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Research Article

In-vitro Investigation of Anti-Coagulation Property of Four Bangladeshi Plants of *Crotalaria* Species and Analysis of their Qualitative Bioactive Compounds

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ABSTRACT

The ischemic stroke caused by thrombosis of the arterial vessels is a growing concern of this world right now. Present treatment for this disease is not satisfactory for all. That is why we aim to run this study with herbal preparations, as because we know herbal preparations are safe enough than any others. An in vitro thrombolytic model was used to check the clot lysis effect of the crude extracts of four Bangladeshi plants, which are, *Crotalaria retusa, Crotalaria alata, Crotalaria prostrata* and *Crotalaria verrucosa*. Streptokinase here was used as a positive control and water as a negative control. In short, venous blood withdrawn from twenty healthy volunteers was allowed to form clots that were weighed and treated with the test plant materials to disrupt the clots. Weight of clot after and before treatment provided a percentage of clot lysis. Through this model we got plants like *C. retusa* and *C. prostrata* showed highest significant potency, 39.16 ± 9.52% and 37.85 ± 7.74%, respectively compared with positive control standard streptokinase (79.55 ± 9.09%) and negative control distilled water (7.41 ± 2.76%). Other extracts showed moderate clot lysis activity, *C. alata* and *C. verrucosa* respectively lysis clots 31.63 ± 6.60% and 35.88 ± 8.94%. Our study suggests that thrombolytic activity of *C. retusa* and *C. prostrata* could be considered as very promising and beneficial for the Bangladeshi traditional medicine and should be proceed for further study regarding this activity.

Keywords*: C. retusa, C. verrucosa, C, prostrata, phytochemical analysis, thrombolytic*

INTRODUCTION

Herbal foods contain significant amounts of bioactive compounds that provides more than desirable health benefits and large-scale nutrition. The World Health Organization (WHO) reported that 80% of total populations of the world beliefs principally on indigenous medicine and that the majority of traditional therapies involve the use of either plants or of their active constituents[1] and more than 25% of modern medicines that we are using worldwide, contains compounds extracted from medicinal plants[2]. The active constituent is different from one plant to another plant, due to their biodiversity, and they produce a definite pharmacological action on the human body that develops interest on their medicinal importance[3]. In past years, there has been a restoration in the use of customary medicinal herbs and therefore, pharmaceutical companies are interested in investing a lot of money in developing herbal products extracted from plants[4]. In Bangladesh thousands of plant species are known to have pharmacological importance[5] and ninety percent of the medicinal plants are wild sourced [6, 7].

Thrombosis is a fatal disease that characterized by the development of a blood clot (thrombus) in the circulatory system due to the malfunction of homeostatic system of the body. It leads to vascular blockade, causes other fatal consequences, like myocardial or cerebral infarction, and lastly lead to death[8]. Thrombosis underlies some acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks and recently these are the major causes of death in developed countries[9-11]. Therefore, anticoagulation or thrombolytic therapy is essential for the management of systemic coagulation, and the proper choice of thrombolytic drugs to decrease platelet aggregation process can be critical. With the modernization in the field of pharmaceuticals, many drugs are available with the purpose of dissolving clots, like as alteplase, anistreplase, streptokinase, urokinase etc.[12]. Out of these drugs, streptokinase and urokinase are most common in practice, because their cost and efficacy are far

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Table 1: Phytochemicals present in the four plants of *Crotalaria* species

Chemicals	*C. retusa*	*C. alata*	*C. prostrata*	*C. verrucosa*
Alkaloid	++	++	+	++
Saponin	-	--	++	++
Steroid	++	++	++	++
Flavonoid	+	-	++	+
Tannin	+	+	++	+
Reducing sugar	++	++	++	++
Phenol	-	++	-	++

Table 2: Percentage yield

Herbs	Final weight (gm)	Percent yield
C. retusa	27.5	5.5 %
C. alata	23.3	4.66 %
C. prostrata	19.5	3.9 %
C. verrucosa	24.7	4.94 %

better than the other thrombolytic drugs[13, 14]. However, due to the weak substrate specificity of these first generation drugs (streptokinase and urokinase) have serious complication like, systemic fibrinolysis, anaphylactic reaction and hemorrhage[15]. Once again, it is prohibited to choose multiple treatments with streptokinase in a given patient, because of immunogenicity[16].

Cardiovascular disease due to thrombus formation is one of major disease that are increasing at an alarming rate in the past few years[17]. Homeostasis regulate the circulation of blood in the system after damaging of the vascular channel[18] and development of thrombus interrupt this with some critical event in the arterial diseases associated with myocardial infarction, anoxia, hypertension[19], stroke, reduction of the blood supply to the liver[20] and venous thromboembolic disorders that account for considerable number of deaths worldwide[21]. Notable efforts have been made towards the discovery and development of natural constituents from various plant and animal sources that have antiplatelet[22,23], anticoagulant[24,25], antithrombotic[26] and thrombolytic activity[27-29]. 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[30].

METHODS AND MATERIALS

Plant Collection

The leaves of four plants of *Crotalaria* species (*C. retusa, C. alata, C. prostrate* and *C. verrucosa*) were collected from different areas of Chittagong hill tracts, Bangladesh. Voucher sample numbers these plants issued from the Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh. These were 49287, 49288, 49289, 49290 and 49291 respectively. The leaves were sun dried for several days and then oven dried for 24 hr at considerably low temperature (not more than 40°C) for better grinding.

Extract Preparation

The desirable dried sample plants were grinded. About 500 g of powdered sample from each plant materials attenuated in 2 L of 95% methanol and kept it for 7 days with rigorous shaking. Then filtered through a cotton plug accompanied by Whatman filter paper number 1. Applying a temperature of 40°C to 45°C and reducing the pressure, the extract was concentrated with the assistance of a rotary evaporator[31].

Chemicals

To the commercially available lyophilized Streptokinase vial (Polamin Werk GmbH, Herdecke, Germany) of 1,500,000 IU, 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 thrombolysis assay.

Blood Specimen

Blood (4 mL) specimen was drawn from healthy human volunteers (n = 20) without a history of anti-inflammatory or anticoagulant therapy using a protocol approved by the Institutional Ethics Committee of IIUC (International Islamic University Chittagong), Chittagong-4203, Bangladesh. An earlier consent, approval number IUC-D/P-05/13, was taken for the purpose of collection of blood samples from human volunteers. A 500 µL of blood was transferred to each of the previously weighed microcentrifugal tubes to form clots.

Thrombolytic Assay

Collected fresh blood was poured in pre-weighed sterile micro-centrifuge tube (500 µL per tube) and incubated at 37°C for 45 min. The observations were taken for three times. After clot formation, the serum was completely removed without disturbing the clot, and each tube having the clot was again weighed to determine the clot weight (Clot weight = Weight of clot containing tube - Weight of tube alone). To each micro-centrifuge tube containing the pre-weighed clot, 100 µL of aqueous solution of different plant extracts such as methanolic extract of *C. retusa*, *C. alata*, *C. prostrata* and *C. verrucosa* were added individually. About 100 µL of Streptokinase considered as the positive control and about 100 µL of distilled water considered as the negative control were individually added to the control tubes. At 37°C for 90 min, all the tubes were incubated and observed for clot lysis. After the incubation process, the excess fluid was removed, and the tubes were again weighed to mark the deviation in weight after clot disruption. Deviation obtained in weight taken before and after clot lysis was conveyed as percentage of clot lysis using the following equation: % of clot lysis = (weight of released clot/clot weight) × 100[32-34].

Phytochemical Screening

A general screening process was carried out with the crude methanol extract of the four plants to find out the primary phytochemicals, following the methods described by Muanda, 2010[35].

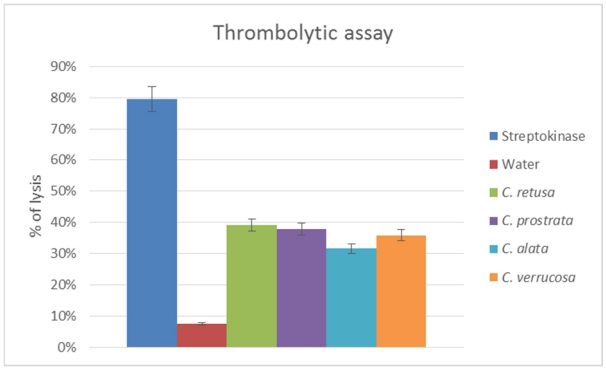

Figure 1: Schematic representation of thrombolytic assay of four plants from *Crotalaria* species; *C. retusa* found highest potent among other species in comparison with standard.

Table 3: Percentage lysis

Herbal preparation	Percent of lysis
Water	7.41 ± 2.76
Streptokinase	79.55 ± 9.09 *
C. retusa	39.16 ± 9.52 **
C. alata	31.63 ± 6.60***
C. prostrata	37.85 ± 7.74***
C. verrucosa	35.88 ± 8.94**

Here percent of lysis represented as mean ± SD; *p<0.001, **p<0.01, ***p<0.004; when compared to water (negative control)

Statistical Analysis

All data analyzed here was made through the statistical software GraphPad Prism 6, and they are represented here as mean ± SD and the null hypothesis set before this study, there is no pharmacological effect in these plants, would be rejected in $p<0.05$.

RESULT

Primary Phytochemicals

The phytochemical screening of the crude extracts of *Crotalaria* species indicates qualitative presence of alkaloid, saponins, steroids, flavonoids, tannins and reducing sugar (Table 1). The percent of the crude extract yields are presented in the (Table 2).

Thrombolytic Activity

Addition of 100 µL SK (positive control) to the clots along with 90 minutes of incubation at 37°C, showed 79.55 ± 9.09% of clot lysis. Water, as negative control, when treated-clots results 7.41 ± 2.76% of clot lysis that is quite negligible. Methanolic extract of *C. retusa* showed the highest (39.16%), with the significant limit of $P< 0.01$, clot lysis activity among the other extracts. All other plant extract of *Crotalaria* species have shown quite similar activity, only the methanolic extract of *C. alata* showed lowest (31.63%) activity. Percent clot lysis obtained after treating the clots with different organic extracts and appropriate controls is shown in Table 3 and represent in Figure 1.

DISCUSSION

A number of plants source, especially study with some fruits and vegetables concluded with their capacity as anti-coagulant, anti-platelet and fibrinolytic activity. There is also some data suggesting that consuming such food can prevent coronary events and stroke[36-38]. In our present study, four different extracts of *Crotalaria* plant species showed the thrombolytic activity among which the crude extracts of *C. retusa* and *C. prostrata* had the significant activity than the other plants. There is evidence bacterial contaminants of plants may present and that can have plasminogen receptors, which binds to plasminogen. This binding can then easily activate to plasmin, which could lead to fibrinolysis[39], it is also true some other herbal extract shows their thrombolytic or fibrinolytic effects by their content of certain fibrinolytic proteases enzymes. However, bacterial plasminogen activator: staphylokinase, streptokinase, act as cofactor molecules that contribute to exosite formation and enhance the substrate presentation to the enzyme. Staphylokinase activates plasminogen to dissolve clots, also destroys the extra-cellular matrix and fibrin fibers that hold cells together[40,41]. Research is going on to find the link between thrombolytic activity of plant content and anti-staphylococcal effects, although some of these plant products are modified further in order to use as thrombolytic drugs which are more site specific and effective[42]. Individual chemical component-activity relationship are the next step of this present study that can explore some of the other new clue of our observed effects of these plants.

CONCLUSION

We have described the thrombolytic activity of some locally available plants. These four plants used by different tribes in different pharmacological purposes. This study with four different plants validated by in vitro blood clots lysis activity of their organic extracts. However, *C. retusa* and *C. prostrata* showed the promising thrombolytic effects and they to be studied further for to suit them in therapeutic applications. Some of the bioactive components present in those plants have been identified primarily, but a quantitative analysis with them is needed. A dose response study in in vivo model is suggested to make to understand their pharmacokinetic property as well. These indigenous sources might be, going to be future hit.

AUTHORS CONSENT

All the authors contributed in this project declared that they have no conflict with this article.

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