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Pharmacological Profiles of Different Solvent Fractions of *Annona Squamosa* Leaves

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Abstract

Annona squamosa, a species from the *Annonaceae* family, is well-known in the multiple indigenous communities in Bangladesh because of its foods and medicinal value. The study aimed to explore the pharmacological potentials and phytochemical analysis (total phenolic content) of *A. squamosa* leaves. The crude methanolic extract (CMESF), and its consecutive fractions, namely petroleum ether-soluble fraction (PSF), dichloromethane-soluble fraction (DSF), and ethyl acetate-soluble fraction (ESF) were generated for the investigation. Among them, the ESF fraction showed the highest phenolic content (24.24 mg GAE/g), followed by PSF (16.87 mg GAE/g), DSF (15.29 mg GAE/g), and CMESF (0.96 mg GAE/g). For the evaluation of thrombolytic activity, ESF demonstrated the highest clot lysis (44.46 %), followed by PSF (26.45 %), CMESF (25.55 %), and DSF (24.66 %). Moderate membrane stabilizing potency has been revealed by the extractives. Among them, the DSF fraction exhibited the highest heat-induced hemolysis (74.78 %), while at hypotonic solution-induced hemolysis, it showed 27.02 % inhibition. In cytotoxicity assay by brine shrimp lethality bioassay, the PSF exhibited the highest lethality (LC_{90} = 0.751 µg/ml), followed by DSF (1.720 µg/ml), and ESF (2.179 µg/ml). The outcomes indicated that the *A. squamosa* leaves have pharmacological potential which needs further *in vivo* validation and genomic studies along with isolation and characterization of its corresponding bioactive molecules.

Key words: *Annona squamosa*, total phenolic content, thrombolytic activity, membrane stabilizing activity, cytotoxicity

Introduction

Annona squamosa L., a significant plant species belonging to the genus *Annona* (Family: Annonaceae) is especially distributed across tropical regions of Asia, Africa, and South America (Pareek *et al.*, 2011). In Bangladesh, it is found in Sylhet and Chittagong (Rahman *et al.*, 2019). The plant holds considerable importance due to its traditional uses as food and medicine for Bangladeshi indigenous communities (Uddin *et al.*, 2017). Leaves are commonly consumed as vegetables, while seeds are sometimes utilized in specific culinary practices (Sarker *et al.*, 2018). In folk medicine, *A. squamosa* has been utilized for treating common ailments, including dysentery, urinary tract infections, and wound-healing agent (Islam *et al.*, 2019). Moreover, the species are well-known in traditional medicine to treat various ailments, including diabetes, hypertension, cancer, and asthma (Shakya *et al.*, 2021). Extracts from various *Annona* species have

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demonstrated strong free-radical scavenging activity (Ogbonnaya and Eleazu, 2013). Certain *Annona* species have shown potential in thrombolytic activity (Usunomena and Samuel, 2016).

Studies have identified a variety of bioactive compounds in these plants, including alkaloids, flavonoids, terpenoids, acetogenins, and phenolic acids (Nandhakumar and Indumathi, 2018; Kumar *et al.,* 2020). For instance, acetogenins such as squamocin and annonacin, isolated from *A. squamosa*, exhibit potent anticancer and antimicrobial activities (Chen *et al.,* 2019). Two prior studies on *A. squamosa* have demonstrated anti-inflammatory and analgesic properties (Begum *et al.,* 2017; Jahan *et al.,* 2019). Moreover, cytotoxic studies have reported that *A. squamosa* leaf fractions inhibit cancer cell proliferation (Sukohar *et al.,* 2020). Furthermore, phenolic and terpenoid constituents with notable cytotoxic and antitumor effects have been identified from *A. squamosa* extracts (Xu *et al.,* 2021).

Despite existing knowledge, significant gaps remain in the comprehensive pharmacological profiling for *A. squamosa* leaves (Mia *et al.,* 2019). Previous studies have often focused on crude extracts without systematic fractionation to identify active fractions (Sultana *et al.,* 2018). Thus, the specific objectives of this study were to determine the total phenolic content activity of different solvent fractions of *A. squamosa* leaf. The research further seeks to investigate the antioxidant, thrombolytic, membrane-stabilizing and cytotoxic potentials of the extract and its fractions. The investigation for plant-based therapeutics may explore a safe and cost-effective alternative of synthetic drugs (Kalomira *et al.,* 2018).

Materials and Methods

Collection of the plant: Fresh leaves of *Annona squamosa* (Family: Annonaceae) were collected from the National Botanical Garden located in Mirpur, Dhaka, Bangladesh (Figure **1**). The plant species was authenticated by a specialist at the Bangladesh National Herbarium in Mirpur, Dhaka and preserved there for future reference (accession number 45362).

Extraction of plant materials: Leaves of *A. squamosa* were shade-dried and ground into a coarse powder using a mechanical grinder. A total of 450 grams of the powdered leaves were placed in a clean 5-liter flat bottom flask for soaking (2.0 liters of methanol) and remained for 15 days with occasional shaking. After that period, the mixture was filtered first through a layer of fresh cotton and then using Whatman No. 1 filter paper. The filtrate was concentrated using a rotary evaporator (BUCHI, Switzerland). This process yielded a thick, and semi-solid paste of crude methanolic extract (CMESF), which was weighed and stored in a properly labeled container for subsequent use.

Partitioning into different solvent fractions: For the fractionation of CMESF, the modified Kupchan partitioning method (VanWagenen *et al*., 1993) was used. The CMESF was sequentially partitioned using solvents of increasing polarity such as petroleum ether (PSF), dichloromethane (DSF), ethyl acetate (ESF), and the remaining fraction was the aqueous-soluble fraction (AQSF).

Chemicals and reagents: Acetyl salicylic acid and streptokinase were collected from renowned pharmaceutical companies of Bangladesh. All reagents and chemicals such as gallic acid, and vincristine sulfate used were of analytical grade.

Total phenolic content analysis: The total phenolic content of each solvent fraction was determined using the Folin-Ciocalteu colorimetric method (Singleton *et al*., 1999). Absorbance readings were measured at 760 nm using a UV-Visible spectrophotometer. A calibration curve was prepared using standard solutions of gallic acid, and the phenolic-content of the samples was calculated based on this curve. Results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract (mg GAE/g).

In vitro thrombolytic activity: The thrombolytic activity of the plant extractives was evaluated using a previously established method (Prasad *et al*., 2006), with streptokinase (SK) (30,000 I.U.) serving as the standard. Here, 1 ml of freshly drawn human blood was dispensed into pre-weighed Eppendorf tubes and

allowed to stand at room temperature until clot formation occurred. After clotting, the serum was carefully removed without disturbing the clot. Subsequently, the samples were added to each clot-containing tube, and incubated at room temperature for 90 minutes. After incubation, the released fluid was discarded, and the tubes were reweighed to assess the reduction in clot weight (Hossain *et al.*, 2025). The degree of clot lysis was calculated as a percentage using the following formula:

$$(\%\ clot\ lysis) = \frac{Weight\ of\ the\ lysis\ clot}{Weight\ of\ the\ clot\ before\ lysis} \times 100\%$$

In vitro membrane stabilizing effect: The membrane-stabilizing activity of the plant extractives were evaluated by examining their protective effects on human red blood cells against two different stress conditions namely by heat-induced and hypotonic solution-induced hemolysis (Shinde *et al*., 1999). Stock preparations of all extractives were initially made at a strength of 1000 µg/mL and subsequently serially diluted to obtain concentrations of 500 µg/mL and 250 µg/ml. A 0.1 mg/mL aqueous solution of acetylsalicylic acid was also formulated. To prepare the erythrocyte suspension, nearly 5 mL of whole blood was treated with EDTA for anticoagulation. The blood cells were washed using an isotonic saline medium (154 mM NaCl) prepared in 10 mM sodium phosphate buffer at pH 7.4. Subsequently, an iso-saline solution was employed to produce a 10% (v/v) red blood cell suspension. Thereafter, 0.5 mL of the erythrocyte suspension, 1 mL of phosphate buffer, and 2 mL of hypotonic saline were dispensed into each experimental tube. For the standard, 1 ml of acetylsalicylic acid solution (0.1 mg/mL) was incorporated into the standard tubes. For the experimental sets, 1 mL of each extractive was added to the respective tubes. All prepared mixtures were maintained at 37°C for 30 min and subsequently centrifuged at 3000 rpm for 10 min. Then, the resulting supernatants were collected and analyzed using a UV–Visible spectrophotometer at a wavelength of 560 nm. The percentage protection against hypotonicity-induced erythrocyte hemolysis was determined using the following equation:

$$\text{Percentage Inhibition of Hemolysis} = \frac{A_{Control} - A_{Test}}{A_{Control}} \times 100\%$$

where, A is the absorbance for each group such as positive control and test compounds.

Two groups of centrifuge tubes were arranged for the experiment. The first group contained 30 µl of erythrocyte suspension, 5 ml of isotonic buffer, and the test samples at a concentration of 1 mg/ml. The second group was prepared in the same manner but without the addition of the samples or standard, serving as the control. Following gentle mixing by inversion, one group of tubes was maintained in a water bath at 54°C (consistent throughout) for 20 min, whereas the corresponding group was placed in an ice bath (0–5°C). After incubation, all samples were centrifuged at 1300 rpm for 3 min, and the absorbance of the collected supernatants was subsequently measured.

The percentage inhibition of hemolysis was estimated using the following formula:

$$\%\ \text{Inhibition of Hemolysis} = \left[\frac{1 - (AD2 - AD1)}{(AD3 - AD1)}\right] \times 100$$

where, AD_1 represents the absorbance of the unheated test sample, AD_2 corresponds to the absorbance of the heated test sample, and AD_3 denotes the absorbance of the heated control sample.

Evaluation of in vitro cytotoxicity: The cytotoxic potential of the CMESF and its extractives was assessed using the brine shrimp lethality bioassay, as described by Meyer *et al.* (1982). DMSO alone was used as the negative control. Briefly, 2 mg/mL of the dried crude test sample was initially dissolved in dimethyl sulfoxide (DMSO), followed by serial dilution to achieve a range of concentrations from 20.0 to 0.03906 µg/ml. Each concentration of the extractives and standard (vincristine sulphate, 0.46 µg/ml) was tested by placing ten viable *Artemia salina* nauplii into test tubes containing the prepared solutions in artificial seawater (3.5% sea salt). The tubes were incubated at ambient room temperature (24°C) under 24-hour continuous illumination. After the incubation period, the number of surviving nauplii in each tube was recorded. The cytotoxic effect of the samples was determined by calculating

the percentage of mortality at each concentration using the following formula:

$$(\%\ of\ mortality) = \frac{Number\ of\ nauplii\ death}{Number\ of\ nauplii\ taken} \times 100\%$$

The cytotoxic activity of the plant extract was quantified by determining the lethal concentration 90 of a test population (LC_{90}), which was derived from a plot of the percentage of nauplii mortality versus the logarithm of the extract concentrations.

Results and Discussion

The current investigation was carried out to assess the total phenolic content, thrombolytic, membrane stabilizing, and cytotoxic activities of various organic solvent-soluble fractions derived from the CMESF. The corresponding outcomes are presented in figures 1 through 4.

The TPC of the CMESF and its solvent fractions of *A. squamosa* demonstrated significant variation, indicating differential distribution of phenolic constituents among the fractions. The ESF exhibited the highest phenolic content (24.24 mg GAE/g extract), suggesting that phenolic compounds in *A. squamosa* are predominantly concentrated in moderately polar solvents. The PSF and DSF also contained appreciable amounts of phenolics, whereas the CMESF showed a markedly lower phenolic content (0.96 mg GAE/g extract) (Figure 1). The enrichment of phenolics in the ESF may be attributed to the favorable solubility of flavonoids, phenolic acids, and related polyphenolic compounds in ESF (Alara *et al.*, 2021). Similar observations have been reported in previous studies on *A. squamosa* in this study, where ESF displayed higher phenolic concentrations than CMESF. Since phenolic compounds are known to possess strong antioxidant, anti-inflammatory, and other pharmacological properties through their ability to donate hydrogen atoms or electrons and neutralize reactive oxygen species, the elevated phenolic content of the ESF may contribute substantially to the biological activities observed in this fraction (Halliwell, 2012). The ESF may be considered a promising source of bioactive phenolic compounds from *A. squamosa*.

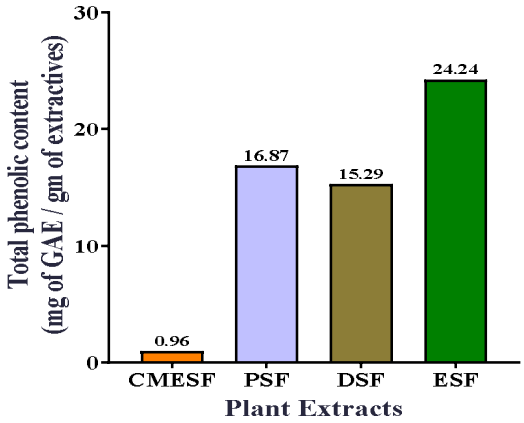

Figure 1. Total phenolic content assay of the CMESF and its fractions.

The *in vitro* thrombolytic assay demonstrated that the extractives of *A. squamosa* possess varying degrees of clot-lysing activity. Among the tested fractions, the ESF exhibited the highest thrombolytic activity (44.46% clot lysis), followed by the PSF (26.45%), whereas the CMESF and DSF showed comparatively lower activities (25.55% and 24.66%, respectively) (Figure 2). Although the activity of the ESF was lower than the standard (66.67%), its moderate clot lysis potential suggests the presence of bioactive phytoconstituents capable of promoting fibrinolysis. Previous studies have reported notable thrombolytic activity in extracts of *A. squamosa*, supporting the traditional medicinal value of the plant and indicating its potential as a source of natural antithrombotic agents (Kiranmayi *et al.*, 2021).

However, the exact compounds responsible for the activity and their mechanisms of action remain to be elucidated through bioassay-guided isolation and *in vivo* investigations.

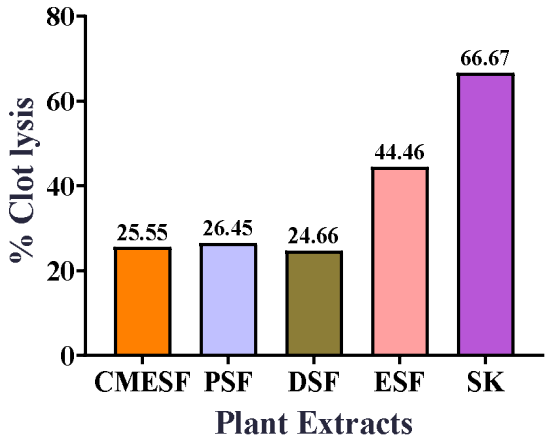

Figure 2. Thrombolytic activity assay of the CMESF and its extractives.

Stabilization of the red blood cell membrane against hemolytic stress suggests the ability of bioactive compounds to prevent the release of lysosomal enzymes and inflammatory mediators at sites of inflammation (Shinde *et al*., 1999). In the present study, all extractives exhibited varying degrees of membrane stabilization under both heat-induced and hypotonic solution-induced hemolytic conditions. Under heat-induced stress, the DSF demonstrated the highest inhibition of hemolysis (74.78%), followed by the ESF (57.79%), while the CMESF and PSF showed moderate activities (Figure 3). Notably, the DSF and ESF exhibited greater protective effects than the standard ASA, indicating the presence of potent membrane-stabilizing constituents within these extractives. Heat-induced hemolysis occurs due to protein denaturation and disruption of membrane integrity, leading to leakage of intracellular components (Lepock *et al*., 1989). Under hypotonic stress conditions, the membrane stabilizing activity of the fractions was comparatively lower. Hypotonic-induced hemolysis results from osmotic swelling of erythrocytes followed by membrane rupture (Pribush *et al.*, 2013). Among the tested fractions, only the DSF (27.02%) showed greater inhibition than ASA (22.56%). This finding indicates that the active constituents present in the DSF may be particularly effective in maintaining membrane integrity against osmotic stress. Further phytochemical investigations are recommended to isolate and characterize the specific compounds responsible for the observed membrane stabilizing effects.

The brine shrimp lethality bioassay is a simple, and cost-effective method widely employed for the preliminary assessment of the cytotoxic potential (Meyer *et al*., 1982). The cytotoxicity results of the different extractives of *A. squamosa* demonstrated marked toxicity. Among the tested samples, the PSF exhibited the highest cytotoxic activity (LC_{90} = 0.751 µg/ml), while the standard's LC_{90} was 1.1571 µg/ml. The DSF and CMESF also showed strong cytotoxic effects (LC_{90} = 1.720 and 1.722 µg/mL, respectively), whereas the ESF displayed comparatively lower activity (LC_{90} = 2.179 µg/mL) (Figure 4). According to the previous study by Apu *et al.* (2013), all tested fractions can be considered highly toxic, indicating the presence of potent bioactive constituents. Previous studies have demonstrated that annonaceous acetogenins isolated from *Annona* species possess remarkable cytotoxic and anticancer activities through inhibition of mitochondrial complex I (Moghadamtousi *et al.*, 2015). Furthermore, extracts and isolated compounds from *A. squamosa* have been reported to exhibit significant antiproliferative effects against various cancer cell lines (Pardhasaradhi *et al.*,

2005). The strong lethality exhibited by the PSF, DSF, and CMESF fractions suggests that non-polar and moderately polar phytochemicals may contribute substantially to the observed bioactivity. Such studies may facilitate the discovery of novel cytotoxic agents from *A. squamosa* with potential pharmaceutical applications.

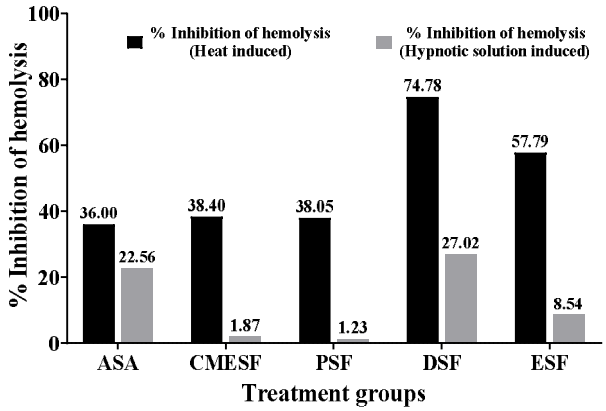

Figure 3. Membrane stabilizing assay of the CMESF and its fractions.

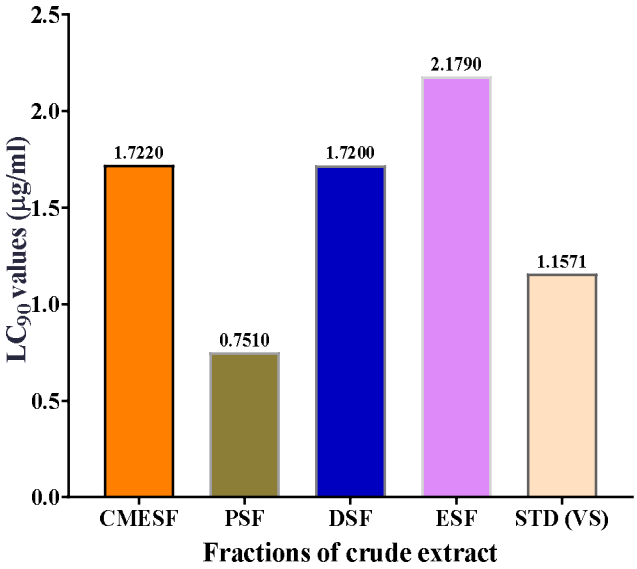

Figure 4. Cytotoxicity assay of the CMESF and its fractions.

Conclusion

The present study demonstrated that the CMESF and its fractions of *Annona squamosa* possess notable biological activities, supporting the medicinal potential of this plant. The ESF exhibited the highest total phenolic content and the strongest thrombolytic activity, suggesting that phenolic constituents may contribute significantly to these pharmacological effects. In membrane stabilizing assays, the DSF showed superior protection against both heat- and hypotonic solution-induced hemolysis, indicating promising anti-inflammatory potential. Furthermore, all fractions displayed marked cytotoxicity in the brine shrimp lethality assay, with the PSF demonstrating the highest cytotoxic activity. Overall, the study highlights that *A. squamosa* as a promising

source of natural therapeutic agents and recommends further phytochemical investigations, mechanistic studies, and in vivo experiments to identify the active constituents and validate their pharmacological potential, mechanistic studies, and *in vivo* investigations to identify the active constituents and validate their pharmacological potential.

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