American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

Stabilizing Ability and Anti-sickling Potentials of *Ganoderma Lucidum* Decoction Extract on Human HbS Erythrocytes Membrane

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Received:07 October 2018 ; | Revised:09 November 2018; | Accepted: 10 December 2018

Abstract

Anti-sickling properties of *Ganoderma lucidum* decoction extract were studied using human HbS erythrocytes. The % lysis, median corpuscular fragility, % stability, polymerisation rate and morphological deformations were determined. The lowest % lysis of (6.76 + 1.63% and 6.15 + 1.96%) were observed in cells treated with 5 mg/ml of extract at 7.0 g/l and 9.0 g/l saline concentrations, while increase in median corpuscular fragility of the erythrocytes were observed, with values of 5.33 + 0.41 g/100g, 5.99 + 0.83 g/100g and 6.08 + 0.51 g/100g for 5 mg/ml, 10 mg/ml and 15 mg/ml of extract. Erythrocytes percentage stability also increased with values of 12.92 + 0.86 %, 26.91 + 1.05 % and 28.81 + 0.94 % for 5 mg/ml, 10 mg/ml and 15 mg/ml of extract, while a pronounced decrease in haemoglobin polymerisation rate was observed at 15 mg/ml of extract from the 2nd to the 3rd minute of analysis with values 0.78 + 0.12 %, 0.47 + 0.07 % and 0.58 + 0.09 % for 2.0, 2.5 and 3.0 minutes. The lowest erythrocytes morphological deformity score was observed at 15.0 mg/ml extract with values of 80.00 + 3.70 %, 56.00 + 2.80 % and 44.00 + 2.20 % at 0.0, 5.0 and 10.0 minutes respectively.

Keywords: Ganoderma lucidum, Decoction extract, Erythrocytes, Stability, Polymerization

1. Introduction

Sickle cell disease is a disorder of the haemoglobin that alters the normal morphology of the erythrocytes thereby producing a crescent shaped or sickled erythrocyte. At the molecular level, this inheritable disease is caused by a mutation of a nucleotide of the β -globin gene from a GAG codon that translates to glutamic acid to a GTG codon which subsequently transcribes into a GUG and translates to valine. This tantamount to

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substitution of glutamic acid at position 6 with valine (if the starting methionine in amino acid numbering of a protein was skipped) or at position 7 (if the methionine was counted as the first amino acid). Though sickle cell disease is more prevalent (about 80 %) in sub-Sahara Africa [1], its occurrence in parts of India, the Arabian peninsula, and among people of African origin living in other parts of the world has been reported [2]. Different health problems such as attacks of pain (sickle-cell crisis), anaemia, bacterial infections and stroke have been associated with sickle cell disease [3]. To a large extent, these disease conditions have been managed with vaccination, pain medication, high fluid intake, preventive antibiotics and folic acid supplementation [4,5]. While advanced measure such as blood transfusion, administration of hydroxycarbamide [5] and bone marrow transplantation [6] have been taken into consideration in the cause of management and treatment of sickle cell anaemia. Irrespective of these advances in sickle cell management and treatment, the mortality rate increased from 113,000 in 1990 to 176,000 in 2013 [7], thereby necessitating the need for more research.

The relevance and rich medicinal history of most mushrooms demands for researches using extracts of such mushrooms in the treatment and management of notable disease conditions such as sickle cell anaemia. Though some of these mushrooms are still very relevant in traditional medicine for treatment of diseases mainly of microbial related infections [8], most of their isolates have shown potential anti-inflammatory and anti-diabetic properties in preliminary studies [9]. Some of their polysaccharides, glycoproteins, proteoglycans and other components are presently under basic research for their potential to modulate immune system responses and inhibit tumor growth [8]. One notable mushroom with a long medicinal history is the *Gernoderma. lucidum* (also known as Reishi or Lingzhi mushroom). *G. lucidum* is a polypore mushroom that grows on wood, and many of its known 80 species are from tropical regions [10]. Scientific studies on the extracts from the fruiting body, mycelia and spores of this mushroom have shown its ability to boost the immune system, work against herpes virus, reduce cholesterol level and stop cell proliferation [11]. Yuen and Gohel [12] and Zaidman et al., [13] also reported that most polysaccharides and triterpenes, extracted from G. lucidum exhibits chemopreventive and tumoricidal effects. This study will determine the possibility of Ganoderma lucidum decoction extract to elicit membrane stabilization and anti-sickling potentials on Human HbS erythrocytes membrane.

2. Experimental section

2.1 Sample collection, preparation and extraction

Fresh *G. lucidum* was obtained from its natural environment within the University of Port Harcourt in Rivers State, South-south Nigeria. A quantity of the mushroom (3.5 kg) was thoroughly washed with distilled water, sliced with a sterilized knife and air dried for a period of one (1) week in a clean dust free environment. The dried samples were ground using a Thomas Scientific, (Model 4) Wiley's mill until a fine smooth powder was obtained. Five hundred grams of ground *G. lucidum* was weighed out and 0.5 dm3 of distilled water was added and mixed properly. The mixture was boiled at 100 0C for 30 min. and after 2 hrs of cooling, the mixture was filtered and the filtrate was concentrated to dryness using a Secador 4.0 autodesicator.

2.2 Preparation of *G. lucidum* sample solution

A volume of 15 mg/ml stock solution of *G. lucidum* decoction extract solution was prepared by dissolving 0.375 g of dried decoction extract of *G. lucidum* in deionized water and adjusted to 0.25 dm3, Volumes of 5.0 mg/ml and 10.0 mg/ml were obtained from the stock solution via serial dilution.

2.3 Collection and preparation of blood sample

A volume of 5.0 ml of HbS venous blood sample was collected by venipuncture from an apparently healthy volunteer using a sterilized 5.0 ml syringe. The blood was immediately transferred into ethylenediaminetetraacetic acid (EDTA) sample bottle. Exactly 1.0 ml of the blood sample was introduced into a 10.0 ml test tube and 5.0 ml of 0.9 % physiological saline was added. The erythrocyte suspension was gently mixed and centrifuged in a Sorvall Instruments GLC-4 General Laboratory Centrifuge at 2500 rotation per minute (r.p.m) for 10 min. and the supernatant was gently removed using a Pasteur pipette. The procedure was repeated twice and the washed erythrocytes were

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suspended in physiological saline and use for analysis within 2 hours.

2.4 Determination of osmotic fragility of erythrocytes

Erythrocyte osmotic fragility was determined using the method described by Dacie et al., [14]. A volume of 5.0 ml each of saline solutions 9.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0 and 1.0 g/l NaCl were placed into 1 x 12cm labeled test tubes, while 5.0 ml of deionized water was introduced into the control test tube. Exactly of 0.5 ml of 5.0 mg/ml of *G. lucidum* extract was added into all the test tubes and 50.0 µl of properly mixed fresh blood was added into each of the tubes. The mixtures were carefully and properly mixed immediately to avoid foaming. After incubation at room temperature for 30 min., the mixtures were carefully mixed again and centrifuged at 1200 rpm for 5 mins. Using the supernatant from test tube 1 (osmotically equivalent to 9.0 g/l NaCl) as the blank, the supernatants were collected and the quantity of hemoglobin released was colorimetrically determined at 540 nm using a Labtech single beam 295 Advanced Microprocessor UV-VIS spectrophotometer. This procedure was repeated for 10.0 mg/ml and 15.0 mg/ml of *G. lucidum* extract. The % lyses was calculated and the results were plotted against the NaCl concentration using the absorbance of deionized water (control test tube) as 100% lysis point.

$$\% \text{ lysis} = \{As / Ac\} \times 100.$$

Where: As = absorbance of sample.
Ac = absorbance of control.

The median corpuscular fraction (MCF) at 50% lysis was extrapolated as described by Dewey et al., [15] and Krogmeier et al., [16] and the % stability was calculated using the method described by Chikezie et al., [17] with slight modification.

$$\% \text{ stability} = \frac{mcf_t - mcf_c}{mcf_c} \times 100.$$

Where: mcft=Median corpuscular fraction of test.
mcfc=Median corpuscular fraction of control.

2.5 Determination of haemoglobin polymerization rate

Haemoglobin polymerization rate was determined using the method of Noguchi and Schechter, [18], as modified by Iwu et al., [19], A volume of 4.40 ml of freshly prepared 2 % w/v Sodium metabisulphite (Na2S2O2) was placed into a 5.0 ml plain sample bottle and 0.1 ml of washed human Hbs erythrocyte haemolysate was added and mixed with 0.5 ml of 5.0 mg/ml of *G. lucidium* extract, while 0.5 ml of deionized water was added in the control bottle. The mixture was gently transferred into a 1 cm cuvette and the absorbance at 700 nm was taken at 30 sec. interval for 3 min. using a Labtech single beam 295 Advanced Microprocessor UV-VIS spectrophotometer. This procedure was repeated using 10.0 mg/ml and 15.0 mg/ml of *G. lucidum* extract. The polymerization rate was calculated and expressed as percentages.

$$\text{Polymerization rate } (P_R) = \frac{a_{fn} - a_{i0}}{t_n}$$

Where: afn = Final absorbance at time (t) = n.
ai0 = Initial absorbance at time (t) = 0.
tn = Time of reaction in minutes.

2.6 Erythrocytes culturing and smear preparation

This was carried out using the method described by Daland and Castle [20], exactly 0.1 ml of washed human HbS erythrocytes was placed in a test tube and mixed with 0.5 ml of 5.0 mg/ml of G. lucidium extract, while 0.5 ml of deionized water was added to the control. Smear of 10.0 µl of the mixture was prepared according to the method described by Brown [21], where a monolayer of erythrocytes was obtained by carefully dispersing a drop of the suspended erythrocytes placed on one end of a slide over the slide's length using a spreader slide. The feathered edge of the monolayer was viewed at 0.0, 5.0 and 10.0 mins. at X400 magnification under an Olympus CX31RTSF microscope and snapped using Olympus E330-ADU1.2X 6K13883 camera. This procedure was repeated for 10.0 mg/ml and 15.0 mg/ml of G. lucidium extract.

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2.7 Statistical analysis

The mean + standard deviations and One Way Analysis of Variance (ANOVA) were calculated electronically using International Business Machine (IBM) Statistical Program for Social Sciences (SPSS) 19 statistics software (SPSS Inc Chicago) at 95% confidence level, using post hoc Duncan [22], multiple range test of significance.

3. Results

The result of this study showed a decrease in lysis rate of the erythrocyte as the concentration of the buffered saline increases. Lowest values of 6.76 + 1.63 % and 6.15 + 1.96 % were observed for 5.0 mg/ml of *G. lucidum* extract concentration at 7.0 g/l and 9.0 g/l of saline concentration, while 10.0 mg/ml of *G. lucidum* extract concentration had 15.87 + 2.71 % and 14.62 + 3.11 % at 7.0 g/l and 9.0 g/l of buffered saline concentration. Values of 17.56 + 2.74 % and 17.70 + 2.61 % were observed for 15.0 mg/ml of *G. lucidum* extract concentration at 7.0 g/l and 9.0 g/l of buffered saline concentration, while the control had 3.90 + 0.59 % and 2.60 + 0.83 % at 7.0 g/l and 9.0 g/l of saline concentration (Fig. 1).

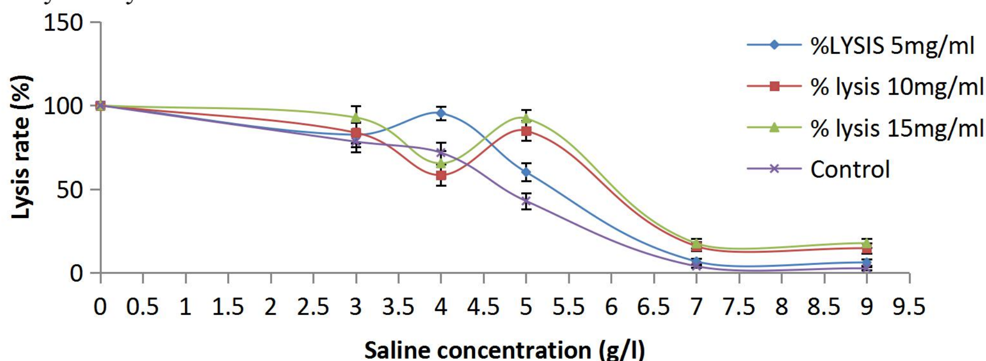

Fig.1. Percentage lysis of erythrocytes treated with different concentrations of *G. lucidum* decoction extract

A continuous increase in median corpuscular fragility of the erythrocyte was observed as the concentration of the *G. lucidum* extract increases. Values of 5.33 + 0.41 g/100g, 5.99 + 0.83 g/100g and 6.08 + 0.51g/100g were observed for 5.0 mg/ml, 10.0 mg/ml and 15.0 mg/ml of *G. lucidum* extract respectively, while the control had a median corpuscular fragility 4.72 + 0.21 g/100g (Fig. 2).

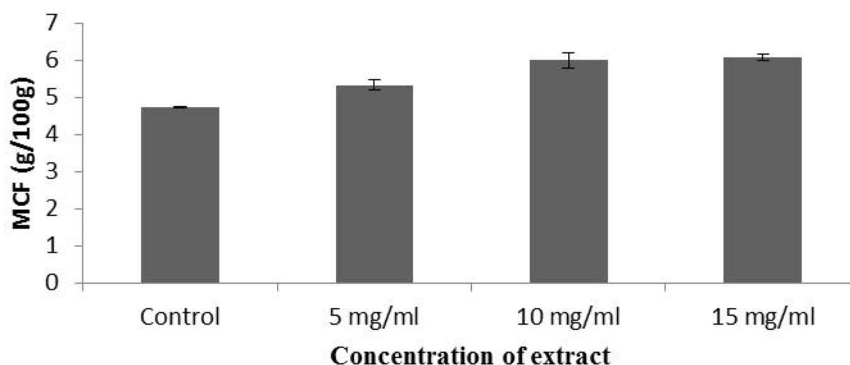

Fig.2. Median corpuscular fragility (MCF) of erythrocytes treated with different concentrations of *G. lucidum* decoction extract

The stability rate (%) of the erythrocytes also increased as the concentration of *G. lucidum* extract increases with values of 12.92 + 0.86 %, 26.91 + 1.05% and 28.81 + 0.94 % for 5.0 mg/ml, 10.0 mg/ml and 15.0 mg/ml of *G. lucidum* extract respectively, while zero stability was observed for the control (Fig.3)

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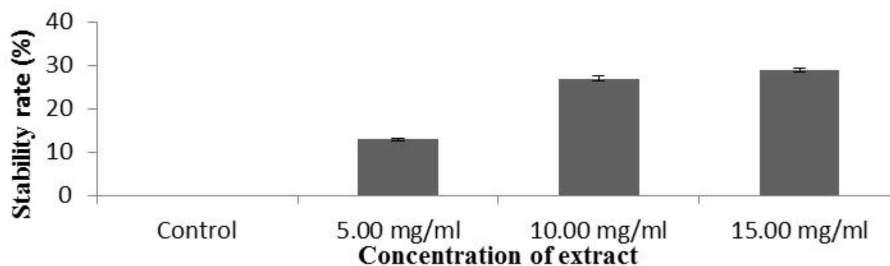

Fig.3. Stability rate (%) of erythrocytes treated with different concentrations of *G. lucidum* decoction extract

A decrease in haemoglobin polymerisation was observed mainly from the second to the third minute with values of 2.27 + 0.64 %, 1.57 + 0.49 % and 1.43 + 0.23 % at 2.0 min, 2.5 min. and 3.0 min for 5.0 mg/ml *G. lucidum* extract. Values 0.63 + 0.10 %, 0.48 + 0.08 % and 0.58 + 0.11% at 2.0 min, 2.5 min. and 3.0 min were observed for 10.0 mg/ml *G. lucidum* extract, while 15.0 mg/ml of *G. lucidum* extract had 0.78 + 0.12 %, 0.47 + 0.07 % and 0.58 + 0.09 % at 2.0 min, 2.5 min. and 3.0 min. respectively (Fig. 4).

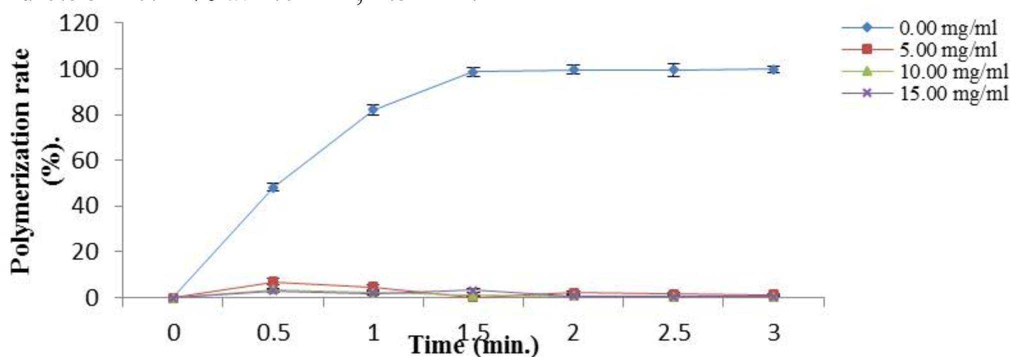

Fig.4. Polymerization rate (%) of erythrocytes treated with different concentrations of *G. lucidum* decoction extract

The lowest morphological deformity scores were observed in the erythrocytes cultured with 15.0 mg/ml of *G. lucidum* extract with values of 80.00 + 3.70 %, 56.00 + 2.80 % and 44.00 + 2.20 % at 0.0 min., 5.0 min. and 10.0 min. respectively, while those cultured with 10.0 mg/ml of *G. lucidum* extract had values of 82.00 + 2.90 %, 78.00 + 3.40% and 71.00 + 4.80 % at 0.0 min., 5.0 min. and 10.0 min. respectively. Values of 86 + 6.10 %, 88.00 + 2.70 % and 82.00 + 3.60 % at 0.0 min., 5.0 min. and 10.0 min. was observed for those cultured with 5.0 mg/ml of *G. lucidum* extract, while the control (those cultured with 0.0 mg/ml) had values of 80.00 + 3.70 %, 93.00 + 3.90 % and 93.00 + 5.20 % for 0.0 min., 5.0 min. and 10.0 min. respectively.

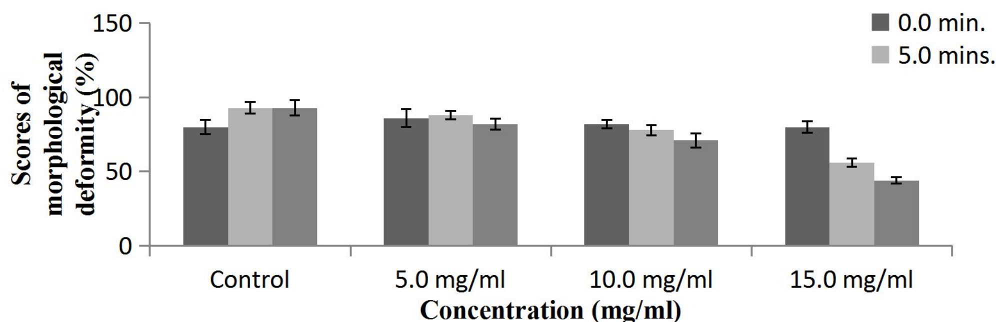

Fig.5. Scores of morphological deformities (%) of erythrocytes treatment with different concentrations of *G. lucidum* decoction extract

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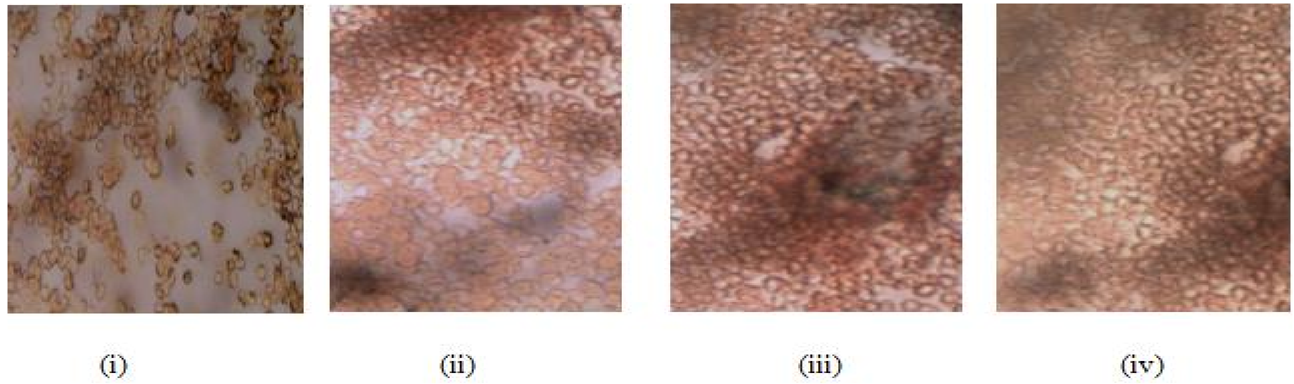

Fig.6a. Morphology of erythrocytes at 0.0 min. of treatment with different concentrations of *G. lucidum* decoction extract (Magnification. X400)

(i). Control (showing clusters of sickled, slightly-sickled and slightly normal erythrocytes). (ii). Erythrocytes treated with 5.0 mg/ml of *G. lucidum* extract (showing clusters of sickled and slightly-sickled erythrocytes). (iii). Erythrocytes treated with 10.0 mg/ml of *G. lucidum* extract (showing sickled, slightly-sickled and few normal erythrocytes). (iv). Erythrocytes treated with 15.0 mg/ml of *G. lucidum* extract (showing sickled, slightly sickled and few normal erythrocytes) .

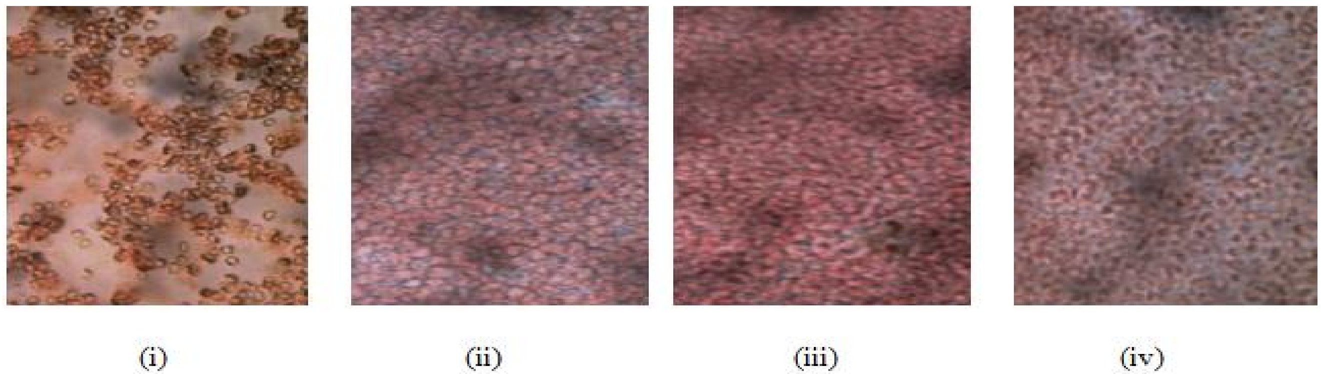

Fig. 6b. Morphology of erythrocytes at 5.0 min. of treatment with different concentrations of *G. lucidum* decoction extract (Magnification. X400)

(i). Control (showing clusters of sickled, slightly-sickled and slightly normal erythrocytes). (ii). Erythrocytes treated with 5.0 mg/ml of *G. lucidum* extract (showing sickled, slightly-sickled and morphologically deformed erythrocytes). (iii). Erythrocytes treated with 10.0 mg/ml of *G. lucidum* extract (showing sickled, slightly-sickled and morphologically deformed erythrocytes). (iv). Erythrocytes treated with 15.0 mg/ml of *G. lucidum* extract (showing few slightly-sickled and normal morphologically stable erythrocytes).

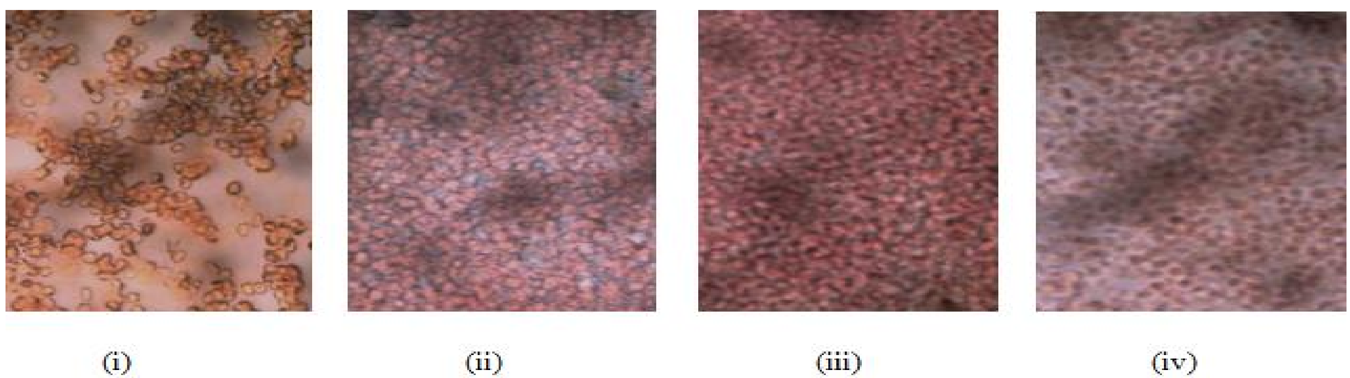

Fig .6c. Morphology of erythrocytes at 10.0 min.of treatment with different concentrations of *G. lucidum* decoction extract (Magnification. X400)

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(i). Control (showing clusters of sickled, semi-sickled and few normal erythrocytes). (ii). Erythrocytes treated with 5.0 mg/ml of *G. lucidum* extract (showing sickled, slightly-sickled and morphologically deformed erythrocytes) (iii). Erythrocytes treated with 10.0 mg/ml of *G. lucidum* extract (showing slightly-sickled and few normal erythrocytes). (iv). Erythrocytes treated with 15.0 mg/ml of *G. lucidum* extract (showing normal and morphologically stable erythrocytes).

4. Discussion

The structural transformation of the erythrocytes has been an important phenomenon that aids blood circulation. Mohandas and Fea [23], reported the ability of the erythrocytes to move through successive stages that spans between biconcave discocyte and complete spherical form. Erythrocytes suspended in hypotonic medium undergo irreversible transformations that tantamount into hemolysis after entering the spherical phase [24]. The sigmoid shape of the normal (AA) % lysis (osmotic fragility) curve observed in fig. 1 indicates that normal erythrocytes may vary in their resistance to hypotonic solutions. The non-significant decrease ($p < 0.05$) in lysis rate observed when 10.0 mg/ml and 15.0 mg/ml of mushroom extract was added to the erythrocytes suspended in 4.0 g/l saline concentration show the ability of this mushroom extract to minimally reduce hemolysis at such hypotonic saline concentration. This marginal decrease indicates that the decoction extract of this mushroom may have either stabilized the membrane composition or preserved the integrity of the cells at such saline concentration but was unable to sustain such stability at higher saline concentration and even at the isotonic concentration of 9.0 g/l. This corroborates the works of [25], [26], [27], where cell membrane composition, membrane integrity and cells' sizes were reported to affect osmotic fragility.

The significant increase ($p < 0.05$) in stability rate observed when 5.0 mg/ml, 10.0 mg/ml and 15.0 mg/ml of mushroom extract was added to the erythrocytes (see fig. 3) shows the extracts of this mushroom may alter the erythrocyte membrane, thereby enhancing the passage of sodium out of the erythrocytes, which may tantamount to increase in percentage stability. The significant decrease in haemoglobin polymerization rate ($p < 0.05$) observed in this study (see fig. 4) shows that some components of this mushroom may have the ability to enhance the binding and transportation of oxygen by reversing the deoxygenating effect of sodium metabisulphite thereby preserving or stabilizing the haemoglobin structure. The low morphological deformity scores observed in the erythrocytes cultured with 15.0 mg/ml and 10.0 mg/ml of *G. lucidum* extract for 5.0 min. and 10.0 min. (see fig. 5) reveals the ability of this mushroom extract to stabilize the structure of the erythrocytes. The morphologically stable erythrocytes observed at 5.0 min. and 10.0 min. when cultured with 15.0 mg/ml of the extract (see figs. 6a – 6c) indicates that compound(s) present in the extract of this mushroom can potentially reduce and possibly reverse erythrocyte sickling in a concentration and time dependent manner. As stated earlier, this low morphological deformity indicates that a certain non-volatile component(s) of this mushroom may have the ability to reverse and sustain the erythrocyte membrane integrity, thereby enhancing erythrocyte capillary passage and possibly reducing sickle cell attributed pains and related crisis.

5. Conclusion

The reversal and stabilization of erythrocyte membrane integrity and morphology observed in this study is a clear indication that non-volatile component(s) present in the decoction extract of this mushroom may be responsible for its anti-sickling properties and isolation of this active component(s) may be of good use in the amelioration sickle cell anaemia and its associated crises.

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