International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

**Research Article** **CODEN: IJPNL6**

# *IN VITRO* AND *IN VIVO* BIOLOGICAL INVESTIGATIONS OF THE WHOLE PLANT EXTRACTS OF *CHROZOPHORA PROSTRATA* (DALZ.)

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**ABSTRACT**

*Chrozophora prostrata* (Family- Euphorbiaceae), it is an herbaceous perennial plant, is found throughout Egypt, Palestine, Syria, Western Arabia, tropical Africa from Senegal to Ethiopia, South Africa (Transvaal) and tropical Asia and it is the most important herb in ayurvedic medicine. The herb has been highly valued for its traditional use as blood purifier which is also used for the treatment of chronic persistent fever, syphilis, gonorrhea, leucoderma as well as its antioxidant properties helps to maintain cell integrity. In the present study *In vitro* thrombolytic activity and membrane stabilizing activity, *In vivo* GI motility and neurophramacological activity of the whole plant extract of *C. prostrata* (Suryavarta, Neel kanthi, Shad, Khudi okra) was evaluated. The plant extract showed mild thrombolytic activity and membrane stabilizing activity. This plant extracts also exhibit significant GI motility and neurophramacological activity.

**Key words:** *Chrozophora Prostrata*, *In-vitro* thrombolytic activity, *In-vitro* membrane stabilizing activity, *In-vivo* GI motility, *In-vivo* neurophramacological activity.

## INTRODUCTION

Over centuries and decades, our ancestors relied on the herbal product as therapeutic which can be traced back for at least 5000 years. According to World Health Organization (WHO), about 80% of the world population depends on the natural product for their health due to minimal side effect and cost effective. After searching various literatures for plants that may have useful properties, we have selected *Chrozophora prostrata (Dalz). Chrozophora* is a genus from *Euphorbiaceae* family endemic to tropical Asia. It consists of approximately 26 species that are typically found growing in these areas. *C. prostrata* (*Dalz.*) (Bengali Name: Khudi okra) is selected for the current study. There remains a possibility that the plant may contain some bioactive compounds essential to treat diseases and so this plant is considered under the current phytochemical and pharmacological studies. Different parts of the plant have extensively been used in the native system of medicine to treat various kinds of ailments. The leaves are presumed to possess depurative properties and the seeds are known to possess laxative and alterative properties. Ash of the root is used to manage cough in children. Decoction made from the plant has been employed to control leprous affections. It is also regarded as blood purifier and used for the treatment of chronic persistent fever, syphilis, gonorrhea as well as leucoderma [1-5]. As a part of our continuing studies on medicinal plants of Bangladesh the organic soluble materials of the plant extracts of *C. prostrata* were evaluated for *In-vitro* thrombolytic activity and membrane stabilizing activity through hypotonic solution and heat induced heamolysis, *in-vivo* neurophramacological activity through open field test and forced swimming test and gastrointestinal motility for the first time [6-13].

## MATERIALS AND METHODS

**Collection and Processing of Plant Samples:** Fresh whole plants of *C. prostrata* were collected from Dhaka, Bangladesh in July 2014 and a sample was

submitted to the Bangladesh National Herbarium for identification (Accession number: DACB- 39671). Plant was sun dried for seven days. The dried plants were then ground in coarse powder using high capacity grinding machine which was then stored in air-tight container with necessary markings for identification and kept in cool, dark and dry place for the investigation.

**Extraction Procedure:** The powdered plant parts (22 gm) were successively extracted in a soxhlet extractor at elevated temperature using 250 ml of distilled methanol (40-60) °C which was followed by ethanol and chloroform. After extraction all extracts kept in refrigerator 4°C for future investigation with their necessary markings for identification.

**Blood Sample:** Blood (n=5) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 5 ml of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

**Streptokinase (SK):** Commercially available lyophilized altepase (Streptokinase) vial (Popular pharmaceutical Ltd.) of 15, 00,000 I.U, was collected and 5 ml sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100 µl (30,000 I.U) was used for in vitro thrombolytic activity evaluation.

**Thrombolytic Activity:** The thrombolytic activity of all extracts of the plants was evaluated by the method developed by Prasad *et al*. using, streptokinase (SK) as the standard [14].

**Membrane Stabilizing Activity:** The membrane stabilization by hypotonic solution and heat-induced haemolysis method was used to assess anti-inflammatory activity of the plant extracts by following standard protocol. Since the erythrocyte membrane resembles to lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane. The membrane stabilizing activity of the extractives was assessed by using hypotonic solution-induced and heat-induced human erythrocyte haemolysis. To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes (containing anticoagulant 3.1% Na-citrate). The blood was centrifuged and blood cells were washed three times with solution (154mM NaCl) in 10mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000g [15-17].

***Hypotonic solution-induced haemolysis*:** The test sample consisted of stock erythrocyte (RBC) suspension (0.5mL) mixed with 5mL of hypotonic solution (50mM NaCl) in 10mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1.0 mg/mL) or acetyl salicylic acid (ASA) (0.1 mg/mL). The control sample consisted of 0.5mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10min at room temperature, centrifuged for 10min at 3000g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

% inhibition of haemolysis = 100 x $(OD_1-OD_2)/OD_1$

Where, $OD_1$= optical density of hypotonic-buffered saline solution alone (control)

$OD_2$= optical density of test sample in hypotonic solution

***Heat-induced haemolysis:*** Isotonic buffer containing aliquots (5ml) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56°C for 30min in a water bath, while the other pair was maintained at (0-5)°C in an ice bath. The reaction mixture was centrifuged for 5 min at 2500g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

% Inhibition of hemolysis = 100 x $[1- (OD_1-OD_2)/ (OD_3-OD_1)]$

Where, $OD_1$= optical density of unheated test sample
$OD_2$= optical density of heated test sample
$OD_3$= optical density of heated control sample

**Experimental Animal:** For the experiment Swiss albino mice of either sex, 4-5 weeks of age, weighing between 10-24 gm were collected from ICDDR, B, Dhaka. Animals were maintained under standard environmental conditions [temperature: (27.0 ±1.0) ºC, relative humidity: (55-65) % and 12 hour light/12 hour dark cycle] and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

**Neurophramacological Study:** To check the neurophramacological effects or side-effects of drug, two types of experiment is carried out which are open field test and swimming test.

***Open field test (OFT):*** According to Gupta *et al*. (1971) [18] with slight modification open field test was performed to monitor behavioral responses in mice that were placed in a novel and bright arena. Rodents tend to stay away from brightly illuminated areas. The experiment also assesses a range of anxiety-induced, locomotor activity and exploratory behaviors. The animals were divided into 8 groups of 5 mice each. The first group was given 10ml/kg of 1% Tween 80 orally and served as control. Group 2 was served 2 mg of bromazepam per kg of body weight and it served as standard. Groups 3, 4 received methanol extract of *C. prostrata* at 100 and 200 mg/kg of body weight and groups 5, 6 received ethanol extract of *C. prostrata* at 100 and 200 mg/kg of body weight. Group 7, 8 received chloroform extracts of *C. prostrata* at 100 and 200 mg/kg of body weight. The open field apparatus is made of hardboard (60cm x 60cm; 40cm walls). Blue lines drawn on the floor divide the floor into thirty six squares 10cm x 10cm squares alternatively colored black and white and Central Square (10cm x 10cm) in the middle clearly marked. The number of squares visited by the animals was calculated for 2 min at 0, 30, 60, 90 and 120 min subsequent to oral administration of the experimental crude extracts.

***Forced swimming test (FST):*** According to Porsolt *et al*. (1978) [19] swimming test was performed with slight modification. Animals were randomly divided into 8 groups with 5 mice on each group. Group 1 was given 10 ml/kg of 1% Tween 80 which served as control and group 2 was given 2 mg of benzyl diazepam per kg of body weight which served as standard. Groups 3, 4 received methanol extracts of *C. prostrata* at 100 and 200 mg/kg of body weight. Groups 5, 6 received ethanol extracts of *C. prostrata* at 100 and 200 mg/kg of body weight. Group 7, 8 received chloroform extracts of *C. prostrata* at 100 and 200 mg/kg of body weight. The forced swim test was carried out on mice individually forced to swim in an open acquire water tank apparatus (29cm x 19cm x 20cm), containing 9 cm of water at 25±1°C. The total duration of immobility during the 4-min test was scored as described. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. The duration of immobility was recorded. Decrease in the duration of immobility during the FST was taken as a measure of antidepressant activity.

**Gastrointestinal Motility Determination:** Forty eight Swiss Albino mice, weighing between (10-20) gm were selected and housed properly for 10 days before performing the experiment. On the test day, the animals were divided into eight groups of six mice each. They were weighed and deprived of food, with free access to water. Three hours after food deprivation, the animals in group 1 received orally by gavages 5 ml/kg body weight of 0.9% NaCl (normal saline) as control group, while those in group 2 received 5 mg/kg body weight of butapen as standard group. The other six groups received their respective doses as shown in the **table 5**. After 90 min, 0.3 ml of an aqueous suspension of 5% charcoal in normal saline was administered to each animal orally by gavages (time 90 min). Sixty minutes later they had free access to food (time 150 min). The animals were observed at 5 min intervals until feces with charcoal were eliminated (maximum time of observation was 300 min). Charcoal was observed on the feces using normal light when it was easily visible, or using a microscope to help the identification of the black spots. The results were based on the time for the charcoal to be eliminated [20].

**Statistical Analysis:** Data was expressed as Mean ±SD (Standard deviation). Significance was measured by t-test (two sample assuming unequal variances). Results below $p<0.05$ and $p<0.01$ were considered as statistically significant.

## RESULTS AND DISCUSSIONS

***In Vitro* Thrombolytic Activity:** The extractives of *C. prostrata* were also assessed for thrombolytic activity and the results are presented in **table 1**. Addition of 100 µl Streptokinase (30,000 I.U.), standard to the clots along with 90 minutes of incubation at 37°C, showed 18.89% clot lysis. Clots when treated with 100 µl sterile distilled water (control) showed only clot lysis 4.97%. The mean difference in percentage of clot lysis between standard & control was found to be statistically significant. The comparison of standard with control clearly demonstrated that clot dissolution does not occur when water was added to the clot. After treatment of clots with 100 µl methanolic, ethanolic and chloroform extracts of *C. prostrata*, clot lysis 28.34%, 23.19%, 22.43% was obtained respectively. In this study, the methanol extract of *C. prostrata* revealed highest thrombolytic activity 28.34%, whereas ethanol, chloroform extract showed 23.19%, 22.43% of clot lysis respectively. However, significant thrombolytic activity was demonstrated by the methanol extract.

**Table 1:** % Clot lysis by different extracts of *Chrozophora prostrata*

| Samples | % of Clot lysis |
|---|---|
| Methanol | 28.34 ± 0.21* |
| Ethanol | 23.19 ± 0.23 |
| Chloroform | 22.43 ± 0.20 |
| Control | 4.97 ± 0.22 |
| Streptokinase (Std) | 18.89 ± 0.25 |

Values are expressed as mean ± S.D. (n=5), ***p<0.05* when compared with the corresponding value of the standard

**Membrane Stabilizing Activity**

The different extracts of *Chrozophora prostrata* at concentration of 1.0 mg/ml significantly protected the lysis of human erythrocyte membrane by hypotonic solution induced haemolysis and heat induced heamolysis as compared to the standard, acetyl salicylic acid (0.10 mg/ml) **table 2.** For hypotonic solution induced haemolysis, at a concentration of 1.0 mg/ml, the ethanolic extract inhibited 76.24% haemolysis of RBCs as compared to 63.20% produced by acetyl salicylic acid (0.10 mg/ml). The methanol and chloroform soluble extracts also revealed significant % inhibition of haemolysis of RBCs in hypotonic solution heamolysis **table 2**. On the other hand, during heat induced condition different organic soluble materials of *Chrozophora prostrata* demonstrated 54.2%, 62.5% and 61.1% inhibition of haemolysis of RBCs, respectively whereas acetyl salicylic acid inhibited 40%. To confirm the membrane stabilizing activity the above mentioned model was observed. Experiments were performed on the erythrocyte membrane. A possible explanation for the stabilizing activity of the extractives due to an increase in the surface area/volume ratio of the cells which could be brought about by an expansion of membrane or shrinkage of the cell and an interaction with membrane proteins. The present investigation suggests that the membrane stabilizing activity of *Chrozophora prostrata* may be playing a significant role in its anti-inflammatory activity.

**Table 2:** Effect of extractives of *C. prostrata* on hypotonic solution (A) and heat induced (B) of erythrocyte membrane

| Samples | Concentration (mg/ml) | % Inhibition of hemolysis | |
|---|---|---|---|
| | | (A) Haemolysis inhibition (%) | (B) Haemolysis inhibition (%) |
| Control | Only hypotonic solution | -- | -- |
| Std. (Acetyl salicylic acid) | 0.1 | 63.20 ± 0.043 | 40 ± 0.026 |
| Methanol extract | 1 | 63.52 ± 0.076* | 54.2 ± 0.080* |
| Ethanol extract | 1 | 76.24 ± 0.059 | 62.5 ± 0.029 |
| Chloroform extract | 1 | 12.75 ± 0.042* | 61.1 ± 0.061* |

Values are expressed as mean ± S.D. (n=5), ***p<0.05* when compared with the corresponding value of the standard

***In Vivo* Neurophramacological Study:**

***Open Field Test (OFT):*** This experiment was performed to assay general locomotor activity levels. After investigation with different extracts of *C. prostrata* following data was observed **figure 1, 2** and **table 3**.

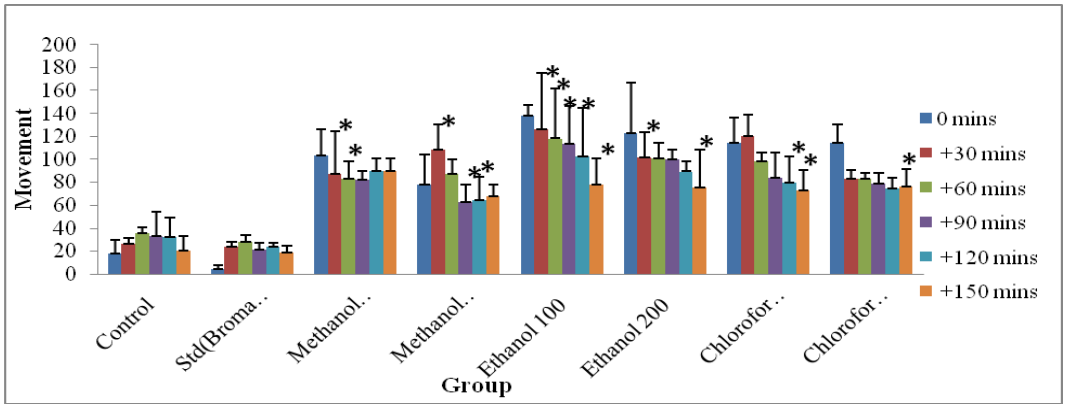


**Figure 1:** Comparative representation of open field test of *Chrozophora prostrata* (movement)
Values are expressed as mean ± S.D. (n=5), ***p<0.05* when compared with the corresponding value of the standard

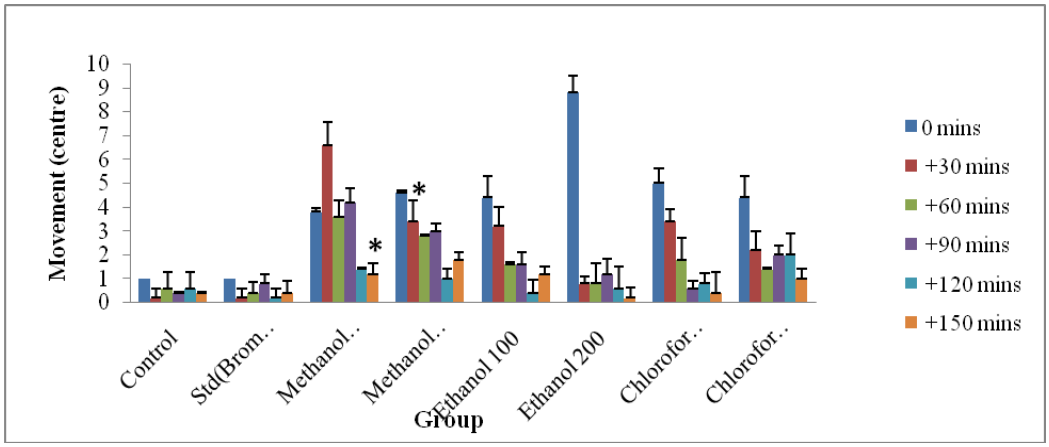


**Figure 2:** Graphical representation of open field test of *Chrozophora prostrata* (centre)
Values are expressed as mean ± S.D. (n=5), ***p<0.05* when compared with the corresponding value of the standard

**Table 3:** Effect of different extracts of *C. prostrata* in open field test (stool)

| **Sample** | **Doses (mg/kg)** | **0 mins** | **+30 mins** | **+60 mins** | **+90 mins** | **+120 mins** | **+150 mins** |
|---|---|---|---|---|---|---|---|
| Control | | 1±0.6 | 0±0 | 0±0 | 0±0 | 0±0 | 1.4±1.2 |
| Bromazepam (Std) | 2 | 0.6±0.4 | 0±0 | 0±0 | 0±0 | 0.2±0.4 | 0.2±0.4 |
| Methanol | 100 | 0±0 | 0±0 | 0.2±0.4 | 0±0 | 0±0 | 0±0 |
| | 200 | 0±0 | 0±0 | 0.6±0.8 | 0.8±1.3 | 0.2±0.44 | 0.2±0.44 |
| Ethanol | 100 | 0.4±0.54 | 0±0 | 0.2±0.44 | 0.8±1.30 | 0±0 | 0±0 |
| | 200 | 0.2±0.44 | 0±0 | 0.2±0.44 | 0±0 | 0±0 | 0.2±0.4 |
| Chloroform | 100 | 0±0 | 0±0 | 0.2±0.44 | 0±0 | 0.2±0.4 | 0±0 |
| | 200 | 0.2±0.4 | 0.2±0.4 | 0±0 | 0.4±0.54 | 0±0 | 0±0 |

Values are expressed as mean ± S.D. (n=5), ***p<0.05* when compared with the corresponding value of the standard

Many reports have validated open field tests as useful measures of emotional reactivity for Turku aggressive mice; others have not found differences in open-field activity despite differences in other anxiety measures e.g., MHC-congenic mice. The standard Open field test is commonly used to assess locomotor, exploratory and anxiety like behavior in laboratory animals (rats/mice). The open field test is designed to examine responses of mice or rats to a new and unfamiliar environment (novel environment). Rodents demonstrate anxiety, fear and curiosity when placed in a new environment. In response to the novel environment the rodents tend to explore the surrounding. The exploration capacity might be considered to be an index of anxiety although it is difficult to separate it from motor activity. However, rodents are also fear to go to the open and illuminated space which is also a sign of anxiety. So the novel environment induces anxiety and fear in rodents which is clearly demonstrated by their rearing, grooming, defecation, locomotor, and so on. These parameters are well utilized to assess anxiety and fear in rodents. Inhibition of such behaviors is indicative of centrally acting depressant or sedatives. 9, 10, 11 tables represent the effect of different extracts of *Chrozophora prostrata* on various parameter of open field test. Methanol extract decreased movement of rodents in a dose dependent manner but could not reach significance; whereas bromazepam decreased movement significantly. But extracts failed to exert any effect on movement and entrance in the center of the open field. The effect of higher dose of methanol 200 mg and chloroform 200 mg on defecation was like bromazepam. The results show that the methanol and chloroform extract has no ability to relieve stress and had an anxiolytic effect on the rodents like bromazepam did.

***Forced Swimming Test (FST):*** Swimming test was performed to evaluate the effect of antidepressant effect of *C. prostrata* plant extracts on mice. After investigation with plant extracts of *C. prostrata* following data was observed (**Table 4**).

**Table 4:** Effect of different extracts of *C. prostrata* in swimming test

| Group | Doses (mg/kg) | Duration of Immobility (s) |
|---|---|---|
| Control | - | 47.4 ± 0.31 |
| Benzyl Diazepam (Std) | 2 | 41.6 ± 0.59 |
| Methanol Extract | 100 | 24.8 ± 0.59 |
| | 200 | 39.8 ± 0.45* |
| Ethanol Extract | 100 | 38.6 ± 0.45* |
| | 200 | 45.6 ± 0.60* |
| Chloroform Extract | 100 | 46.8 ± 0.42* |
| | 200 | 45.8 ± 0.51* |

Values are mean ± SD (n=6), * ($p < 0.05$), significantly different when compared with the corresponding value of standard group, done by independent sample t- test

Different doses of ethanol and chloroform extract (100, 200) ($p<0.05$) decreased immobile phase like diazepam (2 mg/kg). Higher dose of methanol extract also showed the decrease in immobile phase. It indicates the Anti-depressant like effect of these extracts. Literature revealed that the FST was designed by Porsolt *et al.* (1978) [19] as a primary screening test or antidepressants. It is still one of the best models for this procedure. This is a low-cost, fast and reliable model to test potential antidepressant. It has a great sensitivity with all the antidepressant classes and all the mechanisms of action of treatments could be determined, but clinical correlations should be considered very carefully. When rodents are forced to swim in a confined place, they tend to become immobile after vigorous activity (struggling). This stressful inescapable situation can be evaluated by assessing different behavioral strategies and immobility during the test could be an efficient adaptive response to the stress. The

development of immobility when the rodents are placed in an inescapable container of water reflects the cessation of persistent escape directed behavior. The CNS depressant effect of the extracts may be attributed to chemical constitutes other than flavonoids and alkaloids because flavonoids are responsible for the decrease in immobile phase in the swim test and so do alkaloid as well.

***In Vivo Gastro Intestinal Motility Test:*** *In vivo* gastrointestinal motility test was conducted on methanol, ethanol and chloroform extracts on the doses of 100 mg/kg and 200 mg/kg. The duration between charcoal administration and charcoal defecation is measured for gastrointestinal motility determination. Methanol 200 mg/kg, ethanol 200 mg/kg and chloroform 100 mg/kg extracts showed significant ($p<0.05$) increase in gastrointestinal motility comparing the standard drug butapan (**Table 5)**. Among all the test groups, the lowest defecation time was shown by methanol extract 100 mg/kg (129.2 min) and chloroform extracts 100 mg/kg (130 min).

**Table 5:** Time of defecation at gastro intestinal motility activity after administration of doses of control, standard and extracts of *C. prostrata*

| Group | Doses (mg/kg) | Charcoal Defection Time (Min) |
|---|---|---|
| Control | - | 90.2 ± 3.70 |
| Butapan (Std) | 5 | 130 ± 4.53 |
| Methanol Extract | 100 | 129.2 ± 1.92 |
| | 200 | 142.8 ± 3.70* |
| Ethanol Extract | 100 | 134.6 ± 4.16 |
| | 200 | 141.4 ± 2.88* |
| Chloroform Extract | 100 | 130 ± 5.70** |
| | 200 | 146 ± 4.52 |

Values are mean ± SD (n=6), ∗ ($P < 0.05$) ∗∗ ($P < 0.01$) significantly different when compared with the corresponding value of standard group, done by independent sample t-test

## CONCLUSION

All the conducted experiments in the present study are based on crude extract and are considered to be preliminary and more sophisticate research is necessary to reach concrete conclusion about the finding of the present study. Biological investigation of the extract confirms significant thrombolytic and neurophramacological activity. Extracts of *C. prostrata* also showed significant Gastro intestinal motility activity. Therefore, further chemical and pharmacological studies with extract of *C. prostrata* for anti-inflammatory activity, anti-pyretic activity, diuretic effect in mice, hypoglycemic effect in rabbits, acute toxicity test, tail immersion test, isolating new bioactive compounds and evolution of their extract mode of action and chronic toxicity profile might be the next steps to be followed to eventually find new led compounds.

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