ANTIOXIDANT STUDIES ON THE ETHANOLIC EXTRACT OF *ZORNIA GIBBOSA*

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Summary

The ethanolic extract of *Zornia zibbosa,* is widely used in the indigenous system of medicine was studied for its in vitro scavenging activity in different methods viz. DPPH, ABTS radical scavenging, lipid peroxidation, iron chelation activity, superoxide scavenging, total antioxidant capacity and antioxidant haemoglobin glycosylation. The results were analyzed statistically by regression method. Its antioxidant activity was estimated by IC_{50} value and the values are 14.78µg/ml for lipid peroxidation, 40.29 µg/ml for ABTS scavenging, 83.11µg/ml for iron chelating activity, 105.90µg/ml for DPPH scavenging and 97.96µg/ml for superoxide scavenging. Total antioxidant capacity was found to be 17.96 µg/ml. In Haemaglobin glycosylation model the % scavenging was found to be 45.49% and 74.68% at the concentration 0.5 mg/ml and 1.0 mg/ml respectively. In all the methods the extract showed its ability to scavenge free radicals in a concentration dependent manner. The results indicate that *Z. Gibbosa* has significant antioxidant activity.

Keywords: *Zornia gibbosa,* 2, 2-Azino bis (3-ethyl Benzo Thiazoline-6-Sulphonate) (ABTS), Haemoglobin glycosylation, superoxide scavenging, lipid peroxidation

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Introduction

Though oxygen is essential for the aerobic process, cells under aerobic condition are threatened with the insult of reactive oxygen metabolites, a threat which is efficiently taken care by the powerful antioxidant system in human body. Aerobic life is characterized as the continuous production of antioxidant balanced by an equivalent synthesis of antioxidants. The free radicals are capable of independent existence and cause oxidative tissue damage. The non-radical oxidants like hydrogen peroxide and hypochlorous acid, which do not possess unpaired electrons, are also capable of inciting oxidative tissue damage. The improper balance between reactive oxygen metabolite production and antioxidant defence result in “oxidative stress”, which deregulates the cellular function leading to various pathological conditions. Antioxidant principles from natural sources possess multifacetedness in their multitude of activities and provide enormous scope in correcting the imbalance. Therefore, much attention is being directed to harness and harvest the antioxidant principles from natural sources. (1)

Zornia gibbosa belonging to family papilionaceae, is a diffuse herb. Leaves are digitately 2-foliolate; leaflets up to 2.5 cm long, lanceolate, dotted with black glands. Flowers are yellow, enclosed in leafy bracts, four in spikes. Pods are with three to six joints, densely prickly. It is distributed throughout Kanyakumari, Ramanathapuram, South Arcot, Tiruneveli of Tamil Nadu state and also in tropical region of India. Traditionally, herb is used in dysentery and roots as a soporific given to children. (2)

Materials and methods

Chemicals and Instruments: All chemicals and solvents used in the study were analytical grade. DPPH (1, 1- Diphenyl-2-Picryl Hydrazyl) and ABTS (2, 2-Azino bis (3-ethyl Benzo Thiazoline-6-Sulphonic acid) were obtained from Sigma Chemicals (St.louis, Mo, USA). Sodium nitroprusside, ferrous sulphate, trichloroacetic acid, dimethyl sulphoxide (DMSO), ethylene diamine tetra acetic acid (EDTA), sodium hydroxide, potassium chloride and sulphanilamide were obtained from Ranbaxy Fine Chemicals Ltd. India. NBT (Nitro blue tetrazolium chloride) and thiobarbituric acid were obtained from Himedia Laboratories Ltd. Mumbai, India.

UV spectrophotometer (Shimadzu 1650 pc), Micro plate reader (Biotek Instruments, ELx 800 and pH meter (Elico Ltd., India).

Plant Material: The plant material of *Z. Gibbosa* were collected from Manipal, Udupi district, Karnataka, India in November 2007 and was authenticated by Dr. Gopalakrishna Bhat, professor, Department of Botany , Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen has been deposited at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Preparation of ethanol extract: About 500 g of whole plant powder was taken in soxhlet extractor and extracted with ethanol (95%) for four days. The solvent was recovered by distillation in vacuo and residue (yield 40 g), stored in the desiccator was used for subsequent experiment.

Preparation of Z. gibbosa stock solution: Z. gibbosa stock solution was prepared in concentration of 1000µg/ml in methanol. From the stock solution different concentration viz. 2, 4, 8, 16, 32, 64, 128, 256, 512, 1000 µg/ml were prepared in methanol and used for antioxidant studies.

Preparation of stock solution (Standard drugs): Ascorbic acid was used as standard. Ascorbic acid stock solution was prepared in concentration of 1000µg/ml in methanol. From the stock solution different concentration viz. 2, 4, 8, 16, 32, 64, 128, 256, 512, 1000 µg/ml were prepared in methanol and used for antioxidant studies.

Vitamin E (Tocopherol) was used as standard for antioxidant haemoglobin glycosylation method.

Antioxidant invitro methods

DPPH Radical Scavenging

Ethanolic extract of various concentrations were made upto 1ml with methanol, 1ml of DPPH solution 0.1 mM (0.39 mg in 10ml methanol) was added to the test tube. An equal amount of ethanol and DPPH was added to the control. Ascorbic acid was used as the standard for comparison. After 20 minutes of incubation in the dark, absorbance was recorded at 517 nm. Experiment was performed in triplicate. (Fig.1). (3, 4)

ABTS Radical Scavenging

The ABTS radical cation preparation: ABTS 2mM (0.0548g in 50 ml) was prepared in distilled water. Potassium per sulphate 70mM (0.0189g in 1ml) was prepared in distilled water. 200µl of Potassium per sulphate and 50 ml

of ABTS were mixed and used after 2 hrs. This solution was used for the assay.

To the 0.5 ml of various concentration of extract, 0.3 ml of ABTS radical cation and 1.7 ml of phosphate buffer pH 7.4 was added. For control studies, methanol was taken and the absorbance was measured at 734 nm. The experiment was performed in triplicate. (Fig.2)(5, 6)

Iron Chelating Activity

To the 2 ml of various concentration of extract, 1ml of ortho-Phenanthroline (0.005g in 10 ml methanol) and 2 ml ferric chloride (3.24 mg in 100 ml distilled water) were added. The mixture was incubated at ambient temperature for 10 min, and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate. (Fig.3) (7, 8,)

Superoxide Scavenging

To the 0.5 ml of different concentration of extract, 1 ml alkaline DMSO and 0.2 ml NBT 20 mM (50 mg in 10ml phosphate buffer pH 7.4) was added. The absorbance was measured at 560 nm. The experiment was performed in triplicate. (Fig.4) (9)

Lipid Peroxidation Assay

Phospatidylcholine (20 mg) in 2 ml chloroform was dried under vacuum in a rotary evaporator to give a thin homogenous film and further dispersed in normal saline (5 ml) with a vortex mixer. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid into a mixture containing 0.1 ml liposome ,150 mM potassium chloride, 0.2 mM ferric chloride, extract (2 to100 μg/ml). The reaction mixture as incubated at 37°C for 40 min. After incubation, the reaction was stopped by adding 1 ml of ice-cold 0.25 M sodium hydroxide containing 20 % TCA (w/v), 0.4 % TBA (w/v), and 0.05 % BHT (w/v). After incubating in a boiling water bath for 20 min, the samples were cooled to room temperature. The blank was prepared in the same manner without extract. The pink chromogen was extracted with 1ml of methanol. The absorbance was read at 532 nm. (Fig. 5) (10)

Total Antioxidant

0.1ml of extract (10mg/ml) dissolved in water was mixed with 1 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid. (11, 12)

Antioxidant Haemoglobin Glycosylation

The antioxidant activities of different extracts were investigated by estimating degree of non-enzymatic haemoglobin glycosylation measured colorimetrically. Haemoglobin, 60 mg/100 ml in 0.01 M phosphate buffer (pH 7.4) was incubated in presence of 2 g/100 ml concentration of glucose for 72 h, in order to find out the best condition for haemoglobin glycosylation. The assay was performed by adding 1 ml of glucose solution, 1 ml of haemoglobin solution, and 1 ml of gentamicin (20 mg/ 100 ml), in 0.01 M phosphate buffer (pH 7.4). The mixture was incubated in dark at room temperature for 72 h. The degree of glycosylation of hemoglobin in the presence of different concentration of extracts and their absence were measured colorimetrically at 520 nm. (13)

Statistical Analysis

All results are expressed as mean ± S.E.M. Linear regression analysis was used to calculate the IC_{50} values.

Results

Concentrations ranging from 2-1000µg/ml of the ethanolic extract of *Zornia gibbosa* were tested for their antioxidant activity in different *invitro* models. It was observed that free radicals were scavenged by the extract in concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all the models. In DPPH method, the maximum scavenging activity was found at a concentration 512µg/ml and the minimum scavenging activity at a concentration of 2µg/ml (fig.1). IC_{50} value was found to be 40.29 µg/ml in ABTS scavenging method, maximum activity was exhibited at 128 µg/ml with 93.70% scavenging and decline was observed at higher concentration (fig 2). Iron chelating activity, maximum scavenging was found at 1000

μg/ml with IC_{50} of 83.11μg/ml (fig.3). In superoxide scavenging, the maximum activity was observed at 300 μg/ml with scavenging of 97.02% (fig. 4). Maximum scavenging activity was observed at 100 μg/ml with an IC_{50} value of 14.78 μg/ml in lipid peroxidation (fig.5). Antioxidant haemoglobin glycosylation, the % scavenging was found to be 45.49% and 74.68% at concentration 0.5 mg/ml and 1.0mg/ml of extract respectively. Total antioxidant capacity of ethanolic extract of *Z. gibbosa* (10mg/ml) is equivalent to 31.66 mg/ml of ascorbic acid.

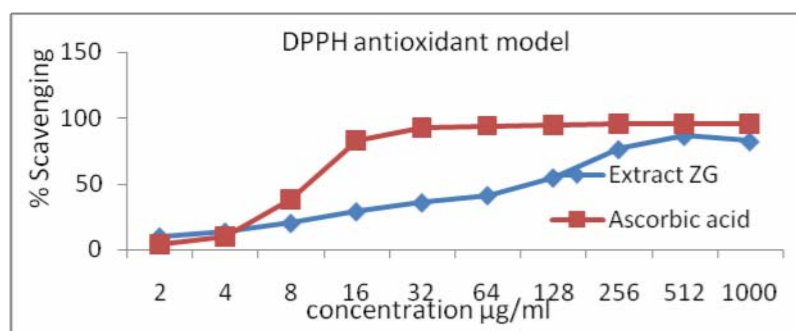

Fig. 1. DPPH radical scavenging activity of different concentrations of *Z. gibbosa* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

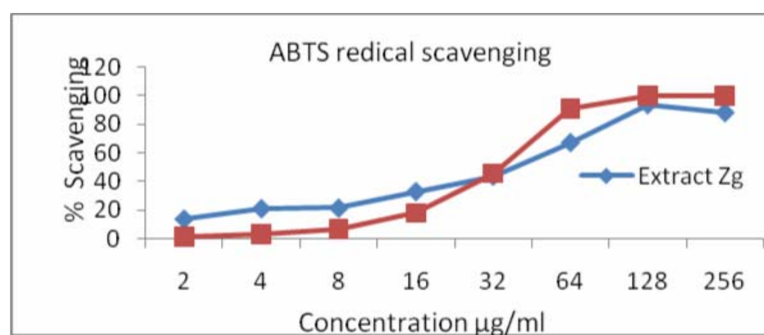

Fig. 2. ABTS radical scavenging activity of different concentrations of *Z. gibbosa* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

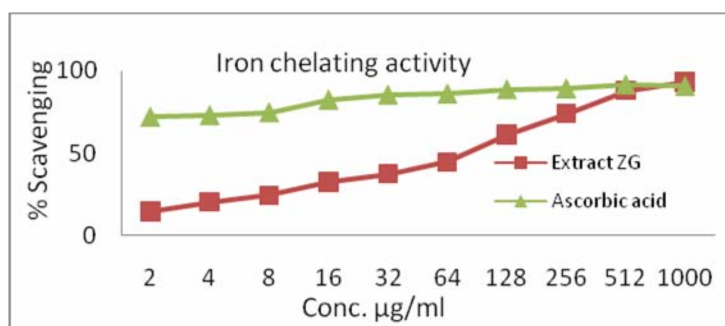

Fig. 3. Reducing power of extract of *Z. gibbosa* and ascorbic acid by spectrophotometric detection of the Fe^{3+} - Fe^{2-} trans-formation. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

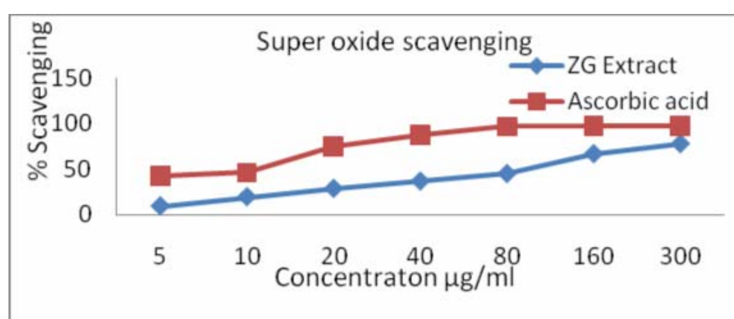

Fig. 4. Super oxide anion radical scavenging activity of different concentrations of *Z. gibbosa* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

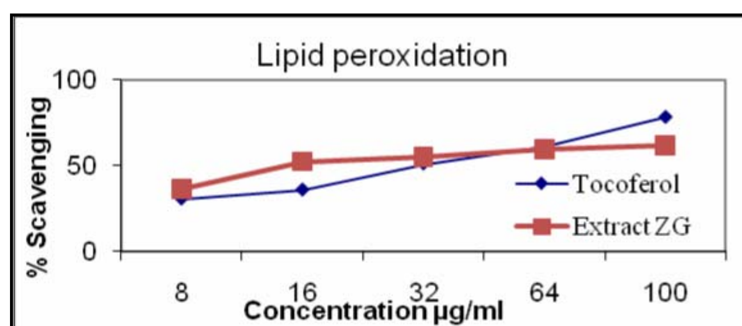

Fig. 5. Antioxidant activity of different concentrations of *Z. gibbosa* and α-tocoferol in lipid peroxidation assay. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ±S.E.M.

Table 1*:* Results of various antioxidant models

Serial No.	Model	IC_{50} µg/ml	
		Ethanol Extract	Ascorbic acid
1	DPPH radical scavenging	105.90	10.36
2	ABTS radical scavenging	40.29	36.42
3	Iron chelating activity	83.11	59.65
4	Superoxide scavenging	97.96	13.00
5	Lipid peroxidation assay	14.78	31.05*
6	Antioxidant haemoglobin glycosylation		
	Extract/ standard	*Concentration(µg/l)*	*% Scavenging*
	Ethanolic extract	0.5	45.49
		1	74.68
	Vitamin –E	0.5	61.53
		1	86.69
7	Total Antioxidant		
	10 mg/ml alcoholic extract of *Zornia gibbosa* = 31.66 mg/ml of ascorbic acid		

* In lipid peroxidation α-tocoferol was taken as standard drug.

Discussion

There is an extensive evidence to implicate free radicals in the development of degenerative diseases. Free radicals have been implicated in the causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity, cancer etc. Together with other derivatives of oxygen, they are inevitable by products of biological redox reactions. Reactive oxygen species such as superoxide anions, hydroxyl radical, and nitric oxide inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation. (14-17)

DPPH is a stable free radical. When antioxidant reacts with this stable radical, the electron becomes paired off and bleaching of the colour stochiometrically depends on the number of electrons taken up. From our finding, it may be postulated that *Z.gibbosa* reduces the radical moderately to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles. (18, 19)

Ortho substituted phenolic compounds may exert prooxidant effects by interacting with iron. o-phenonthroline quantitatively forms complexes with Fe^{2+}, which get disrupted in the presence of chelating agents. The ethanolic extract interfered with the formation of ferrous-o-phenonthroline complex, thereby suggesting that the extract has metal chelating activity. Iron stimulates lipid peroxidation by Fenton reaction and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation. The metal chelating capacity is important, since it reduces concentration of the catalyzing transition metal in lipid peroxidation. It has been reported that the chelating agents, form bonds with a metal are effective as secondary antioxidant because they reduce the redox potential, thereby stimulating the oxidized form of the metal ion. The observed results demonstrate a marked capacity of the extract for iron binding, suggesting that their action as a peroxidation protector may be related to its iron binding capacity. (20-24)

ABTS assay is a decolorizing assay, which involves the direct generation of ABTS radical into monocation, which has a long wavelength absorption spectrum without involvement of any intermediary radical. The antioxidant activity of the extract by this assay implies that the action may be either inhibiting or scavenging radicals since both inhibition and scavenging properties of antioxidant towards this radical have been reported in earlier studies. (25)

Superoxide is a highly reactive molecule that can react with many substrates produce in various metabolic processes including phagocytosis. It can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme, which catalyze the breakdown of superoxide radical. In our study, alkaline DMSO used for superoxide generation indicates that *Z.gibbosa* is a potent superoxide scavenger. (26)

Lipid peroxidation has been implicated in the pathogenesis of number of diseases and clinical conditions. Malondialdehyde and other aldehydes have

been identified as products of lipid peroxidation that reacts with the thiobarbituric acid to give pink coloured species. The aldehyde products are responsible for DNA damage, generation of cancer and aging related diseases. The decrease in the concentration of the malondialdehyde levelled with the increase in the concentrationof *Z.gibbosa* extract indicate the antioxidant role of the extract. (27)

Haemoglobin glycosylation is an *invitro* non-enzymatic method. Being an oxidation reaction, an antioxidant is expected to inhibit the reaction. The degree of haemoglycosylation *invitro* in the presence of different concentration of extract can be measured spectrophotometrically. (13)

Total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically. (28)

Z.gibbosa extract exhibits its antioxidant action in several ways: removal of oxygen, scavenging of ROS, binding of metal ions needed for catalysis of ROS and upregulation of endogenous antioxidant defences.

Our finding shows that the ethanolic extract of *Z. gibbosa* have significant antioxidant potential in all the models and to a lesser extent in DPPH and SOD.

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