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In vitro antisickling activities of two indigenous plant recipes in Ibadan, Nigeria

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Abstract

In view of the prevalence sickle cell disease in Nigeria, the use of herbs for treatment of diseases due to high poverty level and inadequate distribution of health care centres in the country, two indigenous recipes consisting of six ethnobotanicals used in the management of sickle cell anaemia in Nigeria were screened for antisickling activity. Recipe 1 constituents were *Detarium microcarpum* (Guill. and Perr.), *Harungana madagascariensis* (Lam. ex Poir), *Sorghum bicolor* (Linn.), *Tetracera potatoria* (Afzel. ex G. Don) and *Theobroma cacao* (Linn.). Recipe 2 was a monorecipe of *Phyllanthus amarus* (Schum and Thonn). Clinical blood samples of confirmed non-crisis sickle cell patients (male and female) were collected from the Department of Haematology, University College Hospital (UCH), Ibadan, Nigeria. Extracts of the recipes were prepared in 80% ethanol using cold extraction method. Sickling of HbSS red blood cells was induced using sodium metabisulphite. *In-vitro* antisickling activities of the crude extracts of the recipes were evaluated using p-hydroxybenzoic acid and normal saline as positive and negative control respectively. Readings were taken at different incubation time (0 – 180 min). The two recipes showed varied antisickling activities. At 90 min incubation period, Recipe 1 showed maximum percentage inhibition of 71.6 ± 1.21 while Recipe 2 showed 60.0±0.51 percentage inhibition. The inhibitory activity of Recipe 1 at 90 min incubation period was higher than that of the positive control (61.8 ± 2.28) and there was no significant difference ($P < 0.5$) in the inhibitory activity of Recipe 2 and the positive control. All plant constituents of the two recipes contained saponins and tannins (except *Detarium microcarpum*). The two indigenous recipes significantly demonstrated antisickling activity. The results of this study validate the use of the two recipes by indigenous people for the treatment of sickle cell disease. The isolation and identification of active phytochemicals responsible for the antisickling activity of Recipe 2 could lead to the discovery of novel drugs

Keywords: Sickle cell diseases, indigenous recipes, antisickling activity, Phyllanthus amarus, Harungana madagascariensis, Tetracera potatoria, Nigeria.

Introduction

Sickle cell anaemia is an inherited blood disease in which the red blood cells contain haemoglobin S, an abnormal type of haemoglobin. This crystallizes in the capillaries, making red cells sickled-shaped and fragile, and leading to haemolytic anaemia. The abnormal cells are unable to pass easily through tiny blood vessels. The blood supply to organs is blocked intermittently causing sickle cell crises [1]. The effects of sickle cell crises on different parts of the body can cause a number of complications such as hand-foot syndrome; splenic crisis; infections; acute chest syndrome; pulmonary arterial hypertension; delay in growth and puberty in children; stroke; eye problem; priapism (painful and unwanted erections); gallstones; ulcers on the leg and multiple organ failure involving the failure of two out of three major organs (lungs, liver, or kidney) [2].

The World Health Organization reported that each year about 300,000 infants are born with major haemoglobin disorders including more than 200,000 cases of sickle-cell anaemia in Africa. Globally, the high frequency of the sickle-cell gene in certain areas leads to a high rate of affected babies. The sickle-cell gene has become common in Africa because the sickle-cell trait confers some resistance to falciparum malaria during a critical period of early childhood, favouring survival of the host and subsequent transmission of the abnormal haemoglobin gene. In West African countries such as Ghana and Nigeria, the frequency of the sickle cell trait is 15 - 30% [3].

Orthodox mode of treatment of sickle cell anaemia focuses on the ways of inhibiting sickle cell haemoglobin polymerization, prevention or repair of red cell dehydration and interrupting the interaction of sickle cells with the endothelium [4]. The treatment of sickle cell anaemia using orthodox approach has proved difficult and inefficient due to the genetic origin of the disease. The side

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effects of orthodox mode of treatment of sickle cell anaemia with chemo therapeutic agents such steroids have been reported [5].

The use of natural products in treatment and management of sickle cell disease could be as old as the disease, though very few ethnobotanical remedies for its treatment have been reported in the literature due to secrecy attached to the indigenous knowledge of treatments of the disease [6]. However the efficacy of some ethnobotanical remedies in the management of sickle cell disease has been reported. The safety and efficacy of NIPRISAN (a phytomedicine) for the management of patients with Sickle Cell Disorder (SCD) was reported by Wambebe *et al.* [7]. Divanilloylquinic acids isolated from root bark of *Zanthoxylum zanthoxyloides* have antisickling properties [8,9] reported the antisickling potential of *Terminalia catappa* leaves. Adejumo *et al.*,[10] also reported the *in vitro* antisickling activities of crude methanol extracts and aqueous fractions of roots of *Plumbago zeylanica* and *Uvaria chamae*.

Recipe 1 constituents were *Detarium* microcarpum (bark), *Harungana madagascariensis* (bark), *Sorghum bicolor* (leaf), *Tetracera potatoria* (bark), and *Theobroma cacao* (bark). The leaves, stems and root barks of *Detarium microcarpum*, as well as the fruits have found tremendous usage in treatment of various ailments such as tuberculosis, meningitis, itching and diarrhoea. The fruit is edible and rich in vitamin C and the leaves and seeds are also used in cooking [11]. Traditionally, the leaves and stem bark of *Harungana madagascariensis* are used for the treatment of anaemia. The stem bark is also used for nephrosis, malaria, gastro-intestinal disorders and fever [12-14]. *Sorghum bicolor* is reported to be anti-abortive, cyanogenic, demulcent, diuretic and emollient. It is a folk remedy for cancer, epilepsy, flux, and stomach ache [15]. The leaves of *Tetracera potatoria* boiled in its own sap are used for the treatment of gastrointestinal sores [16,17] also reported the antiulcer activity of the methanolic extract of *Tetracera potatoria* roots. *Theobroma cacao* has antiseptic, diuretic, parasiticidal, vulnerary and emmenagogue properties (www.ntbg.org/plants). Recipe 2 is made up of *Phyllanthus amarus* (whole plant) only. In indigenous medicine, *Phyllanthus amarus* is used in the treatment of skin diseases and stomach disorders [18]. It has anti-diarrhea activity [19]. Its anti-viral activity against hepatitis B has been reported [20]. Furthermore, it is used for its antihypertensive, antidiabetic, hepatoprotective, analgesic, antimicrobial and anti-inflammatory properties [21,22].

In view of the fact that Sickle Cell Disease is common in Africa and 80% of Nigerians depend on herbal remedies for the treatment of ailments and diseases due to inadequate distribution of health care centres, this study screened the two indigenous recipes for antisickling activity to ascertain the claim of the indigenous people, provide scientific insight into their efficacy as antisickling herbs and increase the number of candidates of antisickling phytomedicines.

Materials and methods

Identification and preparation of plant materials

Freshly harvested plant parts of *Detarium microcarpum* (bark), *Harungana madagascariensis* (bark), *Theobroma cacao* (bark), *Sorghum bicolor* (leaves), *Tetracera potatoria* (bark) and whole plant of *Phyllanthus amarus* were purchased from a local herbal market in Ibadan, Oyo State, Nigeria. The plants were identified at species level and deposited in the University of Ibadan Herbarium (UIH), Nigeria. The plant samples were washed, cut into small pieces and dried separately at room temperature for three weeks until completely dried. Samples were powdered and stored in air tight glass containers at 4^{o}C for further use.

Extraction of powdered plant samples

Powdered plant components of each recipe were separately measured and mixed thoroughly in a beaker. 150g was poured into a conical flask and macerated in 1000 ml of 80% ethanol for one week with intermittent stirring using a spatula. The conical flasks were covered with foil paper. After one week, the extracts were filtered using Whatman no.1 filter paper and concentrated to dryness using rotary evaporator at 40^{o}C. The extracts were dispensed into labeled sample vials and stored at 4^{o}C for subsequent use.

Blood samples

Blood samples of non-crisis sickle cell anaemia patients were obtained from the Department of Haematology, University College Hospital (UCH), Ibadan (through due process). The 8 subjects were healthy adults of both sexes aged between 20 – 28 yrs. 5ml of blood was obtained by vein puncture from each patient into sodium EDTA bottles and the contents were thoroughly mixed by gently rolling the bottle. Fresh blood was used for experiments.

In-vitro antisickling activities of extracts of the two recipes

The *in-vitro* antisickling activity of the extracts was evaluated using a modified method of Sofowora *et al.* [23]. The fresh blood samples were centrifuged to remove the serum. The resulting packed erythrocytes were washed with 1ml sterile normal saline per 5ml of blood and immediately centrifuged for 5min at a speed of 2000 revolution per minute to remove the supernatant repeatedly three times. 0.5ml of the washed erythrocytes were mixed each with 0.5ml of the different extracts (mg/ml) in uncovered test tubes and mixed together thoroughly. The mixtures were incubated at 37^{o}C for 3 hrs while shaking occasionally. 0.2 ml of 2% sodium metabisulphite were added to deoxygenate the mixture, mixed thoroughly and sealed with liquid paraffin. Samples were taken in duplicates from the mixture below the paraffin level at 0 min, subsequently samples were taken at 45 min interval until 5 readings were obtained. Each sample was smeared on a microscopic slide, fixed with 95% methanol, dried and stained with giemsa stain. Each sample was examined under the oil immersion light microscope and counting at least 500 red blood cells in each from five different fields of view across the slide. The numbers of

both sickled and unsickled red blood cells were counted and the percentage of unsickled cell determined [24]. P-hydroxybenzoic acid (PHBA) a compound known to reverse the sickling in HbSS blood cells was used as reverence standard at a concentration of 5mg/ml while normal saline was used as negative control. The experiment using the blood sample of a particular patient was replicated twice.

Phytochemical screening of plant components of the two herbal recipes

The powdered plant components of recipes 1 and 2 were analysed separately for their phytochemical constituents in the Laboratory of the Department of Pharmacognosy, University of Ibadan following established protocols [23,25].

Alkaloids

The powdered plant sample 1 g each was extracted with 10 ml of 10 % HCl on a water bath for 10 mins. The extract was filtered and the pH of the filtrate adjusted to about 5-7 with ammonia solution. Aliquots of the filtrate were taken each into a test tube and a few drops of Dragendoff's reagents (Potassium bismuth iodide solution) were added and mixed with the filtrate. A precipitate indicated the presence of alkaloids.

Saponins

The powdered sample (2g) was boiled in 20 ml of distilled water in a water bath and filtered. The filtrate (10 ml) was mixed with 5ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously and any formation of an emulsion was observed.

Tannins

The dried powdered sample (0.5g) was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride ($FeCl_3$) were added and formation of a blue-black coloration was observed.

Anthraquinones

The extract solutions 5 ml each was hydrolyzed with diluted concentrated H_2SO_4 extracted with benzene. 1 ml of dilute ammonia (NH_4) was added to it. Rose pink coloration indicated the presence of anthraquinone.

Cardiac glycosides (Keller-Killani test)

The extract (5 ml) was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1ml of concentrated H_2SO_4. A brown ring at the interface indicated a deoxy sugar characteristic of cardenolides.

Cyanogenic glycosides

The powdered sample (0.5g) was moistened with distilled water, and moist sodium picrate paper was suspended at the mouth of the test tube and cork tight, the moistened sample was then placed in water bath for 30 minutes. Emergence of brick red colour on the moist sodium picrate paper shows the presence of cyanogenic glycosides.

Coumarin

The extract of plant samples was dissolved in water; the resulting extracts were now basified with ammonia (NH_3). Observation of blue of green fluorescence in 254nm UV light suggests the presence of coumarin.

Statistical Analysis

Data obtained were expressed as means. The statistical significance of difference was expressed using Analysis of Variance (ANOVA). A one tailed p-value ($P< 0.05$) was considered to be statistically significant.

Results & Discussion

The profile of the test plants is presented in Table 1. The habits of the test plants were 66.6 % tree, 16.7 % shrub and 16.7 % herb. The plant parts used were 16.7 % leaves, 66.6 % barks, and 16.7 % whole plant respectively. The high use-value of barks might be attributed to high bioactive constituents of barks. Table 2 shows how the indigenous people prepare and use the recipes. Table 3 shows the phytochemical components of each plant making up the two recipes. 88.3% of the plants contained tannins and saponins, 66.6 % contained cardiac glycosides and coumarins, 33.3 % contained alkaloids and anthraquinones, and 16.7 % contained cyanogenic glycosides. The phytochemical constituents of the test plants expectedly should contribute to alleviating the multiple health challenges presented in sickle cell disease. Alkaloids have been reported as nerve stimulants [26]. Thus the presence of alkaloids in Recipes 1 and 2 is an indication that they may be useful in alleviating some of the symptoms associated with pains. Anthraquinones act on the gastro-intestinal tract to increase the peristalsis action. Anthranones, anthrones, oxanthrones and dianthrones are all derivatives of anthraquinones [27]. So, the anthraquinones in Recipe 2 may be useful as a mild laxative especially to sickle cell patients who may be constipated. Tannins are phenolics derivatives and are non-nitrogenous plant constituents with astringent properties on mucous membrane [28].

Table 1: Profile of medicinal plants constituents of the two Recipes

Recipe	Botanical Name	Family	Local Name (Yoruba)	Plant Habit	Part used
1	*Detarium microcarpum*	Caesalpinaceae	Ariira	Tree	Barks
	Harungana madagascariensis	Hypericaceae	Amuje	Tree	Barks
	Theobroma cacao	Sterculiaceae	Koko	Tree	Barks
	Sorghum bicolor	Poaceae	Oka Pupa, Poporo	Shrub	Leaves
	Tetracera potatoria	Dilleniaceae	Opon	Tree	Barks
2	*Phyllanthus amarus*	Euphorbiaceae	Eyin olobe	Herb	Whole plant

Table 2: Indigenous Recipes used in the management of sickle cell anaemia

Recipe	Constituents of the Recipes	Herbal combination and dosage	Method of preparation
1	*Detarium microcarpum* bark, *Harungana madagascariensis* bark, *Sorghum bicolor* leaves, *Tetracera potatoria* bark and *Theobroma cacao* bark	The plant parts are washed in clean water, dried and ground into powder and a teaspoonful of the powder is taken as tea with hot water twice daily after meal.	Powder
2	*Phyllanthus amarus (*whole plant)	The fresh plant is washed and ground into paste. The paste is made into small pills and dried. One pill is taken twice daily after meal.	Pills

Table 3: Phytochemical constituents of medicinal plants component of the two Recipes.

Plants	Tannins	Saponins	Cardiac glycosides	Cyanogenic glycosides	Coumarin	Alkaloids	Anthraquinones
Detarium microcarpum	-	+	+	-	+	-	+
Harungana madagascariensis	+	+	+	-	+	-	-
Theobroma cacao	+	+	+	-	+	±	-
Sorghum bicolour	+	±	+	-	-	+	-
Tetracera potatoria	+	+	-	-	-	-	-
Phyllanthus amarus	+	+	-	+	+	+	+

Legend: + = present; ± = Trace; - = absent;

The presence of tannins in Recipes 1 and 2 make it useful in cleansing the surface of the skin ulcers that develop as a result of sickle cell diseases. The presence of cardiac glycosides in Recipe 1 indicates its usefulness in managing cardiac insufficiency, coughs and circulatory problems. Also they may act as good sedatives as they have antispasmodic properties [26].

The antisickling activities of the two recipes are presented in Table 4. The recipes displayed varied degree of antisickling activity compared with the positive control experiment. At 0 min, the %

Table 4: *In-vitro* antisickling activity of extracts of the two indigenous Recipes

Time of incubation (min)	Unsickled red blood cells (%)			
	Normal Saline	PHBA	Recipe 1	Recipe 2
0	53.0 ±0.95 [a*]	50.2 ±2.25 [ab]	50.4 ±0.93 [ab]	45.6 ±0.87 [b]
45	45.0 ±2.59 [a]	56.4 ±1.21 [b]	59.4 ±0.40 [b]	50.8 ±0.97 [c]
90	40.0 ±1.00 [a]	61.8 ±2.28 [b]	71.6 ±1.21 [c]	60.0 ±1.03 [b]
135	37.0 ±1.14 [a]	66.8 ±0.58 [bc]	69.6 ±0.93 [b]	65.4 ±1.54 [c]
180	35.0 ±1.52 [a]	70.2 ±0.58 [b]	60.6 ±0.75 [c]	67.4 ±0.51 [d]

Legend: *Values are means ± standard error
Values in the same column followed by the same letter are not significantly different ($p > 0.05$) from each other. They differ significantly ($p \leq 0.05$) with values that do not share a similar letter.

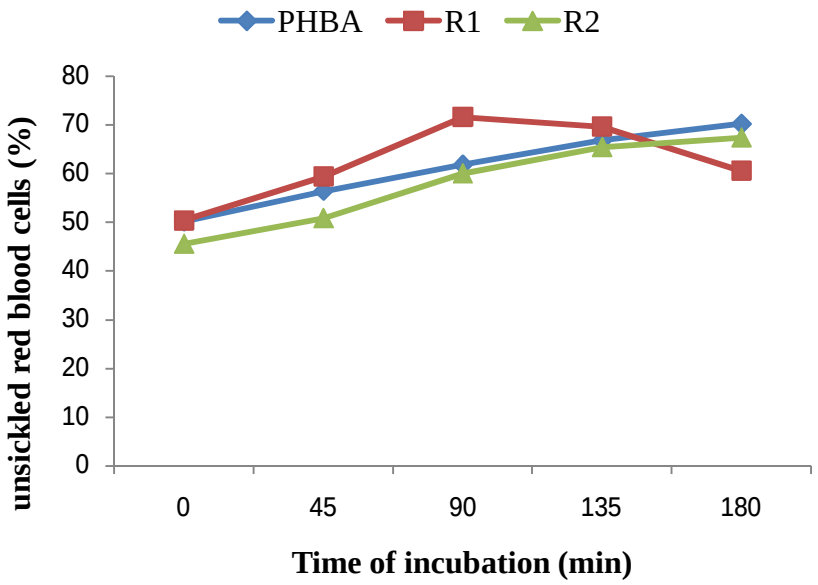

Fig 1: The antisickling effect of the two recipes (R1 and R2) on sodium metabisulphite induced sickled cell with P-hydroxybenzoic acid (PHBA) as positive

unsickled red blood cell of the negative control experiment (normal saline 53.0%) was the highest, there was no significant difference between PHBA (50.2%) and Recipe1 (50.4%) and Recipe 2 gave the least with 45.6%. Subsequently, the % unsickled red blood cell in the negative control experiment decreased with time as expected. In Fig. 1, at 45 min, Recipe 1 (59.4%) gave the highest % unsickled red blood cells; followed by PHBA (56.4%) and Recipe 2 (50.8%) was the least. At 90 min, Recipe 1 (71.6%) gave the highest % of unsickled red blood cells, followed by PHBA (61.8%) and least was Recipe2 (60.1%). At 135 min, Recipe 1 (69.6%) was still the highest, followed by PHBA (66.8%) and Recipe 2 (66.4%) was the least. At the peak of the experiment (180 min), PHBA (70.2%) recorded the highest % unsickled red blood cell, followed by Recipe 2 (67.4%) and the least was Recipe 1 (60. 6%).

Overall the antisickling activity of Recipe 2 increased steadily along the gradient with time whereas Recipe 1 recorded a decrease in antisickling activity at 180 min. Considering the fact that Recipe 1 was made up of 5 plants, the extract of one or more of the plant

constituents of the recipe might have acted on the surface level of the red blood cells to bring about reduction in O_2-tension within the red blood cells hence the observed decrease in the antisickling activity of Recipe 1 at 180 min of incubation. The antisickling effect of Recipe 1 in the present study agrees with the report that the efficacy of an antisickling agent *in-vitro* must act effectively and rapidly, especially in cases of severe crises [28]. However it will be necessary to investigate the sudden drop in its activity at 180 min of incubation. *Sorghum bicolor* and *Detarium microcarpum* are components of Recipe 1. *S. bicolor* has been reported to have antisickling activity [29] and *D. microcarpum* was reported to be part of a traditional recipe used in the management of sickle cell anaemia [28]. As *Harungana madagascariensis*, *Tetracera potatoria*, and *Theobroma cacao* have been reported to be anti-anaemic plants [30], they may be beneficial in the management of sickle cell diseases. Relatively, Recipe 2 showed more significant and stable antisickling activity than Recipe 1 and compared well with the activity of the positive control. In addition to the antisickling potential of *Phyllanthus amarus* recorded in this study, its antimicrobial activity against multiple antibiotic resistant bacteria has been reported [31]. This can help in the management of Sickle Cell Anaemia since people with the disease often have infections due to depressed immune system (especially in children).

Conclusion

Based on the present study, Recipe 2 is a potential antisickling phytomedicine. The isolation and characterization of the active compounds of *Phyllantus amarus* responsible for the observed antisickling activity could lead to the discovery of novel drug.

Author's contributions

Gbadamosi, IT conceived and designed the experiment. She analysed and interpreted the data. She drafted the manuscript and revised it critically for important intellectual content. The funding was collaboration between Gbadamosi, IT and Moody, JO whose laboratory was used for the experiment. Adeyemi, SB and Adeyemi, AA contributed to the screening of the plant samples in the Laboratory and were also involved in the analysis of data. The four authors gave final approval of the version to be published.

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