CHAPTER 4

IN VITRO AND *IN VIVO* ANTIOXIDANT ACTIVITIES OF STEM BARK EXTRACT OF STRYCHNOS HENNINGSII GILG

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This chapter has been published in the Journal of pharmacy and pharmacology

***In vitro* and *in vivo* antioxidant activities of aqueous bark extract of *Strychnos henningsii* Gilg**

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Abstract

The present study assessed the antioxidant activity of aqueous stem bark extract of *Strychnos henningsii* on the occurrence of oxidative stress which plays an important role in chronic complications of diabetes mellitus. The antioxidant and free radical scavenging activity of aqueous extract of this plant was investigated using a spectroscopic method against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions, hydrogen peroxide (H_2O_2), nitric oxide (NO), 2, 2'- azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) and the ferric reducing agent. Total phenols, flavonoids, tannins, flavonols, proanthocyanidins and percentage content of alkaloids, saponins and were also determined to assess their effects on the antioxidant activity of the extract. Free radical scavenging activity of the plant extract against H_2O_2, ABTS and NO was concentration dependent with IC_{50} value of 0.023, 0.089 and 0.49 mg/ml respectively. However, *S. henningsii* exhibited lower inhibitory activity against DPPH with IC_{50} value of 0.739 mg/ml. The reducing power of the extract was found to be concentration dependent. The administration of the aqueous extract at 250, 500 and 1000 mg/kg body weight to Wistar rats significantly increased the percentage inhibition of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) while lipid peroxidation level decreased in hepatotoxic rats induced with CCl_4 treated at the dose of 500 and 1000 mg/kg body weight at the end of 7 days. The extract yielded high phenol content (48 mg/g tannic acid equivalent) followed by proanthocyanidins (8.7 mg/g

catechin equivalent), flavonols (5.5 mg/g quercetin equivalent), tannin (4.2 mg/g tannic acid equivalent) and flavonoids (4.8 mg/g quercetin equivalent) respectively. Percentage contents of alkaloids, saponins and tannins were 1.5 and 6 % respectively of plant sample. A positive linear correlation was observed between these polyphenols and the free radical scavenging activities.

Keywords: *Strychnos henningsii*; enzymes; free radicals; CCl_4; antioxidant activity; phenolics compounds.

INTRODUCTION

Many human diseases are caused by oxidative stress that results from imbalance between the formation and neutralization of pro-oxidants (Hazra et al., 2008). Oxidative stress initiated by free radicals, such as superoxide anions, hydrogen peroxide, hydroxyl, nitric oxide and peroxynitrite, play a vital role in damaging various cellular macromolecules such as DNA, proteins and lipids. These cellular oxidative damages may result into many diseases, including diabetes mellitus, atherosclerosis, myocardial infarction, arthritis, anemia, asthma, inflammation, neurodegenerative diseases and carcinogenesis (Polterat, 1997). However, human cells have an array of protecting mechanisms to prevent the production of free radicals and oxidative damage (Chandra et al., 1994). These mechanisms include enzymic and non- enzymic antioxidants such as superoxide dismutase, catalase, glutathione reductase, ascorbic acid and tocopherol (Niki et al., 1994). The protective roles of these enzymes may be disrupted as a result of various pathological processes and thereby causes damage to the cells. Antioxidant supplement has been reported to reconcile the increase of these radicals by directly reacting and quenching their catalytic metal ions (Robak and Marcinkiewicz, 1995). Several synthetic antioxidant agents such as butylated hydroxylanisole (BHA) and butylated hydroxyltoluene (BHT) are commercially available; however, they are reported to be toxic to animals including human beings (Madhavi and Salunkhe, 1995). Natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity. This activity is mainly due to the presence of phenolic compounds such as flavonoids, phenols, flavonols and proanthocyanidins (Rice-Evans et al., 1995). A vast majority of plants used in traditional medicine in South Africa have currently not been evaluated for their antioxidant potential. One of such plants is *Strychnos henningsii*.

Strychnos henningsii Gilg. (Loganiaceae) is a small evergreen tree or shrub with leathery leaves. The bark is crown compact with dark green, glossy foliage and the fruit is

oblong which turns brown when ripe. The leaves of the plant have a characteristic aromatic-pungent odour with rough texture. It is one of the most widely distributed species of strychnos in east and southern Africa (Leeuwenberg, 1969).

The bark of *S. henningsii* has been recommended for the treatment of various diseases by the traditional health practitioners in southern Africa including rheumatism, gynaecological complaints, abdominal pain, snake bite, gastrointestinal pain, malaria and diabetes mellitus (Hutchings, 1989; Bisset, 1970). In east Africa, the bark has been documented to have significant medicinal uses in the healing of wounds and as a mouth antiseptic. About five compounds, including indolinic alkaloids, strychnine, brucine, curarine and bitter glycoside have been isolated from this plant (Tits, 1982).

Before the commencement of this work, there was no information in scientific literature on the free radical scavenging and antioxidant activity of the aqueous extract of *Strychnos henningsii* bark both *in vivo* and *in vitro*. Therefore, this study was aimed at providing information on the phytochemicals and antioxidant activities of this plant.

MATERIALS AND METHODS

Plant material

The bark of *S. henningsii* was collected in February, 2009 from a thick forest in Amathole District (Eastern Cape, South Africa). The plant was identified by its vernacular name and later authenticated by Prof. DS. Grierson of the Department of Botany, University of Fort Hare. Voucher specimen (Sun MED 2009) was deposited at the Giffen herbarium of the University.

Preparation of extract

The bark material was air-dried at room temperature in the laboratory. The dried material was then pulverized using an electric blender (Waring Products Division, Torrington, USA). About 60 g of the powdered plant material was extracted in 1 L of distilled water (4 ^{o}C) maintained on a mechanical shaker (Stuart Scientific Orbital Shaker, UK) for 48 h. The extract was filtered using a Buchner funnel and Whatman No.1 filter paper. The filtrate was quickly frozen at -40 ^{o}C and dessicated for 48 h using a freeze dryer (Savant Refrigerated vapor Trap, RV T41404, USA) to give a yield of 8.4 g of dry extract. The resulting extract was reconstituted in distilled water to give desired doses (250, 500 and 1000 mg/ml) used in this study.

Animals

Male Wistar rats (*Rattus norvegicus*) with a mean weight of 175g ± 5.2 were obtained from the animal house of the Agricultural and Rural Development Research Institute, University of Fort Hare. They were kept in clean metabolic cages placed in a well ventilated house conditions (temperature 23 ± 1 ^{o}C: photoperiod: 12 h light and 12 h dark cycle throughout the experimental period; humidity: 45 – 50%). The rats were allowed free access to food (Balanced Trusty Chunks, Pioneer Foods (Pty) Ltd, and Huguenot, South Africa) and water. The experiment was carried out after its approval by the Animal Ethics Committee of the University of Fort Hare in accordance with the recommendations for the proper care and use of laboratory animals.

Animal grouping and extract administration

Twenty five male rats were randomized into five groups consisting of five rats each. Group 1 served as control and was given distilled water alone (0.5 ml) per day for seven days with the aid of oropharyngeal cannula. Group 2 animals served as hepatotoxic control; treated with 50 % CCl_4 prepared in olive oil and were orally administered in a single dose of 0.5 ml for seven days. Animals in group 3-5 were treated like the control except that they received 0.5 ml of the extract corresponding to 250, 500 and 1000 mg/kg body weight respectively. Groups 3-5 were also given 0.5 ml of CCl_4 on the seventh day, 6 h after the administration of plant extract. All the animals from each group were sacrificed by ether anesthesia 24 h after their last dose of the extract and distilled water. The liver from each animal was excised, rinsed in ice cold 0.25 M sucrose solution and 10 %w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 12,000 × g for 60 min at 4 ^{o}C. The supernatant obtained was used for the estimation of catalase, superoxide dismutase, lipid peroxidation (TBARS) and reduced glutathione.

Total phenolics

The total phenolics content in the aqueous bark extract of *S. henningsii* was determined spectrophotometrically with Folin Ciocalteau reagent using the modified method of Wolfe et al. (2003). An aliquot of the extract (0.5 ml) was mixed with 2.5 ml of 10 % Folin-Ciocalteu reagent and 2 ml of Na_2CO_3 (75 % w/v). The resulting mixture was vortexed for 15 s and incubated at 40 ^{o}C for 30 min for colour development. The absorbance of the samples was measured spectrophotometrically at 765 nm using Hewlett Packard, UV/visible light spectrophotometer. Total phenolic content was expressed as mg/g tannic acid equivalent from a calibration curve using the equation: $Y = 0.1216x$, $R^2 = 0.936512$, where x was the

absorbance and Y was the tannic acid equivalent (mg/g). The experiment was conducted in triplicate and the results are reported as mean ± SD values.

Total flavonoids

The method of Ordonez et al (2006) was used to estimate total flavonoid contents of the extract solution based on the formation of a complex flavonoid-aluminium. A volume of 0.5 ml of 2 % $AlCl_3$ ethanol solution was added to 0.5 ml of extract solution. After one hour of incubation at the room temperature, the absorbance was measured at 420 nm using UV-VIS spectrophotometer. A yellow colour indicated the presence of flavonoids. All determinations were done in triplicate and values were calculated from calibration curve obtained from quercetin using the equations: $Y= 0.0255x$, $R^2 = 0.9812$, where x was the absorbance and Y the quercetin equivalent (mg/g).

Total flavonols

Total flavonols content was determined by adopting the procedure described by Kumaran and Karunakaran (2007). The reacting mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/L) sodium acetate solution. The absorption at 440 nm was read after 2.5 h at 20 ^{0}C. Total flavonols content was calculated as quercetin (mg/g) equivalent from the calibration curve using the equation: $Y= 0.027x$, $R^2 = 0.9912$, where x was the absorbance and Y the quercetin equivalent (mg/g).

Total proanthocyanidins

Total proanthocyanidins was determined based on the procedure of Sun et al. (1998). To 0.5 ml of 1 mg/ml extract solution was added to 3 ml of vanillin-methanol (4 % v/v), and

1.5 ml of hydrochloric acid and then vortexed. The resulting mixture was allowed to stand for 15 min at room temperature followed by the measurement of the absorbance at 500 nm. Total proanthocyanidins content was expressed as catechin equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.5825x$, $R^2 = 0.9277$, where x was the absorbance and Y is the catechin equivalent (mg/g).

Tannins content

Tannins content was determined according to the method of Swain (1979). About 20 ml of 50% methanol prepared in distilled water was added to 0.2 g of plant sample and covered. The mixture was shaking vigorously on the shaker (Stuart Scientific Orbital Shaker, UK) placed in a water bath at 77^{o}C for 1 h to ensure the mixture is mixed uniformly. Extract was filtered using a Buchner funnel and Whatman no 1 filter paper into 100 ml volumetric flask. Twenty milliliter (20 ml) of distilled water containing 2.5 ml of folin- Denis reagent and 10 ml of 17% Na_2Co_3 were added to the filtrate and properly mixed together. The bluish-green colour developed at the end of the reaction (0.02 - 0.1 mg/ml). The absorbance of the tannic acid standard solution was measured after colour development at 760 nm using UV-VIS spectrophotometer (AJ-C03). Total tannins content was expressed as tannin standard equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.087x - 0.06$, $R^2 = 0.9277$, where x was the absorbance and Y is the catechin equivalent (mg/g).

Total alkaloids

Alkaloids contents in plant sample were quantitatively determined following the method described by Harborne (2005). Two hundred milliliter of 10 % acetic acid prepared in ethanol was added to 5 g of powdered plant sample, covered and allowed to stand for 4 h.

The filtrate was collected and concentrated on a water bath to 1/4th of its original volume. Concentrated ammonium hydroxide was added in drop-wise to the mixture until the precipitation was complete. The precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue was dried, weighed to determine the amount of alkaloids in the plant sample. The percentage alkaloids content was calculated using this equation:

$$\% \text{ Alkaloids} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

Total saponins

The method described by Obadoni and Ochuko (2001) was used for saponins content determination. Twenty gram of the plant sample was added to 100 ml of 20 % ethanol prepared in distilled water and kept in a shaker for 30 min. The plant sample was heated over a water bath at 55 ^{o}C for 4 h. The resulting mixture was filtered and the residue was re-extracted again with 200 ml of 20 % aqueous ethanol. The mixture was reduced to 40 ml over water bath at 90 ^{o}C. The concentrate was transferred into 250 ml separatory funnel, extracted twice with 20 ml diethyl ether. Ether layer was discarded while aqueous layer was retained followed by adding 60 ml of n-butanol. The butanol extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated over a water bath and evaporated to dryness to a constant at 40 ^{o}C. The saponins content was calculated using the following the equation:

$$\% \text{ Saponins contents} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

In vitro antioxidant activity

Determination of reducing power

The reducing power of the extract was evaluated according to the method of Yen and Chen (1986). A volume of 1.0 ml of the extract prepared in distilled water or BHT, Vitamin C or Vitamin E (0 - 5.0 mg/ml) were mixed individually to the mixture containing 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide ($K_3Fe(CN)_6$) (1%w/v). The resulting mixture was incubated at 50 ^oC for 20 min, followed by the addition of 2.5 ml of trichloroacetic acid (10 % w/v), and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of ferrous chloride (0.1 %, w/v). The absorbance was measured at700 nm against a blank sample containing the mixture without the extract. Increased absorbance of the reaction mixture indicated higher reducing power of the plant extract.

DPPH radical scavenging activity

The method of Liyana-Pathiranan and Shahidi (2005) was used for the determination of scavenging activity of DPPH free radical in the extract solution. A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract prepared in methanol containing 0.025 – 0.5 mg of the plant extracts or BHT or rutin. Both BHT and rutin are used as the standard drugs. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation: DPPH radical scavenging activity = {(Abs $_{control}$ – Abs $_{sample}$)}/ (Abs $_{control}$} × 100 where Abs $_{control}$ is the absorbance of DPPH radical + methanol; Abs $_{sample}$ is the absorbance of DPPH radical + sample extract or standard.

ABTS radical scavenging activity

The method of Re et al. (1999) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in equal amounts and allowed to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1ml of freshly prepared ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 nm after 7 min of incubation using spectrophotometer. The percentage inhibition of $ABTS^{\cdot+}$ by the extract was calculated and compared with that of BHT and rutin using the following equation: $ABTS^{\cdot+}$ scavenging activity = {(Abs $_{control}$ − Abs $_{sample}$)}/ (Abs $_{control}$} × 100 where Abs $_{control}$ is the absorbance of ABTS radical + methanol; Abs $_{sample}$ is the absorbance of ABTS radical + sample extract or standard.

Scavenging activity of nitric oxide

The method of Garrat (1964) was used to determine the nitric oxide radical scavenging activity of aqueous extract of *S. henningsii*. A volume of 2 ml of 10 mM sodium nitroprusside prepared in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extract or BHT or rutin at various concentrations (0.025-0.5 mg/ml). The mixture was incubated at 25 ^{o}C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33 % prepared in 20 % glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm. The amount of nitric oxide radical inhibited by the extract was calculated using the following equation: NO radical scavenging activity = {(Abs $_{control}$ – Abs

$_{sample}$)}/ (Abs $_{control}$ } × 100 where Abs $_{control}$ is the absorbance of NO radical + methanol; Abs $_{sample}$ is the absorbance of NO radical + sample extract or standard (BHT and rutin).

Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method of Ruch et al (1989). Plant extract (4 ml) prepared in distilled water at various concentration (0.025-0.5 mg/ml) was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was measured at 230 nm against blank solution containing the plant extract without H_2O_2. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation H_2O_2 radical scavenging activity = {(Abs $_{control}$ – Abs $_{sample}$)}/ (Abs $_{control}$} × 100 where Abs $_{control}$ is the absorbance of H_2O_2 radical + methanol; Abs $_{sample}$ is the absorbance of H_2O_2 radical + sample extract or standard (BHT or rutin).

Determination of catalase activity

Catalase activity was assayed according to the method of Pari and Latha (2004). The percentage inhibition was done spectrophotometrically following decrease in absorbance at 620 nm. The liver was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuged at 5000 rpm. The reaction mixture consisted of 0.4 ml of hydrogen peroxide (0.2 M), 1 ml of 0.01 M phosphate buffer (pH 7.0) and 0.1 ml of liver homogenate (10 % w/v). The reaction of the mixture was stopped by adding 2 ml of dichromate-acetic acid reagent (5 % $K_2Cr_2O_7$ prepared in glacial acetic acid). The changes in the absorbance was measured at 620 nm over 3 min at 1 min interval and recorded. Percentage inhibition was calculated using the equation:

% catalase inhibition = 100 - {Increase in absorbance of the sample × 100}/ Increase in absorbance of the blank.

Determination of superoxide dismutase activity

Superoxide dismutase was assayed following the method of Misra and Fridovich (1972). The assay mixture contained 0.5 ml of hepatic PMS (Protein Microsomal), 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µM nitroblue tetrazolium and 0.2 ml of freshly prepared 0.1 mM hydroxylamine-hydrochloride. The reaction mixture was mixed quickly by inversion followed by the addition of clear supernatant of 0.1 ml of liver homogenate (10 % w/v). The change in absorbance was recorded at 560 nm. Percentage inhibition was calculated using this equation:

$$\% \text{ Superoxide dismutase inhibition} = 100 - \frac{\text{Increase in absorbance of the sample}}{\text{Increase in absorbance of the blank.}} \times 100$$

Determination of reduced glutathione activity

Reduced glutathione was determined using the modified method of Ellman (1951). An aliquot of 1.0 ml of supernatant of liver homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1 % sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was measured at 412 nm. The percentage inhibition of GSH was calculated using the following equation: % reduced glutathione inhibition = 100 - {Increase in absorbance of the sample × 100}/ Increase in absorbance of the blank.

Estimation of lipid peroxidation

Lipid peroxidation in the liver was estimated colorimetrically by thiobarbituric acid reactive substances (TBARS) using the modification method of Niehius and Samuelsson (1968). In brief, 0.1 ml of liver homogenate (10 %w/v) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent (thiobarbituric acid 0.37%, 15 % trichloroacetic acid and 0.25 N HCl). All the tubes were placed in a boiling water bath for 30 min, and cooled. The amount of malondialdehyde formed in each of the samples was assessed by measuring the absorbance of clear supernatant at 535 nm against reference blank. Percentage inhibition was calculated using the equation: % lipids Inhibition = $\{A_o - A_1\}/A_o \times 100$, where A_o is the absorbance of the control and A_1 is the absorbance of the sample extract.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates.

RESULTS

The total phenolics contents in the aqueous extract of *S. henningsii* are shown in Figure 1. The plant extract possessed high phenol contents (48 mg/g tannic acid equivalent) followed by proanthocyandins (8.7 mg/g catechin equivalent), flavonols (5.5 mg/g quercetin equivalent), tannin (4.2 mg/g tannic acid equivalent) and flavonoids (4.8 mg/g quercetin equivalent). The percentage contents of alkaloids and saponins were 1.5 and 6% respectively. Phenolics compounds, especially flavonoids and phenolics compounds have been shown to possess significant antioxidant activity.

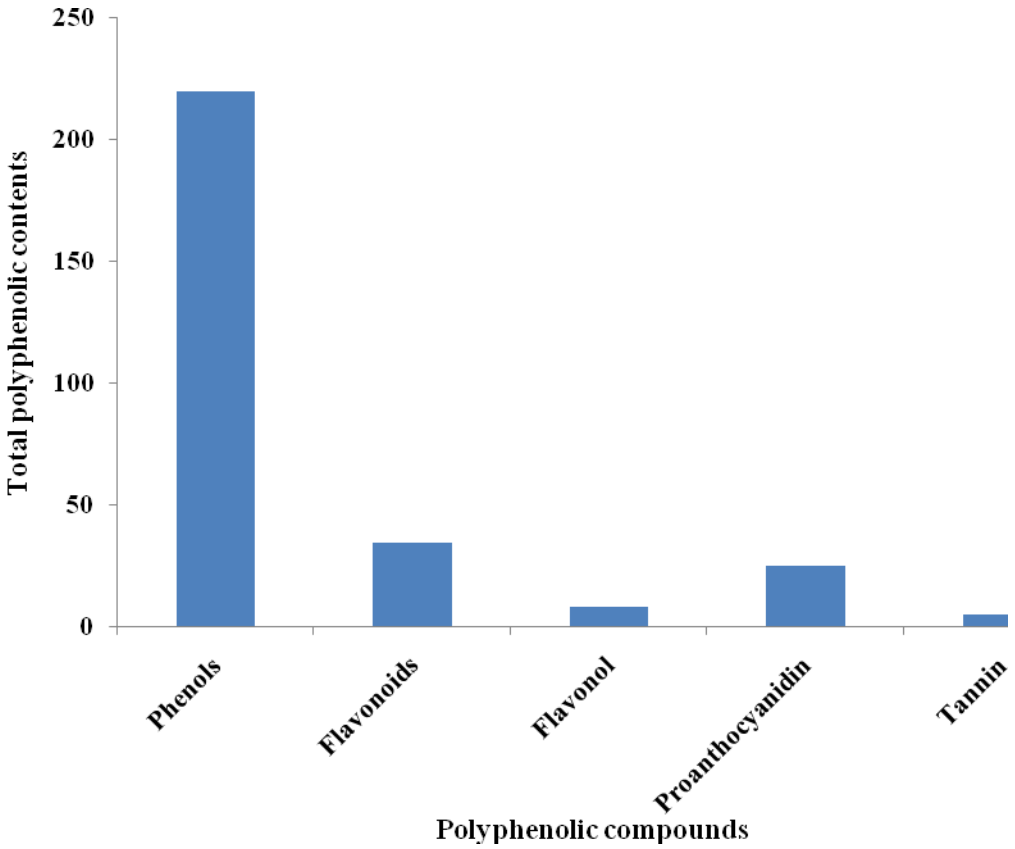

Figure 1: Polyphenolics contents of aqueous stem bark extract of *S. henningsii*.

The antioxidant potentials of the plant extract was estimated from their ability to reduce Fe^{3+} to Fe^{2+}. This was observed from yellow colour of the test solution that changed to various shades of green and blue depending on the concentration of the plant extract. The reducing value of the extract was significantly lower than that of BHT, Vitamin C and Vitamin E used as reference compounds in this study (Figure 2). Even at 0.5 mg/ml, the absorbance of plant extract was still low compared to the reference drugs.

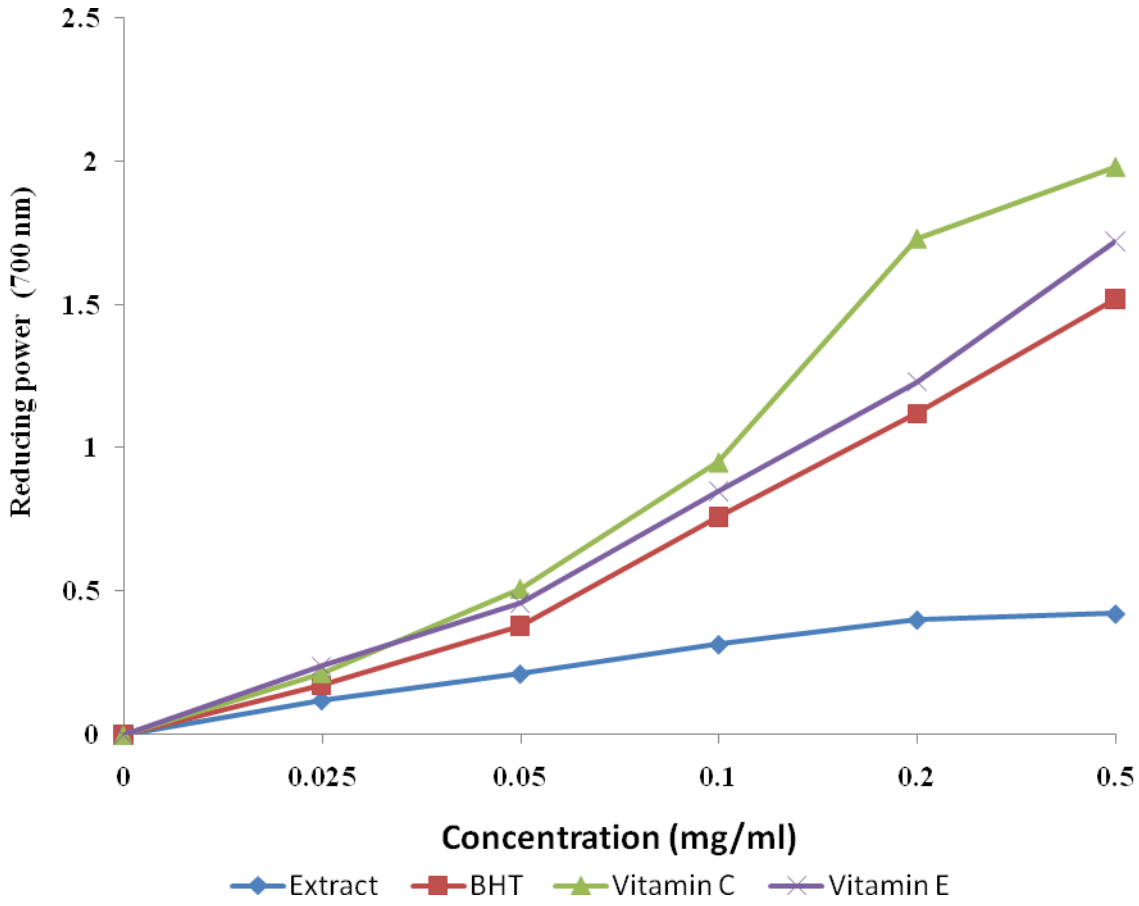

Figure 2: Total ferric reducing potential of aqueous bark extract of *S. henningsii*.

Figure 3 shows the dose-response curve of DPPH radical scavenging activity of *S. henningsii* extract compared with rutin and BHT. It was observed that the extract had DPPH scavenging activity with IC_{50} value of 0.739 mg/ml. The scavenging activity of this plant against DPPH was observed as the weakest among other reactive oxygen species evaluated.

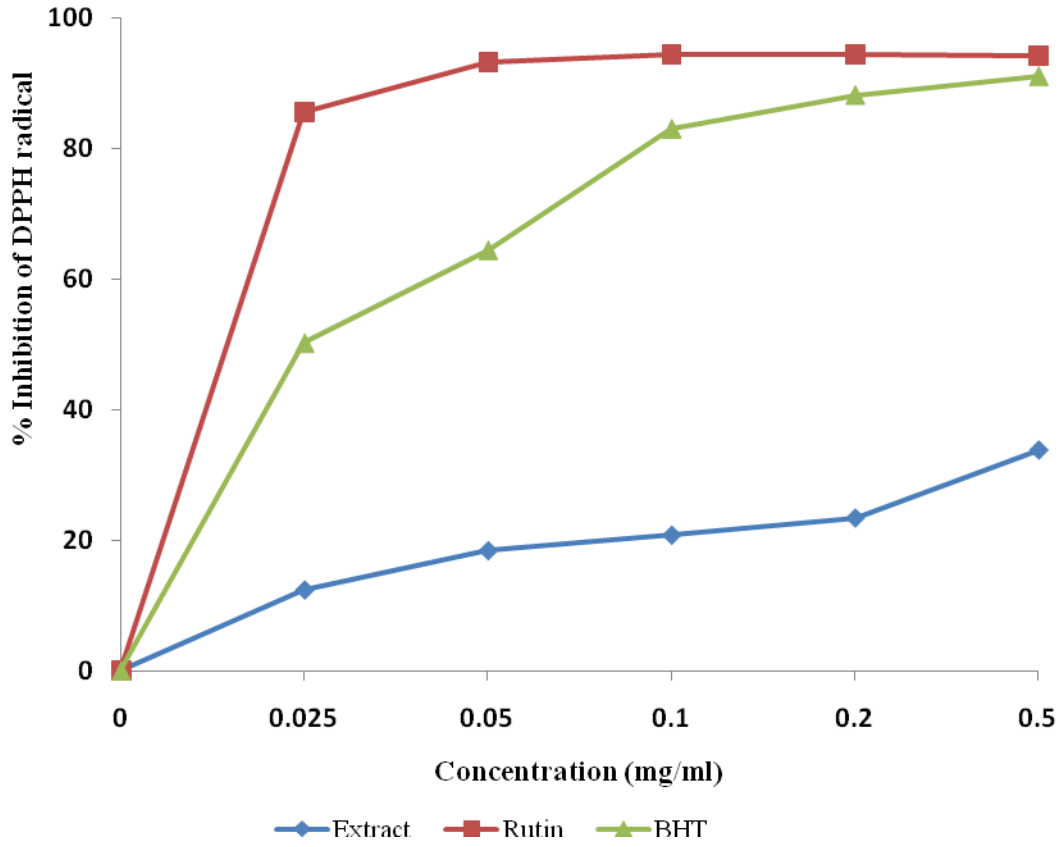

Figure 3: DPPH radical scavenging activity of the aqueous bark extract of *S. henningsii*.

S. henningsii extract was fast and effective scavenger of the ABTS radicals as shown in Figure 4. The scavenging activity of this plant was observed with that of BHT and rutin. The IC_{50} values of the extract, rutin and BHT were 0.089, 0.016 and 0.015 respectively. At 0.5 mg/ml, the plant extract showed strong inhibitory activity in removing ABTS radicals from the reaction system.

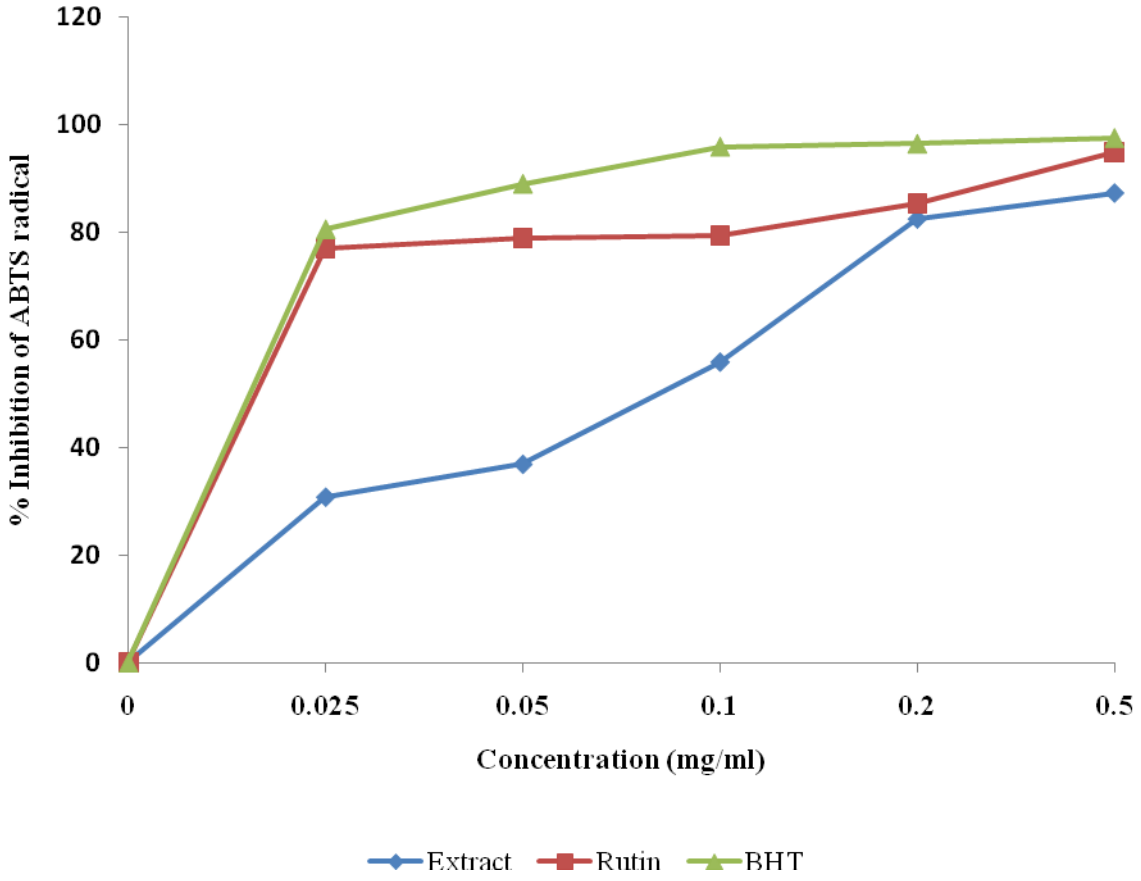

Figure 4: ABTS radical scavenging activity of the aqueous bark extract of *S. henningsii*.

The scavenging activity of aqueous extract of *S. henningsii* compared to BHT and Vitamin C for hydrogen peroxide is shown in Table 1. The results indicated a concentration dependent activity against H_2O_2 with IC_{50} values of 0.023, 0.018 and 0.02 mg/ml for plant extract, BHT and Vitamin C respectively. The percentage inhibition values at 0.5 mg/ml were 92.51, 98.46 and 99.82 % for plant extract, BHT and Vitamin C respectively.

Table 1: Scavenging activity of aqueous extract of *S. henningsii* bark against hydrogen peroxide radical.

Sample	Concentration (mg/ml)	% inhibition	IC_{50}	r^2
Aqueous extract	0.025	54.82		
	0.050	77.80		
	0.100	85.38		
	0.200	87.52	0.023	0.9962
	0.500	92.51		
Vitamin C			0.020	0.9861
BHT			0.016	0.9952

In each group n = 5 rats, Values are expressed as mean ± SD

r^2 – regression co-efficient.

The plant extract caused a moderate dose-dependent inhibition of nitric oxide with an IC_{50} of 0.49 mg/ml as shown in Table 2. The scavenging activity of BHT and Vitamin C showed IC_{50} values of 0.032 and 0.026 mg/ml and percentage inhibition of 85.96 and 94.22% respectively while the percentage inhibition of *S. henningsii* was 50.31 %.

Table 2: Nitric oxide radical scavenging activity of aqueous bark extract of *S. henningsii*

Sample	Concentration (mg/ml)	% inhibition	IC_{50}	r^2
Aqueous extract	0.025	15.80		
	0.050	16.30		
	0.100	28.74		
	0.200	35.56	0.490	0.9982
	0.500	50.31		
Vitamin C			0.026	0.9961
BHT			0.032	0.9946

In each group n = 5 rats. Values are expressed as mean ± SD

r^2 – regression co-efficient.

Table 3 showed the effect of plant extract on the activities of antioxidant enzymes in the liver of control and experimental rats. There was a marked decreased in the percentage inhibition of superoxide dismutase, catalase and the level of GSH in carbon tetrachloride treated rats when compared with normal control group. However, the percentage inhibition of SOD, CAT and the level of GSH were significantly increased followed the oral administration of plant extract at 250, 500 and 1000 mg/kg in a dose dependent manner.

Table 3: Effect of aqueous extract of *S. henningsii* bark on lipid peroxidation, antioxidant enzymes and GSH in carbon tetrachloride induced hepatotoxic rats.

Group	CAT	SOD	GSH	TBARS
Control	89.27 ± 0.24	80.22 ± 0.21	83.55 ± 0.33	45.00 ± 0.35
CCl_4	31.00 ± 0.22	42.60 ± 0.21	31.77 ± 0.31	91.67 ± 0.32
CCl_4 + *S. henningsii* (D1)	37.46 ± 0.34	59.33 ± 0.24	43.33 ± 0.39	47.00 ± 0.43
CCl_4 + *S. Henningsi* (D2)	47.82 ± 0.32	62.11 ± 0.29	48.44 ± 0.36	52.50 ± 0.32
CCl_4 + *S. Henningsii* (D3)	64.00 ± 0.28	67.77 ± 0.26	54.00 ± 0.36	58.33 ± 0.30

D1 = 250 mg/kg; D2 = 500 mg/kg and D3 = 1000 mg/kg. Results are expressed as percentage inhibition of the control. Each value is mean ± S.D (n = 5 rats).

The *in vivo* lipid peroxidation study revealed that rats treated with carbon tetrachloride showed a significant increase ($P < 0.05$) in TBARS when compared with normal control group. Treatment with aqueous extract of *S. henningsii* for 8 days was able to retard the increase in TBARS levels in a dose dependent manner (Table 3).

DISCUSSION

Polyphenols are the major plant compounds with high levels of antioxidant activity. This activity could be due to their ability to adsorb, neutralize and quench free radicals (Duh et al., 1999). Their ability as free radical scavenger could also be attributed to their redox properties, presence of conjugated ring structures and carboxylic group which have been reported to inhibit lipid peroxidation (Rice-Evans et al., 1995).

In the present study, it was found that the aqueous extract of *S. henningsii* contains high levels of phenols that might account for the strong activity observed against ABTS and H_2O_2 radicals. This scavenging activity may be due to the presence of hydroxyl groups attached to the aromatic ring structures and thus help to quench the radicals (Vinson et al., 1998). On the other hand, the weak activity depicted in DPPH and NO radicals scavenging activities may be as a result of lower content of flavonoids which have been reported to possess high antioxidant activity.

The reducing power of aqueous extract of *S. henningsii* obtained in this study was determined by measuring the transformation of Fe^{+3} to Fe^{+2}. The result obtained showed that the extract possessed antioxidant activity in a concentration dependent manner. The reducing power of the extract could be related to electron transfer ability and this may serve as a significant indicator of antioxidant activity observed in this study (Meir et al., 1995). This effect suggests that *S. henningsii* may possess the ability to minimize oxidative damage to some vital tissues in the body (Kojic et al., 1998; Weighand et al., 1999).

The relatively low level of flavonoids might account for the weak activity observed in the DPPH radical scavenging assay as shown in Figure 3. The scavenging of $ABTS^+$ by the plant extract was found to be higher than that of DPPH radical and this could be due to different mechanisms involved in the radical-antioxidant reactions. Factors such as the solubility of the extracts in different testing system, substrate used and quantization method may affect the ability of the plant extract to quench different radicals (Yu et al., 2002). This result corroborate with the report of Wang et al. (1998) found that some compounds with $ABTS^+$ scavenging activity may not exhibit DPPH scavenging activity. As a result, it may be difficult to compare antioxidant activity based on antioxidant assay because of the different test systems used and the substrate to be protected (Frankel and Meyer, 2000).

Hydrogen peroxide is a highly important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radicals in the cell (Gulcin et al., 2003). The scavenging activities of the plant extract were nearly the same as the reference compounds. The result obtained could be due to the presence of phenolics compounds that donate electrons to H_2O_2 and thus neutralize it to water (Mathew and Abraham, 2006).

Nitric oxide (NO) is a reactive free radical generated from sodium nitroprusside in aqueous solution at physiological pH and reacts with oxygen to form nitrite. The plant extract inhibits nitrite formation by directly competing with oxygen, nitric oxide and other nitrogen oxides such as NO_3, N_2O_4 and N_2O_3 in the reaction (Marcocci et al., 1994). The percentage inhibition of NO showed that the extract has a moderate activity as compared with the standard drugs (Table 2).

Carbon tetrachloride is one of the most commonly used hepatotoxins in the experimental study of liver damage (Lee et al., 2001). The hepatotoxic effects are largely based on membrane lipid peroxidation and induction of trichloromethyl radical that results in severe cell damage (Johnson and Kroening, 1998). In our study, the rats treated with a single dose of CCl_4 developed a drastic hepatic damage and oxidative stress, which was observed by a substantial increase in the lipid peroxidation. The inability of an antioxidant defense mechanism to prevent formation of excessive free radicals may be responsible for this observation. Treatment with aqueous extract of *S. henningsii* was able to reduce the level of lipid peroxides in a dose dependent manner as compared with hepatotoxic group.

Superoxide dismutase has been reported as one of the most important enzymes in the enzymatic antioxidant defense system (Curtis and Mortiz, 1972). It removes superoxide anion by converting it to hydrogen peroxide, and thus diminishing the toxic effect caused by this radical. The decrease in percentage inhibition of superoxide dismutase as observed in this

study indicated hepatocellular damage by CCl_4. However, an increase in the percentage inhibition of superoxide by SOD after administration of aqueous extract of *S. henningsii* at the doses investigated suggests that the plant has an efficient protective mechanism in response to reactive oxygen species that are generated in hepatotoxic rats.

Catalase is an antioxidant enzyme widely distributed in the animal tissues. It decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1992). Inhibition of this enzyme may enhance sensitivity to free radical-induced cellular damage. Therefore reduction in the activity of CAT may leads to deleterious effects as a result of superoxide and hydrogen peroxide assimilation. In the present study, the percentage inhibition of catalase was shown to increase after the administration of the aqueous extract at 250, 500 and 1000mg/kg body weight. This indicates the hepatoprotective ability of this plant from liver damage.

Reduced glutathione (GSH) is a tripeptide, non enzymatic biological antioxidant present in the liver. It protects cellular proteins against oxidation in glutathione redox cycle and directly detoxifies reactive oxygen species generated from exposure to carbon tetrachloride (Arivazhagan et al., 2000). Decreased level of GSH is associated with an increased level of lipid peroxidation in CCl_4 treated rats; thus cause liver disorder and injury. Administration of *S. henningsii* extract increased the activity of GSH by the reactivation of hepatic glutathione reductase and thus reduced the level of lipid peroxidation. The reduced glutathione levels of plant extract treated groups are in accordance with the report of Bhandarkar and Khan (2004).

In conclusion, present results demonstrate that aqueous bark extract of *S. henningsii* has both *in vivo* and *in vitro* antioxidant activities due to the presence of phenolic compounds. This result also showed that the plant extract has the ability to prevent the process of initiation and progression of hepatocellular diseases.

REFERENCE

Ariyazhagan, S., Balasenthil, S and Nagini, S (2000). Garlic and neem leaf extracts enhance hepatic glutathione and glutathione dependent enzymes during N-methyl-Nnitrosoguanidine (MNNG)- induced gastric carcinogenesis. Phytother Res. 14: 291-293.

Bhandarkar, M.R and Khan, A (2004). Antihepatotoxic effect of *Nymphaea stellata* Willd, against carbon tetrachloride-induced hepatic damage in albinos rats. J. Ethnopharmacol. 91: 61-64.

Bisset, N.G (1970). The African species of Strychnos. The ethnobotany. Lloydia. 33:201-243.

Chance, B and Greenstein, D.S (1992). The mechanism of catalase actions-steady state analysis. Arch Biochem Biophys. 37: 301-339.

Chandra, A., Samali, S and Orrenius, S (2000). Triggering and modulation of apoptosis by oxidative stress. Free radical Biol. Med. 29: 323-333.

Curtis, J.J and Mortiz, M (1972). Serum enzymes derived from liver cell fraction and response to carbon tetrachloride intoxication in rats. Gastroenterol. 62: 84-92.

Duh, P.D, Tu, Y.Y and Yen, G.C (1999). Antioxidant activity of water extract of Harng Jyur (Chrssanthemum morifolium Ramat). Lebnesmittel-Wissenschaft und technologie. 32: 269-277.

Ellman, G.L (1959). Tissue sulfhydryl groups. Arch Biochem Biophys. 82:70-77.

Frankel, E.N and Meyer, A.S (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J. Sci. Food and Agric. 80: 1925-1941.

Garrat, D.C., 1964. The quantitative analysis of drugs Japan: Chapman and Hall. 3. 456-458.

Gulcin, I., Oktay, M., Kirecci, E and Kufrevioglu, O.I (2003). Screening of antioxidant and antimicrobial activities of anise (Pimpinella anisum L) seed extracts. Food Chem. 83: 371 - 382.

Harborne, J.B (2005). Phytochemical methods: A guide to modern techniques of plant analysis. Chapman A and Hall.

Hazra, B., Santana, B and Nripendranath, M (2008). Antioxidant and free radicals scavenging activity of *Spondias pinnata*. J. BMC Compl and alt med.8: 63.

Hutchings, A (1989). A survey and analysis of traditional medicinal plants used by the Zulu, Xhosa and Sotho. Bothalial. 19:111-123.

Johnson, D.E and Kroening, C (1998). Mechanism of early carbon tetrachloride toxicity in cultured rat hepatocytes. Pharmacol Toxicol. 83:231-239.

Kojic, G., Vlahonic, P and Ravloure, D (1998). The possible importance of the cation binding site for the oxidative modification of liver nucleolidase. Arch. Physiol. Biochem. 106:91-99.

Kumaran, A and Karunakaran, R.J (2007). *In vitro* antioxidant activities of methanolic extracts of Phyllantus species from India. Lebens-Wiss technologie. 40:344-352.

Lee, K.S., Lee, S.J., Park, H.J., Chung, J.P., Han, K.H and Chon, C.Y (2001). Oxidative stress effect on the activation of hepatic stellate cells. *J. Med* ; 42:1-8.

Leeuwenberg, A.J.M (1969). The Loganiaceae of Africa VIII. Strychnos III. Mededdelingen Landbouwhogeschool Wageningen. 69: 1-316.

Liyana-Pathiranan, C.M and Shahidi, F (2005). Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L) as affected by gastric pH conditions. J. Agric and Food Chem. 53: 2433-2440.

Madhavi, D.L and Salunkhe, D.K (1995). Toxicological aspects of food antioxidants in Madhavi, DL., Deshpande, SS and Salunkhe, DK. Food antioxidants. New York: Dekker; 267.

Marcocci, L., Packer, L., Droy-Lefai, M.T., Sekaki, A and Gardes-Albert, M (1994). Antioxidant action of *Ginkgo biloba* extracts EGb 761. Met. enzymol. 234: 462-475.

Mathew, S and Abraham, T.E (2006). In vitro antioxidant activities and scavenging effects of *cinnamomum verum* leaf extract assayed by different methodologies. J. Food. Chem and Toxicol. 44:198-206.

Meir, S., Kanner, J and Akiri, B (1995). Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. J. Agric and Food Chem. 43: 1813-1815

Misra, H.P and Fridovich, I (1972). The role of superoxide anion in the autooxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 247: 3170-3175.

Niehius, W.G and Samuelson, B (1968). Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur. J. Biochem. 6: 126-130.

Niki, E., Shimaski, H and Mino, M (1994). Antioxidantism-free radical and biological defense. Gakkai Syuppn Center, Tokyo. 3-16.

Obadoni, B.O and Ochuko, P.O (2001). Phytochemical studies and comparative efficacy of the crude extract of some homeostatic plants in Edo and Delta states of Nigeria. Global J. Pure Appl.Sci. 8:203-208

Ordonez, A.A.L., Gomez, J.D., Vattuone, M.A and Isla, M.I (2006). Antioxidant activities of *Sechium edule* (jacq). Swart Extracts. Food chem. 97:452-458.

Pari, L and Latha, M (2004). Protective role of *Scoparia dulcis* plant extract on brain antioxidant status and lipid peroxidation in STZ diabetic male Wistar rats. BMC Compl and Alternatl Med. 4:16. doi:10.1186/1472-6882-4-16.

Polterat, O (1997). Anti -oxidants and free radical Scavengers of natural origin. Current Org. Chem. 1:415-440.

Re, R., 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 Biol and Med. 26: 1231-1237.

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M and Pridham JB (1995). The relative activities of plant-derived polyphenolic flavonoid. Free radical Res. 22:375-383.

Robak, J and E. Marcinkiewicz, 1995. Scavenging of reactive oxygen species as the mechanism of drug action. Pol. J. Pharmacol. Pharm. 47: 89-98

Ruch, R.J., Cheng, S.J and Klaunig, J.E (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from chinese green tea. Carcinogen. 10: 1003-1008.

Sinha, A.K (1972). Colorimetric assay of catalase. Anal Biochem. 47: 389-394.

Sun, J.S., Tsuang, Y.W., Chen, J.J., Huang, W.C., Hang, Y.S and Lu, F.J (1998). An ultra-weak chemiluminescence study on oxidative stress in rabbits following acute thermal injury. Burns. 24: 225-231.

Swain, T (1979). Tannins and Lignins. In: Rosenthal, G.A and Janzen, D.H (eds) Herbivores: their interaction with plant metabolites. Academic press. New York.

Tits, M (1982). Contribution a l'etude chimique du Strychnos variabilis, Plante toxique du Bas-Zaires. Isolement et Determination de structure de nouveaux Alcaloides indoliques. Thesis, faculte de medicine, Universite de Liege, Belgium. 44-192.

Vinson, J.A., Yong, H., Xuchui, S and Zubik, L (1998). Phenol antioxidant quantity and quality in foods: vegetables. J. Agric. Food Chem. 46: 3630-3634.

Wang, M., Li, J., Rangarajan, M., Shao, Y., La Voie E.J., Huang, T and Ho, C (1998). Antioxidative phenolic compounds from sage (*Salvia officinalis*). J. Agric. Food Chem. 46: 4869-4873.

Weighand, M.A., Laipple, A and Plascke, K (1999). Concentration changes of malondialdehyde across the cerebral vascular bed and shedding of L selectin during carolide endarterectomy. Stroke, 30:306-311.

Wolfe, K., Wu, X and Liu, R.H (2003). Antioxidant activity of apple peels. J. Agric. Food Chem. 51: 609-614.

Yen, G and Chen, H (1995). Antioxidant activity of various tea extract in relation to their antimutagenicity. J. Agric and Food Chem. 43:7-32.

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J and Qian, M (20020. Free radicals scavenging activities of wheat extracts. J. Agric. Food Chem. 50: 1619-1624.