In vitro erythrocyte membrane stabilization properties of *Carica papaya* L. leaf extracts

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ABSTRACT

Background: *Carica papaya* L. fruit juice and leaf extracts are known to have many beneficial medical properties. Recent reports have claimed possible beneficial effects of *C. papaya* L. leaf juice in treating patients with dengue viral infections. This study aims to evaluate the membrane stabilization potential of *C. papaya* L. leaf extracts using an *in vitro* hemolytic assay. **Materials and Methods:** The study was conducted in between June and August 2010. Two milliliters of blood from healthy volunteers and patients with serologically confirmed current dengue infection were freshly collected and used in the assays. Fresh papaya leaves 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 cold distilled water. Freshly prepared cold water extracts of papaya leaves (1 ml containing 30 µl of papaya leaf extracts, 20 µl from 40% erythrocytes suspension, and 950 µl of phosphate buffered saline) were used in the heat-induced and hypotonic-induced hemolytic assays. In dose response experiments, six different concentrations (9.375, 18.75, 37.5, 75, 150, and 300 µg/ml) of freeze dried extracts of the partly matured leaves were used. Membrane stabilization properties were investigated with heat-induced and hypotonicity-induced hemolysis assays. **Results:** Extracts of papaya leaves of all three maturity levels showed a significant reduction in heat-induced hemolysis compared to controls ($P < 0.05$). Papaya leaf extracts of all three maturity levels showed more than 25% inhibition at a concentration of 37.5 µg/ml. The highest inhibition of heat-induced hemolysis was observed at 37.5 µg/ml. Inhibition activity of different maturity levels was not significantly ($P > 0.05$) different from one another. Heat-induced hemolysis inhibition activity did not demonstrate a linear dose response relationship. At 37.5 µg/ml concentration of the extract, a marked inhibition of hypotonicity-induced hemolysis was observed. **Conclusion:** *C. papaya* L. leaf extracts showed a significant inhibition of hemolysis *in vitro* and could have a potential therapeutic effect on disease processes causing destabilization of biological membranes.

Key words: *Carica papaya* L., erythrocyte, *in vitro*, membrane-stabilization

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INTRODUCTION

Carica papaya L. is the only species within the Caricaceae genus and the palm-like tree has segmented leaves, yellow flowers, and large black seeded yellow to orange fruits.[1] It is widely cultivated for consumption as a fresh fruit, juice, and a dried and crystallized fruit. The extracts of both the leaves and fruit are known to contain several proteins and alkaloids with important pharmaceutical, medical, and industrial applications. Interestingly, *C. papaya* L. fruit juice and leaves extracts have demonstrated anti-cancer,[2] anti-oxidative,[3] anti-inflammatory,[4] and anti-bacterial[5] properties. In addition, nephro-protective[6] and hepato-protective[7] activity against toxins, hypoglycemic, and hypolipidemic effects[8] and anti-sickling properties in sickle cell disease[9] have also been reported. Furthermore, these extracts have effectively been used for the treatment of burns[10] and chronic skin ulcers.[11] *C. papaya* L. has been used them for centuries in ethnomedicine to treat many diseases and symptoms, mature ripe fruits have been used as an effective remedy against ringworms.[12] Green fruits, on the other hand, have been used to lower blood

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pressure, and as an aphrodisiac. Papaya leaves were eaten and used as a heart tonic and analgesic. 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 papaya as a folk remedy for contraception and abortion.[12]

There are specialized cells (laticifers) that secrete a substance known as 'latex' that are dispersed with in most of the tissues of the plant.[13] Latex is a complex mixture of chemical compounds with diverse chemical activities. Cysteine proteinases that constitute as high as 80% of the enzyme fraction in papaya latex are thought to be responsible for nearly all of the medicinal properties of the plant. Among the most studied proteinases from papaya include papain, chymopapain, caricain, and glycyl endopeptidase.[13] The phytochemical analysis of the papaya leaves has shown that they contain saponins, cardiac glycosides, and alkaloids.[14] *C. papaya* leaves are a rich source of mineral elements such as Ca, Mg, Na, K, Fe, and Mn.[14]

There is emerging evidence for possible beneficial effects of the extracts of *C. papaya* L. leaves in the treatments of patients with dengue viral infections.[15] Dengue viral infection caused by a Flavi virus is the most important mosquito borne disease in the tropical and sub-tropical regions and, at present, dengue is endemic in 112 countries in 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%.[16] Some patients with Dengue viral infection develop DHF or Dengue Shock Syndrome (DSS) with plasma leakage as a consequence of increased vascular permeability and enhanced capillary fragility.[16] Thrombocytopenia is one of the key clinical manifestations in dengue viral infections and contributes to the plasma leakage and hemorrhage in DSS/DHF in the presence of enhanced vascular fragility.[16] 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.[17-19]

Interestingly, *C. papaya* L. leaves extracts have demonstrated a positive effect on increasing platelets counts in healthy mice.[20] 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.[21] This study aims to investigate the membrane stabilization potential of *C. papaya* L. leaves extracts using an *in vitro* hemolytic assay.

MATERIALS AND METHODS

Blood samples were collected from healthy volunteers and patients at Professorial Medical unit, Colombo South Teaching Hospital, Kalubowila, Sri Lanka in between June and August 2010. Informed written consent was obtained and ethical approval was obtained from the Ethics Review Committee of the Colombo South Teaching Hospital. *In vitro* testing was done at Industrial Technology Institute, Colombo, Sri Lanka. All chemicals used in the study were purchased from Sigma-Aldrich chemicals (USA) unless otherwise stated.

Total phenolic and flavonoid content of papaya leaf extracts

Total phenolic content in the cold water extract of Papaya leaves at three different maturity stages were determined using the Folin–Ciocalteu method.[22] Six different cold water extracts from each maturity stage was diluted in distilled water and 20 µl from each concentration was incubated with 110 µl of Folin–Ciocalteu reagent and 70 µl of 10% sodium carbonate at room temperature for 30 min, followed by absorbance reading at 670 nm. Gallic acid in five different concentrations (12.5, 25.0, 50.0, 100, and 200 µg/ml) were used to construct the standard curve. Total phenolic content in leaf extracts were estimated as Gallic acid equivalent (GAE) per g of dry matter.

Total flavonoid content was determined using the aluminum chloride method. Six different cold water extracts from each maturity stage was diluted in distilled water and 100 µl from each concentration was incubated with 100 µl of 2% aluminum chloride (in methanol) at room temperature for 10 min, followed by absorbance reading at 365 nm using a SPECTRAmaxPluse384 Microplate reader (Molecular Devices, Inc., USA). Five different concentrations (7.81, 15.62, 31.25, 62.5, and 125.0 µg/ml) of quercetin were used to construct the standard curve. Total flavonoid in leaf extracts were estimated as quercetin equivalent (QE) per g of dry matter.

Preparation of blood samples for membrane stabilization assays

Two millilitres of blood from healthy volunteers and patients with serologically confirmed acute dengue viral infections were freshly collected into K_3EDTA (F.L. Medical s.r.l. Torreglia, Italy) 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 papaya leaf extracts

Fresh papaya leaves of three different stages of maturity (immature, partly mature, and mature) were collected from a healthy Papaya tree at Industrial Technology Institute (ITI), Colombo, Sri Lanka. The leaves 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 papaya leaves were used in the heat-induced and hypotonic-induced hemolytic assays. In dose response experiments, freeze dried extracts of the partly matured leaves were used.

Heat-Induced hemolysis assay

The heat-induced hemolysis of erythrocytes was carried out as was described by Okoli *et al*.,[23] with some modifications. Preliminary tests were done to establish the suitable incubation time for the heat-induced hemolysis. Twenty microlitres (20 µl) of prepared erythrocyte suspension was mixed with 980 µl of pre-incubated buffer in a 1.5 ml micro-centrifuge tube and incubated in a water bath at 55°C (temperature was controlled by a thermostat with an accuracy of ±0.1°C; WiseBath, Daithan Scientific Co. Ltd, Seoul, Korea) 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 at 540 nm was measured using a SPECTRAmax PLUS384™ microplate reader (Molecular Devices, Inc., CA, USA). Following these observations, 20 min of incubation at 55°C was selected to study the effect of papaya leaf extracts on heat-induced hemolysis.

To evaluate the effect on heat-induced hemolysis, 30 µl from papaya leaf 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 papaya leaf extract was corrected with sample negative controls.

To evaluate the dose response effect on heat-induced hemolysis, the freeze dried extract of partly mature papaya leaves 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 papaya leaf 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*.,[24] with some modifications. A reaction volume of 1 ml containing 37.5 µg/ml of papaya extract from partly matured leaves 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 5min and 200 µl of supernatant was transferred to a microtitre plate. The free hemoglobin was measured spectrophotometrically at 540 nm using a SPECTRAmax PLUS384™ microplate reader (Molecular Devices, Inc., US). 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 assay.

Table 1: Total extractable matters, total phenolic content and total flavonoids contents of papaya leaf extracts at different maturity levels

Maturity level	Total extractable matters (mg/ml)	Total phenolic content as GAE/g of extract	Total flavonoid content as QE/g of extract
Immature	13.0 ± 0.5[a]	34.87 ± 2.50[a]	33.79 ± 5.49[a]
Partly mature	13.7 ± 0.8[a]	35.19 ± 2.60[a]	48.67 ± 4.86[b]
Mature	13.5 ± 0.5[a]	33.71 ± 2.08[a]	65.31 ± 5.10[c]

Values are presented as mean ± SEM of six independent replicates. Values in a column with same superscript letters are not significantly different at $P > 0.05$

RESULTS

The total extractable matters, phenolic and total flavonoids contents of papaya leaves of the three different maturity levels used in study are summarized in Table 1.

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 ($P > 0.05$) different from normal cells. Up to 15 min of incubation absorbance was less than 0.2 for both groups of erythrocytes and was not significantly higher than absorbance at 10 min [Figure 1]. 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 papaya leaves at different maturity levels is presented in Figure 2. Compared to the controls, fresh extracts of papaya leaves showed a significant reduction in heat-induced hemolysis in all maturity levels ($P < 0.05$). Inhibition of heat-induced hemolysis of erythrocytes is shown in Table 2. Papaya leaf extracts of three maturity levels showed more than 25% inhibition at 37.5 µg/ml concentration [Table 2]. However, there was no significant difference ($P > 0.05$) among the three maturity stages in their level of inhibition of hemolysis [Table 2]. The results of successive experiments carried out using partly matured leaf extracts on erythrocytes of dengue-infected patients are presented in Table 3. 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 papaya leaf extracts are illustrated in Figure 3. The highest degree of inhibition of heat-induced hemolysis was observed at 37.5 µg/ml of papaya leaf 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 papaya leaf extracts. Hemolysis inhibition activity of papaya leaf extracts did not demonstrate a linear dose response relationship [Figure 3].

Effect on hypotonicity-induced hemolysis

The effect of partly matured papaya leaf extracts on

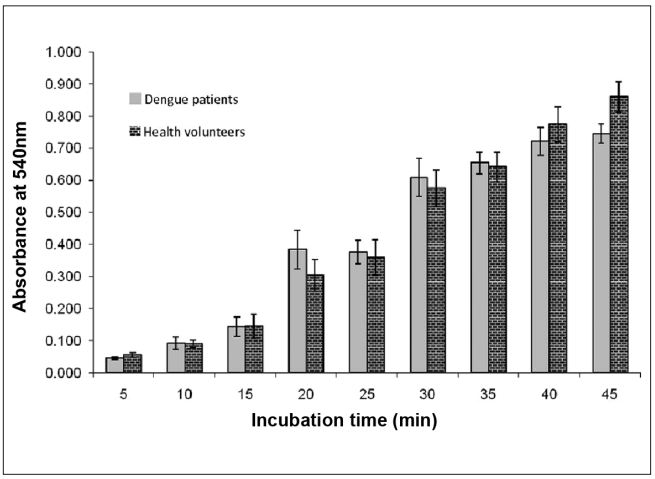

Figure 1: Heat-induced hemolysis of erythrocytes at 55°C at different incubation times

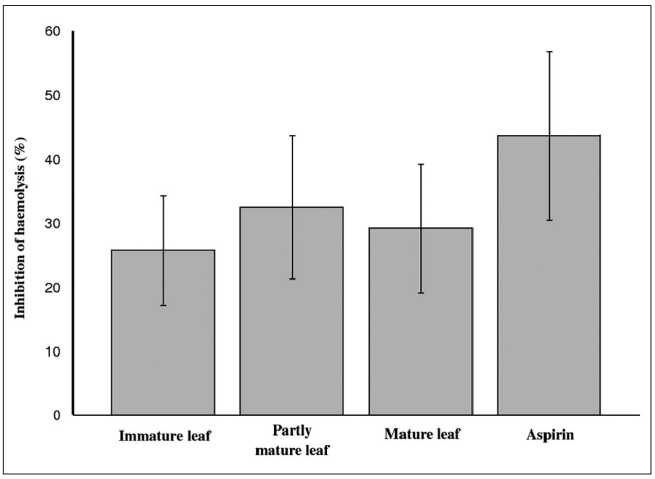

Figure 2: Inhibition of heat-induced hemolysis by papaya leaves at different maturity levels

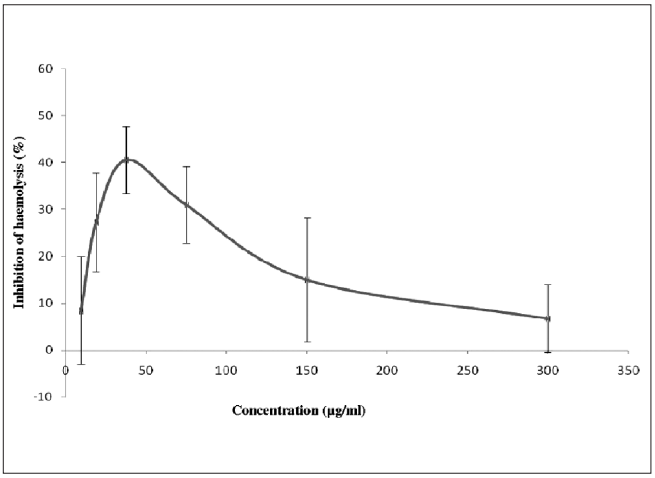

Figure 3: Effect of different papaya leaf extract concentrations on heat-induced hemolysis

hypotonicity-induced hemolysis of healthy volunteers and dengue-infected patient's erythrocytes were studied using five different concentrations [Tables 4 and 5]. Papaya leaf extracts at 37.5 μg/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 *C. papaya* L. leaf extracts. In this study, we demonstrated that *C. papaya* L. leaf extracts inhibit heat-induced and hypotonicity-induced hemolysis of erythrocytes derived from both healthy individuals and patients with dengue viral infections. This indicates that *C. papaya* L. leaf extracts possess biological membrane stabilization properties preventing stress-induced destruction of the plasma membrane.

The exact underlying mechanism for the membrane stabilizing effect of *C. papaya* L. leaf extracts and the chemical constituent(s) responsible for this effect is

Table 2: Effect of papaya leaf extracts on heat-induced hemolysis

Sample	Concentration (μg/ml)	% Inhibition of heat-induced hemolysis	
		Healthy volunteers	Dengue patients
Immature leaves	37.5	35.0 ± 3.4[a]	25.7 ± 7.5[a]
Partly mature leaves	37.5	38.8 ± 5.0[a]	32.5 ± 8.6[a]
Mature leaves	37.5	31.8 ± 5.8[a]	29.2± 7.1[a]
Aspirin	90.0	45.9 ± 3.9[a]	43.6 ± 5-9.4[a]

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

Table 3: Effect of partly mature papaya leaf extract on heat-induced hemolysis of dengue infected subjects

Subject	Control	Papaya (37.5 μg/ml)		Aspirin (90.0 μg/ml)	
	Absorbance	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
6	0.154	0.106	31.169	0.115	25.108
Mean ± SEM	0.223 ±0.031	0.144 ± 0.020	42.08 ± 8.35*	0.139 ± 0.014	44.54 ± 11.50*

*No significant difference

Table 4: Effect of papaya leaf extracts on hypotonicity-induced hemolysis of healthy volunteers

Subject	Control	Papaya (37.5 μg/ml)		Indomethacin	
	Absorbance	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 5: Effect of papaya leaf extracts on hypotonicity-induced hemolysis of dengue-infected patients

Subject	Control	Papaya (37.5 μg/ml)		Indomethacin	
	Absorbance	Absorbance	% Inhibition	Absorbance	% Inhibition
1	0.529	0.353	33.3	0.362	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	65.35 ± 13.01*

*No significant difference

hitherto not known. However, a number of studies have shown that flavonoids[25] and a host of other plant compounds[26] exhibit analgesic and anti-inflammatory effects as a result of their membrane stabilizing ability in various experimental models. It has also been shown that *C. papaya* L. leaf extracts contain flavonoids such as kaempferol, quercetin and *p*-coumaric acid.[27] The production of free radicals, such as lipid peroxides and superoxides, are reported to be accountable for cell membrane destabilization.[28] Flavonoids and other phenolic compounds are reported to act as effective scavengers of free radicals.[29] Thus, it is not unreasonable to postulate that flavonoids and other phenolic compounds in *C. papaya* L. leaf extracts could be responsible for the observed membrane stabilizing effect in this study. Previous studies have shown that *C. papaya* L. also demonstrates anti-sickling properties in a dose-dependent manner. This could be a consequence of the membrane stabilization potential of *C. papaya* L. leaf extracts that speculates a possible use of it as a phytomedicine in sickle cell disease.[8] Furthermore, the reported anti-cancer,[2] anti-inflammatory,[4] and nephro/hepatoprotective properties[6,7] of *C. papaya* L. extracts could well be due to their membrane stabilizing potential.

Our results also highlight that *C. papaya* L. leaf 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.[30] Such dose-response relationships have been reported to occur with a wide range of chemotherapeutics including antibiotics, antiviral, and antitumor agents.[31] 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 *C. papaya* leaf extracts in different disease conditions.

CONCLUSION

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

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