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Phytochemical and Anti-sickling Activities of *Terminalia catappa* Linn.

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Abstract

An ethnobotanical survey revealed that the dried fallen leaves of *Terminalia catappa* Linn. are used locally in various parts of Nigeria for the management of sickle cell anaemia. This research investigated if *Terminalia catappa* leaf interferes with the basic mechanism of erythrocyte sickling. Powdered dried fallen leaves of Terminalia catappa was extracted with 90% methanol by maceration. Bioactivity of the extract was studied by investigating its effect on inhibition of erythrocyte sickling (membrane effect).

Bioactivity guided fractionation showed that the highest antisickling activity in the erythrocyte sickling model resided in its triterpenoids components (fraction F3, $K = 0.0037\ min^{-1}$). This fraction drastically reduced the rate of erythrocyte sickling and is very significantly more active than the control ($P<0.001$). Bioactivity guided separation of the extract was carried out on flash chromatography using RP18 and Silica gel stationary phases respectively. Final separation was done by HPLC on Cyanosilica column. Structural elucidation of bioactive compounds was carried out by Nuclear magnetic resonance (NMR) and mass spectrometry (MS). The most active compounds were established to be two triterpenic acids (1) 2α, 3β ,24 –Trihydroxyurs –12 – en – 28 – oic acid and (2) 2α, 3β, 24 – trihydroxy – 12, 20 (30) – ursandien – 28 – oic acid. This work therefore shows that there is a scientific basis for the application of this extract in the management of sickle cell anaemia in traditional medicine.

Keywords: Terminalia catappa, Sickle cell anaemia , 2α, 3β, 24–trihydroxyurs – 12 – en – 28 – oic acid and (2) 2α, 3β, 24 – trihydroxy – 12, 20 (30) – ursandien – 28 – oic acid.

Introduction

Sickle cell diseases are defined as conditions resulting from the inheritance of two abnormal allelemorphic genes controlling the formation of β chains of haemoglobin, at least one of which is the sickling gene (Boyo, 1979). Sickle cell diseases therefore include Hb-SS, Hb- SC, Hb S/β thalassaemias and other doubly heterozygous conditions. Research works on the development of specific therapy for sickle cell anaemia that goes beyond supportive measures such as analgesics, antibiotics, blood transfusion, etc, (Abraham *et al*, 1991; Akarya *et al*, 1995; Berkowitz *et al,* 1982) involve strategy that interferes with the basic mechanism of erythrocyte sickling (Charache, 1997; Gamaniel *et al*, 2000, Iyamu *et al*, 2002). Such approaches can be divided to three major classes (1) The haemoglobin modifiers (2) The membrane modifiers and (3) The genetic modifiers (Charache, 1997).

Some medicinal plants previously studied for antisickling properties include the following: *Zanthoxylum zanthoxyloides* (Sofowora *et al* 1979; Ekong *et al* 1975), *Cajanus cajan* (Ekeke &

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Shode, 1985), NIPRISAN®, an ethanol/water extract which consists of *Sorghum bicolor, Ptereocarpus osun*, *Eugenia caryophyllus* and *Piper guinense* (Wambebe *et al* 1998; Wambebe *et al,* 2001) and *Terminalia catappa* (Ngbeneme, 1999).

Previous work on *Terminalia catappa* (Combretaceae) showed it contained flavonoids, terpenoids and tannins (Andrew *et al*, 1990; Ant & Rastogi, 1979; Chen *et al*, 2000). Fan *et al* (2004) found that two triterpenoids were responsible for its antimflamatory activities. It is widely used in Nigeria as an alcoholic decoction for the management of sickle cell anaemia. A local concoction of seven different plants that had *Terminalia catappa* as one of its components was recently reported to have *in vitro* antisickling activities (Egunyomi *et al,* 2009). Our current study was to investigate if the dried fallen leaves of *Terminalia catappa* interfere with basic mechanism of erythrocyte sickling by studying its effect on whole erythrocyte sickling under low oxygen tension, isolate and characterise the bioactive compounds.

Materials and Methods

Equipment
High performance liquid chromatography (HPLC Hitachi L 6200) fitted with UV detector (Jasco UVDEC 100V), preparative HPLC column Dynamax 60A CN (25 x 1 cm), NMR, (Varian XL 400 (400MHz), Mass Spectrometer (MS KRATOS 80 Systems.

Extraction and isolation
The dried fallen leaves of *Terminalia catappa* (Combretaceae) was collected from the University of Ibadan and was authenticated by NIPRD taxonomist; Herbarium specimen of the plant was deposited at NIPRD herbarium with the voucher number NIPRD H 5687. 500g of the dried leaves was pulverized and extracted into 90% methanol in water with intermittent warming on water bath over a period of 48 hours. The extract was concentrated by means of a rotary evaporator and weighed.

The methanolic extract (20g) was adsorbed on RP- 18 Lichrosorb gel (1000g). The system was eluted with a gradient of water, methanol and acetone (100 % water $\rightarrow$ 100 % methanol, followed by acetone) on RP-18 Lichrosorb gel. Six major fractions were collected (TC1 - TC6) by combining related fractions using TLC fingerprints. The fractions were screened for antisickling activity as described below. The most active fraction (TC4) was further eluted with silica gel stationary phase with a gradient solvent system of hexane (100 %) $\rightarrow$ ethyl acetate (100 %). Five fractions (F1 - F5) were collected based on similarities from TLC fingerprint. The most active fraction (F3) was finally eluted by HPLC technique. HPLC was performed on a Dynamax – 60A Cyanosilica column monitored on an absorbance detector at 215nm, flow rate at 3 ml/min and a mobile system of 0 – 15 minutes [(80%) hexane (20%) isopropyl alcohol] and 15 – 25 minutes [(50% hexane: 50% isopropyl alcohol)].

Two compounds were separated as F3A (24mg) and F3B (20mg). ^{1}HNMR, 2D NMR and MS data of F3A and F3B were obtained.

Bioassay technique – erythrocyte sickling model
Blood (2 ml) was collected from a sickle cell patient through the vein into EDTA bottle. The blood sample was centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the packed red cell re-dispersed in phosphate buffered saline (PBS) and centrifuged again at 1500 rpm for 10 minutes. This washing procedure was repeated the third time after which the cells were re-suspended in PBS and made up to 10 ml (Ekeke & Shode, 1985). This serves as the stock of red blood cells (erythrocytes).

Erythrocyte in PBS (0.5ml) was mixed with 0.5ml of phosphate buffered saline. The mixture was incubated for 30 minutes at 37 °C in a thermostated water bath. 0.5ml of 2% sodium metabisulphite solution was then added to the mixture for induction of erythrocyte sickling. The percentage of sickled (elongated crescent shaped sickled cell) was monitored by means of microscopical examination at the following time intervals: 0, 10, 20 and 30 minutes. The sample was returned to the thermostated bath immediately after each sampling. The percentage sickled and normal cells were determined by counting 500 cells at each time assessment. Reoxygenation was prevented by use of tight seal glass containers and the application of jelly on the edges of the cover slips immediately before they are used. (The blood used for this study was taken from patients confirmed to be homozygous SS haemoglobin with the patient's signed consent). This served as the control sickling test.

Test of fractions

The same process was repeated for the various samples tested. The only difference was that 0.5ml of PBS in control experiment was replaced by 0.5 ml extract solution in PBS. Fractions tested were: 1). RP-18 fractions (TC1 - TC6), 2). Silica gel fraction (F1 - F5).

Modelling of erythrocyte sickling

Erythrocyte sickling (aggregation of deoxy Hb S) was modelled mathematically as a one phase exponential association (first-order) rate process (Graphpad software, 2004). The rate constants (K) of erythrocyte sickling when incubated with *Terminalia catappa* extracts and in the control experiment were respectively determined by using the (global) one phase exponential association model of non-linear regression curve fitting. Best-fit value of rate constant was then computed for each of the fraction investigated, within 95% confidence limits.

Results

From Reversed phased column fractions, TC4 was shown to be the most active fraction as shown in figure 1. Silica gel fractionation of TC4 gave 5 major fractions (F1-F5). The fractions were tested on the erythrocyte sickling bioassay model and showed varied antisickling activity (Fig 2). From the result, it was shown that F3 is the most active fraction but for a precise quantitative and comparative evaluation of the activity of the fractions to be carried out, there is the need to carry out a mathematical modelling of the sickling process. Hence the result from this experiment was transformed by curve fitting the data to a one phase exponential association model (Fig. 2). This mathematical model is considered most appropriate because the sickling process is truly an association of monomers to form a polymer. The constraint employed in the curve fitting analysis was dictated by the context of the experiment, in which the maximum sickling obtained in the control experiment plateau at 95% sickling. Secondly, the rate constant was constrained to be a positive value (>0) because a negative value will be meaningless in the context of the experiment.

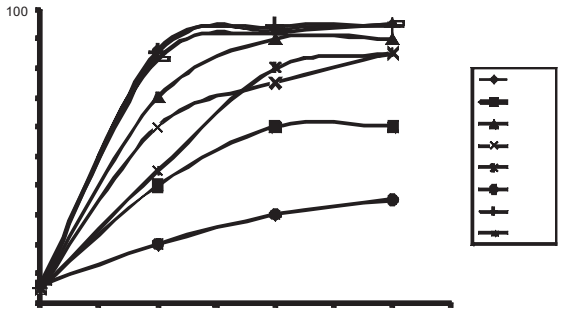


Figure 1: Effect of reversed phase fractions of *T. catappa* on erythrocyte sickling

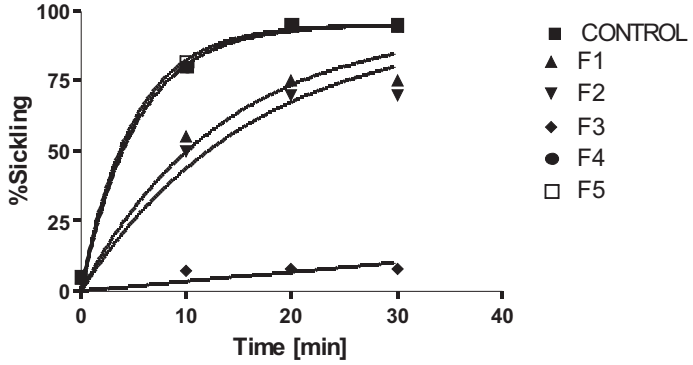


Figure 2: Kinetics of sickling of erythrocytes in the presence of chromatographic fractions of *T. catappa* (Tc4) using the one phase exponential association curve-fitting model.

The results showed that fraction F3 with best-fit K value of 0.0037 min^{-1} (Fig. 3) has the lowest rate constant and hence the most active fraction. Fractions F1 and F2 with rate constants less than half the value obtained for the control experiment also showed reasonable activity (Table 1).

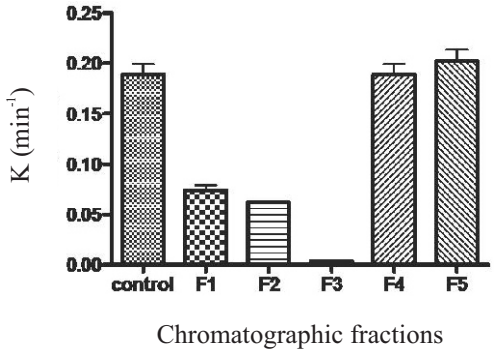


Figure 3: K (min^{-1}) is the antisickling rate constant which is the parameter of assessment of antisickling effects of various fractions of *Terminalia catappa*.

Table 1: Rate constant of erythrocyte sickling kinetics in the presence of chromatographic fractions

Fractions (1 mg/ml)	Best fit value	95 % CI	Rate constant K*(min^{-1}) Std Error	R^2
Control	0.1892	0.1677 -0.2106	0.01016	0.9946
F1	0.0743	0.0639 -0.0847	0.0049	0.9543
F2	0.0620	0.0532 -0.0707	0.00042	0.9387
F3	0.0037	0.0029 -0.0048	0.00049	-6.168
F4	0.1892	0.1677 -0.2106	0.01016	0.9946
F5	0.2024	0.1787 -0.2261	0.01124	0.9950

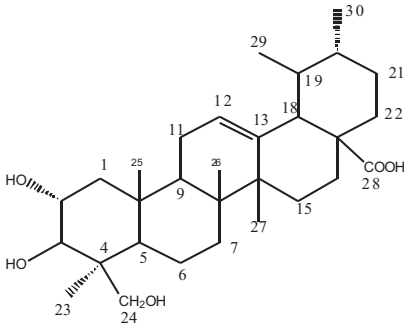
*Curve fitting was performed by a global model of one phase exponential association non-linear regression analysis with the following constraints and statistics:

Constraints
Y_{max} is shared and set at >95%, K>0.0
Statistics
Best fit value ±S.E (S.E. =Standard Error)
Ymax = 95± 2.47%, 95% CI = (95-100.2%),
Goodness of fit
Degrees of freedom = 17, Absolute sum of squares = 453.9, Sy.x=5.167, R^2 = 0.9868

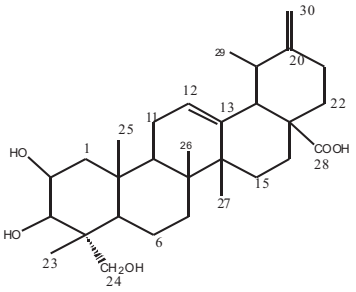
Spectroscopic analysis of F3A and F3B
From mass spectroscopic data, Compound F3A showed a molecular ion of 488.4 which is consistent with the formula $C_{30}H_{48}0_5$ (488). It also showed a base peak with a m/z of 248. This is characteristic of the 12-en-28-oic acid (ursenes). Typical Retro – diels Alder fragmentation leading to the base peak of 248 is a characteristic diagnostic tool for the presence of 12 – 13 double bond in triterpenoids of the α and β amyrin class such as ursenes (Ogunkoya, 1981). HMQC data from NMR showed the olefinic proton at H-12 with a chemical shift of 5.4 and its corresponding carbon (C-12) with a chemical shift of 126.8ppm.

Compound F3B showed a Molecular mass (M^+) at m/z of 486.4 while the base peak is 246.2 .The molecular mass of 486.4 is consistent with the formula $C_{30}H_{46}O_5$ (486). The only difference in the spectra of F3A and F3B was the mass units difference of two in their mass. This is because there is a double bond at 20 (30) – ene position in F3B. This is supported by the base peak of m/z 246.2 instead of 248.4 in F3A.

Spectroscopic data obtained for compounds F3A and F3B agree with previously reported data for (1) 2 α, 3 β, 24 – Trihydroxyurs – 12 – en – 28 – oic acid(F3A) and (2) 2α, 3β, 24 – trihydroxy – 12,20(30) – ursandien – 28 – oic acid(F3B) (Budzikiewicz *et al*, 1963; Doddrell *et al*, 1974).



F3A



F3B

Conclusion
The search for antisickling agents is either through synthesis or isolation of bioactive compounds from medicinal plants. The antisickling property of the extract of *T. catappa* was found in this work to be due mainly to its triterpenoid acid derivatives namely :i) 2α, 3β, 24 – trihydroxy urs–12 – en – 28 – oic acid and ii) 2α, 3β, 24 – trihydroxy – 12,20(30) – ursandien – 28 – oic acid.

Further investigations proposed will involve the isolation of these two triterpenic acids in large quantity to carry out detailed comparative studies of their bioactivity, their effects on transgenic mice models, as well as detailed chronic and acute toxicity studies. It will also be worthwhile to carry out possible structural modifications which could afford the synthesis of new compounds with higher

activities and less toxicity. Overall, this work has provided a scientific justification for the use of *T. catappa* in the management of sickle cell disease.

Acknowledgments

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