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In vitro Thrombolytic and Membrane Stabilizing Studies of *Brassica rapa* subsp. *chinensis* (L.) Hanelt

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Abstract

Brassica rapa subsp. *chinensis* (L.) Hanelt is a popular herb in Bangladesh. In the present studies, the plant has been assessed to evaluate its *in vitro* thrombolytic and membrane stabilizing properties. Initially, the plant was collected, processed and extracted with methanol and the concentrated crude methanol extract was fractionated into petroleum ether, carbon tetrachloride, chloroform and aqueous soluble materials. All extractives, including the parent extract were subjected to thrombolytic and membrane stabilizing bioassays. In case of thrombolytic study, the carbon tetrachloride soluble fraction exhibited highest clot lysis. Here, streptokinase and distilled water were used as positive and negative control, respectively. However, in the assay for membrane stabilizing activity, the crude methanol extract along with other extractives were capable to inhibit hemolysis of erythrocyte membrane in hypotonic solution- and heat- induced conditions, which indicated anti-inflammatory property of the samples. Acetyl salicylic acid was used as standard drug in this assay. This represents the first report of thrombolytic and membrane stabilizing activities of *B. rapa* subsp. *chinensis*.

Key words: *Brassica rapa* subsp. *chinensis*, Brassicaceae, membrane stabilization, thrombolytic, anti-inflammatory

Introduction

Medicinal plants have been proven to be very promising candidates for developing new drugs. Plants have wide range of potentials for treating different types of diseases. Systematic screening of plants might be a good tool for finding and isolating bioactive lead molecules (El-Abhar and Schaalan, 2014; Jin *et al.*, 2014; Naveen *et al.*, 2014).

Thrombosis and inflammation are frequently observed in human body for making very complicated situations. Many drugs have been developed to take care of these problems but side effects still remain as a matter of concern (Fox and Kahn, 2005; Rodriguez *et al.*, 2012; Wilson and Chaikof, 2008). To obtain safer molecules, comprehensive studies are still going on. Natural products might be very useful resources for finding the desired drug.

Bangladesh is in the tropical zone blessed with thousands of plants and people here are accustomed with herbal practices for long time (Chowdhury *et al.*, 2010; Jahan *et al.*, 2010; Rahman *et al.*, 2011). Plants growing here have not been systemically evaluated yet to a significant extent. With this view, *B. rapa* subsp. *chinensis* was selected here to explore its medicinal properties.

B. rapa subsp. *chinensis* (L.) Hanelt (Bengali name: Bati shak) belongs to the Brassicaceae family. The leaves are commonly light green and thin. White-fleshed roots are developed at the base of the leaf petioles. At the top of the raceme the flowers remain as cluster. This plant is usually famous as vegetable, which is very low in calories. It is a very good source of many nutrients, vitamins, minerals and antioxidants (Dominguez-Perles *et al.*, 2014; Siddiqui *et al.*, 2014). Previous phytochemical investigations on *Brassica* genus led to the isolation of various types of phenolics and organic acids (Fernandes *et al.*, 2007; Mucha-Pelzer *et al.*, 2010; Romani *et al.*, 2006; Tenore *et al.*, 2012).

The aim of the current study was to evaluate the *B. rapa* subsp. c*hinensis* for thrombolytic and membrane stabilizing activities for the first time.

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Materials and Methods

Collection of plant materials: The whole plants of *Brassica rapa* subsp. *chinensis* were collected from Gazipur, Bangladesh on June 2013 and a voucher specimen (DACB accession no. 39457) has been deposited at Bangladesh National Herbarium, Mirpur, Dhaka for future reference.

Extraction: The collected plant materials were chopped, dried and powdered. About 500 g of the powdered materials was soaked in 1.5 litre of methanol at room temperature for 7 days. The extract was filtered through cotton plug and concentrated with a rotary evaporator. An aliquot (5 g) of the concentrated methanol extract was fractionated by the modified Kupchan method (VanWagenen *et al.*, 1993) into petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions. Subsequent evaporation of solvents yielded petroleum ether (PE, 0.80 g), carbon tetrachloride (CT, 1.80 g), chloroform (CF 1.20 g) and aqueous (AQ 1.20 g) soluble materials, respectively.

Thrombolytic activity: In case of *in vitro* thrombolytic assay (Prasad *et al.*, 2006; Prasad *et al.*, 2007), 5 ml of venous blood was drawn from healthy volunteers, distributed in different pre-weighed sterile microcentrifuge tubes (0.5 ml/tube) and then incubated at 37 °C for 45 minutes. After clot formation, the serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the weight of the clot. To each tube, each of the extractives (1 mg/100 µl water) was added. Here, 100 µl (equivalent to 30,000 I.U.) of streptokinase (Altepase®, Beacon Pharmaceuticals Limited, Bangladesh) and 100 µl of distilled water were used as positive control and negative control, respectively. After incubation of the tubes at 37 °C for 90 minutes, the release of fluid from the clot was removed and tubes were again weighed to observe the difference in weight after clot disruption. Percentage of clot lysis was expressed as:

% thrombolysis = (weight of clot after treatment /weight of clot before treatment) × 100

Membrane stabilizing activity: The following two methods were used for conducting this *in vitro* membrane stabilizing assay (Shinde *et al.*, 1999; Sikder *et al.*, 2012).

i) *Hypotonic solution-induced hemolysis:* To prepare the erythrocyte suspension, 5 ml of whole blood was obtained from healthy human volunteers in a tube containing dipotassium salt of EDTA (2.2 mg/ml of blood). The blood was centrifuged, supernatant was removed and blood cells were washed three times by sodium chloride isotonic solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation (10 min at 3000 g) using the same volume as supernatant. Finally, it was resuspended in the same volume of this isotonic buffer solution. After that, 0.5 ml of this was mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (2 mg/ml) or reference drug, acetyl salicylic acid (0.1 mg/ml). The control sample was consisted of 0.5 mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the optical density (OD) of the supernatant was measured at 540 nm. The percentage inhibition of hemolysis was calculated using the following equation-

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

ii) *Heat-induced hemolysis:* 5 ml of isotonic buffer containing aliquots (1 mg/ml) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle (5 ml) was used as control in a separate tube. Afterwards, 30 µl erythrocyte suspension (prepared similarly as in hypotonic solution induced hemolysis method) was added to each tube and mixed gently. One pair of the tubes and control samples were incubated at 54 °C for 20 min in a water bath, while the other pair was maintained at 0-5 °C in an ice bath. The reaction mixture was centrifuged for 3 min at 1300 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition or acceleration of hemolysis in tests was calculated according to the equation:

$$\% \text{ Inhibition of hemolysis} = \{1 - (OD_{heated\ test\ sample} - OD_{unheated\ test\ sample}) / (OD_{heated\ control\ sample} - OD_{heated\ test\ sample})\} \times 100$$

Statistical analysis: Three replicates (n = 3) of each sample were used for statistical analysis and the values are reported as mean ± standard deviation (SD).

Results and Discussion

Thrombolytic activity: The thrombolytic activity of the plant extractives are shown in Figure 1. The highest thrombolytic activity (58.57 %) was noticed by the carbon

tetrachloride soluble fraction of the crude methanolic extract. However, moderate clot lysis ability (considering >40 % lysis) was also exhibited by the chloroform (49.39 %) and petroleum ether (43.37 %) soluble fractions. However, the crude methanolic extract and its aqueous soluble fraction showed weaker thrombolytic activity.

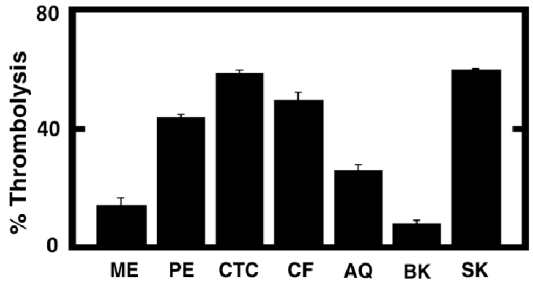

Figure 1: Effect of different extractives of *B. rapa* subsp. *chinensis* on thrombolysis. Here, ME: crude methanolic extract; PE- petroleum ether soluble fraction of methanolic extract; CTC- carbon tetrachloride soluble fraction of methanolic extract; CF- chloroform soluble fraction of methanolic extract; AQ-aqueous fraction; BK-blank (negative control); SK-streptokinase (positive control).

Membrane stabilizing activity: The membrane stabilizing activities are depicted in Figure 2. In case of hypotonic solution induced hemolysis (Figure 2A), the maximum level of membrane stabilizing activity was observed by the crude methanolic extract (78.31 %). Besides, all the partitionates of the crude methanolic extract except petroleum ether soluble fraction displayed more than 70 % of the membrane protecting capabilities.

In case of heat induced hemolysis (Figure 2B), the crude methanol extract and its chloroform soluble fraction exhibited stronger protection to the membrane (>80 % inhibition of hemolysis). Among all other partitionates, the carbon tetrachloride and aqueous soluble extractives showed more than 55 % inhibition of the heat induced hemolysis of membrane.

Bangladesh is a country which is blessed with numerous medicinal plants. For thousands of years, people have been using plants for traditional healing. Many of them are considered beneficial but there is a great lack of systematic evaluation to prove the efficacy of the plant remedies. In this consideration, scientific evaluation is the demand of time to explore the medicinal functional aspects of these plants (Islam *et al.*, 2009; Kabir *et al.*, 2010; Rahman *et al.*, 2008a; Rahman *et al.*, 2008b). *Brassica* is a genus encompassing lot of species. People are used to take this plant as vegetables in Bangladesh and many other countries. Nutritionally these herbs are considered very much exclusive (Dominguez-Perles *et al.*, 2014; Fernandes *et al.*, 2007). Therefore, it is a good opportunity to study this plant for evaluating other health benefits. In the current studies, among many *Brassica* speices, *B. rapa* subsp. *chinensis* (L.) was subjected to the thrombolytic and membrane stabilizing assays for the first time.

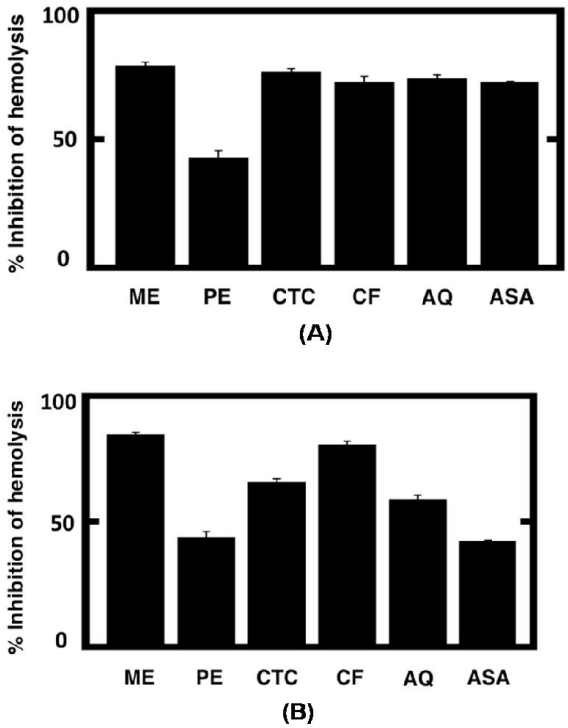

Figure 2: Effect of different extractives of *B. rapa* subsp. *chinensis* on hypotonic solution induced (A) and heat induced (B) hemolysis of erythrocyte membrane. In both cases, ME: crude methanolic extract; PE- petroleum ether soluble fraction of methanolic extract; CTC- carbon tetrachloride soluble fraction of methanolic extract; CF- chloroform soluble fraction of methanolic extract; AQ- aqueous fraction; ASA- acetyl salicylic acid (standard drug).

Thrombus formation in the circulatory system causes vascular blockage leading to death. Currently used thrombolytic agents that include tissue plasminogen activator, urokinase, streptokinase etc. are used all over the world but still associated with risk of hemorrhage, anaphylactic reaction and lack specificity. So, attempts are still ongoing around the world to develop improved thrombolytic agents (Hilleman and Campbell, 2011; Tsikouris and Tsikouris, 2001; van Domburg *et al.*, 2000;

Woo and White, 1993). With this view, this investigation was done on *B. rapa* subsp. *chinensis*. The carbon tetrachloride, chloroform and petroleum ether soluble partitionates of the crude methanol extract showed potent thrombolytic activity (Figure 1). It was previously reported that polyphenols and flavonoids have significant thrombolytic activity (Chen *et al.*, 2012; Fuentes *et al.*, 2014). *B. rapa* subsp. *chinensis* is also rich in polyphenols and flavonoids as secondary metabolites (Fernandes *et al.*, 2007; Romani *et al.*, 2006). This might be a reason to display noticeable thrombolytic activity of this plant.

Inflammation is the pathology to make people suffer. Numerous anti-inflammatory drugs are available in the market but all remain with some side effects more or less. If dietary agents can provide some anti-inflammatory effects, it might be very beneficial in the time of need. Membrane-stabilizing experiment can serve as an indicator to screen out the anti-inflammatory agents. Compounds with membrane-stabilizing properties can prevent the release of phospholipases that initiate the formation of inflammatory mediators (Shinde *et al.*, 1999). In this regard, the experimental herb was subjected to membrane stabilizing assay in order to check its performance to prevent hemolysis by both hypotonic solution- and heat- induction. The extractives of *B. rapa* subsp. *chinensis* (L.) demonstrated significant membrane stabilizing property (Figure 2). It is noteworthy to mention that this plant possesses a lot of phenolics and flavonoids (Fernandes *et al.*, 2007; Romani *et al.*, 2006; Tenore *et al.*, 2012), which may be considered as causative agents to show substantial anti-inflammatory activity (Jean-Gilles *et al.*, 2012; Jin *et al.*, 2010; Pan *et al.*, 2010; Santangelo *et al.*, 2007). Even this plant is taken as vegetable, medicinal properties may add some values over nutritional contribution.

Conclusion

B. rapa subsp. *chinensis* (L.) extractives were evaluated for the first time to check their thrombolytic and membrane stabilization potential. The plant demonstrated potent thrombolytic and membrane stabilization activities. Even though, this herb is generally considered as vegetable, the current studies have proven it to be a useful remedial plant. Further comprehensive investigations are required to isolate the bioactive compounds and to know their detailed underlying mechanism.

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