Free Radical Scavenging Activity and Total Phenolic content of *Zi*ziphus *Mauritiana* Lam.

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Summary

The free radical scavenging potential of the ethanolic and aqueous extracts of the leaves of *Ziziphus mauritiana* Lam, was studied for its *in vitro* scavenging activity by different methods *viz.* DPPH radical scavenging, ABTS radical scavenging, lipid peroxidation assay, superoxide scavenging activity, nitric oxide scavenging activity, total antioxidant capacity and Non-enzymatic Glycosylation of Hemoglobin assay. The results were analyzed statistically by regression method. The percentage scavenging and IC_{50} values were calculated for all models. In all the methods, the extract showed its ability to scavenge free radicals in a concentration dependent manner. The measurement of total phenolic content by Folin-Ciocalteus reagent indicated that ethanolic and aqueous extract contains total phenolic compound 0.122 mg/ml and 0.150 mg/ml equivalent to gallic acid. The results indicate that both ethanolic and aqueous extract of Z. *mauritiana* had significant antioxidant activity.

Keywords: DPPH, Hemoglobin glycosylation, lipid peroxidation, superoxide scavenging, *Ziziphus mauritiana*.

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 of 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 harvest the antioxidant principles from natural sources (1).

Ziziphus mauritiana Lam. (Rhamnaceae) is a small evergreen tree of variable size, up to 15 m or even more in height, found both wild and cultivated throughout the greater part of India. Literature survey reveals that the leaves are eaten with catechu as an astringent. They are regarded as diaphoretic and are prescribed for typhoid in children. They are also used as poultices. Leaves are bitter, cooling, astringent, anthelmintic, diaphoretic and antipyretic, and are useful in stomatitis, ulorrhea, wounds, syphilitic ulcers, asthma, typhoid fever, diarrhoea and obesity (2-4).

Materials and Methods

Chemicals

DPPH (1, 1- Diphenyl-2-Picryl Hydrazyl), ABTS (2,2- azinobis- (3- ethylbenzothiazoline- 6-sulphonate), hemoglobin and L-α-phosphatidyl choline were obtained from Sigma Chemicals, USA. Ferrous sulphate, dimethyl sulphoxide (DMSO), ethylene diamine tetra acetic acid (EDTA), sodium hydroxide and potassium chloride were obtained from Ranbaxy Fine Chemicals Ltd., India. TBA (Thiobarbituric acid), TCA (Trichloro acetic acid), NBT (Nitro blue tetrazolium chloride) and BHT (Butylated hydroxytoluene) were obtained from Himedia Laboratories Ltd. Mumbai, India. All other chemicals and solvents used in the study were of analytical grade.

Plant Material

The fresh leaves of *Z. mauritiana* Lam (Rhamnaceae) were collected from the Manipal, Udupi district, Karnataka, India in November 2007 and were authenticated by Dr. Gopalakrishna Bhat, Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (PP No.562) has been deposited in the museum of the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences for future reference.

Preparation of the extract

The leaves were shade dried at room temperature and powdered. About 500 g of the leaves powder was taken and extracted with chloroform water (1:99) by maceration for seven days with intermediate shaking. The extract evaporated under vacuum gave a dry extract (yield 35 g) and was stored in a desiccator for further study. For ethanolic extract preparation shade dried powdered leaves at room temperature were extracted (500 g) in a soxhlet extractor with ethanol (2 L) for 4 h. The crude extract was concentrated to dryness in rotary flash evaporator (Buchi type) under reduced pressure and controlled temperature (40-50^{o}C). The extract (yield 40 g) was preserved in vacuum desiccators for subsequent use in study.

DPPH radical scavenging assay

To 1 ml of various concentrations (2-1000 µg/ml) of ethanolic and aqueous extract, 1 ml of solution of DPPH 0.1 mM was added. An equal amount of ethanol and DPPH were added to the control. Ascorbic acid was used as the standard for comparison. After 20 minutes incubation in the dark, absorbance was recorded at 517 nm. Experiment was performed in triplicate (5).

ABTS radical scavenging assay

The ABTS radical cation was prepared by the following method.

ABTS 2 mM was prepared in distilled water. Potassium per sulphate 70 mM was prepared in distilled water. 200 µl of Potassium per sulphate and 50 ml of ABTS were mixed and used after 2 h. This solution known as ABTS radical cation was used for the assay.

To 0.5 ml of various concentrations of extract (2-500 µg/ml), 0.3 ml of ABTS radical cation and 1.7 ml of phosphate buffer, pH 7.4 were added. For control, instead of extract, ethanol was substituted for ethanolic extract and water for the aqueous extract. The absorbance was measured at 734 nm. The experiment was performed in triplicate (5).

Lipid peroxidation assay

Egg Phosphatidylcholine (20 mg) in chloroform (2 ml) 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 mixture. The mixture was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid 0.1 ml to a mixture containing liposome (0.1 ml). 150 mM potassium chloride, 0.2 mM ferric chloride (0.1 ml), drug solution (2-64 µg/ml) were added separately in a total volume of 1 ml. The reaction mixture was incubated for 40 min at 37ºC. After incubation, the reaction was terminated by adding 1 ml of ice cold 0.25 M sodium hydroxide containing 20% w/v TCA, 0.4% w/v TBA and 0.05% w/v BHT. After keeping in boiling water bath for 20 min, the samples were cooled. The pink chromogen was extracted with constant volume of n-butanol and absorbance of the upper organic layer was measured at 532 nm. The experiment was performed in triplicate (6-7).

Nitric Oxide scavenging assay

To the 1 ml of various concentrations of the extract (2-1000 µg/ml), 0.3 ml of 5 mM sodium nitroprusside was added in the test tubes. The test tubes were incubated at 25ºC for 5 h. After 5 h, 0.5 ml of Griess reagent was added. The absorbance was measured at 546 nm. The experiment was performed in triplicate **(**5).

Superoxide scavenging assay

To 0.5 ml of different concentration of extract, 1 ml alkaline DMSO and 0.2 ml NBT 20 mM were added. The absorbance was measured at 560 nm. The experiment was performed in triplicate **(**8-9).

Non-enzymatic hemoglobin glycosylation assay

The antioxidant activities of extract were investigated by estimating degree of non-enzymatic hemoglobin glycosylation, measured colorimetrically. The assay was performed by adding 1 ml of 2 % glucose solution, 1 ml of hemoglobin solution 60 mg/100 ml in 0.01 M phosphate buffer (pH 7.4), and 1 ml of gentamycin (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 (10-11).

Total antioxidant capacity

0.1 ml of extract (10 mg/ml) dissolved in water was added to 1 ml of the reagent (0.6M sulphuric acid, 28 mM Sodium Phosphate, 4 mM Ammonium Molybdate) incubated at 95^{o}C for 90 min, and then cooled to room temperature. The absorbance was measured at 695 nm. The antioxidant activity was expressed as the number of equivalents of ascorbic acid. (12-13).

Determination of Total phenolic compounds

Total phenolic compounds were determined according to a protocol similar to that of *Singleton* and *Rossi* (14). From the stock solution (1 mg/ml) of the extract, suitable quantity was taken into a 25 ml volumetric flask and mixed with 10 ml of water and 1.5 ml of Folin Ciocalteu's reagent. After 5 minutes 4 ml of 20 % w/v sodium carbonate solution was added and volume was made up to 25 ml with double distilled water. The Absorbance was recorded at 765 nm after 30 minutes. % of total phenolic was calculated from calibration curve of Gallic acid (50-250 µg) plotted by using the same procedure and total phenolic were expressed as equivalent to Gallic acid.

Statistical Analysis

All results are expressed as mean ± S.E.M. Linear regression (Origin 6.0 version) analysis was used to calculate the IC_{50} values.

Results

Several concentrations ranging from 2-1000 µg/ml of the ethanolic and aqueous extracts of *Ziziphus mauritiana* Lam. were tested for their antioxidant activity in different *in-vitro* models. It was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC_{50} values were calculated for all models. The maximum inhibitory concentration (IC_{50}) in all models *viz.* DPPH, ABTS, lipid peroxidation assay, nitric oxide scavenging and superoxide scavenging activity were found to be 100.38, 12.33, 49.06, 21.77, 15.91 and 16.11, 55.47, 34.99, 126.93 and 25.96 µg/ml respectively for ethanolic and aqueous extract (Table 1). In antioxidant hemoglobin glycosylation, the % scavenging was found to be 52.14% and 65.93% of aqueous extract and 66.50% and 76.96% of ethanolic extract for concentration 0.5 mg/ml and 1.0 mg/ml respectively (Table 2). In total antioxidant capacity assay, it was found that 1 mg of aqueous extract is equivalent to 59 µg of ascorbic acid. Also 1 mg of ethanolic extract is equivalent to 91 µg/ml of ascorbic acid. Total phenolic content for ethanolic and aqueous extract was found to be 0.122 mg/ml and 0.150 mg/ml

equivalent to gallic acid. The antioxidant activity of the extract is close and identical in magnitude, and comparable to that of standard antioxidant compounds used.

Table 1: Comparison of IC_{50} values of extracts with standard

Sr. No.	Model	Ethanolic extract (µg/ml)	Aqueous extract (µg/ml)	Ascorbic acid/ Tocopherol* (µg/ml)
1	DPPH Model	100.38	16.11	9.99
2	ABTS Model	12.33	55.47	35.21
4	Lipid peroxidation model	49.06	34.99	30.67*
5	Nitric oxide scavenging model	21.77	126.93	38.68
6	Superoxide scavenging assay	15.91	25.96	15.79
7	Total antioxidant capacity	10 mg/ml aqueous extract of *Z. mauritiana* is equivalent to 59 µg/ml of ascorbic acid.		
		10 mg/ml ethanolic extract of *Z. mauritiana* is equivalent to 91 µg/ml of ascorbic acid.		

Table 2: Antioxidant study by Non-enzymatic hemoglobin glycosylation assay

Sr. No.	Extract/ standard	Conc. (µg/ml)	% Scavenging
1	Ethanolic extract	0.5	66.50
2		1	76.96
3	Aqueous extract	0.5	52.14
4		1	65.93
5	Vitamin –E	0.5	61.53
6		1	86.68

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 reaction. 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 (15-16). Phenolic compounds and flavonoids are the major constituents in most plants reported to possess antioxidant and free radical scavenging activity (17). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (18).

DPPH is a stable free radical. When antioxidant reacts with this stable radical, the electron becomes paired off and bleaching of the colour stoichiometrically depends on the number of electrons taken up. From our findings, it may be postulated that *Z. mauritiana* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (18, 19) (Fig.1)

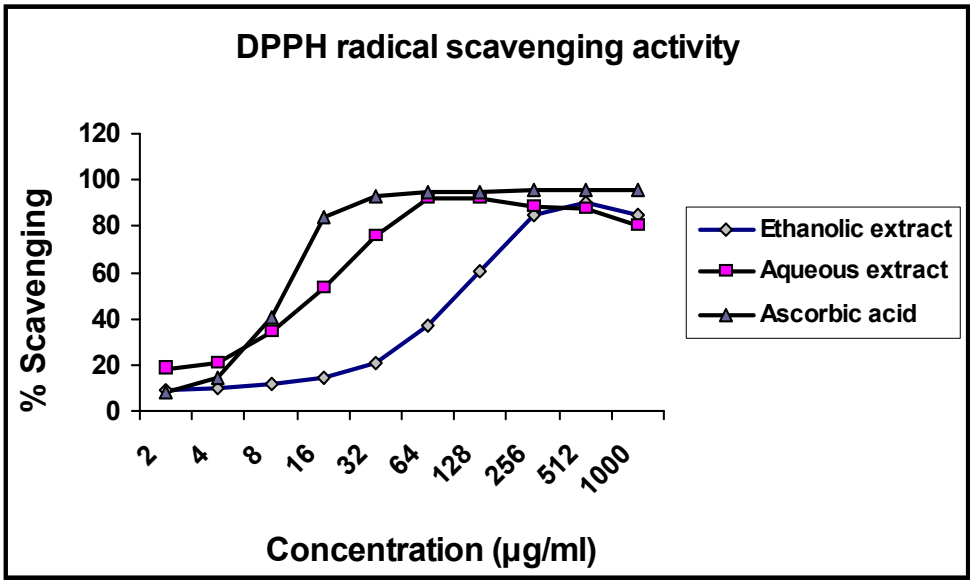

Fig 1. DPPH radical scavenging activity of different concentrations of *Z. mauritiana* extract and ascorbic acid.

ABTS is a decolorisation assay, which involves the direct generation of ABTS radical mono cation, which has a long wavelength absorption spectrum without the involvement of any intermediary radical. The antioxidant activity of the extract by this assay implies that action may be by either inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards this radical have been reported in earlier studies (20) (Fig. 2).

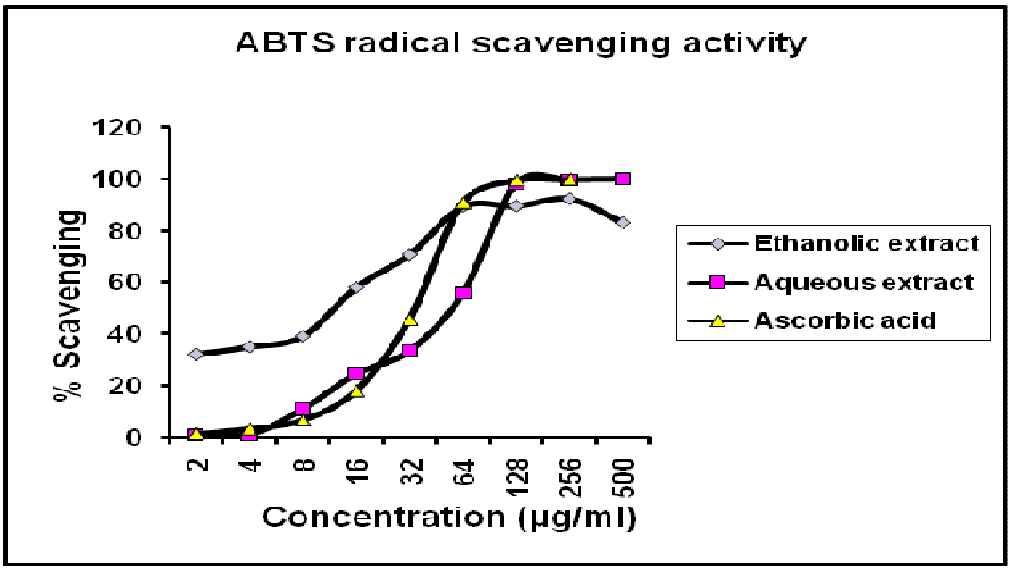

Fig 2. ABTS radical scavenging activity of different concentrations of *Z. mauritiana* extract and ascorbic acid.

Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through **.**OH radical by Fenton's reaction. The Fig. 3 shows the extract inhibited lipid peroxidation in egg phophatidylcholine as a dose dependent manner. The inhibition could be caused by absence of ferrylperferryl complex or by scavenging the ·OH radical or the superoxide radicals or by changing the Fe3+/Fe2+ or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. Iron catalyses the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydro-peroxides is produced (21). Lipid hydro-peroxide can be decomposed to produce alkoxy and peroxy radical which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases (22). Thus the decrease in the MDA level in egg phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant (Fig. 3).

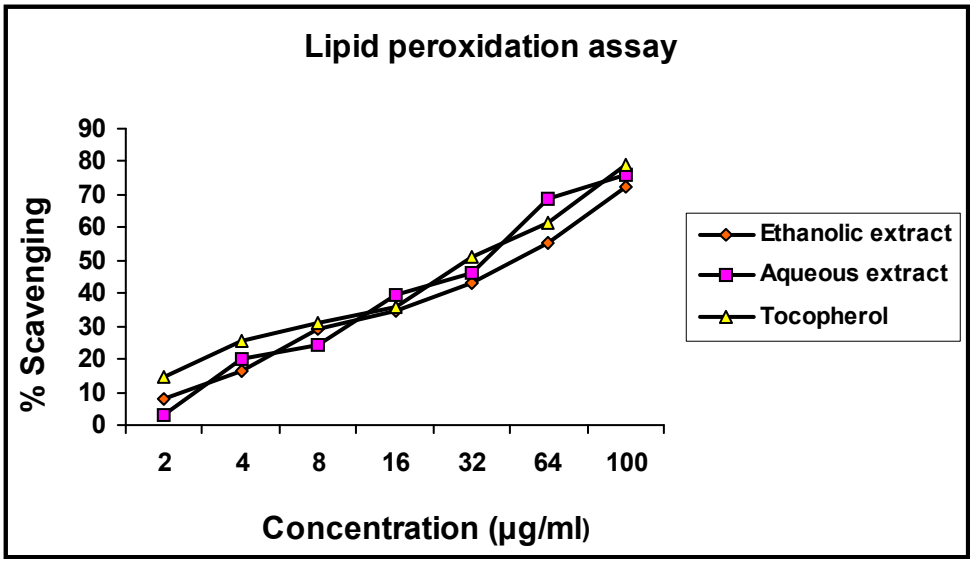

Fig 3. Lipid peroxidation assay of different concentrations of *Z. mauritiana* extract and tocopherol.

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, 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 (23). *Z. mauritiana* inhibit nitric oxide in dose dependant manner (Fig. 4). This may be due to antioxidant principles in the extract, which compete with oxygen to react with nitric oxide.

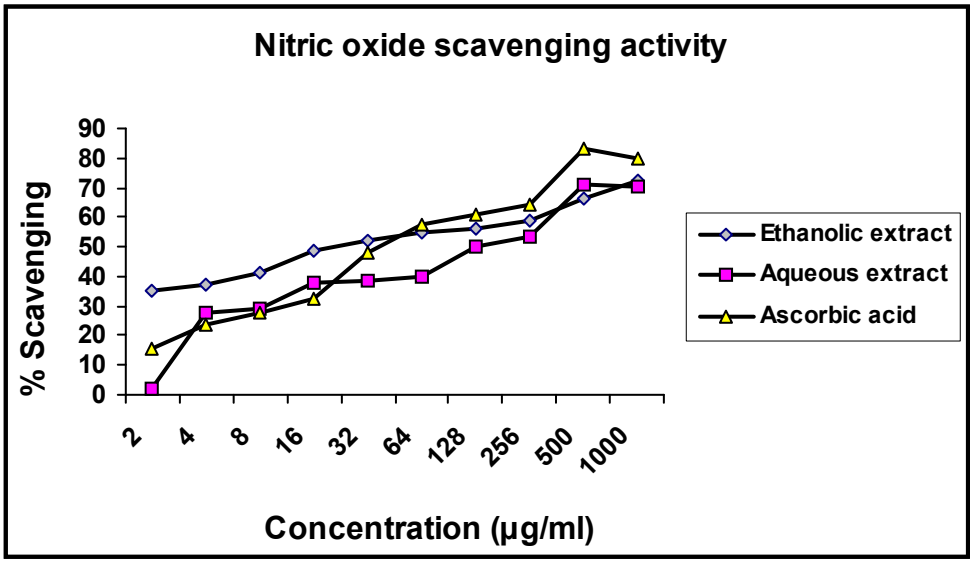

Fig.4. Nitric oxide scavenging activity of different concentrations of *Z. mauritiana* extract and ascorbic acid.

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. (1) In our study, alkaline DMSO used for superoxide generation indicates that *Z. mauritiana* (Fig. 5) is a potent superoxide scavenger (24).

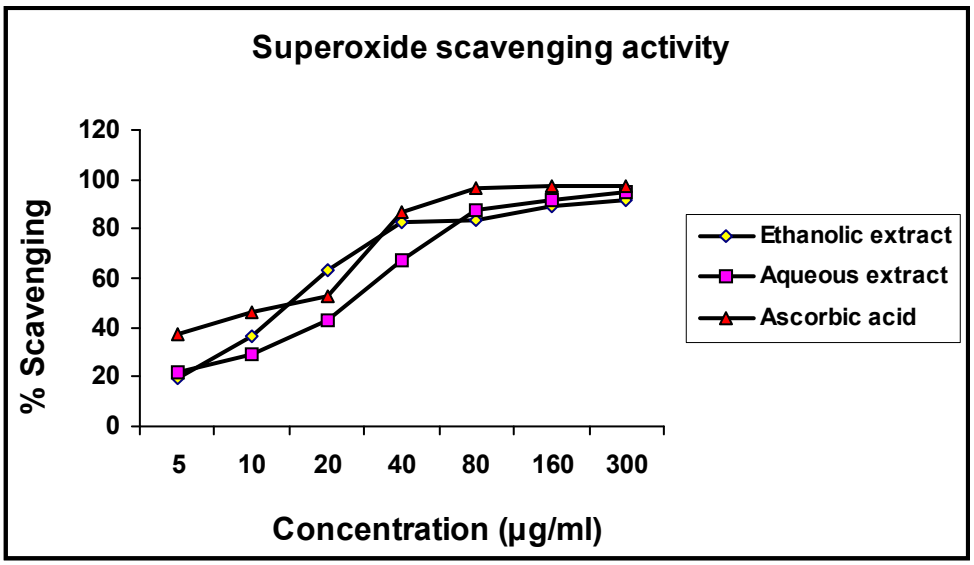

Fig 5. Superoxide scavenging activity of different concentrations of *Z. mauritiana* extract and ascorbic acid.

Haemoglobin glycosylation is an *in vitro* non-enzymatic method. Being an oxidation reaction, an antioxidant is expected to inhibit the reaction. The degree of haemoglycosylation *in vitro*, in the presence of different concentration of extract can be measured spectrophotometrically (10, 11).

Total antioxidant capacity of the extract was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically at 695 nm (13). *Z. mauritiana* extract exhibits its antioxidant action in several ways: removal of oxygen, scavenging of ROS, binding metal ions needed for catalysis of ROS and up regulation of endogenous antioxidant defences. The results of the present study show that the extract of *Z. mauritian* which contains phenolic compounds and exhibits the antioxidant activity through the scavenging of free radicals.

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