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Research Paper

PHARMACOLOGY AND PHYTOCHEMICAL EVALUATION OF TRIUMFETTA RHOMBOIDEA JACQ.

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Triumfetta rhomboidea Jacq is being used in tradidional medicines for various ailments. It is widely distributed throughout tropical and subtropical part of India, and also found in Ceylon, Malay, Africa and America. The aim of the present study was to evaluate the antioxidant and radical-scavenging activities in the fractions of Triumfetta rhomboidea. The extract/fractions solvent systems in the assay included petroleum ether, chloroform, ethyl acetate, n-butanol and methanol. The fractions were investigated by several methods in vitro, such as reducing power assay, 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging assay, nitric oxide radical scavenging assay, superoxide anion radical-scavenging assay, lipid peroxidation assay and deoxyribose degradation (site-specific and non-site-specific) to find out the fractions as hydrogen or proton donor or direct free radical scavenger.

Key words: Triumfetta rhomboidea, Antioxidants; Free radical scavengers

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide (O_2-), hydroxyl radicals (OH-) and hydrogen peroxide (H_2O_2-) form an important factor in the etiology of several pathological conditions such as Alzheimer's disease. Parkinsons's disease, arthritis, haemorrhoids, rheumatism, heart attack, AIDS, immune system and disorders, cataract, stroke, cancer stress, varicose veins, hepatitis, diabetes and several degenerative diseases including aging [1'2&3]. ROS are degraded to non-reactive forms by enzymatic and non-enzymatic antioxidant defense mechanisms.

There is a balance between generation of ROS and antioxidant system in organisms. In pathological condition, ROS are overproduced and result in lipid peroxidation and oxidative stress. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules.

Many antioxidant compounds, naturally occurring from plant sources, have been identified as free radical or active oxygen scavengers.

The plant *Triumfetta rhomboidea* is distributed throughout tropical and subtropical part of India, and also found in Ceylon, Malay, Africa and America. All parts of the plant are used in natural medicine in the tropics. Hence, the present study has been undertaken to investigate the in vitro antioxidant activity of *Triumfetta rhomboidea* using different in vitro models.[4,5]

MATERIALS AND METHODS

Plant material and Extraction

ISSN 2249-3875

International Journal of Pharmaceutical Erudition

The whole plant of Triumfetta rhomboidea Jacq. was collected from Sikar district, Rajasthan, India, and authenticated by Dr. M. K. Singhadiya, Botanical Survey of India (BSI), Jodhpur, Rajasthan, India.

The leaves, stems, roots, flowers and fruits of the plant were separated. Plant material was washed with distilled water to remove epiphytes and dirt particles and dried at room temperature. The dried plant material was manually ground to a fine powder.

ANTIOXIDANT ACTIVITY

The aim of the present study was to evaluate the antioxidant and radical-scavenging activities in the fractions of Triumfetta rhomboidea. The extract/fractions solvent systems in the assay included petroleum ether, chloroform, ethyl acetate, n-butanol and methanol. The fractions were investigated by several methods in vitro, such as reducing power assay, 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging assay, nitric oxide radical scavenging assay, superoxide anion radical-scavenging assay, lipid peroxidation assay and deoxyribose degradation (site-specific and non-site-specific) to find out the fractions as hydrogen or proton donor or direct free radical scavenger.

IN-VITRO ANTIOXIDANT ASSAY

1. DPPH radical scavenging assay

DPPH is nitrogen centered free radical that show strong absorbance at 517 nm. DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable DPPH radical. The free stable radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors; this ability was evaluated by more frequently used discoloration assay, which evaluates the absorbance decrease at 517 nm produced by the addition of the antioxidant to a DPPH solution in methanol. This method is widely used to check the free radical scavenging antioxidants. To evaluate the antioxidant activity, specific compounds or extracts (antioxidant) were allowed to react with a stable radical DPPH in a methanol solution. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of IC50 values [6,7].

Method

DPPH· scavenging potential of different fractions was measured, based on the scavenging ability of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by Triumfetta rhomboidea. The ability of fractions to scavenge DPPH radicals was determined. Briefly, 1 ml of 1 mM methanolic solution of DPPH· was mixed with 1 ml of fractions solution. The mixture was then vortexed vigorously and kept for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH· scavenging activity relative to the control, using the following equation.

ISSN 2249-3875

International Journal of Pharmaceutical Erudition

$$\% \text{ Radical scavenging activity} = \frac{(A_{Control} - A_{Sample})}{A_{Control}} \times 100$$

The percentage inhibition was plotted against the sample fractions concentration in order to calculate the IC50 values, which is the concentration (µg/ml) of fraction that causes 50% loss of DPPH activity [8].

2. Nitric oxide radical scavenging assay

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions [9].

Method

For the experiment, sodium nitroprusside (10mM), in phosphate buffered saline was mixed with different concentration of different fractions dissolved in different solvents and incubated at room temperature for 150 minutes. The same reaction mixture, without the fractions but with an equivalent amount of solvent, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Rutin was used as positive control.

3. Superoxide radical-scavenging assay

The superoxide anion radical scavenging capacity assay was developed to evaluate the ability of hydrophilic antioxidants to directly react with this physiologically relevant radical. The assay measures the ability of a selected antioxidant to complete with a molecular probe, nitroblue tetrazolium (NBT), to scavenge superoxide anion radical generated by an enzymatic hypoxanthine-xanthine oxidase (HPX-XOD) system. NBT has a yellow color that upon reduction by superoxide anion radical forms formazan with a blue color measured at 560nm with a spectrophotometer [6, 7].

Method

Each 3 ml reaction mixture contained 0.05 M phosphate-buffered saline (PBS) (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and 1 ml of test sample solutions (10–100 µg/ml). The tubes were kept in front of a fluorescent light and absorbance was read at 560 nm after 20 minutes. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes containing reaction mixture were kept in the dark and served as blank [10].

4. Ferric reducing/antioxidant power (FRAP) assay

In this method, the reduction of Fe3+-Fe2+was determined by measuring absorbance of the Perl's Prussian blue complex. This method is based on the reduction of (Fe3+) ferricyanide in

stoichiometric excess relative to the antioxidants.

Method

Potassium phosphate buffer (2.5 ml of 0.1 M and pH 6.6) and 2.5 ml of 1% w/v potassium ferricyanide were mixed with 1.0 ml of fractions of varying dilutions. The reaction mixture was incubated at 50°C for 20 minutes, after which 2.5 ml of 10% w/v trichloroacetic acid was added. Water (2.5 ml) and 0.5 ml of 0.1% w/v $FeCl_3$ were then added to 2.5 ml of the reaction mixture, and the solution was incubated at 28°C for 30 minutes to facilitate color development. The absorbance was measured at 700 nm and the amount of gallic acid equivalents in mg per gram fresh material (mg GAE/g) was calculated [11].

5. Lipid peroxidation assay

A thiobarbituric acid-reactive species (TBARS) assay was used to measure the lipidperoxide formed, using egg-yolk homogenates as lipid-rich media. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturatedfatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm [10].

Method

Egg homogenate (500 µl of 10%, v/v in phosphate-buffered saline pH 7.4) and 100 µl of sample (10–250 µg/ml) were added to a test tube and made up to 1 ml with distilled water. Then, 50 µl of $FeSO_4$ (0.075 M) and 20 µl of L-ascorbic acid (0.1 M) were added and incubated for 1 hour at 37°C to induce lipid peroxidation. Thereafter, 0.2 ml of EDTA (0.1 M) and 1.5 ml of TBA reagent (3 g TBA, 120 g TCA and 10.4 ml 70% $HClO_4$ in 800 ml of distilled water) were added in each sample and heated for 15 minutes at 100°C. After cooling, samples were centrifuged for 10 minutes at 3000 rpm using a centrifuge and absorbance of supernatant was measured at 532 nm.

6. Hydroxyl radical scavenging assay

The hydroxyl radical is one of the most reactive species known in the biological system. This assay monitors the ability of the antioxidant sample to compete with a molecular probe, fluorescein (FL), and scavenge pure hydroxyl radical generated by the Fenton-like Fe3+/H_2O_2 system. The hydroxyl radical scavenging activity of fractions from Triumfetta rhomboidea was measured by the competition between deoxyribose and Triumfetta rhomboidea for the hydroxyl radicals generated from the Fe3+/ascorbate/EDTA/H_2O_2 system (nonsite-specific assay) or Fe3+/ascorbate/H_2O_2 (site-specific assay).

Non-site-specific hydroxyl radical-mediated 2-deoxy-D-ribos degradation

The deoxyribose method was used for determining the scavenging effect on hydroxyl radicals. The reaction mixtures contained ascorbic acid (50 µM), $FeCl_3$ (20 µM), EDTA (2

mM), H_2O_2 (1.42 mM), deoxyribose (2.8 mM) with different concentrations of the test fractions in a final volume of 1 ml in potassium phosphate buffer (10 mM, pH 7.4). It was incubated at 37°C for 1 hour and then 1 ml of 2.8% TCA (w/v in water) and 1 ml of 1% thiobarbituric acid (TBA) (w/v) were added. The mixture was heated in a boiling water bath for 15 minutes. It was cooled and absorbance was taken at 532 nm. BHT was taken as positive control [12].

Site-specific hydroxyl radical-mediated 2-deoxy-Dribose degradation

The ability of the fraction to inhibit site-specific hydroxyl radical-mediated degradation was also carried out to understand its role as a metal chelator. The method was the same as described above except that buffer replaced EDTA.

RESULT AND DISCUSSION

The DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical scavenging activities of antioxidants [13]. All the fractions were capable of scavenging DPPH radicals in a concentration-dependent manner. The ethyl acetate fraction showed highest DPPH radical scavenging activity of 95.61% at 100 µg/ml concentrations whereas petroleum ether, chloroform, n-butanol and aqueous fractions showed 75.15%, 63.87%, 73.22% and 85.67% inhibition, respectively, at the same concentration. The DPPH scavenging activities of the fractions, expressed as an IC50 value, ranged from 25.02 to 73.53µg/ml. The IC50 value of various fractions was compared with positive control rutin (Table 1.0). The order of DPPH radical scavenging assay was found to be petroleum ether > ethyl acetate > aqueous > n-butanol > chloroform.

The aqueous fraction showed the highest NO radical scavenging activity of 91.48% at 80 µg/ml concentrations whereas petroleum ether, chloroform, ethyl acetate and n-butanol fractions showed 82%, 61.84%, 88.72% and 76.29% inhibition, respectively, at the same concentration.

The NO radical scavenging activities of the fractions, expressed as an IC50 value, ranged from 26.86 to 60.46µg/ml. The IC50 values of various fractions were compared with positive control rutin (Table 1.0). The order of NO radical scavenging assay was found to be petroleum ether > ethyl acetate > aqueous > n-butanol > chloroform.

The petroleum ether fraction showed highest superoxide radical scavenging activity of 79.92% at 100 µg/ml concentrations whereas chloroform, ethyl acetate, n-butanol and aqueous fractions showed 40.48%, 75.79%, 60.68% and 64.91% inhibition, respectively, at the same concentration.

The superoxide radical scavenging activities of the fractions, expressed as an IC50 value,

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ranged from 16.56 to 65.65µg/ml. The IC50 values of various fractions were compared with positive control rutin (Table 1.0). The order of super oxide anion radical scavenging assay was found to be petroleum ether > ethyl acetate > aqueous > n-butanol > chloroform.

Among the different fractions, ethyl acetate fraction exhibited the maximum reducing power (0.67 ± 1.69) at a dosage of 100µg/ml. The sequence for reducing power was: gallic acid (0.71 ± 2.56)>ethyl acetate (0.67 ± 1.69)> aqueous (0.57 ± 1.25)>petroleum ether (0.46 ± 2.09)> n-butanol (0.373 ± 0.91)> chloroform (0.22 ± 0.92). The ethyl acetate fraction showed maximum lipid peroxidation inhibition of 51.09% at 250 µg/ml concentrations whereas petroleum ether, chloroform, n-butanol and aqueous fractions showed 43.22%,34.75%, 39.08% and 47.5% inhibition, respectively, at the same concentration. The lipid peroxidation assay of the fractions, expressed as an IC50 value, ranged from 229.49 to 316.61µg/ml. The IC50 value of various fractions was compared with positive control BHT (Table 1.0). The order of lipid peroxidation assay was found to be ethyl acetate > aqueous > petroleum ether > n-butanol >chloroform. In site-specific assay, aqueous fraction showed maximum inhibition of 198.4% at 250 µg/ml whereas petroleum ether, chloroform, ethyl acetate and n-butanol fractions showed 165.7%, 139.72%, 182.89 and 148.8% inhibition, respectively, at the same concentration in hydroxyl radical scavenging ability of different fractions.

The IC50values of various fractions were compared with positive control BHT (Table 1).

Table 1: IC50 values obtained in the antioxidant activity assays of different fractions from *Triumfetta rhomboidea.*

	IC_{50} Value (µg/ml)					
	DPPH	**NO**	**SOD**	**LP**	**NSSDRD**	**SSDRD**
Ruti	7.23±0.575	15.02±1.23	15.1019±0.74	-	-	-
BHT	-	-	-	220.823±3.2	32.72 ±	20.37 ±
PE	25.026±3.44	26.86±1.13	16.56±3.979	275.9±1.65	52.53 ±	42.63 ±
CF	73.53±	60.46±	65.653± 4.107	316.61 ±	71.87 ±	61.93 ±
EA	39.780±3.88	32.24±1.6	18.883±0.727	229.49±3.62	36.87 ±	26.31 ±
NB	63.176±	42.89±	38.936± 1.626	298±0.82	60.77 ±	51.558 ±
AQ	50.052±4.68	34.52±1.24	31.728±1.358	244.78±1.83	47.11 ±	34.59 ±

PE, petroleum ether; CF, chloroform; EA, ethyl acetate; NB, n-butanol; AQ, aqueous; BHT, butylated hydroxyltoluene; DPPH, 1,1-diphenyl-2-picrylhydrazyl; NO, Nitric oxide; SOD, Superoxide; LP, Lipid peroxidation; NSSDRD, Non-site-specific deoxyribose degradation; SSDRD, Site-specific deoxyribose degradation.

Each value is expressed as a mean ± standard deviation (n = 3).

The order of non-site specific and site specific hydroxy radical scavenging activities was found to be ethyl acetate > aqueous > petroleum ether > n-butanol > chloroform.

CONCLUSION

In this study, petroleum ether, chloroform, ethyl acetate, n-butanol and aqueous fractions of 95% methanol extract of Triumfetta rhomboidea were initially screened for their in vitro antioxidant assays. Among the fractions, ethyl acetate fraction exhibited highest inhibition of 95.61% and 51.09% in DPPH and lipid peroxidation assays whereas the aqueous fraction exhibited the highest inhibition of 91.48%, 174%, 198.4% in nitric oxide radical, non-site specific and site specific assays, respectively. Petroleum ether fraction was more effective than other fractions in scavenging ability on DPPH, nitric oxide and superoxide radicals, whereas ethyl acetate fraction was more effective in reducing power, lipid peroxidation, non-site-specific and site-specific assays as evidenced by their lower IC50 values. This study clearly indicates that Triumfetta rhomboidea possesses antioxidant effects.

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