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In Vitro Studies on the Effects of Aqueous Extracts of *Syzygium Aromaticum* Linn. (Cloves) and *Zingiber Officinale* Roscoe (Ginger) on Erythrocyte Osmotic Fragility in Sickle Cells

Adamu Laila Ali[1], Gadaka Madu Adamu[1], Abubakar Abdulwasiu[2], Usman Muhammad Ibrahim[3] and Salami Hamza Adegoke[2*]

[1]*Department of Biochemistry, Faculty of Science.*

[2]*Department of Human Physiology, Faculty of Basic Medical Sciences, University of Maiduguri.*

[3]*Department of Biochemistry, Faculty of Science, Yobe State University, Damaturu, Nigeria.*

[*]Correspondence:
Salami Hamza Adegoke, Department of Human Physiology, Faculty of Basic Medical Sciences, University of Maiduguri, Borno state, Nigeria, Tel: +2348067557350.

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ABSTRACT

Sickle cell disease is a genetically inherited blood disease that is characterized by the production of abnormal erythrocytes, shaped like a sickle. Definite cure for sickle cell disease is yet to be established, therefore, palliative methods such as antioxidant agents which reduce occurrence of intravascular haemolysis are employed. Naturally occurring antioxidants are found in Syzygium aromaticum Linn. (Clove) and Zingiber officinale (ginger) as documented by previous researches are therefore tested on the erythrocyte osmotic fragility of sickle cell red blood cells (RBC). Aqueous extracts of clove and ginger were obtained by maceration and antioxidant activity of the extracts was determined by 2, 2,-diphenyl-1-picryl-hydrazyl (DPPH) using ascorbate as standard. Blood samples from twenty five sickle cell disease subjects were collected and divided into five groups. Group one was control, groups two and three were incubated with 1mg/ml and 10mg/ml of clove extract respectively, while groups four and five were incubated with 1mg/ml and 10mg/ml of ginger extract respectively. Erythrocyte osmotic fragility was determined using graded series of hypotonic Sodium Chloride (NaCl) solutions of concentrations ranging from 0.1% to 0.9%. Analysis of Variance (ANOVA) and Turkey test was used to analyze data and test of significance was applied at $p<0.05$. The results revealed the presence of phenols, alkaloids, flavonoid and tannins in both clove and ginger extracts; glycosides were present in ginger but absent in clove. Antioxidant at low concentration of 50µg/ml showed percentage inhibition of clove and ginger were 67.43% and 11.87%, respectively, and that of ascorbate was 20.15%. At high concentration of 250µg/ml, clove and ginger had percentage inhibition of 93.8% and 81.18% respectively, while ascorbate had 84.06%. Erythrocyte osmotic fragility test results showed complete haemolysis at 0.1% NaCl concentration in all groups. At 0.2% NaCl concentration, percentage haemolysis of the group treated with 10mg of clove 69.12 ± 0.05, was significantly lower ($p<0.05$) than the control (98.12 ± 0.05), while the group treated with 10mg ginger extract showed 98.02 ± 0.04 which is not significantly lower ($p<0.05$) than that of the control. At 0.5% NaCl concentration, percentage haemolysis of groups treated with 10mg clove extract and 10mg ginger extract were 8.400.18 and 20.42 ± 0.18, respectively, which were significantly lower ($p<0.05$) than that of the control group (45.90 ± 0.10). The groups treated with 1mg clove extract and 1mg ginger extract showed percentage haemolysis values of 38.04 ± 0.10 and 30.02 ± 0.10 respectively at 0.5% NaCl concentration which were significantly lower ($p<0.05$) than the control group (45.90 ± 0.10). Clove showed higher antioxidant activity than ginger. Both plants also demonstrated a capacity for maintaining erythrocyte membrane stability, with clove being better at lowering erythrocyte fragility than ginger.

Keywords
In vitro Studies, *Syzygium aromaticum* Linn, *Zingiber officinale* Roscoe, Osmotic fragility, Sickle Cells.

Introduction
One of the most devastating with high economic burden among blood diseases is sickle cell anaemia. Being a genetically inherited disorder, most management therapy have proved to be short-lived and abortive with the exception of bone marrow transplant which is also very expensive, not readily available and in most cases unaffordable to the affected population. Sickle cell disease is predominantly common among people of sub- Saharan Africa, India, Saudi Arabia, and Mediterranean descent. The distribution of the disease reflects the fact that the sickle cell trait confers a survival advantage against malaria and that selection pressure due to malaria has resulted in high frequencies of the mutant gene especially in areas with high transmission. In Nigeria, by far the most populous country in sub-Saharan Africa, 24% of the population is a carrier of the mutant gene and the prevalence of the disease is about 20 per 1000 births (i.e. 2%). This translates to about 150,000 children born annually with sickle all disease [1]. Charache et al., [2] in their study showed that sickling is caused by polymerization of molecules of deoxygenated sickle cell heamoglobin into rigid rod-like polymers. The sufferer develops abnormally shaped erythrocytes which are elongated like a sickle. Sickling polymerization rate has been shown to correlate well with the concentration of HBS and cell-free heme released after auto-oxidation [3].

Polymerization of HbS upon deoxygenation leads to a marked increase in intracellular viscosity [4] which causes the red cells to sickle and plug blood vessels, thereby depriving tissues of essential oxygen and nutrients. This causes oxidative stress and derangement of tissue functions. Oxidative stress occurs when the homeostatic processes fail and ROS generation is beyond the capacity of the body defenses, thus promoting cellular injury and tissue damage. This damage may involve DNA and protein content of the cell, lipid peroxidation of cellular membrane, calcium influx, mitochondrial swelling and lysis [5,6]. Meanwhile, haemolysis is a promoter of the complications of sickle cell disease [7]. ROS is highly reactive, it release oxygen metabolite which can induce oxidative damage to the cell membrane and can also form a very stable structure by extracting electron from other sources [8]. The Major defense against this Reactive Oxygen Species (ROS) include enzymatic and non-enzymatic systems. The protective mechanisms of enzymatic are principally antioxidants: super oxide dismutase (SOD), catalase, glutathione peroxidase (GPX) and nitric oxide (NO); and the non-enzymatic antioxidants are: tocopherols, reduced glutathione (GSH), carotenoids, ascorbic acid, lipoic acid, ubiquinols, selenium, riboflavin, zinc, uric acid and metal-binding proteins. The ROS that fail to be neutralized by these two mechanisms can target biological molecules such as DNA, lipids, proteins and carbohydrates, which can result in cell dysfunction and cell death [9]. The definitive cure of sickle cell disease is yet to be established therefore, palliative method were generally adopted in the management of sickle cell disease. One of this palliative aim at increasing anti-oxidant agent to counter the effect of excessive reactive oxygen specie liberation as a result of high turnover of sickle cell erythrocytes which was seen to double the amount produced by normal erythrocytes. Reactive oxygen species are not only potential markers of sickle cell disease severity, but are important targets for antioxidant therapies for sickle cell disease patients [10,11]. Several studies targeting the therapeutic reduction of oxidative stress in SCD patients have shown great promise in bringing relief from symptoms of the disease. L arginine supplementation, which increases antioxidant enzymes level in SCD patients, also showed reduced RBC osmotic fragility that was attributed to increased antioxidant activity [12]. Hydroxyurea increases synthesis of Hb fetal (HbF), thereby reduces the expression of several oxidative stress biomarkers and NO scavenging in SCD patients [13]. Supplementation with zinc [14], tocopherol [15] and Glutamine [16] also play antioxidant roles. All this anti-oxidants in use for the palliative management of sickle cell disease are synthetic, however the effectiveness of using naturally occurring anti-oxidant in some plants have not been documented.

Clove (*Syzygium aromaticium* Linn) and Ginger (*Zingiber officinale* Roscoe) are well studied herbal plants because of their high consumption as spices and their benefits in traditional settings in the management of some disease. Ginger is traditionally used in the management of cough, muscle pain and indigestion [17]. Scientific authentication proved ginger been possess anti-thrombotic, hypolipidemia [18], anti-coagulant and anti-sickling properties [19,20]. Meanwhile clove are used as spices in food and beverages because of it aroma and also as a preservative. Scientifically, clove possess anti-inflammatory, potent anti-analgesic that is effectively been used in the management of vasooculusive crisis of sickle cell disease patient [21]. Based on phytochemical finding of previous study conducted by the united state department of agriculture [22] on anti-oxidant activity of Clove and Ginger, the two plants possess powerful antioxidant activity with oxygen radical absorption capacity (ORAC) scale score of almost 300,000 in Clove while Ginger's was almost 40,000 when dried and ground according to United States Department of Agriculture [22]. In addition to the beneficial effect of clove and ginger in the treatment of various blood and vascular disorder, the antioxidant capacity of these two plants surpasses the synthetic antioxidants presently in use. In view of the potent antioxidants found in both clove and ginger, this study was conducted to test their probable effectiveness in countering oxidative stress on sickle cell erythrocyte *in vitro* using osmotic fragility test.

Materials and Methods
Plant Material
Dried Syzygium aromaticum buds and dried Zingiber officinale rhizomes were purchased from Maiduguri Monday Market, in Maiduguri, Borno state in April, 2019. They were taken to the Department of Biological Sciences, Faculty of Science, University of Maiduguri, for identification and authentication. Voucher Specimens were prepared and deposited in the department.

Preparation of Plant Extracts

Extracts of Syzygium aromaticum and Zingiber officinale were prepared separately by maceration [23] using distilled water as solvent. 1000 grams from each of coarsely ground *Syzygium aromaticum* and *Zingiber officinale* were suspended in 2 liters of distilled water each for 72 hours with regular agitation. The mixtures were sieved, marc was pressed and the liquid were filtered with Whatman filter paper. The filtrates were poured in two separate beakers of 1000ml capacity. The beakers together with contents were placed in an organ bath with temperature 48°C for 48 hours followed by air drying indoors for 72 hours. Dried, powdered extracts were kept in a closed container in a cool, dry place until use. Dry powdered extract was dissolved in distilled water to make two different concentrations of extract solutions (1mg/ml and 10mg/ml).

Subjects

Selection

Random sampling technique was employed in the selection of the subjects that attend the sickle cell clinic in state specialist hospital Maiduguri, Borno state. The potential subjects were in stable state, and have agreed or their parent/guardian have agreed to informed consent. Subjects of both sexes aged 3 to 18 years who met the inclusion criteria were randomly selected.

Inclusion Criteria

The study subjects consisted of 25 sickle cell disease patients with haemoglobin SS (i.e. HbSS) genotype, which was confirmed via a genotype test. Full blood count and malarial parasite tests were conducted. Subjects with Packed Cell Volume (PCV) of 26% or higher and Hb of 8g/dl or higher [24] who tested negative for malaria parasite, are not on medication of any kind, and have agreed to informed consent were recruited for the study.

Exclusion Criteria

The following patients were excluded from the study: any patient that is on any form of medication, patient who have had transfusion in the last 6 months, patient that is in crisis, and patient who withdraws consent.

Ethical Clearance

Approval for the study was obtained from Ethics Committee at State Specialist Hospital, Maiduguri. Consent of each participant and/or their parents/guardians was obtained in accordance with ethical principles [25].

Phytochemical Screening of the Extracts

Test for alkaloids, saponins, glycosides, steroids, flavonoids, reducing sugar and phenols were conducted by standard laboratory procedures as described in previous studies [26-28].

Determination of Antioxidant Activity of the Extracts

DPPH Radical Scavenging Assay

The free radical scavenging activity of aqueous extracts of *Syzygium aromaticum* and *Zingiber officinale* were estimated by 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Olugbami et al., [29]. 0.1mM solution of DPPH in 90% ethanol was prepared and 1ml of this solution was mixed vigorously with 0.05ml of either extracts (*Syzygium aromaticum* and *Zingiber officinale*) at five different concentrations (50, 100, 150, 200 and 250µg/ml). Each mixture was shaken vigorously and allowed to stand for 30 minutes at room temperature. It is expected that when DPPH reacts with an antioxidant which donate hydrogen, it is reduced to diphenyl-picryl-hydrazyl and change its colour from deep purple to muddy orange, which was measured as Absorbance at 518nm using spectrophotometer (UV-VIS, PEC Medical, USA). L-ascorbic acid was used as reference standard and the assay was repeated three times and mean determined. The IC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration was obtained from the linear regression of plots of mean percentage of the antioxidant activity against the concentration of the test extracts (µg/ml). Lower absorbance of the reaction mixture indicated higher free radical activity. The percentage of radical scavenging activity (RSA) was calculated based on the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \text{x } 100$$

Where Absorbance blank and Absorbance sample are the absorbance values (at 518nm) for blank and sample respectively. 90% ethanol (1ml) plus each sample solution (0.05ml) was used as blank. This method was as described by previous study [29] using DPPH method, it also stipulated that DPPH is more reliable and convenient in determination of free radical absorption capacity in plant extracts.

Experimental Design and Sample Collection

A total of 25 SCD patients with HbSS of both sexes and in different ages were randomly recruited in to the study as was described earlier. The subjects were divided into 5 groups. The first group served as negative control (the blood samples were treated with graded NaCl only, no extracts), groups two and three, the blood samples were treated with 10mg/ml and 1mg/ml clove extracts and graded NaCl respectively. Groups four and five bloods were treated with 10mg/ml and 1mg/ml ginger extracts and graded NaCl respectively. The blood samples (5ml) were collected aseptically at State Specialist Hospital by Doctor in charge of sickle cell clinic from each subject between 9:00am and 10:00am at the cubital vein with a syringe, and immediately transferred to anticoagulant bottles containing EDTA. After collection, the blood samples were placed in a cooler containing ice block and transported to the laboratory at the Department of Biochemistry, University of Maiduguri, where they were centrifuged at 2000rpm for 5 minutes to remove plasma. Two blood samples were collected per clinic day for easier determination. All the collected Red Blood Cells from sickle cell patients were then washed 3 times with phosphate buffered saline (PBS) solution. Each wash was centrifuged and supernatant was discarded and the aliquots were incubated [30].

Table 1: Grouping of sickle cell blood for assay.

Groups	Number of samples	Treatments
I	5	Graded NaCl (Control)
II	5	Graded NaCl + 1mg/ml clove extract
III	5	Graded NaCl + 10mg/ml clove extract
IV	5	Graded NaCl + 1mg/ml ginger extract
V	5	Graded NaCl + 10mg/ml ginger extract

Assay of Erythrocyte Aliquots and Determination of Effects of the Extracts on Erythrocyte Osmotic Fragility

Erythrocyte osmotic fragility assay was carried out using the method described by Adalgisa et al.,[31]. Briefly, whole blood sample from each SCD patient were incubated with 5ml of normal saline and served as negative control, group two samples were incubated with 1mg/ml of clove, group three samples were incubated with 10mg/ml of clove extract, group four were incubated with 1mg/ml of ginger extract and five were incubated with 10mg/ml ginger extracts. The samples were centrifuged to separate supernatant from RBCs, while the aliquots were used to determine osmotic fragility. The osmotic fragility of erythrocytes measures the membrane stabilizing effect of the extracts in osmotic stress incubation [32]; osmotic fragility assay principle is based on measuring the efflux of haemoglobin from suspended erythrocytes, concentration of which can then be measured photometrically [33]. The effects of extracts on membrane stability was tested using method described by Faulkner and King, 1970. Briefly, nine test tubes, each containing 5ml of NaCl solution concentrations: 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8% and 0.9% were arranged in a test tube rack. Each set of tubes was used to test one sample. The test tubes were labelled with corresponding NaCl concentration. The aliquot from each sample was pipetted (0.02ml), using a micropipette into each test tube containing varying concentrations of NaCl. The test tubes were inverted twice and then centrifuged at 200rpm for 5 minutes. The supernatants were transferred into glass cuvettes and absorbance of the supernatants were measured using spectrophotometer (UV-VIS, PEC Medical, USA), at 540nm wavelength. The percentage haemolysis for each sample was calculated and plotted against sodium chloride concentration. Thus:

$$\text{Percentage haemolysis}(\%) = \frac{\text{Optical density of test}}{\text{optical density of distilled water}} \text{x}100$$

Erythrocyte osmotic fragility curve was obtained by plotting percentage haemolysis against NaCl concentrations.

Statistical Analysis

The data were expressed as Mean ± SEM (Standard error of mean) and were subjected to One way analysis of variance (ANOVA) and Turkey test using graphpad prism version 4.0 for windows from Graphpad software San diego Carlifonia USA. (www.graphpad.com) values of $P<0.05$ were considered significant.

Results

Phytochemical Constituents of Aqueous Extracts of *Syzygium aromaticum* linn and *Zingiber officinale* Roscoe

Phytochemical constituents of aqueous extracts of *Syzygium aromaticum* (Clove) and *Zingiber officinale* (Ginger) revealed the presence of phenols, alkaloids, tanins, and flavonoids (Table 2). In addition, it was observed that glycosides were detected in ginger extract.

Table 2: Phytochemical constituents of aqueous extracts of Syzygium aromaticum (clove) and *Zingiber officinale* (ginger).

Constituent	Clove		Ginger
Alkaloids	+		+
Flavonoids	+		+
Glycosides	-		+
Phenols	+		+
Reducing sugars	-		-
Saponins	-		-
Steroids	-		-
Tannins	+		+

Key: - Absent; + Present

Absorbance of Aqueous Extracts of *Syzygium aromaticum* L. (Clove) and *Zingiber Officinale* (Ginger) compared with Standard Ascorbic Acid

Figure 1 shows the means of absorbances of aqueous extracts of *Syzygium aromaticum* and *Zingiber officinale* using ascorbate as standard at 518nm from triplicate assay using DPPH scavenging method. The absorbances at 50µg/ml each for *Syzygium aromaticum* and *Zingiber officinale* were 0.1961 and 0.5323, respectively compared with that of standard ascorbic acid (0.4823) at the same concentration (50µg/ml). At the highest concentration (250µg/ml), the least absorbance recorded for *Syzygium aromaticum* was 0.0370, while *Zingiber officinale* and standard ascorbic acid were 0.1137 and 0.0963 respectively. This indicates that extract of clove at both lower and higher concentrations captured more free radicals, resulting in the relatively lower absorbance observed.

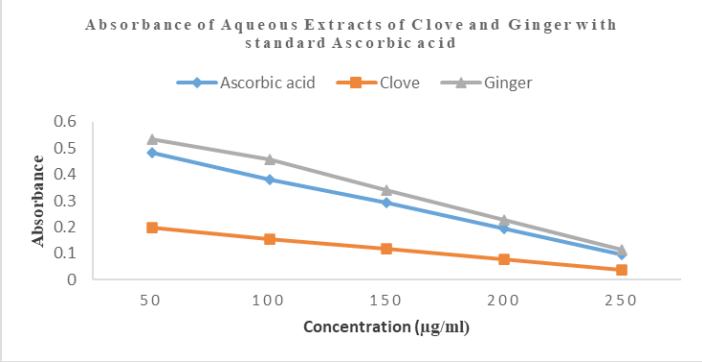

Figure 1: Absorbance of Aqueous extracts *Syzygium aromaticum L.* (Clove) and *Zingiber officinale* (Ginger) with standard Ascorbic acid at 518nm by UV visible Spectrophotometer (DPPH scavenging assay method).

Percentage Inhibition of Aqueous Extracts of *Syzygium aromaticum* (Clove) and *Zingiber officinale* (Ginger)

Figure 2 shows percentage inhibition of aqueous extracts of *Syzygium aromaticum* and *Zingiber officinale* compared with L-ascorbic acid concentration of 50µg/ml, clove possessed high percentage inhibition (67.43%) which is comparably higher than

that of ginger and ascorbic acid (11.87% and 20.15%) respectively. A sequential increase in the concentration of clove, ginger and ascorbic acid resulted in further gradual increase in percentage inhibition, and at highest concentration used (250µg/ml), the percentage inhibition of clove was 93.8% which was higher than those obtained for both ginger and ascorbic acid (81.18% and 84.06% respectively). The results indicated that clove possesses high antioxidant potential than ginger and ascorbic acid, by 20% and 15% respectively.

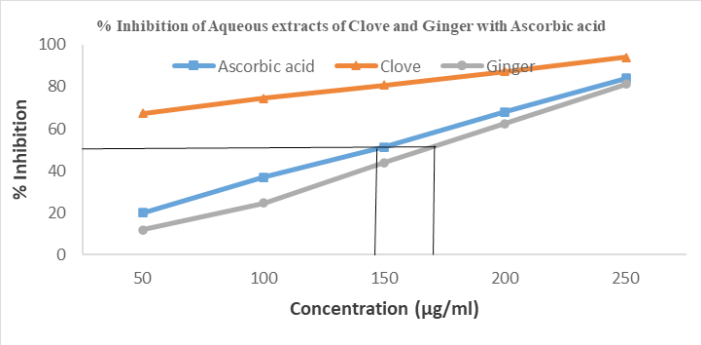

Figure 2: Percentage Inhibition of Aqueous extracts of *Syzygium aromaticum L.* (Clove) and *Zingiber officinale* (Ginger).

The Effects of *Syzygium aromaticum* L. (Clove) and *Zingiber officinale* (Ginger) on osmotic Fragility of Sickle Cells in Stable Condition.

The effects of *Syzygium aromaticum* and *Zingiber officinale* on osmotic fragility of sickle cell erythrocytes in the stable condition at different saline concentrations are shown in figure 3-5. The results recorded gradual decrease in osmotic fragility as NaCl concentration increases in both treated and untreated erythrocytes of sickle cell blood, however, the decrease was more pronounced in treated groups than the RBC of sickle cell untreated group. A complete haemolysis at 0.1% NaCl concentration was observed in all groups while at 0.2% NaCl concentration, the percentage haemolysis of the group treated with 10mg of *Syzygium aromaticum* L. was significantly (P<0.05) lower (69.12 ± 0.35%) than the untreated (98.12 ± 0.05%) and those RBC treated with ginger (98.02 ± 0.04%). It was also observed that at 0.5% NaCl concentration, the haemolysis was significantly (P<0.05) lower (8.40 ± 0.18%) in RBCs treated with 10mg of *Syzygium aromaticum* L. than that of the untreated group (45.90 ± 0.10%); whereas, at 0.9% NaCl concentration, the percentage haemolysis was 0.24 ± 0.19 in the *Syzygium aromaticum* L. group (10mg), the untreated was 2.2 ± 0.05%, the difference was also significant (P<0.05).

Similarly, the group treated with 10mg *Zingiber officinale* recorded a percentage haemolysis of 98.02 ± 0.04% and the untreated recorded 98.12 ± 0.05%, indicating no significant difference (P>0.05) at 0.2% NaCl concentration. However, at 0.5% NaCl concentration, the group treated with 10mg *Zingiber officinale* had percentage haemoysis of 20.42 ± 0.18%, which though significantly lower (P<0.05) than that of the untreated group (45.90 ± 0.10%), was however, significantly higher than that of group treated with 10mg *Syzygium aromaticum* L. (8.40 ± 0.18%) indicating that the latter has higher anti- oxidant protective effect than *Zingiber officinale*, which is consistent with the DPPH antioxidant activity test results. The results also showed that groups treated with 1mg *Syzygium aromaticum* L. and 1mg *Zingiber officinale* had percentage haemolysis values (38.04 ± 0.02% and 30.02 ± 0.1% respectively) that were significantly lower (p<0.05) than the control (45.90 ± 0.10%) at 0.5% NaCl concentration. While at NaCl concentration above or below 0.5% showed no significant difference between treated and untreated sickle cell RBCs. The median mean corpuscular fragility (MCF_{50}) of sickle cell untreated RBC was at 0.47% NaCl concentration (Figure 3). This value (0.47% NaCl) is significantly (P<0.05) higher than sickle cell erythrocytes treated with either 10mg of *Syzygium aromaticum* or 10mg of *Zingiber officinale* (0.27% and 0.37% respectively) as shown in figure 5 and 6. Whereas treatment of sickle cell erythrocytes with 1mg of either *Syzygium aromaticum* L. or *Zingiber officinale* did not produce a significant (P>0.05) decrease when compared to the untreated group (0.45% NaCl and 0.43% NaCl respectively, versus 0.47% NaCl) as shown in figure 3 and 4. Treatment with *Syzygium aromaticum* L. and *Zingiber officinale* also shifted the osmotic fragilligram to the left and it was more pronounced at higher concentration (Figure 3-6).

The median mean corpuscular fragility (MCF_{50}) of sickle cell untreated RBC was at 0.47% NaCl concentration. This value (0.47% NaCl) is significantly (P<0.05) higher than sickle cell erythrocytes treated with either 10mg of *Syzygium aromaticum* or 10mg of *Zingiber officinale* (0.27% and 0.37% respectively) (Figures 5-6). Whereas treatment of sickle cell erythrocytes with 1mg of either *Syzygium aromaticum* or *Zingiber officinale* did not produce a significant (P>0.05) decrease when compared to the untreated group (0.45% NaCl and 0.43% NaCl respectively, versus 0.47% NaCl, Figure 3 and 4,). Treatment with *Syzygium aromaticum* and *Zingiber officinale* also shifted the osmotic fragilligram to the left and it was more pronounced at higher concentration.

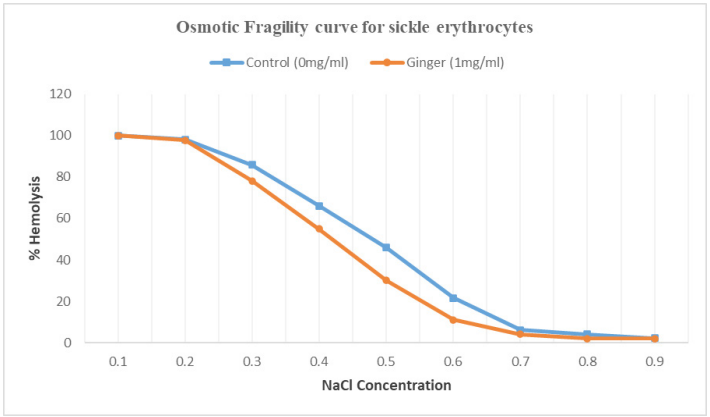

Figure 3: The Effects of 1mg of *Syzygium aromaticum L.* (Clove) on Osmotic Fragility of Sickle Cells in Steady State.

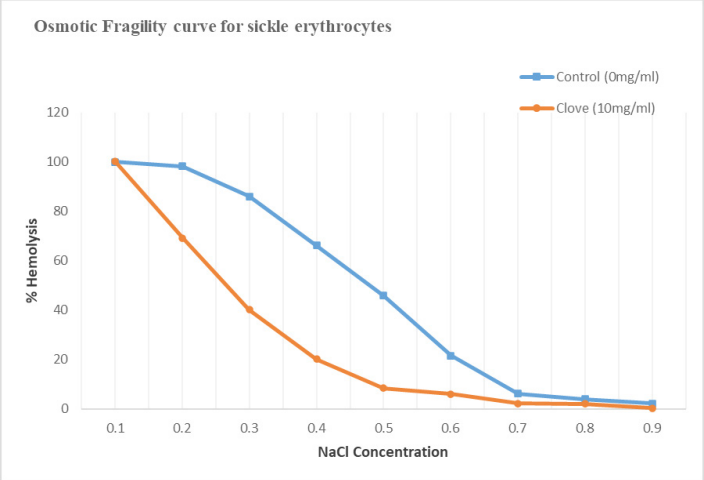

Figure 4: The Effects of 1mg of *Zingiber officinale* (Ginger) on Osmotic Fragility of Sickle Cells in Steady State.

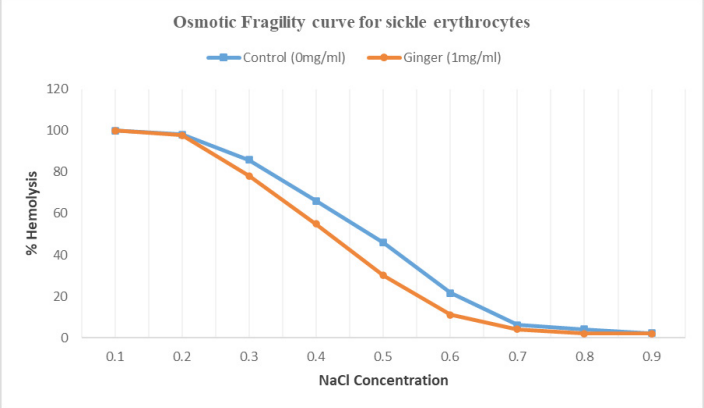

Figure 5: The Effects of 10mg of *Syzygium aromaticum L.* (Clove) on Osmotic Fragility of Sickle Cells in Steady State.

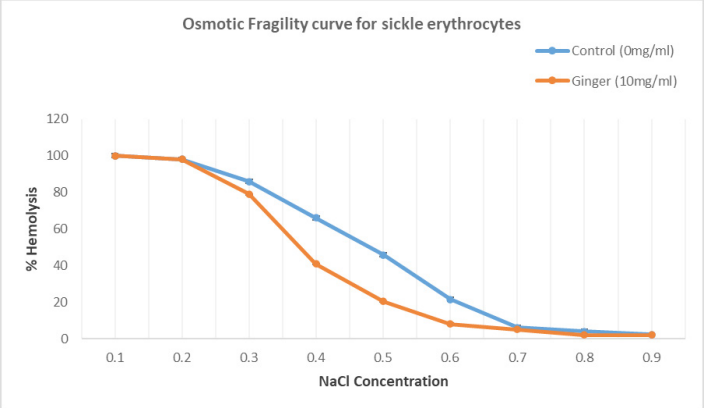

Figure 6: The Effects of 10mg of *Zingiber officinale* (Ginger) on Osmotic Fragility of Sickle Cells in Steady State.

Discussion

Phytochemical qualitative screening from the present study revealed the presence of tannins, alkaloids, phenols and flavonoids in aqueous extracts of both *Syzygium aromaticum* (clove) and *Zingiber officinale*, (ginger). These findings are consistent with previous studies [34-36]. In addition, the study also showed the presence of glycosides in ginger which was not detected in clove may probably be due to nature and environment of soil where the plant was grown. From phytochemical point of view, it seems that both plants have basically the same constituents. Glycosides were detected in ginger but not in clove, while steroids and saponins were not found in either ginger or clove. The presence of secondary metabolites/phytochemical depends on many factors that includes environment (soil composition) and plant variety. Phytochemicals found in these plants have been established to possess high antioxidant properties [36]. This claim is corroborated in this study by the results of the antioxidant assay conducted on both plants. Results from other studies on ginger reported the presence of monoterpenes [37]. *In vitro* antioxidant activity studies were carried out on both plants, using DPPH method. Results obtained from such investigation revealed an interestingly very high antioxidant potential in both plants, supporting previous studies on both plants by United States Department of Agriculture [22]. Based on their findings, clove has an Oxygen Radical Absorbance Capacity (ORAC) scale score of three hundred thousand (300,000) while ginger has an ORAC scale score of forty thousand (40,000). The same observation was also seen in this work in the *in vitro* DPPH antioxidant assay of the two plants which affirmed the presence of high antioxidant activity in both plants. Furthermore, this study showed that clove had high DPPH scavenging activity (93.08% inhibition), which was comparatively higher than that of standard L-ascorbic acid (84.06% inhibition), suggesting that the plant possess potent antioxidants that could quench free radicals in biological systems better than what had been seen in standard L-ascorbic acid. Ginger also showed antioxidant activity as indicated by the high percentage inhibition (81.18% inhibition), however, it was significantly lower than that of clove, and slightly lower than that of the standard L-ascorbic acid. These findings are supported by an earlier study [35] in which conclusive evidence indicated that clove has higher antioxidant activity than ginger. Therefore, both plants could be used to reduce free radicals and reactive oxygen species during its kinetic process and inhibit its proliferation. The relatively higher antioxidant activity observed in clove compared to ginger may be due to quantity rather than quality of the phytochemical contents in the two plants. Clove may have larger quantity of the phytochemicals than are found in ginger. The higher antioxidant activity of clove may also be attributed to the solvent used in extraction, perhaps the phytochemicals in clove are more soluble in water than those found in ginger.

The study also evaluated the effects of antioxidant activity of these two plants (clove and ginger) on erythrocyte osmotic fragility of sickle cell erythrocytes for probable beneficial effects. The hallmark of pathogenesis of sickle cell erythrocytes is their capability of releasing oxidant agents [3,38,39] in amounts that double that of normal RBCs due to exposure of these cells to high oxygen content [40]. Erythrocytes are the main cells in circulation and they are responsible for oxygen transport. Erythrocytes have no nucleus and organelles, and have a particular

membrane structure composition made up of 35.1% lipids. The unique structural composition of the erythrocyte membrane allows it to undergo large deformations as it penetrates tissues while maintaining structural integrity, hence it has the ability to resume its original shape when distorted, this makes the plasma membrane of erythrocytes a good model for erythrocyte osmotic fragility assay, a test based on measuring the efflux of haemoglobin from suspended erythrocytes [33]. The results showed that osmotic fragilligram for both sickle cell RBCs treated with clove or ginger, and untreated sickle cell RBCs showed decrease in haemolysis with increasing concentration of NaCl from 0.1% to 0.9%. However, the decrease was more pronounced in the treatment groups with clove having the highest percentage decrease. The results also noticed that at 0.2% NaCl, a significant decrease from 100% ± 0.02 at 0.1% NaCl to 69.12% ± 0.35 in the percentage haemolysis of the group treated with 10mg of clove. This decrease could be due to high resistance of RBC membranes to osmotic damage. The probable mechanism may be attributed to high antioxidant activity of both clove and ginger, or other protective mechanism in crude water extract of these plants. Moreover, antioxidants should be implicated due to the fact that they neutralized oxidative damage of cell membrane through peroxidation of the lipids in the bilayer. Lipid peroxidation refers to the oxidative degeneration of lipids, which is characterized by the formation of a hydroperoxide group in the lipid tails of cell membrane fattyacids. The hydroperoxide group forms in a chain of reactions which are initiated when a free radical attacks a weak allylic C- H bond. The hydroperoxide lipid derivatives thus formed decompose into secondary products such as cytotoxic 4- hydroxynonenal and malondialdehyde. Extensive lipid peroxidation in cell membranes causes loss of fluidity, lowers phase transition temperature and thinning of the bilayer, leading to increased permeability and eventual rupture. The peroxidation chain reaction is broken and damage to membrane is stopped when antioxidants are introduced [41,42] which neutralize the effects of the oxidants that initiated it. In addition, studies have shown that total antioxidant level in SCD are depressed [43], while total antioxidant status (TAS) level were about 50% lower in the SCD patients [44]. This might have been the reason for the discrepancies in the concentration of NaCl at which certain percentages of haemolysis occurred in treated groups when compared with untreated sickle cell RBCs. The concentration of NaCl at which 50 percent of erythrocytes lyse decreased from 0.47 percent in untreated sickle cell RBCS to 0.26 percent and 0.37 percent when sickle cell RBCs were treated with clove and ginger respectively, indicating that these plants increased the resistance of erythrocytes to lysis. A comparable analysis of DPPH antioxidant assay using ascorbic acid as standard showed higher antioxidant activity are found in clove compared to the standard, while ginger showed no significant difference compared with standard ascorbic acid, indicating probable preference of clove to ascorbic acid. Probably one of the significant findings of this study is the results of DPPH antioxidant assay of aqueous extracts of clove and ginger which correlated well with the osmotic fragiligram of sickle cell RBCs treated with these plants. The results indicated that the osmotic fragility curve shifted to the left, which is more pronounced in sickle cell RBCs treated with clove compared to those treated with ginger. Thus supporting the finding of DPPH antioxidant assay that clove has more potent antioxidant activity than ginger and ascorbic acid. Therefore, clove may be preferable to ascorbic acid based on the findings in this study.

Conclusion

This study has shown that aqueous extracts of *Syzygium aromaticum* (clove) and *Zingiber offinale* (ginger) contain phytochemical compounds which were responsible for the high antioxidant activity observed. The reduced osmotic fragility in sickle cell erythrocytes following administration of *Syzygium aromaticum* (clove) and *Zingiber offinale* (ginger) shows potential for the two plants to be used as potent treatment strategy options for management of SCD.

Recommendations

Based on the findings of this work, it may be recommended that further studies into the effects of *Syzygium aromaticum* and *Zingiber offinale* on erythrocyte osmotic fragility of sickle cell erythrocytes be undertaken *in vivo*. It is also recommended that *Syzygium aromaticum* (clove) be used as alternative treatment strategy for haemolytic crisis in SCD patients which could bring about relief in other forms of crises.

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References

1. WHO (World Health Organization). Sickle cell anaemia report by the secretariat. 59th world health assembly. 2006; A59/9.
2. Charache S, Micheal J, Dover PB, et al. Effect of Hydroxyurea on the Frequency of painful crises in sickle cell Anaemia. New England Journal of Medicine. 1995; 332: 1317-1322.
3. Uzunova V, Pan V, Galkin OH, et al. Free heme and the polymerization of sickle cell hemoglobin. Biophysical Journal. 2010; 99: 1976-1985.
4. Dong C, Chadwick RS, Schechter AN. Influence of sickle haemoglobin Polymerization and membrane properties on deformability of sickle erythrocytes in the microcirculation. Biophysical Journal. 1992; 63: 774-783.
5. Metteis DF, Smith IJ, Reed DJ. "Toxicity of Oxygen" in Molecular and Cellular Mechanisms of Toxicity. CRC Press. Boca Raton Florida U.S.A. 1995; 35-68.

6. Marks DB, Marks AD, Smith CM. "Oxygen Metabolism and Toxicity". Basic Medical Biochemistiy: A chemical Approach. William and Wilkins, Baltimore, Maryland U.S.A. 1996; 327-340.
7. Hebbel RP. Reconstructing Sickle Cell Disease: a data- based analysis of the "hyperhemolysis Paradigm" for pulmonary hypertension from the perspective of evidence- based medicine. American Journal of Haematology. 2011; 86: 123-154.
8. Belcher JD, Beckmen JD, Balla G, et al. Heme degradation and vascular injury. Antioxidants and Redox signaling. 2010; 12: 2333-248.
9. Chirico EN, Pialoux V. Role of oxidative stress in pathogenesis of sickle cell disease. International Union of Biochemistry and Molecular Biology life. 2012; 64: 72-80.
10. Raphael FQ, Emerson SL. Oxidative Stress in sickle cell disease. Rev Bras Hemoter. 2013; 35: 3-17.
11. Nur E, Biemond BJ, Often HM, et al. Oxidative stress in sickle cell Disease: Pathophysiology and potential implications for Disease Management. American Journal of Haematoiogy. 2011; 86: 484-489.
12. Kehinde MO, Ogungbemi SI, Anigbogu CN, et al. L. Arginine Supplementation enhances antioxidant activity and erythrocyte integrity in sickle cell Aneamia Subjects. Journal of Pathophysiology. 2015; 22: 137-142.
13. Silva DG, Belini JB, Torres LS, et al. Relationship between oxidative stress, Glutathione S-transferase polymorphisms and Hydroxyurea treatment in sickle cell anaemia. Blood Cells Molecules and Diseases. 2011; 47: 23-28.
14. Bao B, Prascal AS, Beck FH, et al. Zinc supplementation Decreases oxidative stress, incidence of infection and Generation of inflammatory Cytokines in Sickle Cell Disease Patients. Translational Research. 2008; 152: 67-80.
15. Natta CL, Machlin LJ, Brin M. A Decrease in Irreversibly sickled Erythrocytes in sickle cell Anaemia patients given vitamin E. American Journal of Clinical Nutrition. 1980; 33: 968-971.
16. Morris CR, Kato GJ, Poljakovic M, et al. Dysregulated arginine metabolism, hemolysis associated pulmonary hypertension, and mortality in sickle cell Disease. Journal of the American Medical Association. 2005; 294: 81-90.
17. Yamahara J, Miki K, Chisaka T, et al. Cholagic Effects of Ginger and its Active Constituents. Journal of Ethnopharmacology. 1985; 13: 217-225.
18. Stoilava I, Krastaniv ASA, Denev P, et al. Antioxidant activity of a Ginger Extract. Food Chemistry. 2007; 102: 764-770.
19. Taj E, Elmutalib IM, Hiba MA, et al. An invitro anticoagulant effect of aqueous extract of ginger (zingiber officinale) rhizomes in blood samples of normal individuals. American journal of research communication. 2016; 4: 13-121.
20. Alabdallat NG, Adam I. *In vitro* anti-sickling activity of Zingiber officinale Roscoe (Ginger) Methanolic Extract on sickle all Disease. British Journal of Medicine and Medicinal Research. 2016; 12: 1-7.
21. Ghadage P, Satish HS, Niranjan Y, et al. De -addiction potential of clove in Opiate addiction in sickle cell Disease. World Journal of Pharmaceutical Research. 2017; 6: 1546-1556.
22. USDA: United States Department of Agriculture. Database for the oxygen radical absorbance capacity (ORAC) of selected foods. Nutrient Data laboratory; Beltsville. 2017.
23. Handa SS, Khanuja SPS, Long G, et al. Extraction Technologies for Medicinal and Aromatic Plants 1st edn. United Nations Industrial Development Organization and the International Center for Science and High Technology. Italy. 2008; 66: 70-72.
24. Akinbami A, Dosunmu A, Adediran A, et al. Steady state hemoglobin concentration and packed cell volume in homozygous sickle cell disease patients in Lagos, Nigeria. Caspian Journal of International Medicine. 2012; 3: 405-409.
25. American Psychological Association. Ethical Principles of Psychologists and Code of Conduct, pp. 3-5. Washington, DC. January 1. 2017.
26. Evans HC. Trease and Evans Pharmacognosy, 15th Edition. WB Sanders Company Ltd. London, 2002; 131-139. 230-240.
27. Banu K, Cathrine L. General Techniques involved in Phytochemical Analysis. International Journal of Advanced Research in Chemical Science. 2015; 2: 25-32.
28. Harbone RP. Phytochemical Methods: A guide to modern techniques of plant analysis 13th edition, Chapman and Hall Ltd, London. 1973; 5-15.
29. Olugbami JO, Gbadegesin MA, Odinola, OA. *In vitro* free radical scavenging and antioxidant properties of ethanol extract of Terminalia glaucescens. Pharmacognosy Research. 2015; 7: 49-56.
30. Islah L, Bargaa R, Chabir Y, et al. Study of incubation conditions for Erythrocytes Osmotic Fragility Testing in Dromedary Camel (Camelus dromedaries). International Journal of Research in Environmental Science. 2016; 2: 22-32.
31. Adalgisa IM, Giuseppe AP, Sebastiao DS, et al. Osmotic and morphological effects on red blood cell membrane: action of an aqueous extract of Lantana camara. Brazilian Journal of Pharmacognosy. 2008; 18: 42-46.
32. Pauline N, Cabral BNP, Anatole PC, et al. The *in vitro* anti- sickling and antioxidant effects of aqueous extracts of zanthoxyllum heitzii on sickle cell disorder. BMC complementary and Alternative Medicine. 2013; 13: 162.
33. Pagano M, Faggio C. The use of Erythrocyte fragility to assess Xenobiotic cytotoxicity. Cell Biochemistry and Function. 2015; 33: 351-35.
34. Kahkonen MP, Anu IH, Heikki JV, et al. Antioxidant activity of Plant extracts containing Phenolic Compounds. Journal of Agricultural and Food Chemistry. 1999; 47: 3954-3963.

35. Perez - Jemenez J, Neven V, Vos F, et al. Identification of the 100 Richest Dietary Sources of Polyphenols: an application of the phenol- explorer Database. European Journal of Clinical Nutrition. 2010; 64: S112-S120.

36. Middleton JE. Biological properties of plant flavonoids: an overview. International Journal of Pharmacognosy. 1996; 34: 344-348.

37. Brunneton J. Ginger: Pharmacognosy, Phytochemistry. Medicinal Plants, Lavoiseir Pulishing Co. Inc. 1995; 258-261.

38. Aslan M, Thorley-Brown D, Freeman BA. Reactive Species in Sickle Cell Disease. Annals of the New York Academy of Sciences. 2000; 899: 375-391.

39. Wood KC, Hebbel RP, Lefer DJ, et al. Critical role of endothelial cell derived nitric oxide synthase in sickle cell disease-induced microvascular dysfunction. Free Radical Biology and Medicine. 2006; 40: 1443-1453.

40. Amer J, Ghoti H, Rachmilewitz E, et al. Red blood cells, Platelets and polymorphonuclear Neutrophils of patients with sickle cell disease exhibit oxidative stress that can be ameliorated by antioxidants. British Journal of Haematology. 2006; 132: 108-113.

41. Yun OX, Zeng A, Brennan CS, et al. Effect of pulsed electric field on membrane lipids and oxidative injury of salmonella typhimurium. International Journal of Molecular Sciences. 2016; 17: 1374.

42. Rems L, Marilyne V, Marina AK, et al. The contribution of lipid peroxidation to membrane permeability in electropermeabilization: A molecular dynamics study. Bioelectrochemistry. 2019; 125: 46-57.

43. Kehinde MO, Ogungbemi SI, Anigbogu CN, et al. I-Arginine Supplementation enhances antioxidant activity and erythrocyte integrity in sickle cell Aneamia Subjects. Journal of Pathophysiology. 2015; 22: 137-142.

44. Fasola F, Adedapo K, Anetor J, et al. Total Antioxidant Status and some Haematological value in Sickle Cell Disease Patients in steady state. Journal Medical Association. 2009; 99: 891-894.