10,11].

MATERIALS AND METHODS

Plant material

The shrub *Ichnocarpus frutescens* L. was collected from iron netted fences around deer park in first ghatroad (Tirumala), S.V. dairy farm in Tirupati on high way sides from Erravaripalem to Nerabailu, and was authenticated by Dr. Mahava Chetty, Asst. Professor, Department of Botany, S.V. University, Tirupati, Andhra Pradesh, India, and the sample specimen is stored in Dept. of Pharmacognosy, Luqmqn college of Pharmacy, Gulbarga.

Chemicals and Reagents

All the chemicals and reagents used were of analytical grade includes DPPH (diphenyl picryl hyrazyl), 2-deoxy$_{-D-}$ribose-Aldrich, TBA (Thiobarbituric acid), EDTA (Ethylene diamine tetra acetic acid)-Sd fine chemicals Ltd, Ascorbic acid -Himedia Lab, Mumbai, India.

Extraction and fractionation

Activity guided separation of phytoconstituents [4,5]

Shade dried flowers of *Ichnocarpus frutescens* L. is coarsely powdered, about 250gm of the coarse powder were extracted exhaustively with 95% ethanol in a soxhlet assembly. The extract was concentrated in rotary flash evaporator under reduced pressure at 600C and dried in desiccator over sodium sulphite. The alcoholic extract has shown positive test for the presence of flavonoids so it is screened for antioxidant activity and it shown positive results for antioxidant models. So an attempt is been made to fractionate the alcoholic extract with Petroleum ether, chloroform and ethyl acetate in order to isolate the bioactive flavonoids responsible for antioxidant activity. Further the Petroleum ether fraction,

Chloroform fraction, and Ethyl acetate fraction of Alcoholic extract is screened for antioxidant activity by using different *in vitro* models. The results are tabulated in Table no.01.

Phytochemical screening of the various fractions was carried out. The ethyl acetate fraction has shown positive chemical test for Shinoda, Zinc/HCl and other tests for flavonoids. For further conformation T.L.C. studies was carried out which showed a mixture of flavonoids as it has shown two spots with different Rf values 0.54 and 0.69. For the separation and purification of these components the ethyl acetate fraction was subjected for preparative TLC.

Isolation of flavonoids by preparative TLC

The glass plate measuring 20 x 20cm was used for the preparative TLC, Silica gel G for TLC is used as an adsorbent. Layers of 0.5-2mm were prepared then they are air dried to avoid cracking of thick layers then moved to oven for activation at 110^{O}C for 1 hour. Then the plate were taken out from the oven and spot the sample in the form of band or streaks, and kept the plate for some time for drying, mean while the chamber with solvent was saturated, after that the plate was kept in the chamber and allowed to develop, after development the plate is taken out from the chamber, a portion of the plate was visualized for the localization of compounds by spraying with anisaldehyde sulphuric acid reagent (by covering with a glass plate, and just visualizing an edge (or both edges) of the plate, the corresponding areas were scrapped off with spatula and washed with an appropriate solvent to remove the components. Methanol was used because the components are soluble in methanol. A couple of washing is made to get the entire component from the silica gel. Centrifugation was done for this purpose; supernatant is collected leaving the silica gel behind. The washings or supernatant is concentrated to get pure compound. The compound corresponding to Rf value 0.54 was labeled as F.-I and the compound corresponding to Rf value 0.69 was labeled as F-II.

Antioxidant activity

The various samples selected for the activity are-

1. Alcoholic extract and their fractions such as Petroleum ether fraction, Chloroform fraction and Ethyl acetate fraction.

2. Isolated flavonoids, Flavonoid I(F-I) and Flavonoid II (F-II)

By using DPPH (l,l-diphenyl-2-picrylhydrazyl) assay[6-9]

The samples were prepared in different concentrations i.e. 12.5-75μgm/ml in AR grade methanol. The samples of above concentrations were mixed with 2ml of 90μM of DPPH prepared in AR grade methanol and the final volume was made up to 4ml with same. The absorbance of the resulting solutions and the blank (with same chemicals except sample) were recorded after 1 hour at room temperature, against ascorbic acid as a positive

control. Experiment was performed in triplicate. The disappearance of DPPH was read spectrophotometrically at 517nm using a GENESYS 6 vl.620 Spectrophotometer. Radical Scavenging Capacity (RSC) in percent was calculated by following equation.

$$\mathbf{RSC\ (\%) = 100 \times A_{blank} - A_{sample} \ / \ A_{blank}}$$

From the obtained RSC values the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization or inhibition.

Percentage scavenging capacity and IC_{50} of various extracts, fractions and isolate pure flavonoids were tabulated in Tabe-2.

Scavenging capacity for hydroxyl radicals [10, 11]

By measuring the degradation of 2-deoxyribose

All the solutions and reagents were dissolved in KH_2PO_4 –K_2HPO_4 phosphate buffer. All the sample solutions were prepared in KH_2PO_4 –K_2HPO_4 phosphate buffer (P^H-7.4). In the test tubes different concentrations of sample i.e. 16.6 to 66.66 μgm/ml were prepared in phosphate buffer and mixed with 0.125ml H_2O_2, 0.125 ml $FeSO_4$ and 0.125ml 2-deoxy-D-ribose and filled up with 0.05M phosphate buffer of pH 7.4, to a volume of 3ml and test tubes were kept for incubation at 37^{O}C for 1hour. After incubation for 1 hour 1.5ml of TBA reagent and 0.2ml of 0.1M EDTA were added and the tubes were heated at 100^{O}C for 20min. After cooling the absorbance was read against a blank (containing a buffer solution instead of sample) at 532nm.The absorbance was used for the calculation of the percentage inhibition of deoxyribose degradation by the sample by using the formula.

$$\mathbf{I(\%) = 100 \times A_{blank} - A_{sample} \ / \ A_{blank}}$$

Experiment was performed in triplicates. Ascorbic acid was used as a positive control. Percentage scavenging capacity and IC_{50} of various extracts and fractions were tabulated in Tabe-3.

STATISTICAL ANALYSIS

The data were reported as mean values ± Standard error mean. Values representing the concentration of investigated samples that causes 50% of inhibition (IC_{50}) were determined by the linear regression analysis.

RESULTS AND DISCUSSION

Various extracts fractions and isolated flavonoids of the flowers of *Ichnocarpus frutescens* L were tested for antioxidant property by using DPPH assay, measuring the degradation of 2-deoxyribose with hydroxyl radicals. Amongst the samples studied the flavonoid-I (F-I), isolated from ethyl acetate fraction of alcoholic extract of *Ichnocarpus frutescens* L shown more free radical scavenging activity with an IC_{50} of 33.89 and 19.83.

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TABLE-1

SAMPLE	CONC. µgm/ml	% RSC
Alcoholic extract	75 µgm/ml	22.29
Petroleum ether fraction		35.17
Chloroform fraction		36.58
Ethyl acetate fraction		60.03
Ascorbic acid		97.62

Table No.02: PERCENTAGE FREE RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS, FRACTIONS AND ISOLATED FLAVONOIDS

Conc.mcg/ml	PERCENTAGE FRE RADICAL SCAVENGING ACTIVITY						
	Alc.Ext.	P.E.Fr.	Chl.Fr.	Et.ac.Fr.	F-I	F-II	Asc.acid
12.5	5.32±0.02	10.15±0.04	10.15±0.01	14.73±0.04	37.66±0.08	18.83±0.03	43.05±0.04
25	6.21±0.05	16.75±0.02	21.9±0.05	21.33±0.12	41.13±0.05	25.62±0.07	51.71±0.02
37.5	6.55±0.03	19.41±0.22	26.75±0.09	32.29±0.09	56.65±0.04	35.14±0.02	63.57±0.28
50	9.41±0.2	24.13±0.06	27.79±0.04	41.20±0.05	65.24±0.06	52.64±0.10	77.31±0.15
62.5	20.26±0.3	25.13±0.03	35.18±0.04	49.06±0.02	71.56±0.08	68.42±0.23	89.36±0.01
75	22.29±0.09	35.17±0.04	36.58±0.02	60.03±0.07	78.39±0.19	76.15±0.03	97.62±0.03
IC_{50}	-	-	-	63.30	33.89	48.92	24.95

All values in this table represent the mean ±SEM (n=3). IC_{50} values, from the data, were calculated by regression analysis

Table No.03: PERCENTAGE HYDROXYL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS, FRACTIONS AND ISOLATED FLAVONOIDS

Conc.mcg/ml	PERCENTAGE HYDROXYL SCAVENGING ACTIVITY						
	Alc.Ext.	P.E.Fr.	Chl.Fr.	Et.ac.Fr.	F-I	F-II	Asc.acid
16.6	17.85±0.32	19.82±0.10	30.21±0.37	44.57±0.31	48.2±0.20	46.7±0.30	61.74±0.31
33.33	22.88±0.04	22.60±0.17	33.68±0.18	49.94±0.43	59.79±0.19	56.1±0.11	71.32±0.22
50	26.49±0.09	24.05±0.16	37.62±0.14	59.17±0.36	67.00±0.14	64.28±0.10	83.56±0.16
66.66	29.96±0.03	27.37±0.27	41.07±0.46	68.53±0.20	79.00±0.06	73.13±0.03	91.66±0.14
IC_{50}	-	-	-	32.11	19.83	23.34	11.61

All values in this table represent the mean ±SEM (n=3). IC_{50} values, from the data, were calculated by regression analysis

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CONCLUSION

The pure flavonoidal compound (F-I) isolated from the ethyl acetate fraction of alcoholic extract of flowers of *Ichnocarpus frutescens* L shown maximum antioxidant property as compared to the other extract, fractions and isolated pure flavonoid II(F-II). Hence we conclude that the flavonoidal content in F-I is more bioactive towards antioxidant activity than other samples studied.

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