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Polymerization Studies of Sickle Cell Hemoglobin Incubated in Aqueous Leaf Extract of *Nicotiana tabacum* Product

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ABSTRACT

In vitro studies have revealed that plant extracts interfered and altered the polymerization profile of deoxygenated sickle cell haemoglobin molecules (deoxyHbS). The present study seeks to ascertain the capacity of aqueous extract of *N. tabacum* to alter and interfere with polymerization of deoxyHbS molecules *in vitro*. Spectrophotometric method was used to measure level of sodium metabisulphite induced polymerization of deoxyHbS molecule incubated in aqueous extract of *N. tabacum* for 180 sec. The polymerization profile of deoxyHbS molecules of control and test samples showed increasing level of polymerization with progression of experimental time. At experimental time t>30 sec, level of polymerization of control sample ranged between 60.9±0.76-100±1.05%, whereas, the test samples ranged in the following corresponding order: (*N. tabacum*) = 0.8 mg 100 mL^{-1}, 65.6±0.93-176.0±4.26%, (*N. tabacum*) = 1.0 mg 100 mL^{-1}, 75.7±1.07-192.9±5.03% and (*N. tabacum*) = 2.0 mg 100 mL^{-1}, 135.9±5.04-297.2±19.14%. Activation of deoxyHbS polymerization by aqueous extract of *N. tabacum* increased in a concentration dependent manner and duration of incubation. Specifically, at t =180 sec, (*N. tabacum*) = 2.0 mg 100 mL^{-1} caused highest level of activation of deoxyHbS polymerization (197.2±19.14%); an increase in % polymerization in a ratio of 1:4.5 (approx.) compared with (*N. tabacum*) = 0.8 mg 100 mL^{-1} at t = 30 sec. The study showed that aqueous extract of *N. tabacum* exacerbated polymerization of deoxyHbS molecules in a concentration and time dependent manner. Therefore, *N. tabacum* in the present experimental form did not exhibit therapeutic potentials for management and alleviation of sickling disorder.

Key words: Polymerization, sickle cell haemoglobin, *N. tabacum*, sodium metabisulphite

INTRODUCTION

Globally, Africa, part of Asia, the Arabian Peninsula and part of Southern Europe has been reported as areas of high incidence the sickle cell gene (β^S). In Africa, two major regions of very high frequencies of β^S are in West Africa (Nigeria and Ghana) and Central Africa (Gabon and Zaire) (Wainscoat, 1987; Uzoegwu and Onwurah, 2003). Lower incidence of Sickle Cell Anaemia (SCA) occurs in part of Italy, Greece, the Middle East and India. SCA is a dilapidating disease often associated with organ disorders and complications with a reduced life expectancy (Kutlar, 2005; Frenrtte and Atweh, 2007).

The sickle cell haemoglobin (HbS) variant is caused by a point mutation affecting the coding sequence of the β-globin gene resulting in a substitution of glutamic acid by valine at the sixth position of β-globin chains (Kutlar, 2005; Bianchi *et al.*, 2009). Under low oxygen tension, deoxyHbS molecules polymerize into microfibrils parallel to each other with concomitant reduced solubility (forms gel) and resultant erythrocyte membrane deformity and damage (Xu *et al*., 2002). Therapeutic approaches for the treatment and management of SCA disease include induction of fetal hemoglobin expression (Bianchi *et al*., 2009; Fathallah and Atweh, 2006), bone marrow transplantation (Billings, 1990; Walters *et al*., 2000) and the use of pharmacological agents that interact either non-covalently or covalently with HbS molecules (Xu *et al*., 1999; Abdulmalik *et al*., 2005) to retard or inhibit haemoglobin aggregation and polymerization.

The use of tobacco plant (*Nicotiana tabacum*) for several purposes has been worldwide for centuries. Cigarettes, in spite of the health risk associated with its usage constitute the largest share of manufactured tobacco products in the world, accounting for 96% of total sales. (Adeniyi *et al*., 2010). The chemical compositions of *N. tabacum* leaf extract have been previously reported (Andersen and Kasperbauer, 1973; Siddiqui *et al*., 2004; Chitra and Sivaranjani, 2012). The prospects of utilization of *N. tabacum* products for purposes that do not pose health risk are now widely encouraged and advertised (Mugisha-Kamatenesi *et al*., 2008). There are reports and claims by several authors on the medicinal benefits (Chitra and Sivaranjani, 2012; Nacoulma *et al*., 2012) and insecticidal activities against several insect species (Isman, 2006; Xue *et al*., 2010; Farsani *et al*., 2011) by leaf extracts of *N. tabacum*. Application of herbal remedies for the management of SCA has been widely reported (Ekeke and Shode, 1985; Kade *et al*., 2003; Okpuzor *et al*., 2008; Imaga *et al*., 2010). *In vitro* studies have revealed that plant extracts altered the polymerization of deoxyHbS molecules (Imaga *et al*., 2010; Chikezie, 2011). Therefore, the present study seeks to ascertain the capacity of aqueous extract of *N. tabacum* to alter and interfere with the polymerization of deoxyHbS molecules *in vitro*.

MATERIALS AND METHODS

Collection and preparation of *Nicotiana tabacum* leaf extract: Preparation of aqueous extract of *N. tabacum* was according to the methods earlier described by Chikezie and Uwakwe (2011). Twenty-five grams of processed leaves of *N. tabacum* obtained from the popular cigarette brand Benson and Hedges™ were collected in desiccators and allowed to dry for 72 h to become crispy. The dried specimen was ground in ceramic mortar and pestle into fine powder. The pulverized specimen was suspended in 100 mL of distilled water and allowed to stand for 6 h at 37°C. Aqueous extract of *N. tabacum* was obtained by filtration with Whatman No. 2 filter paper. Finally, the extract was concentrated in a rotary evaporator at 50°C and dried in vacuum desiccators. The yield was calculated to be 5.14% (1.29 g; w/w). The extract was finally suspended in 50 mL Phosphate Buffered Saline (PBS) solution osmotically equivalent to 0.9 g 100 mL^{-1} NaCI (NaCI (9.0 g), $Na_2HPO_4.2H_2O$ (1.71 g) and $NaH_2PO_4.2H_2O$ (0.243 g) 100 mL^{-1}; pH = 7.4) (Ibegbulem and Chikezie, 2012). The extract was kept at 4°C in a refrigerator for at least 24 h before subsequent tests. Concentration equivalents of 0.8, 1.0 and 2.0 mg 100 mL^{-1} aqueous extracts of *N. tabacum* were used for polymerization studies.

Ethics: The institutional review board of the Department of Biochemistry, Imo State University, Owerri, Nigeria, granted approval for this study and all participants involved signed an informed consent form. This study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki. Individuals drawn were from Imo State University, Owerri and environs. The research protocols were in collaboration with registered and specialized clinics and medical laboratories.

Collection of blood samples/preparation of erythrocyte haemolysate: Five milliliters (5.0 mL) of venous blood obtained from the volunteers by venipuncture was stored in EDTA anticoagulant tubes. Blood samples were from patients attending clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic/Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria.

The erythrocytes were separated from plasma and washed by centrifugation method as described by Tsakiris *et al.* (2005) with minor modification according to Pennings *et al.* (1999). Blood volume of 4.0 mL was introduced into centrifuge test tubes containing 4.0 mL of buffer solution pH = 7.4:250 mM tris (hydroxyl methyl) amino ethane-HCl (Tris-HCl)/140 mM NaCl/1.0 mM $MgCl_2$/10 mM glucose and centrifuged at 4000 rpm for 10 min using a centrifuge (B.Bran Scientific and Instrument Company, England). The plasma and buffy coat (supernatant) were carefully removed with Pasteur pipette. This process was repeated until the supernatant became clear. The pelleted erythrocytes were suspended in PBS (pH = 7.4) to obtain approximately 10% haematocrit and stored at 4°C for 24 h. The washed erythrocytes were lysed by freezing/thawing as described by Galbraith and Watts (1980) and Kamher *et al.* (1984). The erythrocyte haemolysate was used for polymerization studies.

Polymerization studies: Sodium metabisulfite ($Na_2S_2O_5$; (BDH, UK)-induced polymerization of HbS molecules was ascertained as described previously by Iwu *et al.* (1988) with minor modification according to Chikezie *et al.* (2010). The underlying principle is that HbS molecules undergo gelation when deprived of oxygen, transiting to deoxyHbS molecules; $Na_2S_2O_3$ was used as the reductant. The level of polymerization was measured by recording increasing absorbance of the assay mixture with progression of time. A 0.1 mL of HbS haemolysate was introduced into a test tube, followed by 0.5 mL of PBS and 1.0 mL of distilled water. The mixture was transferred into a cuvette and 3.4 mL of 2 g 100 mL^{-1} aqueous solution of $Na_2S_2O_5$ was added. The absorbance (λ_{max} = 700 nm) of the assay mixture was measured with a spectrophotometer (SPECTRONIC 20, Labtech-digital Blood Analyzer®) at every 30 for 180 sec (control assay). This procedure was repeated by substituting distilled water with 1.0 mL of corresponding three increasing concentrations (0.8, 1.0 and 2.0 mg 100 mL^{-1}) of *N. tabacum* aqueous extracts (test assay).

Calculations: Percentage polymerization was calculated according to Chikezie *et al.* (2010), thus:

$$\text{Polymerization (\%)} = \frac{A\frac{t}{c} - 100}{A_c\,180\text{th sec}}$$

where, At/c is the Absorbance of test/control assay at time = t s and Ac180th sec is the Absorbance of control assay at the 180th sec.

Cumulative polymerization$_{(\%polymerization.sec)}$ was evaluated using the Simpson's Rule. Thus:

$$f(X_1)h_1+f(X_2)h_2+....+f(X_n)h_n$$

Area under the curve (AUC) of the plot of % polymerization versus time (sec) is given by:

$$\text{AUC(polymerization \% sec)} = \frac{h}{(2)(y_n + 2y_{n-1} + 2y_{n-2} + 2y_{n-3} + ...)} \quad (1)$$

where, h is the time intervals (30 sec) and y is the % polymerization at corresponding time interval. Thus:

$$\text{AUC(polymerization \% sec)} = \frac{h}{(2)(y_6 + 2y_5 + 2y_4 + 2y_3 + 2y_2 + 2y_1)} \quad (2)$$

RESULTS

The results illustrated in Fig. 1 showed that deoxyHbS molecules of the control and test samples showed increasing level of polymerization with progression of experimental time. At experimental time t>30 sec, level of polymerization of control sample ranged between 60.9±0.76-100±1.05%, whereas, the test samples ranged in the following corresponding order: (*N. tabacum*) = 0.8 mg 100 mL^{-1}, 65.6±0.93-176.0±4.26%; (*N. tabacum*) = 1.0 mg 100 mL^{-1}, 75.7±1.07-192.9±5.03% and (*N. tabacum*) = 2.0 mg 100 mL^{-1}, 135.9±5.04-297.2±19.14%. Therefore, the three experimental concentrations of *N. tabacum* caused activation of deoxyHbS polymerization in the order: (*N. tabacum*) = 0.8<1.0<2.0 mg 100 mL^{-1}. Furthermore, cumulative level of deoxyHbS polymerization was in the order: Control<(*N. tabacum*) = 0.8<1.0<2.0 mg 100 mL^{-1} (Table 1).

Table 2 showed that activation of deoxyHbS polymerization by aqueous extract of *N. tabacum* increased in a concentration dependent manner and duration of incubation. Specifically, at t =180 sec, (*N. tabacum*) = 2.0 mg 100 mL^{-1} caused highest level of activation of deoxyHbS polymerization (197.2±19.14%). The value represented an increase of %polymerization in a ratio of 1: 4.5 (approx.) compared with (*N. tabacum*) = 0.8 mg 100 mL^{-1} at t = 30 sec.

When t>30 sec, deoxyHbS incubated in aqueous extract (*N. tabacum*) = 2.0 mg 100 mL^{-1} exhibited %polymerization>100%. Likewise, %polymerization>100% in the presence of 0.8 and 1.0 mg 100 mL^{-1} of *N. tabacum* occurred when t>60 and t>30 sec, respectively.

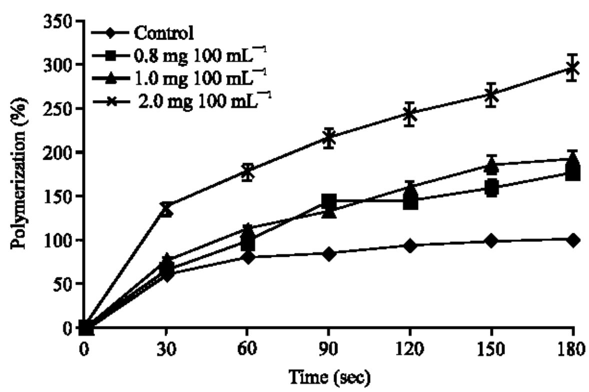

Fig. 1: Levels of DeoxyHbS polymerization of control and in presence of *N. tabacum* with time

Table 1: Cumulative level of polymerization of deoxyHbS within experimental t = 180 sec

(*N. tabacum*) mg 100 mL^{-1}	Control	0.8	1.0	2.0
AUC (% polymerization.sec)×10^3	13.2	20.0	21.3	52.0

AUC: Area under the curve

Table 2: Activation of DeoxyHbS polymerization in the presence aqueous extracts of *N. tabacum* within intervals of t = 30 sec

	Percentage activation of deoxyHbS polymerization					
Time (sec)/(*N. tobacum*) mg 100 mL^{-1}	30	60	90	120	150	180
0.8	4.7±0.93	17.5±1.64	60.3±2.15	50.5±2.03	60.7±3.14	76.0±4.26
1.0	14.8±1.07	30.6±2.08	49.5±3.15	65.3±4.04	86.6±4.16	92.9±5.03
2.0	75.0±5.04	97.6±9.04	133.0±10.04	150.1±17.04	167.1±19.01	197.2±19.14

Values are Means±SD of six (n = 6) determinations

DISCUSSION

The nature and mode of interaction between biochemical agents and contact points of deoxyHbS molecules, in addition to erythrocyte membrane structural components are crucial determining factors in the capability of these agents to retard/inhibit polymerization of deoxyHbS molecules, to alleviate and prevent erythrocyte sickling disorders (Oyewole *et al*., 2008; Chikezie, 2011; Chikezie and Uwakwe, 2011; Ibegbulem *et al*., 2011). Contrary to reports by previous authors and researchers on the anti-polymerization (Imaga *et al*., 2010; Chikezie, 2011) and claims of anti-sickling potencies (Ekeke and Shode, 1985; Kade *et al*., 2003; Okpuzor *et al*., 2008; Imaga *et al*., 2010; Ibegbulem *et al*., 2011) of plant extracts, the present report showed that aqueous extract of *N. tabacum* exacerbated polymerization of deoxyHbS molecules. Chitra and Sivaranjani (2012) have previously reported that processed *N. tabacum* (leaf powder) was devoid of flavonoids and terpenoids. These two phytochemicals have been implicated in inhibiting polymerization of deoxyHbS molecules and by extension exhibited anti-sickling activity on human HbSS erythrocytes *in vitro* (Fall *et al*., 1999; Ibegbulem *et al*., 2011). However, it is worthwhile to note that the biologic action of *N. tabacum* on HbS molecules reported here and the capacity plant extracts to alter erythrocytes physiochemical properties is an additive effect of combination of the plant components (Mikstacka *et al*., 2010).

Haemoglobin polymerization and subsequent sickling of erythrocytes is favored when HbS molecules are deoxygenated (Martin, 1983; Poillon *et al*., 1998). In the present *in vitro* study, the aggravation of polymerization of deoxyHbS molecules by the three experimental concentrations of *N. tabacum* was an obvious reflection of the capability of the plant extract to act in synergy with the hypoxemic agent ($Na_2S_2O_5$), thereby facilitating more rapid rate of polymerization of HbS molecules compared with the control assay (Fig. 1). Conversely, chemical compounds and phytochemicals that serve to stabilize the R-state of HbS molecules with resulting increase oxygen/haemoglobin affinity will cause reduction in the rate and degree of HbS polymerization (Oyewole *et al*., 2008; Chikezie, 2011).

CONCLUSION

Aqueous extracts of *N. tabacum* in the present experimental form did not demonstrate R-state stabilizing property *in vitro* and therefore, did not exhibit therapeutic potentials for the management and alleviation of sickling disorder.

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