Effect of Aqueous Extract of Roselle Calyx (*Hibiscus sabdariffa Linn*) on Hidrogen Peroxide Induced Oxidative Stress of Rat Red Blood Cell Membranes

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Abstract

Hisbiscus sabdariffa Linn or Roselle is traditional herbs are widely used to treat various diseases. Roselle is rich with natural antioxidant agents such as anthocyanins, vitamin C, protocatechuic acid (PCA) and others. High oxygen reactive species (ROS) such as hidrogen peroxide (H_2O_2) cause oxidative stress on rat red blood cell (RBC) membrane and lead to hemolysis. The purpose of this in vitro study is to evaluate the effect of aqueous extract calyx Roselle pre-treated on rats RBC membrane towards oxidative stress induced by H_2O_2. Aqueous extract of calyx Roselle are used on three difference doses of concentration of 0.5, 1.0 and 2.0 mg/ml. The result showed that aqueous extract of calyx Roselle at 0.5 mg/ml concentration as a screening dose was the safest dose based on the reduction about 1.21% of hemolysis. The value was almost near to 1.19% of 0.9% NaCl group (negative control). Furthermore, at dose of 0.5 mg/ml aqueous extract of calyx Roselle studied revealed about 53% reduction via RBC percentage hemolysis when induced by 10 mM H_2O_2. The study was also measured the concentration of malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity towards 10 mM H_2O_2 induction. MDA concentration and SOD activity shows significantly increase as well as the decrease of GSH activity at p value <0.001 compare to 0.9% NaCl control group. Meanwhile, the study on pre-treated RBC with 0.5 mg/ml aqueous extract of calyx Roselle indicated reduction of MDA concentration and SOD activity significantly reduced ($p<0.05$) whereas GSH activity also significantly increase ($p<0.05$) compare with control group. In conclusion, this study found that concentration of dose 0.5 mg/ml aqueous extract of calyx Roselle provide a protective effect against oxidative stress induced damage on RBC membrane by 10 mM H_2O_2 induction.

Key words: Reactive oxygen species, Hibiscuss sabdariffa Linn, trditional herbs, Malaysia

Introduction

The involvement of reactive oxygen species or ROS such as hydrogen peroxide (H_2O_2), organo peroxide, superoxide radicals (O_2^O) and hydroxyl radicals (OH^O) produced in the aerobic metabolism of biological systems and is also produced by ecsogenous sources such as ultraviolet rays, ionized radiation, drugs and pollution systems (Briviba & Sies 1994). It is believed to be the main contributing factor induced damage to cell membranes. According to sources other mechanisms, OH^O formation resulting from the production of H_2O_2 and O_2^O formed in the presence of redox active transition metal (Saumi et al. 1983). The increase in ROS formation prevents high antioxidant activity levels and cause damage to the function and cell structure such as lipid oxidation, nonactivation of enzyme, DNA mutation and thus causes damage to the whole cell (Toykuni 1999). This condition is known as oxidative stress (Halliwell 1994).

Oxidative stress due to ROS imbalance and cause weaknesses of endogenous antioxidant defenses associated with physiological and pathological conditions such as aging, cancer, rheumatoid arthritis, neurodegenerative disorders and diseases antherosclerosis (Knight 1997; Briviba & Sies 1994; Barnhan et al. 2004). Unbalanced oxidative stress is also said to be the cause of the lipid peroxidation (Halliwell & Chirico 1993). In addition, lipid peroxidation processes produce various by products such as malondialdehid (MDA). MDA will increase stiffness and lower permeability of membrane red blood cell (RBC) (Pfafferott et al. 1982).

RBC containing hemoglobin (Hb) functions as a carrier of oxygen (O_2). O_2 is very important as energy transportation to the tissues for growth and other body activities in aerobic metabolism. This process also causes the formation of free radicals such as reactive ROS (Fang et al. 2002). About two or three per cent of oxygen by the respiratory chain is converted to ROS (Chance et al. 1979).

RBC is often used as a primary target by free radicals attack (Sadrazadeh et al. 1984) due to the presence of polyunsaturrated fatty acids (PUFAs) and oxygen transport related to the Hb molecules are found to be able react with ROS. In addition, RBC has very high of containing membrane lipids and proteins (Hoffbrand & Pettit 1999). Free Hb exposed to H_2O_2 will causes the breaking heme by the release of iron ions activates as the entry of free radicals and lipid peroxidation (Puppo & Halliwell 1998). H_2O_2 and ascorbic/Fe^{2+} also promote changes in appearance as a sign of oxidative damage (Srour et al. 2000).

In this study, *Hibiscuss sabdariffa Linn* known as Roselle will be used to investigate the effect on the membrane RBC rats induced by H_2O_2. Calyx Roselle is rich with various chemicals such as alkaloids, Vitamin C, anisaldehid, anthocyanins, β-sitosterl, citric acid, cyanidin-3rutinoside, dephinidin, galaktos, gossypetin, hibiscetin, mucopolysaccharides, pectin, protocatechuic acid, polysaccharides, quercetin, stearic acid and wax. It is have diuretic and choleretic effect of lowering the level of blood viscosity, blood pressure and stimulating intestinal peristalsis (Josiah et al. 2010; Mahadevan et al. 2009). In addition, Roselle is also reported as antihypertensive, antioxidant, anti-cancer, anticlastrogenik, hypolipidemia, hepatoprotective, anti stress, anti-spasmodic, diuretic and anti diareal activities (Joshi & Parle 2006).

Material & Methods

Plant material: the fresh calys of Roselle types UKMR-2 from UKM, Bangi. Prepared extract solution by distilled water and using method of Noridani 2010.

Chemical: 30% Hidrogen peroxide was purchased from Merck. All other chemical and reagent was obtained from lab of Faculty of Health Sciences (FSK), UKM.

Experimental animal: Wistar rat (180-220g) was supplied by the Laboratory Animal Resource Unit, UKM. The blood sample was collected through the orbital sinus puncture using hematocrit tube and according methods by Yang et al. (2006). A total of 4 ml of rat blood will be collected and stored in the lithium heparin tube. Blood samples will be centrifuge at 3000 rpm for ten minutes at 4^{O}C. The cenrifuge is intended to separate the plasma and RBC from buffy coat. After centrifuges, all the supernatant was separated and the remaining blood is remains into a sterile tube container. The remaining blood is washed by five times the volume of blood cells (1:5) with cold 0.9% normal saline. This process is repeated three times. Then, 10% suspension of RBC was used for determination of hemolysis assay and other biochemical analysis such as GSH, SOD, and MDA concentration.

Safety dose study

Determination safety dose of aqueous extract of HR rats Roselle done in advance by hemolysis test method practiced by Tedesco et al. (2000). This test is to determine the suitable and safe of aqueous extract of Roselle dose for rats RBC based on the effect of the percentage of hemolysis. A total of 0.2 mg/ml aqueous extract of Roselle will be pre-treated to 0.02 mg/ml RBC with different concentrations of the Roselle study group which is 2.0 mg/ml, 1.0 mg/ml and 0.5 mg/ml. The mixture was incubated in a shaken water bath for 1 hour at 37^{O}C. After an hour, the mixture solution was centrifuge at 3000 rpm for 10 minutes. The supernatant was mixed with Drabkin solution and measured by using a spectrophotometer at a wavelength of 540 nm. Calculation of percent hemolysis study group compared with distilled water which is considered as 100% hemolysis.

Hemolysis Assay

Hemolysis inhibition of RBC by aqueous extract of Roselle tested in accordance with procedures determined by Tedesco et al. (2000). The safety dose study found that the concentration of the dose 0.5 mg/ml is safe to RBC. The purpose of this test is to measure the protective effect on RBC of dose concentration of 0.5 mg/ml aqueous extract Roselle induced by 10 mM H_2O_2 based on the percentage of hemolysis.10 mM H_2O_2 consider as 100% hemolysis and comparing with each group.

Sample preparation for MDA measurement

The method used to measure the MDA level is based on the method of Hunter and Jamaludin (1986). The principle used is based on the principles cromogen colourmetry in which material is formed from the chemical reaction is measured by spectrophotometry at 532 nm wavelength.

Sample preparation for GSH activity

The method will be used to analyze GSH activities by the methods practiced by Ellman (1959). The composition of GSH reacts with DTNB (5.5 '-dithio-bis-2-nitrobenzoic acid) (Ellman Reagent). Yellow product 5-Thio-2-nitrobenzoic acid (TNB). GSTNB is a mixture disulfit be generated in this reaction and will be reduced by GR to recycle GSH and produce more TNB. TNB formation rate proportional to place the recycling reaction of GSH and GSH activity level in the sample. Therefore, measuring the absorption of TNB at 415 nm wavelength to show the amount of GSH is in the sample.

Sample preparation for SOD activity

SOD activity is measured using the method of Beyer & Fridovich (1987). This method uses riboflavin to be activated by photons and subsequently oxidize electron donors such as L-metionin. Riboflavin semiquinon will be reduced to a decrease O_2. This process will reduce NBT and measured at 560nm wavelength.

Results

Determination of saefety dos extract aqueous roselle in rats red blood cell

Figure 3.1 shows a graph of the determination of safe doses of Roselle. The concentration of each study group based on the percentage of hemolysis was compared with distilled water. The results showed that there is a difference of 0.9% NaCl control group (1.19 ± 0.04%) was significantly ($p<0.001$) compared with the study group 2.0 mg / ml (2.43 ± 0.08%) and the 1.0 mg/ml (1.67 ± 0.05%). The study also found no significant differences between the study group 0.5 mg/ml (1.21 ± 0.03%) with control. Concentrations of the study group 0.5 mg/ml also showed in reduce percentage hemolysis compared to the 2.0 mg/ml and 1.0 mg/ml significantly $p<0.001$. Percentage hemolysis concentration doses 0.5 mg/ml of 1.21% were found to approach the percent hemolysis of the control group. The results showed the concentration dose 0.5 mg/ml is suitable as a safety dose of aqueous extract of Roselle.

Studies on hemolysis effects of extract aqueous roselle in rats red blood cell induced by hydrogen peroxide

Figure 3.2 shows a graph of the effect of aqueous extract of Roselle induction of H_2O_2. As a result of the graph shows there is a difference of 10 mM H_2O_2 (100 ± 4.53%) between the NaCl control group (42.9 ± 0.9%) in significant level of $p<0.001$. Results of aqueous extract of Roselle study of 0.5 mg/ml, is 46.9 ± 1.29% were found a significantly ($p<0.001$) difference with 10 mM H_2O_2 group and no significant differences between the control group. Percentage hemolysis study group 0.5 mg/ml were also found to show a decrease about 53% compared with the 10 mM H_2O_2. Percentage of hemolysis of 0.5 mg/ml is also close to the control group. This show the ability dose concentration 0.5 mg/ml extract aqueous Roselle reduce the percentage hemolysis of RBC induced by H_2O_2.

Studies of MDA concentration

Figure 3.3 shows a graph of the concentration of MDA between the control and study groups. The study found significantly ($p<0.001$) differences in the group 10 mM H_2O_2 (5.61 ± 0.077 nmol/g Hb) compared with the control group is 4.17 ± 0.138 nmol/g Hb. Group study of Roselle induced by 10 mM H_2O_2 also found that the concentration of MDA (4.28 ± 0.076 nmol/g Hb) was significant ($p<0.001$) compared with 10 mM H_2O_2 group. However, no significant difference in Roselle induction of H_2O_2 to the control and the concentration of MDA can be seen approaching the MDA concentration of the control group. The study found that increased concentrations of MDA in the group 10 mM H_2O_2. This shows the aqueous extract of Roselle 0.5 mg/ml reduced the concentration of MDA in RBC induced by 10 mM H_2O_2.

Study of GSH activity

The results shown in Figure 3.4 is a graph showing the activity of GSH are significant differences between the study groups compared to controls. GSH activity level of 10 mM H_2O_2 (0.381 ± 0.0628 μmol/g Hb) showed a significant increase ($p<0.001$) compared with the control of 1.590 ± 0.0722 μmol/g Hb. While the activity of GSH for group Roselle induced by 10 mM H_2O_2 is 0.642 ± 0.009 μmol/g Hb showed a difference significantly ($p<0.05$) compared with the control group and $p<0.001$ with the group 10 mM H_2O_2. The results showed that Roselle induced by 10 mM H_2O_2 showed an increase in GSH activity level. This shows the aqueous extract of Roselle dose 0.5 mg/ml increase GSH activity of 10 mM H_2O_2 induction of RBC.

Study of SOD activity

Figure 3.5 shows the activity of SOD value for the study and control groups differ significantly. The results showed that increased activity of SOD in 10 mM H_2O_2 group of 448 ± 14.75 U/g Hb was significant ($p<0.001$) when compared with the control group 146 ± 12.9 U/g Hb. While the activity of SOD in the induction of Roselle

induced by 10 mM H_2O_2 is 249 ± 22.24 U/g Hb were found to be significant ($p<0.05$) compared with the control group and found to be significantly ($p <0.001$) compared with the 10 mM H_2O_2. The results showed that there was a decrease of SOD activity of Roselle induced by 10 mM H_2O_2 group compared with 10 mM H_2O_2. This shows the aqueous extract of Roselle dose 0.5 mg/ml showed a decreased activity of SOD RBC induction of 10 mM H_2O_2.

Discussion

Ko et al study. (1997) found the effects of oxidative damage to lipid membranes and proteins cause hemolysis and weaknesses of RBC antioxidant defense system. Mechanism of the effect of ROS membranes catalyze oxidative damage in RBC by causing complications such as β-thalassemia, the cell anemia Sickle and a host of other diseases involving hemoglobinopati (Scott et al. 1993).

Determination of the dose concentration of Roselle extract was used as the solvent extracts of aqueous solution. This is consistent with the fact Moller et al. (1999) found that using aqueous extraction plants more nutritious and made relevant for food and has a significant relationship in terms of the certificate and security.

Three dose concentrations were used in this study of 0.5, 1.0 and 2.0 mg / ml. The results showed that doses of 1.0 and 2.0 mg / ml showed a higher percentage hemolysis compared to the control group. Dose of 0.5 mg / ml showed a decrease percent hemolysis approaching the percent hemolysis of 0.9% NaCl. This shows the dose 0.5 mg / ml can be used as a safe dose for RBC. Previous studies by Farombi & Fakoya (2005) also supports the use of Roselle dose of 0.5 mg/ml as antioxidant agents. Suboh et al. (2004) also using 10 mM H_2O_2 on RBC for studying and there increase in MDA levels.

According Bakko (1985), assay hemolysis is used as an indication of the rate of diffusion or concentration of a particular solution of the rate of permeability of RBC. This study suggests increased percent hemolysis dose concentrations of 2.0 and 1.0 mg/ml may be due to the rate of aqueous extract of Roselle dose concentrations in excess of the rate of concentration in RBC. This leads to changes in osmotic where, changes in the concentration gradient and cause crenation and hemolysis of RBC experience. 0.9% NaCl is an isotonic solution. It is because the concentration dose 0.5 mg/ml is equal to the contents of RBC appears to be in equilibrium and the absence of concentration gradients.

The studies on hemolysis effects of extract aqueous roselle in rats red blood cell induced by hydrogen peroxide also showed decrease significantly. This suggests that H_2O_2 directly reacting with molecular oxygen-rich hemoglobin Fe^{2+} in the membranes RBC. The ability of H_2O_2 across membranes RBC causes Fe^{2+} reacts with H_2O_2 to produce Fe^{3+} and radical OH^O through Fenton reaction. Hb has the potential to react with H_2O_2 to form met-Hb. When free Hb is exposed to H_2O_2 also caused hem fragmentation. Polymerize resulting in increased permeability of the membrane and eventually cause a decrease in cellular shape. Changes result in hemolysis of RBC as well as causing overflow components of the cell (Tsean & Collier 1960). The increase

in percent hemolysis of RBC also showed the involvement of fragmentation H_2O_2 induction cause hemolysis in which the enzyme is not working properly. Studies conducted by Shiva et al. (2007) found that the higher induction of H_2O_2 leads the higher percent hemolysis of RBC.

MDA is the main indicators in lipid peroxidation. To study the concentration of MDA formed shows the level of lipid peroxidation can be estimated. The study suggests that induction of H_2O_2 resulted in lipid peroxidation by causing effects on the components of PUFAs degredation. Lipid peroxidation also plays a role in the initiation of oxidative damage. This is because, the ability of free radicals is possible to extract hydrogen atoms. This radical restructuring to achieve stability conjugate to form dienes. Conjugated dienes were attacking other lipid molecules and initiate subsequent chain propagation. Finally, the lipid peroxidation will cause severe damage to the cell membrane and ultimately cause death (hemolysis) (Miki et al. 1987). This finding is consistent with a study conducted by Suboh et al. (2004) on the protective effects of Hibiscus sabdariffa (Roselle) induced by 10 mM H_2O_2 found at 10 mM H_2O_2 increased concentrations of MDA.

According to Albano et al. (1983), the increase in MDA is associated with reduced GSH activity. When the GSH activity decreased, this will increase in lipid peroxidation process that causes MDA accumulation occurs. The study is consistent with studies Karademir et al. (2007), found that lower concentrations of GSH due to oxidative effects. This suggests that as a result of lipid peroxidation in H_2O_2 activity affecting a decrease of GSH activity. H_2O_2 metabolism leads to GSH deplation, GSSG formation and ultimately increase caused a decrease in the ratio of GSH/GSSG. This decrease GSH activity levels may increase metabolite covalent toxic to macromolecular cells and increase cell damage (Mitchell et al. 1973). Luperchio et al. (1996) also supports that the effects of oxidative damage reduction effect concentrations resulting in increased formation of GSH to GSSG oxidation.

Through this study, it can be suggested that the enhancing effect of SOD may be due to effects on cellular oxidative damage through direct exposure to this concentration of 10 mM H_2O_2. In addition, exposure through high doses of H_2O_2 also restricts the tendency of SOD activity may be due to the induction of toxic effects of H_2O_2. This is because, SOD present in large numbers in the SDM (Speranza et al. 1993). An increased activity of SOD also refers to the excessive production of dismutation reaction with superoxide to cause an increase in hydrogen peroxide (Surapaneni & Vishnu 2009). Studies conducted by Adriana et al. (2011) also found high SOD activity values when induced by Clomazon a pesticide. SOD defense can significantly damage on defense mechanisms in preventing the formation of ROS in living cells, such as human RBC (Bukowska 2003).

RBC has been treated with Roselle seen to reduce the hemolysis percentage. This may be related to the composition of the antioxidant activity Roselle action. These studies suggest that the ability of a good donor of hydrogen ions by aqueous extract Roselle showed effective activity as antioxidant agent. This is because the contents of Roselle calyx is rich in vitamin C, anthocyanins, polyphenols and other water-soluble antioxidants (Duke & Atcley 1984). Tee et al. (2002) found antioxidant activity in aqueous extract of Roselle Roselle showed a stronger antioxidant effect than the BHA

or tocopherol in linoleic acid model system. Vitamin C also acts indirectly by free radical-tocopherol to form α-tocopherol at the aqueous surface of the membrane lipid bi-layer, and thus protects the lipid layer and reduce the level of hemolysis of RBC (May 1998). A study conducted by Wang et al. (2000) also supports that the contents of Roselle anthocyanins can increase GSH levels. Jamaludin (2008) also noted that the increase in the ratio of GSH/GSSG is an indication of high levels of GSH for antioxidant activity.

Conclusion

This study shows there are protective effects of aqueous extract of Roselle to reduce oxidative stress damage in vitro. Various studies also demonstrate the ability of antioxidants as antihypertensive and kardioprotektif Roselle (Onyenekwe et al. 1999), hepatoprotective (Tseng et al. 1997), antihyperlipidemia (Hirunpanich et al. 2006), antioxidants (Christian et al. 2006), anticancer (Chang et al. 2006), antibacterial (Mounnissamy et al. 2002) and others. The concentration of aqueous extract of Roselle for the three dose concentrations of 0.5, 1.0 and 2.0 mg/ml dose concentrations found 0.5 mg/ml are safe for rats RBC based on the percent of low hemolysis percentage close to 0.9% NaCl. Dose concentrations of 0.5 mg/ml aqueous extract of Roselle have decreased levels of percentage hemolysis compared with the group 10 Mm H_2O_2. Induction of 10 Mm H_2O_2 can stimulate membrane lipid peroxidation by increasing the concentration of MDA and reduced antioxidant capacity in the membrane of RBC by increasing the concentration of SOD and decreased concentrations of GSH. Dose concentrations of 0.5 mg/ml aqueous extract of Roselle is seen to reduce the peroxidation on lipids by reducing the concentration of MDA and also helps membrane RBC antioxidant defense system by increasing concentrations of GSH and lower concentrations of SOD.

Conflict of Interest: None declared.

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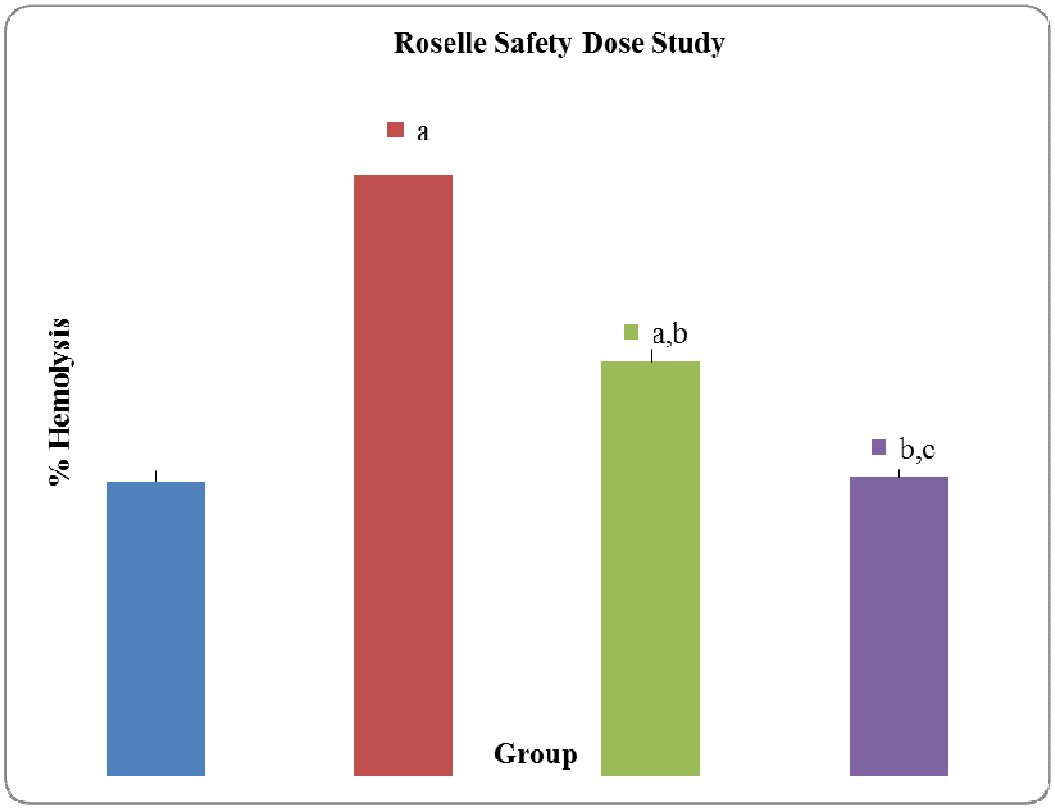

Figure 3.1: Determination a safety dose of aqueous extract of Roselle based on the percent hemolysis (mean ± S.E.M) of each study group.

a: significant compared to the 0.9% NaCl (controls) ($p<0.001$)
b: significant compared to the 2.0 mg/ml Roselle ($p<0.001$)
c: significant compared to the 1.0 mg/ml Roselle ($p<0.001$)

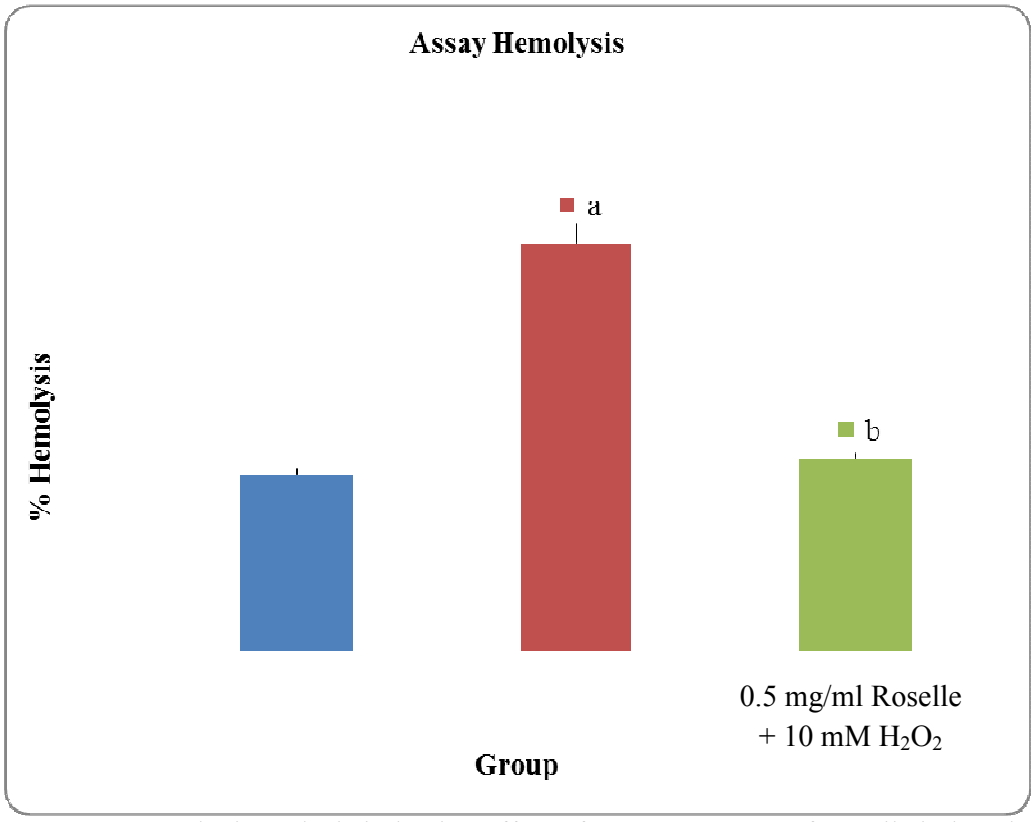

Figure 3.2: The hemolysis induction effect of aqueous extract of Roselle induced H_2O_2 based on percent (mean ± S.E.M) of each study group.

a: significant compared to the 0.9% NaCl (controls) ($p<0.001$)
b: significant compared with the 10 mM H_2O_2 ($p<0.001$)

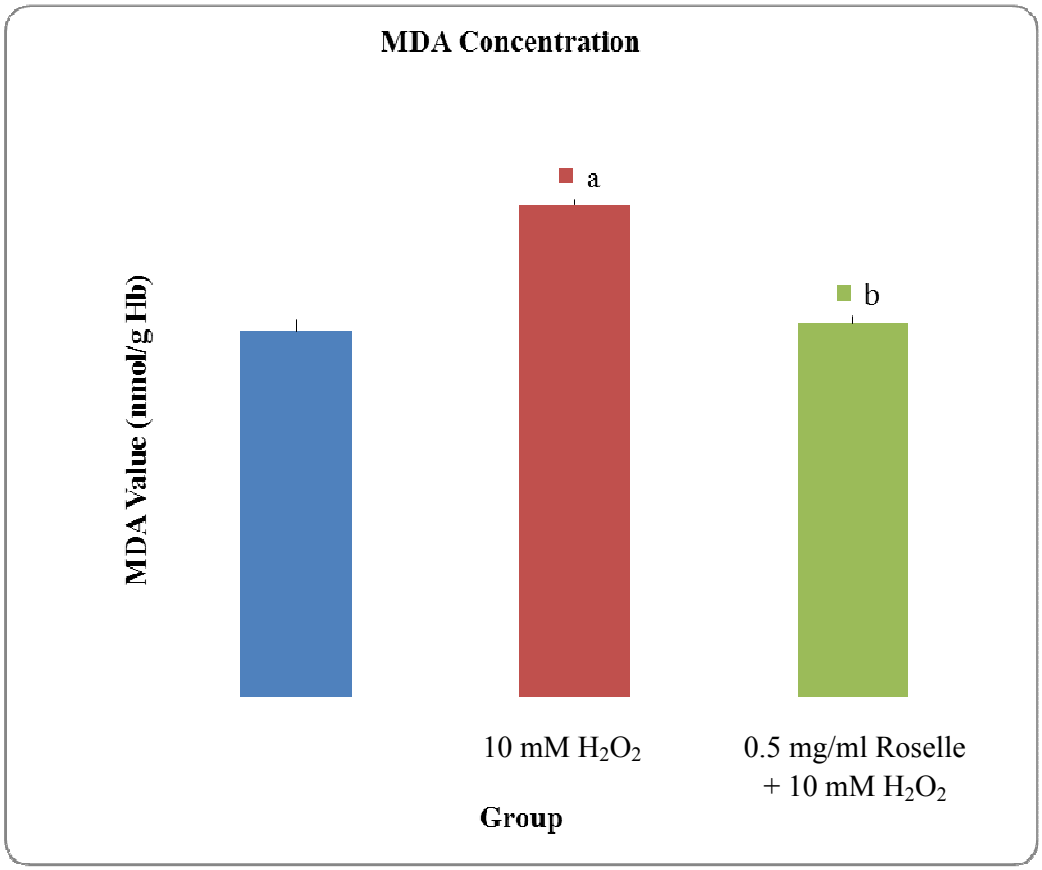

Figure 4.3: The concentration of MDA (mean ± S.E.M) of each study group.

a: significant compared to the 0.9% NaCl (controls) ($p<0.001$)
b: significant compared with the 10 mM H_2O_2 ($p<0.001$).

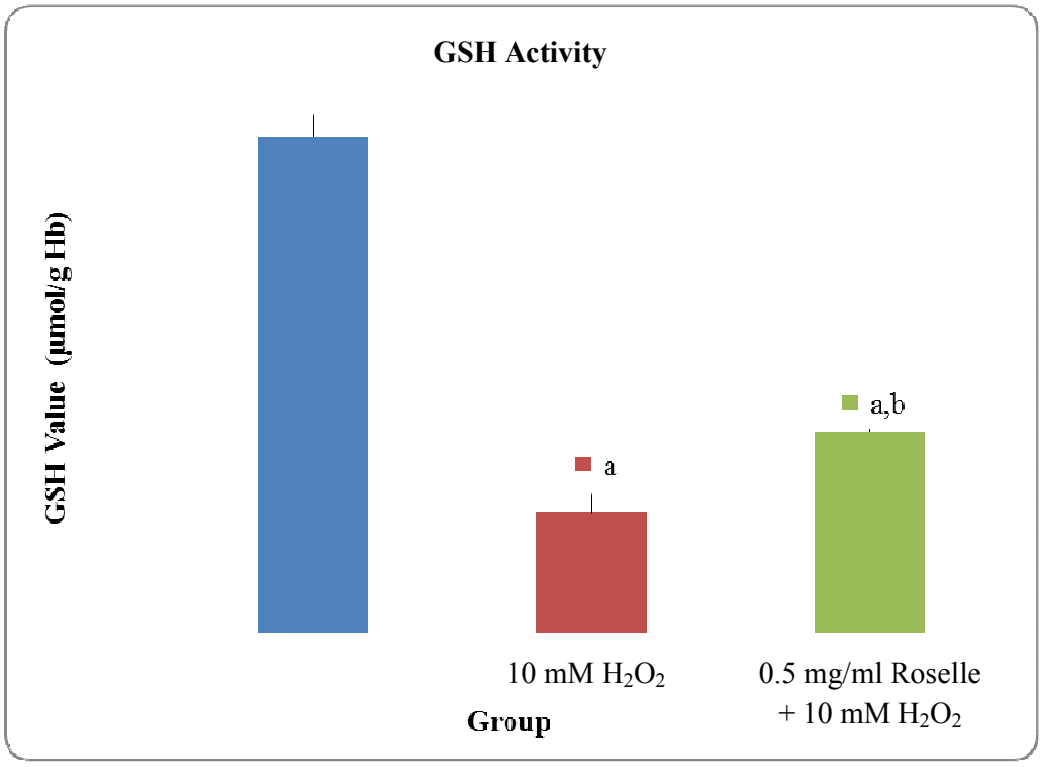

Figure 3.4: The GSH activity (mean ± S.E.M) of each study group

a: significant compared to the 0.9% NaCl (controls) ($p<0.001$)
b: significant compared with the 10 mM H_2O_2 ($p<0.05$)

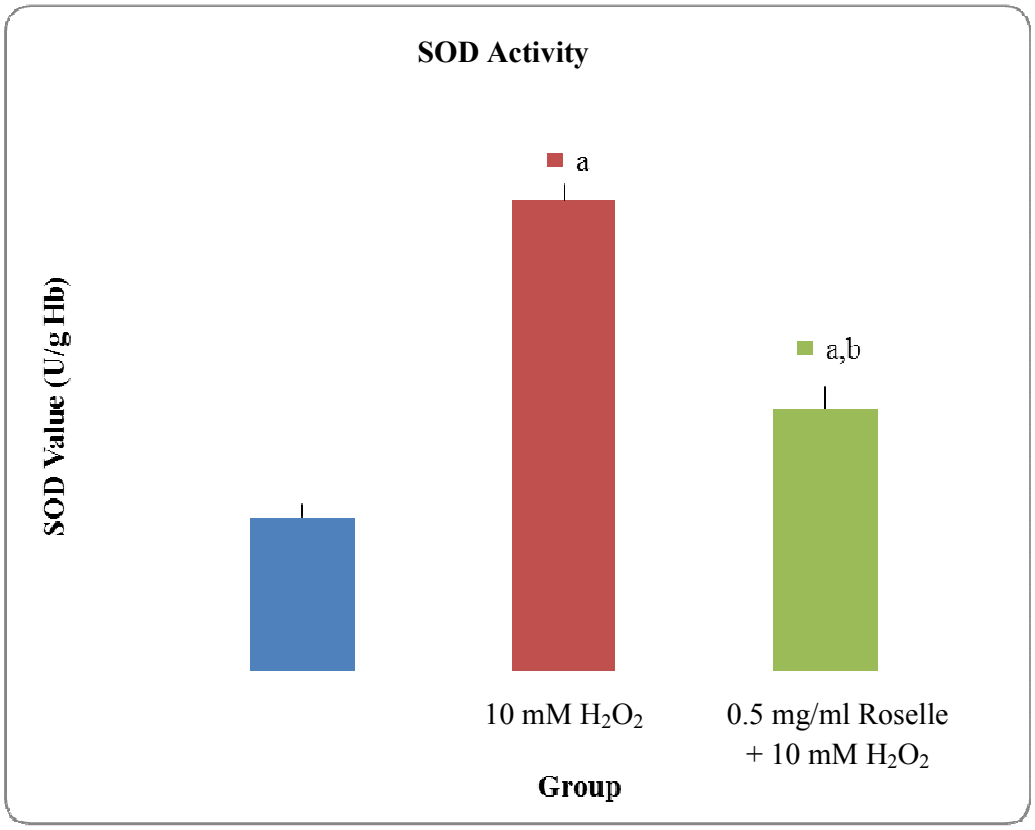

Figure 3.5: The SOD activities (mean ± S.E.M) of each study group

a: significant compared to the 0.9% NaCl (controls) ($p<0.001$)
b: significant compared with the 10 mM H_2O_2 ($p<0.05$)