Membrane stability and methaemoglobin content of human erythrocytes incubated in aqueous leaf extract of *Nicotiana tabacum* product

Chikezie P.C., Akuwudike A. R., Chikezie C. M.

Department of Biochemistry, Imo State University, PMB 2000, Owerri, Nigeria

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ABSTRACT

The present study seeks to ascertain membrane stability and methaemoglobin content (%MetHb) of human erythrocytes suspended in aqueous leaf extract of *N. tabacum*. Erythrocyte membrane stability (%EMS) and %MetHb were measured by spectrophotometric method. Erythrocytes incubated in the five experimental concentrations of *N. tabacum* exhibited higher tendency to haemolyze in phosphate buffered saline solution [PBS] > 0.54 g/100 mL, whereas erythrocytes incubated in aqueous extracts of [*N. tabaccum*] = 0.4 and 0.8 mg/100 mL showed relatively lower level of haemolysis in [PBS] < 0.54 g/100 mL, compared with the control samples. Experimental [*N. tabacum*] = 2.0 mg/100 mL caused destabilization of erythrocyte membrane by 13.3%. %MetHb of control and erythrocytes treated with aqueous extracts of *N. tabacum* ranged between 0.58 ± 0.09 and 1.89 ± 0.12%. Specifically, erythrocytes treated with [*N. tabacum*] = 0.4 mg/100 mL gave %MetHb = 0.59 ± 0.08; $p < 0.05$. Experimental [*N. tabacum*] = 2.0 mg/100 mL engendered a paradoxical reduction in erythrocyte %MetHb = 0.58 ± 0.09; $p > 0.05$. The medium negative correlation ($r = -0.62421$) between MCF and %MetHb was an indication that *N. tabacum* destabilizing effect, exemplified by [*N. tabacum*] = 0.2 mg/100 mL; %MetHb = 0.58 ± 0.09; %EMS = –13.3, was not an oxidative process, but by mechanical perturbation by constituents of the plant extract on erythrocyte membrane structural components. Erythrocyte %MetHb in the presence of the five experimental [*N. tabacum*] = 0.4 – 2.0 mg/100 mL did not cause toxic methaemoglobinemia *in vitro*.

Keywords: Membrane stability, Methaemoglobin, *N. tabacum,* Erythrocytes.

INTRODUCTION

Tobacco is a processed leaf product of the plant genus *Nicotiana* of the *Solanaceae* family with over sixty species. The product is mostly consumed as cigars, cigarettes, snuff, pipe and chewing tobacco but cigarettes constitute the largest share of manufactured tobacco products in the world, accounting for 96% of total sales.[1] The chemical compositions of *Nicotiana tabacum* leaf extract have been documented.[2,3] Despite the deleterious health consequences associated with the use of *N. tabacum* products, which have been widely reported,[4–7] the cultivation of tobacco as a cash crop is still on the increase in Sub-Sahara Africa.[8]

Cultivation of *N. tabacum* for alternative uses having nothing to do with smoking or other harmful tobacco consumption habits will ultimately be beneficial. Accordingly, there are reports that researchers and bioengineering entrepreneurs of Developed Countries have already begun the use *N. tabacum* as hosts for bioengineering processes.[9–13] Likewise, the prospect of utilizing the plant product as biological pesticides in the form of nicotine tartrate,[14–17] and medicinal preparations/decoction have shown promising prospects.[18,19] Although there are concerns about the use of *N. tabacum* extracts as therapeutic agents because of the addictive tendency

***Corresponding Address:**
Chikezie P.C
Phone: +2348038935327.
E-mail: p_chikezie@yahoo.com

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and harmful effects of nicotine content, reports have shown that salt extract of *N. tabacum* leaves are devoid of nicotine.[19] Therefore, exploitation of *N. tabacum* extracts for medicinal purposes will serve to discourage the use of the plant products in the form that promote health risk factors.

Erythrocytes have been used as a model for studies of drugs and other xenobiotics interaction with biomembranes[20–24] oxidation process.[22,25] In this regard, erythrocyte osmotic fragility test offers a good criterion to ascertaining membrane functionality and stability.[26–29] Erythrocyte oxidation process affects haemoglobin and membrane molecular status and functionality.[22,30] Accordingly, erythrocyte methaemoglobin content, altered state of haemoglobin in which the ferrous (Fe^{2+}) iron of haem are oxidized in the ferric (Fe^{3+}) state, serves to establish the oxidation status of erythrocytes.[30–32] Therefore, the present study seeks to ascertain two physiochemical properties (membrane stability and methaemoglobin content) of human erythrocytes suspended in aqueous leaf extract of *N. tabacum*.

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.[29] Twenty five grams (25 g) of processed leaves of *N. tabacum* obtained from the popular cigarette brand Benson & 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. The extract was kept at 4°C in a refrigerator for at least 24 h before subsequent tests. 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 NaCI {NaCI (9.0 g), $Na_2HPO_4.2H_2O$ (1.71 g) and $NaH_2PO_4.2H_2O$ (0.243 g)/100 mL; pH = 7.4}.[33] Portion of the extract was used for phytochemical screening. Concentration equivalents of 0.4, 0.8, 1.2, 1.6, and 2.0 mg/100 mL aqueous extracts of *N. tabacum* were used to ascertain erythrocyte osmotic fragility and methaemoglobin content.

Phytochemical and biochemical contents of *Nicotiana tabacum* leaf

Phytochemical and biochemical screening was carried out for the presence of tannins, carbohydrates, flavonoids, saponin, alkaloids, glycosides and proteins as described by Ayoola *et al.*[34]

Collection and preparation of blood samples

Five milliliters (5.0 mL) of venous blood obtained from healthy male volunteers (23–26 years) by venipuncture was stored in heparinized tubes. The erythrocytes were separated from plasma and washed by centrifugation method as described by Tsakiris *et al.*,[35] with minor modification according to Pennings *et al.*[36] 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 the B.Bran Scientific and Instrument Company, England, centrifuge. The supernatant was carefully removed with a 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.

Erythrocyte osmotic fragility tests

Determination of erythrocyte osmotic fragility was carried out based on the method described by Dewey *et al.*,[37] with minor modifications as reported by Chikezie and Uwakwe.[29] The fraction of erythrocytes lysed when suspended in PBS of varying concentrations was measured by spectrophotometric method. A final volume of 5.0 mL of PBS of dilution equivalents; 0.90, 0.72, 0.54, 0.36 and 0.18 g/100 mL were prepared according to Chikezie *et al.*,[23] and introduced into corresponding 5 test tubes and 5.0 mL of distilled water was added to the sixth test tube. Portion of 0.5 mL of *N. tabacum* aqueous extract of varying concentrations as specified was delivered into each of the given set of test tubes (1–6). To each test tube, 0.05 mL of the erythrocyte suspension was added and mixed thoroughly by inverting the tubes several times. For the control experiment, the same procedure was repeated but devoid of *N. tabacum* aqueous extracts. The suspensions were allowed to stand for 30 min at room temperature (24–27°C) after which the contents were centrifuged at 1200 *rpm* for 15 min. The relative amount of haemoglobin released into the supernatant was measured with a spectrophotometer (Digital Blood Analyzer®; SPECTRONIC 20, Labtech) at maximum wavelength λ_{max} = 540 nm. PBS osmotically equivalent to 0.9 g/100 mL NaCI and distilled water served as blank and 100% lysis point, respectively.

Erythrocyte methaemoglobin content

In a test tube containing 5.0 mL of distilled water, 0.05 mL of erythrocyte, suspended in PBS osmotically equivalent to 0.9 g/100 mL; pH = 7.4, was added. The suspension was allowed to stand for 60 min at room temperature (24–27°C) and absorbance read at two different wavelength maxima λ_{max} = 540 nm and 630 nm using a spectrophotometer (Digital Blood Analyzer®; SPECTRONIC 20, Labtech). Erythrocyte methaemoglobin content in the presence of corresponding concentrations of aqueous extracts of *N. tabacum* was measured by incubating 0.05 mL of the plant extracts in erythrocyte mixture as earlier described. Erythrocyte percentage methaemoglobin content (%MetHb) of total haemoglobin concentration was evaluated using the formula according to Tietz,[38] and Chikezie *et al.*[23]

$$\%\text{MetHb} = \frac{\text{A630}^2 \cdot 100}{\text{A540}^2 + \text{A630}^2}$$

Where: A540 and A630 are absorbance at wavelength maxima λ_{max} = 540 nm and 630 nm, respectively.

Evaluation of cumulative erythrocyte haemolysis and methaemoglobin content

The percentage haemolysis (%haemolysis) in corresponding PBS erythrocyte mixtures (0.90 – 0.18 g/100 mL) is given thus:

$$\%\text{Haemolysis} = \frac{A_{a \cdot 100}}{A_b}$$

Where A_a = Absorbance of test tube (1 – 5) supernatants
A_b = Absorbance of 6th test tube supernatant

Cumulative erythrocyte haemolysis was evaluated using the Simpson's Rule. Thus:

$$f(X_1)h_1 + f(X_2)h_2 + \cdots + f(X_n)h_n$$

Area under the curve (AUC) of the plot of %haemolysis versus PBS concentrations is given by:

$$\text{AUC (\%haemolysis.g.100 mL}^{-1}) = \frac{h}{2}(y_n + 2y_{n-1} + 2y_{n-2} + 2y_{n-3} + \cdots) \quad \textit{Equation 1}$$

Where n = test tube number; h = concentration intervals of PBS (0.18 mg/100 mL); y = %haemolysis at corresponding concentrations of PBS. n = test tube number (1st – 6th).

Thus:

$$\text{AUC (\%haemolysis.g.100 mL}^{-1}) = \frac{h}{2}(y_6 + 2y_5 + 2y_4 + 2y_3 + 2y_2 + y_1) \quad \textit{Equation 2}$$

The corresponding concentration of PBS that caused 50% erythrocyte haemolysis is the mean corpuscular fragility (MCF) value.[27,29]

The relative capacity of aqueous extracts of *N. tabacum* to stabilize or disrupt erythrocyte membrane was evaluated as percentage of erythrocyte membrane stability (%EMS) is given as;

$$\%\text{EMS} = \frac{(\text{MCF}_{control} - \text{MCF}_{test}) \cdot 100}{\text{MCF}_{control}}$$

Statistical analysis

The results were expressed in terms of arithmetic mean (□) ± standard deviation (SD). The correlation coefficients were determined with Excel Software (Microsoft, 2010 version) and data were analyzed by Student's *t*-test as described by Pearson and Hartley.[39] Values of $p < 0.05$ were considered statistically significant.

RESULTS

The phytochemical screening of *N. tabacum* leaf extract showed positive for tannins, flavonoids, alkaloids and carbohydrates.

The results presented in Figure 1 showed that human erythrocytes incubated in 1.6 mg/100 mL of *N. tabacum* aqueous leaf extract exhibited haemolysis level of 96.4 ± 0.19% ([PBS] = 0.18 g/100 mL). At [PBS] = 0.9 mg/100 mL, [*N. tabacum*] = 0.4 mg/100 mL caused 12.4 ± 0.25% haemolysis. The highest haemolysis at [PBS] = 0.9 mg/100 mL was promoted by [*N. tabacum*] = 2.0 mg/100 mL with

Table 1 Phytochemical and biochemical constituents of *N. tabacum* leaf extract

Phytochemicals	Presence
Tannins	+
Alkaloids	+
Steroids	–
Flavonoids	+
Reducing Sugars	–
Carbohydrates	+
Proteins	–
Terpenoids	–

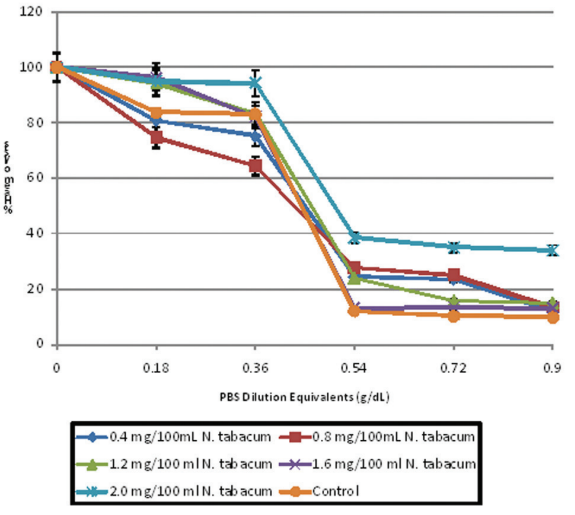

Figure 1. Osmotic fragility curve of human erythrocytes incubated in aqueous extracts of *N. tabacum*. %haemolysis is values of 6 determinations ± S.D (Microsoft Office Excel, 2007 version).

value of 34.1 ± 0.35% haemolysis. Erythrocytes incubated in [PBS] = 0.36 g/100 mL in the presence of [*N. tabacum*] = 2.0 mg/100 mL gave 94.3 ± 0.62% haemolysis. For the control samples, within the experimental range [PBS] = (0-18 – 0.90 g/100 mL), haemolysis was in the order 83.7 ± 0.36, 83.0 ± 0.51, 12.0 ± 0.71, 10.3 ± 0.55 and 9.8 ± 0.23%.

A cursory observation of the pattern of haemolysis (Figure 1) showed that erythrocytes incubated in the five experimental concentrations of *N. tabacum* exhibited higher tendency to haemolyze in [PBS] > 0.54 g/100 mL, compared with the control samples. Conversely, erythrocytes incubated in aqueous extracts of [*N. tabaccum*] = 0.4 and 0.8 mg/100 mL showed relatively lower level of haemolysis in [PBS] < 0.54 g/100 mL, compared with the control samples. The MCF value of human erythrocytes in the absence of aqueous extract of *N. tabacum* was 0.444 ± 0.03% with cumulative haemolysis of 43.90 ± 0.52 %haemoloysis.g/100 mL.

The highest cumulative haemolysis of 59.36 ± 0.91%haemoloysis.g/100 mL was registered by erythrocytes incubated in [*N. tabacum*] = 2.0 mg/100 mL. Whereas [*N. tabacum*] = 0.4 mg/100 mL caused 1.58% erythrocyte membrane destabilization, higher concentrations of 0.8 – 1.6 mg/100 mL of the aqueous extracts stabilized erythrocyte membrane (Table 2). However, the MCF values of erythrocytes incubated in aqueous extracts [*N. tabacum*] = 0.8 – 1.6 mg/100 mL were not significantly different ($p > 0.05$) from the control samples. Experimental [*N. tabacum*] = 2.0 mg/100 mL caused destabilization of erythrocyte membrane by 13.3%. Erythrocytes incubated in [*N. tabacum*] = 2.0 mg/100 mL gave MCF = 0.503 ± 0.04; $p < 0.05$.

Methaemoglobin content

Table 3 showed that %MetHb of control and erythrocytes treated with aqueous extracts of *N. tabacum* ranged between 0.58 ± 0.09 and 1.89 ± 0.12%. Specifically, erythrocytes treated with [*N. tabacum*] = 0.4 mg/100 mL gave %MetHb = 0.59± 0.08; $p < 0.05$. Higher concentrations of aqueous extracts of *N. tabacum* between the range of 0.8 – 1.6 mg/100 mL caused concentration dependent increase in erythrocyte %MetHb that was not significantly different ($p > 0.05$) from the control sample.

Table 2 Fragility indices of human erythrocytes incubated in aqueous extracts of *N. tabacum*

[*N. tabacum*] mg/100 mL	MCF Value (g/100mL)	%EMS	AUC (%haemoloysis.g/100 mL)
Control	0.444 ± 0.03[a]	–	43.90 ± 0.52
0.4	0.451 ± 0.11[a]	1.58[d]	46.84 ± 0.73
0.8	0.431 ± 0.09[a]	2.92[s]	46.93 ± 0.92
1.2	0.461 ± 0.04[a]	3.83[s]	49.47 ± 0.81
1.6	0.443 ± 0.05[a]	0.23[s]	47.05 ± 0.95
2.0	0.503 ± 0.04[b]	13.3[d]	59.36 ± 0.91

MCF values are means of 6 determinations ± S.D (Microsoft Office Excel, 2007 version); Values with superscript of the same alphabet are not significantly different p > 0.05; b: Difference in MCF values is significant (p < 0.05); d: Percentage of membrane destabilization; s: Percentage of membrane stabilization. Correlation coefficient (r) between MCF and cumulative haemolysis = 0.933379.

Table 3 Methaemoglobin content of erythrocytes incubated in aqueous extracts of *N. tabacum*

[*N. tabacum*] mg/100 mL	Control	0.4	0.8	1.2	1.6	2.0
%MetHb	1.89 ± 0.12[a]	0.59± 0.08[b]	1.63± 0.08[a,c]	1.75 ± 0.07[a,d]	1.55 ± 0.10[a,e]	0.58 ± 0.09[bf]

%MetHb values are means of 6 determinations ± S.D (Microsoft Office Excel, 2007 version. Values with superscript of the same alphabet are not significantly different p > 0.05.

Experimental [*N. tabacum*] = 2.0 mg/100 Ml engendered a paradoxical reduction in erythrocyte %MetHb = 0.58 ± 0.09; $p > 0.05$ compared with the control sample. Erythrocyte %MetHb and MCF values in the presence of the five experimental concentrations (0.4 – 2.0 mg/100 mL) of aqueous extracts of *N. tabacum* gave a medium negative correlation coefficient ($r = –0.62421$).

DISCUSSION

The haemolysis profile of human erythrocytes in varying dilution equivalents of PBS (Figure 1) conformed with the pattern earlier reported elsewhere.[29;40;41] The present study used mean corpuscular fragility (MCF) and cumulative haemolysis, defined by the area under the curve (AUC) of the plot of %haemolysis versus dilution equivalents of PBS, as indices for ascertaining the level of erythrocyte fragility. The strong positive correlation ($r = 0.933379$) between $MCF_{(g/100mL)}$ and $AUC_{(\%haemoloysis.g/100\ mL)}$ was an indication that the two parameters could be used in concert and are reliable indices for ascertaining erythrocytes hemolytic properties. Although [*N. tabacum*] = 0.4 – 1.6 mg/100 mL altered erythrocyte membrane stability (Table 2), the capacity of the plant extract to stabilize/destabilize membrane integrity was not statistically significant ($p > 0.05$). Previous studies have reported protective and destabilizing effects of varieties of plant extracts.[29,42,43] They noted that the presence of phytochemicals such as tannins, saponin and related membrane chaotropic agents were responsible for destabilizing effects of certain plant extracts. In the same vein, plant extracts have been widely reported to promote protective effect on biomembrane. There are reports on the protective effects of plant components such as polyphenolic phytochemicals, terpenoids and flavonoids on biomembranes.[43–45] Therefore, the capacity of plant extract to interfere with membrane integrity is the additive effects of combination of plant components.[45]

The medium negative correlation ($r = –0.62421$) between MCF and %MetHb was an indication that *N. tabacum* destabilizing effect, exemplified by [*N. tabacum*] = 0.2 mg/100 mL; %MetHb = 0.58 ± 0.09 (Table 3); %EMS = 13.3^{d} (Table 2), was not an oxidative process, but by mechanical perturbation by constituents of the plant extract on erythrocyte membrane structural components. Furthermore, incubation of erythrocytes in [*N. tabacum*] = 0.2 mg/100 mL did not cause significant ($p > 0.05$) increase in %MetHb level (Table 3); an oxidative stress indicator.[22,32,46] In corroboration with the present report, Levander *et al.*,[47] had posited that erythrocytes from vitamin E deficient lead-poisoned rat exhibited increased mechanical fragility caused, not by oxidative damage, but by erythrocyte membrane 'tanning' effect. The 'tanning' or toughing effect of lead on membrane assemblies renders the erythrocytes less deformable and less resistant to mechanical trauma. Likewise, Cruz Silva *et al.*,[20] noted that 4-Hydroxytamoxifen (OHTAM) promotes perturbation of the biomembrane backbone region due to its strong binding to proteins with consequent formation of membrane paths of permeability to small solutes and retention of large solutes like haemoglobin, followed by osmotic swelling and cell lysis. Custo´ dio *et al.*,[48] had earlier posited that OHTAM strongly partitioned in biomembranes and distributed in the outer region of the lipid bilayer, mainly in the lipid–protein interfaces,[49] may have accounted for membrane structural perturbations reflected in the permeability status.

Erythrocyte %MetHb in the presence of the five experimental [*N. tabacum*] = 0.4 – 2.0 mg/100 mL did not cause toxic methaemoglobinemia *in vitro*. Toxic methaemoglobinemia is diagnostic when erythrocyte %MetHb > 15% of total haemoglobin.[30,50] Table 3 showed that erythrocyte %MetHb was within the normal physiologic range of 1.0-3.0%.[51,52] Thus suggesting that the experimental concentration of *N. tabacum* extracts did not induce methaemoglobinemia *in vitro*. *N. tabacum* extract contains flavonoids (Table 1) which has been widely reported to be an anti-oxidant principle.[53,54] Therefore, the phytochemical constituents of *N. tabacum* served to protect haemoglobin molecules against oxidative events that could elicit methaemoglobinemia *in vitro*. Furthermore, erythrocyte anti-oxidant defense and protective systems[30,32,55–57] were not overwhelmed by oxidizing species that may have been generated during the incubation of erythrocytes in the plant extract.

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