

Original Research Article

Antioxidant activity of the methanolic extract of *Solenostemon monostachyus* (P.Beauv.) Briq. leaves in 2-butoxyethanol-induced rats

ABSTRACT (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Aim: To determine the biological activities of *solenostemon monostachyus* in order to provide a platform for treating hemolytic diseases.

Sample: To correlate hematological parameters and antioxidant activities with the presence induced hemolytic anemia in female rats to validate other screening parameters.

Place and duration of study: The study as carried out in the covenant university animal house and biochemistry laboratory, department of biological sciences, covenant university. **sample:**the animals were obtained from the institute for advanced medical research and training (IMRAT), college of medicine, UCH, Orita-mefa, Ibadan, Oyo state and allowed to acclimatize under a 12-hour light/dark cycle for 3 weeks prior to the commencement of the study between January 2015 and February 2015.

Methodology: The methanolic extract of the leaves was orally administered for 5 days at 150 mg/kg, 200 mg/kg and 250 mg/kg of body weight doses to determine the antioxidant activities and some hematological indices of plasma in the kidney, spleen and liver of 2-butoxyethanol hemolytic-induced rats. **Sample:** we used 49 Wistar albino rats (49 female; weight: 95-120g). Hematological examination (PCV, MCV, RBCs, and hemoglobin [Hb]count) and sICAM levels was done besides antioxidant activities(bilirubin, glutathione, superoxide dismutase, TBARS and peroxidase). Weight of the animals was also calculated.

Results: treatment with 200 mg of extract/kg of body weight significantly increased ($p<0.05$) the weight, blood qualities and restored the status of white blood cells and soluble intracellular adhesion molecule. a dose of 150 mg of extract/kg of body weight significantly increased ($p<0.05$) reduced glutathione in the spleen.

Conclusion: the leaves of *solenostemon monostachyus* had potential healing properties.

These predictors, however, need further work to validate reliability.

Keywords: Plant; *Solenostemon monostachyus*; hemolysis; anemia; antioxidant; inflammation

1. INTRODUCTION (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

Hemolytic anemia may be intravascular or extravascular (macrophages of spleen and/or liver) and is either inherited or acquired according to Berentsen and Sundic (2015). It can sometimes be induced by harmless or life-threatening factors from external and internal sources, such as exposure to chemicals, including drugs (Nayak *et al.* 2014). Common causes of acquired hemolytic anemia are autoimmunity (i.e., immune-mediated hemolysis),

21 which is caused by anti-erythrocyte antibodies that could be secondary to malignancies,
22 autoimmune disorders, drugs, and transfusion reactions. Gehrsand Friedberg (2002)
23 showed that microangiopathy may be of immune origin, secondary to infections or
24 secondary to lesions of the endothelium. Hereditary hemolytic anemia usually arises from
25 disorders of RBC enzymes, membranes, and hemoglobin that are characterized by various
26 morphological abnormalities of the red blood cells according to the report of Dhaliwal *et al.*
27 (2004).

28 Sick cell disease is known to be one of the diseases ravaging most world populations
29 cutting across nations and ethnic divide. Diallo and Tchernia (2002) showed that Africa is
30 believed to be the origin of sick cell disease, and affects an estimated at 200,000 people
31 per year worldwide. Sick cell anemia and thalassemia are hemoglobinopathies that are
32 characterized by chronic hemolysis. The associated morbidity and mortality remain
33 unacceptable despite the recent deployment of advanced scientific techniques (Diallo and
34 Tchernia (2002), Kato 2012). Despite the challenges faced by scientists in developing
35 countries, many sources of constituents with the potential to ameliorate sick cell crises
36 have been investigated to contribute to the search for substances that can effectively solve
37 the sick cell disease problem. Consequently, the anti-sickling effects of many different
38 substances have been investigated, and various researchers have reported the effect of diet
39 on the disease; however, nutrition has been shown to be insufficient to overcome sick cell
40 disease (Nwaoguikpe and Braide (2010). Crude extracts from plants have been used to
41 treat a wide array of diseases since ancient times, although the bioactive components of
42 such plants remain largely unknown. Okpuzor *et al.* (2008) revealed that scientific
43 investigations have been undertaken to authenticate the traditional use of the plants.
44 However, reports relating to sick cell disease remain scarce, although many years have
45 passed since its discovery, because the disease is limited to tropical countries, which are
46 largely developing countries.

47 *Solenostemon monostachyus* (P.Beauv). Briq. is attracting increasing attention because of
48 its numerous medicinal benefits. The leaf is generally consumed as food and medicine to
49 treat arthritis, rheumatism, eye defects, menstrual cycle disorders, paralysis, epilepsy,
50 convulsions, spasm, pulmonary troubles and stomach troubles. It is traditionally used as a
51 diuretic, laxative, pain killer, sedative, and oral treatment, as well as an antiemetic and
52 antidote for venomous stings and bites. Jacques *et al.* (2010) has proposed a study on the
53 clinical importance and pharmacological activities of the leaves of *S. monostachyus* because

54 they are rich in phytochemicals. *S. monostachyus* has been suggested to have the ability to
55 reverse sickle erythrocytes and alleviate SCD symptoms in human sickle cell blood
56 according to

57 Afolabi *et al.* (2012). This implies that this plant has potential applications in the
58 management of sickle cell disease, which is a congenital form of anemia and threatens
59 people with African ancestry.

60 NIH *NTP Technical report* 2000 showed that 2-Butoxyethanol (2-BE) is involved in many
61 biological functions in humans, such as acetate production, and is also used in herbicides,
62 automotive brake fluids, printing inks, spot removers, and cosmetics. 2-BE causes acute
63 hemolytic anemia in male and female rats when it is metabolized to 2-butoxyacetic acid
64 (BAA) (Ghanayem *et al.* (1987), Ramot *et al.* (2007).

65 The aim of this study was to investigate the alleviating potential of *S. monostachyus* in 2-BE-
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117 the disease-alleviating effects of *S. monostachyus*, to account for the effects of antioxidants
118 in the plant, and to determine the differences in HO-1, COX-2 and TNF- α levels after
119 treatment with *S. monostachyus* leaf extract.

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121 *monostachyus*, to account for the effects of antioxidants in the plant, and to determine the
122 differences in HO-1, COX-2 and TNF- α levels after treatment with *S. monostachyus* leaf
123 extract.

124

125 **2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY**

126

127 **2.1 Reagents and chemicals**

128 Rat sICAM-1 ELISA kits, rat COX-2 ELISA kits and rat TNF- α ELISA kits were obtained
129 from Hangzhou Eastbiopharm Co., Ltd (Yile Road, Hangzhou, Zhejiang, China). Rat HO-1
130 ELISA kits were obtained from Cloud-Clone Corp. (Houston, USA). Bilirubin and total protein
131 kits were obtained from RANDOX Laboratories Ltd. (Crumlin, Co. Antrim, United Kingdom),
132 while methanol was obtained from British Drug House Chemicals Limited (Poole, England).
133 All chemicals used in this study were of analytical grade.

134 **2.2 Collection of plant material**

135 *S. monostachyus* (P. Beauv) Brig. (Voucher no: FHI108913) plants were collected for 2
136 weeks from the lawns of Covenant University, Ota, Ogun State. This plant was identified at
137 the Applied Biology Unit of the Biological Sciences Department at Covenant University. The
138 plant samples were reconfirmed and deposited at the Herbarium Unit of the Forest Research
139 Institute of Nigeria (FRIN), Ibadan, Nigeria. The allocated voucher number of the plant
140 deposited at FRIN is as indicated above. The collected leaves were air-dried at ambient
141 temperature (35–37 °C) on the laboratory bench. Plants were turned daily to accelerate the
142 drying process over a period of 2 weeks. The dried plants were then blended to a coarse
143 powder using a blender.

144 **2.3 Preparation of the plant methanolic extract**

145 The methanolic extract of the plants was prepared using the method of Ogunyemi, et al.
146 (2008) with modifications. A total of 90 g of the plant was macerated in 1,200 ml of methanol
147 (BDL chemical) for 72 hours at room temperature. The resulting mixture was filtered twice
148 using cotton wool and Whatman filter paper 1 Cat No 1001 150 and then evaporated to
149 dryness *in vacuo* with a Stuart rotary evaporator (RE 300) at 20 °C. A total of 5.0 g of the
150 dried extract was re-dissolved in 25 ml of absolute methanol. This solution was used as the
151 working anti-hemolytic agent.

152 **2.4 Handling of animals and experimental design**

153 A total of 49 adult female Wistar albino rats 95-120g were used in this study. The animals
154 were obtained from the Institute for Advanced Medical Research and Training (IART),
155 College of Medicine, UCH, Orita-mefa, Ibadan, Oyo State. The animals were housed in
156 metabolic cages and allowed to acclimatize under a 12-hour light/dark cycle for 3 weeks
157 prior to the commencement of the study. They were maintained on a layer diet (produced by
158 Grace Feeds, Ota, Ogun State) and water during the entire period of experimentation. An
159 ethical permit (reference number CU/BIOSCRECU/BIO/2015/010) was obtained from the
160 ethics committee of our institution prior to beginning the study. Handling of the experimental

161 rats was in accordance with the ethical procedures recommended by the Covenant
162 University Ethical Committee.

163 The animals were randomly classified into the following groups, each including 7 animals.
164 The control group (C) was orally administered 5 ml of normal saline/kg of body weight, and
165 the hemolytic anemic group (2-BEA) was orally administered 2-BE only to serve as a
166 negative control. The positive control group (CF-A) was orally administered 10 mg of caffeic
167 acid/kg of body weight (caffeic acid a major bioactive compound in the studied plant) after
168 inducing hemolysis with 2-BE. Treatments 1 (SM-150), 2 (SM-200) and 3 (SM-250) were
169 orally administered 150 mg/kg, 200 mg/kg and 250 mg/kg of the plant extracts, respectively,
170 following the induction of hemolysis by 2-BE administration. The standard group (CKV) was
171 similarly employed as the treatment group, but 2 ml of ciklavit/kg of body weight was used in
172 place of the extract. Ciklavit is drug manufactured by Neimeth International Pharmaceutical
173 Plc., Nigeria, for the management of sickle cell anemia

174 The 2-BE was orally administered to induce hemolytic anemia following the method of
175 Ramot, Lewis, Ortel, Streicker, Moser, Elmore, Ward, Peddada and Nyska (2007), at 250
176 mg/kg of body weight daily for four consecutive days. The methanolic extract of *S.*
177 *monostachyus* plants was also orally administered daily at doses of 150 mg/kg, 200 mg/kg
178 and 250 mg/kg of body weight for five days. The body weights were measured, and all rats
179 were monitored for abnormal clinical signs prior to the administration of 2-BE on day 1 and
180 daily thereafter. The rats were observed twice: 2 hours and 24 hours after each dose.

181 The 2-BE group was sacrificed immediately after administration on the fourth day. Other
182 groups were sacrificed after the fifth day of treatment under diethyl ether anesthesia in an
183 enclosed desiccator. The heart was carefully punctured to drain the blood using a syringe.
184 The blood samples were collected into EDTA bottles or plain bottles, as described by El-
185 Gawad, et al. (2011). The blood samples in EDTA bottles were used for hematological study,
186 and the plasma obtained was used for sICAM analysis. The blood samples in plain bottles
187 were centrifuged at 10,000 rpm for 15 minutes to obtain the serum, which was preserved at -

188 20 °C for further analyses. The livers, kidney and spleen were then excised carefully from
189 the root to avoid rupturing the organ. The organs were rinsed in cold normal saline before
190 preservation.

191 **2.5 Homogenization of organs**

192 A portion (0.2 g) of each organ was excised, weighed and blended in 0.9 ml of
193 homogenizing buffer contained in homogenizing tubes. An additional 0.9 ml of buffer was
194 used to rinse the homogenizer (Emel blender EM-242) per sample. The homogenized
195 tissues were immediately centrifuged at 3,000 g for 10 min, and the supernatants were
196 collected into new tubes and preserved at -20 °C for experimental purposes.

197 **2.6 Analysis**

198 **2.6.1 Procedure for the determination of hematological parameters**

199 The hematology parameters (PCV, MCV, RBCs, and hemoglobin [Hb]) monitored in this
200 study was determined using a Sysmex 8000 hematology auto-analyzer.

201 **2.6.2 Procedure for the determination of bilirubin contents**

202 Direct bilirubin and total bilirubin were determined colorimetrically with the aid of a
203 ThermoScientific Evolution 60S UV-Visible spectrophotometer 23A09015 following the
204 methods described by Jendrassik and Grof (1938), as indicated in the manual provided by
205 the manufacturers of the kit (Randox Laboratories Ltd., Crumlin, Co. Antrim, United
206 Kingdom). Diazotized sulfanilic acid in alkaline medium and caffeine, respectively, were used
207 as substrates. Total and direct bilirubin contents were calculated from the recorded
208 absorbance values using the formulas indicated below.

209 Total bilirubin (mg/dl) = $10.8 * \text{Abs } 578 \text{ nm}$;

210 Direct bilirubin (mg/ml) = $14.4 * \text{Abs } 546 \text{ nm}$.

211 **2.6.3 Procedure for the determination of superoxide dismutase activity**

212 Superoxide dismutase activity was determined spectrophotometrically using a
213 ThermoScientific Evolution 60S UV-Visible Spectrophotometer 23A09015, as described by
214 Sandstrom et al. (1994). An increase in absorbance at 420 nm over approximately 3 minutes

was observed. The change in absorbance (Δ Abs 420 nm/minute) was determined using the maximum linear rate for both the test and the blank. The inhibition was then calculated using the formula presented below.

218

$$\text{Inhibition (\%)} = \frac{\Delta \text{ Abs 420 nm/min of blank} - \Delta \text{ Abs 420 nm/minute of sample} * 100}{\Delta \text{ Abs 420 nm/min of blank}}$$

221

222 **2.6.4 Procedure for the determination of lipid peroxidation**

TBARS was estimated to monitor lipid peroxidation according to the method proposed by Buege and Aust (1978). The absorbance was read at 535 nm using the spectrophotometer (ThermoScientific Evolution 60S UV-Visible Spectrophotometer), and the concentration was calculated based on the observed absorbance using the formula below, where the constant (ξ) is equal to $1.56 * 10^5 \text{ m}^{-1} \text{ cm}^{-1}$.

$$\text{Concentration (m)} = \frac{\text{Absorbance}}{\xi}$$

230

231 **2.6.5 Procedure for the determination of peroxidase activity**

The peroxidase activity of the tissue homogenate was determined in the absence (blank) and presence (sample) of the plant extract according to the method described by Chance and Maehly (1955). The test reagents were mixed by inversion, and the rates of the change in absorbance at 420 nm per 30 seconds were determined for both the test and blank samples. The activity of the enzyme was calculated from the observed change in absorbance using the formula provided below.

$$\text{Units/ml enzyme} = \frac{(\Delta \text{ Abs 420 nm/30 sec of sample} - \Delta \text{ Abs 420 nm/30 sec of blank}) * \text{df}}{12 * 0.03}$$

where df = the dilution factor, 12 = the millimolar extinction coefficient of pyrogallol, and 0.03= the volume (ml) of enzyme used.

242

243 **2.6.6 Procedure for the determination of GSH levels**

244 GSH was determined by the method described by Ellman(1959). GSH reacts with DTNB to
245 produce a yellow-colored product that absorbs at 412 nm.

246 Calculation

247 Enzyme content (mmol/g tissue) = (Absorbance/14.15) *2.44 *dilution factor

248 Where 14.15 mM⁻¹cm⁻¹ is the molar extinction coefficient of DTNB and 2.44 is the dilution
249 factor of the sample in the 1.22ml solution assayed.

250 **2.6.7 Procedure for the determination of sICAM levels**

251 The determination of sICAM in serum was conducted following the instructions of the ELISA
252 kit manual provided by the manufacturer (Hangzhou Eastbiopharm, Hangzhou, China; Cat.
253 No: CK-E30438). Briefly, 50 µl of standard and 50 µl of streptavidin-HRP were loaded into
254 the blank well, and 40 µl of sample, 10 µl of antibody and 50 µl of streptavidin-HRP were
255 loaded into the test wells. The plate was gently shaken and incubated at 37 °C for 60 min.
256 The plate was then washed with washing buffer five times. Fifty-microliter aliquots of
257 chromogens A and B were then added to each well, mixed gently, and incubated for 10 min
258 at 37 °C in the dark. Then, 50 µl of the stop solution was added to each well. After 15 min,
259 the optical density was measured at 450 nm. The standard curve was plotted using Microsoft
260 Excel.

261 **2.7 Statistical analysis**

262 A one-factor randomized complete block design was used for this study. Data were
263 analyzed using analysis of variance (ANOVA) in the MegaStat statistical software package
264 (version 1.0.0.0). Treatments with P<0.05 were considered statistically significant and were
265 subjected to Student's t-test using the same statistical software. The results were expressed
266 as mean ± standard deviation.

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270 **3. RESULTS AND DISCUSSION**

271 The weight of the animals in the SM-200 group significantly increased ($P = .05$) throughout
272 the study period (Figure 7). Table 1 shows the levels of soluble intracellular adhesion

273 molecule (sICAM) and hematological parameters considered in this experiment. The PCV in
 274 the blood of the animals treated with *S. monostachyus* extracts (SM-150 and SM-200) was
 275 significantly higher ($P = .05$) than in the controls. SM-250 generated the lowest PCV. The
 276 hemoglobin levels in the rats treated with SM-150 and SM-200 were significantly increased
 277 ($P = .05$), as were those treated with 2BEA and CF-A, compared to the control and CKV
 278 groups (Table 1). The level of RBCs was also significantly increased ($P = .05$) by the
 279 administration of SM-150, SM-200 and CF-A. All of the extracts (i.e., SM-150, SM-200 and
 280 SM-250) significantly increased ($P = .05$) mean cell volume (MCV) in a manner similar to
 281 that of CF-A and CKV compared with the controls (Table 1).
 282 TBARS and Bilirubin were insignificantly different ($P = .05$) compared to control.
 283

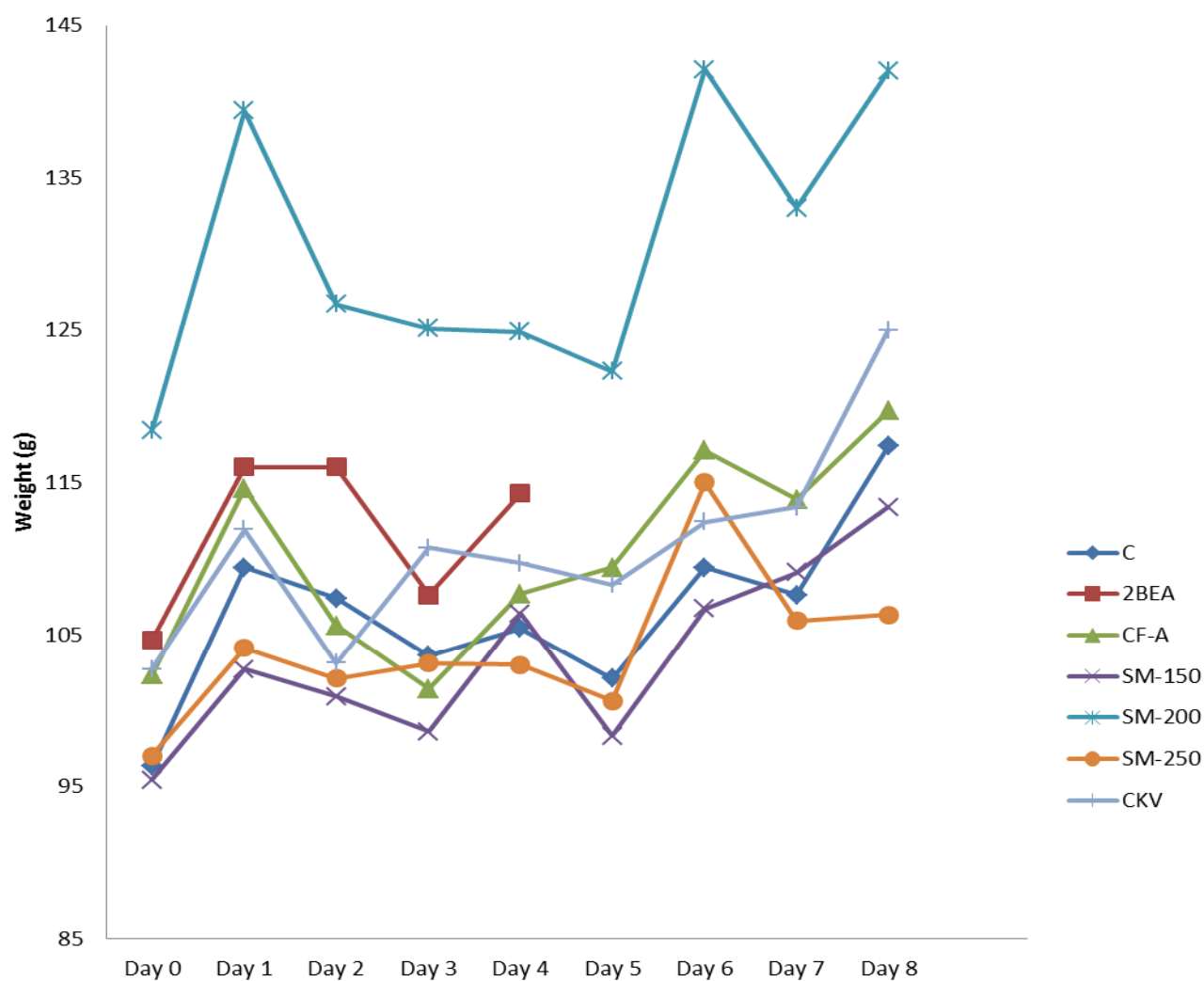


Fig.7: The weight of animals within the days of experiment

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291 Table 1: TABLE SHOWING HEMATOLOGY ANALYSES AND sICAM RESULT

	C	2BEA	CF-A	SM-150	SM-200	SM-250	CKV
PCV (%)	38.0±2.89	44.44±2.30 ^{a,f,g}	43.0±3.06 ^a	42.4±3.31 ^a	47.5±1.87 ^{a,b,c,d,f,g}	39.9±2.61	40.4±2.30
Hb (g/dl)	12.89±1.04	14.53±1.09 ^{a,f}	14.30±0.93 ^{a,f}	14.30±1.08 ^{a,f}	15.50±0.85 ^{a,c,d,f,g}	13.20±0.57	13.76±0.75
RBC/ml	4.26±0.29	4.84±0.28 ^a	4.77±0.36 ^a	4.71±0.36 ^a	5.30±0.20 ^{a,b,c,d,f,g}	4.41±0.30	4.53±0.29
MCV (fL)	76.86±3.840	91.79±2.474	90.10±1.456	89.81±0.558	89.52±0.866	90.20±0.529	89.04±1.367
WBC/ml	7000.0±1655.29	6100.0±2221.11	6614.3±1038.31	6500.0±1721.43	6616.7±1231.94	6500.0±1800.00	6885.7±819.41
sICAM (ng/g)	2.90±0.32	3.00±0.12	3.12±0.41	3.12±0.21	3.13±0.42	3.02±0.42	3.00±0.38

292 Values expressed in Mean ± SD; n=7 Values in each column with different superscripts (a-g) are
 293 significantly different ($P = .05$)
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301 Table 2: Effect of oral administration extract of *Solenostanum monostachyus* plant
 302 extract on some oxidative stress parameters in the liver

Parameters	C	2BEA	CF-A	SM-150	SM-200	SM-250	CKV
SOD	68.61±15.62	68.13±5.98	57.59±13.69	59.11±7.61	71.73±6.63	70.53±7.30	62.14±10.32
TBARS (nmol/g tissue)	3.89±0.76	6.04±1.93	4.74±0.80	5.96±1.27	7.85±2.48	6.12±2.23	4.94±1.31
PEROXIDASE (Unit/ml)	4.92±1.89	3.71±1.78	4.64±2.28	5.76±3.60	4.50±1.48	4.11±1.23	6.32±1.37
GSH (mmol/g tissue)	4.14±0.65	13.75±11.05	3.45 ±0.49	4.27 ±0.47	3.92 ±1.20	4.57 ±1.32	10.91±8.05
BILIRUBIN	1.43±0.34	1.47±0.36	1.98±0.64	1.29±0.63	0.80±0.59	1.49±0.62	1.59±0.37

303 Values expressed in Mean± SD, n=4
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Table 3: Effect of oral administration extract of *Solenostanum monostachyus* plant extract on some oxidative stress parameters in the kidney

Parameters	C	2BEA	CF-A	SM-150	SM-200	SM-250	CKV
SOD (Unit/ml)	47.92±1 3.98	51.76±9. 96	54.15±2.86	53.51±7.5 9	32.03±2 1.16	43.37±1 0.48	54.31±7.23
TBARS (nmol/g tissue)	4.49±1.2 0	2.56±0.2 9	3.93±1.48	13.89±16. 40	4.58±1.2 1	7.71±1.8 7	4.55±1.13
PRX (Unit/ml)	3.71±1.0 0	3.30±1.7 3	2.31±0.91	258.99±67 4.82	3.63±1.7 1	2.23±0.6 6	2.85±0.53
GSH (mmol/g tissue)	0.007±0. 001	0.009±0. 001 a,c,d,e,f	0.008±0.00 1 ^{c,d,e,f}	0.007±0.0 01	0.007±0. 001	0.006±0. 001	0.011±0.001 ^a b,c,d,e,f
BILIRU BIN	1.37±0.4 2	1.13±0.2 2	1.25±0.21	1.21±0.35	0.86±0.5 0	1.13±0.2 9	1.60±0.51

Values expressed in Mean ± SD; n=4. Values in each column with different superscripts (a-f) are significantly different ($P = .05$)

Of the three organs examined, only CKV significantly increased ($p<0.05$) total bilirubin in the spleen (Tables 2–4). 2BEA consistently significantly increased ($p<0.05$) reduced glutathione (GSH) activity in the liver (Table 2), kidney (Table 3), and spleen (Table 4), while CKV significantly reduced ($P = .05$) the activity of GSH in the kidney (Table 3). SM-150 significantly increased ($P = .05$) GSH activity in the spleen (Table 4). SM-200, SM-250 and CKV significantly increased ($P = .05$) direct bilirubin in the spleen (Table 4).

Table 4: Effect of oral administration extract of *Solenostanum monostachyus* plant extract on some oxidative stress parameters in the spleen

Parameters	C	2BEA	CF-A	SM-150	SM-200	SM-250	CKV
SOD	76.76±7.7 3 98	77.56±10.14	79.71±10.57	81.15±7.26	78.43±6.56	73.32±5.21	78.19±8.25
TBARS(nmol/g tissue)	3.78±1.14 1	5.11±0.56	4.97±1.16	3.46±1.38	3.38±1.35	3.24±0.84	3.33±0.79
PEROXIDASE (Unit/ml)	3.20±0.97	4.31±1.66	3.14±1.49	4.87±4.58	4.90±2.37	5.67±6.43	4.04±1.36
GSH(mmol/g tissue)	4.87±1.08	7.85±2.83 ^{a,c} g	5.47±1.07	7.67±2.19 ^a c,g	4.57±2.21	6.08±1.35	4.27±0.62
BILIRUBIN	0.73±0.24	0.88± 0.14	1.09± 0.45	0.93± 0.34	1.12±0.52	1.14±0.22	1.14±0.22

Values expressed in Mean ± SD; n=4 Values in each column with different superscripts (a-g) are significantly different ($P = .05$)

335 4 DISCUSSION

336 In this work, we investigated the antioxidant and anti-hemolytic activities of *S.*
 337 *monostachyus*. We recently reported that in addition to its multiple health benefits, *S.*
 338 *monostachyus* treatment resulted in 98.1 % sickle cell blood reversal in humans [10]. Our
 339 study provides a clue regarding the mode of action of the plant based on some
 340 hematological and biochemical indices. We suggest that this understanding will contribute to
 341 the development of a permanent solution for sickle cell disease and other hemolytic-
 342 associated diseases. The results from the hematology analysis of the blood samples from
 343 the animals are shown in Table 1. The PCV levels were increased by 2BEA and CF-A
 344 treatments. The PCV levels in the SM-150- and SM-200-treated groups increased over
 345 those of the CKV and control groups. The effect of SM-250 on PCV levels was similar to that
 346 of CKV. Additionally, the level of RBCs was increased by SM-150, SM-200 and CF-A
 347 administration (Table 1). This ability of *S. monostachyus* to promote blood synthesis may be
 348 attributed to the presence of iron (95.5 mg/kg dry wt.) and some blood-boosting
 349 biomolecules in the leaf (Afolabi, Akpokene et al. 2012).

350 In addition, the hemoglobin level was remarkably increased with SM-150, CF-A, 2BEA and
 351 SM-200 administration. This increase has been attributed to the release of hemoglobin
 352 during hemolysis (RBC destruction) resulting from exposure to high concentrations of 2BEA
 353 according to the report of U.S Department of Health and Human Services of the National
 354 Institutes of Health(1998). The ability to reverse the induced hemolysis was mainly exhibited
 355 by SM-250 and CKV. The MCV level also increased in all the treatments over that of the
 356 controls. The status of the white blood cells and sICAM were restored by all treatments
 357 (Table 1). The *S. monostachyus* extracts had the ability to reverse hemolysis induced by
 358 2BE, resulting in improved hematological parameters (i.e., PCV, RBC, MCV and
 359 hemoglobin) in this study [Ghanayem *et al.*1987, Ramot *et al.* 2007, Dieter 1993). The fact
 360 that our treatments had no effect on the white blood cell level is in line with previous reports
 361 indicating that 2-BE principally affects RBCs (Lewis *et al.* 2005, Zhang *et al.* 2010). The

362 ability of the plant extracts to cure hemolysis manifested as increased hematologic
363 parameters in the blood of the treated animals (Ghanayem *et al.* 1992). *S. monostachyus*
364 contains moderate cardiac glycosides that may be responsible for the curative effects on
365 hemolysis and other diseases associated with the heart, as reported previously [Jacques *et*
366 *al.* 2010, Pietta 2000).

367 Bilirubin is a product of heme catabolism and a potent antioxidant in biological systems
368 (Cornelius *et al.* 2013, Vitek 2012). It is metabolized to biliverdin, which is subsequently
369 metabolized by heme oxygenase (Santangelo *et al.* 2012, Kapitulnik 2004).

370 The total bilirubin level of the CKV group increased in the spleen over that of the controls
371 and other treatment groups. Additionally, bilirubin was increased in the liver by 2BEA, CF-A,
372 SM-250 and CKV to a greater extent than in the control group, while SM-150 and SM-200
373 decreased the bilirubin levels in the liver. In the kidney, similar increased bilirubin levels were
374 manifested by the CKV group, while decreased bilirubin levels were observed in the other
375 groups relative to the controls (Table 2). The increased levels of bilirubin resulting from the
376 administration of CKV in all the organs and CF-A and SM-250 in the liver, indicate the ability
377 of these treatments to reduce stress, whereas the administration of SM-150 and SM-200
378 tended to increase stress, mainly in the liver. This may be because of the availability of more
379 antioxidants at the highest concentration (i.e., SM-250) of *S. monostachyus* (Cornelius *et al.*
380 2013, Vitek 2012, Santangelo *et al.* 2012, Kapitulnik 2004). SM-200, SM-250 and CKV
381 treatments increased the level of direct bilirubin in the spleen (Table 4). Hence, *S.*
382 *monostachyus* doses of 200–250 mg/kg of body weight may be capable of protecting the
383 spleen from stress and other malignancies (Wu *et al.* 2010)

384 Lipid peroxidation is a well-established mechanism of cellular injury in both plants and
385 animals and is used to indicate oxidative stress in cells and tissues. Lipid peroxides are
386 unstable, and thus, they are liable to decompose and form a complex series of compounds,
387 including reactive carbonyl compounds (Aiyegoro and Okoh 2010). Polyunsaturated fatty
388 acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon

decomposition, which are used to indicate lipid peroxidation (Esterbauer *et al.* 1991). A decrease in thiobarbituric acid reactive substance (TBARS) suggests that the plant extract is rich in antioxidants able to neutralize the effects of free radical reactive species.

The effects of the three extracts (i.e., SM-150, SM-200 and SM-250) and the other treatments (i.e., CF-A and CKV) on the oxidative stress parameters (i.e., TBARS, GSH, peroxidase, bilirubin and superoxide dismutase [SOD] activity) in the liver were not better than that of 2BEA (Table 2). The results of bilirubin, GSH, TBARS, peroxidase activity and SOD activity in the kidney were comparable to those of the control group (Table 3). The integrity of TBARS, SOD activity and peroxidase activity in the spleen were restored by the extracts and other treatments (Table 4).

GSH is a freely available antioxidant that acts directly as an antioxidant and facilitates the catalytic cycles of several antioxidant enzymes, such as glutathione peroxidase, glutathione reductase and glutathione-S-transferase (Circu and Aw 2008, Biswas and Rahman 2009). GSH activity was generally increased during hemolysis induced by 2BEA in the liver (Table 2), kidney (Table 3) and spleen (Table 4). An increase in GSH activity therefore indicates hemolysis in this study. A similar increase in GSH activity manifested in the spleen of the SM-150 administered groups (Table 4). This implies that the concentration of the SM-150 extract was not able to provide sufficient antioxidants to prevent stress in the spleen. As expected, the hemolysis was cured by the anti-hemolytic and anti-sickling drug (i.e., CKV), which was able to reduce GSH activity in the kidney (Table 3). The other extracts with higher concentrations (i.e., SM-200 and SM-250), including CF-A, mainly restored the integrity of the spleen by restoring the antioxidant status that was degraded during the induced hemolysis. The antioxidant properties of phenolic acids, such as caffeic acid, enhance their protective effects (Abbas *et al.* 2014). Thus, *S. monostachyus* extract at 200–250 mg/kg of body weight, CF-A and CKV are good inhibitors of the oxidative stress associated with hemolysis [NIH NTP Technical report 2000, Ghanayem *et al.* 1992).

415 In conclusion, this study revealed that *S. monostachyus* dosages of 150–200 mg/kg of body
 416 weight were most appropriate for treating hemolytic anemia-related diseases, such as SCD,
 417 because such treatments enhanced or restored the hematological and inflammatory
 418 activities and oxidative stress parameters. In the future, the molecular effects of the plant
 419 extract at these doses (150–200 mg/kg of body weight) should be investigated by
 420 sequencing the affected genes to further understand the therapeutic mechanism of action
 421 against hemolytic-related diseases.

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550 **DEFINITIONS, ACRONYMS, ABBREVIATIONS**

551 **Abbreviations**

552 SM-150: Group orally administered 150 mg/kg extract of Solenostemon monostachyus; SM-
553 200: Group orally administered 200 mg/kg extract of Solenostemon monostachyus; SM-250:
554 Group orally administered 250 mg/kg extract of Solenostemon monostachyus; 2BE: 2-
555 Butoxyethanol; 2-BEA: 2-BE induced hemolysis; CF-A: Caffeic acid; CKV: Ciklavit; sICAM:
556 Soluble intracellular adhesion molecule.

557