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IN-VITRO ANTI-SICKLING POTENTIAL OF CATECHIN AND THE FUNCTIONAL CHEMISTRY AND METABOLIC PATHWAYS ANALYSIS OF HUMAN SICKLE ERYTHROCYTES

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ABSTRACT: Sickle cell disease (SCD) treatment and management remain a challenging puzzle, especially among developing Nations. We evaluated catechin's sickling-suppressive properties using *in-vitro* and bioinformatics approaches in human sickle erythrocytes. Sickling was maximally induced (76%) using 2% sodium metabisulfite (SMS) at 3h. Addition of catechin prevented the sickling by SMS at 1mM (81.19%) and reversed the same at 1mM (84.63%), with IC_{50} values of 1.026µM and 1.103µM, respectively. Based on functional chemistry, catechin alters the functional groups of certain notable compounds within erythrocytes, favouring its anti-sickling effects, as indicated by the observed bends and shifts. From GC-MS and LC-MS analyses, it was observed that catechin treatment favours fatty acid alkyl monoesters (FAMEs) production with concomitant shutting down effects on selenocompound metabolism. Pathway enrichment and topology analyses revealed activation of fatty acid biosynthesis, linoleic acid metabolism and steroid hormone biosynthesis pathways upon catechin treatment. Thus, sickling-suppressive effects of catechin could potentially be associated with modulation of oxygenated and deoxygenated haemoglobin via alteration of human sickle erythrocytes' functional chemistry and metabolic pathways implicated in SCD crisis.

INTRODUCTION: Sickle cell disease (SCD) is one of the most prevalent hemoglobinopathies worldwide. It has been hypothesized that this disease originated millions of years ago, in the sub-Saharan countries in mid-western Africa, eastern Asia, and some regions of India.

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Today, SCD is not restricted to Africa and parts of India, but is found in the America and Europe, mainly as a result of migration and racial intermingling.

In the United States, the disease afflicts approximately 1:500 Afro-American and 1:4000 Hispanic-American neonates [2]. Sickle haemoglobin (HbS) shows peculiar biochemical properties, which lead to it polymerising when deoxygenated. HbS polymerisation is associated with a blood cell membrane deformability and cell-to-cell adherence, adhesion of sickle red blood cells to the endothelium, high production of reactive oxygen

species (ROS), decreasing lactate dehydrogenase activity and increasing Fe2+/Fe3+ ratio of HbS [3-9]. In sickle cell hemoglobin (HbS), the normal sequence of Valine-Histidine-Leucine-Threonine-Proline-Glutamicacid-Glutamic acid-Lysine is changed to Valine-Histidine-Leucine-Threonine-Proline-Valine-Glutamic acid-Lysine, with the amino acid valine substituting for the glutamic acid in the β6 (codon 6) site [11]. The gene defect is a known mutation of a single nucleotide of the β-globin gene and hemoglobin with this mutation is referred to as hemoglobin S (HbS) as opposed to the more normal adult hemoglobin A (HbA). In HbS, replacement of the hydrophilic glutamic acid at position 6 in the β-globin chain by the hydrophobic valine residue makes that this last one establishes hydrophobic interactions with other hydrophobic residues on the β-globin chain of another deoxy-HbS molecule [12, 17]. Sickle cell crises have been investigated by researchers all around the world in order to explore effective therapy towards solving the sickle cell disease problem. However, some researchers tend to treat people with sickle cell disease with different treatments including the use of polyphenolic compounds having targeted metabolic pathways. Antisickling agents are nutrients, drugs, phytochemicals and ions which by their actions inhibit polymerization of sickle red blood cells or the pathophysiological mechanisms leading to sickling in the vasculature. These agents nonetheless exhibit pharmacokinetic and pharmacodynamic properties [21].

MATERIAL AND METHODS:

Chemicals and Reagents: Catechin, a synthetic flavonoid with molecular weight of 254.24 g/mol, 284–286oC melting point and 97% purity was acquired from Sigma Aldrich, St Louis, Missouri, USA. All other chemicals/reagents utilized were of analytical grade until otherwise stated.

Collection of Blood Samples: The blood samples used in this study were collected with the consent of the patients involved at the Ahmadu Bello University Teaching Hospital, Zaria, Nigeria (ABUTH/HREC/UG/6). A written informed consent was read and signed by all the patients participating in the study. All the research procedures have received the approval of the scientific and health research ethics committee of Ahmadu Bello University Teaching Hospital Zaria, Nigeria. The patients' genotypes were further analyzed for confirmation. The blood samples were collected in sodium EDTA tubes, prepared by centrifugation at 5000 rpm for 10mins and washed three times in normal saline (Sigma Aldrich St . Louis, Missouri, USA) and stored at -22℃ for future use.

***In-vitro* Induction of Sickling:** Red blood cells (RBCs) were obtained from five milliliters of the venous blood collected from the sickle cell disease patients by centrifugation at 5000 rpm for 10mins and washed three times in normal saline (Sigma Aldrich St. Louis, Missouri, USA). The RBCs were finally suspended in the normal saline and used for the analysis according to a previously described method (Joppa *et al.*, 2008). 100µL of the blood cell suspensions was mixed with 100µL of 2% sodium metabisulphite solution and incubated at 37°C. Sickling induction was monitored microscopically at different time points (3,4,5,6 and 9 hours). The number of sickled cells was counted at each time point and the percentage of sickled cells was calculated using the formula:

% sickling = (Number of sickled cells) / (Total number of cells) x 100

Evaluation of Anti-Sickling property of Catechin: Catechin anti-sickling activity was investigated as described previously by Oduola et al., 2006. Briefly, half a milliliter (0.5 mL) of the washed erythrocytes was mixed with 0.5mL of freshly prepared 2% sodium metabisulphite in a clean test tube. The mixture was incubated in a water bath at 37℃ for 30 minutes. A drop of the mixture was placed on a microscope slide and viewed under a light microscope. Equal volumes of normal saline and the test compound (catechin) were added to the blood-metabisulphite mixtures in separate test tubes, respectively, and incubated at 37°C for another 30 minutes. Aliquots were taken from each of these test tubes at 30 minutes intervals, for up to 2 hours to confirm induction of sickling as described below.

Smear Preparation and Counting of Sickled and Unsickled Cells: The method described by (Egunyomi *et al.*, 2009) was adopted for smear preparation and counting of sickled and unsickled cells. Briefly, each sample was smeared on

microscope slide, fixed with 95% methanol, dried, and stained with Giemsa stain. It was then examined under an oil immersion microscope and the RBCs were viewed and counted from different fields (4 fields) across the slide. Both sickled and unsickled red blood cells were quantified and the percentage of the unsickled cells was determined using the following formula:

% of unsickled cells = (Mean of sickled cells at 0 min-mean of sickled cells at time t) / (Mean of sickled cells at 0 min) x 100

FTIR Analysis: Equal volumes of the test sample and sickled erythrocytes were incubated for 24h at 37°C, as described by Muhammad *et al.*, 2016. The samples were then dried under pressure and sandwiched between potassium bromide (KBr) cells. They were then scanned on FTIR spectrophotometer at room temperature (250C–28°C) at 300–4500 cm^{-1} spectral range. The peak heights were used in determining the functional groups present in the sample by comparison to the IR spectroscopy correlation table.

GC-MS Analysis: The treated sample was subjected to GC-MS analysis to identify the various secondary metabolites present. Shimadzu GC-MS QP2010 ULTRA (Labstock Nigeria ltd) with column: Optima 5MS (5% diphenyl and 95% dimetylpolysiloxane); length: 30 m; thickness: 0.25 mm; ID: 0.25 mm; column flow: 1.34 mL/min; gas: helium was used for GC-MS analysis. Flow rate was set at 1.34 mL/min, and temperature of column maintained at 60°C for 2 min and then raised to 200°C (15°C/min) followed by 9 min at 280° C (5o C/min). The National Institute of Standards and Technology mass spectral program 2011 was used for mass spectral survey. The concentrations of the identified compounds were calculated using area normalization over flame ionization detector response method.

Liquid Chromatography–mass Spectrometry Analysis: The samples were analyzed using liquid chromatography tandem mass spectrophotometry (LC-MS) as previously reported [19] with little modifications. Briefly, the samples were reconstituted in Methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. After filtration, the filtrate (10.0 µl) was injected into the LC system and allowed to separate on Sunfire C18 5.0µm 4.6mm x 150 mm column. The run was carried out at a flow rate of 1.0 mL/min, Sample and Column temperature at 25°C. The mobile phase consists of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with the gradients as shown **Table 3.**

The ratio of A/B 95:5 was maintained for further 1 min, then A/B 5:95 for 13min to 15min, then A/B 95:5 to 17min, 19min and finally 20min. the PDA detector was set at 210-400nm with resolution of 1.2nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100 – 1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative); probe temperature 600°C; flow rate 10 mL/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The data was processed with Empower 3. The compounds were identified on the basis of the following information, elution order, and retention time (tR), fragmentation pattern, and Base m/z. The metabolites were identified by direct comparison of the mass spectra data with those from the Human Metabolome Database [20].

Metabolic Pathway Analysis: The relevant metabolic pathways were identified by subjecting the identified metabolites to pathway analysis using the MetaboAnalyst 4.0 (21). The hypergeometric test and relative-betweenness centrality were utilized for over representation and pathway topology analyses, respectively of the metabolites. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway library was selected for metabolite mapping.

Statistical and Bioinformatics Analyses: Data were analyzed using one-way Analysis of Variance (ANOVA) at $p \leq 0.05$ followed by post-hoc t-test at $p \leq 0.01$, where necessary. Venn diagrams were constructed from the Bioinformatics & Evolutionary Genomics site http://bioinformatics.psb.ugent.be/webtools/Venn/

RESULTS: 2% sodium metabisulphite optimally induces erythrocytes sickling at 3 h post incubation period. To establish the optimal incubation period for sickling induction by 2% sodium metabisulphite

(SMS), equal volumes (0.5 ml) of SS erythrocytes (not in crisis) and 2% SMS were mixed and incubated at 37 °C for 1, 2, 3, 4, 5, 6, 9 and 10 hours. After these periods, smears of the mixtures were prepared, fixed, stained using 10% Giemsa stain and observed under a light microscope. Our results demonstrate an optimal sickling at 3 h incubation period (76% sickling) with 2675:866 severity ratios **Table 1** and **Fig. 2.**

TABLE 1: PERCENTAGE OF SICKLED AND UNSICKLED CELLS AFTER TREATMENT WITH 2% SODIUM METABISULPHITE

Groups	Sickled Cells	Unsickle	Total Cells	%Sickle Cells	% Unsickled Cells	Ratio
1 hour	192	149	341	56.30	43.70	192:149
2 hours	291	137	428	67.99	32.01	291:137
3 hours	4905	404	5309	92.39	7.61	4905:404
4 hours	3701	916	4617	80.16	19.84	3701:916
5 hours	2438	711	3149	77.42	22.58	2438:711
6 hours	2313	669	2982	77.57	22.43	771:223
9 hours	2871	839	3710	77.39	22.61	2871:839
10 hours	3090	907	3997	77.31	22.69	3090:907

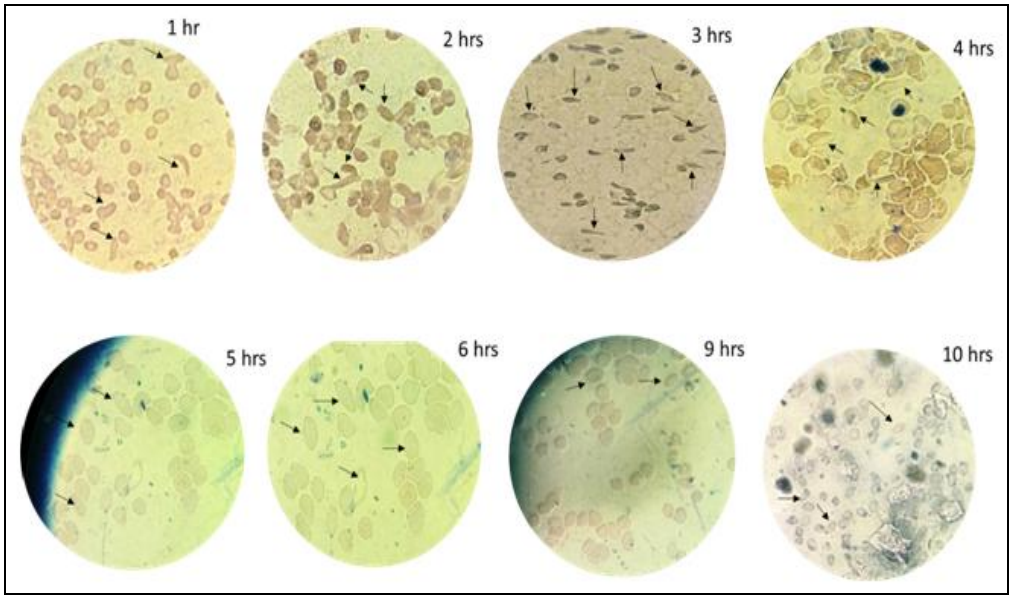

FIG. 1: CATECHINEXERTS ANTI-SICKLING EFFECT ON HUMAN ERYTHROCYTES

To understand the curative and preventive effects of catechin on erythrocyte sickling, we treated the cells with this compound at varying concentrations before and after sickling induction, respectively. In **Table 2**, the cells were pretreated with catechin before induction of sickling using 2% SMS. Strikingly, the number of sickled cells after sickling induction decreases with increasing concentration of catechin **Table 2**. The most anti-sickling effect of catechin was recorded at 1 mM concentration where only about 19% of the cells were sickled, unlike the noncatechin-treated group that presented about 68% sickled erythrocytes. On the other hand, the erythrocytes were treated with 2% SMS first before introducing the varying concentrations of catechin, respectively. Similarly, the number of sickled erythrocytes decreases with increasing catechin concentration **Table 3**. Interestingly, the most anti-sickling effect was also observed at 1 mM catechin concentration where only about 19% of the cells remained sickled at 30 minutes post catechin treatment. At this time point, about 62% of the cells in the control group were sickled. The 1 mM catechin concentration generally had the least unsickled to sickled erythrocyte ratio **Table 1**. We further determined the half maximum inhibitory concentration (IC_{50}) of the compound and found it to be 0.0257µM and 0.00275µM for SS-after and SS-before respectively. This value was considered in the subsequent experiments where appropriate. Taken together, these results demonstrate the preventive and curative effects of catechin on erythrocyte sickling.

TABLE 2: EFFECT OF CATECHIN TREATMENT BEFORE INDUCTION OF SICKLINGIN HUMAN ERYTHROCYTES

Catechin concentration	Sickle cells	Non-sickle cells	Total cells	% sickle	% non-sickle
0.0 µM	1039	129	1168	88.96	11.04
0.1 µM	1311	198	1509	86.88	13.12
1.0 µM	1424	761	2185	65.17	34.83
10.0 µM	1524	1721	3245	46.96	53.04
100.0 µM	627	1998	2625	23.89	76.11
1.0 mM	301	1972	2273	13.24	86.76

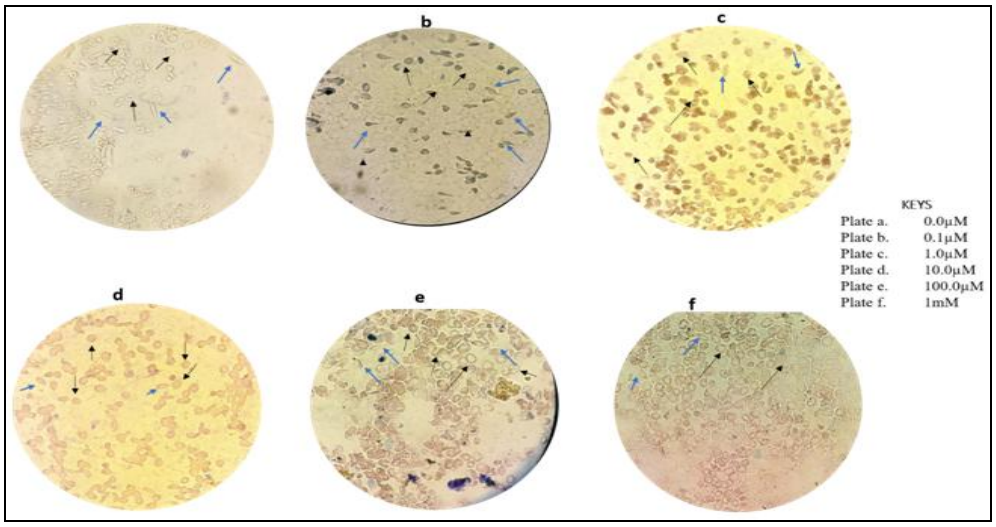

FIG. 2: MICROSCOPIC OBSERVATIONS OF ERYTHROCYTES SICKLING FOLLOWING TREATMENTS WITH DIFFERENT CONCENTRATIONS OF CATECHIN USING PREVENTIVE APPROACH. BLACK ARROWS POINT TO UNSICKLE CELLS WHILE BLUE ARROWS SHOWS SICKLED CELLS

TABLE 3: EFFECT OF CATECHIN TREATMENT AFTER INDUCTION OF SICKLINGIN HUMAN ERYTHROCYTES

Catechin concentration	Sickle cells	Non-sickle cells	Total cells	% sickle	% non-sickle
0.0 µM	1009	104	1113	90.66	9.34
0.1 µM	1210	239	1449	83.51	16.49
1.0 µM	1308	892	2200	59.45	40.55
10.0 µM	1102	1521	2623	42.01	57.99
100.0 µM	817	2494	3311	24.68	75.32
1.0 mM	408	2352	2760	14.78	85.22

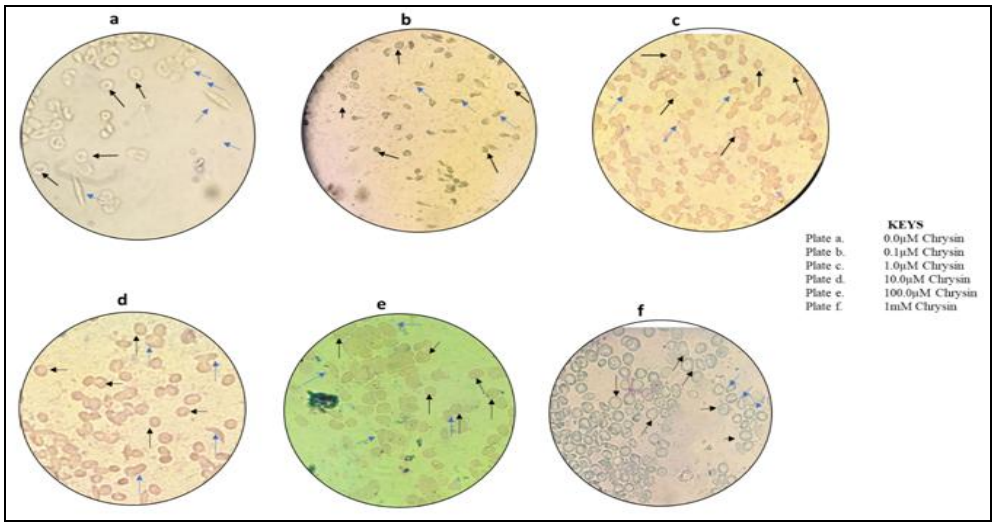

FIG. 3: MICROSCOPIC OBSERVATIONS OF ERYTHROCYTES SICKLING FOLLOWING TREATMENTS WITH DIFFERENT CONCENTRATIONS OF CATECHIN USING CURATIVE APPROACH. BLACK ARROWS POINT TO UNSICKLE CELLS WHILE BLUE ARROWS SHOWS SICKLED CELLS

FTIR and GC-MS Reveal Alterations in the Functional Chemistry of Erythrocytes after Catechin Treatment: To further gain a clue into the molecular mechanism of catechin function in ameliorating erythrocyte sickling, we set to dissect whether catechin alters some functional groups on the sickle erythrocytes in the course of restoring their lost functions. The samples were first subjected to Fourier-Transform Infrared Spectroscopy (FTIR) to investigate some possible changes in the hydrophobic macromolecules within the cells. **Table 4** presents the FTIR results of the various treatments. The 9th, 13th and 15th rows show functional group changes due to catechin treatment. The 9th row shows that catechin treatment supplies hydroxyl group to an alkane molecule, thereby improving its solubility in water. The 13th row shows the ability of catechin to introduce carbon-carbon double bond (C=C) into a polysaccharide in the cells which suggest that catechin acts as an oxidizing agent here by withdrawing electrons from this compound. Interestingly, catechin removes C=C bond from a given lipid as seen from the 15th row, most likely by supplying electron pair into this position to convert the double bond to a single one **Fig. 5-6**. These results suggest that catechin could function as a redox compound to prevent/ameliorate erythrocytes sickling.

TABLE 4: FTIR RESULTS SHOWING ALTERATIONS IN THE FUNCTIONAL CHEMISTRY OF ERYTHROCYTES IN THE VARIOUS SAMPLES

1	Characteristic absorptions, cm^{-1}	Functional Groups and Bond Type	AA Treated	AA Untreated	Curative	Preventive	Induced	Assignment of probable Biomolecules
2	3842.97	O-H Stretching	+	+	+	+	-	Carboxylic group
3	3432.93	C-H Stretching	+	+	+	+	+	Carbohydrate
4	2929.85	N-H Stretching	+	+	+	+	+	Protein
5	3063.90	C-H Stretching	+	+	+	+	-	Lipid
6	2870.91	C=C Stretching	+	+	+	+	-	Alkene
7	2258.84	N-O Stretching	+	+	+	+	+	Nitro compound
8	2197.94	C-H Stretching	+	+	+	+	+	Conjugate alkene
9	1349.18	O-H Bending	+	-	+	+	-	Alkane (methyl group)
10	1199.91	C-N Bending	+	-	+	+	+	Phenol
11	944.85	C-O Bending	-	+	+	+	+	Alcohol
12	958.82	C-O Stretching	+	+	+	+	+	Primary Amine
13	842.81	C=C Bending	+	-	+	+	-	polysaccharide
14	1028.86	C-Cl Stretching	+	-	-	-	+	Halo compound
15	1099.88	C=C Bending	-	+	-	-	+	Lipid

Catechin Treatment Favors Fatty Acid Alkyl Monoesters (FAMEs) Production: Considering the above FTIR results, we tried to identify some compounds that may have been altered following catechin treatment. The various test samples were therefore subjected to GC-MS to identify the compounds present in them. The compounds identified in the various samples are presented in **Table 2** and **Fig. 7-8**. Six of these compounds were found to be common in both SS-before and SS-after treatments **Fig. 4, Table 5**. Interestingly, all of these compounds are fatty acid alkyl monoesters (FAMEs) except 4H-1-Benzopyran-4-one which is a flavone (note that catechin is also a flavone). Also note that n-hexadecanoic acid is also present in the induced sample. Of all the catechin-treated samples, only SS-before and SS-after contain FAMEs; AA-treated contains only methyl tetradecanoate as a FAME, which is also present in AA-untreated. This suggests that catechin recruits FAMEs to exert its anti-sickling function.

TABLE 5: GC-MS-IDENTIFIED COMPOUNDS ACROSS GROUPS

Treatment	Peak No.	Similarity Index	Compound
AA-untreated	9	99	Methyl tetradecanoate
	18	89	5.alpha.-Dihydroprogesterone
	26	78	Hexahydropyridine
	28	76	2-Myristynoyl-glycinamide
AA-treated	2.84	95	Cetene
	9.65	94	Hexadecane

	2.15	99	E-14-Hexadecenal
	7.25	97	Heptadecane
	38.51	98	Catechin
	21.90	98	Methyl tetradecanoate
	39.59	98	Hexadecanoic acid
	2.09	99	11-Octadecenoic acid
	2.09	99	cis-13-Octadecenoic acid
Induced (SS-untreated)	1	85	9,12-Octadecadienoyl chloride
	3	88	Hexadecanoic acid
	4	95	Cyclopentadecanone, 2-hydroxy
	5	92	10-undecenyl ester, 2-Methylenecyclohexanol
	6	94	heptadecyl ester, 9,12-Octadecadienoyl chloride
	7	87	trimethylsilyl ester
	32	79	Ethyl 2-butyramido-3,3,3-trifluoro-2, propionate
SS-before	1	98	Hexadecanoic acid, methyl ester
	2	99	n-Hexadecanoic acid
	4	94	Methyl 9-cis,11-trans-octadecadienoate.
	5	97	9-Octadecenoic acid
	7	92	Methyl stearate
	8	99	Oleic Acid
	11	87	Glycerol 1-palmitate
	15	98	Catechin
	9	99	Methyl tetradecanoate
	18	95	5.alpha.-Dihydroprogesterone
	19	99	Ethyl-2-butyramido-3,3,3-trifluoro-2-propionate
	22	96	Cyclopentadecanone, 2-hydroxy
	14	90	4H-1-Benzopyran-4-one, 5,7-dihydroxy-3-phenyl-
SS-after	1	99	Hexadecanoic acid
	2	98	n-Hexadecanoic acid
	4	99	Methyl 9-cis,11-trans-octadecadienoate
	5	99	9-Octadecenoic acid
	7	87	Methyl stearate
	9	99	Methyl tetradecanoate
	11	92	Glycerol 1-palmitate
	14	90	4H-1-Benzopyran-4-one, 5,7-dihydroxy-3-phenyl
	15	98	Catechin
	18	97	5.alpha.-Dihydroprogesterone

Catechin Shuts Down Seleno-Compound Metabolism to Improve Erythrocytes Integrity and Function: To unveil the various compounds whose metabolisms are affected by catechin treatment, we subjected the samples to Liquid Chromatography-Mass Spectrometry (LC-MS) analysis **Fig. 9-10**. **Table 6** presents the various compounds identified in each of the samples analyzed. A number of metabolites have been identified in the different samples. From the results, it is notably observed that catechin activates the biosynthesis of oxalosuccinate (an intermediate of the Krebs cycle) in sickle erythrocytes. Considering the fact that few reactions of oxalosuccinate produce NAD (a redox cofactor) which plays important roles in redox reactions and the lifespan of a cell [24] and that isoforms of TCA cycle enzymes could be found in mature erythrocytes [25], we suggest that catechin activates these enzymes to provide adequate energy and improve the lifespan of the sickle cells. Interestingly, we also found that the syntheses of L-cystine (an oxidized dimer of cysteine) and selenomethionine (selenomethionine and selenocysteine are cytotoxic at certain concentrations) were shut down in all the catechin-treated samples **Table 6.**

To support the above role of catechin in preventing seleno-compound-mediated cytotoxicity, we analyzed the various pathways that are impacted in the erythrocytes due to catechin treatment. We found out that many different pathways are impacted following catechin treatment before/after sickling induction. Consistent with the results above, the pathway for seleno-compound metabolism has zero impact in all the catechin-treated samples (AA-treated, preventive and curative) while it has an impact score of more than

0.15 in the catechin-untreated samples (AA-untreated and Induced) **Fig. 5.** Taken together, these results demonstrate that catechin blocks seleno-methionine-mediated cytotoxicity to improve the RBC integrity and function.

TABLE 6: IDENTIFIED METABOLITES BY LC-MS ANALYSIS

Metabolite	AA-untreated	AA-treated	Preventive	Curative	Induced
Selenocystathionine	+	+	+	+	+
5,7-Dihydroxyisoflavone	-	+	+	+	-
Phenylalanine	+	+	+	+	-
Triglyceride	+	-	+	+	+
1-Palmitodiolein	+	+	-	+	+
Orotic acid	+	+	+	+	+
L-Cystine	+	-	-	-	+
Selenomethionine	+	-	-	-	+
Selenomethionine se-oxide	+	+	-	-	-
Linoleic acid	-	+	+	+	-
Acetylphenol sulfate	+	-	-	-	+
Inosine triphosphate	+	-	-	+	+
Uric acid	+	+	-	-	-
Dihydrothymine	+	+	+	+	-
Norepinephrine sulfate	+	-	-	-	-
Ribose 1,5-bisphosphate	-	+	-	-	-
Glycerate 1,3-biphosphate	-	+	-	-	-
Lysine	-	+	+	+	-
(R)-Lipoic acid	-	+	-	-	-
3-Methoxytyrosine	-	+	-	-	-
Dihydrouracil	+	+	-	-	-
Sphingosine	+	+	+	+	-
Phosphatitylinositol	-	+	-	-	-
Cardiolipin	-	+	-	-	-
L-Aspertyl-4-phsphate	-	+	-	-	-
2-Phosphoglycerate	-	+	-	-	-
Carbovir triphosphate	-	+	+	-	-
D-Glucuronate 1-phosphate	-	-	+	-	-
Oxalosuccinate	-	-	+	+	-
Uridine 5'-diphosphate	-	-	+	-	-
5-Hydroxyisourate	-	-	+	-	-
Guanosine triphosphate	-	-	+	-	-
Galactaric acid	-	-	+	-	-
3'-Ketolactose	-	-	+	-	-
Guanosine 3', 5'-bis(diphosphate)	-	-	+	-	-
DOPA sulfate	-	-	-	+	+
6-Thiourate	-	-	-	+	-
Glyceraldehyde 3-phosphate	-	-	-	+	-
Inosine	+	-	-	-	+
6-Thioguanosine monophosphate	-	-	-	+	-
Lentinic acid	-	-	-	-	+
Isopentenyl pyrophosphate	-	-	-	-	+
Phosphohydroxypyruvate	-	-	-	-	+
Adenosine triphosphate	-	-	-	-	+
Acetylcholine	-	+	+	+	-
Stearate	-	+	+	+	-

In each case, + indicates the presence of a given metabolite; - indicates the absence of a given metabolite.

Catechin Employs Different Mechanisms for Prevention and Reversal of Erythrocyte Sickling: Pathway enrichment and topology analyses were performed to analyze and compare the metabolomics profiles from the various samples. The enriched pathways and their respective impact scores from the AA- and SS-genotype erythrocytes metabolomics data are shown in **Fig. 6-11**. When AA-untreated metabolomics data were analyzed, the steroid

hormone biosynthesis pathway was found to be significantly enriched with an impact score of 0.02 **Fig. 7**. In the case of AA-treated and induced (SS-untreated) samples, three enriched pathways were identified from each of the two treatments **Fig. 8** and **9**. Of these pathways, only fatty acid biosynthesis pathway is significantly activated (with an impact score of 0.01 each). Four pathways were identified from SS-after samples **Fig. 10** where only linoleic acid metabolism and fatty acid biosynthesis pathways were activated with impact scores of 1.00 and 0.01, respectively. Similarly, for the samples treated with catechin before induction (SS-before), four enriched pathways were also recorded of which steroid hormone and fatty acid biosynthesis pathways were active with impact scores of 0.02 and 0.01, respectively **Fig. 11**. Fatty acid biosynthesis pathway is activated in almost all the samples including the controls. Strikingly, steroid hormone biosynthesis and linoleic acid metabolism pathways were activated in SS-before and SS-after treatments, respectively **Fig. 10** and **11**. Treatment of the SS erythrocytes with catechin before induction of sickling activated the same pathway as in AA-untreated control, suggesting the efficacy of catechin in restoring the defects of the SS erythrocytes. On the other hand, linoleic acid metabolism pathway is activated when the SS erythrocytes were treated with catechin after induction of sickling **Fig. 10**. Collectively, these findings reveal that catechin uses different mechanisms to prevent and reverse erythrocytes sickling, via activation of steroid hormone biosynthesis and linoleic acid metabolism pathways, respectively.

DISCUSSION: Conventional drugs established so far for SCD management focus principally on indicative breather of pain and crisis alleviation coupled with their biological side effects and the burdensome associated with their availability and affordability particularly in developing countries [26]. Catechin, a 5, 7-dihydroxyflavone, was reported in honey and propolis, *Passiflora caerulea*, *Passiflora incarnata*, *Oroxylum indicum* in addition to fruits and vegetables [27]. We have evidently, reported the antioxidant, anticlastogenic and DNA-protective properties of catechin from our laboratory [28]. Catechin has also been reported with optimal bioavailability favouring its anti-inflammatory and protein stability effects that could be advantageous to SCD associated oxidative stress, inflammation and decreased HbS stability [29]. We have equally reported the sickling-suppressive effects without deciphering the potential mechanism behind the said effects. We have in the present study investigated the preventive and reversal potentials of the bioactive flavone, catechin, on erythrocyte sickling, and went further to unveil its mechanism of action. Our data reveal that 84.63% of the SS erythrocytes pretreated with catechin retained their normal morphology after induction of sickling and 81.19% of sickle SS erythrocytes were reversed to their normal biconcave shapes when subjected to catechin treatment, demonstrating the potentials of catechin in preventing and reversing erythrocytes sickling, respectively. Sickling in SCD patients is characterized by continuous polymerization of erythrocytes which is the resultant effect of oxidative stress due to inadequate oxygen supply [30]. Flavonoids are well-known of their antioxidant properties [31]; as such, catechinlikely relieved these blood cells of the devastating oxidative stress resulting in the stability of their membranes. This is similar to the cancer chemopreventive effect of quercetin (another member of the flavonoid family) which confers pro-apoptotic effect in tumor cells, as well as the effects of rutin and quarcetin previously reported [32].

Our findings show that the levels of oxygenated haemoglobin in the two groups were similar but significantly higher than that of SS erythrocytes that were sickling-induced but not catechin-treated (the ‘induced’ control) **Fig. 1A**. The similar haemoglobin levels in the two treatment groups were, however, lower than those of catechin-treated and untreated AA-genotyped erythrocytes. The two AA groups have significantly similar haemoglobin levels, suggesting that catechin treatment does not exert any negative consequential effect on the erythrocytes function and integrity. However, the level of deoxygenated haemoglobin in the ‘induced’ control group was significantly higher than those of the catechin-treated SS groups, which suggests that catechin treatment restores the loss-of-function of sickled erythrocytes.

Put together, our data suggest that catechin confers anti-sickling property through restoration of haemoglobin function. This agrees with a previous study [33] indicating that the percentage of MetHb

was significantly decreased ($P < 0.05$) after administration of curcuminoids for 12 months, while there were no changes in Hb levels, but in our study, there was improvement of haemoglobin concentrations in sickle cells treated with catechin. This variation in the concentration of oxygenated and deoxygenated Hb maybe credited to differences in reagents and experimental timings. Interestingly, increased oxygenated Hb concentration and improved anemia may be caused not only by vitamins E and C treatment but also by vitamin A supplementation, since according to previous studies, vitamin A supplementation increases haemoglobin concentrations in children with poor vitamin A status [34, 35]. Our osmotic fragility results relate that catechin possesses the potential to sustain the integrity of the erythrocyte membrane under sickle condition. These results indicate that the normal and catechin-untreated erythrocytes (AA-untreated) were significantly more resistant to osmotic fragility than the SS-untreated (induced) ones **Fig. 2.**

However, treatment of the SS erythrocytes with catechin significantly reduced the osmotic fragility of the cells as compared to the untreated ones, although the hemolysis levels were still higher than those of the AA erythrocytes. As usual, there is no significant difference in the percentage fragility between the samples treated before and those treated after sickling induction. Likewise, the treated and untreated normal erythrocytes (the AA erythrocytes) did not present any significant difference. Based on these results, we conclude that catechin treatment improves the membrane stability of sickle human erythrocytes. Although the exact mechanism of this function was not demonstrated in this study, our findings are contrary to some previous studies [36, 37], most likely due to polymerization, which subsequently leads to irreversible sickling of these cells as well as oxidative damage which compromises cell integrity and cause hemolysis [38].

In our attempt to check whether catechin treatment could alter the level of 2,3-DPG in the sickle erythrocytes, we discovered in this present study that catechin treatment significantly lowers the high level of 2,3-DPG observed in the sickle erythrocytes irrespective of whether the catechin is applied before or after induction of sickling. Whereas for SS-before, SS-after, AA untreated, AA treated and Induced groups, the concentrations were observed to be 53.74±0.75, 50.32±0.24, 36.79±0.16, 35.37±0.32 and 80.47±0.71 correspondingly indicating an improvement in oxygen affinity of the haemoglobin. This agrees with our data presenting that catechin restores haemoglobin function in sickled erythrocytes **Fig. 1**. Catechin treatment did not affect the concentration of 2,3-DPG in the normal erythrocytes. Contrariwise, a previous study reported increased concentrations of 2,3-DPG in the RBCs of insulin-dependent diabetes mellitus (IDDM) patients with elevated HbA1c [39], although this could be attributed to the fact that these patients had no hypoxic stress as observed in the samples used for the present study. A previous study reported similar data for patients without ketoacidosis [40]. These disagreements could be related to nerve tissue degeneration observed in diabetic and SCD patients.

To further gain a clue into the molecular mechanism of catechin function in ameliorating erythrocyte sickling, we set to dissect whether catechin alters some functional groups on the sickle erythrocytes in the course of restoring their lost functions. The samples were first subjected to fourier-transform infrared spectroscopy (FTIR) to investigate some possible changes in the hydrophobic macromolecules within the cells. The FTIR analysis revealed polar charged amines as the primary functional groups **Table 5**, indicating fewer negative surface charges than normal erythrocytes. These polar charged functional groups may be responsible for the abnormal adherence of sickle erythrocytes to vascular endothelial cells, thus eliciting painful vaso-occlusive event [41]. The formation of hydrophobic functional groups **Table 5** on treatment with the catechin depicts a change in the polar surface charge of the sickled erythrocytes.

Thus, implying a potential of catechin to suppress the occurrence of a vaso-occlusive episode by attenuating abnormal adherence of sickle erythrocytes to vascular endothelial cells [42]. On the other hand, our GC-MS analysis of both the treated and untreated erythrocytes revealed the presence of some major constituents of most essential oils such as cis-13-Octadecenoic acid, Methyl

tetradecanoate, cetene, hexadecane, E-14-Hexadecenal, Heptadecane, Cyclopentadecanone, 2-hydroxy, 10-undecenyl ester, 2-Methylenecyclohexanol, heptadecyl ester, 9,12-Octadecadienoyl chloride, trimethylsilyl ester and Ethyl 2-butyramido-3,3,3-trifluoro-2, propionate. Some of these identified constituents has been investigated to possess reasonable levels of antioxidant properties [43, 44].

When AA-untreated metabolomics data were analyzed, a pathway was identified to be significantly enriched with relatively large impact score greater than 0.016 **Fig. 6**.

The pathway was found to be a steroid hormone biosynthesis activation pathway. Moreso, the LC-MS results indicate changes induced in the erythrocytes upon treatment with catechin. Some of the observed metabolites include Seleno-cystathionine, Triglyceride, 1-Palmitodiolein, L-Cystine, Selenomethionine, Selenomethionine se-oxide, Inosine triphosphate, Uric acid, Norepinephrine sulfate, Ribose 1,5-bisphosphate, Glycerate 1,3-biphosphate, (R)-Lipoic acid, 3-Methoxytyrosine, Phosphatitylinositol, Cardiolipin, L-Aspertyl-4-phsphate, 2-Phosphoglycerate, Carbovir triphosphate, D-Glucuronate 1-phosphate, Oxalosuccinate, Uridine 5'-diphosphate, 5-Hydroxyisourate, Guanosine triphosphate, Galactaric acid, 3'-Ketolactose,Guanosine 3', 5'-bis(diphosphate), DOPA sulfate, 6-Thiourate, Glyceraldehyde 3-phosphate, 6-Thioguanosine monophosphate, Lentinic acid, Isopentenyl pyrophosphate, Phosphohydroxypyruvate, Adenosine triphosphate across the different groups of treatments. These findings agree with similar studies on other flavonoids like rutin and quercetin that exhibited strong affinities to deoxy-haemoglobin and 2,3-bisphophoglycerate mutase. [45].

By implication, this might have revealed catechin's tendency to allosterically bind to haemoglobin with good binding affinity that may potentially alter oxygen's affinity to haemoglobin towards oxygenation due to supportive potential inhibitory effects on 2,3-bisphophoglycerate mutase by catechin[45]. Interestingly, for the first time, we have been able to demonstrate in this study that catechin indeed possesses sickling-suppressive property corroborating other findings on the antisickling activities of some flavonoids [6].

The observed antisickling activities were found to be associated with a favourable modulation of osmotic fragility, redox homeostasis and functional chemistry of erythrocytes as clearly seen from our repetitive experiments. This is in addition to the observed decrease on the number of sickle erythrocytes upon treatment with catechin *in-vitro*, which was a similar observation based on other studies with rutin and quercetin [45]. To this end, the observed changes of the functional chemistry may possibly be related to membrane soothing effects of catechin in an induced versus treated erythrocytes by virtue of the fact that erythrocytes like other cells are composed of lipids, proteins and carbohydrates peripherally, integral or/and intracellularly [45].

CONCLUSION: Findings from this provide more details on *in-vitro* underlying mechanism of sickling-suppressive effects of catechin which could potentially be associated with modulation of oxygenated and deoxygenated haemglobin via alteration of human sickle erythrocyte's functional chemistry and metabolic pathways implicated in SCD crisis. This was evidently characterized by catechin's ability to ameliorates erythrocytes sickling by restoring haemoglobin function, lowering the osmotic fragility, reduction on the level of 2,3-diphosphoglycerate, stimulation of fatty acid alkyl monoesters production coupled with inactivation of selenocompound metabolism in human sickle erythrocytes. Comparatively, we found that catechin does not exert any significant effects on AA-genotype human erythrocytes. However, further studies are needed to validate the biochemical contribution of catechin using *in vivo* models.

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REFERENCES:

1. Weatherall DJ: "Genetic variation and susceptibility to infection: The red cell and malaria". British Journal of Haematology 2008; 141(3): 276–286.
2. Bonds DR: "Three decades of innovation in the management of sickle cell disease: The road to understanding the sickle cell disease clinical phenotype". Blood Reviews 2005; 19(2): 99–110.

3. Bunn H and Forget BG: "Hemoglobin: Molecular, genetic and clinical aspects", Philadelphia, PA, USA: WB Saunders 1986; 123.
4. Hebbel RP: "Adhesive interactions of sickle erythrocytes with endothelium". J Clin Invest 1997; 100(11): 83-6.
5. Hebbel RP: "Adhesive interactions of sickle erythrocytes with endothelium", J Clin Invest 1997; 100(11): 83-6.
6. Stuart MJ and Nagel RL: "Sickle cell disease". Lancet 2004; 364: 1343-60.
7. Shalev O and Hebbel RP: "Extremely high avidity association of Fe (III) with the sickle red cell membrane". Blood 1996; 88: 349-352.
8. Nwaoguike RN and Uwakwe AA: "The antisickling effects of dried fish (tilapia) and dried prawn (Astacus red)". J Appl Sci Environ 2005; 9(3): 115-119.
9. Dahmani O, Belcaid A, Ouafa EA and Hayate H: "Biosynthèse Et Rôle Physiologique De L'hémoglobine''. 2009; 36.
10. Nwaoguikpe RN, Ekeke GI and Uzogara SG: "Amino acids in the management of sickle cell disease", Msc Thesis, University of PortHarcourt, Nigeria 1993; 70-72.
11. Maciaszek JL, Andemariam B and Lykotrafitis G: "Microelasticity of red blood cells in sickle cell disease". Jstarin Analysis 2011; 46: 368-374.
12. Edelstein, SJ, Telford JN and Crépeau RH: "Structure of fibers of sickle cell hemoglobin". Proc Natl Acad Sci USA 1973; 70: 1104-7.
13. Angastiniotis M, Modell B, Englezos P & Boulyjenkov V: Prevention and control of haemoglobinopathies. Bull World Health Organ 1995; 73: 375–386.
14. Modell B: A second look at the distribution and frequency of the hemoglobinopathies. Ann. N. Y. Acad Sci 1985; 445: 268–273.
15. Weatherall DJ: Phenotype genotype relationships in monogenic disease: lessons from the thalassaemias. Nature Reviews Genetics 2002; (1): 245–255.
16. Diallo D & Tchernia G: Sickle cell disease in Africa. Current Opinion in Hematology 2002; 9: 111–116.
17. Bunn HF: Pathogenesis and Treatment of Sickle Cell Disease. N Engl J Med 1997; 337: 762–769.
18. Muhammad A: Sickling-suppressive effects of catechin may be associated with sequestration of deoxy-haemoglobin, 2,3-bisphosphoglycerate mutase, alteration of redox homeostasis and functional chemistry of sickle erythrocytes. Hum Exp Toxicol 2020; 39:537–546.
19. Hsia CC: Respiratory function of hemoglobin. New England Journal of Medicine 1998; 338: 239–248.
20. Poillon WN & Kim BC: 2,3-Diphosphoglycerate and intracellular pH as interdependent determinants of the physiologic solubility of deoxyhemoglobin S. Blood 1990; 76: 1028–1036.
21. Nwaoguikpe RN, Ekeke GI & Uzogara SG: Amino acids in the management of sickle cell disease. (M. Sc. Thesis, Uniport 1993.
22. Batra P & Sharma AK: Anti-cancer potential of flavonoids: recent trends and future perspectives 2013; 3: 439–459.
23. Pereira OR, Domingues MRM, Silva AMS & Cardoso SM: Phenolic constituents of Lamium album: Focus on isoscutellarein derivatives. Food Research International 2012; 48: 330–335.
24. Zheng X, Meng WD, Xu YY, Cao JG & Qing FL: Synthesis and anticancer effect of catechin derivatives. Bioorganic & Medicinal Chemistry Letters 2003; 13: 881–884.
25. Oduola T, Adeniyi FA, Ogunyemi EO, Bello IS & Idowu TO: Antisickling agent in an extract of unripe pawpaw (Carica papaya): Is it real? African Journal of Biotechnology 2006; 5.
26. Egunyomi A, Moody JO & Eletu OM: Antisickling activities of two ethnomedicinal plant recipes used for the management of sickle cell anaemia in Ibadan, Nigeria. African Journal of Biotechnology 2008; 9.
27. Szigeti B: Estimation of oxyhaemoglobin and of methaemoglobin by a photoelectric method. Biochemical Journal 1940; 34: 1460–1463.
28. Drabkin D: Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. J Biol Chem 1935; 112: 51–65.
29. Imaga NOA: Antisickling property of Carica papaya leaf extract. African J of Biochemistry Res 2009; 3: 102–106.
30. Muhammad A: Induction of Haemolysis and DNA Fragmentation in a Normal and Malarial-Infected Blood by Commonly - used Antimalarial Drugs in the North-Western Region of Nigeria. Drug Metabolism Letters 2016; 10: 49–55.
31. Piovesana S: Recent trends and analytical challenges in plant bioactive peptide separation, identification and validation. Analytical and Bioanalytical Chemistry 2018; 410: 3425–3444.
32. Wishart DS: HMDB 3.0 The Human Metabolome Database in 2013. Nucleic Acids Research 2013; 41: 801-807.
33. Chong J: Metabo Analyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Research 2018; 46: 486–494.
34. Rainey PB, Brodey CL & Johnstone K: Biological properties and spectrum of activity of tolaasin, a lipodepsipeptide toxin produced by the mushroom pathogen Pseudomonas tolaasii. Physiological and Molecular Plant Pathology 1991; 39: 57–70.
35. Macdonald RL & Barker JL: Pentylenetetrazol and penicillin are selective antagonists of GABA-mediated post-synaptic inhibition in cultured mammalian neurones. Nature 1977; 267: 720–721.
36. Di Stefano M & Conforti L: Diversification of NAD biological role: the importance of location. The FEBS Journal 2013; 280: 4711–4728.
37. Nemkov T, Hansen KC & D'alessandro A: A Three-Minute Method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. Rapid Commun Mass Spectrom 2017; 31: 663–673.
38. Erukainure O: *Monodora myristica* (African nutmeg) modulates redox homeostasis and alters functional chemistry in sickled erythrocytes. Human & Experimental Toxicology 2017; 37: 458–467.
39. Wang J: Catechin suppresses proliferation, migration, and invasion in glioblastoma cell lines *via* mediating the ERK/Nrf2 signaling pathway. Drug Design, Development and Therapy 2018; 12: 721–733.
40. Babangida S: The role of molecular modelling strategies in validating the effects of catechin on sodium arsenite-induced chromosomal and DNA damage. Human and Experimental Toxicology 2018; 1–11 doi:10.1177/0960327117751233.
41. Mani R & Natesan V: Catechin: Sources, beneficial pharmacological activities, and molecular mechanism of action. Phytochemistry 2018; 145: 187–196.
42. Muhammad A: Sickling-preventive effects of rutin is associated with modulation of deoxygenated haemoglobin, 2,3-bisphosphoglycerate mutase, redox status and alteration of functional chemistry in sickle erythrocytes. Heliyon 2019; 5: 01905.

43. Panche AN, Diwan AD & Chandra SR: Flavonoids: an overview. Journal of Nutritional Science 2016; 5.
44. Gibellini L: Quercetin and Cancer Chemoprevention. Evidence-Based Complementary and Alternative Medicine 2011; 053 https://www.hindawi.com/journals/ecam/2011/591356/ (2011).
45. Kalpravidh RW: Improvement in oxidative stress and antioxidant parameters in beta-thalassemia/Hb E patients treated with curcuminoids. Clin. Biochem 2010; 43: 424–429.
46. Mwanri L, Worsley A, Ryan P & Masika J: Supplemental vitamin A improves anemia and growth in anemic school children in Tanzania. J Nutr 2000; 130: 2691–2696.
47. Zimmermann MB: Vitamin A supplementation in children with poor vitamin A and iron status increases erythropoietin and hemoglobin concentrations without changing total body iron. Am J Clin Nutr 2006; 84: 580–586.
48. Nolan VG, Wyszynski DF, Farrer LA & Steinberg MH: Hemolysis-associated priapism in sickle cell disease. Blood 2005; 106: 3264–3267.
49. Rother RP, Bell L, Hillmen P & Gladwin MT: The Clinical Sequelae of Intravascular Hemolysis and Extracellular Plasma Hemoglobin: A Novel Mechanism of Human Disease. JAMA 2005; 293: 1653–1662.
50. Kato GJ, Hebbel RP, Steinberg MH & Gladwin MT: Vasculopathy in sickle cell disease: Biology, pathophysiology, genetics, translational medicine, and new research directions. Am J Hematol 2009; 84: 618–625.
51. Roberts AP, Story CJ & Ryall RG: Erythrocyte 2,3-bisphosphoglycerate concentrations and haemoglobin glycosylation in normoxic Type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1984; 26: 389–391.
52. Triolo G: [Glycosylated hemoglobins and erythrocyte 2,3-diphosphoglycerate in diabetes mellitus]. Arch Sci Med (Torino) 1981; 138: 179–185.
53. Kaul DK, Finnegan E & Barabino GA: Sickle red cell-endothelium interactions. Microcircul 2009; 16: 97–111.
54. Erukainure OL: Dietary fatty acids from leaves of clerodendrum volubile induce cell cycle arrest, downregulate matrix metalloproteinase-9 expression, and modulate redox status in human breast cancer. Nutr Cancer 2016; 68: 634–645.
55. Grattan BJ: Plant sterols as anticancer nutrients: evidence for their role in breast cancer. Nutrients 2013; 5: 359–387.
56. Silva TM: Changes in the essential oil composition of leaves of *Echinodorus macrophyllus* exposed to γ-radiation. Revista Brasileira de Farmacognosia 2013; 23: 600–607.
57. Muhammad A: Sickling-suppressive effects of catechin may be associated with sequestration of deoxy-haemoglobin, 2,3-bisphosphoglycerate mutase, alteration of redox homeostasis and functional chemistry of sickle erythrocytes. Hum Exp Toxicol 2020; 39: 537–546.

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