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Quantitative Analysis of γ-Globin Gene Expression in Human Erythroleukaemic K562 Cells Treated with *Theobroma cacao* Bark Ethanolic Extract

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ABSTRACT: Therapeutic reactivation of foetal haemoglobin (HbF) can alleviate clinical complications associated with sickle cell anaemia (SCA), a severe monogenic disorder that is prevalent in sub-Saharan Africa. Traditional medicine practitioners in Nigeria utilize the stem bark of *Theobroma cacao* as a haematinic for SCA patients, but its potential to induce HbF remains uncertain. Here, we investigated the cytotoxic effects of ethanol extract of *Theobroma cacao* bark (TCBEE) using the MTT assay and quantified in real-time, γ-globin gene expression levels in human erythroleukaemic K562 cells treated with TCBEE (1, 5, 10 and 30 µg/mL) or 200 µM hydroxyurea (HU) for 24 hours, alongside untreated cells. Total RNA (A_{260}/A_{280} > 1.9) isolated from un/treated cells was converted to cDNA for RT-qPCR analysis, and the gene expression data was normalized with GAPDH as a reference gene. TCBEE demonstrated moderate cytotoxicity against K562 cells, which are of leukaemic origin, with an IC_{50} value of 101.5 µg/mL in the MTT assay, suggesting that TCBEE might have good anti-cancer components. The relative quantification of the γ-globin gene revealed a decrease in fold changes as the concentration of TCBEE increased. Notably, treatment with 1 µg/mL TCBEE showed a significant (p < 0.0001) 1.08-fold change compared to HU-treated cells which could be beneficial for individuals with haemoglobinopathies, such as SCA. Therefore, this study pinpoints the therapeutic potential of TCBEE in SCA management and recommends further research to identify the bioactive compounds in TCBEE responsible for naturally inducing γ-globin gene expression.

Keywords: Foetal haemoglobin, *Theobroma cacao*, K562 cells, RT-qPCR, Sickle cell disease

Introduction

Normal adult haemoglobin (HbA) consists of two pairs of α-like and β-like globin while foetal haemoglobin (HbF) comprises two pairs of α-like and γ-like globin subunits. HbF is indispensable for delivering oxygen from the mother's circulation to the foetus during gestation due to its higher affinity for oxygen. Shortly after birth (~ 6 months), a switch from HbF to HbA, brought about by the silencing of γ-globin gene expression and the reciprocal activation of adult β-globin gene expression, occurs (Makani *et al*., 2013; Paikari and Sheehan, 2018). Consequently, HbA becomes the predominant form of haemoglobin (Hb) and individuals with β-globin gene disorders such as sickle cell disease (SCD) would gradually begin to show clinical complications of the disease. The defining mutation of SCD occurs at the 6th position of the first exon of the β-globin gene ($\beta^{6Glu\text{-}Val}$) located on chromosome 11p15.5. This point (A to T) mutation leads to the formation of a crescent or sickle-shaped haemoglobin variant (HbS) that can polymerize during periods of deoxygenation and hypoxemia, consequently causing acute and chronic complications in several organs of the body (Inusa *et al*., 2019; Sundd *et al*., 2019).

Because HbF inhibits the intracellular polymerization of HbS, the search for drugs that could reactivate and modulate HbF expression in adult SCD individuals led to the discovery of hydroxyurea (HU) over three decades ago, and subsequent approval by the FDA in 1998 (McGann and Ware, 2015). Although HU is relatively inexpensive and plays a significant role in reducing acute painful episodes, preventing acute chest syndrome events and, more importantly, increasing HbF levels (which lack β-globin chains), its use comes with various reported side effects. Moreover, concerns have been raised regarding the potential carcinogenic or leukemogenic effects associated with prolonged use of HU (Platt, 2008; Strouse *et al*., 2008). Previous studies have shown that treating human erythroleukaemic K562 cells with synthesized compounds such as HU or compounds from natural sources increased the expression of embryonic (ε) and HbF genes (Bianchi *et al*., 2009). Various medicinal plants used to manage SCD in a Nigerian population have been reported and such plant species are gaining much attention in ethnopharmacology (Amujoyegbe *et al*., 2016).

Nigerian traditional medicine practitioners administer the stem bark of *Theobroma cacao* as a haematinic to individuals suffering from sickle cell anaemia (SCA, HbSS), which is the most prevalent and clinically severe form of SCD (Odugbemi, 2008; Gbadamosi, 2015). *Theobroma cacao* L. also known as cacao or cocoa, is a member of the Malvaceae plant family. The cocoa tree is a perennial tree that grows to a height of 8 to 15 m in natural habitats including the tropical regions of South and Central America and the Amazon basin rain forest. The oval-shaped fruit contains 30 to 40 seeds embedded in a mucilaginous pulp that has an acid-sweet taste. The ellipsoid or ovoid cocoa seeds, also known as cocoa beans, are essential ingredients for chocolate production (Lima *et al*., 2011; de Souza *et al*., 2018). Cocoa beans are rich in polyphenols such as catechins or flavan-3-ols, anthocyanins and proanthocyanidins, as well as hundreds of important bioactive compounds that confer the antioxidant, anti-inflammatory, anti-hypertensive, chemopreventive, neuroprotective and cardioprotective properties to humans (Aprotosoaie *et al*., 2016). To the best of our knowledge, there is a paucity of information on the potential of *T. cacao* stem bark ethanol extract (TCBEE) to induce γ-globin mRNA in K562 cells. The present gene expression studies could address such a dearth of information and contribute knowledge in this regard.

Materials and methods

Plant material: The dried stem bark of *Theobroma cacao* was bought from a local herb vendor at Oyingbo Market in Lagos, Nigeria, and identified by Dr. Nodza George at the Herbarium of the Department of Botany, University of Lagos and a voucher specimen (LUH 9514) was deposited afterwards. The plant material was thoroughly rinsed with distilled water, air-dried completely and finely ground in an electric blender. Subsequently, 1.5 kg of the ground plant material was macerated in 10 litres of 70% ethanol for 72 hours with intermittent shaking. The resulting ethanol extract was filtered through a layer of cotton wool and re-filtered with Whatman filter paper (15 mm). The filtrate was concentrated to dryness using a rotary evaporator (Bibby Stuart RE300, UK) and a water bath set at 40 °C. The dried *Theobroma cacao* bark ethanol extract (TCBEE) was then stored in a clean, airtight container. A 10 mg/mL stock solution was prepared by first dissolving the semi-solid TCBEE in DMSO overnight, followed by additional dissolution in PBS to achieve a final DMSO concentration of 0.1%. This was then filter-sterilized under the laminar flow hood using a syringe filter (0.22 µM) and stored at 4 °C until it was needed to be used.

Cell culture: The human erythroleukaemic K562 cell line was purchased from Cell Line Services (CLS, Germany) and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma Aldrich, USA), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin as described by (Iftikhar *et al*., 2022). Cells were grown in a humidified 5% CO_2 incubator regulated at 37 °C. The medium was changed three times per week and passaged when confluence was reached.

Cell viability assay: The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was used to evaluate the cytotoxicity of varying concentrations of TCBEE extract in K562 cells. Cells seeded at a density of 2 x 10^5 cells per mL in a 96-well plate were treated with TCBEE in a two-fold dilution series starting from a concentration of 5000 µg/mL. RPMI medium only served as the blank. After 24 h, a 10 µL aliquot of MTT (5 mg/mL) solution prepared in PBS was added to all 96 wells and incubated at 37 °C for another 3 hours. DMSO (100 µL) was then added to each well and incubated for another 15 minutes. Changes in absorbance of the resulting purple-coloured formazan product, which reflects the number of viable cells, were measured on a microplate reader (Varioskan LUX) at a reference wavelength of 540 nm. The assay was done in triplicates and the half-maximal inhibitory concentration (IC_{50}) was calculated using log-probit regression analysis (Langdon, 2003; Gao *et al*., 2012; Riss *et al*., 2016).

Gene expression studies: K562 cells were seeded at a density of 7 x 10^5 per mL in a 6-well plate and treated with four concentrations of TCBEE (1, 5, 10 and 30 µg/mL) lower than the calculated IC_{50} values. Cells treated

with 200 µM HU (Glentham Life Sciences, UK) were the positive control while untreated cells served as the normal control. After 24 h, total RNA was isolated from (un)treated cells with Zymo Quick-RNA™ MiniPrep Kit (R1054) according to the manufacturer's instructions. RNA integrity check was achieved by measuring the optical density (OD) absorption ratio between 260 nm and 280 nm on a Nanodrop. cDNA was then synthesized from 1 µg pure RNA (>1.9) using the ProtoScript II First Strand cDNA synthesis kit (New England Biolabs, E6560S) following the manufacturer's instructions. Real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was performed on the obtained cDNA template with Luna Universal qPCR Master Mix (New England Biolabs, M3003S) on a Bio-Rad CFX96 real-time PCR system with the SYBR Green fluorescence chemistry according to the following conditions; an initial denaturation step at 95 °C for 60 seconds, followed by 45 cycles of denaturation (at 95 °C for 15 seconds) and extension at 60 °C for 30 seconds. Subsequently, a melt-curve program was executed, gradually increasing the temperature from 60°C to 95 °C. Furthermore, the RT-qPCR experiments were conducted with two technical replicates for each primer and negative template control (NTC) reactions were performed simultaneously without using a template DNA to ensure the reliability of the results. The primer information for the target gene (γ-globin) and the reference gene (GAPDH) as reported by Iftikhar *et al*. (2022) are presented in Table 1.

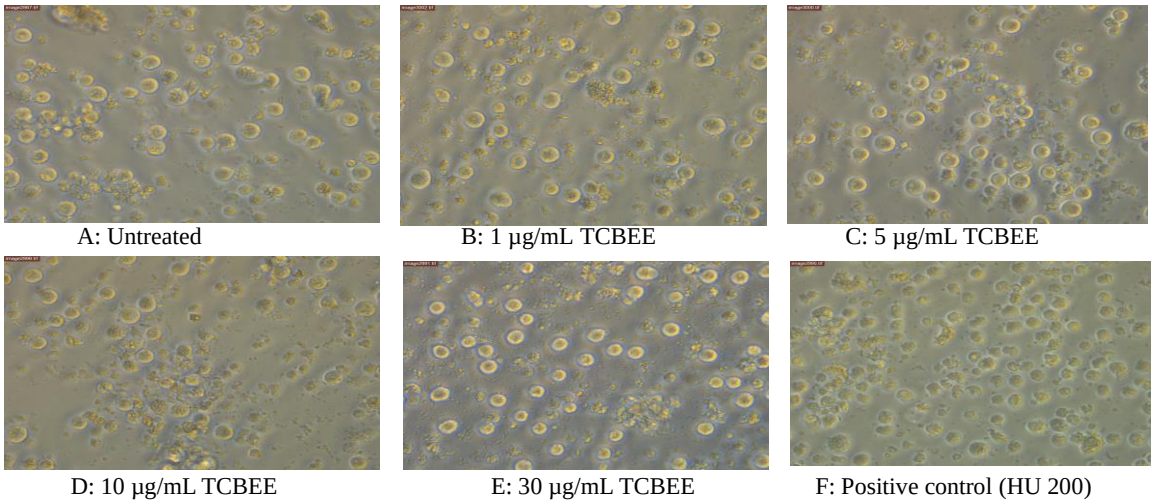
Table 1: Primer Sequences for RT-qPCR analysis

Target Gene	Primer	Nucleotide Sequence (5'-3')	Bases
Human γ-Globin	Forward	TTCCTGGCAGAAGATGGT	18
	Reverse	AGCTCTGAATCATGGGCAGT	20
Human GAPDH	Forward	CCAGAACATCATCCCTGCCT	20
	Reverse	CCTGCTTCACCACCTTCTTG	20

Statistical analysis: All data from this study were analyzed using GraphPad Prism Software (version 9.0), and the results are presented as mean ± standard deviation (S.D). The relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method after normalizing the data with that of the expression levels of the reference gene, GAPDH (Livak and Schmittgen, 2001). One-way ANOVA was performed to compare differences between the fold changes of treated and untreated groups. *P*-values less than 0.0001 were considered statistically significant.

Results

Morphology of K562-treated cells: Figure 1 illustrates the morphological changes observed in K562 cells after exposure to TCBEE or HU. Micrographs depicting cells treated with varying concentrations of TCBEE (Fig. 1b-1e) did not reveal discernible alterations in cellular morphology compared to untreated cells (Fig. 1a). In contrast, cells treated with HU exhibited cytotoxic effects, manifested by an increased presence of dead cells or cells of reduced size (Fig. 1f). These observations highlight the varying effects of TCBEE and HU on K562 cell morphology, indicating distinct cellular response to the two treatments.



A: Untreated B: 1 µg/mL TCBEE C: 5 µg/mL TCBEE

D: 10 µg/mL TCBEE E: 30 µg/mL TCBEE F: Positive control (HU 200)

Figure 1: Representative morphology images of K562 cells (A-F) treated with TCBEE (*Theobroma cacao* bark ethanol extract) or HU (Hydroxyurea) for 24 hours, and untreated cells. Magnification: x100.

Cytotoxicity of TCBEE on K562 cells: The half-maximal inhibitory concentration (IC_{50}) of TCBEE on K562 cells, estimated from the dose-response curve in Fig. 2, is 101.5 µg/mL. The regression analysis shows the concentration range at which TCBEE exerts significant growth inhibitory effects on the K562 cell line.

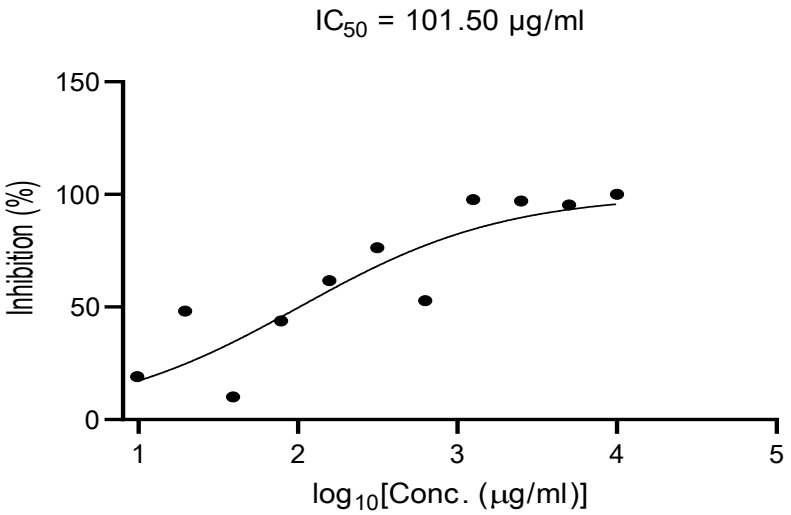


Figure 2: Determination of the half-maximal inhibitory concentration (IC_{50}) of *Theobroma cacao* bark ethanol extract (TCBEE) on K562 cells.

γ-Globin mRNA expression analysis: Figure 3 depicts the relative fold changes in γ-globin mRNA expression levels in K562 cells following a 24-hour exposure to varying concentrations of TCBEE or HU. HU treatment resulted in a fold change of 0.5, while 1 µg/mL of TCBEE yielded a fold change of 1.08. In comparison, untreated cells exhibited a fold change of 0.1. Notably, as the concentration of TCBEE increased, there was a decrease in fold changes, indicating a dose-dependent effect on γ-globin mRNA expression levels.

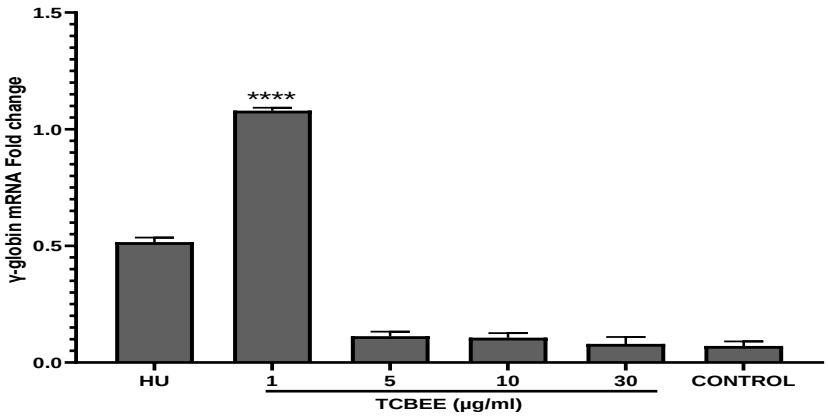


Figure 3: Relative fold changes in γ-Globin mRNA expression in K562 cells after 24 hours of exposure to TCBEE (Theobroma cacao bark ethanol extract) or HU (hydroxyurea). Gene expression data were normalized to GAPDH Levels. $P < 0.0001$ between the treated and untreated groups was considered statistically significant.

Discussion

Sickle cell disease (SCD) remains a deadly monogenic disorder globally, with a higher prevalence in sub-Saharan Africa to date (Adigwe *et al*., 2023). The high costs associated with managing SCD often compel individuals with limited income and resources to opt for traditional medicine, which is generally perceived to be less toxic than synthetic drugs and linked to fewer side effects (Imaga, 2013; Shah *et al*., 2020; Lubega *et al*., 2021). In Nigeria, the stem bark of *Theobroma cacao* is one of the traditional remedies for managing SCA. This

study investigated the efficacy of *Theobroma cacao* stem bark ethanol extract (TCBEE) in stimulating the production of γ-globin mRNA in K562 cells.

The haemoglobin phenotype of the human erythroleukaemic K562 cell line established by Lozzio and Lozzio (1975) has been characterized and shown to contain all globin chains (embryonic ∈- and ζ-, fetal γ-, α-, and δ-globin chains) except the human β-globin gene (Mookerjee *et al*., 1992). Consequently, K562 cells serve as a valuable *in vitro* model for studying the effect of various synthetic and natural compounds on the expression of γ- and other globin genes (Bianchi *et al*., 2009; Testa, 2009). Evaluation of the effects of four concentrations of TCBEE (1, 5, 10, and 30 µg/mL) on K562 cell morphology under a microscope revealed no significant alterations compared to the untreated control group (Figure 1a-e). In contrast, treatment with hydroxyurea (HU) resulted in a slight reduction in cell size and reduced cell viability (Figure 1f).

To assess TCBEE's effect on K562 cell viability and cytotoxicity, we employed the MTT assay and obtained an IC_{50} value of 101.5 µg/mL (Figure 2). The MTT colorimetric assay relies on the enzymatic activity of succinate dehydrogenase, found in the mitochondria of viable cells, or other intracellular enzymes such as NAD(P)H-dependent oxidoreductases that can facilitate the reduction of MTT (a yellow tetrazolium salt) into a purple formazan product that is soluble in DMSO (Damiani *et al*., 2019). The IC_{50} value of 101.5 µg/mL indicates the concentration at which 50% inhibition of cell growth occurs with the extract. According to the US NCI plant screening program, a crude extract is typically considered to exhibit *in vitro* cytotoxic activity if its IC_{50} value is below 20 µg/mL whereas for pure compounds, it has to be less than 4 µg/mL (Lee and Houghton, 2005). The IC_{50} value of 101.5 µg/mL obtained for TCBEE falls above the threshold associated with strong cytotoxicity, suggesting a moderate level of cytotoxicity on K562 cells which could be beneficial for SCD individuals as the goal is not to induce cell death but to increase the production of normal erythrocytes (Elufioye *et al*., 2017). Baharum *et al*. (2014) investigated the *in vitro* cytotoxicity of methanolic extracts of *T. cacao* bark against the human estrogen receptor-positive (MCF-7) breast cancer cell line and six other cell lines. They obtained an IC_{50} value of 72.0 ± 9.3 µg/mL against MCF-7 cells which is somewhat similar to TCBEE's IC_{50} value of 101.5 µg/mL obtained in this study against K562 cells but observed a weak cytotoxic activity (IC_{50} ~ 1000 µg/mL) against the six other cell lines in their study. Plant extracts with high toxicity against cancer cells and low toxicity towards normal cells, such as TCBEE, have good anti-cancer components and usually exhibit cytotoxic activity on cancer cells without killing normal cells (Baharum *et al*., 2014).

Numerous studies have explored the botanical composition and health benefits of various parts of *T. cacao*, especially the fruits and seeds (Baharum *et al*., 2016; Jean-Marie *et al*., 2022). However, only a few studies have utilized the stem bark of *T. cacao* in their research (Baharum *et al*., 2014; Barreto *et al*., 2017; Kosoko *et al*., 2017; Oyeleke *et al*., 2018; George-Opuda *et al*., 2021). TCBEE contains essential phytochemicals like carbohydrates, saponins, phlobatannins, tannins, glycosides, resins, and alkaloids with potential pharmaceutical applications (Abulude *et al*., 2022).

Treating K562 cells with varying concentrations of TCBEE (1, 5, 10, and 30 µg/mL) for 24 hours resulted in a dose-dependent decrease in γ-globin gene expression fold changes as the concentration increased (Figure 3). This response pattern, known as biphasic dose response, has been observed in certain phytochemicals or hormetic compounds that induce biologically opposite effects at different doses (Jodynis-liebert and Kujawska, 2020). The highest fold change of γ-globin gene expression was seen in cells treated with 1 µg/mL TCBEE, showing a significant ($p < 0.0001$) 1.08-fold change compared to HU-treated cells. This finding aligns with a similar fold change of 1.06 ± 0.17 observed after treating K562 cells with vascinol (25 µM), a compound isolated from *Adhatoda vasica*, for 5 days (Iftikhar *et al*., 2022). Given the short exposure duration of TCBEE (24 hours) in our study, it might be possible to observe increased gene expression levels over a longer exposure duration. Further identification of the compound(s) in TCBEE responsible for enhancing γ-globin gene expression could potentially lead to overexpression of the gene.

In conclusion, our study demonstrates the therapeutic potential of a low dose of TCBEE to induce **γ**-globin gene expression in K562 cells, which could alleviate the severity of symptoms in individuals with haemoglobinopathies such as sickle cell anaemia and thalassemias through increased HbF levels. Further research is necessary to identify the bioactive compounds responsible for this induction as this could potentially provide a rationale for future drug development. The precise mechanisms underlying TCBEE's effect on HbF production also need to be elucidated before clinical applications can be considered.

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References

Abulude FO, Ogunkoya MO, Adenibuyan GB, Arifalo KM, Akinusotu A, Samuel A, Kenni AM, Bello LJ: Phytochemical assessment of the extracts of stem (bark) and leaves of *Theobroma cocoa* materials: experimental procedure and its comparison to literature. Asean J Sci Eng Mater, 1(2): 85-92. 2022.

Adigwe OP, Onoja SO, Onavbavba G: A critical review of sickle cell disease burden and challenges in sub-Saharan Africa. J Blood Med, 14:367-376. 2023.

Amujoyegbe OO, Idu M, Agbedahunsi JM, Erhabor JO: Ethnomedicinal survey of medicinal plants used in the management of sickle cell disorder in Southern Nigeria. J Ethnopharmacol, 185:347-360. 2016.

Aprotosoaie AC, Miron A, Trifan A, Luca VS, Costache II: The cardiovascular effects of cocoa polyphenols—an overview. Diseases, 4(4):39. 2016.

Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R: In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of *Theobroma cacao*. Molecules, 19(11):18317-18331. 2014.

Baharum Z, Md Akim A, Hin TYY, Hamid RA, Kasran R: *Theobroma cacao*: review of the extraction, isolation, and bioassay of its potential anti-cancer compounds. Trop Life Sci Res, 27(1): 21–42. 2016.

Barreto LS, Tokumoto MS, Guedes IC, de Melo HG, Amado F, Capelossi VR: Study and assessment of the efficiency of the cocoa bark extracted from the *Theobroma cacao* as an inhibitor of the corrosion of carbon steel in substitution of benzotriazole. Mat Res, 21(1):e20160309. 2017.

Bianchi N, Zuccato C, Lampronti I, Borgatti M, Gambari R: Fetal hemoglobin inducers from the natural world: a novel approach for identification of drugs for the treatment of β-thalassemia and sickle-cell anemia. Evid Based Complement Alternat Med, 6(2):141-151. 2009.

Damiani E, Solorio JA, Doyle AP, Wallace HM: How reliable are *in vitro* IC_{50} values? Values vary with cytotoxicity assays in human glioblastoma cells. Toxicol Lett, 302:28-34. 2019.

de Souza PA, Moreira LF, Sarmento DHA, da Costa FB: Cacao—*Theobroma cacao*. In Exotic fruits, Academic Press. pp. 69-76. 2018.

Elufioye TO, Abdul AA, Moody JO: Cytotoxicity studies of the extracts, fractions, and isolated compound of *Pseudocedrela kotschyi* on cervical cancer (HeLa), breast cancer (MCF 7) and skeletal muscle cancer (RD) cells. Pharmacogn Res, 9(1):46-50. 2017.

Gao Z, Kang X, Hu J, Ju Y, Xu C: Induction of apoptosis with mitochondrial membrane depolarization by a glycyrrhetinic acid derivative in human leukemia K562 cells. Cytotechnology, 64(4):421-428. 2012.

Gbadamosi IT: An inventory of ethnobotanicals used in the management of sickle cell disease in Oyo State, Nigeria. Bot Res Int, 8(4):65-72. 2015.

George-Opuda IM, Adegoke OA, Oluwatayo OB, Odeghe OB, Ndibe J: Effect of stem bark hydroalcoholic extract of cocoa (*T. cacao*) on liver function of Wistar albino rats induced with anaemia. J Appl Sci Environ Manage, 25(9):1715-1720. 2021.

Iftikhar F, Rahman S, Khan MBN, Khan K, Khan MN, Uddin R, Musharraf SG: In vitro and in vivo studies for the investigation of γ-globin gene induction by *Adhatoda vasica*: A pre-clinical study of HbF inducers for β-thalassemia. Front Pharmacol, 13:797853. 2022.

Imaga NA: Phytomedicines and nutraceuticals: Alternative therapeutics for sickle cell anemia. Sci World J, 2013 (269659):1-12. 2013.

Inusa BPD, Hsu LL, Kohli N, Patel A, Ominu-Evbota K, Anie KA, Atoyebi W: Sickle cell disease-genetics, pathophysiology, clinical presentation and treatment. Int J Neonatal Screen, 5(2):20- 35. 2019.

Jean-Marie E, Jiang W, Bereau D, Robinson JC: *Theobroma cacao* and *Theobroma grandiflorum*: Botany, composition and pharmacological activities of pods and seeds. Foods, 11(24):3966. 2022.

Jodynis-liebert J, Kujawska M: Biphasic dose-response induced by phytochemicals: experimental evidence. J Clin Med, 9(3):718. 2020.

Kosoko AM, Olurinde OJ, Akinloye OA: Doxorubicin-induced neuro- and cardiotoxicities in experimental rats: Protection against oxidative damage by *Theobroma cacao* stem bark. Biochem Biophys Rep, 10:303-317. 2017.

Langdon SP: Cancer Cell Culture. https://doi.org/10.1385/1592594069. 2003.

Lee CC, Houghton P: Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J Ethnopharmacol, 100(3):237-243. 2005.

Lima LJR, Almeida MH, Rob Nout MJ, Zwietering MH: *Theobroma cacao* L., "The food of the gods": Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. Crit Rev Food Sci Nutr, 51(8):731-761. 2011.

Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods, 25(4):402-408. 2001.

Lozzio CB, Lozzio BB: Human chronic myelogenous leukemia cell-line with positive philadelphia chromosome. Blood, 45(3):321-334. 1975.

Lubega M, Osingada CP, Kasirye P: Use of herbal medicine by caregivers in the management of children with sickle cell disease in Mulago National Referral Hospital - Uganda. Pan Afr Med J, 39(163). 2021.

Makani J, Ofori-Acquah SF, Nnodu O, Wonkam A, Ohene-Frempong K: Sickle cell disease: New opportunities and challenges in Africa. Sci World J, 2013(193252):1-16. 2013.

McGann PT, Ware RE: Hydroxyurea therapy for sickle cell anemia. Expert Opin Drug Saf., 14(11):1749-1758. 2015.

Mookerjee B, Arcasoy M, Atweh G: Spontaneous delta- to beta-globin switching in K562 human leukemia cells. Blood, 79(3):820-825. 1992.

Odugbemi TA: A textbook of medicinal plants from Nigeria. University of Lagos Press, University of Lagos, Lagos, Nigeria. pp. 299-317. 2008.

Oyeleke SA, Ajayi AM, Umukoro S, Aderibigbe AO, Ademowo OG: Anti-inflammatory activity of *Theobroma cacao* L. stem bark ethanol extract and its fractions in experimental models. J Ethnopharmacol, 222:239-248. 2018.

Paikari A, Sheehan VA: Fetal haemoglobin induction in sickle cell disease. Br J Haematol, 180(2):189. 2018.

Platt OS: Hydroxyurea for the treatment of sickle cell anemia. N Engl J Med, 358(13):1362-1369. 2008.

Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L: Cell Viability Assays. In: Markossian S, Grossman A, Arkin M, *et al.* (Eds.). Assay Guidance Manual. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. 2016. Available from: https://www.ncbi.nlm.nih.gov/books/NBK144065/

Shah N, Bhor M, Xie L, Paulose J, Yuce H: Medical resource use and costs of treating sickle cell-related vaso-occlusive crisis episodes: A retrospective claims study. J Health Econ Outcomes Res, 7(1):52-60. 2020.

Strouse JJ, Lanzkron S, Beach MC, Haywood C, Park H, Witkop C, Wilson RF, Bass EB, Segal JB: Hydroxyurea for sickle cell disease: a systematic review for efficacy and toxicity in children. Pediatrics, 122(6):1332-1342. 2008.

Sundd P, Gladwin MT, Novelli EM: Pathophysiology of sickle cell disease. Annu Rev Pathol, 14:263-292. 2019.

Testa U: Fetal hemoglobin chemical inducers for treatment of hemoglobinopathies. Ann Hematol, 88(6):505-528. 2009.