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Antioxidant, cytotoxic and thrombolytic activity of leaves of *Kalanchoe pinnata* (LAM.) PERS.

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Abstract
The study was aimed to evaluate the, antioxidant, cytotoxic and thrombolytic activity of ethanol extract of the leaves of *Kalanchoe pinnata*. *In vitro* antioxidant activity of extract was studied using DPPH radical scavenging, reducing power determination assays. The antioxidant activity of the extracts was found promising. The reducing powers of this crude extract increase with the increase of concentration. IC_{50} values of DPPH scavenging activity was 282.136±0.46µg/ml. The cytotoxic activity of crude extract was determined using brine shrimp lethality bioassay and LC_{50} values of the sample was 51.88 ± 0.52µg/ml whereas for standard vincristine sulphate was 8.50 ± 0.16µg/ml as a positive control. The plant's extract showed (15.73 ±1.31%) clot lytic as compared to standard streptokinase's (30.26% ± 0.67) clot lytic activity in case of thrombolysis assay

Keywords: *Kalanchoe pinnata*, antioxidant, cytotoxic, thrombolytic activity, ethanol extract

1. Introduction
Plants, the valuable gift of nature, have been used as medicines for hundreds of years. The World Health Organization reported that 80 % of the world populations rely chiefly on indigenous medicine and that the majority of traditional therapies involve the use of plant extracts or of their active constituents [1] and over 25% of modern medicines that are commonly used worldwide contains compounds extracted from medicinal plants [2]. In Bangladesh there is abundant of medicinal plants and ninety percent of the medicinal plants are wild sourced [3, 4]. Although advanced allopathic medicines are available, a large majority (75-80%) of the population of this country still prefer using traditional medicine in the treatment of most of their diseases. Out of approximately 5,000 species of indigenous and naturalized phanerogamic and pteridophytic plants growing in the country, more than a thousand of them, including many food, vegetable, beverage, spice and ornamental plants, contain medicinally useful chemical substances [5].
Limitations of synthesized compounds in the treatment of chronic diseases and the potential of plant based medicine as a more effective and cheaper alternative was probably responsible for the fast growing industry of herbal medicine [6].
Oxidative stress occurs when the generation of free radicals or reactive oxygen species (ROS) exceeds the antioxidant capacity of a biological system [7]. Excess free radicals and ROS attack biological molecules such as lipids, proteins and nucleic acids that lead to tissue or cellular injury [8, 9]. Oxidative stress has already been implicated in atherosclerosis, cancer, diabetes, arthritis, reperfusion damage and inflammation [10]. Recent researches have shown that the antioxidants of plant origin with free-radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress. Antioxidants can be broadly defined as "any substance that, when pre- sent at low concentrations compared to those of an oxi- dizable substrate, significantly delays or prevents oxida- tion of that substrate" [11, 12]. Antioxidants are recognized for their potential in promoting health and lowering the risk for cancer, hypertension and heart disease [13, 14]. The uses of natural antioxidants from plant extracts have experience growing interest due to some human health professionals and consumer's concern about the safety of synthetic antioxidants in foods [15, 16]. Antioxidant activities in plants have been identified by many researchers [17, 18, 19].
Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and parasitic disease [20].

Extracts of medicinal plants are believed to contain a wide spectrum of polyphenolic, flavonoids, alkaloids, terpenoids and saponin compounds, which might have therapeutic properties and hinder cancer formation [21]. Over 60% of current cytotoxic agents have been derived from natural sources including plants, marine organisms and microorganisms, either directly or by chemical synthesis based on natural lead compounds [22, 23]. Therefore, natural products have a wide application in cancer chemotherapy [23].

Cardiovascular disease caused by blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in the recent years [24]. Homeostasis maintains the integrity of circulatory system after damaging of the vascular channel [25]. Thrombus development is a critical event in the arterial diseases associated with myocardial infarction, anoxia, hypertension [26], stroke, reduction of the blood supply to the liver [27] and venous thromboembolic disorders that account for considerable number of deaths worldwide [28]. Remarkable efforts have been made towards the discovery and development of natural constituents from various plant and animal sources which have antiplatelet [29, 30] anticoagulant [31, 32], antithrombotic [33] and thrombolytic activity [34, 35, 36].

Thrombolytic agents are used to dissolve clot and in the management of thrombosis in patients [37]. Thrombolytic agents such as tissue plasminogen activator (t-PA), Urokinase (UK), streptokinase (SK)[38] etc, are used all over the world for the treatment [39] but their use is associated with hyper risk of haemorrhage [40], anaphylactic reaction and lacks specificity [25, 40]. Because of the shortcomings in the existing thrombolytic agents, a number of researches are underway to improve the variants of these drugs for their better effective nature [41].

Kalanchoe pinnata (syn. *Bryophyllum calycinum*), *Bryophyllum pinnatum*, also known as the patharkuchi, Air Plant, Life Plant, Miracle Leaf, Goethe Plant, the Katakataka (Filipino) and also called "Wonder of the World" in the English speaking Caribbean. It is a succulent plant native to Madagascar. It is distinctive for the profusion of miniature plantlets that form on the margins of its leaves, a trait it has in common with the other members of the Bryophyllum section of the Kalanchoe genus.

Kalanchoe pinnatum that belongs to the Crussulaceae family is a smooth skinned more or less erect fleshy herb [42]. It grows up to about 1- 2m tall. Its lower leaves are simple and the upper complex in formation. Its flowers are light green (and turn yellowish) and dull brownish red. The leaves and bark are bitter tonic, astringent to bowels, analgesics, carminative and useful in diarrhoea and vomiting. Antiulcer [43] activities of the leaf were also reported. Several other biological activities have been reported for *Kalanchoe pinnata*. The plant has hepatoprotective activity and is also used to increase vascular integrity [44], to treat hypertension and kidney stones [45] and to enhance the dropping of umbilical cord of a newly born baby [46]. It also has been used for hypertension and treatment of sore feet, but generally it is most commonly used for treating the common cold [47]. The leaves are boiled in water and the extract is given as a sedative for asthma and palpitation [48], but according to the best of our knowledge there is not any scientific detailed report on antioxidant, cytotoxic and thrombolytic activities. So we have selected the ethanol extract of leaves of *K. pinnata* to see the antioxidant, cytotoxic, thrombolytic potentials.

2. Materials and methods

2.1 Chemicals

Lyophilised streptokinase vial (1 500 000 IU) was purchased from Square Pharmaceuticals Ltd, Bangladesh. DPPH (1,1-diphenyl, 2-picryl hydrazyl) trichloroacetic acid, ferric chloride were obtained from Sigma Chemical Co. USA. Ascorbic acid was obtained from SD Fine chem. Ltd. India and potassium ferricyanide from May and Backer, Dagenham, UK. Ethanol was purchased from Merck, Germany. Normal saline solution was purchased from Beximco Infusion Ltd. All chemicals used were of analytical reagent grade.

2.2 Plant materials

Fresh leaves of *K. pinnata* for this study were collected from the local area of Chittagong, Bangladesh and were authenticated by Dr. Sheikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.3 Preparation of crude extract

The collected leaves were dried for a period of 2 weeks under shade and ground. The ground leaves (250 gm) were soaked in sufficient amount of ethanol for one week at room temperature with occasional shaking and stirring. The sediments were filtered and the filtrates were dried at 40 °C in a water bath. The solvent was completely removed by filtering with Whatman number-1 filter paper. The solvent was evaporated under reduced pressure at room temperature to yield semisolid. The extract was then preserved in a refrigerator till further use [49].

2.4 Antioxidant activity

2.4.1 DPPH radical scavenging activity

Free radical scavenging activity of the ethanol extract of *K. pinnata* leaves, based on the scavenging activity of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Braca *et al* [50]. Crude extract (0.1 mL) was added to 3 mL of a 0.004% alcohol solution of DPPH. Absorbance at 517 nm was determined after 30 min and the percentage inhibition activity was calculated by using the equation:

$$\% \text{ scavenging activity} = [(A_0-A_1)/A_0] \times 100.$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. Lower the absorbance, the higher is the free radical scavenging activity [51]. The curves were prepared and the IC_{50} value was calculated using linear regression analysis.

2.4.2 Reducing power

Reducing power was determined according to the method described by Srinivas *et al.* [52]. The different concentrations of extract (125, 250, 500 and 1 000 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide- K_3 Fe $(CN)_6$ (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3 000 r/min for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl3 (0.5 mL, 0.1% w/v) and the absorbance was measured at 700 nm. The increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the reference and phosphate buffer (pH 6.6) was used as blank solution.

2.5 Thrombolytic test

This test was performed according to the method described by Prasad *et al.* [53]. In the commercially available lyophilised streptokinase vial (1 500 000 IU) 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. Five milliliter of venous blood was drawn from the healthy volunteers (n=10) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each ten previously weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to form the clot. After the clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. A volume of 100 µL of ethanol extract (10 mg/ mL) was added to each micro centrifuge tube containing pre weighed clot. As a positive control, 100 µL of streptokinase and as a negative control 100 µL of distilled water were separately added to the control tube numbered. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis [54].

2.6 Brine shrimp lethality assay

The assay was carried out according to the principle and protocol previously described by [55, 56, 57], with slight modifications. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. Dried cysts of *Artemia salina* were collected from an aquarium shop (Chittagong, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. After hatching, active naupli free from egg shells were collected from brighter portion of the hatching chamber and used for the assay.

The test sample (extract) were prepared by dissolving them in DMSO (not more than 50 µL in 5 mL solution) plus sea water (3.8% NaCl in water) to attain concentrations of 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml. A vial containing 50 µL DMSO diluted to 5 mL was used as a control. Vincristine sulphate [58] was used as positive control. After 24 hours the number of survival of nauplii was counted and percentage of mortality was determined using the equation:

% mortality = (no. of dead nauplii/ initial no. of live nauplli) x 100.

Statistical method of probit analysis (Finney's table) [59] was used to calculate LC_{50}. Criterion of toxicity for fractions was established according to Déciga-Campos M *et al* [60]. LC_{50} values > 1000 µg/mL (non-toxic), ≥ 500 ≤ 1000 µg/mL (weak toxicity) and < 500 µg/mL (toxic).

3. Results

DPPH radical scavenging activity of leaves of *K. pinnata* was found to increase with increasing concentration of the extract (Figure 1). This assay was based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. IC_{50} values of DPPH scavenging activity was 282.136 µg/ml. The extract also displayed significant reducing power which was found to increase with the increasing concentration(Figure 2).The results and methods of analysis of antioxidant activities concurred with other studies [61,62,63]. The lethality of the crude extract of *K. pinnata* leaves to brine shrimp was determined on *Artemia salina* after 24 h of exposure the samples, the negative control DMSO and sea water and vincristine sulphate used as standard. This technique was applied for the determination of general toxic property of the plant extract. The LC_{50} value (Figure 3) of the extract was 51.88 µg/mL and that for standard vincristine sulphate was 8.50µg/mL. No mortality was found in the control group, using DMSO and sea water. The plant extract showed moderate clot lysis activity (15.73%) as compared to standard streptokinase's clot lysis (30.26%) activity (Figure 4).

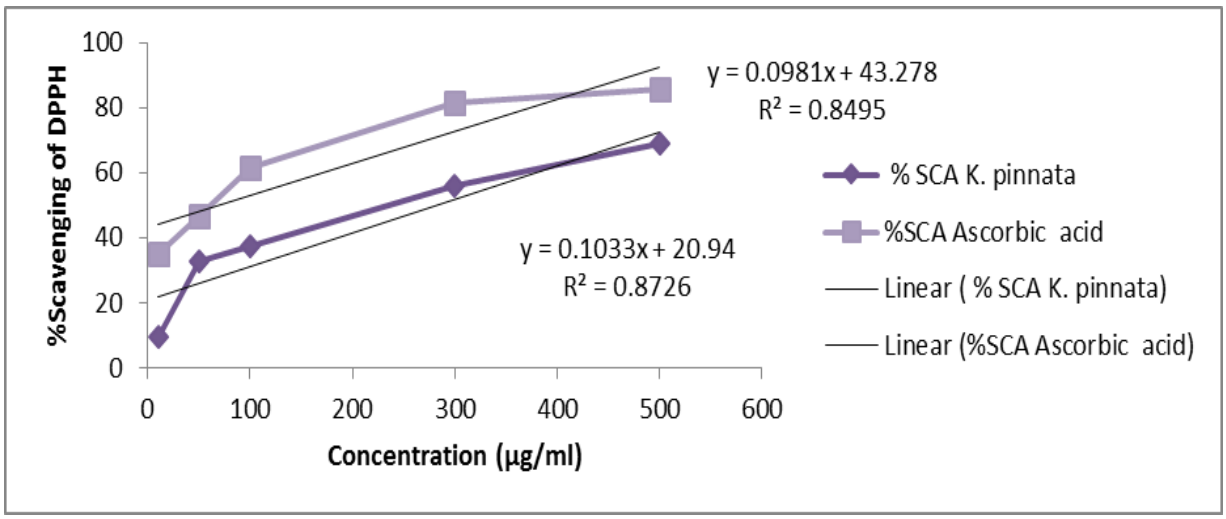

Fig 1. The free radical scavenging activity of *K. pinnata* and ascorbic acid by DPPH.
Results are mean±SEM of three measurements

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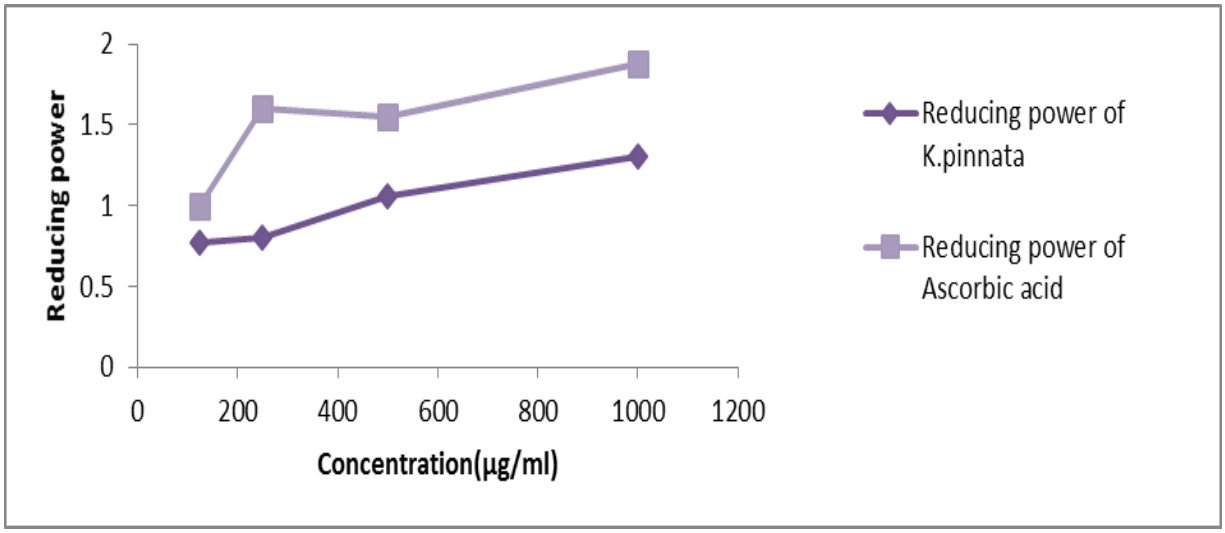

Fig 2. Reducing capacity of *K. pinnata* extract. Values are mean±SEM of three experiments

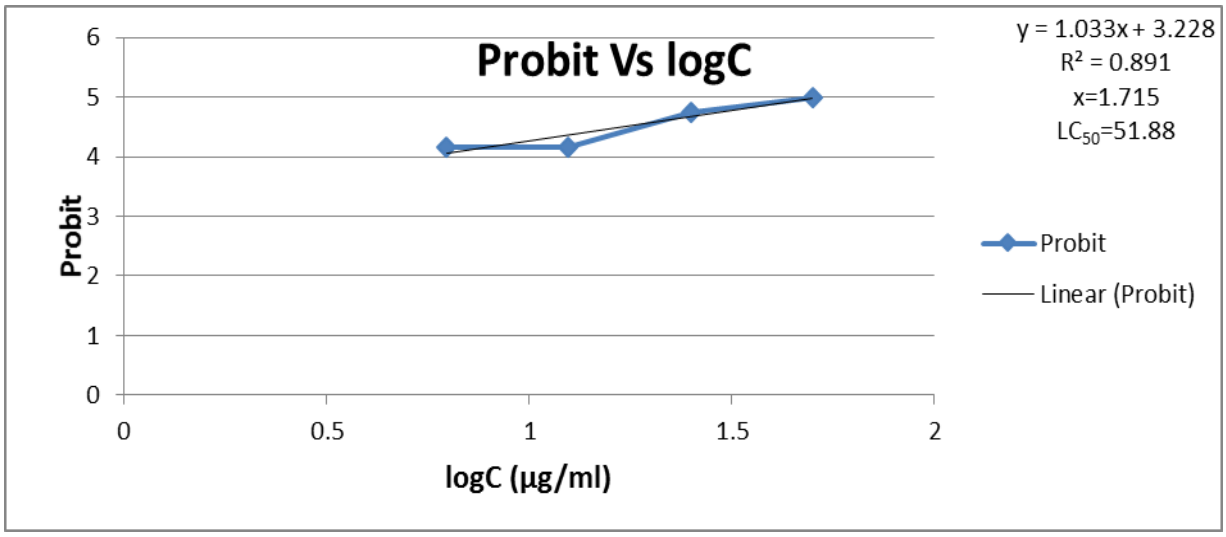

Fig 3. Toxicity assay of *K. pinnata* on brine shrimp. The results are expressed as mean±SEM of three measurements

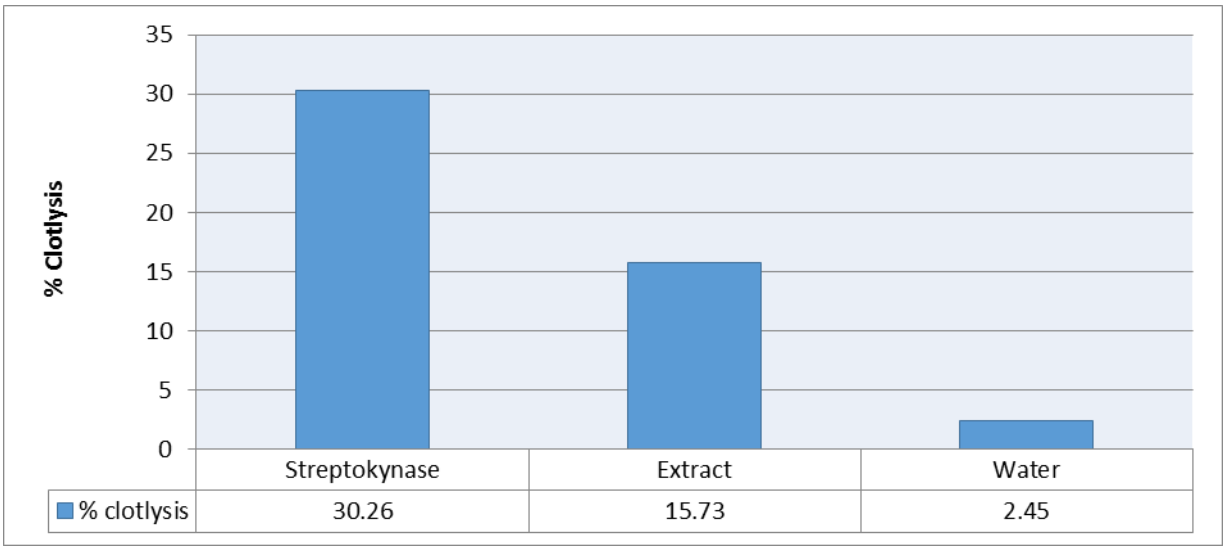

Fig 4. The clot lysis activity of *K. pinnata* extract and streptokinase. All results are mean±SEM of three consecutive experiments

3.1 Statistical analysis
All the results obtained by *in vitro* experiment were expressed as mean±SEM of three measurements followed by Dunnet's test where $P<0.01$ was considered as statistically significant.

4. Discussion
Antioxidants are helpful in reducing and preventing damage from free radical reactions because of their ability to donate electrons which neutralize the radical without forming another. Crude extracts of fruits, herbs, vegetables, cereals,

and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [64]. The antioxidative effect is mainly due to phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes [65]. Typical phenolics that possess antioxidant activity have been characterized as phenolic acids and flavonoids [66].Over the past three decades, the free radical theory has greatly stimulated interest in the role of dietary antioxidants in preventing many human diseases including cancer, atherosclerosis, stroke, rheumatoid arthritis, neurodegeneration and diabetes [67] Most thrombolytic agents work by activating the enzyme plasminogen, which clears the cross-linked fibrin mesh. This makes the clot soluble and subject to further proteolysis by other enzymes, and restores blood flow over occluded blood vessels. Thus thrombolytic agents are useful for the treatment of myocardial infarction, thromboembolic strokes, deep vein thrombosis and PE to clear a blocked artery and avoid permanent damage to the perfused tissue (e.g. myocardium, brain, and leg).

Ideally, any agent useful in the treatment of cancer should not be toxic to normal cell. However, in reality, anticancer agents are often toxic to normal cells, particularly towards rapidly growing cells [68]. It is necessary to test this extract in low concentration to evaluate its potency and also against various cancer celllines as well as normal cell line so justify the potential to further investigate this plant for anticancer activity.

Hence this study was conducted by crude extract of *K. pinnata*, further advanced studies should be carried out for compound isolation and it is necessary to observe which compounds are actually responsible for specific effects.

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