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Determination of the potentials of aqueous and ethanolic extracts of *Buchholzia coriacea* in preventing sodium metabisulphite-induced oxidation of some hemoglobin SS erythrocyte membrane proteins

Olasunmbo Oluwaseun Afolayan, Muhammad Nurudeen Nurudeen, Ayorinde Babatunde James[1], Abayomi Olugbenga Okanlawon

Abstract:

BACKGROUND: The sustenance of the erythrocyte membrane integrity is crucial to ensuring a continuous lamina flow of erythrocytes through narrow blood vessels. So it is imperative to find a substance to attenuate the oxidation of erythrocytes that cause the membrane structure remodelling.

OBJECTIVES: To investigate the hydrating strength and modulatory effect of *Buchholzia coriacea* seed extracts on erythrocyte membrane proteins degradation.

MATERIALS AND METHODS: Aqueous and ethanolic extracts of *B. coriacea* were obtained following standard procedures. Blood samples (5.00 mL) from five known hemoglobin SS individuals in steady state were subjected to electrophoresis to establish the genotype of the individuals. The osmotic fragility assay was performed with 2.50, 5.00, 10.00 and 20.00 mg/mL of the extracts in the presence of different concentrations of phosphate-buffered saline. The graph of percentage hemolysis was plotted against saline concentration, and the mean corpuscular fragility (MCF) determined. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was also done using the same concentrations of extracts.

RESULTS: There was no statistically significant difference ($P > 0.01$) between the average MCF in all the extract concentration and the control. Protein 4.1 and Ankyrin did not appear on the electrophoretic lanes of all the test samples when compared with the control. However, spectrin and ankyrin bands were visible with regular size and intensity.

CONCLUSION: *B. coriacea* seed extracts possess antihemolytic character. However, it does not have the ability to maintain the erythrocyte membrane model despite its acclaimed antioxidant character.

Keywords:

Corpuscular, denaturing, electrophoresis, fragility

Departments of Anatomy and [1]Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Lagos, Nigeria

Address for correspondence:
Dr. Muhammad Nurudeen Nurudeen, Department of Anatomy, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, P.M.B. 12003, Lagos, Nigeria.
E-mail: nurudeen muhammadn@gmail.com

Introduction

Buchholzia coriacea is a tropical plant used in traditional medicine (Ejikeugwu *et al*, 2015). It is a shrub, evergreen, with a dense crown, large glossy, and leathery leaves (Mbata *et al*., 2009; Enechi *et al*., 2013). It is also described as an evergreen understorey tree of forests (lowland rainforest). The tree is about 10–20 m high (Enechi, 2013; Umeokoli, 2016). It has

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a smooth and blackish stem bark with simple branching racemes. The bark slash of the seed is deep red and the sap exudes with a violently spicy pungent smell that causes sneezing (Lapshak *et al*., 2016. The flowers are cream in color, fruits are yellowish when ripe and seeds are blackish and spicy when tasted with deep red color when sliced with a knife. Considering the variety of antisickling agents acting at different levels of the sickling mechanism, there is still paucity of potent antisickling medicine (Abere and Okpalaonyagu, 2015). This situation inspired the setting up of this study on the reactivity of *B. coriacea* seeds extracts with erythrocytes obtained from subjects with sickle cell anemia.

The survival of practically all other cells in the body depends on the proper functioning of erythrocytes (Smith, 1987). A structurally balanced erythrocyte with membrane-bound hemoglobin (Hb) is required for the rapid oxygenation of Hb in the lungs and deoxygenation in the tissues supplied. Erythrocytes' ability to survive depends on the ability of the membrane to remain intact and flexible (Lux, 2015). Certain conditions such as sickle cell and hemolytic anemia that affect the functional and structural integrity of the erythrocytes' membrane may lead to an ineffective and short-lived cell (Weed and Reed, 1966; Dzierzak and Philipsen, 2013). According to the fluid mosaic model of the membrane structure proposed by Singer and Nicolson (1972), the plasma membrane is composed of a lipid bilayer of phospholipids arranged into a sheet that is two molecules thick and 45°A wide (Ballas and Mohandas, 2004). The phospholipid molecules are oriented in such a way that the hydrophobic nonpolar groups of the two layers are directed toward one another, forming lipid–lipid interactions (Singer and Nicolson, 1972). The hydrophilic polar groups are directed outward on both the extracellular and intracellular surfaces. Cholesterol is intercalated between the phospholipid molecules. The phospholipid bilayer forms a liquid-crystalline matrix or core of the red cell membrane (Ballas and Mohandas, 2004). The erythrocyte membrane contains approximately equal amounts of lipids and proteins (Ballas and Mohandas, 2004). The erythrocyte membrane also consists of two domains, a lipid bilayer and the cytoskeleton. The lipid domain is similar structurally to that found in most mammalian cells (Mankelow *et al*., 2012). However, the cytoskeleton differs from what is considered cytoskeleton in other cells because it does not contain the structural protein tubulin and is not involved in cell motility or phagocytosis (Smith, 1987). In essence, the architecture of this membrane skeleton as well as the nature of its intermolecular contacts determine the mechanical properties of the skeleton and confer the characteristic biconcave shape of red cells (Nans *et al*., 2011). The erythrocytes membrane of sickle Hb SS are osmotically and mechanically more fragile than those of Hb AA; hence, sickled red blood cells (RBCs) are easily destroyed and removed from circulation in the spleen thus causing anemia and subsequent splenomegaly (Parker and Orringer, 1990) (Vojdani *et al*., 2008). Hb S polymerization concentration also affects the integrity of the RBC membrane. Human erythrocyte skeleton is built in the form of a complex array of spectrin and actin which are linked to the membrane by protein 4.1 and ankyrin. The linking proteins also bind to glycophorin A and B and band 3 via the β subunit of Spectrin (Franco and Low, 2010). The overall arrangement of membrane and skeletal proteins is quite unique. Many transmembrane proteins are transporters but the skeletal framework of erythrocytes membrane is formed by the spectrin–actin complex which is highly durable and elastic (Lux, 2015). The interactions between spectrin and protein 4.1 are known to involve the ends of spectrin molecules on the opposite side of the oligomer-binding site. The crucial role of protein 4.1 in membrane stabilization and cell shape regulation is evident in erythrocyte hereditary spherocytosis and elliptocytosis (Alaarg *et al*., 2013). Its activities may be hindered by alteration in the normal amount or a total depletion. Ankyrin formerly known as "Protein 2.1" (Bennett and Stenbuck, 1979) is a globular phosphoprotein of molecular weight 215,000 Da by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) with a particular degree of hydrophobic residues intermediate between spectrin and band 3. It is also referred to as a polypeptide that connects the spectrin–actin network to the cytoplasmic part of band 3. Hence, it acts as a direct linkage between the membrane skeleton and the lipid bilayer (Franco and Low, 2010).

Wide spread claim of the antisickling qualities of various plants have not lead to the substantive use of a single plant material to prevent or reverse erythrocytes sickling. Similarly, the popular belief among Nigerians that *B. coriacea* plant part (s) is capable of providing cure for all ailments has ignited this probe into its antisickling potentials. This study is a pioneer finding on the reaction of sodium metabisulfite-induced sickling of Hb SS erythrocytes with *B. coriacea* seeds extract.

Materials and Methods

Chemicals/reagents

All the chemicals used in this study were of analytical grade and products of AnalaR-BDH Chemicals Ltd., Poole, England, May and Baker Chemicals Ltd., as well as Sigma Aldrich, USA.

Ethical approval

The research protocol used in this study was reviewed by the Health Research Ethics Committee (HREC) of the College of Medicine of the University of Lagos.

Thereafter, an approval letter with file number: CMUL/HREC/O7/17/216 was issued before the commencement.

Plant material

The seeds of *B. coriacea* were obtained from medicinal roots and herb traders in Mushin market, Mushin Local Government Area of Lagos state, Nigeria. Some of the fresh seeds were submitted to Dr. O. O. Oyebanji, a taxonomist in the Lagos University Herbarium (LUH), Department of Botany, Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria for identification and authentication. The plant was issued herbarium number LUH 7630. Voucher specimen was deposited at the LUH and the Department of Pharmacognosy, Faculty of Pharmacy of the University of Lagos, Lagos, Nigeria. The seeds were washed with clean tap water and afterward with distilled water to remove humus and sand materials attached to them. A sharp knife was used to slice the seeds into small cubes, air-dried at room temperature for 14 days during which they were constantly turned to avoid decay. The dried seed grains were grinded into fine powder with an electric grinding machine and kept in an air-tight container (Rao *et al*., 2008).

Aqueous and ethanolic extraction

Nine hundred grams each of the powdered seeds was separately soaked in 3 L of distilled water and ethanol. The mixtures were allowed to stand for 72 h during which they were regularly stirred. At the end of this period, the mixture was separated with Whatman No. 1 filter paper. The filtrate was concentrated using rotary evaporator and oven. The extract obtained was weighed and stored in a refrigerator before eventual use for analysis (Richardson and Harborne, 1990).

Percentage yield of extracts

The percentage yield of the aqueous extract of *B. coriacea* seeds were determined by weighing the seeds before extraction and the concentrated extract obtained after extraction and then calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Weightof extract}\,(\text{g})}{\text{Weight of pulverised seeds}\,(\text{g})} \times 100$$

(Enechi *et al*., 2013)

Preparation of plant extracts for analysis

Stock solutions (20 mg/mL) of the plant were freshly prepared by simple dilution of the concentrated extract in distilled water in the presence of 1% dimethyl sulfoxide.

Experimental design

The use of Hb SS erythrocytes in this study was based on six groups:

i. Control – erythrocytes + normal saline
ii. Group A – erythrocytes + 10.00 mg/mL of potassium thiocyanate (KSCN) (Malomo and Oyewole, 2008)
iii. Group B – erythrocytes + 2.50 mg/mL of extract
iv. Group C – erythrocytes + 5.00 mg/mL of extract
v. Group D – erythrocytes + 10.00 mg/mL of extract
vi. Group E – erythrocytes + 20.00 mg/mL of extract (Ejikeugwu *et al*., 2015.

Informed consent and blood sample collection

Sickle cell disease patients in steady state were recruited from the Sickle Cell Foundation of Nigeria, Surulere, Lagos. The individuals were informed adequately of the objectives and the design of the study as well as the level of commitment required. All individuals were allowed to make the decision to participate after carefully going through the informed consent form without coercion. The consent form approved by the University of Lagos HREC was prepared in duplicate for reference purposes by both researcher and participants. Thus, all experiments were performed in line with appropriate ethical considerations and in accordance with the standards laid down in the 1964 Declaration of Helsinki.

The criteria for selection of the experimental individuals include the following:

i. No blood transfusion in the last 4 months preceding blood collection day
ii. Not currently on blood factor-enhancing drugs
iii. History of known stable Packed cell volume (PCV), and
iv. Not currently on any natural health supplements.

Blood samples obtained from ten individuals were made to pass through Hb electrophoresis on cellulose acetate gel to confirm their status.

Erythrocytes preparation

The 5.00 mL blood samples obtained from the ten sickle-cell disorder patients were centrifuged at 5000 rpm for 10 min and washed in saline, pH 7.4 thrice to obtain the erythrocytes (Imaga, 2012).

The erythrocytes were resuspended in normal saline before using them for the membrane stability and the membrane remodeling assays.

Erythrocyte membrane stability activity (osmotic fragility)

i. Four milliliters of different concentrations (0.25%, 0.45%, 0.65% and 0.85%) of phosphate-buffered saline (PBS) with pH of 7.4 was added to 0.50 mL each of the graded concentration of aqueous and ethanolic extracts as well as KSCN in duplicates
ii. Hb SS erythrocytes (0.50 mL) were added to the mixture above
iii. The resulting mixtures were incubated at room temperature (25°C) for 24 h and centrifuged at 3000 rpm for 15 min

iv. The absorbance of the supernatant was read at 540 nm against blank made of 0.85% PBS concentration
v. The mean corpuscular fragility (MCF) was obtained (from the concentration of saline causing 50% hemolysis of the RBC) through a plot of percentage (%) hemolysis versus PBS concentration (Abere *et al*., 2015).

$$\text{Percentage hemolysis} = \frac{\text{Absorbance of supernatant in each tube}}{\text{Absorbance of supernatant in tube with } dH_2O} \times 100$$

Preparation of sample for sodium dodecyl sulfate polyacrylamide gel electrophoresis

i. Hb SS erythrocytes (0.10 mL) were mixed with 0.10 mL graded concentrations of 2.50, 5.00, 10.00, and 20.00 mg/mL (each in duplicate) of the aqueous and ethanolic extracts as well as KSCN
ii. At zero time, 0.10 mL of 2% $Na_2S_2O_5$ was added to each of the mixtures
iii. The protein sample buffer (2 × SDS) was added to the mixtures prepared
iv. The samples were mixed thoroughly and boiled in a water bath at 95°C for 10 min
v. The samples were spun using a table top centrifuge for 1 min
vi. The samples were thereafter loaded onto the gel.

Detection of protein

Coomassie blue staining was used to mark the proteins of interest.

Identification of membrane proteins

The gel was snapped with a digital camera and edited with J software (1.51n, Wayne Rasband, National Institute of Health, USA). Proteins were identified with respect to their relative mobility in the gel and stain patterns following standard criteria and descriptions (Ballas, 1978; Singh and Rajini, 2008). Membrane and cytoskeletal proteins were scored according to the size and stain intensity of the bands as: absent (−), present (+), low intensity and size (±), and high intensity and size (++). However, because of the subjectivity inherent in these parameters, protein bands were also scored only as present or absent.

Statistical analysis

All data were analyzed by the one-way ANOVA with Bonferroni's posttest using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Values of $P < 0.01$ were considered significantly different.

Results

Erythrocytes membrane stability activity (osmotic fragility)

The graphs showing the MCF is represented by Figures 1-4.

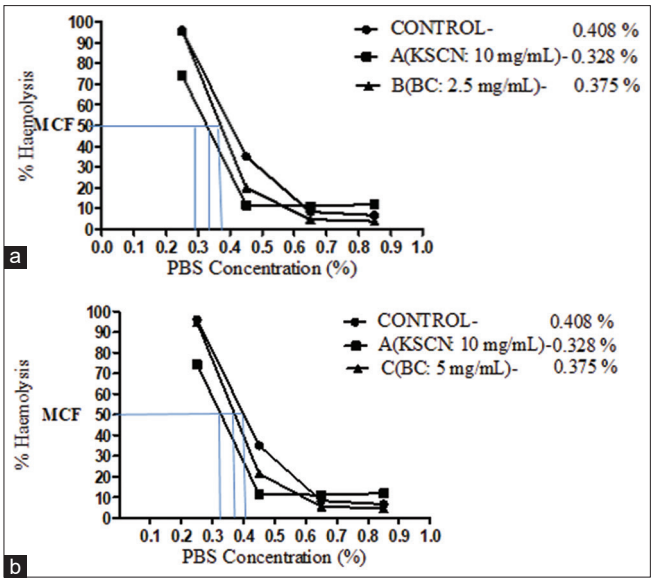

Figure 1: Osmotic fragility curves (a) treatment Group B with MCF of 0.375%; and (b) treatment Group C with MCF of 0.375%. There is no significant difference between the MCF in 2.5 and 5 mg/mL of the aqueous extract when compared with control and 10 mg/mL of KSCN. PBS - Phosphate-buffered saline, MCF - Mean corpuscular fragility, KSCN - potassium thiocyanate

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The image of the electrophoretic gel which showed the banding patterns of specific membrane proteins is represented by Figure 5.

Discussion

The antihemolytic effects of the various concentrations of aqueous and ethanolic extracts of *B. coriacea* and 10 mg/mL of KSCN on Hb SS erythrocytes are presented in Figures 1-4. The curves revealed the maintenance of the erythrocytes fragility throughout the period of incubation with graded concentrations of aqueous and ethanolic extracts of *B. coriacea* seeds. Similarly, the difference between the MCF of 10.00 mg/mL KSCN (0.282%) and the MCF of the control (0.408%) that is not significant suggests that SCN also possess antihemolytic quality in addition to its known antisickling function. The average MCF of 0.408% ± 0.01% obtained for the Hb SS erythrocytes samples treated with only PBS (that is without extract/KSCN) lie within the 0.400%–0.420% normal range for Hb SS erythrocytes under steady state. This result eliminates the presence of dehydration in the Hb S erythrocytes used since the dehydration is subject to the body's physiological condition, especially deviation from the vaso-occlusive crisis state. Moreover, it suggests that phytochemicals other than anthocyanins (phenolic compound) present in *B. coriacea* seeds extract have good antihemolytic qualities. Mpiana *et al.*, (2013) also reported the antihemolytic effect of *Dicliptera colorata* C. B. Clarke, *Euphorbia hirta* L., and *Sorghum bicolor* (L.) extracts on Hb

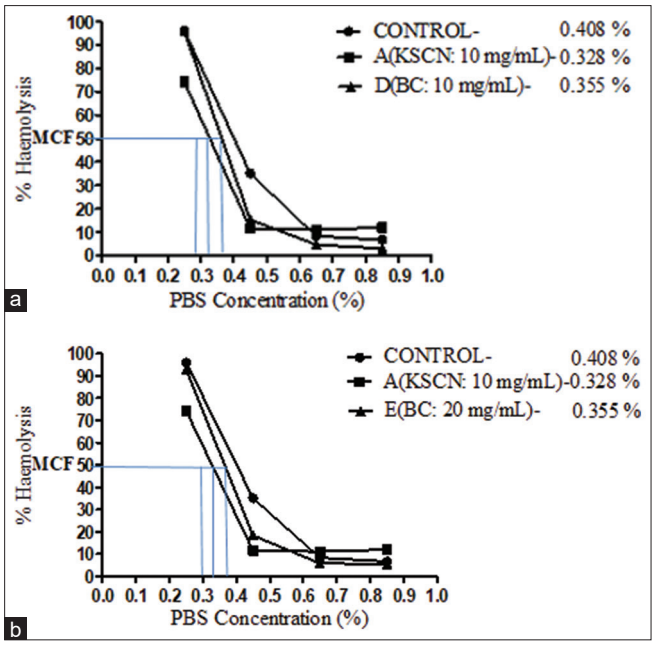

Figure 2: Osmotic fragility curves (a) treatment Group D with MCF of 0.355%; and (b) treatment Group E with MCF of 0.355%. There is no statistically significant difference between the MCF in 10 and 20 mg/mL of the aqueous extract when compared with control and 10 mg/mL of KSCN. PBS - Phosphate-buffered saline, MCF - Mean corpuscular fragility, KSCN - potassium thiocyanate

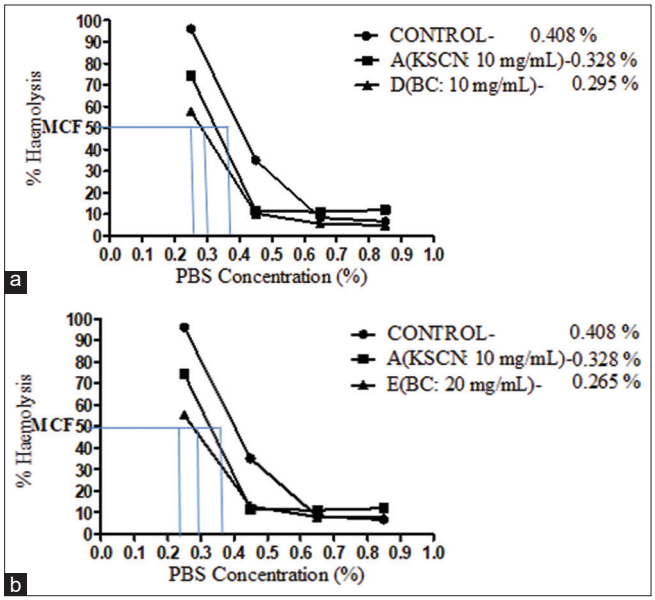

Figure 4: Osmotic fragility curves (a) treatment Group D with MCF of 0.295%; and (b) treatment Group E with MCF of 0.265%. There is no statistically significant difference between the MCF in 10 and 20 mg/mL of the ethanolic extract when compared with control and 10 mg/mL of KSCN. PBS - Phosphate-buffered saline, MCF - Mean corpuscular fragility, KSCN - potassium thiocyanate

S erythrocytes, although exclusively attributed to the presence of anthocyanins.

The SDS PAGE result in Figure 5 indicated that the bands of the main structural membrane proteins (spectrin and actin) were preserved in all the test groups as well as in the control. The size and intensity of the spectrin band

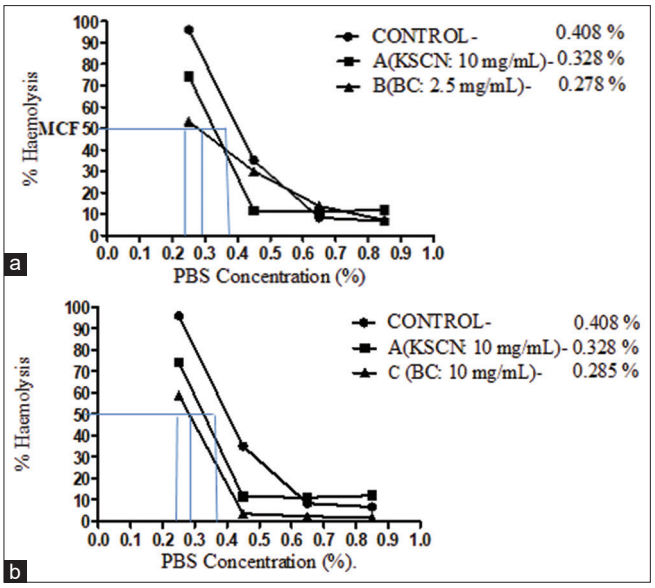

Figure 3: Osmotic fragility curve (a) treatment Group B with MCF of 0.278%; and (b) treatment Group C with MCF of 0.285%. There is no statistically significant difference between the MCF in 2.5 and 5 mg/mL of the ethanolic extract when compared with control and 10 mg/mL of KSCN. PBS - Phosphate-buffered saline, MCF - Mean corpuscular fragility, KSCN - potassium thiocyanate

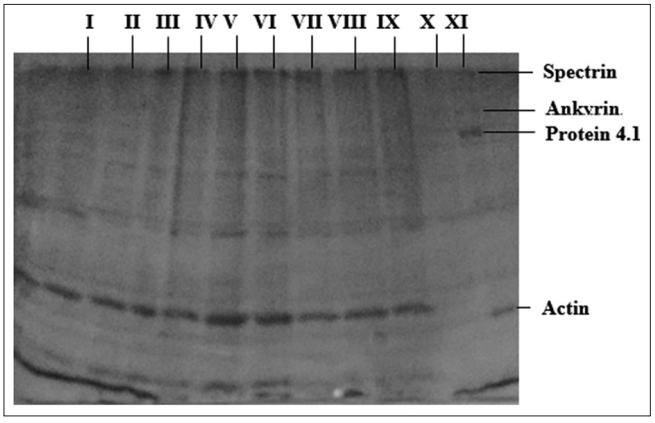

Figure 5: SDS-PAGE of remodeled erythrocyte membrane. Aqueous - (i) 2.50 mg/mL, (II) 5.00, (III) 10.00, (IV) 20.00 mg/mL; Ethanolic - (v) 2.50, (VI) 5.00, (VII) 10.00, (VIII) 20.00 mg/mL; (IX) KSCN, (x) $Na_2S_2O_5$, (XI) protein molecular weight standard. SDS PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, KSCN - potassium thiocyanate

was uniform in most of the groups except in groups treated with 2.5 mg/mL, 5 mg/mL, and 10 mg/mL of the aqueous extract. Actin-banding intensity and size was uniform throughout the groups. Hence, it was assumed that the submembranous cytoskeletal meshwork usually formed by the aforementioned duo remained intact (Banderas Tarabay *et al.*, 2015). However, the bands of the other two proteins, i.e., ankyrin and protein 4.1 which link spectrin and actin with the lipid bilayer were diminished and so did not appear in the electrophoretic lanes of all the test groups except the group treated with normal saline. The nonappearance of these proteins bands may be as a result of the inability of the aqueous and ethanolic extracts to modulate the

injury and remodeling of the erythrocyte membrane induced by sodium metabisulfite through the creation of hypoxic environment and reduction in the rate of hydrolysis for the erythrocytes. Thus, it could be inferred that both extract could not inhibit the oxidation of membrane proteins that normally help to keep the lipid bilayer in proper shape (Dupire *et al*., 2012). Despite the fact that extracts of plants containing polyphenols such as *C. Mexicana* extracts and others like it have been reported to perform anti-oxidant characters on membrane proteins (Tarabay *et al*., 2015), the aqueous and ethanolic extracts of *B. coriacea* could not achieve the same feat despite the presence of the polyphenolic compounds. Some information known about the function of ankyrin and protein 4.1 established their role in maintaining erythrocytes shape by binding with spectrin and actin (Low *et al*., 2002). The altered structure of the degraded ankyrin and protein 4.1 suggests that the actin present could not secure a binding site that would have produced a spectrin–actin–protein 4.1 ternary complex (Branton, 1981). Ultimately, this causes a defect in the normal erythrocytes membrane stability and an eventual collapse in structure and alteration in form and function of the membrane predominantly in sickle cell anaemia and occasionally in hereditary spherocytosis. This observation may also have been responsible for the inability of the extracts to effectively prevent and reverse the sickling of erythrocytes by impeding the evolution of hypoxic condition and a reduction in the rate of hydrolysis.

Conclusion

Available evidence from this study indicates that B. coriacea seeds extracts lacks the ability to attenuate sodium metabisulphite induced oxidation, disruption and alteration of the morphology and mechanical stability of erythrocytes membrane proteins.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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