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Original Article

In vitro Erythrocyte Membrane Stabilization Properties of *Solanum aethiopicum* L. Fruit Extracts

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ABSTRACT: This study aims at evaluating the membrane stabilization potential of *Solanum aethiopicum* L. fruits extracts using an *in vitro* hemolytic assay. 2 mL of blood from healthy volunteers and patients with serologically confirmed current dengue infection were freshly collected and used in the assays. Fresh garden eggs fruits at three different maturity stages (immature, partly matured and matured) were cleaned with distilled water, crushed and the juice was extracted with 10 mL of ice cold distilled water. Freshly prepared cold water extracts of garden eggs fruits were used in the heat-induced and hypotonic-induced hemolytic assays. Extracts of garden egg fruits of all three maturity levels showed a significant reduction in heat-induced hemolysis compared to controls ($P < 0.05$). Garden egg fruits extracts of all three maturity levels showed more than 25% inhibition at a concentration of 37.5µg/mL. Inhibition activity of different maturity levels was not significantly ($P > 0.05$) different from one another. *S. aethiopicum* L. fruit extracts showed a significant inhibition of hemolysis *in vitro*.

KEYWORDS: *Solanum aethiopicum* L., erythrocyte membrane stabilization, dengue infection, hemolytic assays, membrane stabilization.

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INTRODUCTION

Solanum aethiopicum (African garden egg) is a fruiting plant of the genus Solanum and family Solanaceae. It is widely cultivated for consumption as a fresh fruit, juice and a dried and crystallized fruit and it is mostly found in Asia and tropical Africa, grows about 2.5m tall, and bears fruits varying in shapes and colors. Some West African species bear edible fruits; they include *S. aethiopicum, S. melogena, S. macrocarpon* and *S. muricatum.*[1] The fruits of these species are awakening a growing interest in the specialty and exotic vegetable markets of the world. This is probably associated with the increase in the interest for African cuisine and with African immigration and the potentials of the fruits to improve nutrition.[2] The fruit turns bright red when ripe. The fruits are usually harvested while still green before the skin becomes thick.[3]

Interestingly, *S. aethiopicum* L. fruit juice and leaves extracts have demonstrated anti-cancer,[4] anti-oxidative,[5] anti-inflammatory[6] and anti-bacterial[7] properties. In addition, nephro-protective[8] and hepato-protective[9] activity against toxins, hypoglycemic, and hypolipidemic effects[10] and anti-sickling properties in sickle cell disease[11] have also been reported. Furthermore, these extracts have effectively been used for the treatment of burns[12] and chronic skin ulcers.[13] *S. aethiopicum* has been used for centuries in ethnomedicine to treat many diseases and symptoms, mature ripe fruits have been used as an effective remedy against ringworms.[14] Green fruits, on the other hand, have been used to lower blood pressure, and as an aphrodisiac. In folk medicine, they were used to reduce inflammation and pain due to their analgesic properties. Women in India, Bangladesh, Pakistan, Sri Lanka, and other countries have long used green garden eggs as a folk remedy for contraception and abortion.[14]

There are specialized cells (laticifers) that secrete a substance known as 'latex' that are dispersed with in most of the tissues of the plant.[15] The phytochemical analysis of the garden eggs has shown that they contain saponins, cardiac glycosides, and alkaloids.[16] There is emerging evidence for possible beneficial effects of the extracts of *S. aethiopicum* fruits in the treatments of patients with dengue viral infections.[17] Dengue viral infection caused by a Flavi virus is the most important mosquito borne disease in the tropical and sub-tropical regions of the world. Annually, 100 million cases of dengue fever and half a million cases of Dengue Hemorrhagic Fever (DHF)

are reported worldwide with a mortality rate of 5%.[18] Thrombocytopenia in dengue is considered to be an immune related, molecular mimicry involving dengue viral particles and the platelet leads to auto-destruction of the platelets by Immunoglobulin M (IgM) antibodies.[19,20] Interestingly, *S. aethiopicum* fruits extracts have demonstrated a positive effect on increasing platelets counts in healthy mice.[21,22] However, the underlying mechanism for this is hitherto unexplored. Any compound or drug having a stabilization effect on the plasma membrane may effectively enhance survival of platelets with a potential morbidity and mortality benefits in patients with dengue viral infections. Erythrocytes membrane is the model system used for many *in vitro* investigations of drug and membrane interactions.[23] This study aims to investigate the membrane stabilization potential of *S. aethiopicum* L. fruit extracts using an *in vitro* hemolytic assay.

MATERIALS AND METHODS

Blood samples were collected from healthy volunteers and patients in between June and August 2014. Informed written consent was obtained and ethical approval was obtained from the Ethics Review Committee. All chemicals used in the study were purchased from Sigma-Aldrich chemicals unless otherwise stated.

Preparation of blood samples for membrane stabilization assays

Two milliliters of blood from healthy volunteers and patients with serologically confirmed acute dengue viral infections were freshly collected into K_3 EDTA tubes. All the blood samples were stored at 4°C for 24 h before use. An aliquot of 1.0 mL of blood from healthy and dengue volunteers were separately transferred into 1.5 mL micro-centrifuge tubes and was centrifuged at 2500 rpm for 5 min and the supernatant was removed. The cell suspension was washed with sterile saline solution (0.89% w/v NaCl) and centrifuged at 2500 rpm for 5 min. This was repeated three times till the supernatant was clear and colorless and the packed cell volume (PCV) was measured. The cellular component was reconstituted to a 40% suspension (v/v) with phosphate buffered saline (10 mM, pH 7.4) and was used in the assays.

Preparation of garden egg fruits extracts

Fresh garden egg fruits of three different stages of maturity were collected from a healthy *S. aethiopicum* plant. The fruits were cleaned with distilled water, crushed, and the extract was collected with 10 mL of cold distilled water. The extract was filtered and centrifuged at 10,000 rpm. Freshly prepared cold water extracts of garden egg fruits were used in the heat-induced and hypotonic-induced hemolytic assays. In dose response experiments, freeze dried extracts of the partly matured fruits were used.

Heat-induced hemolysis assay

The heat-induced hemolysis of erythrocytes was carried out as described by Okoli *et al*.[24] with some modifications. Preliminary tests were done to establish the suitable incubation time for the heat-induced hemolysis. 20 µL of prepared erythrocyte suspension was mixed with 980 µL of pre-incubated buffer in a 1.5 mL micro-centrifuge tube, incubated in a water bath at 55°C and monitored by calibrated mercury thermometer. Tubes were drawn from the water bath after 5, 10, 15, 20, 25, 30 35, 40 and 45 min of incubation and centrifuged at 5000 rpm at 4°C for 5 min. Absorbance of the supernatant was measured at 540 nm. Following these observations, 20 min of incubation at 55°C was selected to study the effect of garden egg fruits extracts on heat-induced hemolysis.

To evaluate the effect on heat-induced hemolysis, 30 µL from garden egg fruits extracts and 20 µL from erythrocytes suspension (40%) was mixed with pre-incubated buffer (950 µL) in a 1.5 mL microcentrifuge tube and incubated in a water bath at 55°C for 20 min. Then samples were centrifuged at 5000 rpm at 4°C for 5 min and absorbance of the supernatant was recorded at 540 nm. Aspirin (90.0 µg/ mL) was used as the positive control and phosphate buffered saline was used as the negative control. Any influence on absorbance by the garden egg fruits extract was corrected with sample negative controls.

To evaluate the dose response effect on heat-induced hemolysis, the freeze dried extract of partly mature garden eggs fruits were dissolved in distilled water and diluted to serve six different concentrations (9.375, 18.75, 37.5, 75, 150, and 300 µg/mL) before using in the assay as described previously. Blood samples from six different dengue subjects were used in this assay and the degree of hemolysis inhibition of the garden egg fruits extracts was calculated using the following formula:

$$\%\ \text{inhibition of hemolysis} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Hypotonicity-induced hemolysis

The hypotonicity-induced hemolysis was carried out as was described by Umapathy *et al*.,[25] with some modifications. A reaction volume of 1 mL containing 37.5 µg/mL of garden eggs extract from partly matured fruits and 950 µL of phosphate buffered saline was mixed with 20 µL of 40% (v/v) erythrocyte suspension. The samples were incubated for 1 h at room temperature (30°C) and subsequently centrifuged at 5000 rpm for 5 min and 200 µL of supernatant was transferred to a microtitre plate. The free hemoglobin was measured spectrophotometrically at 540 nm. Indomethacin was used as the standard. The negative and positive controls of 0% and 100% lysis were determined by incubating cells with phosphate buffered saline 0.1% (w/v) and distilled water, respectively. The experiment included triplicates at each concentration. The degree of hemolysis inhibition was calculated using the same formula as for the heat-induced hemolysis.

RESULTS

Effect on heat-induced hemolysis

Absorbance (at 540 nm) of supernatant in dengue-infected subjects and healthy volunteers' erythrocyte suspensions at different time intervals of incubation at 55°C are presented in Figure 1. Both dengue patients and healthy volunteers erythrocytes showed a similar pattern in heat-induced hemolysis and at each time point absorbance of dengue patients erythrocytes was not significantly ($P >$

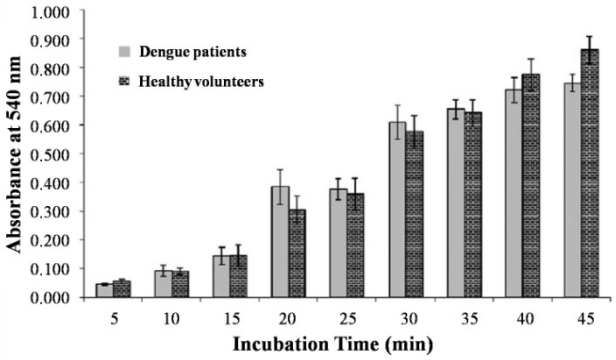

Figure 1. Heat-induced hemolysis of erythrocytes at 55℃ at different incubation times.

Table 1. Effect of garden egg fruits extracts on heat-induced hemolysis.

Sample	Concentration (µg/mL)	% Inhibition of heat-induced hemolysis	
		Healthy volunteers	Dengue patients
Immature fruits	37.5	35.0 ± 3.4[a]	25.7 ± 7.5[a]
Partly mature fruits	37.5	38.8 ± 5.0[a]	32.5 ± 8.6[a]
Mature fruits	37.5	31.8 ± 5.8[a]	29.2 ± 7.1[a]
Aspirin	90.0	45.9 ± 3.9[a]	43.6 ± 9.4[a]

*Values presented are mean ± SE of five replicates. Values in a column with the same superscript letters are not significantly different (P > 0.05).

Table 2. Effect of partly mature garden egg fruits extract on heat-induced hemolysis of dengue infected subjects.

Subject	Control Absorbance	Garden egg (37.5 µg/mL)		Aspirin (90.0 µg/mL)	
		Absorbance	% Inhibition	Absorbance	% Inhibition
1	0.185	0.134	27.568	0.165	19.730
2	0.281	0.179	36.180	0.157	44.247
3	0.334	0.208	49.934	0.153	71.542
4	0.173	0.091	76.636	0.087	80.062
5	0.213	0.147	30.986	0.157	26.526
Mean ± SEM	0.223 ± 0.031	0.144 ± 0.020	42.08 ± 0.020*	0.139 ± 0.014	44.54 ± 11.50*

*No significant difference

0.05) different from normal cells. Absorbance was significantly increased at 20 min than at 15 min for both groups and the reading was around 0.4 (P < 0.05). Absorbance at 25 min was not significantly higher than at 20 min. Both dengue-infected subjects and healthy volunteers' erythrocytes showed marked heat-induced hemolysis at 20 min of incubation at 55°C. Hence, 20 min of incubation at 55°C was selected as the suitable incubation time for the experiments.

Inhibition of heat-induced hemolysis by cold water extracts of garden eggs fruits at different maturity levels is presented in Table 1. Compared to the controls, fresh extracts of garden eggs fruits showed a significant reduction in heat-induced hemolysis in all maturity levels (P < 0.05). Garden egg fruits extracts of three maturity levels showed more than 25% inhibition at 37.5 µg/mL concentration (Table 1). However, there was no significant difference (P > 0.05) among the three maturity stages in their level of inhibition of hemolysis (Table 1). The results of successive experiments carried out using partly matured fruits extracts on erythrocytes of dengue-infected patients are presented in Table 2. The repeated experiments showed similar results as was in the previous assay.

The inhibition of heat-induced hemolysis in dengue-infected patients at different concentration of partly matured garden egg fruits extracts are illustrated in Figure 2. The highest degree of inhibition of heat-induced hemolysis was observed at 37.5µg/mL of garden egg fruits extracts. This was not statistically significant in terms of the level of inhibition of hemolysis compared to 18.75 µg/mL and 75 µg/mL concentrations of garden egg fruits extracts. Hemolysis inhibition activity of garden egg fruits extracts did not demonstrate a linear dose response relationship (Figure 2).

Effect on hypotonicity-induced hemolysis

The effect of partly matured garden egg fruits extracts on hypotonicity-induced hemolysis of healthy volunteers and dengue-infected patient's erythrocytes were studied using five different concentrations (Table 3, 4). Garden egg fruits extracts at 37.5µg/mL concentration showed a marked inhibition of hypotonicity-induced hemolysis in both groups.

DISCUSSION

This is the first report on the *in vitro* membrane stabilization potential of *S. aethiopicum* fruits extracts. In this study, we demonstrated that *S. aethiopicum* fruits extracts inhibit heat-induced and hypotonicity-induced hemolysis of erythrocytes derived from both healthy individuals and patients with dengue viral infections. This indicates that *S. aethiopicum* fruits extracts possess biological membrane stabilization properties preventing stress-induced destruction of the plasma membrane.

The exact underlying mechanism for the membrane stabilizing effect of *S. aethiopicum* fruits extracts and the chemical constituent(s) responsible for this effect is hitherto not known. However, a number of studies have shown that flavonoids[26] and a host of other plant compounds[27] exhibit analgesic and anti-inflammatory effects as a result of their membrane stabilizing ability in various experimental models. It has also been shown that *S. aethiopicum* extracts contain flavonoids such as kaempferol, quercetin and *p*-coumaric acid.[28] Thus, it is not unreasonable to postulate that flavonoids and other phenolic compounds in *S. aethiopicum* fruits extracts

Table 3. Effect of garden egg fruits extracts on hypotonicity-induced hemolysis of healthy volunteers.

Subject	Control Absorbance	Garden egg (37.5µg/mL)		Indomethacin	
		Absorbance	% Inhibition	Absorbance	% Inhibition
1	0.468	0.2527	46.0[a]	0.171	63.4[a]
2	0.410	0.3360	18.0[a]	0.262	36.0[a]
3	0.561	0.5013	10.6[a]	0.403	28.2[a]
4	0.544	0.3417	37.2[a]	0.245	55.0[a]
5	0.182	0.0983	46.0[a]	0.081	55.7[a]
Mean ± SEM	0.433 ± 0.076	0.306 ± 0.073	31.57 ± 8.17*	0.232 ± 0.059	47.67 ± 7.41*

*No significant difference, values in a column with the same superscript letters are not significantly different ($P > 0.05$)

Table 4. Effect of garden egg fruits extracts on hypotonicity-induced hemolysis of dengue-infected patients.

Subject	Control Absorbance	Garden egg (37.5µg/mL)		Indomethacin	
		Absorbance	% Inhibition	Absorbance	% Inhibition
1	0.529	0.353	33.3	0.363	31.6
2	0.217	0.063	71.0	0.032	85.2
3	0.336	0.082	75.6	0.044	86.9
4	0.405	0.132	67.4	0.081	79.9
5	0.334	0.208	37.8	0.190	43.1
Mean ± SEM	0.364 ± 0.051	0.167 ± 0.059	57.03 ± 9.93*	0.142 ± 0.062	63.35 ± 13.01*

*No significant difference

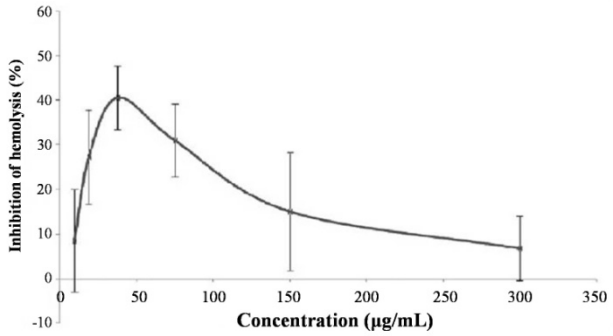

Figure 2. Effect of different garden egg fruits extract concentrations on heat-induced hemolysis.

could be responsible for the observed membrane stabilizing effect in this study. Our results also highlight that *S. aethiopicum* fruits extracts do not demonstrate a linear dose-response relationship. Instead the observed dose-response relationship forms hormetic dose-response relationship (a left-shifted bell shaped curve) where the beneficial effects observed at low doses are absent at higher concentrations.[31] Such dose-response relationships have been reported to occur with a wide range of chemotherapeutics including antibiotics, antiviral, and antitumor agents.[32] We were unable to evaluate a dose-response effect on hypotonicity-induced hemolysis due to the small number of samples. Further studies are required for the isolation of active constituent(s) and elucidation of mechanism(s) of action. We recommend further *in vitro* and *in vivo* studies to evaluate the clinical efficacy of *S. aethiopicum* fruits extracts in different disease conditions.

CONCLUSION

Solanum aethiopicum extracts from partly matured fruits demonstrated a significant inhibition of hemolysis *in vitro*. The inhibition effect shown by crude extracts of the *S. aethiopicum* fruits at comparatively lower concentrations (37.5µg/mL) was comparable with that of standard anti-hemolysis compounds such as aspirin and indomethacin. This experimental evidence indicates that *S. aethiopicum* L. fruits extracts could have a potential therapeutic efficacy in disease processes causing destabilization of biological membranes.

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