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Phytochemical, antimicrobial and pharmacological studies on 'siculine' – a herbal remedy for sickle cell anemia

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Abstract

Siculine syrup – a traditionally prepared plant extract used in sickle cell anaemia pain crisis has been certified in a previous study to possess a dose-dependent analgesic and RBC-sickling inhibitory activities. In the present study, the phytochemical and antimicrobial screening as well as effect on food intake, water intake and urinary output was carried out. Standard methods were used to test for the presence of phytochemical metabolites, while the antimicrobial effect was tested on standard organisms' isolates of *Staphylococcus aureus* NCTC 6571, *Escherichia coli* NCTC 10418 and *Pseudomonas aeruginosa* NCTC 6750. Twenty four hourly daily measurements of food intake, water intake and urinary output of rats placed inside metabolic cages was used to assess for changes in these parameters. The results showed that siculine contained cardiac glycosides, flavonoids, terpenes and sterols and it also demonstrated antimicrobial activity against *S. aureus*, but not against *P. aeruginosa* and *E. coli*. Siculine did not affect food intake, but water intake was substantially increased, but not significantly ($P > 0.05$; Student's t-test). However, there was significant diuretic effect due to siculine ($P < 0.05$; Student's t-test).

Keywords: Siculine syrup; Sickle cell anemia; Antimicrobial; Food intake; Water intake; Urinary output

Introduction

Sickle cell anaemia (Hb-SS genotype) is a genetic disease that had been reported to occur in about 3% of the population in Nigeria (Moody *et al*., 2003). It is a life-long problem characterised by chronically reduced RBC oxy-haemoglobin binding affinity that results in formation of RBCs with defective haemoglobins. Such red blood cells are usually fragile and easily deformed into inactive sickle-shaped forms leading to anaemia (March of Dimes, 2006). The principal therapeutic management of sickle cell anaemia has been to attempt to inhibit and/ or reverse the sickling process associated with incessant thrombo-infarctive pain crises among other serious clinical conditions (Konotey-Ahulu, 1991; Sinou, 2003; ASCAA, 2006). However, there is presently no available specific therapy of either the western or the traditional type of medicines that prevents or reverses the *in vivo* sickling process (ASCAA, 2006). The only available orthodox practice of handling sickle cell anaemia conditions had been to provide a short indirect and intermittent sickling-inhibitory effect so as to prolong the life-span of red blood cells using water and / or blood infusions, haematinics, analgesics and antibiotics. The reversal of sickling by the

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ether extract of *Fagara zanthoxyloides* root reported by Sofowora and Isaacs (1971) was only an *in-vitro* effect. Siculine syrup is a traditional herbal preparation with ethnomedical claims and use in traditional remedies for sickle cell pain crisis. In an earlier study, Siculine syrup was found to inhibit the sickling of RBCs and/ or pain crisis, but it did not reverse the already sickled RBCs (Ejiofor *et al*., 2007). It was also found to inhibit the spontaneous contraction of the heart and skeletal muscle and to contract various isolated smooth muscles (Kwanashie *et al*., 2008). The present study is to ascertain the phytochemical constituents, the antimicrobial activities and the effect of siculine on food intake, water intake and urinary output in rats as part of the study to map out the pharmacological profile of this plant extract.

Experimental

Animals. Adult Wistar rats (male and female) were used for the food intake, water intake and urinary output study. The animals were bred locally in the Animal House of the Department of Pharmacology and Clinical Pharmacy, Ahmadu Bello University (ABU) Zaria. Ethical standards were complied with as stipulated in the Guiding Principles in the Use of Animals in Toxicology by the Society of Toxicology (1989).

Microbial organisms. Standard cultures of *Escherichia coli* NCTC 10418, *Staphylo-coccus aureus* NCTC 6571 and *Pseudomonas aeruginosa* NCTC 6750 obtained from the stock of the Department of Pharmaceutical Microbiology, ABU, Zaria, were used.

Drugs and chemicals. Siculine syrup (a herbal drug used by the local populace for sickle cell pain crisis with product information as 'an aqueous-extract of a sicul vegetable') freely commercially available in 2.5 L white plastic bottles in pharmacy shops across the country, was freeze-dried to obtain dilutions of desired working concentrations. The list of drugs and chemicals used include: Ampicillin (Abtek Biologicals Ltd, U.K.); Normal saline (0.9% $^{w}/_{v}$ NaCl, M & B, England; Distilled water (Pharmaceutical laboratory, ABU, Zaria); Nutrient broth (Oxoid Ltd, U.K.); and molten nutrient agar (Oxoid Ltd, U.K.);

Phytochemical screening. Siculine syrup was tested for the presence of sugars, starch, tannins, phlobatannins, glycosides, cardiac glycosides, cardenolide aglycones, terpene, sterol, steroids, flavonoids or alkaloids using standard procedures as described by Sofowora (1993) and Evans (1996).

Antimicrobial activity. The Cup Plate method was used to test for the susceptibilities of *Escherichia coli* NCTC 10418, *Staphylo-coccus aureus* NCTC 6571 and *Pseudomonas aeruginosa* NCTC 6750 to 1% and 5% siculine respectively. Eighteen hour broth cultures of the organisms were diluted to contain 10^6 cells / ml in saline. One millilitre of each diluted culture was mixed with 19 ml molten nutrient agar and poured into Petri-dishes and allowed to set. Three Petri-dishes were prepared for each culture. After setting, wells measuring 0.9 cm in diameter were made with number 4 cork borer in each of the three Petri-dishes. The wells in each of the Petri-dishes were filled with 0.2 ml of siculine (1% and 5%) and ampicillin respectively and allowed to stand for 2 hours after which the Petri-dishes were incubated at 37˚C for 18 hours and the inhibition zones of ampicillin and siculine were measured respectively. The experiment was repeated and the mean inhibition was taken.

Food intake, water intake and urinary output. The observational and activity assessment described by Irwin (1968) was used to ascertain whether siculine causes any changes in food intake, water intake and urinary output that were different from that of control animals. The Food Intake (FI) in g / 100 g rat / 24 h and Water Intake (WI) in ml / 100 g rat

/ 24 h were measured before and during drug pretreatment. Four (4) male and Four (4) female Wistar rats weighing between 300 – 333 g were divided into two (2) treatment-(control and test) groups of 2 rats per group for each sex into four metabolic cages (Techniplast, Italy). The animals were acclimatized to the experimental condition for 7 days during which the pattern of feeding and water supply was introduced. The animals were fed for 2 hours every day outside the metabolic cages with a certain quantity of food and both the amount of food supplied to each group and the amount of scraps not eaten after the two hours of feeding were weighed and noted every day for the 7 days. Water was supplied to the rats *ad libitum* except for the 2 hours of feeding. The volume of water given and the volume left including the time of removing and replacing the water bottles were noted each day. The urinary output (UO) were also measured daily in ml / 100 g rat / 24 hours for each group of the rats. The rats were then pretreated orally daily with 0.5 ml normal saline for control and 0.5 ml siculine respectively from the 8^{th} day for another 7 days. The mean of the food and water intake and the urinary output for the 7 days before drug administration was then compared with that during siculine pretreatment and with the control groups of rats. The differences in those of the male and female rats were also assessed.

Results

Phytochemical screening. The phytochemical tests (Table 1) showed that siculine did not contain free reducing sugars, starch, tannins, phlobatannins, anthraquinones, saponins and alkaloids. On the other hand, there were presence of other constituents such as cardiac glycosides, flavonoids, terpenes and sterols.

Antimicrobial activity. Of the three bacterial organisms tested for inhibition namely *E. coli*, *P. aeruginosa* and *S. aureus*, only *S. aureus* was sensitive to siculine and this was to 5% concentration (Table 2). In contrast, all three organisms were sensitive to 0.002% ampicillin. Thus, siculine had slight antimicrobial activity.

Food intake, water intake and urinary output. There was no statistically significant difference in the food intake or water intake between the male and female rats both before and during siculine administration ($P > 0.05$). There was also very little or no change in food intake that could be ascribed to the drug (Table 3). However, during siculine treatment, water intake increased by 23.5% in the male rats and by 17.6% in the female rats. This increase in water intake during siculine treatment was not statistically significant from the water intake before treatment ($P > 0.0.5$, Student's t-test), but was however substantial. An almost ten-fold increase in urinary output was obtained in both the male and female groups of rats indicating a clear diuretic effect due to siculine.

Discussion

Phytochemical screening. The various phyto-chemical constituents of siculine may have acted synergistically to contribute to the inhibition of sickling and pain observed with this herbal preparation in the previous studies (Ejiofor *et al.*, 2007). Cardiac glycosides generally often described as digitalis are a group of cardiotonic agents used in various heart diseases especially congestive heart failure (CHF) to improve blood flow and / or perfusion of tissues, and they are also used secondarily as diuretics (Hoffman and Bigger, 1980). They produce direct increase in renal flow (diuresis) and indirect secondary diuretic effect. Thus, the diuretic activity of siculine may be related in part to its cardiac glycoside content.

Table 1: Phytochemical constituents of Siculine

Constituent	Observation
Free reducing sugars	-
Starch	-
Tannins and Phlobatannins	-
Anthraquinones	-
Saponins	-
Cardiac glycosides	++
Terpenes and sterols	+
Alkaloids	-
Flavonoids	++

Key: ++ = Present in high amount + = Present in low amount - = Absent

Table 2: Antimicrobial activity of siculine

Sample	Mean ± SE Inhibition Zones (cm)		
	E. coli	*P. aeruginosa*	*S. aureus**
1% Siculine	0	0	0
5% Siculine	0	0	2.25 ± 0.18
0.002% Ampicillin	2.0 ± 0.07	2.25 ± 0.18	2.90 ± 0.07

Table 3: Effect of siculine on food intake, water intake and urinary output following sub-acute administration in rats

Treatment group.	Mean ± SE 7 days before and 7 days during treatment for - Food Intake (g / 100 g / 24 h)			
	Male		Female	
	Before	During	Before	During
Saline control (0.5 ml)	5.82 ± 0.69 100%	7.21 ± 0.56 100%	6.52 ± 0.73 100%	7.48 ± 0.62 100%
Siculine (0.5 ml)	5.66 ± 0.24 97.3%	7.33 ± 0.58 101.7%	6.09 ± 0.56 93.4%	6.88 ± 0.76 92.0%
Water Intake (ml / 100 g / 24 h)				
Saline control (0.5 ml)	7.29 ± 0.75 100%	6.72 ± 0.52 100%	8.31 ± 0.24 100%	7.36 ± 0.52 100%
Siculine (0.5 ml)	7.66 ± 0.87 105.1%	8.64 ± 0.44 128.6%	8.55 ± 0.23 102.9%	8.87 ± 0.66 120.5%
Urinary Output (ml / 100 g / 24 h)				
Saline control (0.5 ml)	4.11 ± 0.22 100%	4.28 ± 0.15 100%	3.55 ± 0.11 100%	4.93 ± 0.56 100%
Siculine (0.5 ml)	3.97 ± 0.17 96.6%	8.13 ± 0.35* 190. 0%	3.69 ± 0.27 103.9%	9.48 ± 0.45* 192.3%

*Statistically significant urinary output during siculine treatment in both male and female rats ($P < 0.005$, Student's t-test)

Flavonoids are a group of water soluble compounds said to have vitamin activity beneficial for certain types of haemorrhagic diseases (Danford and Munro, 1980). Many flavonoids act as antioxidants (Robins, 1980) and so the flavonoid content of siculine might be a useful tool in reducing the oxidant stress of the RBCs and / or pain. *In vitro*, flavonoids have been reported to have anti-inflammatory and smooth muscles

antispasmodic effects and also to inhibit DNA synthesis, insulin secretion, lymphocyte/ fibroblast glucose uptake, endo- / exo-cytosis and platelet aggregation (Middleton, 1988; Attaway, 1992). The inhibition of aggregation of platelet which is the thrombocyte clotting part of blood ultimately would reduce RBC polymerisation or the sickling process that produces pain. Flavonoids also tend to correct abnormal capillary permeability and fragility associated with variety of diseases (Middleton, 1988), the effect of which enhances inhibition of sickling and pain. Flavonoids also chelate metal ions of trace elements particularly copper which has not only been noted to be of higher serum level in sickle cell anaemia than in Hb-AA and Hb-AS genotypes, but also associated with formation of irreversible sickled RBCs (Olatunbosun, 1975). The chelates of copper-flavonoid complexes so formed are said to stabilise structural proteins including the haemoglobin of red cells as well as strengthen fragile RBC membranes (Kuhnau, 1976). Such chelates however tend to inhibit hyaluronidase enzymes that promote absorption of fluid (Kuhnau, 1976). Thus, the diuresis produced following siculine administration in this study may somewhat be due to its flavonoid constituent.

Terpene-hydrocarbons and their oxygenated terpenoid compounds (limonene, citral, retinoids, geraniol, menthol, cineole etc.) also called volatile oils, essential oils or ethereal oils are colourless, odorous volatile principles of plants and animal sources primarily used as flavouring and perfuming agents and also in medicinals as anthelmintics, diuretics, antiseptic, counter local-irritant or local anaesthetic, sedative, analgesic agents etc (Kokate *et al*., 2002; Karl-Heinz and Ibrahim, 2003). The analgesic and diuretic activity of siculine may also have been partially due to its terpene content.

Phytosterols [beta-sitosterol (BSS), stigmasterol, campesterol etc] are steroid alcohols with a hydroxyl group in the 3-position of the A-ring (Sterols & Sterolins, 2007). Plant sterols are beneficial unsaturated fat-like substances (amphipathic lipids) that naturally occur in vegetables and fruits as free sterols, acylated (sterol esters), alkylated (steryl alkyl esters) or sulphated (cholesterol sulphate) (Goldberg *et al*., 2006). Plant sterols are said to be natural protectors of the heart and bone marrow (Heart-choice.com, 2006). Beta-sitosterol is particularly reported to modulate immune function by controlling the production of inflammatory cytokines and reducing the Cortisol / stress pathway and / or inflammatory pain levels (Sterols & Sterolins, 2007). Natural sterol in their glycosylated form of about 4:1 ratio phytosterols / sterolins strengthens the T-helper lymphocyte (TH-1 and TH- 2) cells of the thymus gland to fight stressful events (Sterols & Sterolins, 2007). Thus, the sterol content of siculine may be beneficial to both its antimicrobial and pain-oxidative stress inhibitory effects.

Antimicrobial activity. It is noteworthy that *Staphylococcus aureus* which was sensitive to siculine is a major cause of infections in sickle cell anaemia patients. *S. aureus* infections including abscesses, bacteremia, endocarditis, pneumonia, meningitis, osteomylitis and others that include influenza and measles are very common in sickle cell anaemia (Sande and Mandell, 1980). Infection or bacterial contamination decreases oxygen tension and accelerates sickling of RBCs and most often are the major cause of death in children with sickle cell disease (Okuonghae *et al*., 1992; March of Dimes, 2006). Cardiotonic glycosides, flavonoids and terpenoids contained in siculine are all plant-derived compounds with antimicrobial activity (Vanden Berghe *et al*., 1986). The antimicrobial activity demonstrated by siculine was possibly too weak to be of any significance in the management of serious

bacterial infections being that it was observed only at the high siculine concentration of 5%. Since the infections associated with sickle cell crisis are usually such that require more powerful antibiotics, the slight antimicrobial effect of siculine when used serves an adjunct effect to other antibiotics.

Food intake, water intake and urinary output. Food intake was not affected by siculine and it has been found that many nutrient deficiencies that occur in sickle cell anaemia sufferers was not necessarily because of reduced dietary habits, but due to an increased need for many nutrients (Mayo Clinic, 2007). Bone marrow needs nutrients to make new red blood cells in place of the sickled or destroyed cells. Siculine caused an increase in water consumption and this effect is of great benefit in sickle cell anaemia because plasma-volume contraction often occurs in association with the frequent pain crises resulting in output of fluid in excess of input with consequent loss of body weight (Hatch and Diggs, 1965). There is also dehydration from potassium leakage and calcium influx common in sickle cell anaemia (Konotey-Ahulu, 1991; ASCAA, 2007); thus, Fluids are required during episodes of pain crisis to maintain the intra-erythrocytic electrolyte balance.

The diuretic effect due to siculine was well pronounced and the producers of siculine may have been aware (in practice), of this diuretic effect, necessitating its being contraindicated in diabetes mellitus-sicklers as specified in its product label to avoid further dehydration. The diuretic activity of cardiac glycosides is well known and often employed in the relief of oedema; and terpenes also have diuretic effects (Kokate *et al*., 2002). These together with a possible formation of copper-flavonoid complexes that inhibit testicular-hyaluronidase fluid uptake promotory effect (Kuhnau, 1976) may have caused the observed diuresis.

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