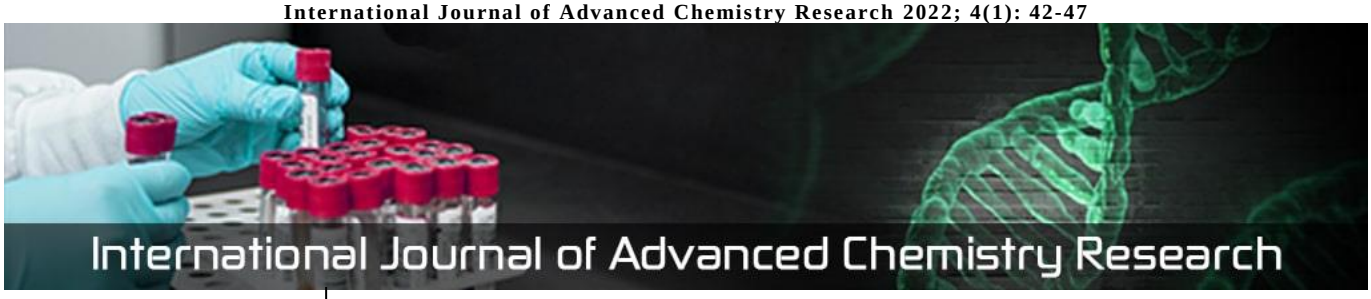

ISSN Print: 2664-6781
ISSN Online: 2664-679X
Impact Factor: RJIF 5.32
IJACR 2022; 4(1): 42-47
www.chemistryjournals.net
Received: 21-12-2021
Accepted: 22-01-2022

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Phytochemical, anti-sickling and polymerization inhibition studies of the crude methanol leaf recipe extracts of three medicinal plants

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DOI: https://doi.org/10.33545/26646781.2022.v4.i1a.46

Abstract
This research focuses on the evaluation of herbal recipe made from *Carica papaya* linn, *Psidum guajava* linn. and *Terminalia catappa* linn which are used individually and as a recipe for the treatment of Sickled cell disease (SCD) in northern Nigeria. The research seek to validate the traditional application of the recipe in the management of sickle cell disease and to further identify its phytoconstituents (qualitatively), anti-sickling and polymerization inhibition potential in an in-vitro system. The results of the phyto-evaluation revealed the presence of alkaloids, cardiac glycosides, flavonoids, tannins, terpenoids, phenolics, and saponins, while the sickling inhibition studies showed a significantly higher inhibition level in dose dependent manner of up to 71.32% and 85.71%. The results for sickling reversal studies showed a similar trend with reversal levels of up to 92.03% and 94.51% and a polymerization inhibition effect of 79.84% to 99.18% respectively. Thus, the recipe formulated from the three commonly used plants may Served as an excellent candidate for the development of plant drug for the management of SCD, may Served as a prophylactic agent that will reduced the frequency of crisis in sickle cell patients.

Keywords: phytochemical, polymerization, methanol leaf recipe

Introduction
Sickle cell anemia is a severe hereditary form of anemia in which a mutated form of hemoglobin distorts the red blood cells into a crescent shape at low oxygen levels [1]. The disease is among the leading cause of deaths in developing countries. According to the world health organization about three hundred thousand (300,000) neonates are born with this disorder globally, and about hundred fifty thousand (150,000) of these births are recorded in Nigeria [2]. There is still no permanent solution to this disorder, however, several therapeutic options (orthodox and traditional) are in used but the successes in the management of the disorder is still challenging due to the difference in the manifestation of symptoms among patients. Various therapeutics approaches that have been employed to inhibit against polymerization of haemoglobin (Hb) which is believed to be the primary patho-physiological events that leads to the sickling of the red blood cells [1]. Currently hydroxyurea and erythropoietin are the most prescribed orthodox options for the prevention of the occurrence of crisis in SCD by inducing the production of fetal haemaglobin [3]. The above approaches did not give the much-needed beneficial effects based on the reduction of painful crisis as the criterion for successful treatment [3]. Other option though not feasible in development countries such ours is the bone marrow transplantation which has been found to be an efficient for controlling the scourge of the disease by completely changing the genotype of the patient to either HbAS or HbAA depending on the genotype of the donor. However, the cost implications, expertise and the problems of finding suitable donors are the major obstacle associated this approach [4-5]. Furthermore, there has been a tremendous increase in research towards finding natural products capable of ameliorating the frequencies of crisis and painful episodes, preventing the loss of water from red blood cells (RBCs) and inducing the level of fetal haemoglobin in the patients [6]. The successes of such effort lead to the discovery of Ciklavit (a herbal recipe made from plants and potash) which was champion by Nigerian pharmaceutical research and development NIPRID [7].

Several current research activities are focused on identifying new drugs that are capable of preventing the loss of water from red blood cells (RBCs) or increasing the level of fetal haemoglobin, a variety of haemoglobin that prevents the sickling of red blood cells (RBC). Clotrimazole, hydroxyurea and erythropoietin (a genetically engineered hormone that stimulates fetal haemoglobin and RBC production) were proposed in this regard [8-11]. Furthermore, plant formulations and recipes currently in circulations for the treatment of sickle cell disease in Nigeria are *Jobelin* (jubi), *Ajawaron HF*, etc [12-13].

The three medicinal plants (*Carica papaya, Psidium guajava and Terminalia catappa*) selected for study were carefully picked after a painstaking review of their ethnomedicinal, ethnopharmacological efficacies, availability and application in the management and treatment of sickle cell disease from sources such as: West African Herbal Pharmacopoeiae, Anti-sickling Herbs, An Inventory of Ethnobotanicals Used in the Management of Sickle Cell Disease in Nigeria, Useful Medicinal Plants of West Africa, Medicinal Plants of Ghana among others [14-19]. Thus, this research will focus on the evaluation of herbal recipe made from *Carica papaya* linn, *Psidum guajava* linn. and *Terminalia catappa* linn which are used individually and as a traditional recipe for the treatment of SCD in northern Nigeria with a view to validate its traditional application, further identify its phytoconstituents qualitatively and quantitatively.

Methodology Plant Collection

The three plants specimens were collected at the State Low Cost Area in Maiduguri, Borno State. The sampling area is located on 11051'40"N1309'39"E, while the samples collected was identified by a Taxonomist at the Department of Biological Sciences, University of Maiduguri, Nigeria and a voucher number 09903 allocated to it.

Sample Preparation, Extraction and Partitioning

The three plant samples (leaves) collected was air dried under shade and pulverized using wooden mortar and pestle. The pulverized plant materials were mixed in equal ratio and extracted with 80% methanol using maceration technique. The extracts was concentrated at low temperature to minimize the degradation of thermolabile components [20]. The resulting mass was then weighed and kept in a desiccator until required for analysis. The concentrated recipe was subjected evaluated quantitative, qualitative phyto-evaluation and was further subject to Invitro antisickling assays and *in vivo* toxicity study pharmacologically

Phytochemical Evaluation

Preliminary phytochemical screening will be carried out to confirm the secondary metabolites using standard procedures as described by [21-25] for the presence of Alkaloids, Flavonoids, Anthraquinones, Saponins, Tannins and Cardiac Glycosides

Anti-Sickling Assay Sample Collection (Blood)

The blood samples used in this study was collected with the informed consent of stable adolescent SCD patients attending State Specialist Hospital Maiduguri, which had not been transfused in the last three months with Hb AA blood as described earlier by [26].

Sickling Reversal Test

The ability of the recipe to reverse the sickling state of the RBCs was performed in accordance with the previously described by procedure [27]. The blood sample was washed twice in five volumes of phosphate buffered saline (1 mL of blood in 5 mL of PBS) with pH 7.4 by centrifugation at 1200g. Into a clean Eppendorf tube, 100 µL of the washed red blood cells and 100 µL of freshly prepared 2% sodium metabisulfite (Na2S2O3) was added and incubated for 2 h at 37 °C. Then 100 µL of anti-sickling agent (1% w/v) was added and incubated for another 2 h at 37 °C. Ten micro liters (10 µL) of the incubated cells was taken and transferred to a hemocytometer or Neubauer's counting chamber, and the cells will be viewed and counted using a microscope at 40× magnification. A control test will be performed by replacing 100 µL of drug/extract with 100 µL of PBS. The cells were classified as normal or sickled by observing their shapes. A biconcave or disk-like shape was taken to be normal while the elongated, star-like, or wrinkled shape was considered sickled. The percentage sickled cells was calculated using the following formula [26]:

$$\text{Percentage sickling (\%)} = \left(\frac{\text{number of sickled cells}}{\text{the total number of counted cells}}\right) \times 100$$

Sickling Inhibition Test

0.2 ml of homozygous sickle cell anaemia whole blood was placed in a test tube (in duplicates). Then, 0.2ml phosphate buffered solution plus 0.2 ml of the aqueous extracts was added and the mixture immediately covered with 1 ml liquid paraffin and incubated in a thermostat water bath at 370C for four hours. After the 4-hour incubation period, 0.6 ml of freshly prepared 2% sodium metabisulphite solution was carefully added under the liquid paraffin to the incubation mixture and the final mixture was thoroughly but carefully mixed by rolling the test tube between the palms. The mixture was incubated for another 11/2 hours at 37 ºC in the water bath. At the end of the incubation period, the liquid paraffin was carefully removed by the use of a Pasteur pipette and the remaining mixture is fixed in 3 ml of 95% methanol solution and reserved until ready for counting. The control experiments consisted of 0.2 ml saline solution to replace the extract. The percentage inhibitory activity for each sample is then calculated from the results and presented as duplicate means for all the samples, including the experimental control [28]. The fixed cell mixtures were each centrifuged and the supernatant decanted. With a capillary tube, one or two drops was applied on a microscope slide, carefully covered with a cover slip and viewed with a high power microscope (x100), 400 cells will be counted and the percentage sickled cells calculated.

Polymerization Inhibition Test

The polymerization inhibition test was carried out following the method of [29]. This procedure involves the measurement of the turbidity of the polymerizing solution of RBCs at a wavelength of 700 nm at 26 °C. Freshly prepared 2% sodium metabisulfite (0.88 mL) was transferred into a cuvette followed by 0.1 mL of PBS and 0.02 mL of SS blood. Absorbance was be read at 700 nm immediately and at every 2 min for 30 min. This serves as control test. For the inhibition test, 0.1 mL of phosphate buffered saline (PBS) was replaced by 0.1 mL of plant extracts. The rate of

polymerization in percentage was calculated using the earlier method of [26] as presented below:

$$\text{Rate of polymerization (RP)} = \left[\frac{(\text{final absorbance} - \text{initial absorbance})}{30}\right] \times 100$$

Results and Discussions

Table 1: Phytochemical Constituents of Extractives of Crude Methanol Leaf *Carica papaya, Psidum guajava, Terminalia catappa Recipe*

Phytochemical Constituents	Extractive
	Recipe
Alkaloids	+
Cardiac glycosides	+
Flavonoids	+
Tannins	+
Terpenoids	+
Phlobatannins	-
Saponins	+

Key: (+) = Present and (-) = Absent
RECIPE = Crude Methanol Leaf Extract of the three plants

Table 2: Result of the Sickling Inhibition Study of the Crude Methanol Extract of *Recipe*

Extract/ CONC.	Percentage Sickled and Percentage Inhibition Per Unit Time (min)				
	30	60	90	120	MEAN±SEM
Recipe/*12.5mg/ml*					
% Sickled	2.95	8.90	27.75	45.27	21.22±9.60
% Inhibition	95.12	86.52	66.92	36.74	71.32
Recipe/*25mg/ml* % Sickled	17.57	6.91	7.69	6.06	9.56±2.69
% Inhibition	70.95	89.52	90.83	91.53	85.71
Control (N.S 0.5ml) % Sickled	60.47	65.99	83.89	71.57	70.48±5.01

Table 3: Result of the Sickling Reversal Test of the Crude Methanol Extract of Recipe

Extract /CONC.	Percentage Sickled and Percentage Reversal Per Unit Time (min)					
	30	60	90	120	150	MEAN±SEM
Recipe/ (*12.5 mg/ml*)						
% Sickled	4.05	7.01	5.67	7.80	3.67	4.91±0.80
% Reversal	93.30	89.38	93.24	89.10	95.14	92.03
Recipe/ (*25 mg/ml*) % Sickled	2.26	2.63	5.02	5.49	4.58	4.00±0.65
% Reversal	96.26	96.01	94.02	92.32	93.94	94.51
Control (N.S 0.5 ml) % Sickled	60.47	65.99	83.89	71.57	75.53	71.49±4.01

Table 4: Result of the Polymerization Inhibition Study of the Crude Recipe Extract

Extract/Concentration	ROP	RPP	RPI
Recipe			
12.5 mg/ml	0.74	20.16	79.84
25 mg/ml	0.03	0.82	99.18
Control			
Control (N.S 0.5ml)	3.67	100	0.00

ROP= Rate of Polymerization = (ΔOD/Time) *100
RPP= Relative Percentage Polymerization = (ROP of Test/ ROP of Control) *100
RPI= Relative Polymerization Inhibition = (Control-Test/Control)*100

Discussion

The results of the qualitative phytochemical study of the methanol crude extract of the formulated recipe revealed that the recipe formulated from the three medicinal plants *Carica papaya*, *Psidum guajava* and *Terminalia catappa* contained some valuable bioactive principle such as; alkaloids, cardiac glycosides, flavonoids, tannins, terpenoids, phenolics, and saponins as presented in the Table 1. According to Akakpo-Akue [30] and Kiefmann [31] reported that Flavonoid (quercetin) has shown to provide protection against haemoglobin oxidation and other cellular modifications promoted by peroxides. Likewise, Kplé, [32] and Akojie and Fung [33] revealed that the anti-sickling effects of *cajanus cajan, J. secunda*, *P. nigrescens* and *J. gossypiifolia* were as a result of additive and synergestic effects of some of these plants metabolites. Thus, the effects manifested by the recipe could be due the possible effects of these phyto-constituents.

Similarly, Railson [34] reported that variety of plants metabolites elicits some physiological activities and medicinal properties. These include among others; promotion of tissue regeneration, decreasing the permeability of blood capillaries, anti-sickling and strengthening their resistance to hemolysis. Moreover, Moody [54] attributed the anti-sickling potential of *Cissus populnea* to the presences of flavonoids, cardiac glycosides, Saponins and steroids which are all inherent in the recipe

under study. Hence, their presence could be the probable reason for their efficacies as plant antisickling drug.

The result of the pharmacological investigation revealed that the recipe is indeed a promising agent for mitigating the sickling crises in an *in vitro* system as presented in Table 2 and 3 for the two concentrations of the recipe and the control group**.** The results indicated a significant ($P<0.05$) potential of the recipe in inhibiting the sickling of RBCs viewed under microscope. The cells with elongated or spike-like shapes were considered sickled while normal RBCs appeared biconcave or disk-like. To quantitatively compare the anti-sickling properties of the crude recipe extract, the cells counting method of [26] Nurain *et al*. (2017) [26] was adopted. The number of sickled cells in the control group was significantly higher ($P<0.01$) corresponding to 70.84% percentage sickled, while in the presence of the crude recipe, the extract acted in dose dependent manner thus reducing the percentage sickled cells to 21.22% at 12.5 mg/ml and 9.56% at 25 mg/ml corresponding to 71.32% and 85.71% inhibition as presented in Table 2. Similarly, these findings are inline with the earlier work of Samuel [36] who investigated the potential of powdered dried fallen leaves of *Terminalia catappa* and confirmed that the plant had highest antisickling activity as it drastically reduced the rate of erythrocyte sickling to very significant level ($P<0.001$). Likewise, in the work of Oduola *et al*. Imaga *et al.* Naiho *et al*. Nurain *et al*. Olomola *et al*. Cyril-Olutayo *et al*. [37-41, 26] corroborated the efficacies of plants and their potentials in inhibiting the sickling of RBC and also suggested that their administration during crisis could probably re-oxygenates red cells, restore their shape and deformability due to the high level of phytochemicals inherent in plants.

The reversal potentials of the crude recipe extracts was evaluated following the assertion of Poillon and Kim [43] had earlier reported that the primary cause of the clinical symptomatology of sickle cell anaemia is the intracellular polymerization of sickle haemoglobin (HbS) that occur when sickle erythrocytes are partially deoxygenated under hypoxic conditions of the microcirculation. This was corroborated by Mojisola *et al*. [44] in her work on the sickling reversal and polymerisation inhibition potential of *Moringa oleifera* and had also affirmed that the polymerisation will in turn make HbSS red blood cells deformed resulting in microvascular occlusions and haemolytic anaemia. However, according to Oder [45], sickled cell could be reversed to their normal biconcave shape upon re-oxygenation.

The results obtained from the sickling reversal assay in Table 3, shows that the crude recipe extract was able to significantly reversed the percentage sickled cells to their normal biconcave shape in a dose-dependent manner. Consequently, it could be suggested that the observed effects of the extracts may be as a result of the secondary plants metabolites bounding allosterically to the HbSS red blood cells under hypoxic condition to give a more stable conformer as earlier suggested by Olomola *et al*. [46] Thus, restoring red blood cells (RBC) shape, flexibility, longevity and smooth movement through micro human blood vessels.

From the experimental results, the extract exhibited different percentage of sickled cells; thus, corresponding to different percentage reversal. The number of percentage sickled cells in the control group was 71.49% after 150 minutes, while the treatment at the dose of 12.5 mg/ml Recipe was 92.03% and 94.51%, 25 mg/ml respectively. These results were obtained from the average of five repeated experiments for each antisickling agent and the control. Similarly, the findings of this study is similar to the earlier findings of Cyril-Olutayo *et al*. [47] which established both antisickling and reversal of sickling activities of the extract of an unripe pawpaw. Likewise in another publication Cyril-Olutayo, and co-workers [42] affirmed that the combination of two plants could have synergistic effect since one or more components of each have antisickling properties, hence, the higher values observed with the recipe for both reversal and inhibitory activities could be due to the synergy of its components.

Furthermore, Table 4 shows the presentation of the effect of the crude recipe extract in preventing RBC polymerization. The results of the recipe had significantly inhibited the RBC polymerization in dose dependant manner from 79.84% to 99.18% respectively. From the data analysed, it is clear that the crude recipe extract possesses a very significant haemoglobin polymerization inhibition effects. This finding is in conformity with earlier reports of Oyewole *et al*. [48] in whose work, the measure of inhibition of deoxyHbS polymerization inhibition of some plant extracts that was found to be in dose dependent. More so, Chikezie; Okpuzor *et al*; Imaga *et al*. [49-51] had reported and proposed the suitability of the use of herbal preparations as candidate for management of sickle cell disease.

In conclusion, the recipe formulated from the three commonly used plants may serve as an excellent candidate for the development of plant drug for the management of SCD, may serve as a prophylactic agent that will reduced the frequency of crisis in sickle cell patients.

Acknowledgement

The authors wish to acknowledge support from the Tetfund (IBR grant), Ramat Polytechnic Maiduguri, Nigeria,

State Specialist Hospital Maiduguri and the Department of Medical Laboratory Science (Haematology Department) University of Maiduguri for an opportunity to do part of this research in their facilities.

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