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Research Article

An *in vivo* and *in vitro* Evaluation of Anti-inflammatory Action of Seeds of *Vigna unguiculata* Available in Bangladeshi Region

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Abstract

Background and Objective: The *V. unguiculata* always considered as a potential source of new drug development. Seeds of *V. unguiculata* have found almost all over the world but their pharmacological and nutritional value varies with geographical variability. The aim of the present study was to determine the membrane stabilizing activity of methanolic extract of *V. unguiculata* Linn (seed) in both *in vitro* and *in vivo* study model, while Swiss albino mice were employed as experimental animal. **Materials and Methods:** The *V. unguiculata* seeds were initially collected, air-dried by using mechanically graded aluminum foil, finally ground powder was extracted with methanol. During *in vitro* evaluation, five different concentrations of methanolic extract of *V. unguiculata* were subjected for determination of anti-inflammatory activity. *In vivo* evaluation of anti-inflammatory effect was tested by carrageenan induced paw edema method on Swiss albino mice model. **Results:** During examination of *in vitro* assay for membrane stabilizing activity, five different concentration of crude methanol extracts were capable of inhibiting hemolysis of erythrocyte membrane dose dependently in both hypotonic solution and heat-induced conditions, whereas *in vivo* anti-inflammatory test produced significant ($p < 0.05$) inhibition of edema diameter at both doses (200 and 400 mg kg⁻¹) level, which indicates the anti-inflammatory property of the samples. **Conclusion:** From the above results, it is clear that *V. unguiculata* Linn. seeds methanolic extract showed significant anti-inflammatory potentials in both *in vitro* and *in vivo* study model. So, it will be very much possible source for an isolating lead compound for curing the inflammatory disorder.

Key words: *V. unguiculata*, hypotonic solution, heat-induced, carrageenan induced

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There is enormous evidence that medicinal plants were promising candidates for developing new drugs. Plants are a 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 molecules¹⁻³. 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 disease⁴.

V. unguiculata is most usually known as "cowpea"⁵. It is an edible legume and belongs to family Fabaceae with high protein extent⁶. The seeds of *V. unguiculata* varies in size, color and shape. There is 10-20 seeds found in each pod⁷. The seeds are sweet in nature and they have, laxative, astringent anthelmintic, anti-bacterial, diuretic and galactagogue properties⁸. The seeds also help in liberating the conditions like constipation, inflammation, jaundice, anorexia, general debility and so on^{5,8}.

So because of their biomedical importance *V. unguiculata* always considered as a potential source of new drug development. Seeds of *V. unguiculata* have found almost all over the world but their pharmacological and nutritional value varies with geographical variability. In Bangladesh, different varieties of *V. unguiculata* are consumed in large quantity as a source of dietary protein and other nutrients, due to their recent finding on their importance on biomedical aspect⁹. So this is become an important issue to disclose their all biomedical importance. By took this responsibility on our end it conduct this present study to determine anti-inflammatory activity of seeds of *V. unguiculata* available in Bangladeshi region. Inflammation is one of the important processes. Inflammatory cells induce a complex mixture of growth and differentiation of cytokines as well as physiologically active arachidonate metabolites^{10,11}. In the human body, it is found that inflammation is frequently responsible for initiating some complicated situations¹¹. Many drugs have been developed to take care of these problems but their untoward effect still remains as a matter of concern¹²⁻¹⁵. 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).

MATERIALS AND METHODS

Materials: Standard Acetyl Salicylic Acid (ASA) and ibuprofen were 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 in April 2015. After collection seeds were thoroughly washed with water. The seeds were then air-dried by using mechanically graded aluminum foil and finally kept at room temperature for 14 days. The plant was identified by an expert of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Accession number-37752).

Preparation of methanolic extract of *V. unguiculata* seeds:

For preparing methanolic extract of seeds of *V. unguiculata*, 500 g of the dried and powdered sample was soaked in 1000 mL of methanol (99.8%). After 15 days the solution was collected through filtration by using filter cloth and Whatman® filter paper¹⁵. 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, 3, 5, 7 and 9 mg mL⁻¹. A dose of 0.1 mg mL⁻¹ was prepared for standard Acetylsalicylic acid.

Red Blood Cells (RBC) collection:

Human red blood cells (RBCs) were collected for the study. About 2 mL of blood was collected from each of the healthy Bangladeshi male human volunteers (aged 20-23 years) without a history of anti-coagulant therapy and free from diseases (using a protocol approved by Institutional Ethics Committee). Blood was collected in tubes with EDTA (Ethylenediaminetetraacetic acid) as anticoagulant. The RBCs were separated after centrifuging the samples for 10 min at 3000 g.

Experimental animals:

Swiss albino mice, used for this study were collected from Jahangir Nagar University, Savar, Dhaka, Bangladesh and were kept in polypropylene cages exposing them to alternate cycle of 12 h dark and light at temperature 25±20°C and relative humidity 55±10%. Mice were fed with standard laboratory pellet diet and water at

libitum and were allowed to acclimatize for 7 days to the laboratory conditions before the experiment. Mice were given adequate human care throughout the experimental period. For determining analgesic and anti-inflammatory activities 20 experimental healthy mice were randomly selected for and divided into four groups with five mice in each group.

For the determination anti-inflammatory activity following groups have been constructed:

Group 1: Control group, where all of the mice were fed with normal food and water

Group 2: Ibuprofen at dose of 10 mg kg⁻¹ b.wt., i.p., has been administered

Group 3: Mice were treated with methanolic extract of 200 mg kg⁻¹ b.wt.

Group 4: Mice were treated with methanolic extract of 400 mg kg⁻¹ b.wt.

***In vitro* evaluation of anti-inflammatory activity:** *In vitro* membrane stabilizing the activity of the extractives was performed by using hypotonic solution and heat-induced hemolysis of erythrocyte membrane by the method developed by Sen *et al.*¹⁶ and modified by Shinde *et al.*¹⁷.

***In vivo* evaluation of anti-inflammatory activity**

Carrageenan-induced rat paw edema assay: The selected samples showing promising average (activity in all solvents) COX-2 selective activities were evaluated for *in vivo* anti-inflammatory studies using carrageenan induced rat paw edema animal model. The mice were divided into four groups each containing five mice. The extract (200 and 400 mg kg⁻¹), normal saline (1 mL kg⁻¹) as vehicle and Ibuprofen (10 mg kg⁻¹) as the referral agents were administered orally. After 1 h of administration, acute inflammation was induced by injecting 0.1 mL of 1% carrageenan into the plantar surface of the rat hind paw method developed by Sikder *et al.*¹⁸. The paw volume was measured at 1, 3 and 5 h using a plethysmometer (Orchid Scientific Laboratory) to determine

the diameter of edema. The difference between the readings at time 1 h and different time interval was taken as the thickness of edema.

Statistical analysis: All the results were expressed as Mean ± SEM. The p-value was calculated by one-way ANOVA using SPSS software, version 22.0 (IBM Corporation, New York, NY, U.S.A.). Where p ≤ 0.05 was considered as statistically significant.

RESULTS

***In vitro* anti-inflammatory effect:** 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 1 and 2, respectively. The 9 mg mL⁻¹ concentration of methanolic extract inhibited maximum hemolysis of RBCs (69.49 and 42.27%) (p < 0.05) in both heat and hypotonic solution induce hemolysis condition, whereas 1, 3, 5 and 7 mg mL⁻¹ concentration of methanolic extract also showed their membrane stabilizing properties in dose-dependent manner while compared to standard acetyl salicylic acid. Although result induced by hypotonic solution is superior to result found from heat induce condition.

***In vivo* anti-inflammatory effect:** The results of the oral administration of *V. unguiculata* plant extracts showed promising anti-inflammatory activity by reducing the carrageenan induced mice paw edema volume (Table 3). It was observed that at the dose of 400 mg kg⁻¹ the crude methanolic extract of *V. unguiculata* exerted a significant (p < 0.05) decrease in edema volume after 1 h, while the standard Ibuprofen also showed a significant (p < 0.05) inhibition of edema. Moreover, at the same dose of *V. unguiculata* considerable reduction in the edema volume was also observed after 3 h (p < 0.05), respectively. Similar trend of results of edema volume reduction also observed

Table 1: Hypotonic solution induced hemolysis of erythrocyte membrane by methanolic extract of *V. unguiculata* Linn. (seed)

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

Values are expressed as Mean ± SD, *when p < 0.05, **when p < 0.01 and ***when p < 0.005 (n = 3)

Table 2: Inhibition of hemolysis of erythrocyte membrane on a heated and unheated solution by methanolic extract of *V. unguiculata* Linn. (seed)

Treatments	Concentration (mg mL ⁻¹)	OD of sample (±SD)		
		Heated solution	Unheated solution	Inhibition (%) of hemolysis
Control	1	1.093±0.035		
Methanolic extract (1 mg mL ⁻¹)	1	0.88±0.107	0.86±0.011	9.7±3.31
Methanolic extract (3 mg mL ⁻¹)	3	0.74±0.004	0.62±0.017***	23.82±1.13
Methanolic extract (5 mg mL ⁻¹)	5	0.70±0.014	0.52±0.022 **	31.14±3.42
Methanolic extract (7 mg mL ⁻¹)	7	0.59±0.082	0.30±0.342**	35.62±2.06
Methanolic extract (9 mg mL ⁻¹)	9	0.53±0.008	0.11±0.021	42.27±1.87
Acetyl salicylic acid	0.10	0.67±0.025	0.13±0.029***	56.32±4.08

Values are expressed as Mean ±SD, *when p<0.05, **when p<0.01 and ***when p<0.005 (n = 3)

Table 3: Effect of methanolic extract of the *V. unguiculata* on carrageenan-induced paw edema in mice

Groups	Dose (mg kg ⁻¹)	Edema diameter (mm)			Inhibition (%)		
		1 h	3 h	5 h	1 h	3 h	5 h
Group 1	Vehicle	4.05±0.62	4.03±0.66	4.01±0.05			
Group 2	10	4.66±0.74*	2.53±0.96**	2.38±1.06*	42.83	84.18	95.80
Group 3	200	4.75±0.56	3.22±0.61**	3.06±0.43**	30.91	47.52	55.22
Group 4	400	4.31±0.22*	2.89±0.93**	2.21±0.35***	37.34	49.14	95.02

Probability values (calculated as compared with control using one way ANOVA followed by Dunnett's test): *p<0.05, **p<0.01, ***p<0.005, all values are mean of individual data obtained from four mice (n = 4), ±: Standard error mean, Group 3 and 4 were treated with 200 and 400 mg kg⁻¹ b.wt. (p.o.) of the methanolic extract of *V. unguiculata*, respectively

with the doses of 200 and 400 mg kg⁻¹ after 5 h whereas the standard (Ibuprofen) at 10 mg kg⁻¹ dose showed most significant (p<0.05) result.

DISCUSSION

Our present finding showed that methanolic extract of seeds of *V. unguiculata* seeds showed significant inhibition of hemolysis at *in vitro* model and significant reduction of oedema volume in case of swiss albino mice model. These *in vitro* and *in vivo* methods were more time-saving, flexible and convenient in other ways. So this observation have also found consistent with some previous allocation who suggested that the breakdown of bio-membranes leads to the formation of free radicals which in turn enhance cellular damage^{19,20}. It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection of cell membrane against injurious substances^{21,22}. Some research works were able to reveal the name of some responsible chemical components (flavonoid and phenolic compounds) present in the extracts, which are well known for their anti-inflammatory activity^{23,24}. Both *in vitro* and *in vivo* studies in experimental animals showed that the flavonoids exert stabilizing effects largely on lysosomes²⁵ while tannin and saponins are also capable of stabilizing the erythrocyte membrane with an ability of binding with cations and other biomolecules^{24,26}. Our present seeds methanolic extract of cowpea also found to contain flavonoid compounds in a significant amount⁷, maybe that is why our plant methanolic

extract showed membrane stabilizing activity in both *in vivo* and *in vitro* model. The results obtained demonstrated that methanolic extract of *V. unguiculata* can significantly and dose-dependently inhibited HRBC hemolysis and carrageenan induced oedema in both *in vivo* and *in vitro* model. The crude methanolic extracts of seeds of *V. unguiculata* showed anti-inflammatory activity towards carrageenan-induced paw edema in mice. This is very consistent with many of the previous findings²⁷⁻³⁰. However, further laboratory study and chemical isolation of this plant might confirm an effective drug molecule in pharmacological aspects effectively for this area of medical science. As our present investigation suggested that good ability of methanolic extract to resist the cell lysis and reduction of edema size in small concentration as compared to the standard drug (acetyl salicylic acid and ibuprofen). So, it will be very much possible source for an isolating lead compound for curing the inflammatory disorder. So necessary initiative should be taken to exploit their molecular pathway to this particular field of medical science. This plant may become a tremendous source of lead compounds in order to cure disorder like inflammation in successful manner.

CONCLUSION

From the findings, it may be concluded that all methanolic extracts of the *V. unguiculata* plant have moderate to high membrane stability effect hence, effective anti-inflammatory activity. However, further laboratory study

and chemical isolation of this plant might confirm an effective drug molecule in pharmacological aspects effectively for this area of medical science. Though as far there is no clinical trial study has been conducted for this plant but we think it is an urgent need to carry out *in vivo* studies regarding this membrane stability activity.

SIGNIFICANCE STATEMENT

This study discover the anti-inflammatory potentiality of seeds methanolic extract of *V. unguiculata* have proven its biomedical importance that can be beneficial for medical science to discover drugs from plant source with anti-inflammatory potentiality with least amount of side effects. This present study will help the researcher to uncover the critical areas of biomedical pathways that many researchers were not able to explore.

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