Phytochemical screening, Antioxidant, Thrombolytic, α-amylase inhibition and cytotoxic activities of ethanol extract of Steudnera colocasiifolia K. Koch leaves

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ABSTRACT
Objective: Present study aims to investigate both qualitative and quantitative phytochemicals presence, antioxidant, thrombolytic, α-amylase and cytotoxic activity of a Bangladeshi plant Steudnera colocasiifolia K. Koch leaf ethanolic extract. Methods: Phytochemical screening was accomplished by using established methods. Thrombolytic assay was conducted through clot lysis method. Alpha amylase inhibition assay was made by modified enzyme inhibitory action and cytotoxicity was studied by brine shrimp lethality test. Data was analyzed by One Way Analysis of Variance (ANOVA) using statistical software Statistical Package for Social Science (SPSS, Version 22.0, IBM corporation, NY) followed by student ‘t’ test with GraphPad Prism Data Editor for Windows, Version 6.0 (GraphPad software Inc., San Diego, CA). Results: Primary screening shows the presence of major metabolites such as alkaloid, flavonoid, tannin, saponins, steroid, quinone, cellulose and glycosides while the quantitative analysis elicits the presence of magnificent amount of flavonoid (70.60 ± 0.23), proanthocyanidin (64.48 ± 0.98), flavonol (71.22 ± 0.07) and total antioxidant (66.40 ± 0.28) in the extract. The thrombolytic effect of S. colocasiifolia was found to be 35.16% and this extract had good α-amylase inhibitory activity (IC50=2.16 ± 0.06 mg/ml) as compared to Acarbose. The extract was found to have a LC50 value of 305.2 μg/ml in Brine shrimp lethality bioassay. Conclusion: Results demonstrate the Steudnera colocasiifolia has a very good prospect to be studied further for its extended antioxidant and α-amylase inhibitory effects.

Key word: Steudnera colocasiifolia, Antioxidant, Thrombolytic, α-amylase, Cytotoxic activity.

INTRODUCTION
Plants and plant-derived sources not only provide us foodstuff, shelter but also they provide remedies for many years. Different chemical constituents contained in plant exhibit different activities for alleviating abnormal health of human or animals. Therefore, traditional medicine practitioners appreciate to use different parts of plant having several chemical constituents. It is progressively being understood that a hefty portion of today’s diseases are because of the ‘oxidative stress’ which produces enormous free radicals, causing tumor, atherosclerosis and cardiovascular illnesses. All human cells ensure themselves against free radical harm by catalysts such as ascorbic acid, tocopherol and glutathione. However, frequently these defensive systems are becoming upset by different neurotic techniques, and cell reinforcement supplements are imperative to battle oxidative harm. This is because much consideration has been controlled towards the improvement of ethnomedicine with solid cell reinforcement properties with low cytotoxicities. Thrombus formed in the circulatory system due to the loss of homeostasis causes vascular blockage, atherothrombotic sicknesses, myocardial or cerebral localized necrosisultimately prompting death. Due to the associated high risk of bleeding intracranial hemorrhage, severe anaphylactic reaction and lacks specificity gastrointestinal bleeding or hypertension Streptokinase and Urokinase, tremendous efforts have also been directed towards the discovery and development of natural products such as the oral anticoagulants are derived from coumarin which is found in numerous plants like warfarins.

Alpha-amylase is a well-known enzyme found in the pancreatic juice and saliva which breaks down large insoluble starch molecules to lower the levels of postprandial hyperglycemia. Several inhibitors of α-amylase have been isolated from different medicinal plants to serve as an alternative drug with increased potency and lesser adverse effects than existing synthetic drugs. However, the α-amylase inhibitory effect of Steudnera colocasiifolia is still undiscovered. Additionally, the toxicity profile of even a potent plant source is inevitable to be recorded for fruitful use.

S. colocasiifolia K. Koch (family: Araceae) is an evergreen, creeping and ascending usually 30-50 cm long herb which is persistently blade paler. It is habituated in dense forests, wet meadows, by streams, seasonally moist lowland forest. It is distributed in Bangladesh, India, Myanmar, Thailand and China. Locally it is used to treat injuries, cuts, snake and insect bites and skin ulcers. Whole plant extract of S. colocasiifolia has been found to show anti-arthritic and anti-inflammatory activities. This research investigated the phytochemical presence, antioxidant potential, thrombolytic effect, α-amylase inhibitory action and cytotoxic effect of S. colocasiifolia ethanolic extract.

MATERIAL AND METHODS
Chemicals and reagents
All the chemicals and reagents used in this research were of analytical grade. Ethanol, chloroform, sulfuric acid and hydrochloric acid were purchased from Merck (India). Gallic acid, Folin-Ciocalteau reagent and trichloroacetic acid (TCA) were procured from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). A 2, 2-diphenyl-1-picryl-hydrazine (DPPH) free radical was used in the study. All the chemicals and reagents used in this research were of analytical grade. Ethanol, chloroform, sulfuric acid and hydrochloric acid were purchased from Merck (India). Gallic acid, Folin-Ciocalteau reagent and trichloroacetic acid (TCA) were procured from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). A 2, 2-diphenyl-1-picryl-hydrazine (DPPH) free radical was used in the study. Ethanol, chloroform, sulfuric acid and hydrochloric acid were purchased from Merck (India). Gallic acid, Folin-Ciocalteau reagent and trichloroacetic acid (TCA) were procured from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). A 2, 2-diphenyl-1-picryl-hydrazine (DPPH) free radical was used in the study.
hydrazyl (DPPH) and aluminium chloride were purchased from Fluka (Fluka chemie GmbH, CH-9471 Buchs). Ascorbic acid, Quercetin and Tannic acid were purchased from BDH Chemicals (BDH Chemicals Ltd, Poole, England). Ferric chloride, potassium ferricyanide, sodium hydroxide and sodium nitrite were purchased from Riedel-De Haen Ag, Seelze-Hannover- Germany. Shimadzu Biospec 1601 UV visible spectrophotometer (Shimadzu, Japan) was used to measure the absorbance. Lyophilized streptokinase vial (1500000 IU) was purchased from Square Pharmaceuticals Ltd (Kalaiakor, Gazipur, Bangladesh). Vincristine sulfate (2 mg/vial) was donated by Techno Drugs Limited Bangladesh.

**Plant material**

Fresh leaves of *S. colocasiifolia* were collected from Alu Tila, Khagrachari, Chittagong, Bangladesh in the month of September, 2014. It was authenticated by Dr. Sheikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh. A voucher specimen of the plant has been preserved to the departmental Herbarium with the ID. No: 1316 CTGUH.

**Preparation of Extract**

The leaves were dried for a period of 10 days under shade and ground with a locally available electrical hand grinder. The resulting powder (250 g) was soaked in 800 ml of 98.5% ethanol for a week at room temperature with occasional stirring. The whole mixture was filtered with double layered cheese-cloth followed by a final filtration with Whatman filter paper (No.1) to obtain the filtrate which was concentrated and the filtrate thus obtained was concentrated using a rotary evaporator (RE 200, Bibby Sterling Ltd., UK) to get a viscous mass known as crude extract (2.8 g. 7% yield) preserved at 4°C until in need for further use.

**Phytochemical Screening**

The freshly prepared crude extract was qualitatively tested for the presence of secondary metabolites especially saponins, flavonoids, steroids, alkaloids, carbohydrates, terpenoids, tannins, quinine, cellulose and glycosides through established methods.7

**In vitro Antioxidative Activity**

**DPPH free radical scavenging activity**

Free radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl, DPPH) was carried out using the method of Brand-Williams.4 Different concentrations (400, 200, 100, 50, 25 and 12.5 μg/mL) of *S. colocasiifolia* extract were dissolved in methanol and placed in different test tubes, and 3 mL of a 0.004% w/v methanol solution of DPPH was added to each test tube. Absorbance at 517 nm was determined after 30 min against a blank, and the percent inhibition activity was calculated from [(A0-A1)/A0]×100, where A0 is the absorbance of the control and A1 is the absorbance of the sample. Ascorbic acid was used as a reference standard and dissolved in methanol to make the stock solution with the same concentration. The control sample was prepared containing the same volume without any extract or reference drug. Methanol served as a blank. The absorbance vs. concentration was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid was taken as a reference standard. Phosphate buffer (pH 6.6) was used as a blank solution.

**Determination of total phenolic content**

The total phenolics content of the extract was estimated according to the method described by Singleton and Rossi.13 The phenolic concentration of extracts was evaluated from a gallic acid calibration curve. To prepare a calibration curve, 0.5 mL aliquots of 12.5, 25, 50, 100, 200, and 400 μg/mL methanolic gallic acid solutions were mixed with 2.5 mL Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. After incubation at 25°C for 30 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank. Methanol was served as blank. The Total content of flavonoid compounds in *S. colocasiifolia* extract was expressed in mg/g quercetin equivalent (QE).

**Total flavonol**

Total flavonol content was determined by adopting the protocol described by Bright et al.15 One ml of *S. colocasiifolia* extracts or standard of different concentrations was taken in a test tube and 3 mL of methanol was added. Then 200 μl of 10% aluminium chloride solution was added into the same test tube followed by the addition of 200 μl of 1M potassium acetate. Finally, 5.6 mL of distilled water was mixed with the reaction mixture. The reaction mixture was then incubated for 30 min at room temperature to complete the reaction. Then the absorbance of the solution was measured at 415 nm using a spectrophotometer against blank. Methanol was served as blank. The Total content of flavonoid compounds in *S. colocasiifolia* extract was expressed in mg/g quercetin equivalent (QE).

**Determination of total proanthocyanidin**

Total proanthocyanidin was determined based on the procedure of Oyedemi et al.11 To 0.5 ml of 1 mg/mL of the extract solution was added 3 ml of vanillin–methyl alcohol (4% v/v) and 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was taken at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/ml and expressed as catechin equivalent (mg/g) using the calibration curve equation: Y =0.0255 x, R² = 0.9812, where x is the absorbance and Y is the catechin equivalent.

**Total tannin content**

The tannins were determined using the Folin–Ciocalteu Phenol reagent as reported by Amorim.14 Briefly, 0.1 ml of the sample extract is added with 7.5 ml of distilled water and then added 0.5 ml of Folin–Ciocalteu Phenol reagent, 1 ml of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well, kept at room temperature
for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of tannic acid is read against a blank. The results of tannins are expressed in terms of tannic acid in mg/g of extract. Total tannin content was determined as mg of tannic acid equivalent per gram using the equation obtained from a standard tannic acid calibration curve $Y = 4.5692x - 0.2538$, $R^2 = 0.9953$, where $y$ is the absorbance and $x$ is the tannic acid equivalent.

**Total antioxidant capacity**

Total antioxidant activity of the fractions was evaluated by the Phosphomolybdate method using Ascorbic acid (AA) as a standard. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the solution was measured at 695 nm against blank using a spectrophotometer. Methanol (0.3 mL) in the place of extract is used as the blank. Ascorbic acid equivalents were calculated using standard graph of AA. The experiment was conducted in triplicates and values are expressed as equivalent of ascorbic acid in mg per g of extract.

**In vitro Thrombolytic Activity**

**Blood sample collection**

Twenty milliliters of whole blood (vein) were drawn from young and healthy human volunteers ($n=20$) without a history of oral contraceptive or anticoagulant therapy using a protocol approved by the Institutional Ethical Review Board. An earlier consent form was supplied to the volunteers for collection of blood samples from human volunteers. A 500 μl of blood was transferred to each of the eight previously weighed micro centrifuge tubes to form clots.

**Clot lysis**

Clot lysis test was performed according to the method described by Prasad et al. In the commercially available lyophilized 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 milliliters of venous blood was drawn from the healthy volunteers ($n=20$) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each twenty 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. Control test tubes were subjected to DMSO in artificial seawater at the same concentration as it was made for samples. After 24 h incubation at 25-30°C, the number of viable nauplii was counted using a magnifying glass. The percent (%) mortality was calculated using the following formula:

$$% \text{ Mortality} = \frac{N_t}{N_0} \times 100$$

Where, $N_t = \text{Number of dead nauplii after 24 hrs of incubation}$, $N_0 = \text{Number of total nauplii transferred (n=20)}$. The Median lethal concentration ($IC_{50}$) was then determined.

**Statistical analyses**

All analyses were carried out in triplicates and values were presented as Mean ± SEM. Data was analyzed by One Way Analysis of Variance (ANOVA) using statistical software Statistical Package for Social Science (SPSS, Version 22.0, IBM corporation, NY) followed by student ‘t’ test with GraphPad Prism Data Editor for Windows, Version 6.0 (GraphPad software Inc., San Diego, CA). P<0.05 was considered as statistically significant.

**RESULTS**

**Primary phytochemical Screening**

Data for primary phytochemical screening are shown in Table 1. Results indicated that the extract contains alkaloids, carbohydrates, phenols, tannin, flavonoids, saponins, steroids, quinone, cellulose and glycosides.

**In vitro Antioxidant Activity**

**DPPH radical scavenging activity**

Results for the free radical scavenging activity of ethanol extract of *S. colocasiifolia* are shown in Figure 1. The extract showed a dose dependent radical scavenging effect in DPPH assay. The half inhibition concentration ($IC_{50}$) for free radicals achieved by the extract is 39.01 μg/ml which is statistically significant compared to that ($IC_{50}$ 8 μg/ml) of ascorbic acid.

**Reducing capacity**

Results showed a proportional increase of reducing power with the increase of extract concentration (Figure 2). Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample.
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**Figure 1:** DPPH radical scavenging activity of *S. colocasiifolia* leaves; (a) Concentration vs. scavenging effect, (b) Log concentration vs. scavenging effect (IC$_{50}$ was calculated from this plot).

**Figure 2:** Reducing capacity of the ethanolic extract of *S. colocasiifolia* leaves; (a) Reducing capacity vs. concentration, (b) Reducing capacity vs. Log concentration (IC$_{50}$ was calculated from this plot.

**Figure 3:** Thrombolytic activities of ethanolic extract of *S. colocasiifolia* leaves. Values are presented as mean ± SEM for 20 volunteers. Data were processed by paired t-test analysis by using SPSS for windows, version 22.0, followed by Dunnet test as compared to control (positive and negative). *a-c* superscript letters on outside-end of the line bars are significantly different from each other.
Phytochemical analysis of leaf extract of *S. colocasiifolia*

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Ethanol extract</th>
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<tbody>
<tr>
<td>Alkaloids</td>
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<tr>
<td>Carbohydrate</td>
<td>+</td>
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<tr>
<td>Phenol</td>
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<td>Tannin</td>
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<td>Flavonoid</td>
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<td>Quinone</td>
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<tr>
<td>Cellulose</td>
<td>+</td>
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<tr>
<td>Glycosides</td>
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Note: (+) = Present, (-) = Absent.

Quantitative determination of phytochemical contents

Data for total phenolic, total flavonoid, total flavonols, total proanthocyanidin, total tannin and total antioxidant capacity has been summarized in Table 2. Data shows that highest amount of total flavonols, total flavonoids and total antioxidants are present in the extract. However, very promising amount of proanthocyanidin was also existed in *S. colocasiifolia* ethanol extract (SCEEx).

In vitro thrombolytic activity

In thrombolytic activity assay, addition of 100 μl streptokinase as positive control to the clots showed 75 ± 1.08 % lysis of clot. On the other hand, distilled water treated as negative control exhibited a negligible percentage of lysis which was 4.19%. The mean difference in clot lysis percentage between positive and negative control was found statistically very significant (P<0.05). In this study, the crude SCEEx exhibited 35.16 ± 0.97% of thrombolytic activity. The data are shown in Figure 3.

In vitro α-amylase inhibitory activity

Alpha amylase inhibitory action of SCEEx is shown in Table 3. It is noted that both SCEEx and acarbose significantly inhibited α-amylase activity in a dose dependent manner.

In vitro Brine Shrimp lethality bioassay

Cytotoxic effect of the SCEEx is summarized in the Figure 4. The LC₅₀ values for SCEEx and Vincristine sulfate were found to be 305.20 μg/ml and 0.74 μg/ml, respectively.
DISCUSSION

Plants polyphenols greatly contribute to defend the common complications involved with free radicals or reactive oxygen species. Structural and quantitative variations of phenolic classes strongly differentiate their effects as free radical scavengers and potentials as food antioxidants. 

This study reflects the quantitative variations of phenolics as total flavonol > total flavonoid > total antioxidant > total proanthocyanidin > total phenol > total tannin indicating a strong radical scavenging, hydrogen benefactors, and singlet oxygen quenching capability of SCEEx.

The electron donation ability of natural products can be measured by 2,2-diphenyl-1-picyrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test. In the present study SCEEx showed significantly higher inhibition percentage and positively correlated with total phenolic content. Results of this study suggest that the plant extract contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

The activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, reducing capacity and radical scavenging. The antioxidants action of SCEEx has been reflected through their reduction of Fe$^{3+}$-ferricyanide complex to the ferrous form making an evidence of the reducing power. The ferric reducing power activity of SCEEx seems to be due to the presence of existence of polyphenols. The reducing capacity of plant extract may serve as a significant indicator of its potential antioxidant activity.

Thrombolytic drugs block the pathway of thrombus formation with the help of plasmin, a natural fibrinolytic agent, that lyzes clot by breaking down the fibrinogen and fibrin contained in a clot. Commercially available thrombolytic drugs especially streptokinase forms a 1:1 stoichiometric complex with plasminogen fostering the conversion of plasminogen to plasmin and thereby increasing clot lysis. Scientists reported that phytoc hemical metabolites especially flavonoids affect thrombosis and cardiovascular disease by interfering with platelet activation, a potential risk factor for cardiovascular disease. High content of flavonoids (70.60 ± 0.23 mg Quercetin/g) of SCEEx is very consistent with previous documents as well as basic hypothesis indicating SCEEx the role of flavonoids for clot lysis activity in this research.

The inhibition of α-amylase, which hydrolyzes starch and related polysaccharides, involved in the breakdown of starch has been suggested to be a useful approach to the management and prevention of type 2 diabetes. Amylase inhibitors contain substances that prevent dietary starch from being absorbed by the body. In recent years, extensive investigation on pharmacological actions of plant metabolites focuses that the main protective effect of plant substances has been attributed to the presence of antioxidants such as flavonoids, isoflavone, flavones, anthocyanin, catechin and isocatechin. Strong antioxidant effect led by highest content of flavonols and flavonoids in the studied plant extract might be the contributory factors for α-amylase action of SCEEx.

The brine shrimp lethality bioassay is found to have a good correlation with cytotoxic activity in some human solid tumors and with pesticidal activity, and led to the discovery of new class of natural pesticides and active antitumoral agents. Hypothetically LC$_{50}$ values less than 250 µg/ml is usually considered toxic and very potential for further investigation to verify the effect of the extract as anticancer, antitumor and pesticidal agent. However, SCEEx was found to have a LC$_{50}$ value of 305.2 µg/ml, which is not toxic for formulation as therapeutics.

CONCLUSION

The results stated above showed that the ethanolic extract of S. colocasiifolia possessed significant antioxidative effects in all the models. Among other activities it is evident that S. colocasiifolia could be prospective to be investigated further for its extended α-amylase inhibitory effects in vivo system.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS USED

SCEEx: S. colocasiifolia ethanol extract; DPPH: 2,2-diphenyl-1-picyrylhydrazyl radical; QE: Quercetin equivalent; LC: Lethal concentration; IC: Inhibition concentration.

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