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Elucidation of membrane stabilizing potentials of methanolic extract of *Vigna unguiculata* (cowpea) linn (seed)



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ABSTRACT

Different concentration of methanolic extracts of seeds of *Vigna unguiculata* (cowpea) Linn collected from the local area of Noakhali, Bangladesh were studied for membrane Stabilizing Assay. *V. unguiculata* Linn seeds were initially collected, processed and extracted with methanol. Then, five different concentration (1mg/ml, 3mg/ml, 5mg/ml, 7mg/ml, 9mg/ml) of methanolic extract of Cowpea (*V. unguiculata*) were subjected for determination of membrane stabilizing activity. In the assay for membrane stabilizing activity, five

different concentration of crude methanol extract capable to inhibit hemolysis of erythrocyte membrane dose dependently in hypotonic solution and heat- induced conditions, which indicates the anti-inflammatory property of the samples. Where, Acetyl salicylic acid was used as standard drug. From the above discussion it is clear that *Vigna unguiculata* Linn seeds methanolic extract showed significant anti-inflammatory potentials. So, it will be very much possible source for isolating lead compound for curing inflammatory disorder.

Key Words: Concentration, *V. unguiculata* (Cowpea), Anti-inflammatory, Hypotonic solution, Heat- induced.

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INTRODUCTION

There is enormous evidence that, Medicinal plants were promising candidates for developing new drugs. Plants are very promising source of potential compounds for treating different types of diseases. Systematic screening of plants might be a vital tool for discovering and isolating pharmacologically active lead molecules.¹⁻³ The practice of complementary and substitute medicine is now on the increase in raising countries in response to WHO (World health organization) mandate and in this, the folk medicine is playing a vital role to treat various disease.⁴

V. unguiculata which is most usually known as “cowpea”. It is an edible legume and belongs to family *Fabaceae* with high protein extent. It is a pairing jungly glabrous, anniversary plant. The fruit legumes augment up to 90 cm in length and the pods are slightly chapfallen between the seeds. There is 10 – 20 seeds found in each pod. The seeds are varies in size, colour and shape. The seeds are sweet in nature and they have, laxative, astringent anthelmintic, antibacterial, diuretic and galactagogue properties. The seeds also help in liberating the conditions like constipation, jaundice, anorexia, and general debility.⁵

Inflammation is the one of the important processes. Inflammatory cells induce a complex mixture of growth and differentiation of cytokines as well as physiologically active arachidonate metabolites.^{6,7}

Inflammation are frequently noticed in human body for making very complicated situations. Many drugs have been developed to take care of these problems but their untoward effect still remain as a matter of concern.⁸⁻¹⁰ To obtain safer molecules, extensive studies are still going on. Natural products might be very vital resources for discovering the desired drug.

The aim of the present study was to determine the membrane stabilizing activity of methanolic extract of *V. unguiculata* Linn (seed) for the first time.

MATERIALS AND METHODS

Materials

Standard Acetyl Salicylic Acid (ASA) or Aspirin was purchased from square pharmaceuticals limited. Other chemicals needed for this investigation was provided by pharmacognosy laboratory of Noakhali Science and Technology University.

Collection of plant

For this present investigation *V. unguiculata* Linn (seeds), were collected from Noakhali, Bangladesh on april, 2015. After collection seeds were thoroughly washed with water. After collection seeds were thoroughly washed with water. The plant was identified by expert of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. (Accession number-37752).

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Preparation of methanolic extract of *V. unguiculata* seeds

500 g of the dried and powdered sample was soaked in 1000ml of methanol (99.8%). After 15 days the solution was collected through filtration by using filter cloth and Whatman® filter paper No. 1. The resulting filtrates were then evaporated in rotary evaporator below 40°C to dryness and thus a concentrated semisolid mass of the extract was obtained.

Preparation of the extract

Amount of different fractions were properly calculated and mixed with solvent (methanol) to make a concentration of 1 mg/ml, 3mg/ml, 5mg/ml, 7mg/ml, 9mg/ml

Red Blood Cells (RBC) Collection

Human RBCs were collected for the study. RBCs collected from the human was male, 65 kg, fare complexion and free from diseases. The collected RBCs were kept in a test tube with an anticoagulant EDTA under standard conditions of temperature 23±2°C and relative humidity 55±10%.

Preparation of Phosphate Buffer Solution

A buffer is an aqueous solution that has a highly stable pH. The buffer was prepared at pH 7 using monosodium phosphate and its conjugate base, disodium phosphate.

Calculation of Phosphate Buffer

A pH of about 7.4 with buffer strength of 10 mM was obtained using 0.0352% monosodium phosphate dehydrate and 0.1099% disodium phosphate anhydrate. The buffer was made by adding 0.352gm monosodium phosphate dehydrate and 1.099gm disodium phosphate anhydrate to 1000 mL water.

- pH: 7.4
- Buffer strength: 10.00 mM
- Monosodium phosphate, dehydrate: 0.0352%
- Disodium phosphate, anhydrate: 0.1099%

Preparation of Isotonic Solution

A solution that has a concentration of electrolytes, nonelectrolytes or a combination of the two that will exert equivalent osmotic pressure as that solution with which it is being compared. Either 0.16M sodium

chloride (NaCl) solution (approximately 0.95% salt in water) or 0.3M nonelectrolyte solution is approximately isotonic with human red blood cells. For the preparation of 500 ml isotonic solution of 154 mM strength, 4.5045 gm NaCl was added and mixed.

Calculation for Isotonic Solution

1000 ml solution of strength 1 M contain = 58.5 gm NaCl
 500 ml solution of strength 1 M contain = 58.5/2 gm NaCl
 500 ml solution of strength 1000 mM contain = 58.5/2 gm NaCl
 500 ml solution of strength 154 mM contain = 58.5 × 154/2 × 1000 gm NaCl
 = 4.5045 gm NaCl

Preparation of Hypotonic Solution

A solution of lower osmotic pressure than that of a reference solution or of an isotonic solution is called hypotonic solution. For the preparation of 500 ml hypotonic solution, having strength of 50 mM, 1.4625 gm NaCl was added and mixed.

Calculation for Hypotonic Solution

1000 ml solution of strength 1 M contain = 58.5 gm NaCl
 500 ml solution of strength 1 M contain = 58.5/2 gm NaCl
 500 ml solution of strength 1000 mM contain = 58.5/2 gm NaCl
 500 ml solution of strength 50 mM contain = 58.5 × 50/2 × 1000 gm NaCl
 = 1.4625 gm NaCl

EFFECT ON HEMOLYSIS

Erythrocyte Suspension

Whole blood was collected from male human under standard condition. EDTA was used to prevent clotting. The blood was washed three times with isotonic solution (154 mMNaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifuge action for 10 min at 3000 g. Thus the suspension finally collected was the stock erythrocyte (RBC) suspension.

Hypotonic Solution- Induced Hemolysis

The experiments were carried out with hypotonic solution. The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) with 5 ml of hypotonic solution (50 mM NaCl) in 10mM sodium phosphate buffer saline (pH 7.4) containing either the different methanolic extract (1.0 mg/mL) or Acetyl Salicylic Acid (0.10 mg/mL). The Acetyl Salicylic Acid was used as a reference

Table 1 Preparation of test samples

Sample name	Concentration				
1. Control	1 mg/ml				
2. Methanolic Extract	1mg/ml	3mg/ml	5mg/ml	7mg/ml	9mg/ml
3. Acetyl salicylic acid	0.10 mg/ml				

Table 2 Hypotonic solution induced hemolysis of Erythrocyte membrane by methanolic extract of *V. unguiculata* Linn (seed)

Treatment	Concentration	Mean ± SD	% of inhibition of hemolysis ± SEM
Control	1mg/ml	3.974 ± 0.011	
Methanolic extract (1mg/ml)	1mg/ml	3.658 ± 0.108	8.32 ± 1.877%
Methanolic extract (3mg/ml)	3mg/ml	3.238 ± 0.164***	18.50 ± 2.385%
Methanolic extract (5mg/ml)	5mg/ml	2.595 ± 0.171**	34.69 ± 2.486%
Methanolic extract (7mg/ml)	7mg/ml	2.107 ± 0.110***	47.09 ± 1.596%
Methanolic extract (9mg/ml)	9mg/ml	1.212 ± 0.093***	69.49 ± 1.349%
Acetyl Salicylic Acid	0.10mg/ml	1.032 ± 0.058***	74.01 ± 0.848%

Here, n=3, values are expressed as mean ± SD, * when p<0.05, ** when p<0.01 and *** when P<0.005

Table 3 Inhibition of haemolysis of erythrocyte membrane on heated and unheated solution by methanolic extract of *V. unguiculata* Linn (seed).

Treatment	Concentration	OD of sample ±SD		% inhibition of haemolysis
		Heated Solution	Unheated Solution	
Control	1mg/ml	1.093±0.035		
Methanolic extract (1mg/ml)	1mg/ml	0.879±0.107	0.856±0.011	9.7±3.31%
Methanolic extract (3mg/ml)	3mg/ml	0.735±0.004	0.623±0.017***	23.82±1.13%
Methanolic extract (5mg/ml)	5mg/ml	0.695±0.014	0.515±0.022 **	31.14±3.42%
Methanolic extract (7mg/ml)	7mg/ml	0.587±0.082	0.307±0.342**	35.62±2.06%
Methanolic extract (9mg/ml)	9mg/ml	0.525±0.008	0.109±0.021	42.27±1.87%
Acetyl Salicylic Acid	0.10mg/ml	0.672±0.025	0.129±0.029***	56.32±4.08%

Here, n=3, values are expressed as mean ± SD, * when p<0.05, ** when p<0.01 and *** when P<0.005

standard. The mixtures were incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance (O.D.) of the supernatant was measured at 540 nm using Shimadzu UV spectrophotometer.

The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ inhibition of hemolysis} = 100 \times \{ (OD1 - OD2) / OD1 \}$$

Where,

OD1 = Optical density of hypotonic-buffered saline solution alone (control) and

OD2 = Optical density of test sample in hypotonic solution.

Heat Induced Hemolysis

Aliquots (5 ml) of the isotonic buffer, containing 1.0 mg/ml of different extracts of the plant were put into two duplicate sets of centrifuge tubes.¹² The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension (30 µL) was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 54 °C for 20 min in a water bath. The other pair was maintained at 0-5 °C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 g and the absorbance of the supernatant was measured at 540 nm using UV spectrometer. The percentage inhibition or acceleration of hemolysis in tests and was calculated using the following equation:

$$\% \text{ inhibition of hemolysis} = 100 \times \{ (OD2 - OD1) / (OD3 - OD1) \}$$

Where,

OD1 = test sample unheated,

OD2 = test sample heated and,

OD3 = control sample heated

RESULTS

The membrane stabilizing activity of methanolic extract of *V. unguiculata* (seed) was assessed by evaluating their ability to inhibit hypotonic solution and heat induced hemolysis. Results are represented in table 2 and table 3 respectively. It was noticed that, different concentration of methanolic extract of *V. unguiculata* protected the hemolysis of RBC induced by heat and hypotonic solution significantly at a comparable level with the standard acetyl salicylic acid (0.1mg/ml). The 9mg/ml concentration of methanolic extract inhibited haemolysis of RBCs 69.49 % and 42.27% respectively in heat and hypotonic solution induce haemolysis condition compared to 74.01% and 56.32% by acetyl salicylic acid respectively. Where 1mg/ml, 3mg/ml, 5mg/ml, 7mg/ml concentration of methanolic extract also showed their membrane stabilizing properties in dose dependent manner (Figure-1). Where, most of the data found to statistically significant while p- value stated at less 0.05.

DISCUSSION

Since human blood cells membrane are very much close with lysosomal membrane components. Thus the prevention of hypotonicity and heat

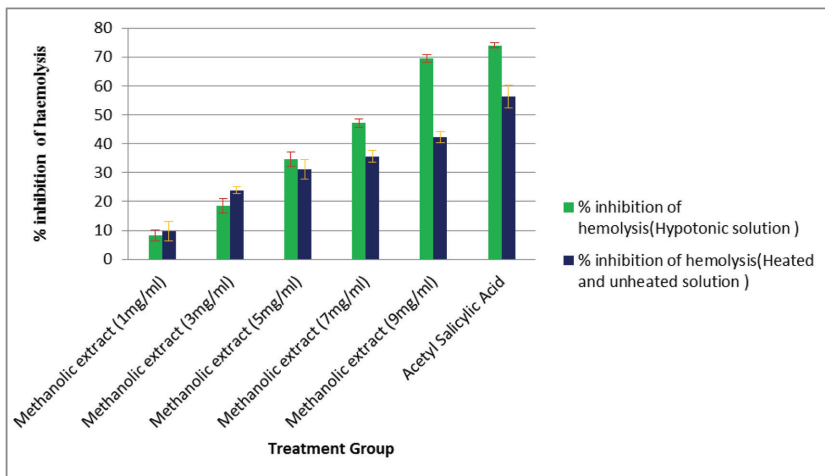


Figure 1 Effect of different concentration of methanolic extract of *V. unguiculata* on heat and hypotonic solution

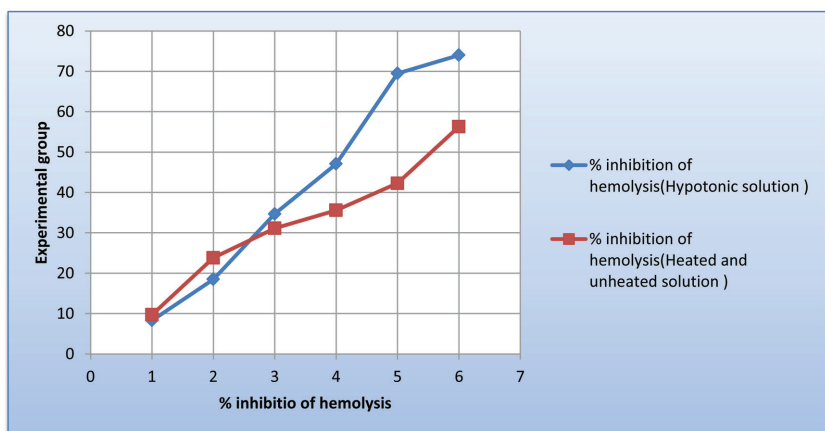


Figure 2 Graphical comparison of % of inhibition of hemolysis by methanolic extract of *V. unguiculata* at two different condition (hypotonic solution and heat induce solution)

induced HRBC membrane lysis was taken as a derminate of anti-inflammatory activity of drugs. It is now established truth that the vitality of cells depends on the integrity of their membranes.¹³ When red blood cell is exposed to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin.^{13,14} Excessive accumulation of fluid within the cell is related to the haemolytic effect of hypotonic solution thus resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical -induced lipid peroxidation.^{13,14} This observation is also consistent with the breakdown of bio-membranes leads to the formation of free radicals which in turn enhance cellular damage.^{15,16} It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances.^{17,18} Some research works were able

to reveal the name of some responsible chemical components present in the extracts, which are well known for their anti-inflammatory activity.^{19,20} Both in vitro and in vivo studies in experimental animals showed that the flavonoids exert stabilizing effects largely on lysosomes²¹ while tannin and saponins are also capable of stabilizing the erythrocyte membrane with an ability of binding with cations and other biomolecules.²² Our present plant extract also found to contain flavonoid compounds in significant amount,²³ may be that is why our plant methanolic extract showed membrane stabilizing activity. The results obtained demonstrated that Methanolic extract of *V. unguiculata* can significantly and dose dependently inhibited HRBC haemolysis. This In vitro method was more time saving, flexible and convenient in other ways. The investigation suggested that good ability of Methanolic extract to resist the cell lysis in small concentration as compared to the standard drug acetyl Salicylic Acid 0.1mg/ml.

CONCLUSION

From the study of this experiment, it may be concluded that all Methanolic extracts of the *V. unguiculata* plant have moderate to high membrane stability hence, effective anti-inflammatory activities. However further laboratory study and chemical isolation of this plant might confirm an effective drug molecule in pharmacological aspects effectively, in both type of pharmaceutical area.

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CONFLICT OF INTEREST STATEMENT

None declared.

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