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Determination of thrombolytic, antioxidant and analgesic activity of methanolic extracts of *Rudbeckia hirta*

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ABSTRACT

Objective: This study was carried out to evaluate the thrombolytic, antioxidant and analgesic activity of plant extract of *Rudbeckia hirta*.

Materials and Methods: For thrombolytic activity, a standard *in vitro* method was applied. Antioxidant activity was measured by employing method of Folin–Ciocalteu reagent (FCR) or Folin's phenol reagent or Folin–Denis reagent, also called the *gallic acid equivalence method* (GAE) in which the total phenolic content of methanol extract was determined. Analgesic potential of the methanolic extract was tested using the model of acetic acid induced writhing in mice.

Results: The thrombolytic activity measured by a standard method revealed that this plant extract has a dose dependent thrombolytic activity. GAE method showed that the total phenolic content of methanol extract of *Rudbeckia hirta* was 24.56 mg of GAE/gm of extract. The analgesic activities of the plant extract were significant ($p< 0.05$) at the dose of 500 mg/kg-body weight in comparison with control animals; however, the activity was less than that of diclofenac Na (standard).

Conclusion: Methanolic extractof *Rudbeckia hirta* leaves have moderate thrombolytic, antioxidant and anti-inflammatory properties.

Keywords: thrombolytic activity, antioxidant activity, analgesic activity.

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INTRODUCTION

Since archaic time, vegetables, fruits, medicinal herbs, etc have been used to cure many diseases. Synthetic drugs nowadays are readily available and highly effective in curing various diseases, but people still prefer using traditional medicines because of their less harmful effects. There is an immense diversity of compounds, minutely secondary metabolites, found & isolated from plants and these compounds have anticancer, antibacterial, analgesic, anti-inflammatory, antitumor, antiviral and many other activity in a lesser or greater extent.[1,2] Even 50% of anticancer drugs have been isolated from plants.[3]

Resins, rubbers, gums, waxes, dyes, flavors, fragrances, proteins, amino acids, bioactive peptides, phyto hormones, sugar, flavonoids and bio pesticides are found in plants.[4] WHO assessment shows that about 80% of world population depends on medicinal plants for their health care needs and more than 30% of the pharmaceutical preparations are based on plants.[5]

Due to the imbalance of homeostatic system of physiological procedure thrombosis is occurred which is characterized by the formation of blood clots in the circulatory system. Thrombosis propagates troublesome situation in the arterial disease specially when connected with acute coronary disorder such as pulmonary emboli, deep vein thrombosis, strokes, heart attacks & venous thromboembolic disorders that estimate for abrupt morbidity and mortality.[6,7] Clots that formed in the blood vessels are dissolved by using thrombolytic agents; however these agents sometimes can lead to dignified and catastrophic consequences.[8]

Singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide (are Reactive Oxygen Species (ROS), often generated as byproduct of biological reaction.[9] Molecules that are found in living cells including DNA react with ROS and exert oxidative damage.[10] Oxidative stress is a condition that is caused by steady increase of free radicals in cells, wherein free radicals oxidize blood vessel walls, DNA, carbohydrates, & lipids.[11] If antioxidant system is unable to eliminate the excess ROS, result in high level of free radicals and lipid peroxides that account for pathogenesis of degenerative diseases like atherosclerosis, carcinogenesis, diabetes, cataract, ageing, and so forth.[12]

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" according to the

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International Association for the Study of Pain (IASP).[13] Analgesic drugs such as aspirin, morphine have been widely used in recent decades, particularly opioids and Nonsteroidal Anti-Inflammatory drugs (NSAIDs), can only relieve 50% of the pain in about 30% of patients.[14] Pain and edema is reduced by using Nonsteroidal Anti-Inflammatory drugs (NSAIDs), NSAIDs act through any of two processes, one is suppressing the formation of prostaglandins and another one is inhibiting the activity of the enzyme Cyclooxygenase (COX-1 and COX-2). Many synthetic compounds that act by the same mechanism have been developed and are associated with serious adverse effects such as ulceration, gastrointestinal bleeding, additive potential, respiratory disorders, drowsiness, nausea etc.[15,16]

In this condition, we need to evaluate bioactive compounds from natural products especially from medicinal plants for use as alternative analgesics with little or no side effects.

Rudbeckia hirta, (Family: Asteraceae) commonly known as black-eyed Susan. It is a North American floweringplant in the sunflower family. It is native to Eastern and Central North America and naturalized in the Western part of the continent as well as in China. It has now been found in all 10 Canadian Provinces and all 48 of the states in the contiguous United States.[17,18,19] *Rudbeckia hirta* has synonyms such as brown-eyed Susan, brown betty, gloriosa daisy, golden Jerusalem.[17,20,21] English bull's eye, poor-land daisy, yellow daisy, and yellow ox-eye daisy.[18,22]

In several tribal nations the plant is also a traditional Native American medicinal herb ; believed in those cultures to be a remedy, among other things, for colds, flu, infection, swelling and (topically, by poultice) for snake bite (although not all parts of the plant are edible).[23] Parts of the plant have nutritional value. Other parts are not edible.

Rudbeckia hirta is an upright annual (sometimes biennial or perennial) growing 30–100 cm (12–39 in) tall by 30–45 cm (12–18 in) wide. It has alternate, mostly basal leaves 10–18 cm long, covered by coarse hair, with stout branching stems and daisy-like, composite flower heads appearing in late summer and early autumn. In the species, the flowers are up to 10 cm (4 in) in diameter, with yellow ray florets circling conspicuous brown or black, dome-shaped cone of many small disc florets.[24] However, extensive breeding has produced a range of sizes and colors, including oranges, reds and browns.[25]

This study was carried out to evaluate the thrombolytic effects, antioxidant activity and analgesic effects of plant extract of *Rudbeckia hirta*.

MATERIALS AND METHODS

Chemicals

Streptokinase (S-kinase, Popular Pharmaceuticals Ltd, Bangladesh), methanol (100%), 0.9% NaCl solution, 3.1% sodium citrate solution, 2% calcium chloride, Polyoxyethylene sorbitan monooleate (Tween 80) &acetic acid was purchased from Merck, Darmstadt, Germany, methanol, Folin-Ciocalteu reagent.

Collection and identification of plants sample

Aerial parts of *Rudbeckia hirta were* collected from saver, Dhaka in December 2017, and were identified by the Bangladesh National Herbarium, Mirpur, in Dhaka. One voucher specimen was deposited in Bangladesh National Herbarium.

Drying and grinding of plant materials

At first leaves of *Rudbeckia hirta* were cut into small pieces and placed under the sun about 15 days for proper drying. After complete drying, the leaves were pulverized into crush powder with the help of a grinding and blending machine.

Extraction of Plant materials

The powder of *Rudbeckia hirta* leaves (250 gm) was extracted with 2000ml methanol (100%) in a flat bottom container, thorough shaking and stirring. After two weeks the extract was filtered through the cotton at first and then through Whitman filters paper (Bbby RE200, Steriling Ltd, Uk). The filtrate (methanolic extract) was evaporated using rotary evaporation machine. Finally, we got methanolic extract and transferred to an airtight 10ml vial for use and protection.

In vitro Thrombolytic activity

For the management of cerebral venous sinus block thrombolytic drugs are being widely used. To develop clot lysis activity of thrombolytic drugs there are several in vitro models have been established, but all these models have certain limitations. So, there is need of an appropriate model to check the clot lytic efficacy of thrombolytic drugs.[26] 600 mg of crude methanolic extract of stem of the plant was taken in a volumetric flask and a stock solution of 20 mg/mL was made using 0.9% NaCl, the final volume being 30 ml. The prepared stock solution was used to make different concentrations of root extract in isotonic saline solution: 2.5, 5, 10 and 20 mg/mL. Venous blood (5 ml) was drawn from healthy human volunteers (n =10). 500 µl of blood was transferred to each of the previously weighed micro centrifuge tubes to form clots. Venous blood drawn from healthy volunteers

(n = 10) was immediately citrated using 3.1% sodium citrate solution and then was transferred in different pre-weighed sterile micro centrifuge tube (500 µl/tube). 200 microliter of 2% calcium chloride was then added to each of these tubes, mixed well and incubated at 37°C for 45 minutes for clotting to occur. After clot formation, serum was completely removed (aspirated out without disturbing the clot formed) and each tube having clot was again weighed to determine the clot weight (clot weight = weight of clot containing tube – weight of tube alone). Each micro centrifuge tube containing clot was properly labeled and five hundred microliter of different concentrations of the plant extract, 2.5 mg/mL (n = 10), 5 mg/mL(n = 10), 10 mg/mL (n = 10) and 20 mg/mL (n = 10) or saline (negative control) (n =10) or 30,000 I.U. or 15000IU of streptokinase [(S-kinase, Popular Pharmaceuticals Ltd, Bangladesh), reference drug (n = 10)] was added to tubes with clots. All the tubes were incubated at 37°C for 90 min. The fluid left was then carefully removed and the tubes were weighed again. The difference in weight before and after clot lysis was expressed as % clot lysis. % of the lysis of the plant extract expressed as,

(weight of release clot)/(weight of clot)×100

Antioxidant screening

The total phenolic content of leaves of *Rudbeckia hirta* was measured by employing method of Folin–Ciocalteu reagent (FCR) or Folin's phenol reagent or Folin–Denis reagent, also called the *gallic acid equivalence method* (GAE).[27] It is named after Otto Folin, Vintilă Ciocâlteu, and Willey Glover Denis. This reagent is part of the Lowry protein assay, and will also react with some nitrogen-containing compounds such as hydroxylamine and guanidine.[28] The reagent has also been shown to be reactive towards thiols, many vitamins, the nucleotide base guanine, the trioses glyceraldehyde and dihydroxy acetone, and some inorganic ions. Copper complexation increases the reactivity of phenols towards this reagent.[29]

2 mg of the extract was taken and dissolved in the distilled water to get a sample concentration of 2mg/ml in every case. To 0.5 ml of extract solution (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na_2CO_3 (7.5 % w/v) solution was added. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer and using the standard curve prepared from gallic acid solution with different concentration and the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of GAE (gallic acid equivalent) / gm of the extract.

In vivo analgesic potential

Analgesic potential of the methanolic extract of *Rudbeckia hirta* leaves were tested using the model of acetic acid induced writhing in mice. The experimental animals were randomly selected and divided into four groups denoted as group1, 2, 3, & 4.Each mouse was weighed properly and the doses of the test samples and control materials were adjusted accordingly. Each group received a particular treatment i.e. Control, positive control (standard Diclofenac Na) and two doses (250 and 500 mg/kg-body weight) of the extract solution. Positive control group was administered at the dose of 25 mg/kg-body weight and control group was treated with 1% tween80 in water at the dose of 15 ml/kg body weight. Test samples, standard drug and vehicle were administered orally 30 min before intra peritoneal administration of 1.5% acetic acid. After an interval of 15 min, the mice were observed writhing for 5 min.The percent analgesia can be expressed as,

Percent analgesia = 100 – [No. of writhing in tested animals/No. of writhing in control animals] × 100.

Statistical analysis

The results ware expressed as mean ± SEM (standard error of the mean)and Statistical comparisons were made using one-way ANOVA with t test &differences between means were considered to be significant when $p < 0.05$.

RESULT

In vitro Thrombolytic activity

Effect of methanolic crude extracts of stem of *Rudbeckia hirta* on blood clot lysis of human blood in vitro (mean± SEM) is given below,

Antioxidant screening

The methanol extract of *Rudbeckia hirta* was tested for evaluating the antioxidant activity. The absorbance of the sample and total phenolic content of extracts is given below

In vivo analgesic potential

Different animal group ware taken for describing analgesic activity of plant extract of *Rudbeckia hirta*. Writhing count (Mean±SEM) and % of writhing inhibition by plant extract is shown in the following table,

DISCUSSION

Many researches indicate that herbs and natural products posses thrombolytic activity.[30] Tissue plasminogen activator (t-PA), Urokinase (UK),

Table 1 ***In vitro*** **thrombolytic activity**

Concentrations of plant extracts, control and standard	n	Mean % of Blood clot Lysis
0.9% NaCl solutions	10	5.35±1.01*
Streptokinase (30,000 I.U.)	10	47.21±1.15*
Streptokinase (15,000 I.U.)	10	24.73±1.12*
Leaves extract 20 mg/mL	10	19.50±1.25*
Leaves extract 10 mg/mL	10	11.44±1.25
Leaves extract 5 mg/mL	10	9.57±1.28
Leaves extract 2.5 mg/mL	10	9.55±1.50

* Determines significance level ($p<0.05$)

Table 2 **Total Phenolic Content of methanol extract of plant of** ***Rudbeckia hirta***

Extract	Absorbance of the sample	Total Phenolic Content (mg of GAE / gm) of Extracts
Methanol Extract	0.185	24.56

Table 3 **Analgesic effect of methanol extract of plant of** ***Rudbeckia hirta***

Animal group	Treatment	*Rudbeckia hirta*	
		Writhing count (Mean ± SEM) (% Writhing)	% Writhing inhibition
Group 1 (n=4)	1% Tween 80 solution in water (orally)	11.88+-0.40	–
Group 2 (n=4)	Diclofenac sodium (25 mg/kg) Orally	8.8+-0.65 **	40.55
Group 3 (n=4)	Methanol extract (250 mg/kg) orally	10.4+-0.65*	34.11
Group 4 (n=4)	Methanol extract (500 mg/kg) orally	9.88+-0.69*	42.78

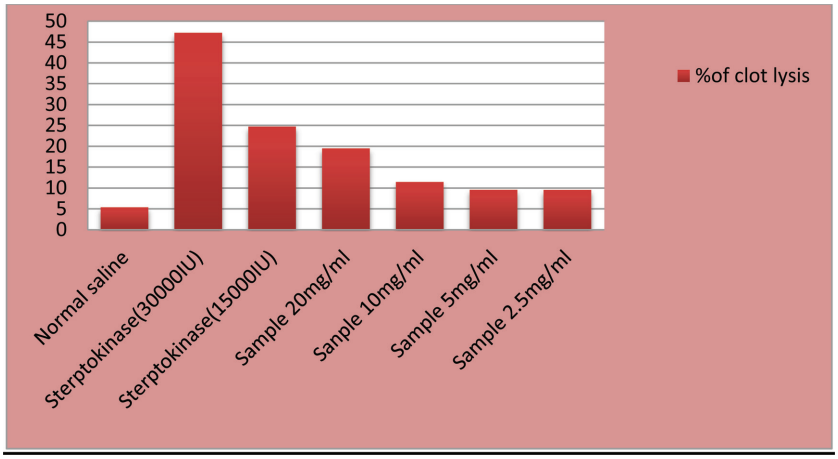

Figure 1 % clot lysis of plant extract

Streptokinase (SK), etc are used globally as thrombolytic agents.[31] Recently, various thrombolytic agents in practice are being used to dissolve the clots that have already formed in the blood vessels; but these drugs have certain limitations and can lead to potential fatal consequences in some cases.[32] Evaluation of thrombolytic activity of *Bougainvillea spectabilis* leaf extract shows that concentrations of leaf extract enhanced in the clot lysis in dose dependent manner along with the incubation time factor. In another similar nature of study conducted in other species of *Bougainvillea glabra* also showed clot lysis ability.[33] Leaf extract of *Bougainvillea spectabilis* has 31.12% clot lysis at 200µg/mL, 42.57% clot lysis at 400µg/mL, 65.74% clot lysis at 600µg/mL, 84. 24% clot lysis at 800 µg/mLin 72 hrs of incubation which also indicates dose dependent thrombolytic activity.

Our study of thrombolytic activity of leaves extract of *Rudbeckia hirta*is dose dependent. In this study when the concentration of standard streptokinase and leave extract was reduced the percent of blood clot lysis was also reduced such as 19.50%, 11.44%, 9.57%, 9.55% while the dose of the leaf extract was 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL respectively (Table 1). On the basis of these result, it may be concluded that *Rudbeckia hirta* leaf extract has a dose dependent thrombolytic activity.

Many studies indicate a linear relationship between total phenolics and antioxidant activity.[34,35,36] Phenolic compounds are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants.[37] The total phenolic content experiment is governed by Folin-Ciocaltau reaction. The reagent measures the reducing capacity of the test sample by reacting with the reducing substance in the test samples. Phenolate ions present in the samples reduce $Mo^{(VI)}$ to $Mo^{(V)}$ by transfer of electron to produce a blue color whose absorbence were measured at 765 nm. Total phenolic content of *Sensevieria cylindrical* leaves extract shows that the content of phenolic compounds is dependent on the polarity of the solvent used; higher the polarity of the solvent, higher the content of phenolic compounds. The maximum phenolic content of *Sensevieria cylindrical* leaves extract was found in methanol fraction (86.2±2.6). In the hexane and chloroform fractions, phenolic compounds could not be detected.[38]

In this survey the total phenolic content of methanol extract of *Rudbeckia hirta* was 24.56 mg of GAE/gm of extract (Table 3).

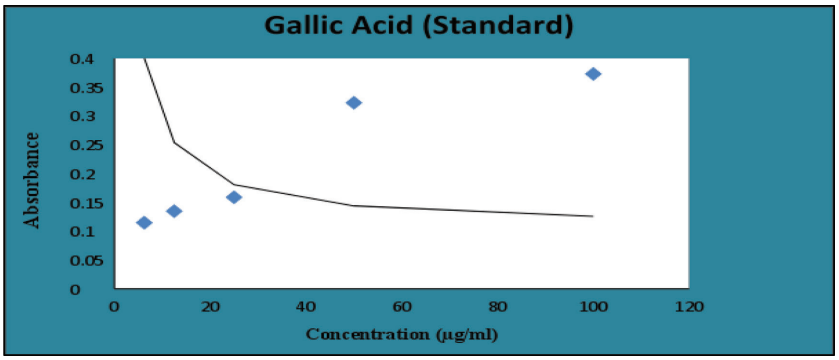

Figure 2 Total Phenolic Content of Gallic Acid (Standard)

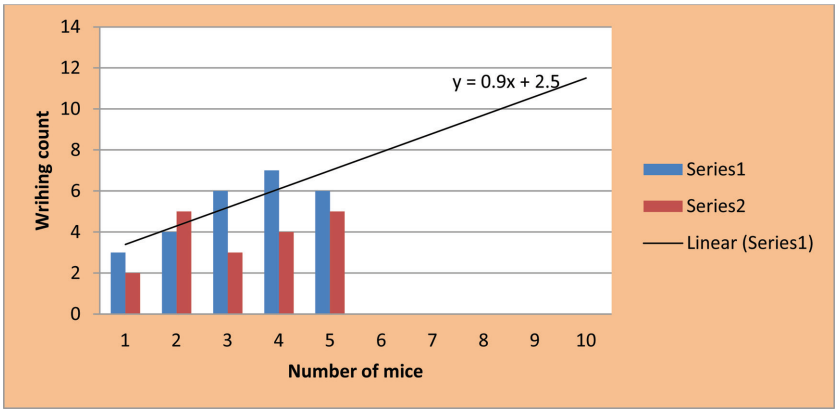

Figure 3 Writhing Count on four groups of mice (n=5)

Acetic acid induced writhing in mice imposed visceral pain finds much attention of screening analgesic drugs.[17] The crude extracts of *Rudbeckia hirta* showed significant analgesic action compared to the reference drug diclofenac sodium but group 4 was found to exhibit higher analgesic activity than group 3 against acetic acid induced pain in mice at two dose levels i.e. 500 mg/kg & 250 mg/kg b. wt. The analgesic activities of the plants were significant ($p < 0.05$) at the dose of 500 mg/kg-body weight in comparison with control animals; however, the activity was less than that of diclofenac Na (standard).

CONCLUSION

From the present study we revealed that the crude extract of *Rudbeckia hirta* has various pharmacological activities such as thrombolytic, antioxidant and analgesic activity. This investigation has opened up the possibility of the use of this plant in future for drug development and various herbal formulation having less side effects.

CONFLICT OF INTEREST

Authors have no conflict of interest.

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