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Evaluation of the Antisickling Property of a Hydro-Alcohol Root Extract of *Rauwolfia vomitoria* Afzel (Apocynaceae)

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ABSTRACT

Background and Purpose: Sickle cell disease (SCD) is a genetic disorder of the haemoglobin molecule with high morbidity and mortality. Parts of *Rauwolfia vomitoria* Afzel (Apocynaceae) are used in herbal medicine for the treatment of several ailments including SCD. This study evaluated the antisickling property of its hydro-alcohol root extract (HAE).

Methods: The roots were pulverised and extracted in ethanol and distilled water (1:1.2) to obtain 5.62% w/w of HAE. The antisickling property of HAE was evaluated using blood samples from SCD patients who were not in crisis by reduction in the percentage of sickled red blood cells (RBCs) and percentage of haemoglobin polymerization as parameters. In vitro and in vivo antioxidant properties of HAE were evaluated using standard methods.

Results: The extract significantly (P<0.005) reduced the percentage of sickled RBCs over a time range of 45 to 180 min and at concentrations ranging from 0.313 mg/mL to 2.5 mg/mL. The concentration of 1.25 mg/mL gave the highest relative haemoglobin polymerisation inhibition between 12th and 24th min and values were significant when compared with those obtained with para-hydroxybenzoic acid, the standard. HAE showed in vitro antioxidant property but less than ascorbic acid which was used as the standard. Only an acute dose of 200 mg/kg of HAE significantly increased serum catalase activity.

Conclusion: The extract possesses antisickling property which may not be dependent on its antioxidant property.

INTRODUCTION

Sickle cell disease (SCD) is the general name for all diseases associated with the haemoglobin molecule. The disease attracts global attention as a major threat to public health because of its impact on individual sufferers and society at large (Ashley-Koch *et al*., 2000). The abnormal characteristics of the red blood cell has been shown to be because of genetic mutation of the sickle beta-haemoglobin molecule at the 6th position where glutamic acid is replaced by the amino acid valine (Adewoyin, 2015) thus reducing the haemoglobin-oxygen affinity (Mpiana et al., 1996) with consequent increase in the red cell deformability (Pierre *et al*., 2015). The African continent is regarded as the origin and a major hub of SCD burden especially the tropical regions of West and Central Africa, although epidemiological evidence shows that the number of individuals suffering from SCD among persons of Mediterranean, Caribbean, South and Central American, Arab, and East Indian ancestry are very significant (Piel *et al*., 2013; Mpiana *et al*., 2014). As millions of people are afflicted with the disease globally, the number of babies born with SCD worldwide is estimated to be around 300,000 and this figure is likely to increase to 400,000 by the year 2050 (Piel *et al*., 2013; Uyoga *et al*., 2019). In Nigeria, SCD prevalence is said to be at 20-30 per 1000 live births (Adegoke *et al*., 2015) and about 45 million Nigerians or about 25% of the country's population are "carriers" of the sickle cell trait (Fleming *et al*., 1984). It has been reported that approximately 2% of neonates born in Nigeria are born with sickle cell anaemia and over 100,000 infant deaths are recorded annually due to the disease (Galadanci *et al*., 2013; Oyenike *et al*., 2019).

While many claims of cure for SCD abound, none is officially acceptable or listed. Nevertheless, there are useful drugs for the management of pain and other complications of the disease with appreciable clinical outcome and cost effectiveness. Synesthetic and formulated therapeutic antisickling agents act at unique stages in the sickling mechanism (Dash *et al*., 2013) and they are all associated with toxicities to various degrees (Abere *et al*., 2014) that adversely impact on sufferers' quality of life. Findings have revealed that many medicinal plants have both antisickling and antioxidant properties (Abere *et al*., 2014; Afolabi *et al*., 2016). It has also been reported that extracts of stem and root barks of *R. vomitoria* possess antioxidant activities (Yondo *et al*., 2009; Erasto *et al*., 2011) and inhibitory effects on lipid peroxidation (Okoli *et al*., 2011) thus suggesting the potential of R. vomitoria as an antisickling medicinal plant. The plant R. vomitoria or poison devil's pepper is known by many indigenous names such as *asofeyeje* by the Yorubas in western Nigeria, *akkemta* by the Igbos, wada by the Hausas, *mmoneba* by the Efiks, and *utoenyin* by the Ibibios (Okereke *et al*., 2017) and is found mainly in the wild in Africa. While the search for effective medicines for SCD is a continuous process, the present study was aimed at determining whether a hydro-alcohol extract of *R. vomitoria* possessed antisickling properties and the likely mechanisms that could be involved.

MATERIALS AND METHODS

The roots of *R. vomitoria* were collected between August and September 2015 from the Botanical Garden of University of Ibadan, Ibadan, Oyo State, Nigeria. The specimen was authenticated by Dr Grace Ugbabe of Department of Medicinal Plant Research and Traditional Medicine, National Institute of Pharmaceutical Research and Development (NIPRD), Idu, Abuja. A voucher specimen (NIPRD/H/6715) was kept in the NIPRD herbarium, Abuja. The roots were carefully cleaned, shade-dried and pounded to coarse powder using mortar and pestle before further pulverisation with a milling machine.

Preparation of Hydro-alcohol Extract (HAE) of *R. vomitoria*

The method of Egunyomi et al. (2009), with slight modification, was used. The root powder of *R. vomitoria* (800 g) was soaked in a mixture of solvents consisting of 3 L of distilled water and 3.6 L of 95% ethanol inside a 5-L sealed flat bottom flask for five days with intermittent stirring using a spatula. The extract was thereafter filtered with Whatman filter paper, grade 1 (pore size = 11 µm). The filtrate was concentrated to dryness over a water bath at 70ºC to obtain the hydro-alcohol extract (HAE) of 5.62%w/w (yield), and subsequently stored in a labelled sample bottle at 4ºC.

Animals

Wistar rats of weigh range 168 ± 29 g (Mean ± SD) and of both sexes were purchased from the Animal House of the Faculty of Pharmacy, University of Benin, Benin City, Nigeria. The animals were kept in standard plastic cages and had free access to water and feeds (Livestock Feeds Ltd, Ibadan, Nigeria). All the animals were exposed to natural lighting and room temperature and were handled according to international protocols for the use of laboratory animals (National Institute of Health, USA: Public Health Service Policy on Humane Care and Use of Laboratory Animals, 2002). Ethical approval with reference number: EC/FP/021/01 was obtained from the Ethics Committee, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. The animals were randomly assigned to experimental groups.

Ethical Approval for Human Blood Sample Collection

Ethical approvals with reference numbers FHREC/ 2015/01/05/04-02-15 and FCT/UATH/HREC/PR/574 were obtained from Ethical Review Committee, Human and Health Services Secretariat of Federal Capital Territory (FCT) Administration, Abuja, Nigeria, and University of

Abuja Teaching Hospital, Gwagwalada, Nigeria, respectively.

Preparation of Erythrocyte Suspension

The preparation of erythrocyte suspension was carried out using the method described by Egunyomi *et al.* (2009). Blood samples (3 - 5 mL) were collected from the median cubital vein of 10 stable, not-in-crisis homozygous sickle cell patients, who were attending the Sickle Cell Clinic at the University of Abuja Teaching Hospital, Gwagwalada, Nigeria, by a certified physician, into sodium EDTA bottles. The samples were centrifuged (Eppendorf, Germany) at 4000 rpm for 5 min to sediment the red blood cells (RBCs). The supernatant plasma was then carefully pipetted out of each bottle and the sediment of RBCs was washed three times with normal saline before re-suspending in saline solution equivalent to the pipetted plasma volume and stored at between 2 - 4°C in refrigerator for subsequent use.

***In Vitro* Antisickling Experiments**

Preparation of Stock Solutions for Antisickling Tests

A quantity (400 mg) of HAE was reconstituted in 20 mL of distilled water to obtain a 20 mg/mL extract solution from which dilutions of 5, 2.5, 1.25, 0.625 and 0.313 mg/mL were prepared.

Microscopic Characterization of Red Blood Cells

The microscopic characterization of red blood cells was carried out using the methods described by Moody *et al.* (2003) and Egunyomi *et al.* (2009) with slight modification. An aliquot (0.5 mL) of washed RBCs was gently mixed with 0.5 mL of the various concentrations of HAE (0.313, 0.625, 1.25, 2.5, and 5 mg/mL) in uncovered test tubes. Each mixture was mixed thoroughly with 0.2 mL of 2% sodium metabisulphite to deoxygenate the system, sealed with liquid paraffin, and incubated at 37^{o}C. At 45, 90, 135, and 180 min of incubation, 0.2 mL of the deoxygenated mixture of washed RBCs, HAE and sodium metabisulphite was taken to prepare thin smears in triplicates on frosted microscopic slides. The remaining mixture was incubated again at 37^{o}C each time after obtaining smear on frosted microscopic slides. The smear samples were fixed with 99.5% methanol, allowed to air-dry, stained with Giemsa and viewed under oil immersion light microscope (x 1000 magnification). The percentage of unsickled RBCs was calculated by counting the number of both sickled and unsickled RBCs from five different fields until at least 500 unsickled RBCs were counted. For the positive and negative controls, 0.5 mL of para-hydroxybenzoic acid (5 mg/mL) and normal saline were used respectively instead of HAE and blood smear samples were also obtained in triplicates at 45, 90, 135 and 180 min as was done for HAE. The mean percentage of sickled RBCs obtained in smear samples of HAE was compared to that of the controls.

Determination of Haemoglobin Polymerization Inhibition Potential

A slight modification of the method of Ojiako *et al.* (2012) was employed. About 3 mL of distilled water was pipetted into a cuvette, shaken gently and inserted into spectrophotometer (Jenway, UK). The absorbance was read at 700 nm and the instrument calibrated. A mixture of 2% sodium metabisulphite solution (4.4 mL) and HAE (0.5 mL of the various concentrations) were carefully mixed with 0.1 mL of washed RBC suspension, in an open test tube. An aliquot (3 mL) of this mixture was quickly (within one minute of mixing) pipetted into a cuvette, shaken gently, and absorbance was read at 2 min interval for 30 min. This process was repeated for positive control: a mixture of 0.5 mL of para-hydroxybenzoic acid and 2% sodium metabisulphite (4.4 mL) gently mixed with 0.1 mL of washed erythrocyte haemolysate, and the negative control: normal saline. All absorbance values were means of two readings. The percentage polymerization inhibition relative to normal saline was estimated for various concentrations of HAE and para-hydroxybenzoic acid according to the following equation (Chikezie *et al*., 2011):

$$\%\ Polymerization\ =\ \frac{\left\{\left(\frac{At}{Ac}\right)\times 100\right\}}{A_{T30}}$$

At = Absorbance of test substance at time t; Ac = Absorbance of normal saline at correspondent time; A_{T30} = Absorbance of normal saline at 30th min.

Evaluation of In Vitro Antioxidant Properties of HAE

Diphenyl-2-picryl-hydrazyl (DPPH) assay was done using the method described by Gulcin and Alwasel (2023). A quantity (50 µL) each of concentrations 10, 20, 40, 60 and 80 µg/mL of HAE were added to 1.0 mL of a methanol solution of DPPH, gently mixed at room temperature for 30 min in the dark and the absorbance of the mixture was determined by spectroscopy (T80+ UV/VIS Spectrophotometer, PG Instruments Ltd, UK) at 518 nm against a solution of DPPH in methanol (1.05 mL) as control. The radical scavenging activity was calculated as follows:

$$Radical\ Scavenging\ Activity\ (\%)\ =\left(\frac{AbsControl-\ AbsTest}{AbsControl}\right)\times 100$$

AbsTest = absorbance of extract in methanol solution of DPPH.

ABTS Radical Cation Decolourization Assay

ABTS radical cations ($ABTS^{+}$) were generated by reacting ABTS solution (7 mM) with 2.45 mM potassium persulfate (1:1). The reacting mixture was stored in the dark at room temperature for 12-16 h before use. The $ABTS^{+}$ solution was diluted with methanol to obtain an absorbance of 0.700

at 745 nm. Aliquots (50 µL) of various concentrations of the HAE (10, 20, 40, 60 and 80 µg/mL) were added to 300 µL of $ABTS^+$ solution in 1 mL methanol. The mixture was left to stand for 30 min and its absorbance read at 745 nm (T80+ UV/VIS Spectrometer, PG Instruments Ltd, UK). Methanol (1 mL) was used as blank, and all measurements were carried out in triplicates. The percentage inhibition of absorbance at 745 nm was calculated using the formula below (Re *et al*., 1999; Shirwaikar *et al.,* 2006):

$$ABTS^+ \text{ scavenging effect } (\%) = \left(\frac{AbsControl - AbsTest}{AbsControl} \right) \times 100$$

AbsControl = absorbance of $ABTS^+$ solution in methanol, AbsTest = absorbance of extract in methanol solution of $ABTS^+$.

Hydroxyl Radical Scavenging Assay

A reaction mixture containing 0.1 mL of 2.8 mM deoxyribose, 0.1 mL of 0.1 mM $FeCl_3$, 0.1 mL of 0.1 mM EDTA, 0.1 mL of 1 mM H_2O_2, 0.1 mL of 0.1 mM ascorbate, 0.1 mL of KH_2PO_4 - KOH buffer (20 mM, pH 7.4) and 20 µL of HAE (20-80 µg/mL) was added to 1.0 mL of methanol and incubated at 37°C for 1 h. After incubation, the mixture was boiled with 1.0 mL of thiobarbituric acid (TBA) at 95°C for 20 min. The mixture was cooled and the absorbance read at 745 nm using a spectrophotometer (T80+ UV/VIS Spectrometer, PG Instruments Ltd, UK). Ascorbic acid was used as positive control, while methanol served as the blank. The percentage hydroxyl radical scavenging activity was estimated as shown below (Kunchandy and Rao, 1990):

$$OH^{\bullet} \text{ scavenging effect } (\%) = \left(\frac{AbsControl - AbsTest}{AbsControl} \right) \times 100$$

AbsControl = absorbance of ascorbic acid with thiobarbituric acid reactive substances (TBARS); AbsTest = absorbance of extract with TBARS.

Ferric Reducing Antioxidant Power Assay

The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM $FeCl_3 \bullet 6H_2O$ in ratio 10:1:1 at 37°C. Aliquots (50 µL) of HAE of different concentrations (20 to 80 µg/mL) were allowed to react with 39.95 mL of the FRAP reagent for 30 min in the dark. The absorbance of the coloured product formed was read at 593 nm (T80+ UV/VIS Spectrometer, PG Instruments Ltd, UK) against a reagent blank (39.95 mL FRAP reagent + 50 µL distilled water) after 30 min incubation at 37°C. A calibration curve was prepared by plotting the absorbance at 593 nm versus the different concentrations of the extract. The concentrations of the extract were in turn plotted against concentrations of the standard antioxidant (ascorbic acid). The values of FRAP were expressed as mM ascorbic acid/g dry mass of extract (Benzie and Strain, 1996; Prior *et al*., 2005).

***In Vivo* Antioxidant Experiments**

Experimental Groups and Obtaining of Sera

Thirty Wistar rats comprising 15 males and 15 females were randomly allotted to five groups (n=6; male = female). Group A served as the negative control and received 0.5 mL of normal saline orally while group B served as the positive control and was given 100 mg/kg of ascorbic acid orally. The animals in groups C, D, and E were given 50, 100, and 200 mg/kg of HAE. All the animals were fasted overnight before the commencement of the experiment. One hour after treatment and under anaesthesia, blood samples were collected from the abdominal aortae using a 5 mL syringe fitted with 21G needle, into plain bottles. Each blood sample was allowed to clot for 30 min and then centrifuged at 2500 rpm for 15 min and the clear serum was harvested with the aid of a Pasteur pipette (Yesufu *et al*., 2010). All sera were stored at -20°C overnight and used for the *in vivo* antioxidant assays.

Estimation of Thiobarbituric Reacting Substances (Malondialdehyde Assay)

The estimation of thiobarbituric reacting substances (TBARS) was carried out using the thiobarbituric acid (TBA) method described by Varshney and Kale (1990). Briefly, 2 mL of the TBARS mixture containing trichloroacetic acid (15 g/100 mL), thiobarbituric acid (0.375 g/100 mL) and hydrochloric acid (0.25 N) was boiled with 1.0 mL of serum for 15 min in a test tube and allowed to cool. The cooled mixture was centrifuged at 3500 rpm for 10 min; the supernatant was removed with the aid of Pasteur pipette and its absorbance read at 535 nm. For the blank, 2 mL of TBARS mixture was mixed with 1.0 mL of distilled water and the absorbance read at 535 nm.

Estimation of Catalase Activity

A reagent mixture containing 2.5 mL of hydrogen peroxide and 0.5 mL sulphuric acid was reacted with 0.25 mL of rat serum in a test tube. An aliquot (3.5 mL) of potassium permanganate was added to the reacting mixture in the test tube, the absorbance was read immediately and after 3 min at 480 nm. For the blank, 0.25 mL of phosphoric acid (H_3PO_4) was substituted for rat serum (Susmitha *et al*., 2015).

Estimation of Superoxide Dismutase (SOD) Activity

An aliquot (0.3 mL) of adrenaline was added to a mixture of carbonate buffer (2.5 mL, 0.05 M $NaHCO_3$) and 0.2 mL of serum sample. The absorbance was read immediately after adding adrenaline at 0, 30 and 60 s at 420 nm. A mixture containing carbonate buffer and adrenaline was

used as a blank. The SOD activity was calculated as shown below (Misra and Fridovich, 1972):

$$SOD\ Activity = \frac{\%INHIBITION}{50y}$$

y = mg protein in the volume of sample.

Statistical Analysis

All results are expressed as the mean ± standard error of mean (SEM). Comparisons were made between groups by use of one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. All data were analyzed using GraphPad Prism software version 5 (GraphPad Prism, USA). $P < 0.05$ indicates a statistically significant difference.

RESULTS

Antisickling Effect of HAE on Human Red Blood Cells

Percentage of Sickled Red Blood Cells in the Presence of HAE

The mean percentage of sickled RBCs produced at the end of 180 min was 29.39±0.50, 20.50±0.17, 20.61±0.30, 20.18±0.21, 20.51±0.27, and 19.17±0.14 for normal saline, para-hydroxyl-benzoic acid, 0.313, 0.625, 1.25, and 2.5 mg/mL of extract respectively. The extract and positive control (para-hydroxyl-benzoic acid) showed significantly (P<0.0001) lower percentage of sickled RBCs in a concentration dependent manner compared to normal saline at the same time points (Figure 1). There was no significant difference in the level of inhibition between the concentrations except for 2.5 mg/mL of extract that showed significantly (P<0.005) higher inhibitory activity against RBC sickling compared to 1.25 mg/kg at all the time points. The percentage of sickled RBCs estimated for same concentration of extract at different time points was not significantly different from one another.

Effect of HAE on Haemoglobin Polymerization

Figure 2 shows that the mean percentage inhibition of polymerization by 0.625 mg/mL HAE was the highest for the first 10 min within the range of 40.3 and 39.06 % in 8 min (between 2nd and 10th min) before taking a sharp decline at 10th min to reach 32.8% at 18th min and thereafter remained relatively constant till the 30th min. The polymerization inhibitory effects of 0.313 mg/mL of HAE increased by 2.2% in 8 min (between 2nd and 10th min) before taking a steady decline to reach 34% at 30th min. However, the inhibitory effects of 1.25 and 2.5 mg/mL HAE was relatively constant from the 10th min to the 30th min values of 1.48% and 0.78% decline respectively. The maximum percentage inhibition of 39.22±0.18 occurred with 1.25 mg/mL HAE at 2 min reaction time while 0.625 mg/mL demonstrated least inhibition at 16 min against polymerization of haemoglobin in RBCs. The mean percentage inhibition of polymerization for PHBA (positive control), 0.313, 0.625, 1.25, and 2.5 mg/mL of HAE was 36.14±0.21, 36.60±0.05, 36.18±0.37, 37.94±0.12, and 36.41±0.06 respectively. The mean percentage inhibition of polymerization (relative to normal saline) by various concentrations of HAE after 30 min of incubation was significant (P<0.0001) and the inhibitory effects due to 1.25 mg/mL was significantly (P<0.05) higher compared to 2.5 and 0.625 mg/mL of HAE.

In Vitro Antioxidant Properties

Effects of HAE on DPPH Radical

The inhibition of DPPH radical by HAE was concentration dependent with an EC_{50} value of 5.08 µg/mL while that of standard was 1.95 x 10^{-5} µg/mL (Figure 3). Although the standard had a lower EC_{50} value, its scavenging effect only increased marginally with increasing concentration as 1% increase in concentration only produced 0.05% increase in scavenging activity whereas HAE caused 0.51% increase in scavenging activity for every 1% rise in concentration.

Effects of HAE on Hydroxyl Ion Radical

Figure 4 shows that the hydroxyl ion scavenging activity of HAE and ascorbic acid (positive control) was concentration dependent. The minimum scavenging activity by HAE and ascorbic acid was 43.12% and 39.81% respectively. HAE possessed highest scavenging activity of 81.38% at 80 µg/mL. The graph also shows that at a concentration of approximately 60.00 µg/mL of either HAE or ascorbic acid exhibited 70.37% scavenging activity. Although HAE had a higher IC_{50} value (1.06 µg/mL versus 0.15 µg/mL) and consequently lower scavenging activity, its hydroxyl ion scavenging effect seems to be more sensitive to concentration change compared to ascorbic acid after 60 µg/mL concentration.

Effects of HAE Extract on ABTS Radical

Figure 5 shows that both HAE and ascorbic acid inhibited ABTS radical formation in a concentration-dependent manner. However, the inhibitory effect of ascorbic acid on ABTS radical was higher than that of HAE (IC_{50} of 4.46 x 10^{-5} µg/mL versus 1.24 x 10^{-2} µg/mL.

Ferric Reducing Antioxidant Power (FRAP) of HAE

The ferric reducing antioxidant power of HAE appears to have remained constant between 20 and 60 µg/mL (log 1.301 and log 1.778 respectively) and later increased marginally at 80 µg/mL (log 1.903) but was not concentration dependent. On the other hand, the FRAP of ascorbic acid was concentration dependent reaching about 225% at 80 µg/mL (log 1.903). The calculated IC_{50} for HAE and ascorbic acid was 9.45x10^{-11} µg/mL and 6.19x10^{-4} µg/mL respectively. HAE demonstrated higher ferric reducing power compared to reference standard and this seems unaffected by increase in concentration (Figure 6).

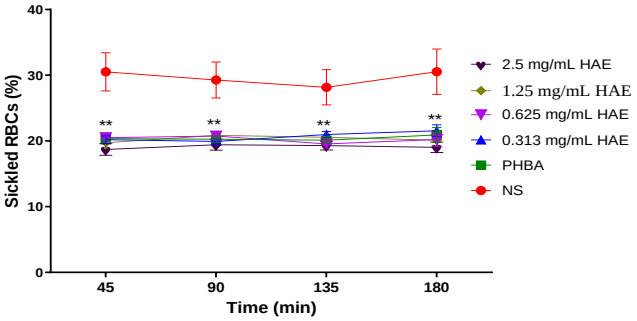

Figure 1: Antisickling effect of hydro-alcohol root extract (HAE) of *R. vomitoria* on human red blood cells (RBCs). ***P*<0.005 versus normal saline (NS) at the same time points. PHBA, p-hydroxyl benzoic acid. n = 4 replicates.

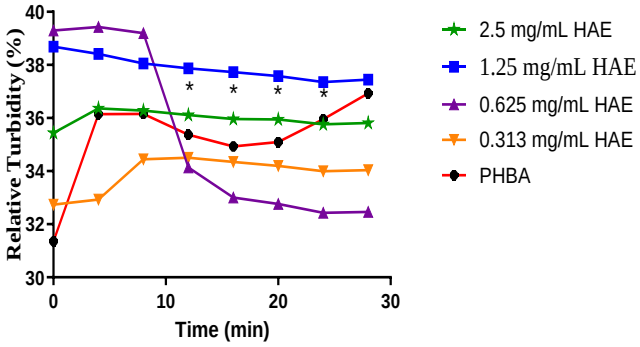

Figure 2: Effect of hydro-alcohol extract of *R. vomitoria* on haemogbobin polymerization in sickled red blood cells. PHBA: p-hydroxyl benzoic acid (PHBA). **P*<0.05 relative to normal saline. n = 4 replicates.

In vivo Antioxidant Properties

Effect of HAE on Thiobarbituric Reactive Substances

The mean level of thiobarbituric acid reactive substances (TBARS) in the serum of rats treated with normal saline or ascorbic acid was 2.50±0.25 x 10^{-3} mg/mL and 2.4±0.22 x 10^{-3} mg/mL respectively. Groups of rats given 50, 100, and 200 mg/kg had 2.20±0.09 x 10^{-3} mg/mL, 2.2±0.22 x 10^{-3} mg/mL and 2.6±0.54 x 10^{-3} mg/mL respectively in their serum samples. The highest level of TBARS (2.6±0.54 x 10^{-3} mg/mL) was recorded among the group given 200 mg/kg of HAE while the group given 50 mg/kg had lowest level of 2.2±0.09 x 10^{-3} mg/mL of TBARS. There was no statistically significant difference in serum levels of TBARS among the treated groups and the controls (Figure 7).

Effect of HAE on catalase activity

Figure 8 shows that the catalase activity in groups of rats given normal saline and ascorbic acid was 0.51±0.08 and 0.84±0.19 Units/mL respectively. The animals in groups C, D and E (50 mg/kg, 100 mg/kg and 200 mg/kg) had mean catalase activities of 0.28±0.14, 0.39±0.10 and 0.45±0.07 Units/mL respectively in their serum samples. The animals that received 200 mg/kg of HAE (Group E) possessed the highest catalase activity (0.45±0.07 Units/mL) while animals that received 50 mg/kg (Group C) had the least catalase activity (0.28±0.14 Units/mL). All the investigated doses possess higher catalase activity compared to normal saline and ascorbic acid. The differences in catalase activity of all investigated doses of HAE compared to normal saline were not statistically significant. However, the catalase activity of 50 mg/kg dose of HAE was significant compared to ascorbic acid ($P < 0.05$), although, the catalase activity of the 100 mg/kg and 200 mg/kg doses were not significant compared to ascorbic acid

Effect of HAE on Superoxide Dismutase Activity

The mean serum SOD activity in rats given normal saline and ascorbic acid (Groups A and B) was 1.69±0.04 and 1.70±0.03 Units/mL respectively. The rats in group C, D and E (50 mg/kg, 100 mg/kg and 200 mg/kg) had mean SOD activity of 1.71±0.05, 1.62±0.08 and 1.66±0.06 Units/mL respectively. The animals that received 50 mg/kg of HAE possessed highest serum SOD activity (1.71±0.05 Units/mL) while group D animals (100 mg/kg) had the least serum SOD activity (1.62±0.08 Units/mL). There were no significant differences in serum SOD activity between each group and control (Figure 9).

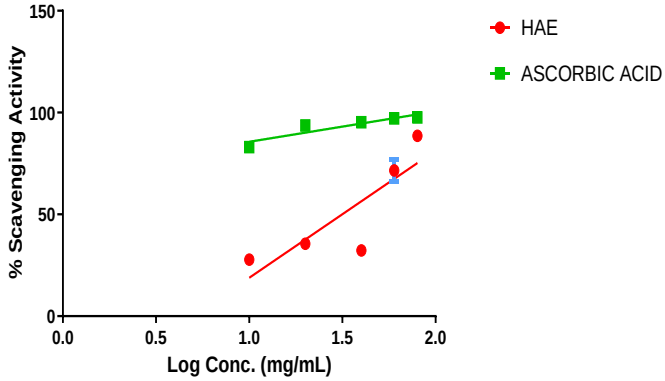

Figure 3: Scavenging activity of HAE of *R. vomitoria* on DPPH radical. n=4 replicates.

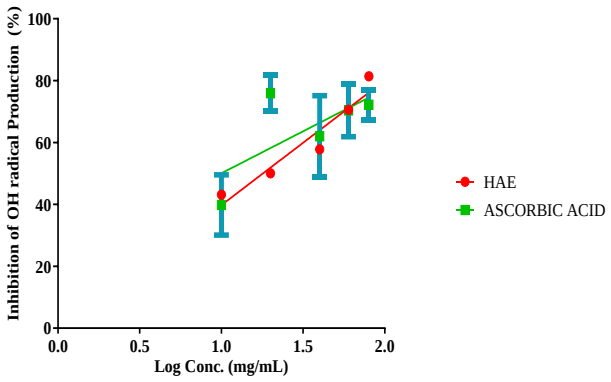

Figure 4: Inhibitory activity of HAE of *R. vomitoria* on hydroxyl radical production. n=4 replicates.

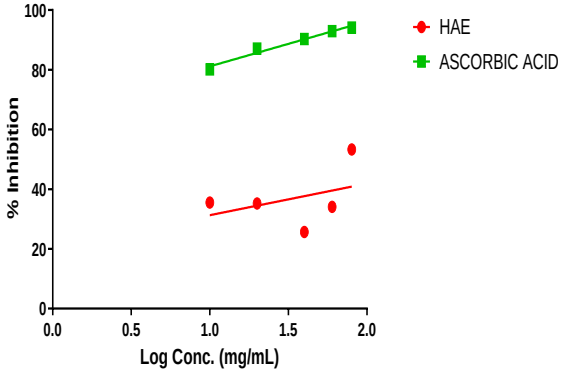

Figure 5: Inhibitory activity of HAE on ABTS radical production. n=4 replicates.

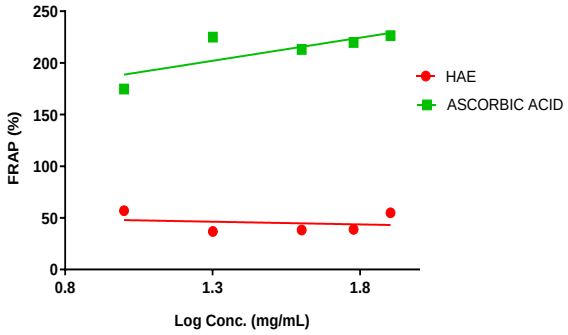

Figure 6: Ferric reducing antioxidant power of HAE. n=4 replicates.

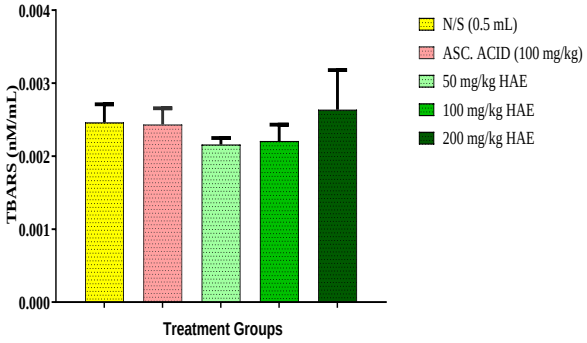

Figure 7: Effect of HAE on serum TBARS levels. No significant difference in mean TBARS level in all the treatment groups. n=4 replicates.

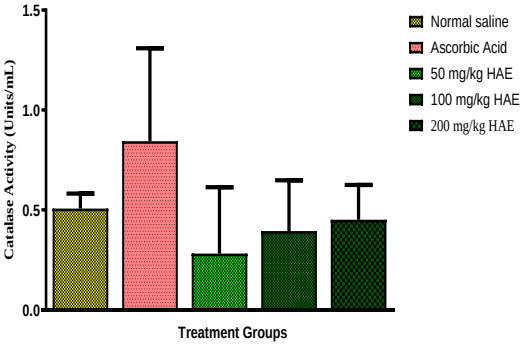

Figure 8: Serum catalase activity of rats administered with HAE of *R. vomitoria*. n=4 replicates.

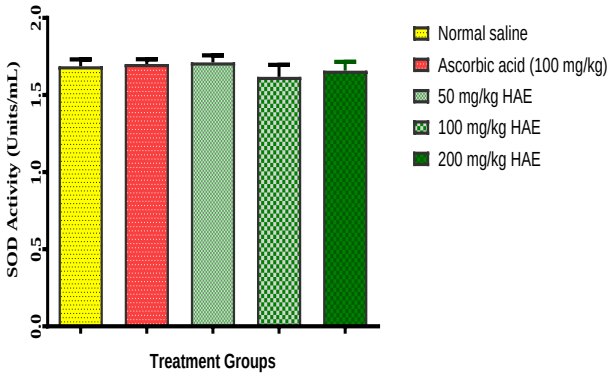

Figure 9: Effect of HAE serum SOD activity in rat. No significant difference in SOD activity values in all the treatment groups. n=4 replicates.

DISCUSSION

The findings from this study showed that the hydro-alcohol root extract (HAE) of *R. vomitoria* possesses significant antisickling activity based on its ability to inhibit sickling of red blood cells and polymerization of sickled haemoglobin. The extract of *R. vomitoria* also exhibited *in vitro* antioxidant activity (higher DPPH and ferric reducing power effects) compared to ascorbic acid. However, the *in vivo* antioxidant activity was not significant in all the investigated doses when compared with normal saline and ascorbic acid.

The observed antisickling activity in this study is consistent with the results obtained from previous study that evaluated the antisickling activity of *R. vomitoria* leaves (Abere *et al*., 2014). The non-concentration dependence of the antisickling activity observed in our study may be due to solubility/stability of the constituent phytochemicals in HAE thus antisickling phytochemicals could not interact with the 'sticky patch' contact points of the deoxyHbS to cause its dissolution to reverse the sickling of RBCs (Chikezie *et al.*, 2011). The observed decline in the antisickling activity of the extract towards the 180 min mark differs from the findings of Egunyomi *et al.* (2009) in which the antisickling activities of an herbal remedy that contained *R. vomitoria* root as one of the components showed increased antisickling activity as incubation time progressed. The reason for the difference in observation may be due to the multiple medicinal plants in the herbal remedy. The mechanism of antisickling effects of various medicinal plants has been investigated and documented (Ohiagu *et al*., 2021), but our study seems to be the first attempt to investigate the mechanism of antisickling effects of *R. vomitoria.*

Findings from this study revealed that the ability of HAE to scavenge hydroxyl, DPPH, and ABTS radicals was lower in comparison with ascorbic acid but was concentration dependent. This observation compares with the results obtained by Adu *et al.* (2015) who reported lower scavenging activity of DPPH radical by *R. vomitoria* bark and leaf extracts when compared to ascorbic acid. However, another study reported that the alcohol extract of *R. vomitoria* root exhibited significantly higher hydrogen peroxide scavenging activity and ferric reducing capacity when compared to ascorbic acid (Okolie *et al.*, 2011). These findings differ from the ones in this study as the hydro-alcohol root extract of *R. vomitoria* demonstrated lower ferric reducing antioxidant power compared to ascorbic acid, in a concentration dependent manner. The different observations in both studies could be due to the solubility and relative abundance of active antioxidant principles in the hydro-alcohol extract (Javid *et al*., 2024). The implication of these observations is that HAE possesses *in vitro* antioxidant properties which may contribute to its anti-sickling effects (Egunyomi *et al*., 2009).

The disparity between systemic production and build-up of reactive oxygen species vis-à-vis efficiency of the intrinsic detoxification process has been implicated in the pathophysiology of sickle cell disease (Möckesch *et al.*, 2017). Reports show that patients with sickle cell anaemia have higher levels of thiobarbituric reacting substance (malondialdehyde levels), low levels of catalase, superoxide dismutase, nitric oxide, and total antioxidant capacity compared to individuals who are not suffering from the disease (Ama Moor *et al*., 2016; Okorie *et al*., 2018). The changes in the concentrations of these indicators of oxidative stress can be monitored and used to test the suitability of any agent or extract for potential clinical benefits as antioxidant therapy against sickle cell disease. The *in vivo* antioxidant analysis indicated that antioxidant properties of *R. vomitoria* root extract was not significant in doses investigated in the study. This observation also suggests that HAE exhibited more *in vitro* rather than *in vivo* antioxidant activity due to pharmacokinetic challenges, whereby the plasma concentration of antioxidant principles in rat serum was insufficient to exert any appreciable effect. This assertion is supported by the fact that the animals were sacrificed one hour after being administered with the extract in which time absorption may have been incomplete and/or the induction of antioxidant enzymes may not have begun. The inference of this observation is that HAE may not exert antioxidant effects when given in *statim* doses; on the other hand, the antioxidant effects of the extract may be cumulative, suggestive of repeated use before any appreciable clinical benefit. This latter assertion seems plausible when considered against the background of instruction given to clients to take herbal remedy containing *R. vomitoria* every day even up to five times daily (Egunyomi *et al*., 2009).

In conclusion, the hydro-alcohol extract (HAE) of *R. vomitoria* possesses antisickling property as deduced by its effects on red blood cells and haemoglobin polymerization. The demonstrable *in vitro* and *in vivo* antioxidant activity of *R. vomitoria* is insufficient to explain its antisickling property. However, the antisickling effects may be maximised by repeated administration

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTION

VOA, RIO, and OAS developed the concept. VOA carried out the antisickling experiments, and the antioxidant studies were carried out by VOA and DOU. All the experiments were supervised by RIO and OAS. All the authors were involved in data collation and analysis. VOA drafted the manuscript which was revised by OAS and RIO. All the authors read and approved the final version of the manuscript before it was submitted for publication.

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AUTHORS' DECLARATION

We, the authors declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by us.

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