Assessments of membrane stabilizing and clot lysis capacity of Canna indica flower aqueous extract: In vitro study

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Abstract: This study aims to investigate the membrane-stabilizing and clot-lysis activities of an aqueous extract derived from the flowers of *Canna indica* (CIFA) on human blood *in vitro*. In this experiment, erythrocyte lysis was induced by a hypotonic solution, and clot formation was created by incubating the blood sample at 37°C for 45 minutes. We conducted experiments using varying amounts of acetyl salicylic acid 6.25–100 µL and a fixed amount of streptokinase (SK) 100 µL as the positive control groups. The group designated as the negative control treated the vehicle. CIFA was treated at doses ranging from 6.25 to 100 µL. CIFA demonstrated dose-dependent membrane stabilizing and clot lysis abilities. The CIFA exhibited a wide range of membrane stabilizing capabilities, with a percentage of 71.03 ± 0.02 at a concentration of 100 µL. The EC_{50} of the test extract was calculated to be 47.43 ± 1.02 µL. In contrast, CIFA demonstrated clot lysis capabilities of 60.38 ± 2.37% at the same concentration, with the EC_{50} of the test extract calculated at 91.42 ± 1.86 µL. CIFA demonstrated significant membrane stabilizing and clot lysis activity in human blood, suggesting that they could be promising candidates for the treatment of atherothrombosis.

Keywords: Canna indica, Clot lysis, Artherothrombosis, Flower extract

1. Introduction

Inflammation is an immunological response initiated by harmful stimuli, such as infections, damaged cells, toxic substances, or radiation exposure (Megha et al., 2021). Inflammatory processes activate coagulation, diminish the effectiveness of endogenous anticoagulant systems, and impede the functionality of the fibrinolytic system (Rajput et al., 2024; Miceli et al., 2022). Inflammatory cytokines play a significant role as the primary mediators in the activation of coagulation (Ahmad et al., 2022). Natural anticoagulants serve to mitigate the increase in cytokine levels. Regardless of the underlying factors, persistent inflammation can give rise to various complications such as extensive tissue damage, rapid progression of atherosclerosis, and the formation of blood clots (Abdel-Bakky et al., 2022; Tsoupras et al., 2019; Gusev et al., 2023). This condition has the potential to result in portal hypertension, diminished hepatic blood flow, cardiac stroke, anoxia, and other related consequences (Xanthopoulos et al., 2019). However, thrombosis has been found to be associated with several medical conditions in humans, including pancreatitis, cirrhosis, diverticulitis, cholangiocarcinoma, and maybe even mortality (Neuberger et al., 2022; Conticchio et al., 2023). Therefore, thrombosis poses a substantial global burden.

Acetyl salicylic acid (ASA) and SK are critical in managing thrombosis, each with unique benefits and drawbacks (Di Bella et al., 2022; Motta et al., 2023). ASA works by acetylating cyclooxygenase -1 (COX-1) in platelets, which decreases thromboxane A2 production, impairs platelet aggregation, and may enhance fibrinolysis through increased tissue-type plasminogen activator (t-PA) release (Fijałkowski et al., 2022; Bruno et al., 2023; Tang et al., 2023; Moore et al., 2023). However, ASA can lead to gastrointestinal discomfort, kidney issues, and cardiovascular problems (Barry et al., 2020; Rognes et al., 2022). On the other hand, SK activates plasminogen, resulting in the dissolution of fibrin clots, but its use is limited by high costs and potential adverse effects, such as anaphylaxis and bronchospasm (Amani et al., 2024; Napolitano et al., 2023; Zia et al., 2020). Thus, there is a need for an effective and economical alternative that minimizes side effects.

Medicinal plants continue to hold promise as a viable reservoir of contemporary medicinal compounds for a wide range of ailments (Abdallah et al., 2023; Bhuia et al., 2023a). Approximately 80% of the global population is believed to rely on traditional medicines as their primary option of treatment for a wide range of ailments (Ozioma et al., 2019). The potential reason for this might be attributed to the minimal adverse reactions and significant safety records observed in both animals and humans (Gunnarsson et al., 2019; Bhuia et al., 2023b). Additionally, it is characterized by its affordability and minimal or negligible concerns regarding antimicrobial resistance and the exploration of medicinal resources (Ochwang'I et al., 2018).

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(a) (b) (c) (d)

Fig. 1. Different parts of *Canna indica* a. Rhizome, b. Leave, c. Flower, d. Seeds.

The membrane stabilizing and clot lysis tests are crucial for evaluating the therapeutic potential of natural compounds (Miah et al., 2018; Akbor et al., 2024). The membrane stabilizing test assesses a compound's ability to prevent cell lysis, indicating potential anti-inflammatory and cytoprotective properties, which are essential in the treatment of inflammation-related conditions (Leláková et al., 2020). Meanwhile, the clot lysis test evaluates the thrombolytic activity, determining the compound's efficacy in breaking down blood clots, which is vital for treating cardiovascular disorders like stroke and myocardial infarction (Lichota et al., 2020; Nikitin et al., 2021). Together, these tests offer insights into the compound's broader pharmacological activities and potential clinical applications. *C. indica* is a tropical herbaceous plant that falls under the family Cannaceae (Reddy et al., 2020) and is often known as KOLABOTI in the Bengali language (Fig. 1). C. indica is indigenous to the tropical parts of the Americas; however, it is also distributed in several other tropical nations globally (Karungamye et al., 2022; Chawla et al., 2022). The flowers have a red coloration and are found either in solitary form or arranged in pairs. Additionally, the bract, measuring around 1.3 cm in length, is present. *C. indica* has been extensively utilized in traditional medicine for the management of several ailments, including malaria, diarrhea, fever, eye disorders, dropsy, and dyspepsia (Kaur et al., 2021; Sarje et al., 2019). The phytochemical examination of the flower of *C. indica* revealed the presence of a diverse range of phytochemicals, encompassing alkaloids, carbohydrates, proteins, anthocyanin, flavonoids, terpenoids, cardiac glycosides, steroids, tannins, saponins, and phlobatannins (Kumbhar et al., 2018; Chigurupati et al., 2021; Patil et al., 2021). The pharmacological investigations revealed that different parts of *C. indica* had several therapeutic properties, including anticancer (Ifandari et al., 2018), antidiabetic (Yadav and Ss, 2020), antibacterial (Indrayan et al., 2011), antiviral (Kaur et al., 2021), anthelmintic (Sarje et al., 2019), anti-inflammatory, antioxidant (Ayusman et al., 2020), hemostatic, hepatoprotective (Pandey and Bhandari, 2021), neuroprotective (Ojha et al., 2022), and analgesic and anti-diarrheal effects (Sultana et al., 2022). In their study conducted in 2023, Alma et al. found that the methanolic extract of *C. indica* leaves exhibited clot lysis and membrane stabilizing action *in vitro* (Alma et al., 2023). However, the membrane stability and clot lysis properties of *C. indica* flower extract remain unclear in the literature currently under publication. This is why this study aims to examine the *in vitro* membrane stabilization and clot lysis capabilities of the aqueous extract of *C. indica* flowers.

2. Materials and methods

2.1. Collection, identification, and extraction of a test sample

The collection of mature, healthy, and fresh flowers of *canna indica* was conducted on August 20, 2023, inside the premises of ''Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj 8100, Bangladesh. These flowers were subsequently recognized by a taxonomist at the Bangladesh National Herbarium located in Dhaka. The fresh flowers, weighing 50 grams, were subjected to a thorough washing using running tap water. Subsequently, the flowers were carefully chopped into small, manageable pieces. Later, the diminutive fragments of the flower were subjected to grinding by the use of appropriate mortar and pestles. The raw extract underwent an initial filtration process using a surgical cotton plug, followed by filtration via Whatman No. 1 filter paper. The filtrate acquired through this procedure was utilized directly for the evaluation of membrane stabilizing and clot lysis activities.

2.2. Chemicals and reagents

Ethanol, Tween 80, and various other chemicals and reagents were procured from Merck (India). SK (Durakinase) was sourced from Dongkook Pharmaceutical Co. Ltd. (South Korea), and acetylsalicylic acid was supplied by ACME Laboratories Ltd., (Bangladesh).

2.3. Ethical concerns

This study received approval from the Department of Pharmacy at ''Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj 8100, Bangladesh (#BSMRSTU/RC2023 (18PHR049)).

2.4. Membrane stabilization test (HRBC model)

This study utilized the methodology adapted from Shinde et al. (1999) with modifications. Initially, 5 mL of fresh blood was collected from a healthy human donor and anticoagulated with dipotassium EDTA (final concentration: 2.2 mg/mL). The blood cells were separated by centrifugation at 3000 × g for 10 minutes and washed three times with isotonic saline (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The resulting cell suspension was centrifuged again at 3000 × g for 10 minutes and resuspended in an equal volume of isotonic buffer. Subsequently, 0.5 mL of the cell suspension was added to a mixture of 5 mL of hypotonic solution (50 mM NaCl) and 100 µL of either a test or standard solution (6.25, 12.5, 25, 50, and 100 µL) in 10 mM sodium phosphate buffered saline (pH 7.4), as specified. The control tube contained only 0.5 mL of cell suspension, 5 mL of hypotonic solution, and 100 µL of distilled water (DW) in the same buffer. The reaction mixtures were incubated for 10 minutes at room temperature and then centrifuged at 3000 × g for 10 minutes. Subsequently, the absorbance of the supernatant was measured at 540 nm using a colorimeter (AE-11M, Japan) (Shinde et al., 1999). The percentage inhibition of hemolysis was calculated using the formula:

$$\% \text{ inhibition of hemolysis} = \{(OD_{control} - OD_{test\ samples})/OD_{control}\} \times 100$$

The half maximal effective concentration (EC_{50}) determined using non-liner regression analysis performed with Graphpad Prism software

2.5. Clot lysis test

This *in vitro* study followed the methodology established by Prasad et al. (2006). Fresh blood samples (0.5 mL) were distributed into pre-weighed microcentrifuge tubes obtained from non-anticoagulated donors. After incubating the blood at 37°C for 45 minutes, serum was carefully removed without disturbing the clot, and the tubes were reweighed. Each tube then received 100 µL of the test sample at various concentrations (6.25, 12.5, 25, 50, and 100 µL). Positive control tubes received 100 µL of SK (equivalent to 30,000 IU), while negative control tubes received 100 µL of DW. Following an additional 90-minute incubation at 37°C, the supernatant from each tube was gently aspirated, and the tubes were reweighed (Prasad et al., 2006). The percentage of clot lysis was calculated using the formula:

%Clot lysis = (Weight of clot after treatment ÷ Weight of clot before treatment) × 100

The EC_{50} value for the test sample was also determined, as mentioned above.

2.6. Statistical analysis

Values are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, followed by Newman-Keuls *post hoc* t-test, using GraphPad Prism software (version 6.5). A significance level of p <0.05 was considered, with a 95% confidence interval.

3. Results

3.1. Membrane stabilizing test

The various doses of CIFA exhibited a significant protective effect against the lysis of the human erythrocyte membrane induced by a hypotonic solution in comparison to the standard ASA (**Table 1**). The CIFA exhibited a diverse array of membrane stabilizing capabilities, with values ranging from 11.84 ± 0.01 to 71.03 ± 0.02. The concentration of 100 µL demonstrated the most significant degree of membrane stabilizing action, with a recorded value of 71.03 ± 0.02. The standard, ASA, had a membrane stabilizing capacity of 71.30 ± 0.02 at a concentration of 100 µL. In contrast, the negative control demonstrated a low membrane stabilizing capacity of 1.54 ± 0.01. The study successfully identified the EC_{50} of the test extract to be 47.43 ± 1.02 µL, accompanied by confidence of interval (CI) spanning from 37.19 to 0.61 µL. The co-efficient of determination (R^2) for this computation was determined to be 0.91.

3.2. Clot lysis test

The concentration-dependent clot lysis action of CIFA was observed in human clotted blood (**Table 2**). The CIFA demonstrated a range of percentage clot lysis capacities, varying from 7.72 ± 2.09 to 60.38 ± 2.37. The maximum observed the percentage of clot lysis was 60.38 ± 2.37 at a concentration of 100 µL. The clot lysis capacity of the standard, SK, was found to be 86.19 ± 0.04%, whereas the negative control showed a minimal clot lysis capacity of 1.13 ± 0.02%. The EC_{50} of the test extract was determined to be 91.42 ± 1.86 µL, with CI ranging from 87.10 to 103.60 µL. The R^2 for this calculation was found to be 0.91.

4. Discussion

Natural plants are recognized as significant reservoirs of secondary metabolites and other phytochemicals that provide substantial therapeutic potential, hence contributing to the management and treatment of a wide range of diseases (Cock et al., 2022; Bhuia et al., 2023c). Various phytochemicals derived from different plant sources have been shown to participate in the breakdown of blood clots, as demonstrated both *in vitro* and *in vivo* (Sikder et al., 2022; Dunn et al., 2006). Inflammation is a multifaceted physiological condition. Chronic inflammation has been observed to potentially correlate with various health conditions, including aging, cancer, adipogenesis, diabetes, cardiovascular disorders, and lung illness (Taube et al., 2012; Ghaben and Scherer, 2019; Furman et al., 2019). The membrane stabilization assay of erythrocytes is a widely utilized method for examining the anti-inflammatory properties of plant extracts (Debnath et al., 2013; Chaity et al., 2016). When erythrocytes are exposed to hazardous substances, such as a hypotonic solution, it leads to the lysis of the cell membranes (Anosike et al., 2012; Oroojalian et al., 2021).

Table 1. Membrane stabilizing capacity of CIFA and controls.

Sample/controls	Concentration (µL)	%Membrane protection	EC_{50} [CI, R^2]
Control (Vehicle)	100	1.54 ± 0.01	-
ASA	6.25	8.09 ± 0.03*	47.43 ± 1.02 µL [37.19 – 0.61 µL; 0.91]
	12.5	18.68 ± 0.01*	
	25	28.41 ± 0.01*	
	50	54.72 ± 0.02*	
	100	71.30 ± 0.02*	
CIFA	6.25	11.84 ± 0.01*	59.21 ± 0.04 µL [67.09 – 86.63 µL; 0.90]
	12.5	14.26 ± 0.02*	
	25	20.26 ± 0.01*	
	50	26.31 ± 0.02*	
	100	71.03 ± 0.02*	

Values are mean ± SD (standard deviation) (n = 3), One-way ANOVA followed by t-student post hoc test; *p <0.05 when compared to the control (vehicle) group; CIFA: Canna indica flower aqueous extract; ASA: Acetyl salicylic acid; EC_{50}: Half maximum effective concentration; CI: Confidence of interval; R^2: Co-efficient of determination.

Table 2. Clot lysis capacity of CIFA and controls.

Sample/controls	Concentration (µL)	%Clot lysis	EC_{50} [CI, R^2]
Control (Vehicle)	100	1.13 ± 0.02	-
SK (15,00,000 U/Vial/10ml)	100	86.19 ± 0.04*	-
CIFA	6.25	7.72 ± 2.09*	91.42 ± 1.86 µL [87.10 – 103.60 µL; 0.91]
	12.5	16.39 ± 3.86*	
	25	23.43 ± 2.55*	
	50	42.99 ± 2.20*	
	100	60.38 ± 2.37*	

Values are mean ± SD (standard deviation) (n = 3), One-way ANOVA followed by t-student post hoc test; *p <0.05 when compared to the control (vehicle) group; CIFA: Canna indica flower aqueous extract; SK: Streptokinase; EC_{50}: Half maximum effective concentration; CI: Confidence of interval; R^2: Co-efficient of determination.

The oxidation and decomposition of hemoglobin accompany this process (Trotta et al., 1981). This phenomenon leads to the extravasation of serum proteins and fluids into the surrounding tissues, thereby initiating an inflammatory response (Bekassy et al., 2022; Krishnan and Davidovitch, 2006). The compounds that possess the ability to stabilize membranes may be highly suitable as agents for reducing inflammation (Vijayakumar et al., 2021). Previous research revealed that the *C. indica* leaf's methanolic extract had membrane stabilizing capabilities (Alma et al., 2023). The present experimental investigation demonstrated that CIFA has a notable protective impact against the lysis of the human erythrocyte membrane caused by a hypotonic solution, as compared to the conventional ASA and control (vehicle). The CIFA exhibited a greater capacity for stabilizing the membrane, with a recorded value of 71.03 ± 0.02, when tested at a concentration of 100 µL. In comparison, the standard ASA has a slightly higher value of 71.30 ± 0.02 under the same conditions. On the other hand, the negative control had a relatively poor capacity for membrane stabilization, measuring at 1.54 ± 0.01. The EC_{50} value of the test extract is determined to be 47.43 ± 1.02 µL.

However, prolonged inflammation can lead to the development of atherosclerosis, which causes the formation of plaques and constriction of blood vessels, resulting in the formation of blood clots inside the veins (Zhang, 2008; Rudijanto, 2007; Sprague and Khalil, 2009). The formation of a thrombus can give rise to many vascular complications, such as myocardial infarction, stroke, deep vein thrombosis, renal vein thrombosis, portal vein thrombosis, and other conditions that may ultimately lead to mortality (Lichota et al., 2020; Hossain et al., 2014). Currently, there are several thrombolytic medicines, such as tissue plasminogen activator, urokinase, and SK that are utilized for the treatment of thrombosis (Roohvand, 2018; Altaf et al., 2021). However, there is still a need for more effective thrombolytic agents in order to meet the demands of the present time (Gumbinger et al., 2014). The previous study revealed that the *C. indica* leaf's methanolic extract demonstrated clot lysis activity (Alma et al., 2023). In addition, *C. indica* significantly decreased the clotting time, bleeding time, and abdominal capillary permeability (Kanase et al., 2018). In the present experimental study, it is shown that CIFA (100 µL) exhibited a greater percentage of clot lysis capacities, specifically measuring at 60.38 ± 2.37. In comparison, the standard, SK, demonstrated a clot lysis capacity of 86.19 ± 0.04%. In contrast, the negative control had a limited ability to clot lysis, with a measured capacity of 1.13 ± 0.02%. The EC_{50} value of the test extract was calculated to be 91.42 ± 1.86 µL. So, CIFA has significant membrane stabilizing and clot lysis activities.

The *C. indica* plant has been previously documented to possess noteworthy anti-inflammatory and antioxidant properties (Chen et al., 2013; Ayusman et al., 2020), while its methanolic extract has demonstrated clot lysis efficacy. The plant exhibits thrombolytic action, which contributes to the maintenance of a healthy cardiovascular system (Alma et al., 2023). Previous studies have found that flavonoids exhibit notable effects on stabilizing cell membranes and promoting thrombolytic activity (Labu et al., 2015; Lakhanpal and Rai, 2007). Moreover, tannins and saponins possess the ability to stabilize the erythrocyte membrane and exhibit thrombolytic properties (Oyedapo et al., 2010; Khan et al., 2013). The plant *C. indica* contains flavonoids, tannins, and saponin compounds (Al-Snafi, 2015), which might be a reason for its potential to produce membrane stabilizing effects and exhibit thrombolytic capabilities. Therefore, CIFA has significant membrane stabilizing and clot lysis activities and is a potential source for the development of new thrombolytic drugs.

5. Conclusion

The findings of our investigation indicate that CIFA exhibits noteworthy properties of membrane stabilization and clot lysis activity inside the test systems. CIFA presents itself as a promising option for botanical candidates that could potentially be effective in treating cardiovascular diseases, including atherothrombosis. However, it should be noted that this study was conducted on the flower aqueous extract *in vitro*. Therefore, further research is needed to isolate the key bioactive components from *C. indica* and to explore the mechanisms underlying its membrane stabilizing and clot lysis effects. This study might be helpful in further research.

Conflict of Interest

The authors declared that they have no conflict of interest.

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Authorship Contributions

All authors made a significant contribution (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing) to the work. All authors have read and agreed to the published version of the manuscript.

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