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Protective effects of ginger (*Zingiber officinale Roscoe*) against cadmium chloride-induced oxidative stress in the blood of rats

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Cadmium is a heavy metal of wide occupational and environmental contamination. In recent years, however, cadmium has been implicated in the pathogenesis of several clinical disorders. Generation of oxidative stress is one of the plausible mechanisms for cadmium-induced diseases. The aim of the study was to investigate the effect of ginger on oxidative stress in rats exposed to cadmium (Cd) of a dose (10 mg/kg b.w.). Ginger was administered orally (500 mg/kg b.w.). After 26 days, significant increases in methemoglobin% (metHb%), carboxyhemoglobin% (HbCO%), glutathione peroxidase (GPx) activity, malondialdehyde (MDA) concentration and hemolysis% were observed in cadmium exposed rats compared to control group ($P < 0.05$), while glutathione-S-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) showed insignificant changes. Cadmium treatment of rats caused a significant decrease in oxyhemoglobin% (HbO_2%) and total blood hemoglobin (Hb) concentration ($P < 0.05$). Ginger treatment of cadmium exposed rats significantly lowered metHb% ($P < 0.05$), while significantly increased HbO_2% ($P < 0.05$) and total Hb concentration ($P < 0.01$), compared to cadmium alone group. Also ginger treatment significantly increased GPx and G6PD activities of cadmium exposed rats compared to cadmium alone group ($P < 0.05$). The treatment of Cd-exposed animals with ginger lowered MDA concentration and hemolysis% by 20% and 17%, respectively. From these findings it can be concluded that ginger is a strong antioxidant plant that protects the blood of rats against the adverse harmful effects of cadmium chloride exposure as well as cadmium chloride-induced oxidative stress.

Key words: Cadmium, ginger, oxidative stress, erythrocytes, hemolysis, hemoglobin derivatives, rats.

INTRODUCTION

Cadmium (Cd) is an important industrial and environmental pollutant that currently ranks seventh on

the list of hazardous substances developed by the Agency for Toxic Substances and Disease Registry

jointly with the U.S. Environmental Protection agency (EPA) and available at ATSDR website (ATSDR, 2013). In the environment, Cd is dangerous because humans consume both plants and animals that absorb Cd efficiently and concentrate it within their tissues (ATSDR, 1999). The flow of Cd in ecological systems increases through major sources as mining, smelting and industrial use. Sources of human exposure to this metal include food, water, cigarette smoke and alcoholic beverages (Jarup et al., 1998). Normally, absorption of Cd is increased if the animals are on a low calcium diet. Once absorbed, Cd associates with cysteine residues of the low molecular weight protein, metallothionein and accumulates in the body. Since the metabolism of Cd is closely related to zinc metabolism, metallothionein binds and transports both Cd and zinc. Cadmium displaces zinc in many vital enzymatic reactions, such as causing disruption or cessation of enzyme activities (Bauer et al., 1980; Casalino et al., 2002).

Cadmium causes poisoning in various tissues of humans and animals (Swiergosz et al., 1998; Stohs et al., 2000). In the blood, Cd stimulates the formation of metallothioneins and reactive oxygen species (ROS) thus causing oxidative damage in erythrocytes and in various tissues, which results in a loss of membrane functions (Sarkar et al., 1995; Simpkins et al., 1998). It has been reported that chronic treatment with cadmium, induced oxidative damage in erythrocytes of rats, causing destruction of cell membranes and increased lipid per-oxidation, as well as alteration of the antioxidant enzyme system, energy metabolism and the appearance of anemia (Kostić et al., 1993; Zikić et al., 1997; Ognjanović et al., 2000; Pavlovic et al., 2001; Zikić et al., 2001; Kanter et al., 2005). Rats administered cadmium chloride orally showed decreased values of erythrocyte counts, hemoglobin and hematocrit, as well as decreased mean cell volume (MCV), mean cell hemoglobin (MCH) and reduced mean cell hemoglobin concentration (MCHC), suggestive of microcytic and hypochromic anemia (Bersenyi et al., 2003; Dallak, 2009; Onwuka et al., 2010). Marked alterations of antioxidant system were found in erythrocytes of Cd-treated rats. Activities of catalase and glutathione peroxidase (GPx) as well as the total glutathione (GSH) contents in erythrocytes were significantly decreased, whereas the activity of superoxide dismutase (SOD) was significantly increased (Messaoudi et al., 2010).

Antioxidants are the natural defense mechanism exis-ting in our system and these are capable of scavenging the deleterious free radicals (Matés et al., 2011). A number of dietary antioxidant compounds have been shown to influence the membrane characteristics such as fluidity, stability and susceptibility to membrane oxidative

damage (Halliwell and Gutteridge, 1990). Recently, a great deal of attention has focused on the protective biochemical functions of naturally occurring antioxidants in biological systems against toxic heavy metals (Matés et al., 2010). Thus, it is believed that antioxidant should be one of the important components of an effective the-rapy of Cd poisoning. Ginger, which is the underground stem or rhizome of the plant *Zingiber officinale Roscoe*, contains polyphenol compounds (6-gingerol and its derivatives), which have a high antioxidant activity (Chen et al., 1986; Herrman, 1994). There are more than 50 antioxidants isolated from rhizomes of ginger (Kikuzaki and Nakatani, 1993; Masuda et al., 2004). Among them, 12 compounds exhibited higher antioxidant activity than α-tocopherol. Ginger and its constituents are stated to have antiemetic, antithrombotic, antihepatotoxic, anti-inflammatory stimulant, cholagogue, androgenic and antioxidant effects (Miller et al., 1993; Khaki et al., 2009). The main pharmacological actions of ginger and compounds isolated there, include immuno-modulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-lipidemic and anti-emetic actions (Ali et al., 2008). It is considered a safe herbal medicine with only few and insignificant adverse/side effects (Ali et al., 2008).

Previous results demonstrated that chronic administration of ginger at the dosages of 500, 1000 and 2000 mg/kg body weight by a gavage method for 35 days was not associated with any mortalities and abnormalities in general conditions, behavior, growth, and food and water consumption in both male and female rats (Rong et al., 2009). Except for dose-related decrease in serum lactate dehydrogenase activity in males, ginger treatment induced similar hematological and blood biochemical parameters to those of controlled animals. Previous results have indicated that ginger is a potent scavenger of a variety of ROS including superoxide anion radicals ($O_2^{.-}$), hydroxyl radicals ($^.OH$) and peroxyl radicals ($ROO^.$) (Stoilova et al., 2007; Baliga et al., 2013). Ginger reduced H_2O_2-induced apoptotic signals and the levels of intracellular ROS (Oh et al., 2012). Ahmed et al. (2000) and Prabhu et al. (2013), found that ginger significantly lowered lipid peroxidation by maintaining the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in the blood of healthy rats. Ginger administration has been reported to prevent the lead-induced oxidative stress to erythrocytes in rats (Attia et al., 2013). The protective effects of ginger against lead-induced hepatotoxicity are well documented and have been attributed to its intrinsic antioxidant properties (Attia et al., 2013).

The antioxidant effects of vitamin E, taurine and curcumin against cadmium chloride-induced oxidative

stress to erythrocytes and hematotoxicity in rats have been studied (Ognjanović et al., 2003; Sinha et al., 2008; Kanter et al., 2009). However, effects of ginger, as a powerful antioxidant, on cadmium chloride-induced oxidative stress to erythrocytes and hematotoxicity in rats have not yet been studied. Therefore, the aim of this study was to investigate the antioxidant effects of ginger against cadmium chloride-induced perturbations in oxidative bio-markers of blood such as levels of hemoglobin derivatives, plasma malondialdehyde (MDA) level, GPx, glutathione reductase (GR), glutathione-S-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PD) activities in erythrocytes hemolysate and erythrocytes hemolysis test, in rats.

MATERIALS AND METHODS

Eighteen non-pregnant female albino rats (age: 6 to 8 weeks and about 80 to 105 g body weight) were obtained from the animal house, National Research Center, Cairo, Egypt. All animals were treated in accordance to the principles of Laboratory Animal Facilities of World Health Organization, Geneva, Switzerland (2003). The animals were fed a standard pellet diet and had free access to water. The standard diet contained 50% wheat, 21% corn, 20% soybean, 8% concentrated proteins and 1% a mixture of salts, vitamins and dicalcium phosphate. The nutritional content was 5% fat, 21 % protein, 55% nitrogen free extract and 4% fiber (w/w) with adequate minerals and vitamin contents. The rats were housed in stainless steel cages in a temperature-controlled room (25 ± 2 °C) with a 12 h light and 12 h dark exposure.

Grouping of animals and treatment

The animals were randomly divided into three groups of 6 animals each, control, cadmium chloride alone, and cadmium chloride with ginger. All groups were given a standard rat chow and water. Rats in cadmium alone and cadmium with ginger groups were given treatments orally by gavage needle for 26 days. Rats of cadmium alone group were given daily 2 ml dose of a solution containing 10 mg/kg body weight of monohydrated cadmium chloride orally. While, rats of cadmium with ginger group were treated orally and daily with 2 ml of an aqueous solution containing 500 mg/kg body weight of ginger in which a concentration of 10 mg/kg body weight of monohydrated cadmium chloride was dissolved. The dose of ginger used in this study was selected on the basis of previous studies (Al-Amin et al., 2006; Rong et al., 2009; Jeena et al., 2011). Ginger, which is the underground stem or rhizome of the plant *Z. officinale Roscoe*, was purchased in a powder form from Elgabry Company for Medicinal Herbs, Giza, Egypt.

Preparation of blood samples

The experiments lasted for 26 days. At the end of the experimental period, blood samples were collected from all animals from the retro-orbital venous plexus. The blood samples were collected into heparinized tubes. The plasma obtained after centrifugation (3000 rpm for 10 min at 4 °C) was used for MDA determination. Erythrocytes was washed three times in phosphate buffered saline (PBS) solution. Lysed erythrocytes were prepared by addition of four volumes of ice-cold distilled water. Cell membranes were removed by centrifugation at 8,500 rpm for 20 min, and the supernatant was used for the assays of antioxidant enzymes activities.

Biophysical assays

Levels of hemoglobin derivatives (sulfhemoglobin, "SHb", methemoglobin, "metHb", carboxyhemoglobin, "HbCO", and oxyhemoglobin, "HbO_2") and the total hemoglobin (Hb) concentration in the blood of rats were determined by the multicomponent spectrophotometric method described previously (Attia et al., 2011a). According to this method, the absorbance measurements for plasma-free diluted Hb solutions were made at four wavelengths (λ = 500, 568, 576 and 620 nm), using a Cary UV/VIS double-beam spectrophotometer (model 100 UV-VIS), manufactured by Varian Inc. company Sydney, Australia. The total blood Hb concentration (in g/dL) was calculated by multiplying the concentration of diluted samples by the dilution factor (DF= 170.66) and the conversion factor for mmol L^{-1} to g dL^{-1} (1.6114). Percentages of erythrocytes hemolysis were determined according to the method of Attia et al. (2011b). According to this method, hemolysis% was determined by the measurement of Hb released from the cells, relative to the total cellular Hb. The absorbance of released Hb was measured at the isobestic point of metHb and HbO_2 (λ = 522 nm).

Biochemical assays

The kits for biochemical parameters were purchased commercially from Biodiagnostic Company, Cairo, Egypt. Plasma MDA concentration was determined spectrophotometrically by the method of Satoh (1978). Thiobarbituric acid (TBA) reacts with MDA in acidic medium at temperature of 95°C for 30 min to form thiobarbituric acid reactive product. The absorbance of the resulting pink product can be measured at 534 nm. GPx activity was determined spectrophotometrically by the method of Paglia and Valentine (1967). The assay is an indirect measure of the activity of cellular GPx. Glutathione disulfide (GSSG), produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme GR. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring GPx enzyme activity. To assay GPx, erythrocytes hemolysate is added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is initiated by adding the substrate, hydrogen peroxide and the A_{340} is recorded. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

GST activity was determined spectrophotometrically by the method of Habig et al. (1974). The Biodiagnostic GST assay kit measures total GST activity (cytosolic and microsomal) by measuring the conjugation of 1- chloro- 2,4- dinitrobenzene (CDNB) with reduced glutathione (GSH). The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample. GR activity was determined spectrophotometrically by the method of Goldberg and Spooner (1983). GR catalyzes the reduction of oxidized glutathione (GSSG) in the presence of NADPH, which is oxidized to $NADPH^+$. The decrease in absorbance at 340 nm directly proportional to GR activity is measured. G6PD activity was determined spectrophotometrically by the method of Kornberg (1955). The enzyme activity is determined by measurement of the rate of absorbance change at 340 nm due to the reduction of $NADP^+$.

Statistical analysis

Data were presented as the mean ± standard error (SE) values. One-way ANOVA was carried out, and the statistical comparisons among the groups were performed with Post Hoc and the least significant difference (LSD) tests using a statistical package program (SPSS version 14). $P < 0.05$ was considered as statistically

statistically significant.

RESULTS

Blood hemoglobin derivatives

Table 1 shows the levels of inactive hemoglobins (SHb, metHb, HbCO), active HbO$_2$ and total Hb concentration in all groups. After 26 days, significant increases in metHb% and HbCO% were observed in cadmium-exposed rats, compared to the control group ($P < 0.05$), while cadmium treatment had no significant effects on SHb%. Cadmium treatment of rats caused a significant decrease in HbO$_2$% and total hemoglobin (Hb) concentration (P < 0.05), compared to controls. Ginger treatment of cadmium exposed rats significantly lowered metHb% (P < 0.05), while significantly increased HbO$_2$% (P < 0.05) and total Hb concentration (P < 0.01), compared to the cadmium alone group.

Plasma MDA concentration

Table 2 shows the concentration of MDA in plasma of all groups. After 26 days, significant increase in MDA concentration was observed in cadmium exposed rats compared to control group ($P < 0.05$). Ginger treatment of cadmium exposed rats lowered MDA concentration ($P < 0.01$, -20%) compared to cadmium alone group.

Erythrocyte antioxidant enzyme activities

Table 2 shows the activities of GPx, GST, GR and G6PD in erythrocytes of all groups. Marked alterations of antioxidant enzyme system were found in Cd-exposed rats. GPx activity significantly increased ($P < 0.05$) in cadmium compared to control group, while GST, GR and G6PD showed insignificant changes. Ginger treatment significantly increased GPx and G6PD activities of cadmium exposed rats, compared to cadmium alone group ($P < 0.05$), while it has no effect on GR and GST activities. However, GPx, GR and G6PD activities of cadmium+ ginger group are significantly higher ($P < 0.05$) than controls.

Percentages of erythrocytes hemolysis

Figure 1 shows the hemolysis % of erythrocytes in all groups. The hemolysis test indicates that intoxication by cadmium significantly increases the hemolytic effect ($P < 0.05$), whereas after treatment with ginger, it decreases by 17%.

DISCUSSION

Cd is a toxic metal that is widely used in different industries.

Cd promotes an early oxidative stress and afterward contributes to the development of serious pathological conditions because of its long retention in some tissues (Bagchi et al., 2000). The present results have clearly demonstrated the ability of Cd to induce oxidative stress in rat blood as evidenced by increased lipid peroxidation after 26 days of Cd treatment. This finding is in agreement with several reports demon-strating that Cd induces oxidative stress by altering the antioxidant status in several tissues (Ognjanović et al., 2003; Sinha et al., 2008; Kanter et al., 2009; El-Sokkary et al., 2009; Onwuka et al., 2011; Tarasub et al., 2011; Tarasub et al., 2012). The mechanism by which Cd induces ROS is not yet known (Ercal et al., 2001). Pre-vious studies have indicated that treatment of cells with Cd, results in specific mitochondrial alterations (Wang et al., 2004). Cadmium exposure also leads to mitochondrial dysfunction in the renal cortex of rats (Tang and Shaikh, 2001). Cadmium inhibits the mitochondrial electron transfer chain and induces ROS (Wang et al., 2004).

The inactive components of Hb (SHb, metHb and HbCO) are unable to transport oxygen, while HbO$_2$ is the active Hb. When erythrocytes reach the end of their life due to aging or defects, Hb molecule is broken up and the iron gets recycled. When the porphyrin ring is broken up, the fragments are normally secreted in liver bile. This process also produces one carbon monoxide (CO) molecule for every heme molecule degraded (Hardison, 1996); this is responsible for the normal blood levels of CO and HbCO. This may explain the higher HbCO levels that accompany the higher hemolysis rate after Cd-treatment of rats. When the iron atom is in the ferrous form, the protein is active and can bind oxygen reversibly. The oxidation to the ferric form (metHb) leads to an in-active protein. Methemoglobin is unable to carry oxygen. High oxidative stress in red blood cells of cadmium exposed animals can account for the increase in metHb% produced through HbO$_2$-autoxidation reactions (Waltkins et al., 1985) and its improvement after treatment with ginger can account for the decrease in metHb% and increase in HbO$_2$% observed in the present study.

Cadmium exposure is known to cause interference with antioxidant enzymes, inhibition of energy metabolism, membrane damage, altered gene expression, and apoptosis (Habeebu et al., 1998; Ikediobi et al., 2004). Another target is erythrocytes, which contain hemoglobin that transports oxygen and carbon dioxide between the lungs and all tissues of the body. After exposure, Cd enters the blood, binds the erythrocyte membrane, and stimulates formation of the ROS (Bauman et al., 1993), leading to alterations in the antioxidant system of erythrocytes and imposing oxidative damage upon the membrane (Sarkar et al., 1995; Sarkar et al., 1997; Sarkar et al., 1998; Simpkins et al., 1998). Anemia is one of the characteristic clinical manifestations of chronic Cd intoxication (Horiguchi et al., 1996) as Cd is known to reduce red blood cell count and hematocrit value as well

Table 1. Effects of cadmium chloride and ginger on levels of inactive, active hemoglobins and total Hb concentration in blood of rats.

Parameter (%)	Control	Cadmium chloride	Cadmium chloride+ ginger
SHb	0.235±0.050	0.265±0.068	0.356±0.035
metHb	1.155±0.056	1.480±0.069[a]	1.029±0.074[b]
HbCO	2.418±0.056	2.816±0.041[a]	2.650±0.034
HbO_2	96.190±0.127	95.445±0.103[a]	95.965±0.097[b]
Hb (g/dl)	13.038±0.409	12.210±0.289[a]	13.546±0.210[a,c]

Data are presented as mean ± S.E. [a] significantly different from control- $P < 0.05$. [b] significantly different from cadmium alone treatment group- $P < 0.05$. [c] significantly different from cadmium alone treatment group- $P < 0.01$.

Table 2. Effects of cadmium chloride and ginger on concentrations of plasma MDA, GPx , GST, GR and G6PD activities in erythrocyte hemolysate in rats.

Parameter	Control	Cadmium chloride	Cadmium + ginger
MDA (nmol/ml)	9.9±0.453	10.407±0.360[a]	8.307±0.567[a,d]
GPx (mU/ml)	110.776±12.07	161.463±8.185[a]	187.185±11.766[b,c]
GST (U/L)	456.622±21.443	516.986±101.29	424.753±41.915
GR (U/L)	11.856±0.214	11.883±0.365	12.165±0.221
G6PD (U/g Hb)	8.775±0.359	8.511±0.615	9.793±0.763[a,c]

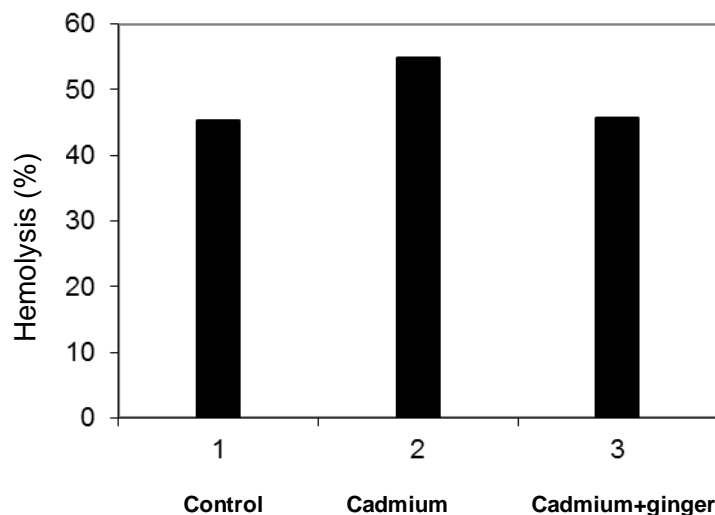Data are presented as mean ± S.E [a] significantly different from control- $P < 0.05$.
[b] significantly different from control- $P < 0.01$. [c] significantly different from cadmium alone treatment group- $P < 0.05$. [d] significantly different from cadmium alone treatment group- $P < 0.01$.

as hemoglobin concentration (Ognjanović et al., 2003; Onwuka et al., 2010). The reduction in Hb content may be due to increased rate of destruction or reduction in the rate of formation of erythrocytes. Long-term Cd exposure induces reduction in the rate of formation of erythrocytes in rats through hypo-induction of erythropoietin in the kidneys (Horiguchi et al., 1996). In addition, the reduction in Hb content may be attributed to hyperactivity of bone marrow that leads to production of erythrocytes with impaired integrity that are easily destructed in the circulation (Tung et al., 1975).

This study showed that erythrocytes hemolysis in Cd treated animals is higher than controls. These results are consistent with a previous study (Kanter et al., 2009). This high rate of Cd-induced hemolysis decreases by 17% after ginger treatment. The high rate of hemolysis of cadmium exposed rats can account for the low Hb concentration induced by Cd treatment. On the other hand, the significant decrease in the rate of hemolysis of ginger treated animals can account for the markedly increased concentration of total Hb induced by ginger treatment of Cd exposed rats. Previous results showed that ginger treatment of Cd-exposed rats significantly improved hematological indices (Onwuka et al., 2011). Ginger contains polyphenol compounds (6-gingerol and its derivatives), which have a high antioxidant activity (Chen et al., 1986; Herrman, 1994). This high antioxidant activity of ginger can account for the decrease in lipid peroxidation and erythrocyte hemolysis after ginger treatment of Cd- exposed rats, observed in this study.

The results obtained in our present study show that treatment with Cd, induces an increase level of lipid peroxidation product, MDA, in the blood of rats, which were accompanied by increased formation of ROS (Ognjanović et al., 2003; Sinha et al., 2008; Kanter et al., 2009). As a consequence of enhanced lipid peroxidation, DNA damage, altered calcium and sulfhydryl homeostasis as well as marked marker disturbances of antioxidant defense system occurred (Hiruku and Kawanishi, 1996). Treatment with ginger was effective in decreasing oxidative damage induced by Cd which resulted in markedly lower MDA concentration. Ginger was capable of inhibiting formation of ROS which caused hemolysis, through its high antioxidant activity (Chen et al., 1986; Kikuzaki and Nakatani, 1993; Herrman, 1994; Masuda et al., 2004). It is assumed that except of therapeutic intervention by using potent chelating agents capable to mobilize intracellularly bound Cd (Eybl et al., 1984; Jones and Cherian, 1990), ginger as antioxidants may be important components of an effective Cd intoxication treatment. The inhibitory effect of ginger on lead-induced lipid peroxidation in blood was demonstrated by Attia et al. (2013), who suggested that ginger provided a protection against lipid peroxidation and hemolysis of RBCs induced by lead treatment of rats.

Marked alterations of antioxidant system were found in erythrocytes of Cd-treated rats. In animals exposed to Cd, the activity of GPx in RBC was significantly increased (Table 2). These results are consistent with previous studies demonstrating that Cd induced an increase in SOD

Figure 1. Percentages of erythrocytes hemolysis in all groups. Hemolysis test was carried out after incubation of erythrocytes in phosphate buffered saline (PBS), pH 7.4, for 3 days at room temperature. [a] significantly different from control- $P < 0.05$, [b] significantly different from cadmium alone treatment group- $P < 0.05$.

and GPx activities in erythrocytes of Cd-exposed rats (Zikič et al., 2001; Ognjanovič et al., 2003; Kanter et al., 2005; Kanter et al., 2009; Attia et al., 2014). It is known that Cd induces the formation of superoxide anion radicals in erythrocytes and it is reasonable to expect an increased activity of SOD. Cadmium induced an increase in GPx activity which may be explained by their influence on hydrogen peroxide as substrate which is formed in the process of dismutation of superoxide anion radicals (Shaikh et al., 1999). The treatment with ginger of Cd exposed rats caused an increased GPx activity, indicating that this substance eliminates the toxic effects of Cd on the activity of this enzyme. The treatment with antioxidants helped to elevate the GPx activity and erythrocyte GSH content (Sinha et al., 2008). In erythrocytes and other tissues, the enzyme GPx, containing selenium as a prosthetic group, catalyzes the destruction of H_2O_2 and lipid hydroperoxides by GSH, protecting membrane lipids and hemoglobin against oxidation by peroxides (Murray et al., 1996). Thus, the increase in GPx activity induced by ginger treatment of Cd-exposed rats can account for the ginger-induced reduction of Hb oxidation and oxidative damage of RBC observed in this study. GPx is a preventive antioxidant, which reduces the rate of chain initiation of lipid peroxidation reaction (Murray et al., 1996). The increase in its activity can account for the decrease in the level of lipid peroxidation product, MDA, in the blood of rats induced by ginger treatment.

Preclinical studies carried out in the past decade have shown that ginger possesses cytoprotective effects against diverse xenobiotics such as alcohol, acetaminophen, fungicides, heavy metals, and organophosphorus compounds. The protective actions are shown to be mediated through its free radical-scavenging antioxidant, cytoprotective, and anti-inflammatory effects. In contrast to the results of this study, ginger was shown to induce the detoxifying enzymes GST in the presence of xenobiotics, thus offering protection to the liver (Shivashankara et al., 2013). GST catalyzes the conjugation of GSH, via a sulfhydryl group, to electrophilic centers on a wide variety of substrates in order to make the compounds more soluble (Douglas, 1987; Oakley, 2011). This activity detoxifies endogenous compounds such as lipid peroxides and also enables the breakdown of xenobiotics. GST efficiently conjugates the end products of lipid peroxidation (Leaver and George, 1998), reducing the level of MDA and thus contributing to the protection of the cell integrity (Grose et al., 1987; Ognjanović et al., 1995). GR catalyzes the reduction of GSSG to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell (Meister, 1988; Deponte, 2013). Therefore, the increase in GR activity can account for the ginger-induced reduction in oxidative stress, as observed in this study. G6PD is in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells (such as erythrocytes) by maintaining the level of the co-enzyme, nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turns maintains the level of GSH in these cells that helps protect the erythrocytes against oxidative damage, which causes acute hemolytic anemia. G6PD reduces nicotinamide adenine dinucleotide phosphate (NADP) to NADPH while oxidizing glucose-6-phosphate (Aster et al.,

2010). G6PD deficiency is very common worldwide, and causes acute hemolytic anemia (Cappellini and Fiorelli, 2008). Therefore, the increase in G6PD activity can account for the ginger-induced reduction in oxidative damage and hemolysis of erythrocytes, as observed in this study.

The high antioxidant activity of ginger is attributed to its high content of polyphenol compounds (6-gingerol and its derivatives), which have a high antioxidant activity (Chen et al., 1986; Herrman, 1994). The isolated antioxidants of ginger are divided into two groups; gingerol related compounds and diarylheptanoids. The nonvolatile fraction of the dichloromethane extract of ginger rhizomes exhibited a strong antioxidant activity. The fraction was purified by chromatographic techniques to provide five gingerol related compounds and eight diarylheptanoids (Kikuzaki and Nakatani, 1993). Among them, 12 compounds exhibited higher antioxidant activity than α-tocopherol. The activity was probably dependent upon side chain structure and substitution patterns on the benzene ring. The oleoresin (gingerols and shogaols), responsible to the pungent flavor of ginger, varies from 4.0 to 7.5% and also possesses substantial antioxidant activity (Kikuzaki and Nakatani, 1993; Balachandran et al., 2006). Ginger is a strong anti-oxidant plant and may either mitigate or prevent generation of free radicals. The alcohol ginger extract inhibited the hydroxyl radicals ($^{.}$OH) 79.6% at 37 °C and 74.8% at 80 °C, which showed a higher antioxidant activity than quercetin (Stoilova et al., 2007). All major active ingredients of ginger such as zingerone, gingerdiol, zingibrene, gingerols and shogaols have antioxidant activity (Zancan et al., 2002; Min-Ji et al., 2012; Baliga et al., 2013). Zingerone scavenges superoxide anion radicals ($O_2^{.-}$). 6-gingerol and zingerone are reported to be good scavengers of peroxyl radicals (ROO$^{.}$). 6-shogaol also inhibited the production of nitric oxide (NO). 6-Gingerol is the major bioactive constituent responsible for the antiinflammatory, antitumour and antioxidant activities of ginger (Nagendra et al., 2013).

Conclusion

It can be concluded from the presented results that cadmium induced oxidative damage in erythrocytes, leading to loss of membrane function by enhanced lipid peroxidation as well as alteration of the activity of GPx antioxidant enzyme. Moreover, the results of multi-component spectrophotometric analysis showed an increase in the level of inactive methemoglobin (metHb) and a decrease in HbO$_2$% and total Hb concentration. Ginger expressed protective role against toxic influence of cadmium on all affected parameters in rats. Ginger may exert its protective actions against cadmium-induced hematotoxicity in rats possibly through its antioxidant mechanisms. The results raise the possibility of ginger being considered as one of the component of the regular diet of the people in the areas, where they may have

chances of exposure to cadmium occupationally or environmentally.

Conflict of interest

Authors declare no conflict of interest.

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Full Length Research paper

Phytochemical screening and hypoglycemic effect of methanolic fruit pulp extract of *Cucumis sativus* in alloxan induced diabetic rats

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Phytochemical screening and hypoglycemic effect of methanolic fruit pulp extract of *Cucumis sativus* on alloxan induced diabetic rats was investigated. The phytochemical analysis of the methanolic fruit pulp extract of *C. sativus* indicated the presence of saponins, glycosides, terpenes, phenolics, alkaloids, flavonoids, and tannins. The methanolic fruit pulp extract of *C. sativus* was administered orally to the alloxan induced diabetic rats and it significantly decreased (P<0.05) the fasting blood glucose concentration (mg/dl) from 231.25±1.11 to 82.25±1.55 at 500 mg/kg body weight. The standard antidiabetic drug (Glibenclamide) administered orally at 5 mg/kg body weight also significantly decreased (P<0.05) blood glucose concentration from 189.00±2.42 to 61.00±2.48. This study therefore revealed that the methanolic fruit pulp extract of *C. sativus* contains active substances with hypoglycemic activity and could be used in the treatment and management of diabetes mellitus.

Key words: *Cucumis sativus*, hypoglycemic activity, phytochemical screening, alloxan, methanolic.

INTRODUCTION

Diabetes mellitus is a heterogenous group of chronic disorders of carbohydrate, lipid and protein metabolism characterized by high blood glucose levels due to relative or absolute deficiency of insulin (Eiselein et al., 2004).

Diabetes mellitus affects more than 100 million people worldwide and the number of people with diabetes is increasing due to population growth, aging and increasing prevalence of obesity and physical inactivity (Nair et al., 2006). In 2010, World Health Organization (WHO) estimated that 285 million people were living with diabetes (corresponding to 6.4% of the world's adult population). About 7 million people develop the disease each year and 3.9 million deaths were attributed to diabetes yearly (Shaw et al., 2010). Current predictions estimate that the prevalence of diabetes will reach 438 million by 2030 (corresponding to 7.8% of the adult population) and that 80% of prevalent cases will occur in the developing world (Roglic and Unwin, 2010).

Phytochemicals are substances found in edible fruits and vegetables that exhibit a potential for modulating human metabolism in a manner beneficial for the prevention of chronic and degenerative diseases (Tripoli

et al., 2007). Plants usually produce such low-molecular-weight ingredients for their protection against pests and diseases, for the regulation of their growth, or as pigments, essence, or odor. Scientists have identified thousands of phytochemicals, including flavonoids, anthraquinone, glucosinolates (isothiocyanates and indoles), phenolic acids, phytates, and alkaloids in vegetables, fruits, grains, legumes, and other plant sources (Perez et al., 2006).

Some phytochemicals in diverse plants have beneficial health effects such as anti-obesity, lipid-lowering, and antidiabetic properties. The vast study done on cucumber proved that the plant has many important phytoconstituents like glycosides, flavonoids, terpenes, sterol, saponins, and tannins (Kren and Ludmila, 2001). These compounds were found to be responsible for the pharmacological activity (Jony et al., 2013). Terpenoids are known to possess medicinal potency against inflammation, cancer, malaria, cholesterol synthesis inhibition, viral and bacterial agents (Mahato and Sen, 1997). Also, the alkaloids are used as anaesthetic agent. Although, variations have been noticed in the leaf alkaloid content of androgenic diploid plants of *Datura innoxia* (Herouart et al., 1988, Kukreja and Maclaren, 1999). Phenolic compounds are known to decrease cholesterol and triglyceride levels in rats (Gutierrez-Lugo et al., 1996, Arguetta, 1994). The tannins are known to inhibit microbial activities, the mechanism is based on its ability to bind proteins thereby inhibiting cell protein synthesis (Ayepola and Adeniji, 2008). The treatment of diabetes with synthetic drugs many years back has not led to any proper drug for the treatment of diabetes, because they are not affordable and have side effects (Sharmin et al., 2013). For this reason, traditional medicinal practitioners have described a number of plants used as complementary herbal antidiabetic drugs, mostly with less dangerous side effects and low cost (Singh et al., 2009). The hypoglycemic effect of several of such plants belonging to families Leguminoseae, Cucurbitaceae, Asteraceae, Moraceae, Rosaceae, Euphorbiacea and Araliaceae (Bnouham et al., 2006) has been confirmed, and the mechanisms of hypoglycemic activity of these new bioactive drug constituents are also being studied (Jung et al., 2006). Plant possesses various phytoconstituents which seems to be the active hypoglycemic principles, suggests different sites of action within the body. Most of the researches are carried out to evaluate the therapeutic effect of the plants along with their mode of action (Colaguiri, 2010). Plants show antidiabetic activity with various mechanism, like alteration of glucose metabolism, insulin-like effect, improve glucose tolerance, reduction of absorption of glucose from intestine, enhancing insulin signal pathway, hypoglycemia through increase glucose uptake and glycogen synthesis, generation of beta cell in pancreas (Banshidhar and Deepmala, 2013).

Cucumber is a widely cultivated plant in the gourd family Cucumbitaceae. It is a creeping vine that bears cylindrical fruits that are used as culinary vegetables. Within the varieties of cucumber, several different cultivars emerged. The plant has large leaves that form a canopy over the fruit. They have an enclosed seed, hence they are classified as accessory fruits. Much like tomatoes and squash, they are prepared and eaten as vegetables (Tindall, 1975).

Several plants of the Cucurbitaceae family are established for their hypoglycemic properties. Cucumber (*Cucumis sativus*) originated in India, but now cultivated in different parts of the world. In Mexico, cucumber is one of the edible plants with hypoglycemic potentials. The plant part in use currently against diabetes includes the seeds, pulp and the fruit itself. Antihypoglycemic study of this plant was studied in healthy rabbits which significantly lowered the blood glucose level (Stano et al., 2002). In addition, the plant has medicinal potency against hypercholesterolemia.

C. sativus is an important medicinal plant with diverse pharmacological activities such as antibacterial, antifungal, antidiabetic, cytotoxic, antacid and carminative activity, hepatoprotective activity, wound healing activities; hence, this plant provides a significant role in the prevention and treatment of a disease (Jony et al., 2013). This study will therefore determine the phytoconstituents and hypoglycemic effect of the methanolic extract of *C. sativus* fruit pulp in alloxan induced albino rats.

MATERIALS AND METHODS

Apparatus

The apparatus used were glucometer (Accu-check Active-Mannheim Germany), glucose strips (Accu-check strips), Reflux extractor, water bath and blender.

Sample collection and preparation

C. sativus was purchased in January from old market in Lokoja, Kogi State. The collected sample was washed, cut into small pieces, dried completely under the controlled mild sun temperature of 25°C and ground with an electric grinder. 50 grams of the powdered fruit pulp of *C. sativus* was weighed and 400 ml of methanol was measured in a round bottom flask and placed into a reflux extractor at 65°C for 3 h after which it was filtered through filter paper (Whatman filter paper no. 1). The methanolic solution was allowed to evaporate using water bath until dried extract was obtained (Sokeng, 2007).

Chemicals and reagents

The chemical used were methanol (AR), alloxan, chloroform, Wagners reagent and acetic anhydride.

Experimental animals

Albino rats of either sexes weighing between 130 and 220 g were

housed in plastic cage and the rats were acclimatized in the laboratory for a period of two weeks with adequate food and water.

Experimental design

Sixteen rats were randomly grouped into four groups with four rats in each group and designated as:
Group 1: Non induced; Group 2: Alloxan-induced diabetic rats not treated; Group 3: Alloxan- induced diabetic rats treated with 500 mg/kg body weight of extract; Group 4: Alloxan-induced diabetic rats treated with glibenclimide. All the treatments were administered in a suitable vehicle of 1% dimethyl sulphoxide (DMSO).

Experimental rats' induction

The fasting blood glucose concentration (mg/dl) of the rats was tested before induction, then a prepared solution of alloxan monohydrate (100 mg/kg body weight) was administered intraperitoneally into groups 2, 3 and 4. After two days interval of induction, the rats were tested and rats with blood glucose levels above 150 mg/dl (60% of the rats) were used to confirm diabetes. Treatment with extract and glibenclamide followed for a period of twenty one days (Adeneye and Agbaje, 2008).

Phytochemical screening

The *C. sativus* fruit pulp extract was screened for phytochemical properties by Trease (1983) and Sofowara (1983).

Anthraquinone test

Five milliliters of the extract was added to 10 ml of benzene. Five milliliters of 10% $NH_{3(aq)}$ was added and mixed. The presence of anthraquinone is indicated by a pink/violet color in the ammonia phase at the bottom of the test tube (Sofowara, 1983).

Alkaloid test

Two milliliters of the extract was treated with 10 ml of 1% HCL in water bath for 30 min. The solution was then treated with few drops of Wagners reagent and the colour change was observed. The presence of alkaloid is indicated with precipitate formation (Sofowora, 1983).

Cardiac glycoside test

Two milliliters of chloroform was used to dilute 0.5 ml of extract. Sulphuric acid was carefully added to the solution drop wise. At the chloroform/sulphuric acid interphase, a reddish coloration indicates the presence of cardiac glycosides (Trease, 1983).

Saponin test

0.5 ml of extract was mixed with 10 ml of distilled water. Frothing which persist on warming of the test tube confirms the preliminary evidence for the presence of saponin (Sofowora, 1983).

Steroid test

Five drops of concentrated sulphuric acid were added to 2 ml of the extract. A reddish brown coloration indicates the presence of steroids (Trease, 1983).

Phenol test

Two milliliters of extract was added to 2 ml of ferric chloride solution. A deep bluish green solution was formed with the presence of phenol (Sofowora, 1983).

Tannin test

Three milliliters of the extract was added to 5 ml of distilled water and then heated in water bath. To this solution, $FeCl_3$ solution was added. A blue-black or green precipitate indicated the presence of tannins (Trease, 1983).

Flavonoid test

One milliliter of the extract was added to 5 ml of distilled water and then filtered to obtain 2 ml of the filtrate. A few drops of 10% ferric chloride solution were added, blue-violet coloration was an indication of the presence of flavonoid (Trease, 1983).

Terpene test

One milliliter of the extract was added to 5 ml of chloroform in a test tube. 3 ml of acetic anhydride was added to the chloroform-extract to which 2 ml of concentrated sulphuric acid was also added. The formation of a ring at the interphase between the two immiscible liquids is a preliminary evidence of the presence of terpenes (Trease, 1983).

Oral administration of plant extract

500 mg/kg body weight of the extract was administered to group 3 alloxan induced diabetic rats and 5 mg/kg body weight of standard drug (glibenclamide) to group 4 alloxan-induced diabetic rats once each day.

Determination of fasting blood glucose level

The fasting blood glucose was determined using glucometer kit (accucheck) after overnight fasting for about 8 to 10 h. The tail was punctured and the blood from the tail was dropped on the strip which had been inserted into the glucometer to obtain the blood glucose concentration in mg/dl for each rat in all the groups at an interval of days 0, 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21.

Statistical analysis of results

Statistical comparisons were performed by one-way analysis of variance (ANOVA), and Dunnett's t-test for comparison. Results were considered significant when p values were less than 0.05 ($p < 0.05$).

RESULTS

The results are as shown in Table 1 and Figure 1, respectively. Table 1 shows the phytoconstituents of methanolic extract of *C. sativus* fruit pulp and Figure 1

Table 1. Phytoconstituents of methanolic extract of *Cucumis sativus* fruit pulp.

Compound	Inference
Anthraquinones	-
Alkaloids	+
Cardiac glycosides	+++
Saponins	+
Steroids	-
Phenols	++
Tannins	+
Flavonoids	+
Terpenes	+++

+: Slightly present, ++: Moderately present, +++: Highly present, -:Not detected.
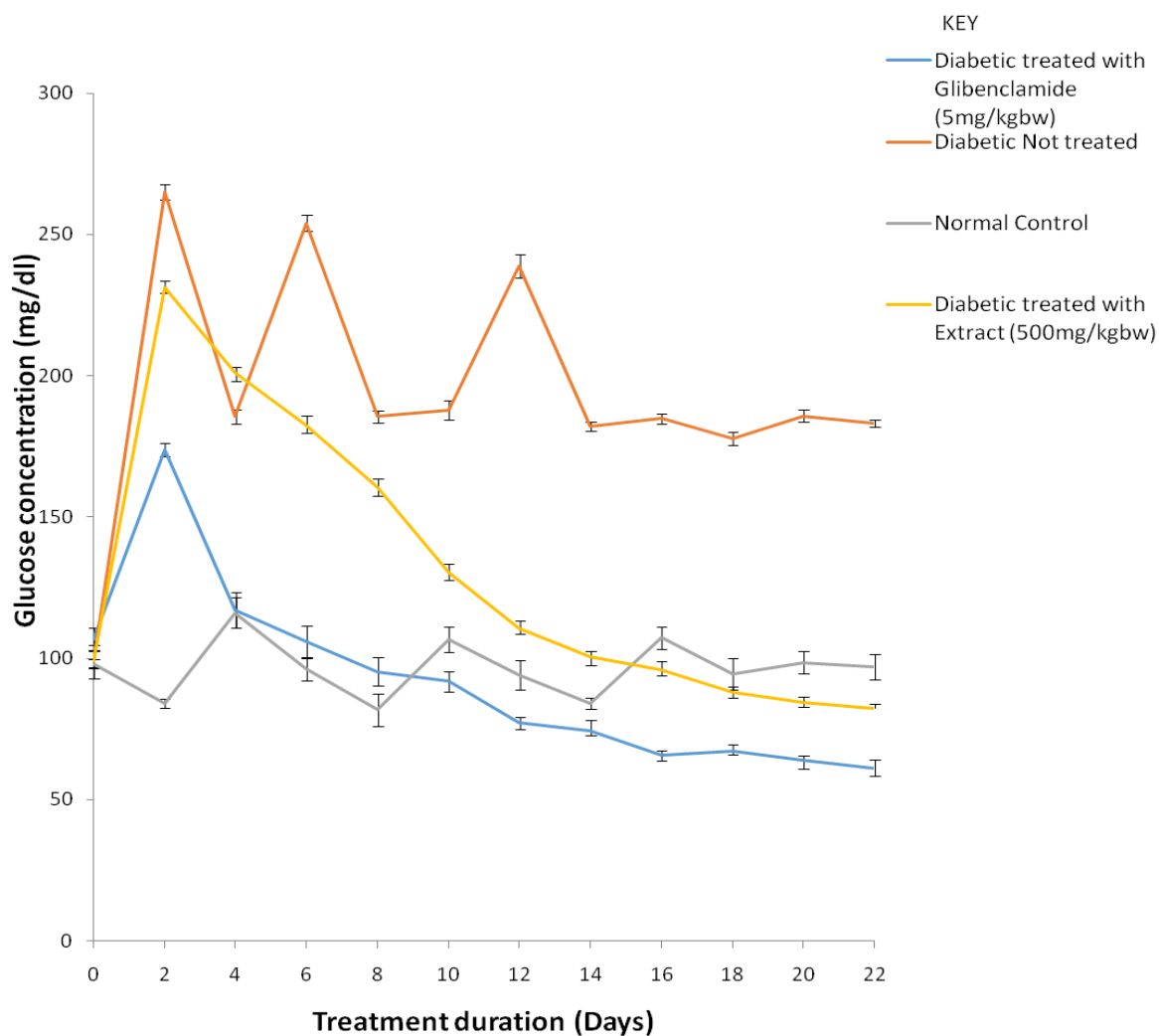
Figure 1. Fasting blood glucose concentration (mg/dl) of diabetic rats not treated and non induced, treated with extract and standard drug.

shows the fasting blood glucose levels of diabetic rats not treated and non induced, treated with extract and standard drug.

DISCUSSION

Table 1 reveals that the phytochemical analysis of

methanolic fruit pulp extract of *C. sativus* contains important phytoconstituents like tannins, saponins, terpenes, glycosides, alkaloids, flavonoids and phenols, while anthraquinone and steroids are absent. The seed extracts of *C. sativus* have also been reported to contain these phytochemicals (Ankita et al., 2012). Flavonoid and tannins have been reported to cause regeneration of damaged pancreatic islets, stimulate calcium and glucose uptake (Tapas et al., 2008, Kumar and Clark, 2002). These compounds are known to be responsible for the hypoglycemic activity of the plant as compared with other hypoglycemic plants which contains similar phytoconstituent found in *Luffa acutangula* fruit extract (Pimple et al., 2011) and methanolic root bark extract of *Acacia albida* (Salisu et al., 2009). It has been reported that *C. sativus* seeds are found as suitable food for medicinal purposes against some diseases such as diabetes, hyperlipidemia, hypertension, gall bladder stones, constipation, dyspepsia in Asian traditional remedies (Trease and Evans, 2002; Roman-Romos et al., 1995; Amin, 2005).

The results in Figure 1 reveals that oral administration of 500 mg/kg body weight of *C. sativus* methanolic fruit pulp extract caused a significant decrease (p<0.05) in fasting blood glucose concentration of alloxan-induced diabetic rats from 231.25±1.11 to 82.25±1.55 and oral administration of 5 mg/kg body weight of standard drug (glibenclamide) caused a significant decrease (p<0.05) in fasting blood glucose concentration of alloxan-induced diabetic rats from 189.00±2.42 to 61.00±2.48, respectively, while the induced not treated remained hyperglycemic from 265.00±2.86 to 183.00±1.30 and the non induced from 98.00±1.47 to 97.00±4.52 after twenty one days. It has been reported that fractions of *C. sativus* seed extract were effective to cause hypoglycemia in normal group even after prolonged treatment during sub-acute phase of the study.

This corroborated the findings by Chandrasekar et al. (1989) who investigated blood glucose lowering effect of eight plants of Cucumbitaceae family including *C. sativus* fruit extract. This study suggests that the methanolic extract of *C. sativus* fruit pulp has hypoglycemic effect by reducing fasting blood glucose concentration and could be as effective as glibenclamide used as hypoglycemic standard drug.

This study reveals similar hypoglycemic effects with the ethanol extracts of Cucurbitaceae family fruits (Sharmin et al., 2013) and *Viscum album* extract (Shahaboddin et al., 2011) on alloxan-induced diabetic rats.

Conclusion

From this study, it can be concluded that the methanolic fruit pulp extract of *C. sativus* at 500 mg/kg body weight is an active hypoglycemic remedy and may be used in the treatment and management of diabetes mellitus since it has been demonstrated in rats. However, further

research should be carried out to uncover the precise mechanism of action.

Conflict of Interest

The authors declare no conflict of interest.

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Full Length Research Paper

Antioxidant activity of leaf and fruit extracts of Jordanian *Rubus sanguineus* Friv. (Rosaceae)

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The *in vitro* antioxidant activity of crude ethanolic, methanolic and aqueous extracts of the leaf and fruit of *Rubus sanguineus* were investigated. The antioxidant activities were performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, nitrogen oxide (NO) scavenging, hydroxyl radical scavenging and lipid peroxidation. The methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruit demonstrated higher antioxidant potentials than the methanolic, ethanolic and aqueous extracts of its leaves in concentration-dependent manner. This study verified that the methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits and leaves have strong antioxidant activities which might be correlated with high level of phenolics and flavonoids. These extracts can be used as a source of potential antioxidant or or added to food materials to give it additional functions.

Key words: Antioxidant, *Rubus sanguineus*, lipid peroxidation, DPPH radical scavenging activity, NO scavenging activity, hydroxyl radical scavenging activity.

INTRODUCTION

Free radicals have a harmful effect by certain synthetic compounds having many side effects. This makes scientists to keep exploring natural sources of antioxidants with multifunctional potential as alternatives for toxic synthetic antioxidants, to avoid the metabolic pathways any oxidation (Orhan et al., 2012). The plant *Rubus sanguineus* Friv. (Rosaceae) that is also known as Holy Bramble or Blackberry, is a wild shrub with edible fruits found near river banks, by springs and swamps in the Mediterranean and West Irano-Turanian, extending into west Siberian regions (Zohary, 1972). It is called Ulaiq in Arabic. In Jordan, this plant is reputed traditionally for its use to treat different infections (Oran and Al-Eisawi, 1998). The species *Rubus sanguineus* has antimicrobial capacities that were studied by Zeidan et al. (2013). The antioxidant activity of *Rubus* from different species was evaluated many times with different techniques and showed different antioxidant effects

(God-evac et al., 2009; Ska et al., 2009; Liu et al., 2005; Gao et al., 2011). This study is aimed to assess the antioxidant potentials of Rubus sanguineus.

MATERIALS AND METHODS

Plant

The plant leaves and fruits of *R. sanguineus* were collected from different areas along Jordan valley and later identified by Sawsan Oran (Professor of Plant Biosystematics at the University of Jordan, Faculty of Science, Department of Biological sciences, Amman, Jordan).

Preparation of plant extracts

Collected plant materials (leaves and fruits separated) were air dried for approximately two weeks. Dried plant samples were grounded using a grinder (Ambar, Liban) and then 50 g of the dried powdered plant were soaked separately in 1 L of ethanol and methanol. After soaking for two weeks, they were filtered using Whattman no. 1 filter paper. All filtrates were evaporated using rotary evaporator (Janke & Kunkel, Germany) and left to dry at room temperature for 24 h and weighed. The air dried stock extracts were then reconstituted in 25% dimethylsulphoxide (DMSO) solution to get 25 mg/ml concentrations and sterilized by filtration (mini pore filter 0.22 µm) and stored in refrigerator at 4°C prior to determination of antimicrobial activities of the extracts (Othman et al., 2011; Rawani et al., 2011). Simple thin layer chromatography (TLC) experiment was done and found that *Rubus sanguineus* has flavonoids, terpenes and alkaloids that appear as bands.

DPPH radical scavenging activity assay

The free radical scavenging activity of the plant extracts was examined using the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) by the method described by Chan et al. (2007) with slight modification. Different dilutions of the plant extracts (2, 5, 8, 10, and 15 mg/ml) were prepared. DPPH solution was also prepared by dissolving 6.0 mg of DPPH in 100 ml methanol. Then, 1 ml of extract from each dilution was added into the test tube containing 2 ml of DPPH solution. Control was prepared by adding 1 ml of methanol to 2 ml of DPPH solution. Ascorbic acid was used as standard. The mixture was shaken vigorously and was left to stand in the dark for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. This wavelength enabled the measurements of the stable free DPPH radical without interference. The scavenging activity of each extract on DPPH radical was calculated using the following equation [1 - (A1) / A0] × 100, where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard.

Nitric oxide radical inhibition assay

Nitric oxide radical inhibition was estimated using Griess Illosvory reaction. In this experiment, Griess Illosvory modified reagent was used. The reaction mixture contain 2 ml of 10 mM sodium nitroprusside (SNP), 0.5 ml saline phosphate buffer and 0.5 ml of standard solution or methanolic and ethanolic fruit and leaves extracts (2, 5, 8, 10,15 mg/ml) with a total volume of 3 ml that were then incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of the modified Griess Illosvory and then allowed to stand for 30 min at 25°C. The concentration of nitrite was assayed at 546 nm and was calculated with the control absorbance of the standard nitrite solution (without extracts or standards, but the same condition should be followed). Buffers were used as blank solution and ascorbic acid, and were taken as standard solution (Green et al., 1982). The scavenging % was calculated as follows:

Nitric Oxide scavenged % = (A control – A test) / (A control) × 100

Hydroxyl radical scavenging assay

Scavenging of hydroxyl radical was measured using the 2-deoxyribose assay as reported by Halliwell (1987a, b) with some modification. All solutions were prepared freshly by mixing 100 µl of 2-deoxy-2-ribose (2.8 mM) that was dissolved in phosphate buffer saline (pH 7.4), 500 µl of methanolic and ethanolic extract of *Rubus* leaves and fruits (2, 5, 8, 10, 15 mg/ml), 100 µl of H_2O_2 (1 mM), 100 µl FeCl$_3$ (100 µM), 100 µl of EDTA (104 µM) and 100 µl of ascorbic acid (100 µM) to form the reaction mixture with a final volume of 1 ml. Then the solutions were incubated for one hour at 37°C. Then 1 ml of trichloroacetic acid (TCA) and 1 ml of thiobarbituric acid was added to the reaction mixture. The resulting mixture was heated for 15 min at 100°C. After cooling on ice, absorbance was measured at 532 nm (Awah et al., 2010). The scavenging (%) was calculated as follows:

Hydroxyl scavenged % = (A control – A test) / (A control) × 100

Lipid peroxidation assay

The lipid peroxidation test was done using peroxi detect sigma kit (PD1). The procedure is based on the fact that peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic pH. The Fe^{3+} ion will form a colored adduct with xylenol orange (XO, 3,3¢-bis[N,N bis(carboxymethyl)aminomethyl]-o-cresolsulfonephthalein, sodium salt), which is observed at 560 nm (Jiang et al., 1991). This kit use two concepts: First, determination of aqueous peroxides that is done by following the reaction scheme (Table 1) for the H_2O_2 standard curve by placing 0, 10, 20, 40, 60, and 80 ml of the 100 mM standard hydrogen peroxide solution in labeled tubes. The final volume in each tube was then brought to 100 ml with water. Then the tested sample was placed up to 100 ml in a test tube such as the H_2O_2 with a starting concentration of 15 mg/ml, then 1 ml of the working color reagent (prepared by mixing 100 volumes of aqueous peroxide color reagent with one volume of ferrous ammonium sulfate reagent) was added to each tube, mixed and incubated at room temperature (25°C) for ~30 min until color formation was completed. Then each standard and test sample was read at 560 nm in a spectrophotometer using water as a reference. Finally, a standard curve of nmoles of H_2O_2 against A560 and calculation of the nmoles of peroxide in the test sample was plotted in order to calculate the nmoles of peroxide in the test sample. Second, determination of organic or lipid hydroperoxides that is done by following the reaction scheme (Table 2) for the tert-butyl hydroperoxide standard curve by placing 0, 5, 10, 20, 40, 60, and 80 ml of the 200 mM tert-butyl hydroperoxide standard solution in

Table 1. Reaction scheme for H_2O_2 standard curve.

100 µM H_2O_2 Solution (µl)	nmoles per reaction volume
0	0.0
10	1.0
20	2.0
40	4.0
60	6.0
80	8.0

Table 2. Reaction scheme for tert-BuOOH standard curve.

200 µM t-BuOOH Solution (µl)	nmoles per reaction volume
0	0
5	1
10	2
20	4
40	8
60	12
80	16

labeled tubes. Then the final volume in each tube was brought to 100 ml with 90 or 100% methanol. The tested sample was placed up to 100 ml in a test tube such as the tert-BuOOH with a starting concentration of 15 mg/ml. Then 1 ml of the working color reagent (prepared by mixing 100 volumes of reconstituted organic peroxide color reagent with one volume of ferrous ammonium sulfate reagent) was added to each tube, mixed and incubated at room temperature (25°C) for ~30 min until color formation was

completed. Then each standard and sample was read at 560 nm in a spectrophotometer using 90 or 100% methanol as a reference. A standard curve of nmoles of t-BuOOH against A560 was plotted in order to calculate the nmoles of peroxide in the test sample. The calculations done using the following equation:

$$\text{nmole peroxide/ml} = \frac{[A560(\text{sample}) - A560(\text{blank})] \times \text{dilution factor}}{[A560(1 \text{ nmole peroxide})] \times \text{sample volumea}}$$

Dilution factor = factor for dilution of original sample; [A560 (1 nmole peroxide)] = calculated from H_2O_2 or t-BuOOH standard curve; sample volume = volume of sample (ml).

IC$_{50}$ calculations

To calculate the IC_{50}, a scatter graph in excel were plotted (where X axis is concentration and Y axis is % activity) then the slop equation for the graph ($Y = mx + c$ or $Y = mx - c$) was gotten. For IC_{50} value in equation $Y = 50$. M and C values were presented in the equation itself. The value of X will be IC_{50} value for that graph. IC_{50} was calculated according to cell biology protocols.

RESULTS

DPPH radical scavenging activity assay

In this present study, the antioxidant activity of the ethanolic, methanolic and aqueous extracts of *R. sanguineus* leaves and fruits were investigated using the DPPH scavenging assay. DPPH radical scavenging activity was observed with all the tested extracts (Figures 1 and 2), these tested extracts showed higher activity compared to ascorbic acid (Figure 3). There was more than 50% DPPH activity inhibition at 8 mg/ml extract

concentrations. The results also showed that DPPH activity inhibition at 10 mg/ml concentration exceeds 80%.

The highest DPPH activity was shown by the ethanolic and methanolic extract of *R. sanguineus* leaves at the concentration of 15 mg/ml that was about 99%. Similar percentage was recorded with the same concentration of fruit ethanolic extract of *R. sanguineus* whereas the methanolic fruit extract was at the same concentration, the DPPH scavenging was 95%. The lowest DPPH activity was shown by the aqueous leaf extract (83%) compared to that of the organic extracts at concentration of 15 mg/ml while the fruit extract caused higher DPPH activity (90%) than that of the leaf extract although this activity still less than that of the organic extracts and the ascorbic acid at equal concentration (Figures 3 and 4). As shown, all extracts showed different percentages of inhibition of the DPPH scavenging activity on the concentration-dependent approach. To support the results recorded from DPPH scavenging assay, the IC_{50} was calculated for each extract and then compared to standard ascorbic acid. As for the IC_{50} (Table 3), the lowest concentration was shown by *R. sanguineus* fruit ethanol extract, followed by fruit methanol extract and then ethanol leaf extract, with significant differences

Table 3. IC_{50} of *R. sanguineus* fruits and leaves extracts for DPPH assay.

Plant part	Extract	IC_{50}
	Methanol	0.42
Fruit	Ethanol	0.35
	Aqueous	4.88
	Methanol	5.08
Leaf	Ethanol	2.64
	Aqueous	7.93
Ascorbic acid	Standard	1.9

Table 4. IC_{50} of *R. sanguineus* fruits and leaves extracts for NO scavenging activity.

Plant part	Extract	IC50 mg/ml
	Methanol	17.6
Fruit	Ethanol	9.5
	Aqueous	5.9
	Methanol	6.7
Leaf	Ethanol	7.3
	Aqueous	7.8
Ascorbic acid	Standard	3.5

between the leaf and fruit extracts.

Nitric oxide radical inhibition assay

The nitric oxide scavenging activity of ethanolic, methanolic and aqueous extracts of *R. sanguineus* leaves and fruits is shown in Figures 4 and 5. The extracts possessed NO scavenging activity at concentrations ranging between 2 and 15 mg/ml. The highest NO scavenging activity was recorded for the ethanolic fruit extract of *R. sanguineus* giving 92% at 15 mg/ml concentration compared to the standard ascorbic acid 66% at the same concentration as shown in Figure 6. The ethanolic and methanolic leaf extracts of *R. sanguineus* had shown approximately nearby NO scavenging activity at the tested concentrations, while there were almost high differences between NO scavenging activities of the methanolic and ethanolic fruit extracts at the same concentrations. The aqueous extracts of both *R. sanguineus* leaf and fruit also possessed NO scavenging activity. The aqueous extracts of both *R. sanguineus* leaf and fruit possessed NO scavenging activity. Although the activity is higher than standard ascorbic acid, it was considered low as

compared to organic extracts at the same concentrations. The inhibition of NO scavenging activity by all extracts was observed to be concentration dependent. To support the results recorded from NO scavenging activity, the IC_{50} was calculated for each extract and then compared to the standard ascorbic acid (Table 4).

Hydroxyl radical scavenging assay

The antioxidant activity of ethanolic, methanolic and aqueous extracts of *R. sanguineus* leaves and fruits were tested also using Hydroxyl radical scavenging activity. The results are shown in Figures 7 and 8. The highest hydroxyl scavenging activity was recorded for the aqueous fruit extract of *R. sanguiuneus* (92%) at concentration of 15 mg/ml. Then the activity of methanolic fruit extract was 89% and the lowest was ethanolic fruit extract hydroxyl scavenging activity (82%).

Compared to the fruit extracts, the leaf extracts have a hydroxyl activity of about 88, 87 and 80% for methanolic, ethanolic, and aqueous extracts, respectively at 15 mg/ml concentration. To support the results recorded from NO scavenging activity, the IC_{50} was calculated for each extract (Table 5).

Table 5. IC_{50} of *R. sanguineus* fruits and leaves extracts for Hydroxyl radical scavenging activity.

Plant part	Extract	IC_{50} mg/ml
	Methanol	10.32
Fruit	Ethanol	6.09
	Aqueous	4.00
	Methanol	5.1
Leaf	Ethanol	3.3
	Aqueous	6.9
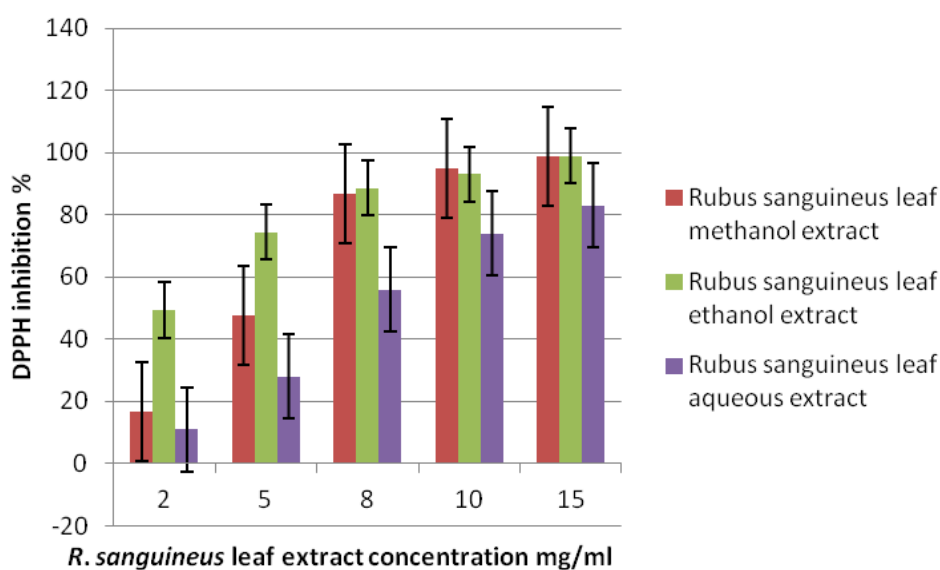
Figure 1. Inhibition % of DPPH by methanolic, ethanolic and aqueous extracts of *R. sanguineus* leaves.

Lipid peroxidation assay

The measurement of peroxides in biological systems is an important factor in determining the degree of free radicals present in specific tissues (Carone et al., 1993). According to the kit (Peroxi Detect kit, Sigma-Aldrich) used to evaluate the lipid peroxidation of the extracts, a standard curve (Figure 9) was plotted of nmoles of H_2O_2 against absorbance at 560 nm and the peroxide was detected in nmoles in the tested samples as described in Materials and Methods. Figures 10 and 11 showed the results of lipid peroxidation assay of *R. sanguineus* methanolic, ethanolic and aqueous leaves and fruit extracts that were measured to determine the aqueous peroxide in these extracts. As shown, the nmoles of aqueous peroxide decreases with the increase of the plant extract concentration. The ethanolic fruit extract showed the highest percentage of inhibition of the

peroxide in the reaction mixture with concentration of 12 mg/ml followed by the methanolic and then the aqueous extract of the fruit. Leaf extractions, however, showed a smaller amount of peroxide inhibition. For the determination of organic or lipid hydroperoxides a standard curve (Figure 12) of nmoles of t-BuOOH against absorbance at 560 nm standard curve was plotted in order to calculate the nmoles of peroxide in the tested samples. Figures 13 and 14 showed the results of lipid peroxidation assay of *R. sanguineus* methanolic, ethanolic and aqueous leaves and fruits extracts measured for determination of lipid or organic hydroperoxide. As shown in Figures 13 and 14, there were notable decreases in the nmoles of organic peroxides with the increase in the concentration of the tested extracts. The highest decrease in the nmoles of organic hydroperoxide was shown by the ethanolic extract of leaf followed by its methanolic and aqueous extracts at the concentration of 12 mg/ml. In contrast, the
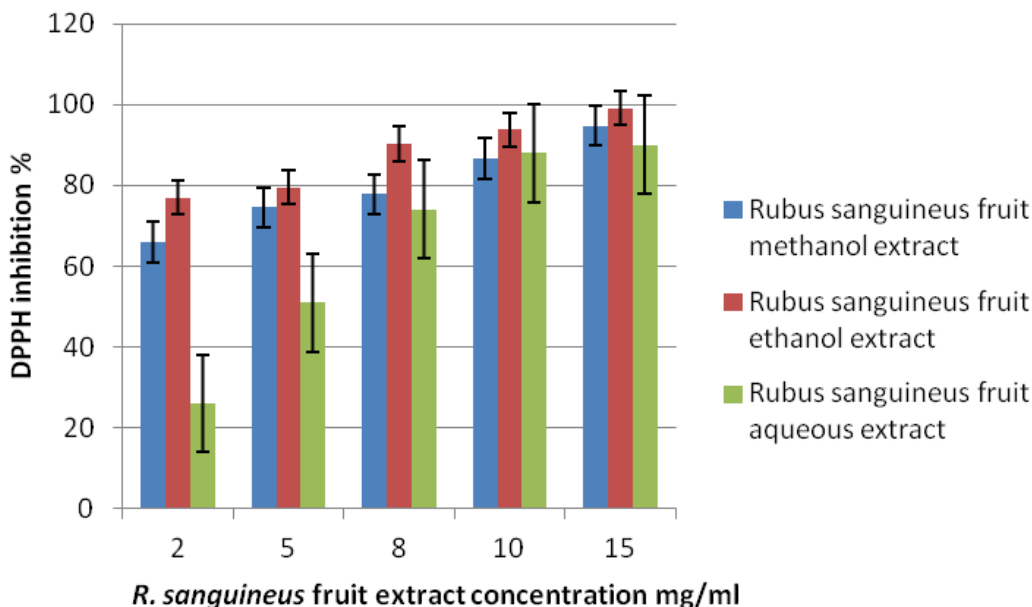
Figure 2. Inhibition % of DPPH by methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.
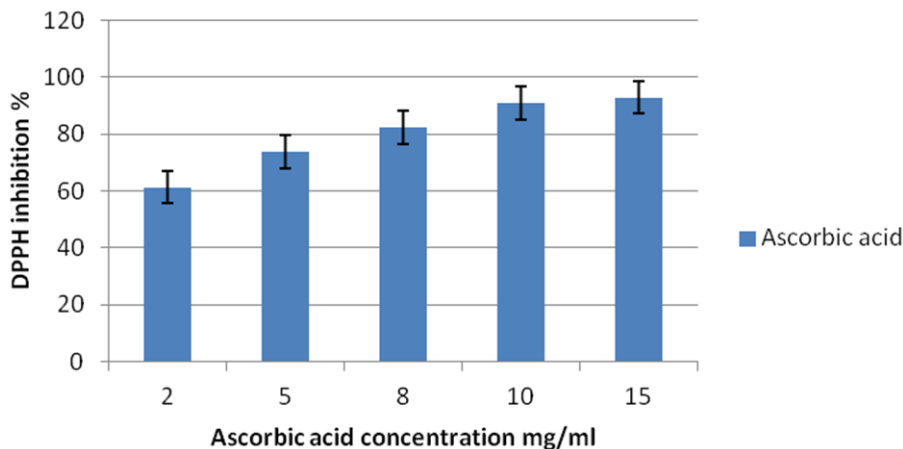

**Figure 3.** Inhibition of DPPH by ascorbic acid.

fruit ethanolic extract showed also a nearby value in decreasing the nmoles of organic hydroperoxide at 12 mg/ml concentration followed by the methanolic and then the aqueous extract of the fruit.

DISCUSSION

DPPH radical scavenging activity assay

The free radical scavenging activities of *R. sanguineus*

leaf and fruit extracts were investigated using DPPH assay (Figures 1 and 2). The results showed that the DPPH radical scavenging activities of *R. sanguineus* increased gradually as the concentration increased, also it could be observed that the complete inhibition was never reached. The explanation of the higher value of the DPPH radical scavenging activity found in the experiment was because the sample used was a crude extract. Decrease in absorbance of DPPH solution (that is, from purple to yellow) depends on intrinsic antioxidant activity of antioxidant as well as on speed of reaction between DPPH and antioxidant.
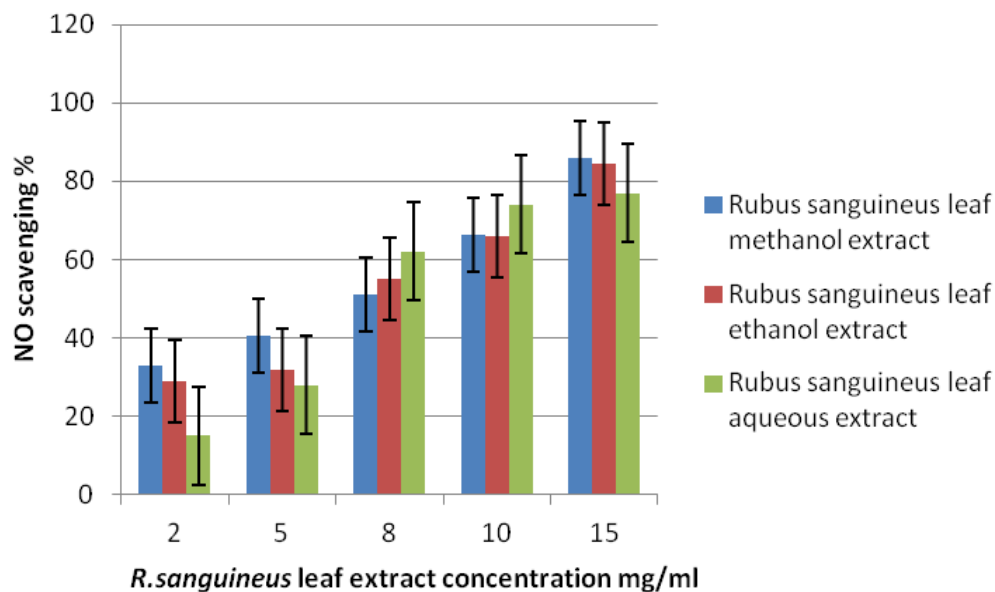
Figure 4. Nitric oxide (NO) scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* leaves.
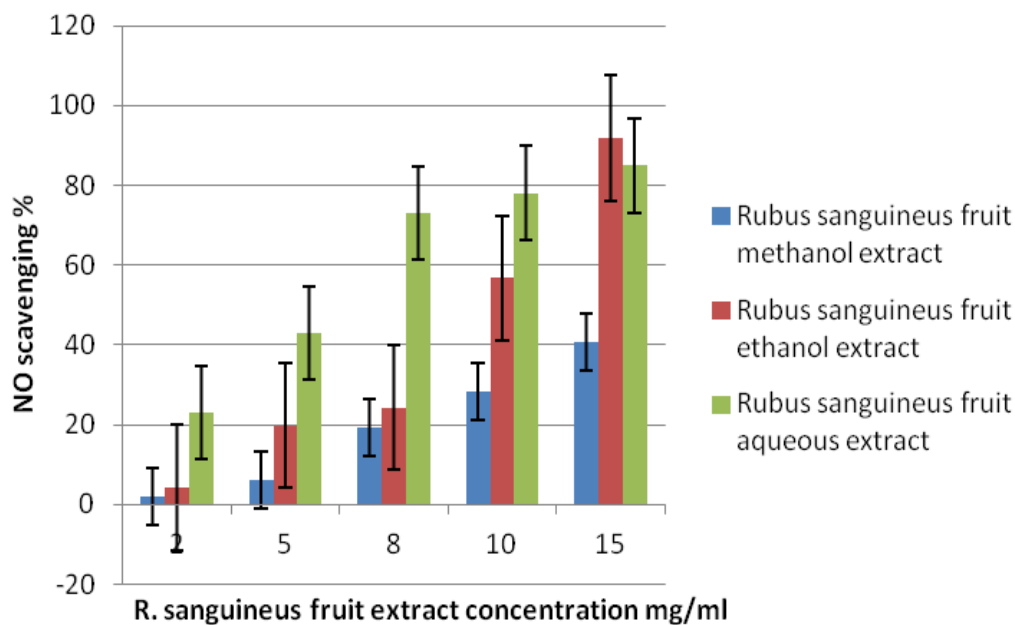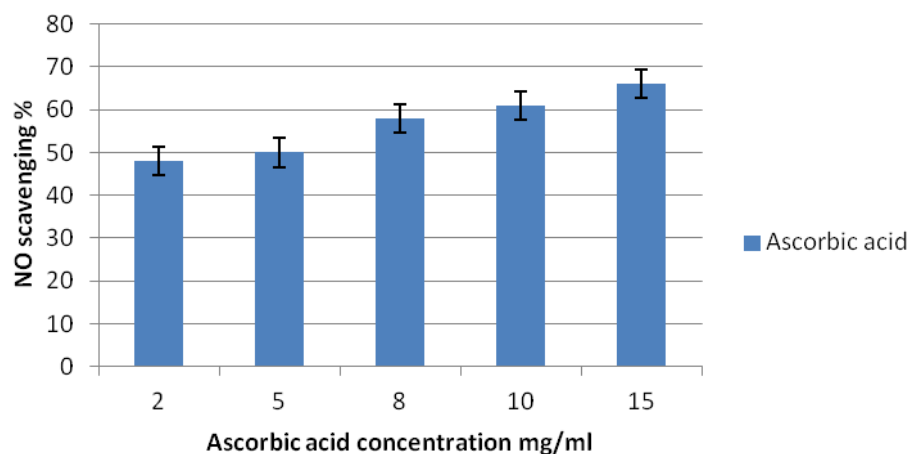

Figure 5. Nitric oxide (NO) scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.

Nitric oxide radical inhibition assay

This assay is based on the fact that sodium nitroprusside in an aqueous solution at a physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent (Ebrahimzadeh et al., 2010). In the presence of tested crude extracts, which are scavengers,

Figure 6. Nitric oxide (NO) scavenging activity of ascorbic acid.
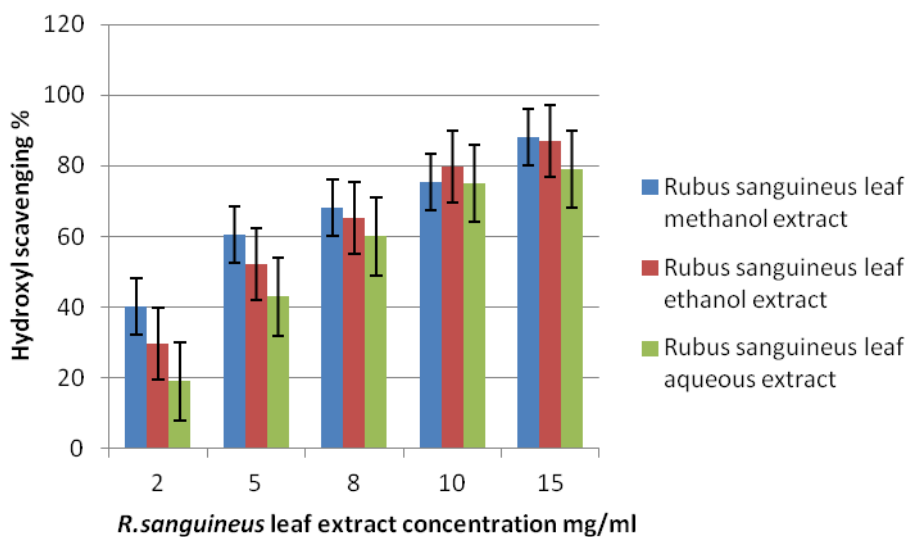
Figure 7. Hydroxyl scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* leaves.

the amount of nitrous acid decreased. The extent of decrease reflects the extent of scavenging. The percentage inhibition of methanolic, ethanolic and aqueous extracts of the leaves and fruits of *R. sanguineus* fruits and leaves are shown in Figures 4, 5 and 6. On the basis of results obtained, it may be concluded that *R. sanguineus* different crude extracts showed the potent radicals scavenging activity and metal ion chelating activity. The data also indicated that *R. sanguineus* extracts may contain phenolic compounds, anthocyanins and ascorbic acids, and appeared to be responsible for

their antioxidant activities.

Hydroxyl radical scavenging assay

Hydroxyl radicals (HO$^•$) are produced through the reductive decomposition of H_2O_2. HO$^•$ reacts with a large number of cellular components like pigments, proteins, lipids and DNA. This reaction can cause lipid peroxidation, DNA damage, protein modification and degradation (Cadenas, 1989; Halliwell, 1987a; b; Sudha
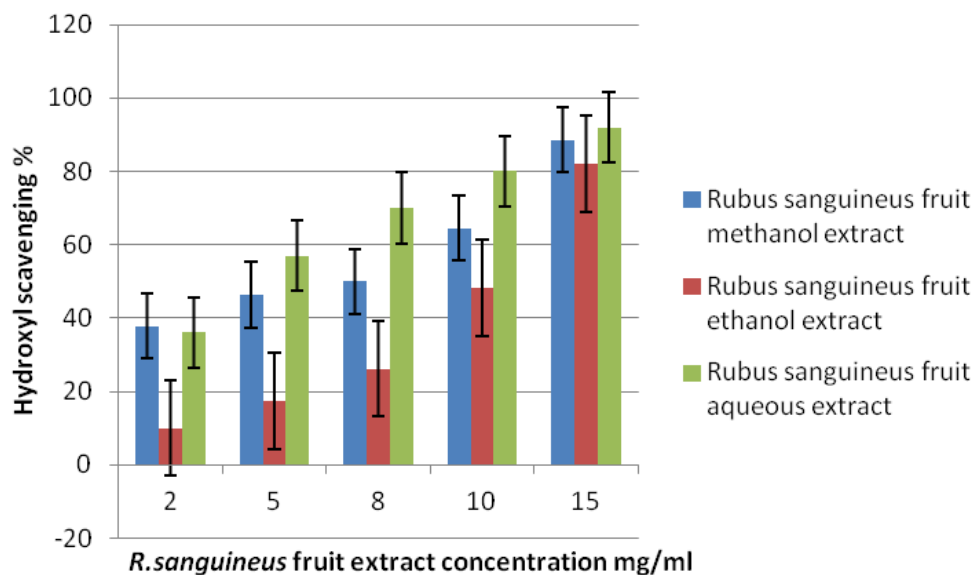
Figure 8. Hydroxyl scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.
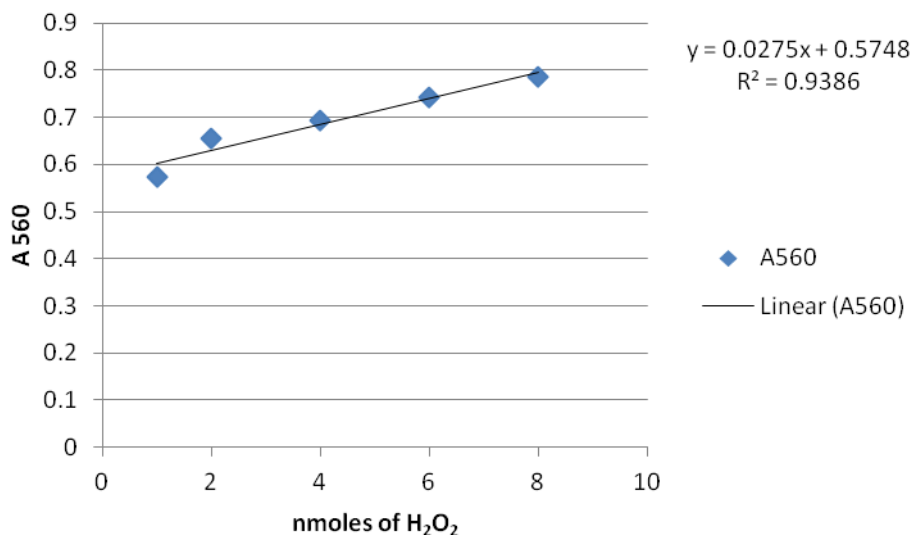
Figure 9. Standard curve of nmoles of H_2O_2 against absorbance at 560 nm.

were incubated with the mentioned reaction mixture, to determine whether they could reduce hydroxyl radical generation by determining the degree of deoxyribose degradation, an indicator of thiobarbituric acid–trichloroacetic acid (TBA–TCA) adduct formation. Hydroxyl radical are known to be capable of abstracting hydrogen atoms from membrane and bring about peroxidic reactions of lipids. From this point, it was expected that the *R. sanguineus* extracts demonstrate

the antioxidant effects against lipid peroxidation to scavenge the hydroxyl radicals and superoxide anions at the stage of initiation and termination of peroxyl radicals.

Lipid peroxidation assay

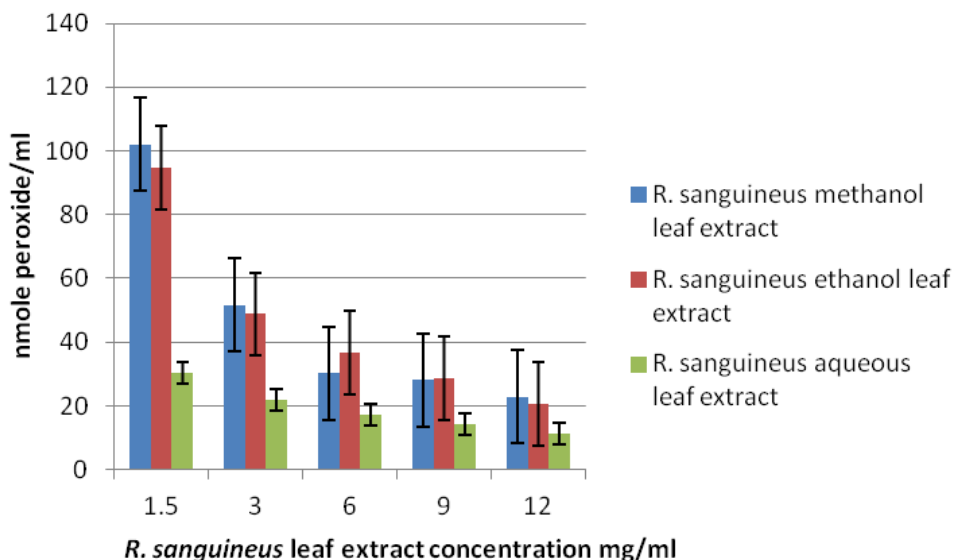Lipid peroxidation is a consequence of reactive oxygen species production as non-enzymatic peroxidation or

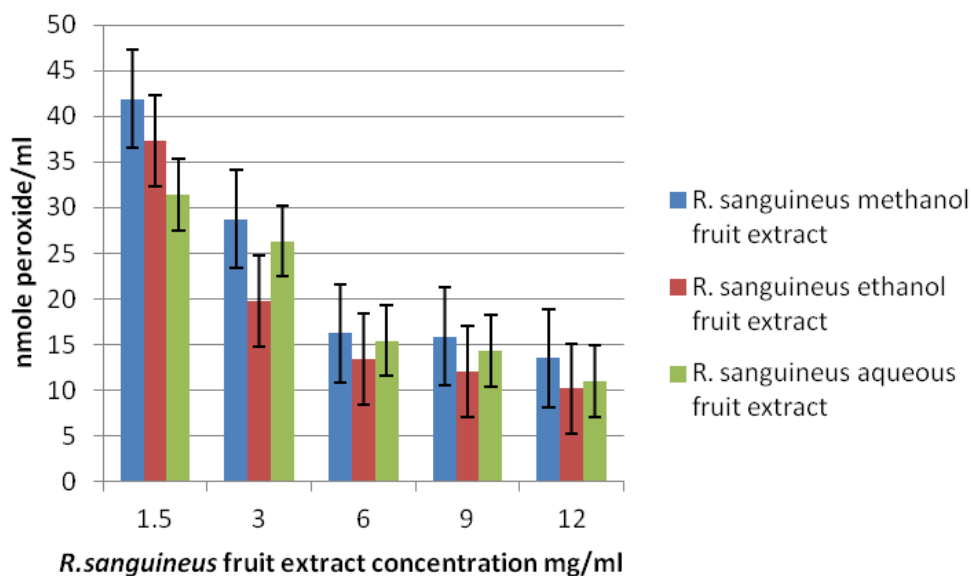**Figure 10.** nmole of aqueous peroxide in *R. sanguineus* leaf extracts.

Figure 11. nmole of aqueous peroxide in *R. sanguineus* fruit extracts.

through occurrence as enzymatic reaction (Wasternack, 2007), which has been considered to be involved in various pathophysiological cell and tissue abnormalities. The lipid peroxidation assay that was performed to test the *R. sanguineus* extracts showed that the inhibition of both aqueous and organic peroxides nmoles/reaction volume was concentration dependent. Lipid peroxidation

was induced with ferrous ammonium sulfate reagent and the degree of lipid peroxidation was assayed by estimating the aqueous and organic peroxide color reagent-reactive substances, while inhibition of lipid pero-xidation was assessed in the presence of sample extracts as described in Sigma peroxi detect kit. Inhibitions of lipid peroxidation (Figures 10, 11, 13 and 14) by methanolic,
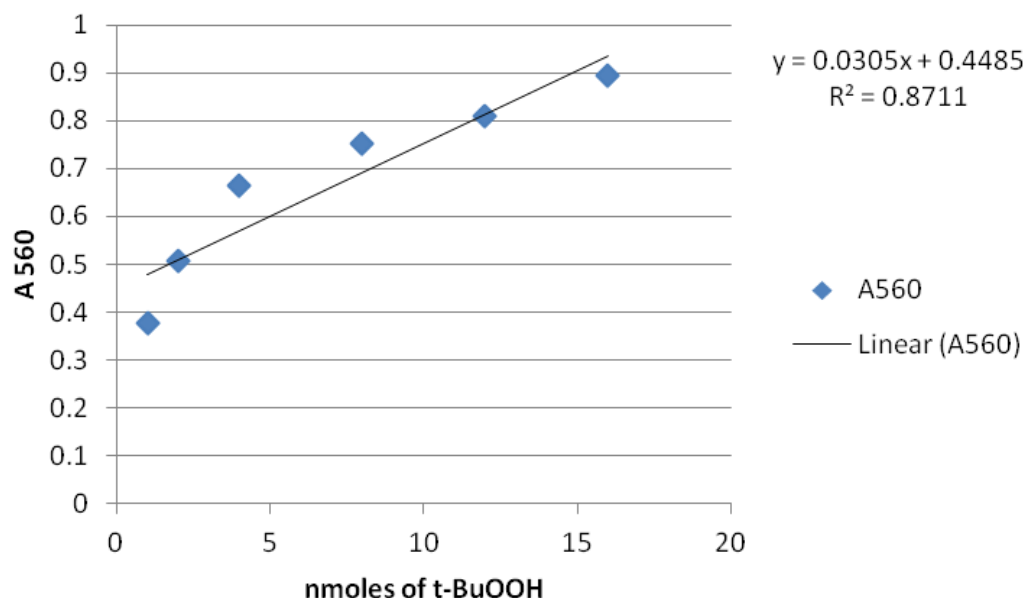
$y = 0.0305x + 0.4485$
$R^2 = 0.8711$

Figure 12. Standard curve of nmoles of t-BuOOH against absorbance at 560 nm.
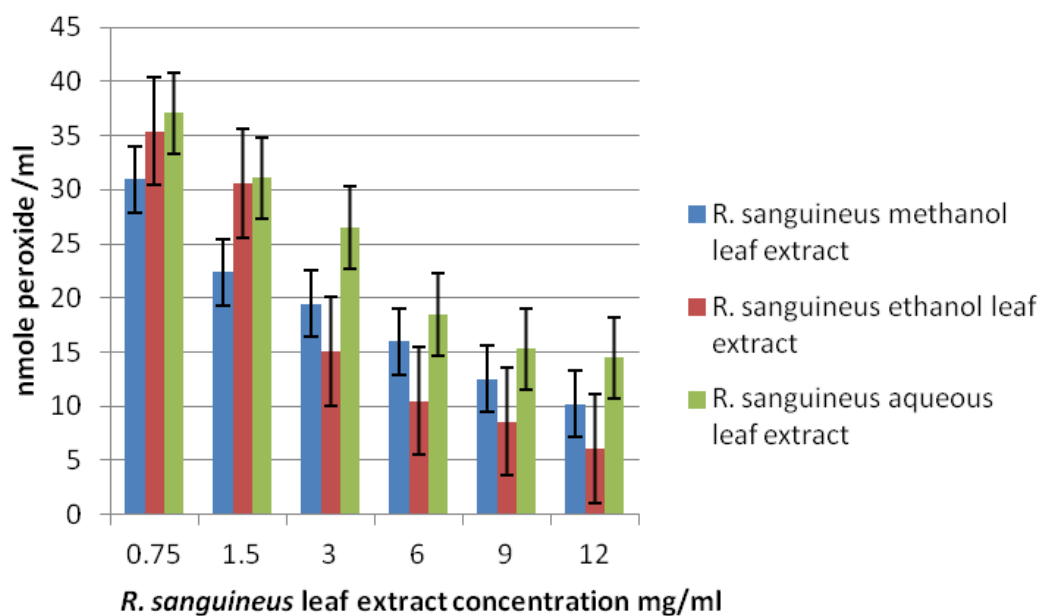

Figure 13. nmole of organic peroxide in *R. sanguineus* leaves extracts.

ethanolic and aqueous extracts of *R. sanguineus* fruits and leaves were observed to be high in magnitude. The highest inhibition of aqueous peroxide was performed by for the ethanolic extract of both *R. sanguineus* fruits and leaves followed by the methanolic and then the aqueous extracts. For the organic peroxide the highest inhibition was recorded for the ethanolic *R. sanguineus* leaf and fruit extracts and the least was for the aqueous fruit extract. Thus, it can be concluded that ethanolic, methanolic and aqueous extracts of *R. sanguineus* fruits
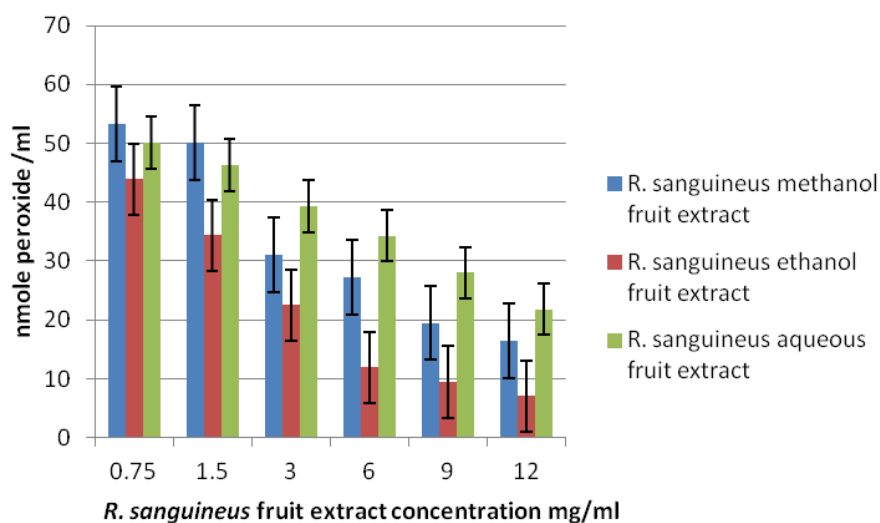
Figure 14. nmole of organic peroxide in *R. sanguineus* fruits extracts.

and leaves extracts can be used as an accessible source of natural antioxidants with consequent health benefits. It can be also concluded that *R. sanguineus* extracts chelates iron and has reducing power. These *in vitro* assays indicate that this plant extracts are significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

Conflict of Interest

Authors have not declare any conflict of interest.

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