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Phytochemical, Toxicity, *In-vitro* Anti-Sickling and UV Polymerization Inhibition Studies of the Crude Methanol Leaf Extract of *Terminalia catappa* Linn.

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ABSTRACT

This research seeks to validate the potential of crude methanol leaf extract of *Terminalia catappa* in the management of sickle cell disease. The evaluations were carried out using already established standard procedures. The result of the qualitative phyto-constituents evaluation of the crude methanol leaf extracts of *Terminalia catappa* revealed the presence of cardiac glycosides, flavonoids, tannins, terpenoids and saponins but absence of alkaloids and phlobatannins. Similarly, the result of the quantitative phytochemical evaluation of crude methanol leaf extract of *Terminalia catappa* Linn revealed the percentage of the amounts of flavonoids as 11.0; 0.85 (phenols); 6.0 (tannins); and 3.60 (saponins). The toxicity thresholds LD_{50} of the crude extract was calculated to be 774 mg/kg bd.wt intraperitoneally and 5000 mg/kg bd.wt orally. The results of the *in-vitro* pharmacological studies revealed that the extracts have significantly inhibited the sickling of the HbSS RBC in non-dose dependent manner with the highest percentage inhibition of 92.83%. The sickling reversal activity of the extract revealed highest percentage reversal of 89.40% and the polymerization inhibition values of up to 97.28% respectively. Therefore, the methanol leaf extract of *Terminalia catappa* Linn. contained phyto-constituents capable of mitigating the sickling of HbSS blood in an *in vitro* model.

Keywords: Phytochemicals, anti-sickling, methanol, crude extract, sickle cell disease, *Terminalia catappa*

INTRODUCTION

The lack of awareness, premarital counselling and poverty have contributed to the prevalence of sickle cell disorder in northern Nigeria. The disorder occurs as a result of the beta globin chain mutation at the sixth position causing the replacement of glutamic acid by valine in the human haemoglobin[1]. This mutation at low oxygen leads to gelation, or fibre formation which leads to the polymerization of the mutant haemoglobin. Hence, resulting in the disorientation of the cell from their normal biconcave shape to sickle shape, thereby hindering the systemic circulations of red blood cells [1]. The estimated cases of sickle cell disease was approximately 300,000 children globally and about half of this number are born in Nigeria. The poor healthcare services in Nigeria have contributed to the high mortality of patience with Sickle Cell Disease (SCD) forcing patients and their families to seek alternative approach to the treatment and management of the disorder. There is still no affordable and accessible permanent cure to this disorder in Nigeria. Thus, the use of medicinal plants is considered as an alternative therapy for the treatment and management of the disorder.

Terminalia catappa is a large tropical tree of the *Combretaceae* family. The plant has various traditional medicinal applications among which are; the treatment of pain, inflammation, liver disease, dysentery and headache for the leaf. Its bark is used as diuretic and cardiotonic; stems bark and seeds have been used for sexual dysfunction [2-4]. A preliminary research by Samuel *et al.* [5] revealed that the dried fallen leaves of *Terminalia catappa* extract exhibited anti-sickling activity to a significant level. Moreover, Pawar and Pal [6] reported significant antimicrobial activity against gram-positive and gram-negative micro-organism of different fractions of the plant. Likewise, Fan *et al.* [7]; Khan *et al.* [8]; Ratnasooriya *et al.* [9] and Aimola *et al.* [10] affirmed that plants possessed anti-inflammatory, analgesic, wound healing and ability to induce the production fetal haemoglobin in sickle cell patients. Similarly, Wen *et al.* [11] in an *in vivo* study revealed that plant has inhibitory effect on the growth as well as the metastasis of Lewis Lung Carcinoma (LLC) cells and its mutagenicity. Accordingly, Wun [12] and Arjariya *et al.* [13] classified the alcoholic leaf extract as practically non-toxic with no changes in haematological, haematopoiesis and renal parameters etc were observed due to the administration of its extract. However, regional variability and weather are factors in the synthesis of natural products, resulting in the production of different constituents from what was established in literature. This leads to either synergistic or potentiation effect and sometimes increase the pharmacological efficacy or the toxicity of the plant.

Therefore, since no study was carried out on the plant's sickling reversal and polymerization inhibition effect from the sampling location. Thus, this research was aimed at screening the methanol crude leaf extract of *Terminalia catappa* for phytoconstituents (qualitatively and quantitatively), evaluate its toxicities (orally and intraperitoneally) and pharmacologically determine its sickling inhibition and reversal effect as well as it UV polymerization inhibition potential using *in-vitro* model**.**

MATERIALS AND METHODS

Plant Collection

The plant sample was collected at Abbaganaram Housing Estate Area in Maiduguri, Borno State, Nigeria. The sample collected was identified by a Taxonomist at the Department of Biological Sciences, University of Maiduguri, Nigeria, and a voucher number 09903C assigned to it.

Sample Preparation and Extraction

The collected leaf sample was air dried under shade and ground into coarse powder using wooden mortar and pestle. The powdered plant sample was weighed and extracted with 80% methanol using maceration technique. In order to avoid the degradation of the thermolabile compounds the extract was dried at low temperature [14]. The concentrated crude methanol leaf extract was subjected to qualitative phytochemical evaluation, polymerization inhibition, *in-vitro* sickling reversal and inhibition assays.

Phytochemical Evaluation

Preliminary phytochemical screening was carried out using standard procedures as described by Harbone, [15]; Trease and Evans, [16]; Sofowora [17] Bruneton, [18]; Silva *et al.* [20] for the presence of alkaloids, flavonoids, anthraquinones, saponins, tannins and cardiac glycosides.

Quantitative Phytochemical Evaluation

Total Tannins

The tannic acid content of the sample was determined calorimetrically by the method of Price and Butler [21]. The Absorbance was measured at 720 nm within 10min and the concentration was calculated in mg/cm^3 using the formula below:

$$\text{Tannins} = \frac{\text{Absorbance of sample x concentration of standard}}{\text{Absorbance of standard}} \times 100 \quad (1)$$

Total Saponin Content

Saponin content was determined according to the method of Birk *et al.* [20], as modified by Hudson and El-Difrawi [22]. The percentage saponin was calculated using the formula:

$$\% \text{ Saponins} = \frac{W3 - W2}{W1} \times 100 \quad (2)$$

Where,

W_3 = weight of beaker and residue after evaporation to dryness

W_2 = weight of beaker alone

W_1 = weight of sample

Total Phenolics Content

The total phenolic content of the extract was determined by adopting the method as described by Wolfe *et al.* [23].

Total Flavonoid

Flavonoid content was determined by the method of the Association of Official Analytical Chemists [24]. Percentage flavonoids was calculated using Equation 3 below:

$$\% \text{ flavonoids} = \frac{W3 - W2}{W1} \times 100 \quad (3)$$

Where,

W_3 = weight of beaker and flavonoids

W_2 = weight of beaker alone W_1 = weight of sample

100 = scaling factor to convert to percentage

Acute Toxicity Study

A total of 24 rats with body weights of 100-125 g were used for this study and the analysis were carried out using the methods of Lorke's [25]. The extract was administered using two routes of administration (orally and intraperitoneally) in two phases. The animals were monitored for 24 hours and later 14 days for mortality and general behaviour changes. The medium lethal dose (LD_{50}) was calculated using the formular below:

$$LD_{50} = \sqrt{(a^2 + b^2)} \quad (4)$$

Where a = least dose that kills a rat while b= highest dose that does not kill any rat.

Anti-Sickling Assay

Sample Collection (Blood)

The blood samples used in this research 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 Nurain *et al.* [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 procedure by Pauline *et al.*[27]. The percentage sickled cells were calculated using the following formula:

$$\text{Percentage sickling (\%)} = \left(\frac{\text{number of sickled cells}}{\text{the total number of counted cells}}\right) \times 100 \quad (5)$$

Sickling Inhibition Test

The potentials of the extract to inhibits the sickling of the red blood cells in accordance with the method previously described by Pauline *et al.* [27]. 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].

Polymerization Inhibition Test

The polymerization inhibition test was carried out following the method of Nwaoguikpe *et al*., [29]. This procedure involves the measurement of the turbidity of the polymerizing solution of RBCs at a wavelength of 700 nm at 26 ^{0}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 method of Nurain *et al.* [26] as presented:

$$\text{Rate of polymerization (RP)} = \left[\frac{(\text{final absorbance} - \text{initial absorbance})}{30}\right] \times 100 \quad (6)$$

RESULTS AND DISCUSSION

Table 1 shows the result of the qualitative phytochemical evaluation of crude methanol leaf of *Terminalia catappa* Linn screened using standards methods, the result revealed the presence of flavonoids, phenols, terpenoids, tannins and saponins.

Table 1: Phytochemical Constituents of Extracts of Crude Methanol Leaf *Terminalia catappa* Linn.

PHYTOCHEMICAL CONSTITUENTS	EXTRACT
	Methanol Crude Extract of *Terminalia catappa*
Alkaloids	-
Cardiac glycosides	+
Flavonoids	+
Tannins	+
Terpenoids	+
Phlobatannins	-
Saponins	+

Key: (+) = Present and (-) = Absent

Table 2 shows the results of the quantitative phytochemical evaluation of crude methanol leaf extract of *Terminalia catappa*, the results revealed the following composition (%) of metabolites: flavonoids, 11.80; phenols, 0.85; tannins, 6.00 and saponins, 3.60 as presented.

Table 2: Quantitative Phytochemical Contents of Crude Methanol Leaf Extract of *T. catappa*

Total Phyto-Constituents	Quantity
Flavonoids (%)	11.80
Phenol (%)	0.85
Tannins (mg/cm^3)	6.00
Saponins (%)	3.60

The result of the *intraperitoneal* (ip) administration of the crude methanol leaf extracts of *Terminalia catappa* conducted in two phases revealed that the extract has *intraperitoneal* (ip) LD_{50} of 774 mg/kg bd.wt.

Table 3: Median Lethal Dose (LD_{50}) of Methanol Leaf Extract of *Terminalia catappa* (*ip*)

PHASE I

Group	Dose (Mg/Kg)	Number of Rats	Clinical Sign	Mortality
A	10	3	None	0/3
B	100	3	None	0/3
C	1000	3	None	1/3

PHASE II

Group	Dose (Mg/Kg)	Number of Rats	Clinical Sign	Mortality
D	600	1	None	0/1
E	1000	1	Weakness	1/1
F	1600	1	Weakness	1/1
G	2900	1	Weakness	1/1

Intraperitoneal $LD_{50} = \sqrt{(a*b)} \Longrightarrow \sqrt{(600 * 1000)} = \sqrt{(600{,}000)}$

$LD_{50} = 774$ mg/kg

The result of the oral (po) toxicity evaluation of the methanol crude leaf extract of *Terminalia catappa* conducted in two phases revealed that the oral administration of the crude extract is safe up to the dose of 5000 mg/kg bd.wt as presented in Table 4.

Table 4: Median Lethal Dose (LD_{50}) of Methanol Leaf Extract of *T. Catappa* (*po*)

PHASE I

Group	Dose (Mg/Kg)	Number of Rats	Clinical Sign	Mortality
A	10	3	None	0/3
B	100	3	None	0/3
C	1000	3	None	0/3

PHASE II

Group	Dose (Mg/Kg)	Number of Rats	Clinical Sign	Mortality
D	1600	1	None	0/1
E	2900	1	None	0/1
F	5000	1	None	0/1

No mortality was recorded up to the dose of 5000 mg/kg

The result of the mean comparison of crude methanol leaf extract of *Terminalia catappa* revealed that the extract revealed a significant inhibition against the sickling of the HbSS RBC in an *in-vitro* model as presented in Table 5.

Table 5: Mean Comparison of Crude Methanol Leaf Extracts of *Terminalia catappa* as Sickling Inhibition Agents

Concentration	Mean Percentage Sickled (Mean±SEM)	Percentage Inhibition (%)
12.5 mg/ml	5.09±1.20[a]	92.83
25 mg/ml	10.11±2.86[a]	86.24
Control (N.S 0. 5ml)	70.48±5.01[b]	0

Key: Values along same column differently superscripted differ significantly (P<0.05)

The result of the sickling reversal effect of the extract revealed that the crude methanol leaf extract of *Terminalia catappa* has significantly reversed the sickled HbSS RBC up to 89% in an *in-vitro* model as presented in Table 6.

Table 6: Mean Comparison of Crude Methanol Leaf Extract of *Terminalia catappa* as Sickling Reversal Agents

Concentration	Mean Percentage Sickled (Mean±SEM)	Percentage Reversal (%)
12.5 mg/ml	7.86±1.76[a]	89.40
25 mg/ml	10.30±1.91[a]	85.72
Control (N.S 0. 5ml)	71.49±4.01[b]	0

Key: Values along same column differently superscripted differ significantly (P<0.05)

The result of the UV polymerization inhibition study of the crude extract was evaluated and presented in Table 7 below. The result highlights the ability of the crude methanol leaf extract of *Terminalia catappa* to serve as an agent capable of preventing the process that is leading to the sickling of the HbSS RBC.

Table 7: The Results of the Polymerisation Inhibition Potential of Crude Methanol Leaf Extract of *Terminalia catappa*

Concentration	ROP	RPP	RPI
12.5 mg/ml	0.10	2.72	97.28
25 mg/ml	0.18	4.90	95.10
Normal Saline (Control) (N.S 0.5ml)	3.67	100	0.00

Key: ROP= Rate of Polymerisation = (ΔOD/Time)*100, RPP= Relative Percentage Polymerisation = (ROP of Test/ROP of Control) *100 RPI= Relative Polymerisation Inhibition = (Control-Test/Control) *100

The results of the qualitative study of phytochemical constituents of the crude methanol leaf extract of *Terminalia catappa* (Table 1) gave an insight on the nature of bioactive principle inherent in the plant. The result revealed the presence of some phytoconstituents earlier implicated to be responsible for plants anti-sickling activities. These phytoconstituents include: cardiac glycosides, flavonoids, tannins, terpenoids and saponins but devoid of alkaloids and phlobatannins. This result is similar with the findings of Dan'azumi *et al*., [30]; Wun [12]; Mustapha *et al*. [31] and Terças *et al.* [32]. According to Akakpo-Akue *et al.* [33] and Kiefmann *et al.* [34] flavonoids (quercetin) was reported to provide protection against haemoglobin oxidation and other cellular modifications promoted by peroxides. Likewise, Kplé *et al*. [35] and Akojie and Fung [36] revealed that the anti-sickling effects of *Cajanus cajan, Justicia secunda, Parquetina nigrescens* and *Jatropha gossypiifolia* were as a result of additive and synergistic effects of plants metabolites such as alkaloids, flavonoids, polyphenols, catechin, tannins, sterols and polyterpenes. Thus, the effects manifested by this extract could be due to the possible effects of these phyto-constituents.

Table 2 shows the result of the quantitative phyto-evaluation of some of the metabolites in the crude methanol leaf extract of *Terminalia catappa*. The result revealed that the extract contains the following percentages of: flavonoids (11.0%), phenols (0.85%), tannins (6.0%) and Saponins (3.60%). These percentages of metabolites are capable of eliciting pharmacological response in an *in-vivo* and *in-vitro* model.

The results of the intraperitoneal and oral LD_{50} study of the methanol crude extract of *T. Catappa* as presented in Tables 3 and 4 revealed different toxicity levels, with the LD_{50} values of 774 and 5000 mg/kg bd.wt for intraperitoneal and oral administration respectively. According to Wun [12] the active compounds found in medicinal plants may be toxic in high doses and could even results in mortality at higher concentration. Similarly, Marlinda *et al.,* [37] pharmacological agent (herbs and orthodox drugs) could be toxic such that the body could not tolerate leading to the manifestation of toxic effects. Hence, the mortality observed in the intraperoneal administration of the extract could be as a result of the toxic effect of some of the metabolites present.

According to Fernández *et al.* [38] and Saputri [39], flavonoidal compounds function as depressants, could be used as sedative in animals, hence, if administered in high dose could lead to mortality. Likewise, Wun [12] in his work attributed high flavonoids content as responsible for the depression of the central nervous system and respiratory function which may result in the death of experimental animals due to respiratory failure. Thus, the mortality observed could be as a result of the high flavonoids content in the crude extract. Thus, resulting in respiratory failure by causing gaseous imbalance in the system leading to the death of the animals.

Similarly, saponins were also reported to cause death of experimental animals. This occurs as a result of the acute hypoglycaemic effects of saponins as was reported by Diwan et al. [40]. The crude methanol leaf extract of *Terminalia catappa* contain an appreciable percentage of saponins and could consequently results in acute hypoglycaemia in the animals, hence, resulting in the mortality of the experimental animals. Likewise the route of administration of the crude extract could also be attributed to be responsible for the high toxicity observed. This is in line with the findings of Al-Shoyaib *et al.* [41] that interperitoneal administration is the fastest and more complete absorption route as compared to oral routes. According to Turner *et al,* [42] (*ip)* administration is a critical determinant of the pharmacokinetics, pharmacodynamics as well as toxicity of pharmacological agents.

The extract may have directly gone into the peritoneal cavity which is an excellent portal of entry into systemic circulation for substances and the vast blood supply facilitate rapid absorption of metabolites after *ip* administration as described by Al-Shoyaib *et al.* [41] and Kuzlan *et al.* [43] respectively. However, going by the classification of Walum [44] (LD_{50}>500 ≤ 2000 mg/kg) is not toxic, the crude methanol leaf extract of *Terminalia catappa* is considered not harmful and safe on this basis, since both its (ip) and (po) LD_{50} are greater than 500 mg/kg.

The mean comparison of crude methanol leaf extract of *Terminalia catappa* revealed that the extract have significantly inhibited the sickling of the HbSS RBC in an *in-vitro* model (Table 5). The *Terminalia catappa* crude extact acted in non-dose dependent manner, with the lowest percentage inhibition of 86.24% at 12.5 mg/ml and the highest percentage inhibition of 92.83% at 12.5 mg/ml respectively. Likewise, the mean comparison of the crude methanol leaf extract of *Terminalia catappa* revealed that the extract have significantly reversed the Sickling of HbSS RBC in non-dose dependent manner as presented in Table 6. The lowest percentage reversal of 85.72% and highest percentage reversal of 89.40% were observed. Similarly, the UV polymerization inhibition analyses of HbSS RBC of crude methanol leaf extracts of *Terminalia catappa* revealed the extract have extensively prevented the polymerization of HbSS RBC in non-dose dependent manner with the lowest polymerisation inhibition of 95.10% at 25 mg/ml and the highest polymerisation inhibition of 97.28% at 12.5 mg/ml respectively. Accordingly, Fulata *et al.* [44] reported that for any herbal or orthodox drug to qualify as anti-sickling drug it must have ability to stall the process leading to polymerization of the abnormal haemoglobin, and further enhances the oxygen affinity of the haemoglobin molecule. In line with this finding, Cyril-Olutayo *et al.* [28] reported that the efficacy of plants extract in inhibiting the sickling of red blood cells under stress and suggested that the plants contain some bioactive compounds capable of preventing oxidative stress. Furthermore, the efficacy of the crude extract could be ascribed to its constituent's ability to interact with RBC membrane or the amino acids that are involved in the polymerization process as suggested by Nurain *et al.* [26] and Oyewole *et al.* [46]. Thus, the extract may have acted via one of the two mechanisms.

CONCLUSION

Based on the findings of this research, it can be concluded that, the methanol leaf extract of *Terminalia catappa* Linn contained sufficient quantity of vital phyto-constituents capable of mitigating the sickling of HbSS blood in an *in-vitro* model, and the leaf extracts is considered safe for oral administration but caution should be observed when administering it intraperitoneally.

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Conflict of Interest

The authors declared no competing interests exist.

Authors Contribution

All authors contributed to the study. AMF, AD and AB acquired data. AMF, MAT and HU prepared the draft. HU, AD, MAT, AB, KBI and MG revised the manuscript critically for important intellectual content and AMF submitted it. All read and confirmed the article ready for publication.

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