

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 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 Ulaq 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

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(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 Whatman 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 $[1 - (A_1) / A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 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:

$$\text{Nitric Oxide scavenged \%} = (A_{\text{control}} - A_{\text{test}}) / (A_{\text{control}}) \times 100$$

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:

$$\text{Hydroxyl scavenged \%} = (A_{\text{control}} - A_{\text{test}}) / (A_{\text{control}}) \times 100$$

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-cresolsulfonaphthalein, 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 A_{560} 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 H₂O₂ standard curve.

100 μ M H ₂ O ₂ Solution (μ l)	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 μ M t-BuOOH Solution (μ l)	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:

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

Dilution factor = factor for dilution of original sample; [A560 (1 nmmole peroxide)] = calculated from H₂O₂ or t-BuOOH standard curve; sample volume = volume of sample (ml).

IC₅₀ calculations

To calculate the IC₅₀, 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 IC₅₀ value in equation Y = 50. M and C values were presented in the equation itself. The value of X will be IC₅₀ value for that graph. IC₅₀ 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 IC₅₀ was calculated for each extract and then compared to standard ascorbic acid. As for the IC₅₀ (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. IC₅₀ of *R. sanguineus* fruits and leaves extracts for DPPH assay.

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

Table 4. IC₅₀ of *R. sanguineus* fruits and leaves extracts for NO scavenging activity.

Plant part	Extract	IC ₅₀ mg/ml
Fruit	Methanol	17.6
	Ethanol	9.5
	Aqueous	5.9
Leaf	Methanol	6.7
	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 IC₅₀ was calculated for each extract and then compared to the standard ascorbic acid (Table 4).

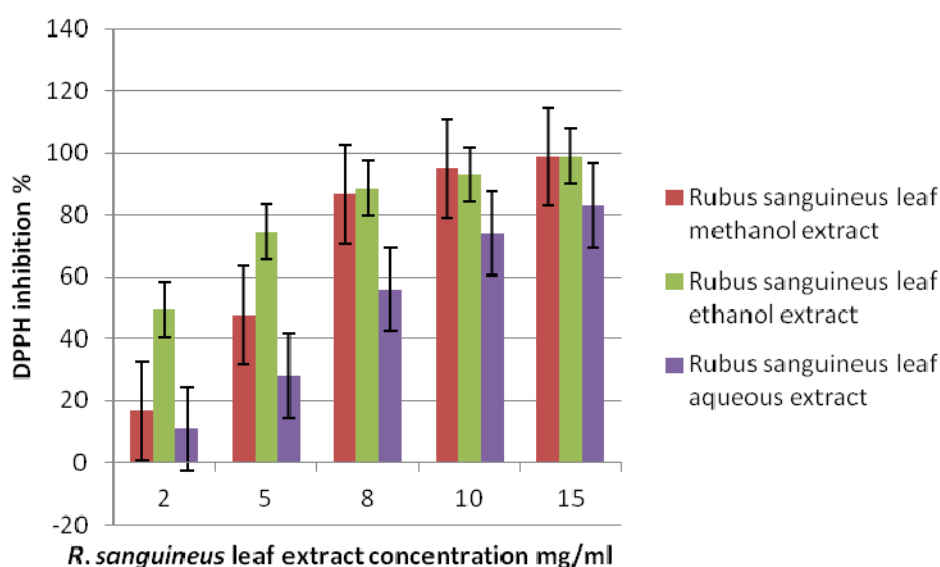
Hydroxyl radical scavenging assay

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. sanguineus* (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 IC₅₀ was calculated for each extract (Table 5).

Table 5. IC₅₀ of *R. sanguineus* fruits and leaves extracts for Hydroxyl radical scavenging activity.

Plant part	Extract	IC ₅₀ mg/ml
Fruit	Methanol	10.32
	Ethanol	6.09
	Aqueous	4.00
Leaf	Methanol	5.1
	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 H₂O₂ 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

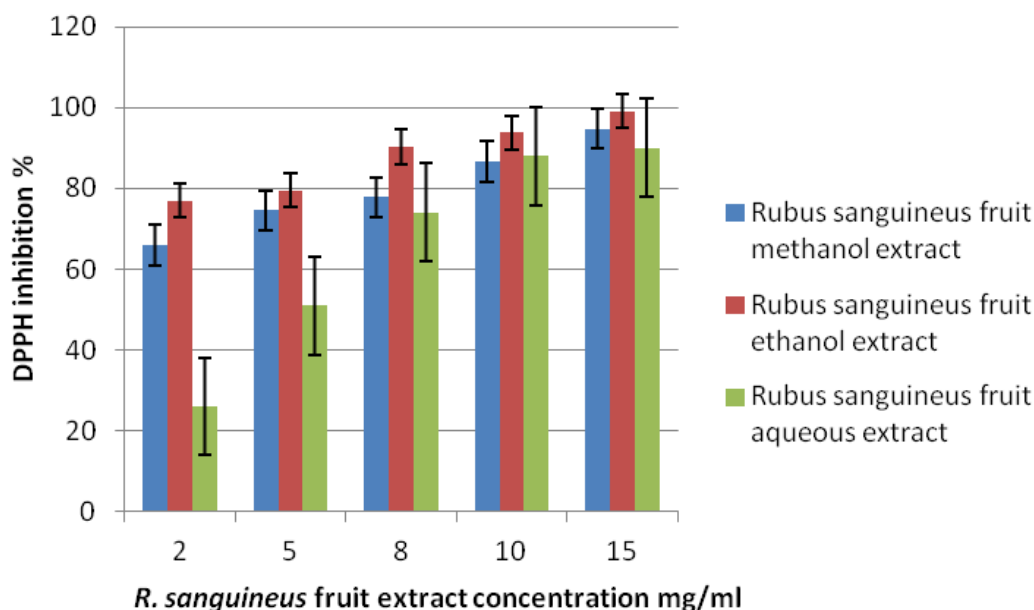


Figure 2. Inhibition % of DPPH by methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.

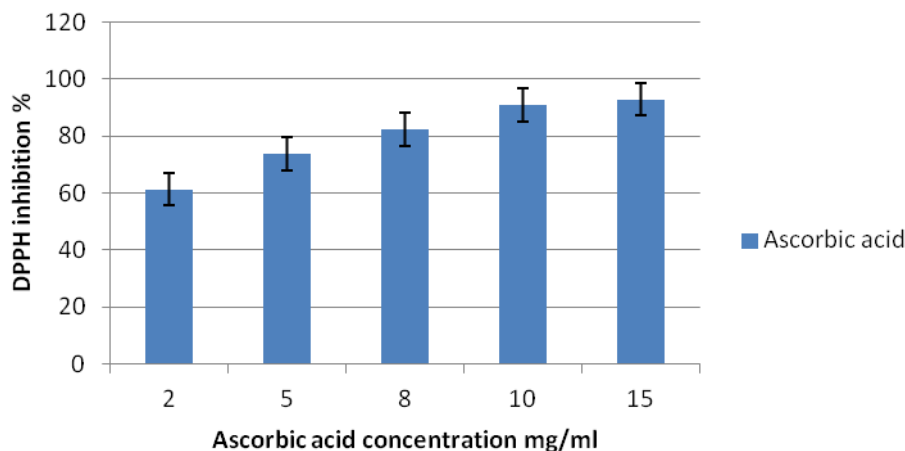


Figure 3. Inhibition of DPPH by ascorbic acid.

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.

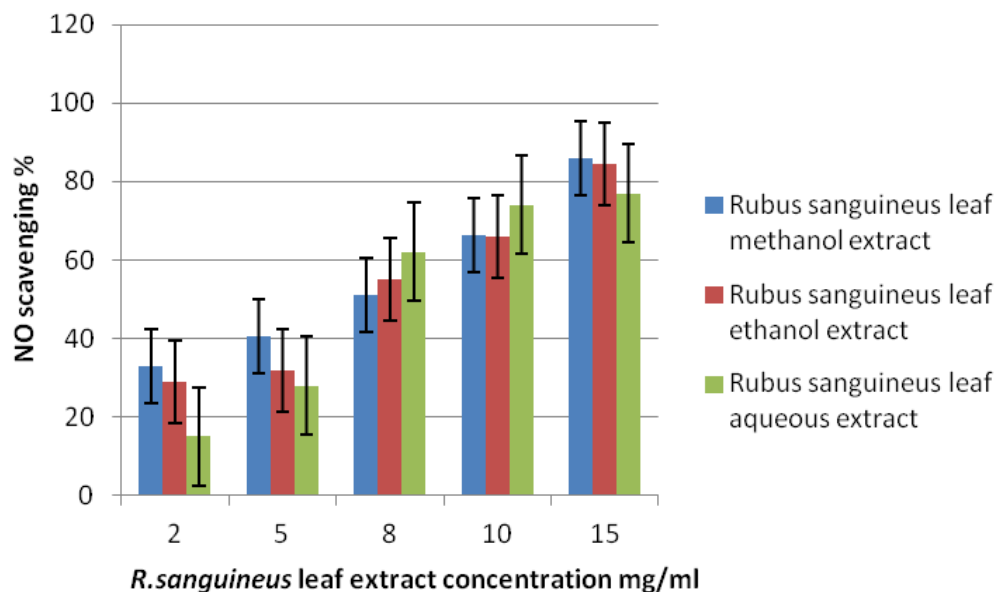


Figure 4. Nitric oxide (NO) scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* leaves.

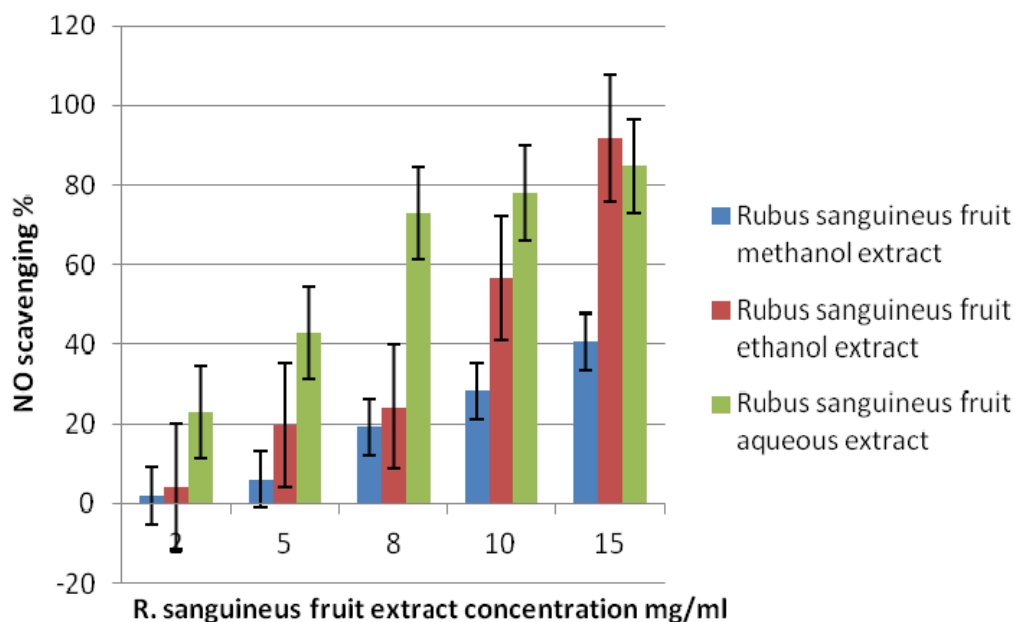


Figure 5. Nitric oxide (NO) scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.

Nitric oxide radical inhibition assay

This assay is based on the fact that sodium nitroprusside in an aqueous solution at a physiological pH spontaneously

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,

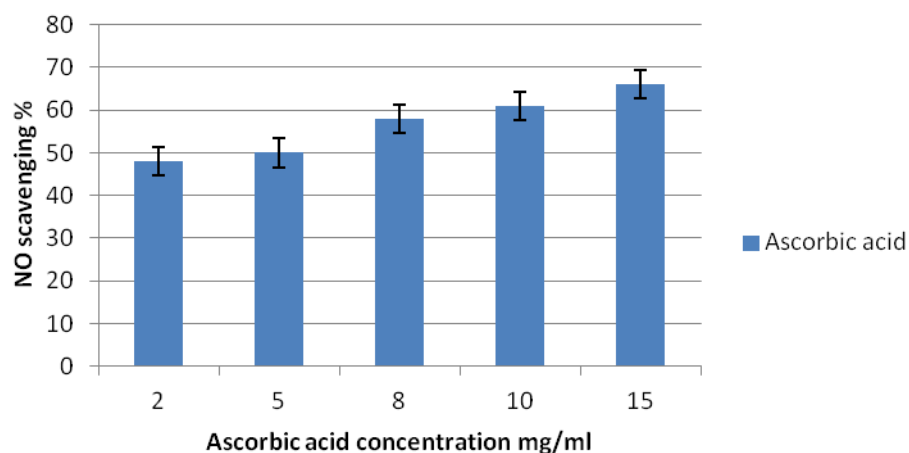


Figure 6. Nitric oxide (NO) scavenging activity of ascorbic acid.

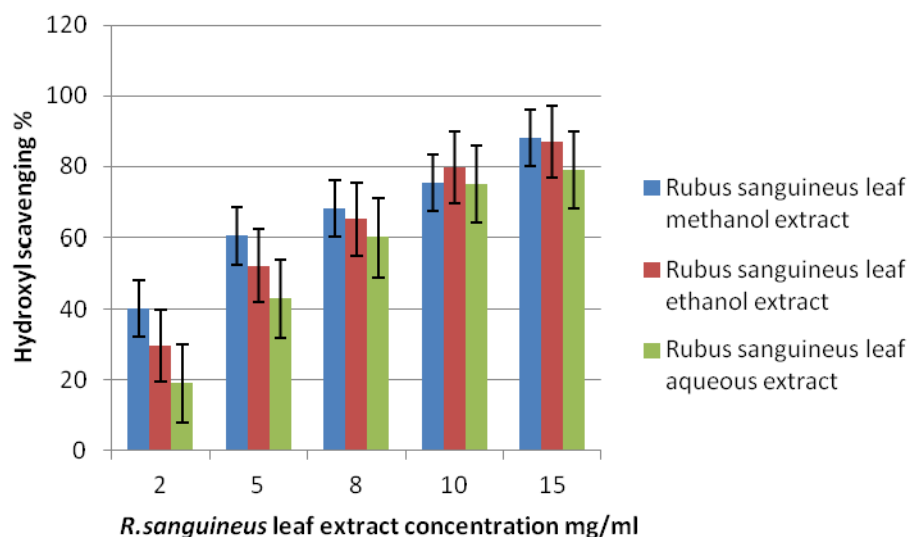


Figure 7. Hydroxyl scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* leaves.

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^\bullet) are produced through the reductive decomposition of H_2O_2 . HO^\bullet 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

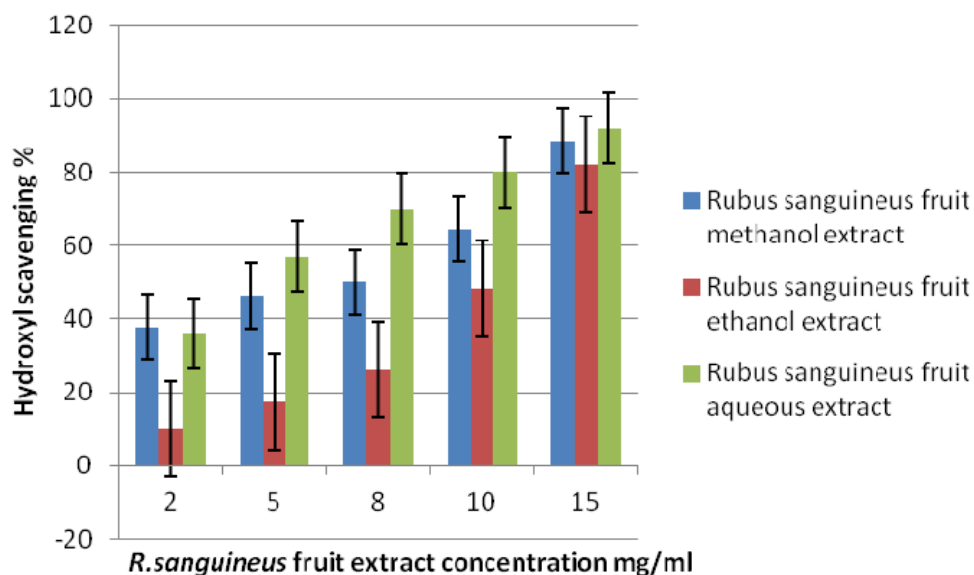


Figure 8. Hydroxyl scavenging activity of methanolic, ethanolic and aqueous extracts of *R. sanguineus* fruits.

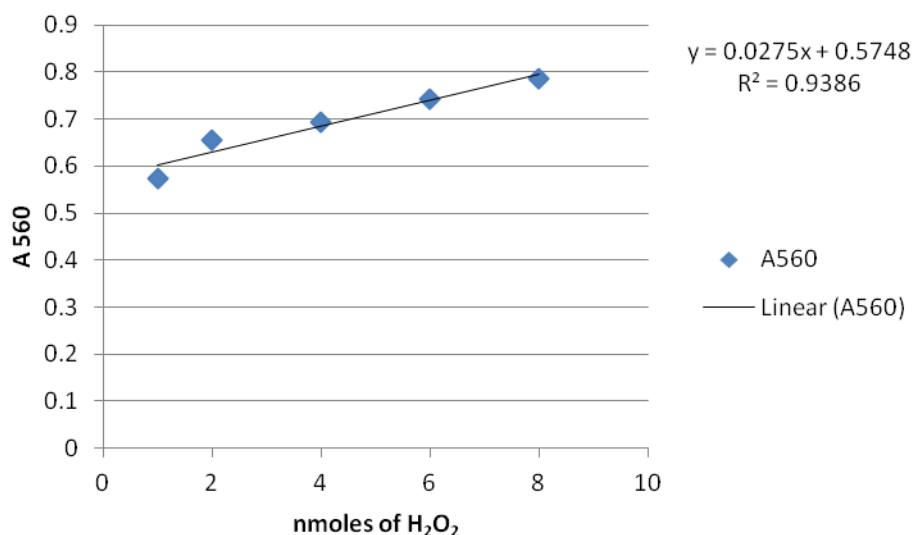


Figure 9. Standard curve of nmoles of H_2O_2 against absorbance at 560 nm.

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 peroxy radicals.

Lipid peroxidation assay

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

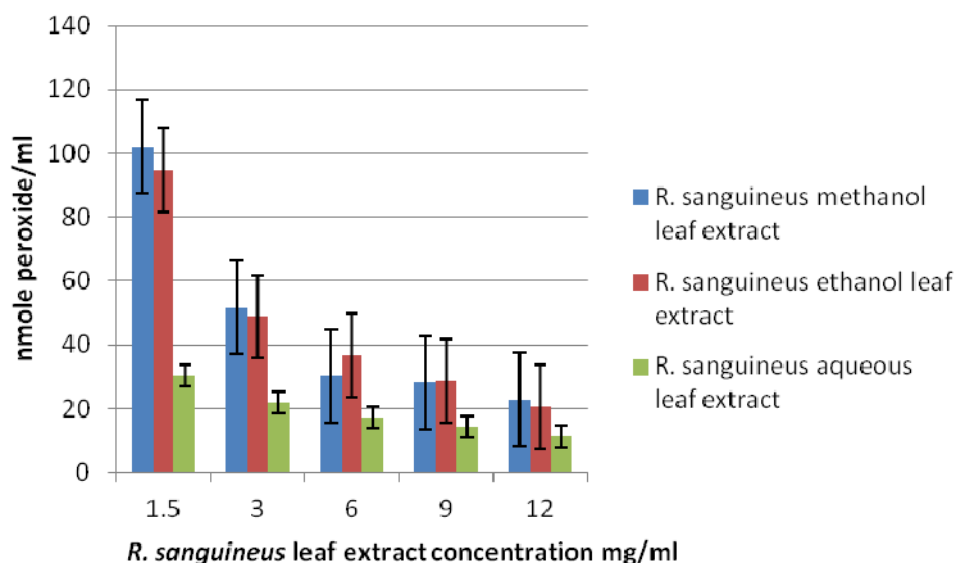


Figure 10. nmole of aqueous peroxide in *R. sanguineus* leaf extracts.

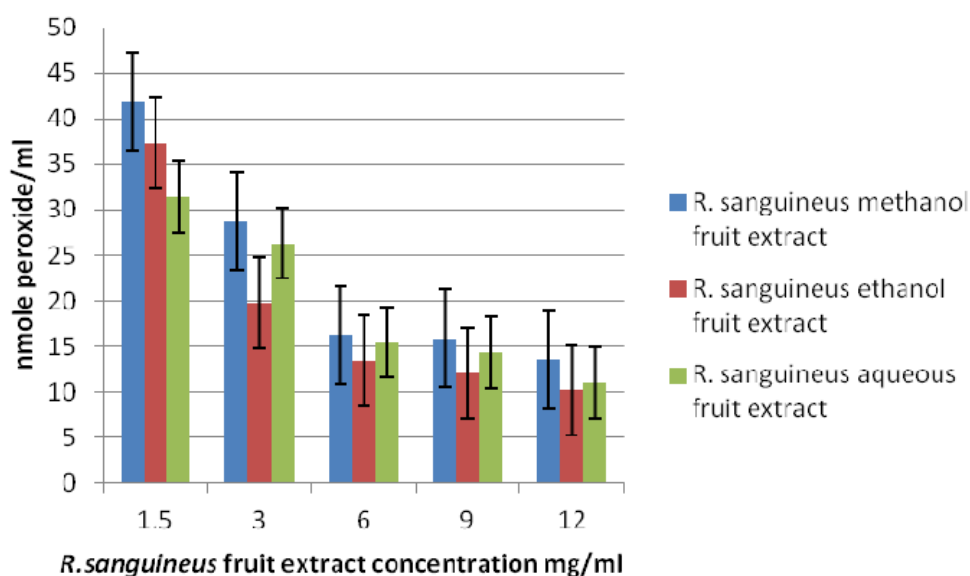


Figure 11. nmole of aqueous peroxide in *R. sanguineus* fruit extracts.

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 peroxidation 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,

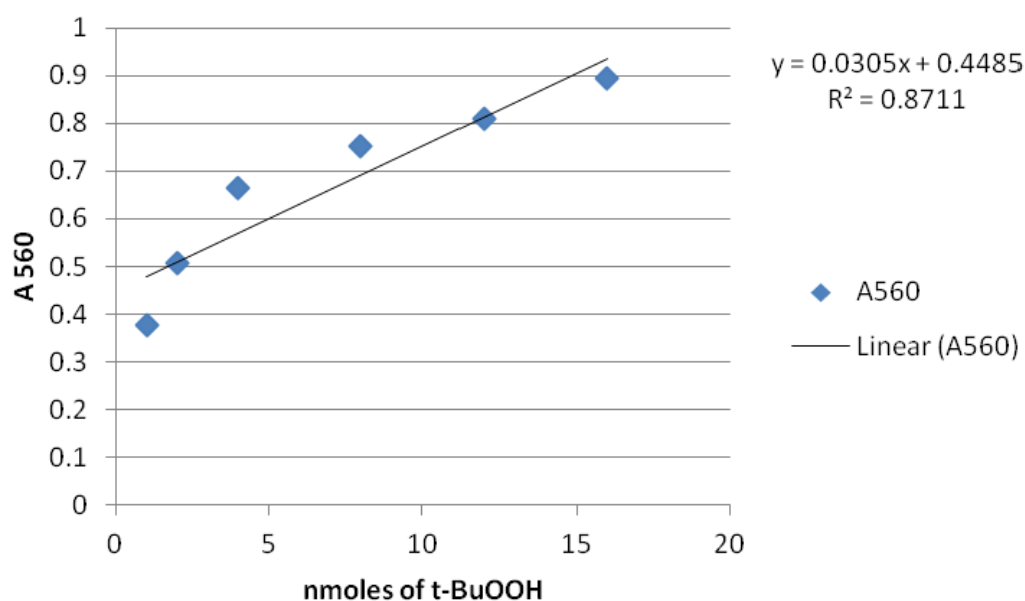


Figure 12. Standard curve of nmoles of t-BuOOH against absorbance at 560 nm.

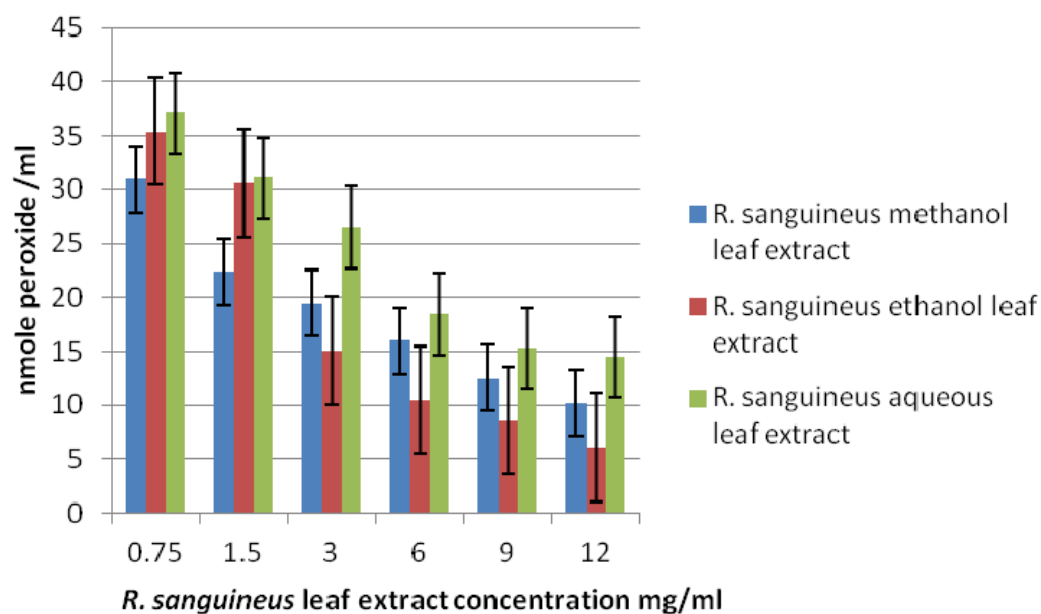


Figure 13. nmole of organic peroxide in *R. sanguineus* leaves extracts.

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

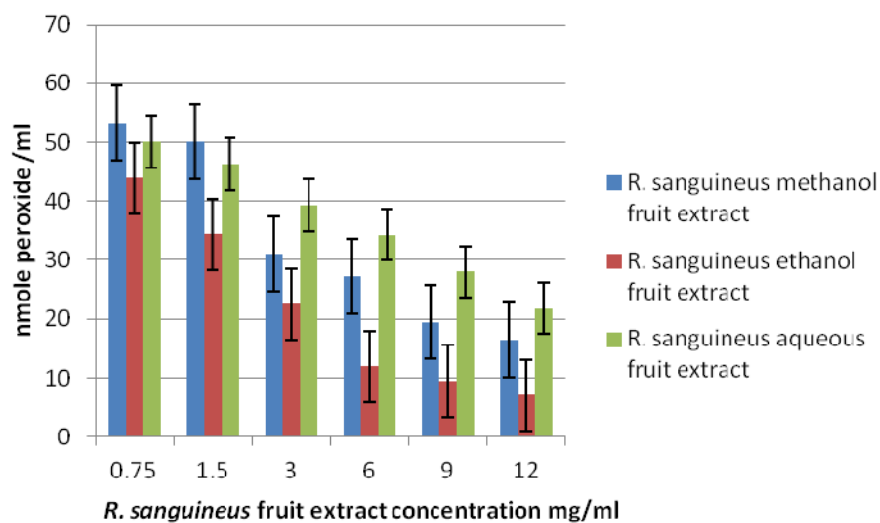


Figure 14. nmole of organic peroxide in *R. sanguineus* fruits extracts.

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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