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Research Article

EFFECTS OF *ATHROSPIRA PLATENSIS, MONODORA MYRISTICA* AND *HELIANTHUS ANNUUS* ON THE RATE OF POLYMERIZATION, SICKLE CELL REVERSION AND OXYGEN AFFINITY OF SICKLE CELL HEMOGLOBIN

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ABSTRACT
The *in vitro* effects of the cyanobacteria (*Athrospira platensis*), spices (*Monodora myristica*) and the achene (*Hellianthus annuus*) on the rate of polymerization, sickle cell reversion and oxygen affinity of sickle cell hemoglobin (HbS) was studied spectophotometrically using hemolysates of HbS containing erythrocytes treated with sodium metabisulphite in the presence of the different plant extracts. All fractions of the three plant extracts showed statistically significant decreases ($p< 0.05$) in the rate of polymerization therefore inhibited the rate of sickling with increasing time. The Benzene Soluble Extract (BESE) fraction of *Hellianthus annuus* demonstrated the highest antisickling activity (89.15% inhibition of HbSS), while the least was by the BESE fraction of *Athrospira platensis* (45.16% inhibition of HbSS). The crude aqueous extract (CAE) fraction of *Athrospira platensis* showed the highest anti-oxidant effect, while CAE fraction of *Monodora myristica* gave the least. The water soluble extract (WSE) fraction of *Hellianthus annuus* expressed maximum rate of sickling reversion. Alcohol extracts of these plants were found to exhibit a more significant antisickling activity relative to the aqueous extracts which is attributable to the presence of some lipophillic amino acids present in the alcoholic fraction of the extracts, and it could also be responsible for the observed high reversion capacity but low antioxidant effect of the lipophillic fraction of the plant extracts.
Keywords: Sickle cell disease; Plant extract; *Athrospira platensis*; *Hellianthus Annuus*; *Monodora Myristica*

INTRODUCTION

Sickle-cell disease (SCD) or sickle-cell anemia (SCA) or depranocytosis, is an autosomal recessive genetic blood disorder with over dominance, characterized by red blood cells that assume an abnormal, rigid, sickle shape[1]. Sickling decreases the cells flexibility and results in a risk of various complications. The sickling occurs because of a mutation in the hemoglobin gene and life expectancy becomes shortened[1]. Sickle-cell disease, usually presenting in childhood, occurs more commonly in people (or their descendants) from parts of tropical and sub-tropical regions where malaria is or was common. One-third of all indigenous inhabitants of Sub-Saharan Africa carry the gene, because in areas where malaria is common, there is a fitness benefit in carrying only a single sickle-cell gene (sickle cell trait). Those with only one of the two alleles of the sickle-cell disease, while not totally resistant are more tolerant to the infection and thus show less severe symptoms when infected[2-4]. Sickle-cell anaemia is the name of a specific form of Sickle-cell disease in which there is homozygosity for the mutation that causes HbS[1]. Sickle-cell anaemia is also referred to as "HbSS", "SS disease", "haemoglobin S" or permutations thereof. In heterozygous people, who have only one sickle gene and one normal adult haemoglobin gene, it is referred to as "HbAS" or "sickle cell trait". Other, rarer forms of Sickle-cell disease include sickle-haemoglobin C disease (HbSC), sickle beta-plus-thalassaemia (HbS/β+) and sickle beta-zero-thalassaemia (HbS/β0). These other forms of sickle-cell disease are compound heterozygous states in which the person has only one copy of the mutation that causes HbS and one copy of another abnormal hemoglobin allele. The term disease is applied, because the inherited abnormality causes a pathological condition that can lead to death and severe complications. Not all inherited variants of haemoglobin are detrimental, a concept known as genetic polymorphism. An application of phytomedicine can serve as succor to sufferers[1,5]. Owing to important culinary and medicinal properties of *Arthrospira platensis*, *Helianthus annuus* and *Monodora myristica*, this study therefore seeks to investigate the following:

- the possible anti–sickling effects of alcohol extracts of these species
- the possible anti-sickling effects of aqueous extracts of these species
- the possible anti-sickling effects of Benzene extracts of these species and a comparative performance assessment among the various extract types.

MATERIALS AND METHODS

Chemicals: The chemicals / reagents used include: Butanol (BDH chemicals, May and Baker LTD, Dagenham England), Sodium metabisulphite (BDH chemicals LTD, Poole England) and Hydrochloric acid (Sigma Chemical Company, Poole Dorset, UK), Chloroform and Methanol (Lilikem Hospilab Services LTD. No. WWLS 211 Ariaria Intl. MKT. Aba, Abia State), Anticoagulant (EDTA tube nycodenz), distilled water (saint john's laboratory Owerri, Imo state), 0.9 % phosphate buffer (chemiscience, Owerri, Imo state), Sodium dihydrogenorthophosphate ($NaH_2PO_4.2H_2PO_4.2H_20$) (lab. Tech. chemicals), Benzene (Hopkin and Williams, Chadwell heath Essex England) Sodium chloride (Nacl) (BDH), disodium hydrogen orthophosphate anhydride (Na_2HPO_4) (Kentin Limited, 13 A Okigwe Road, Owerri, Imo State).

Blood samples

Blood samples were collected from confirmed HbSS patients by the personnel of the Hematology unit of the Federal Medical Center Owerri after due permission for its usage was granted by the Medical Council of Nigeria.

Plant samples

Athrospira platensis was purchased from emeforaku Nigeria Limited at suit 18 C/o 55 sangana street, mile one diobu Port Harcourt Nigeria. *Hellianthus annuus* was harvested from a farm in Choba Port Harcourt, Rivers State of Nigeria and were confirmed by a taxonomist of the department of plant Science University of Portharcourt, Choba, Rivers State, Nigeria.

Spice samples

Monodora myristica was purchased from Eke Ukwu Owerri Main Market in Imo State of Nigeria and was confirmed by a botanist of Imo State University Owerri.

Sample Preparation

Preparation of plant extract: The samples were separately blended, weighed and two hundred (200 g) grams samples divided into two equal parts (100 g). One portion of each of the divided samples was used for crude aqueous extract ion (CAEI) process and the others were subjected to batch-extraction procedures with butanol[6,7]. For the crude aqueous extraction of *Helianthus annuus*, one hundred (100) grams of the sample were soaked in 500 ml distilled water at 100 degrees centigrade for 12 hours, while for crude extracts of *Arthrospira platensis* and *Monodora myristica*, one hundred (100) grams of the sample were soaked in 200 ml distilled water at 100 degrees centigrade for 12 hours. The solutions were filtered separately using whatman paper no. 1 and the filtrate centrifuged at 600 rpm for 10 mins. The supernatant were collected in vials and concentrated at 100 degrees centigrade to get the crude aqueous extract (CAEs). The other portions of the samples were separately soaked in 200 ml of chloroform for twenty four hours (24 h) to detect them and generate the fat soluble (FAS) fraction by filtration. The residues after evaporation to dryness were suspended into 200 ml of methanol for 24 hours. The filtrates were centrifuged and decanted to obtain the methanol water extract[8,9]. Butanol water partitioning was done with the methanol extract of each of the samples. Exactly 20 ml of distilled water and 20 ml of butanol was added to the methanol extracts after concentrating them. This was left to stand for 24 hours and the two-phase liquid separated into the butanol-soluble (BUS) and water soluble (WAS) fractions respectively. The BUS and WAS fractions were concentrated by evaporation at 80 degrees and 100 degrees centigrade respectively. The volumes were equally recorded. Spectrophotometric readings were taken from UV-spectrophotometer. For benzene extracts of the sample, one hundred (100) grams of the individual samples were separately dissolved in 200 ml of chloroform for twenty four hours (24 h). The residues after evaporation to dryness were suspended into 200 ml of methanol for 24 hours. The filtrates were centrifuged and decanted to obtain the methanol water extract. Benzene soluble extract was done with the methanol extract of each of the samples by adding 20 ml of benzene to the extracts and left to stand for (24 h) hours. Preparation of the blood samples: Portions (0.20 ml) of the whole blood samples were used for the Fe^{2+}/Fe^{3+} ratio and the sickling reversion experiment while the remaining portions were collected into EDTA tubes. Erythrocytes were isolated from the blood samples by centrifugation at 10,000 rpm for fifteen seconds (15 sec.) using the bench centrifuge (Nickel-Electro centrifuge). Following careful siphoning of the plasma with Pasteur pipette, the erythrocytes were by repeated inversion suspended in a volume of isotonic saline (0.9% NaCl) equivalent to the siphoned plasma. The erythrocyte suspension was then frozen at $0^{o}C$ and subsequently thawed to produce a hemolysate for the hemoglobin polymerization experiment.

Sickle Cell Hemoglobin (Hbss) Polymerization Inhibition Experiment

The method of Noguchi and Schetcher[10] was used for the HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the solution (polymerizing mixture) at 700 nm, using 2% solution of sodium metabisulphite as deoxygenating agent[11]. Then 4.4 ml of 2% sodium metabisulphite ($Na_2S_20_3$), 0.5 ml normal saline (0.9% NaCl) and 0.1 ml heamoglobin were pipetted into a cuvette, shaken and absorbance reading taken in a spectrophotometer (Unicam-spectronic 20) at 700 nm, every two minutes for 30 min. This represents the control. Distilled water was used as blank for all assays. For the test assays, 4.4 ml of 2% $Na_2S_20_3$, 0.5 ml of each extract and 0.1 ml hemoglobin (HbSS) solution were pipetted in the cuvette and readings taken as above. The rates of hemoglobin polymerization for the extracts or fractions were estimated by calculating the change in optical density (OD) versus time in minutes. The rates were expressed as percentages with respect to the control.

Determination Of The Fe^{2+}/Fe^{3+} Ratio

The Fe^{2+}/Fe^{3+} ratio was determined by the methods of Davidson and Henry[12], while the oxygen affinity of hemoglobin and methemoglobin were measured at 540 nm and 630 nm respectively. The approach employs lysing 0.02 ml whole blood in 5.0 ml of distilled water and 0.02 ml normal saline. The absorbance of hemoglobin (Hb) and methemoglobin (mHb) were measured at 540 nm and 630 nm to determine the %Hb and % mHb respectively. This represents the control. In determining the effect of the extract on Fe^{2+}/Fe^{3+} ratio, 0.02 ml of each extract was added to 5.0 ml of distilled water and 0.02 ml of blood added and incubated for 60 min in a test tube.

Sickle Cell Reversion Experiment

Sickle cell reversal experiment was done as described by Ekeke *et al*[13]. Freshly collected HbSS blood were diluted in 1:2 ratio with 0.9% normal saline / phosphate buffer (0.2 M, pH. 7.2) solution and then incubated with 2% freshly prepared sodium metabisulphite in a ratio 1:2 for one hour. At the end of the time, 1.6 ml of the pre-sickled blood in polystyrene tube was mixed with 0.4 ml of the extract. Cells were counted, taking a sample at an interval with the help of a dropping pipette on a slide and then covered with a cover slip. Finger pressure was used to form a thin layer and the slides cover observed using x 40 objective lens by counting the number of sickled and unsickled cells in different fields on the slide. This procedure was repeated for all the samples. The antisickling rate was estimated by measuring the sickling rate for an hour at 10 mins intervals, starting from zero time, which is immediately after stirring.

Percent (%) sickling was calculated as

$$\frac{\text{No of sickled cell}}{\text{Total no of cells counted}} \times \frac{100}{1}$$

The rate of sickling reversion was calculated as the slope of the Curve at zero time.

Percentage fall (%) / Time taken (mins)

NB: The time required to reverse 50% sickled cells was calculated from the curve by extrapolation from the 50% relative sickled cell axis to the time axis.

RESULTS AND DISCUSSION

From the results represented in Tables 1, 2, 3 and 4 Benzene soluble extracts (BESE) of *Athrospira platensis* showed the highest rate of polymerization, while the fat soluble extract (FSE) fraction showed the least. Butanol soluble extract (BUSE) fraction of *Monodora myristica* showed highest rate of polymerization, while the WSE fraction of the extract has the least. BUSE fraction of *Hellianthus annus* showed the least rate of polymerization.

Table 1: The Rates of polymerization, relative percent polymerization and relative percent inhibition of HbSS by the fractions of the sample

Samples	Fraction	Rates of Polymerization	Relative% polymerization	Relative % inhibition
Control		$0.0341^{a} \pm 0.001$	100	0
Athrospira platensis	FSE	$0.0169^{a} \pm 0.000$	49.56	50.44
Athrospira platensis	CAE	$0.0133^{a} \pm 0.000$	39	61
Athrospira platensis	WSE	$0.0114^{a} \pm 0.001$	33.43	66.57
Athrospira platensis	BUSE	$0.0096^{a} \pm 0.000$	26.4	73.62
Athrospira platensis	BESE	$0.0180^{a} \pm 0.000$	54.84	45.16
Monodora myristica	FSE	$0.010^{b} \pm 0.000$	30.49	69.51
Monodora myristica	CAE	$0.007^{b} \pm 0.000$	20.53	79.47
Monodora myristica	WSE	$0.004^{b} \pm 0.000$	12.9	87.1
Monodora myristica	BUSE	$0.0165^{a} \pm 0.002$	48.39	51.61
Monodora myristica	BESE	$0.0044^{b} \pm 0.001$	12.91	87.11
Helianthus annuus	FSE	$0.0065^{b} \pm 0.002$	19.06	80.94
Helianthus annuus	CAE	$0.0090^{a} \pm 0.000$	26.39	73.61
Helianthus annuus	WSE	$0.0070^{b} \pm 0.003$	21.41	78.59
Helianthus annuus	BUSE	$0.0098^{a} \pm 0.001$	28.74	71.26
Helianthus annuus	BESE	$0.0037^{c} \pm 0.000$	10.85	89.15

Values are means ± SE (n = 3). Different superscript letters in a group are significantly different at $P \leq 0.05$

While the BESE extract of *Arthrospira platensis* has the highest rate of polymerization and relative percent polymerization but least relative percent inhibition, while the BESE fraction of *Helianthus annuus* has the least rate of polymerization and relative percent polymerization, but highest relative percent inhibition. Among the various potential antisickling agents tested, hydroxyurea (HU) has been the most effective compound used for the treatment of patients with sickle cell disease (SCD). NIPRISAN (Nix-0699), an ethanol/water extract from indigenous plants, has a strong antisickling effect[14]. The kinetics of polymerization on addition of 0.05 microgram/ml Nix-0699 caused a six-fold prolongation of the delay time prior to deoxy-Hb S polymerization when compared with that of untreated Hb S samples[14-16]. Expressed anti-sickling activity on the inhibition is comparable to the findings of Platt[17]. The herbal formula (HF) aqueous extract showed the highest anti-sickling activity on a weight by weight basis of all the extracts and fractions tested, giving a 71.4% inhibition of sickling at the end of 180 min incubation when compared with the normal saline control. CAE fraction of *Monodora myristica* has maximum Fe^{2+}/Fe^{3+} ratio, while CAE fraction of *Athrospira platensis* has the least (Table 2) which is in agreement with the findings of Uwakwe and Nwaoguikpe[15] that reported highest antioxidant activity in CAE fraction of M myristica.

Table 2: Fe^{2+}/Fe^{3+} ratio

Sample	BESE	CAE	WSE
Control	$2.13^{a} \pm 0.11$	$2.13^{a} \pm 0.11$	$2.13^{a} \pm 0.11$
Athrospira platensis	$1.72^{b} \pm 0.09$	$1.55^{b} \pm 0.17$	$1.51^{b} \pm 0.13$
Monodora myristica	$1.66^{b} \pm 0.07$	$1.78^{b} \pm 0.23$	$1.71^{b} \pm 0.09$
Helianthus annuus	$1.75^{b} \pm 0.18$	$1.77^{b} \pm 0.07$	$1.79^{b} \pm 0.04$

Values are means ± SE (n = 3). Different superscript letters row-wise are significantly different at $P \leq 0.05$

WSE fraction of *Athrospira platensis* has highest rate of reversion while CAE fraction of *Helianthus annuus* has the least and WSE fraction of *Athrospira platensis* showed highest rate of reversion, while CAE extracts of *Helianthus annus* has the least. As stated by Mpiana *et al*.[18] and Adams *et al*.[19], the seeds aqueous extracts exhibited a higher percentage reversal of sickling of all the tested parts. Charache *et al*. also repoted increased painful crises[20].

Table 3: Rate of sickle cell reversion

Sample	BESE	CAE	WSE
Athrospira platensis	1.625 a ± 0.07	1.585 a ± 0.04	1.805 a ± 0.13
Monodora myristica	1.444 a ± 0.06	1.287 a ± 0.11	1.451 a ± 0.05
Helianthus annuus	1.566 a ± 0.07	1.250 a ± 0.03	1.701 a ± 0.06

The CAE fraction of *Athrospira platensis* has the highest % decrease, while CAE fraction of *Monodora myristica* has the least (Table 4).

Table 4: % Decrease in Fe^{2+}/ Fe^{3+} in studied samples

Sample	BESE	CAE	WSE
Control	$100.00^{a} \pm 8.01$	$100.00^{a} \pm 8.01$	$100.00^{a} \pm 8.01$
Athrospira platensis	$74.20^{b} \pm 4.99$	$72.77^{b} \pm 5.67$	$72.03^{b} \pm 6.93$
Monodora myristica	$80.45^{c} \pm 6.77$	$83.57^{c} \pm 7.23$	$79.98^{c} \pm 5.89$
Helianthus annuus	$81.37^{c} \pm 6.48$	$83.09^{c} \pm 7.07$	$80.12^{c} \pm 6.44$

CONCLUSION

Benzene soluble extracts of *Helianthus annuus* and *Monodora myristica* were found to exhibit a more significant antisickling activity relative to their aqueous and alcohol extracts and all extracts of *Athrospira platensis*, which could be attributed the presence of the soluble steroidal amino acids present in the benzene soluble extract. It also exhibited high reversion capacity, while the crude aqueous extracts of *Athrospira platensis* showed highest antioxidant effect relative to the benzene and alcohol fractions of *Helianthus annuus*, and *Monodora myristica*.

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