

ANTI-OXIDANT ACTIVITIES OF LEAF EXTRACTS OF *ZIZIPHUS MUCRONATA*

T.E Kwape and *P. Chaturvedi

Department of Biological Sciences

University of Botswana, Gaborone, Botswana

*Author for Correspondence

ABSTRACT

Natural anti-oxidants are the good source for scavenging and reducing free radicals generated in the body after multifold oxidative stress. The present study was aimed to evaluate in vitro anti-oxidant properties of different types of leaf extracts of *Ziziphus mucronata*, a tree found in Botswana. Gallic acid was used a reference control for all experiments. Different types of extracts were prepared with 100% hexane; 50% hexane/chloroform; 100% chloroform; 50% methanol/chloroform; 100% methanol and 70% methanol/water solvent system. The anti-oxidant studies included DPPH-TLC-Semi quantitative assay, DPPH radical scavenging activities, ABTS free radical scavenging, superoxide radical and hydrogen peroxide scavenging activities. Total phenol content was also determined. The total phenol content was seen to increase from the less polar solvent to the more polar solvent and the maximum was shown in 70% methanol extract. It is concluded that different extracts of leaf had free radical scavenging activities and extent of scavenging increased from extract obtained from low polarity solvent to the extract obtained from high polarity solvent.

Key Words: Anti-oxidants, Super oxide radical, Hydrogen Peroxide

INTRODUCTION

Living style of modern society has lead to the consumption of fast food, alcohols, soft drinks and so on. Consumption of pain killers and anti-biotics has become very common nowadays. Oxidation of all these consumables results into the formation of free radicals that ultimately results into oxidative stress. Oxidative stress depletes the anti-oxidants present in the body and hence there is a need to supplement the anti-oxidant system because prolonged state of oxidative stress culminates to the development of diseases like arthritis, diabetes and cardiovascular complications (Yu, 1994). Best way to get rid of the imbalance is the supplementation of food with the diet rich in anti-oxidants. Green vegetables, leaves and fruits are the natural source of anti-oxidants. *Ziziphus mucronata* (Rhamnaceae) is locally known as “mokgalo”. is an indigenous tree to Botswana (Braam van and Piet van, 1997). Leaves are ovate to broadly ovate, glossy dark green above the surface and lower surface slightly hairy. Flowers are in auxiliary cluster, small and yellowish green. Fruits are drupe, subglobose, up to 30 mm in diameter, shiny reddish to yellowish brown. All the parts of the tree is usually consumed for its medicinal value. The root is usually dried, boiled and the water decoction is used as a pain reliever (Koeven, 2001). The fruits of this plant are eaten because of their sweet taste. The fruits are usually dried and crushed to powder and in this form they are used as tea substitute. The leaves are also cooked traditionally and though they are not very delicious, are eaten for their high nutritive content or eaten as a spinach alternative (Veronica, 1998). The plant is used to heal illnesses like dysentery, swellings, chest pains, toothache, eye diseases, swollen and open wounds. The roots and leaves are made in to a poultice and used for the treatment of skin infections, gastrointestinal conditions, swollen glands and inflammation (Koeven, 2001). The prominent usage of the plant has been observed among the bushman of the Okavango river bank, Ovambo, Kavango and Zulu tribes in southern Africa (Koeven, 2001). *Ziziphus mauriana* has been reported to decrease the levels of

Research Article

catalase, glutathione peroxidase, glutathione reductase and superoxide dismutase against alcohol induced toxicity (Veronica, 1998).

Anti-oxidant property of this plant has not been yet reported and it is proposed that high medicinal value of this tree is might be due to its antioxidant content. Therefore the present study was aimed to evaluate the anti-oxidant property of the leaf of this plant.

MATERIALS AND METHODS

Plant Material and preparation of extracts

The leaves, roots, bark and fruits were collected around Gaborone. The plant authentication was performed by Mr M. Muzila, University of Botswana Herbarium. They were then washed with distilled water and sun dried. The dried plant materials were crushed with a blender or laboratory grinder to obtain 500 g of powder. The powder was soaked and extracted successively at room temperature in the following solvent system: 100% hexane; 50% hexane/chloroform; 100% chloroform; 50% methanol/chloroform; 100% methanol and 70% methanol/water. The extracts were evaporated in a rota vapour. The 70% methanol/water extract was freeze dried. Yields obtained were as follows: 100% hexane (16% yield) 50% hexane/chloroform (32% yield), 100% chloroform (28% yield) 50% methanol/chloroform (34 yield) 100% methanol (35% yield) and 70% methanol/water (38% yield). The extracts were kept in a refrigerator until required.

DPPH-TLC-Semi quantitative assay

The method described by Yeboah and Majinda (2009) was used to measure the antioxidant activities of plant extracts. 1mg/ml stock solutions of both samples and standard (gallic acid) were prepared. The spraying reagent was prepared by dissolving 2 mg of DPPH in 50 ml conical flask. The mixture was shaken well and kept in a refrigerator for about 20 minutes before use. Different amounts (0.1 μ L, 0.5 μ L, 1.0 μ L, 5.0 μ L, 10.0 μ L) of test samples and standards were taken from the stock solution using micro liter syringes and finely spotted on the TLC plates (Choi *et al.*, 2002). The solvent on the TLC plates was left to evaporate in the fume hood before spraying. The spotted TLC plates were sprayed with the DPPH forming a purple background on the plates. The radical scavenging activities of the extracts were observed after 30 minutes.

Spectrophotometric measurement of free radical scavenging activities of extracts by using 2, 2-diphenyl-1-picryl hydrazyl radical (DPPH)

The radical scavenging activity was determined by the method of Stoilova *et al.*, (2007). 1 ml from 0.3 mM alcohol solution of DPPH was added to 2.5 ml samples from different extracts of *Ziziphus mucronata*. The samples were kept at room temperature in the dark and after 30 min the optical density was measured at 518 nm. The percentage antiradical activity (AA) was determined according to the following formulae:

$$AA\% = 100 - \{[(\text{Abs.}_{\text{sample}} - \text{Abs.}_{\text{empty sample}}) * 100] / \text{Abs.}_{\text{control}}\}$$

Where empty samples- 1ml methanol + 2.5 ml from various concentrations of extract; control sample- 1 ml 0.3 mM DPPH + 2.5 ml methanol.

2-Azobis-3-ethyl benzothiazoline-6-sulphonic acid (ABTS) radical scavenging activity

ABTS radical scavenging activity was determined by the method described by Pellegrini *et al.*, (1999). The ABTS radical cations are produced when ABTS (7 mM) reacts with potassium persulfate (2.45 mM) when incubated at room temperature in the dark for 16 hrs. The solution thus obtained was further diluted with phosphate buffer saline (PBS) to give an absorbance of 1.000. Different concentrations of the test sample in 50 μ l were added to 950 μ l of ABTS working solution to give a final volume of 1 ml. The absorbance was recorded immediately at 734 nm. Gallic acid was used as reference standard. Inhibiting concentrations of extracts were tested at 2.5, 5, 10, 25, 50 and 100 μ g/ml. Reference standard (gallic acid) was tested at 1, 2, 4, 8 and 16 μ g/ml. The percent inhibition was calculated from the following equation:

$$\% \text{ inhibition} = [(\text{Absorbance of control} - \text{Absorbance of test sample}) / \text{Absorbance}]$$

Research Article

Control] x100.

Determination of total phenol content

The total phenol content (TPC) was determined by the method described by Stoilova *et al.* (2007), using Folin-Ciocalteu reagent. 1 millilitre of extract or standard solutions (Gallic acid) (0-500 mg/l) was added to a mixture of 10 ml deionising water and 1.0 ml of Folin - Ciocalteu phenol reagent. After 5 minutes, 2.0 ml of 20% sodium carbonate was added to the mixture. After 1 hour of incubation at room temperature in darkness the absorbance was measured at 750 nm. The TPC was calculated from the linear regression equation of the standard curve, from this equation, the concentration of gallic was determined for each extract and converted to mg of gallic acid equivalents/g of dry extract (mg GAE/g).

Determination of superoxide radical scavenging activity

Superoxide radical scavenging activity was measured as described by Bahramikia (2009) in PMS-NADH superoxide generating system (Chaturvedi et al, 2011). The different concentrations of extracts (2 to 10 mg/ml) was added to the mixture of Tris-HCL buffer (3ml), 50 μ M NBT (1 ml), 78 μ M NADH (1ml) and 10 μ M phenazine methosulphate (1 ml) in phosphate buffer. The mixture was incubated for 5 minutes and absorbance was recorded at 560 nm. Gallic acid was used as standard. Percentage inhibition was calculated using the following:

Percentage scavenging activity= [-absorbance of sample/absorbance of control] x 100

Hydrogen peroxide scavenging

Hydrogen peroxide scavenging of the extract was determined by the method of Iihami, 2005). Different concentrations of methanol leaf extracts (0.06-1mg/ml) in methanol were added 0.6 ml of H₂O₂ (40 Mm in phosphate buffer). After 10 minutes, absorbance of H₂O₂ was measured against a blank containing only phosphate buffer at 230nm after 15 minutes. H₂O₂ was used as control. Percentage scavenging of H₂O₂ was calculated using the following fomula:

Percent scavenging of H₂O₂= [{AC-AS}/AC] X 100

RESULTS AND DISCUSSION

Total phenol content of different types of leaf extracts of *Z. mucronata*

Total phenol content of different types of leaf extract is presented in Table 1. The Table shows that leaf extracts demonstrated high phenol content. The highest estimated phenol content was 271 mgGAE/g in 100% methanol extract, followed by 260, 120, 60, and 45, 20 mgGAE/g in 70% methanol/water, 70% methanol/chloroform, 100% chloroform, 50% hexane/chloroform and 100% hexane respectively

Antioxidant activity of leaf extracts of *Ziziphus mucronata* by TLC-Semi quantitative assay

Results of measurement of antioxidant activity of different types leaf extracts is presented in Figure 1. DPPH is a free radical stable at room temperature and produces purple color in methanol. With anti-oxidants, this free radical is reduced and purple color with methanol becomes color less or yellow. 0.1 μ L, 0.5 μ L, 1.0 μ L, 5.0 μ L, 10.0 μ L concentrations of the leaf extracts from different solvents spotted on the TLC sheet showed high activity as indicated by the yellow color over the purple DPPH background.

Table 1: Total phenol content of the ZMLE.

Leaf extract	TPC (mgGAE/g)
70% methanol	260 $\pm$ 3.6
100% methanol	271 $\pm$ 2.4
50% methanol/ chloroform	120 $\pm$ 1.6
Chloroform	60 $\pm$ 0.4

Research Article

50% Hexane chloroform	45 ± 1.8
Hexane	20 ± 0.2

*Values are the average of three trials ± standard deviation

Table 2 *In vitro* ABTS free radical scavenging activity of various extracts of *Ziziphus mucronata*.

Plant part	Test extract	IC ₅₀ (µg/ml)
Leaf extract	100% methanol	8.12
	50% methanol/chloroform	60.00
	100% chloroform	68.22
	50% hexane/chloroform	85.13
	100% hexane	88.48
Reference standard	gallic acid	1.85

* The values are mean of three replicates ± standard error. Extracts tested at 2.5, 5, 10, 25, 50 and 100µg/ml. Reference standard (gallic acid) tested at 1, 2, 4, 8 and 16 µg/ml.

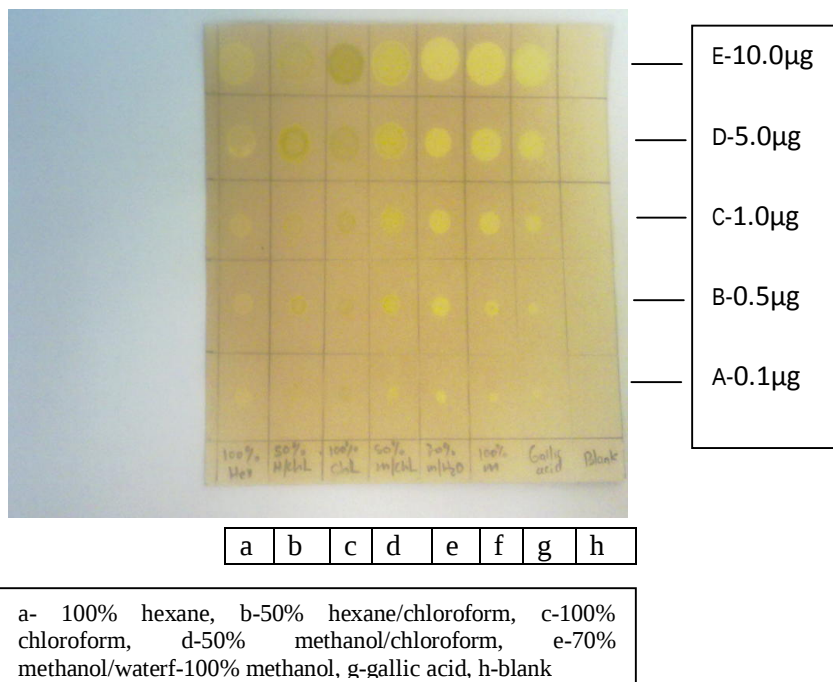


Figure 1: TLC-DPPH assay showing the anti-oxidant activities of different concentrations of ZMLE spotted on a TLC sheet.

Research Article

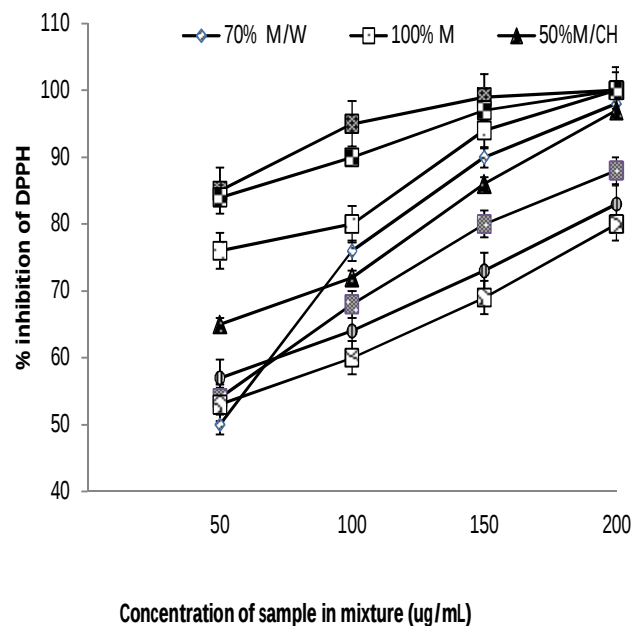


Figure 2: Percent DPPH inhibition versus concentration for the ZMLE and standards observed after 30 minutes.

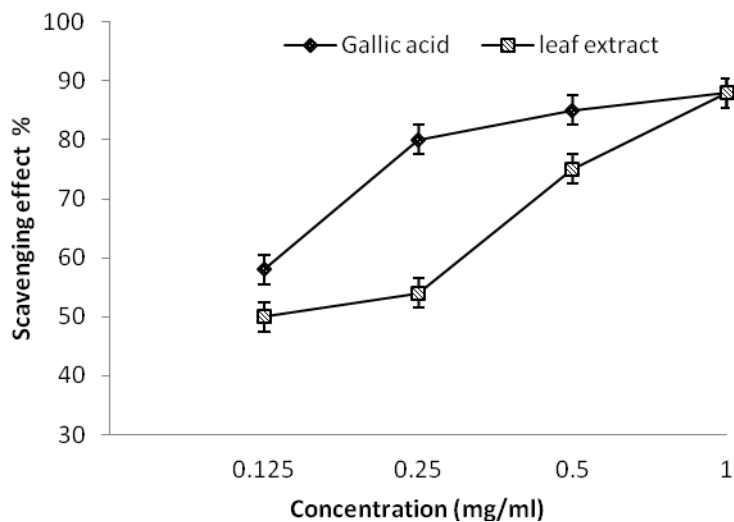


Figure 3. Superoxide scavenging activity of methanol leaf extract

Research Article

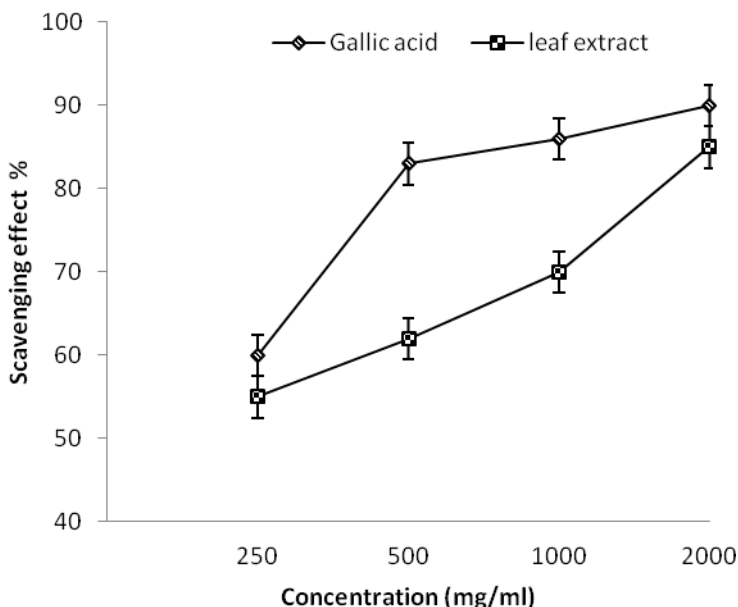


Figure 4. Hydrogen peroxide scavenging activity of methanol leaf extract

100% methanol extract, 70% methanol/water extract, 70% methanol/chloroform extract, 100% chloroform extract, 50% hexane/chloroform extract and 100% hexane extract changed to yellow color on the purple DPPH background. The extent of yellow coloration of all the extracts were comparable to that of the gallic acid standard except 50% hexane/chloroform and 100% hexane solvent extracts. These extract showed less anti-oxidant activity.

Free radical scavenging activities of extracts by DPPH by spectrophotometric method

Free radical scavenging activities of different types of leaf extracts by spectrophotometric methods using DPPH as free radical are presented in Figure 2. Different extracts of ZM have significant scavenging effects on free radicals. The increase in the antioxidant activities occurred in concentration dependent manner. The extent of % inhibition of DPPH increased from a low polarity solvent to a high polarity solvent, 100% methanol, 70% methanol/ chloroform, 100% chloroform, 50% hexane/chloroform, 100% hexane respectively. The standards gallic acid and ascorbic acids showed 100 % inhibition

In vitro antioxidant activity by ABTS

As presented in Table 2. Effect of ABTS free radical scavenging activity of 100% methanol, 50% methanol/chloroform, 100% chloroform, 50% hexane/chloroform, 100% hexane extracts of *Z. mucronata* leaf was assayed at various concentrations from 2.5, 5, 10, 25, 50 and 100 µg/ml. ABTS was used as a free radical to evaluate antioxidant activity of extracts. The method was based on the ability of antioxidant molecules to quench the long lived ABTS radical cation (ABTS). Significant ABTS scavenging activity was evident in 100% methanol extract Its IC_{50} was 8.12 µg/ml. A slight scavenging activity was observed in the 50% methanol/chloroform, 100% chloroform, 50% hexane/chloroform, 100% hexane extracts.

Superoxide scavenging activity of leaf extract

The results presented in figure 3, show a concentration dependent increase in the % scavenging activity of the methanol leaf extract. 1 mg/ml showed a significant scavenging activity of 88% comparable to the Gallic acid standard.

Research Article

Hydrogen peroxide scavenging activity of leaf extract

Hydrogen peroxide scavenging activities of the leaf extract are presented in figure 4 can possibly be as a result of the presence of phenolics, which can donate electrons to H_2O_2 . The extract was able to scavenge hydrogen peroxide in a concentration dependant manner. A Higher concentration displaying a higher scavenging activity, almost comparable to that of gallic acid. Although H_2O_2 itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radical in the cell, it is thus worth removing H_2O_2 throughout food systems.

Taking all the results into consideration, it can be proposed that all the extracts from leaf possess anti-oxidant activities but the extract obtained using polar solvents are more effective than the extract obtained from less polar solvents in vitro condition. Any substance can be considered as anti-oxidant if it scavenge free radicals. DPPH is considered as proton free radical and scavenging of proton free radical is one of the mechanisms to reduce oxidants in the biological system. Leaf extracts of ZM were able to scavenge free radical as observed from TLC-semiquantitative method and spectrophotometric method. The increase in the antioxidant activity occurred in a concentration dependent manner and the extent of percentage inhibition increased from a low polarity solvent to a high polarity solvent.

In ABTS assay method, ABTS reacts with potassium persulphate to produce ABTS⁺ which gives a blue green chromogen. Presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS, the absorbance of which is measured at 734nm. In the present study, the percentage inhibition of ABTS⁺ radical increased from a low polarity solvent to a high polarity solvent for 100% methanol, 70% methanol/ chloroform, 100% chloroform, 50% hexane/chloroform, 100% hexane respectively.

The anti-oxidant effects of plants are attributed in part, because of their phenol content. These compounds interrupt the propagation of chain reaction of lipid per-oxidation by contributing a hydrogen atom with the formation of relatively stable compound that prevents the propagation of oxidation process. Leaf extracts of ZM are found to be rich in phenol content. The total phenol content was seen to increase from the less polar solvent to the more polar solvent and the maximum was shown in 70% methanol extract.

ACKNOWLEDGEMENTS

We are thankful to Office of Research and Development, University of Botswana, for providing fund to carry out this work

REFERENCES

- Bahramikia S, Ardestani A and Yazdanparast R, (2009).** Protective effects of four Iranian medicinal plants against free radical- mediated protein oxidation. *Food Chemistry* **115** (1) 37-42.
- Braam van W. Y. K. and Piet van W. Y. K, (1997).** Field guide to trees of Southern Africa. Strunick publishers, Cape Town: South Africa.
- Chaturvedi P, Khare KB, Kwape TE and Moses M, (2011).** Antioxidant properties of two edible mushrooms, *Agaricus bisporus* and *Pleurotus ostreatus* grown in Botswana. *Mushroom research*; **20** (1): 1-6
- Choi CW, Kim SC, Hwang SS, Choi BK, Ahn HJ, and Lee MY, (2002).** Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science* **163** (6) 1161-1168.
- Dahiru D and Obdoa O, (2008).** Evaluation of antioxidant effects of *Ziziphus mauritiana*, leaf extracts against chronic ethanol induced hepatotoxicity in rat liver. *African Journal of Traditional and Complementary Alternative Medicine* **5** (1) 39-45
- Iihami GI, Hacı AA and Mehmet C, (2005).** Determination of in vitro antioxidant and radical scavenging activities of Propofol. *Chemical and Pharmaceutical Bulletin* **53** (5) 281-285
- Koeven E, (2001). *Medicinal and poisonous and edible plants in Namibia*. Klause Hess Publishers/verlag Windhoek: Namibia.

Research Article

Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* **26** (9-10) 1231-1237.

Stoilova I, Krastanov A, Stoyanova A, Denev P, and Gargova S, (2007). Antioxidant activity of a ginger extracts (*Zingiber Offinale*). *Food Chemistry* **102** (3) 764-770.

Veronica R, (1998). Trees and shrubs of Okavango Delta: *Medicinal uses and Nutritional value*. Briza Publication, Arcadia: South Africa.

Yeboah EMO. and Majinda RT, (2009). Radical scavenging activity and total phenolic content of extracts of the root bark of *Osyris lanceolata*. *Natural product communications* **4** (1): 89-94.

Yu Bp, (1994). Cellular defence against damage from reactive oxygen species. *Physiological Reviews*. **74** (1) 139-162.