ORIGINAL ARTICLE

Phytochemical and *in vitro* thrombolytic activity evaluation of *Cassia siamea* L., Leguminosae leaf extracts, and pyrogallol

M. Vani[1], P. Vijaya Rani[1], O. Madhuri[1], M. Vijaya Sai Sree[1], L. Sai Ramya[1], M. Gnana Chandrika[1], K. Padmalatha[2], J. Supriya[1]

[1]Department of Pharmacognosy and Phytochemistry, Vijaya Institute of Pharmaceutical Sciences for Women, Vijayawada, Andhra Pradesh, India, [2]Department of Pharmacology, Vijaya Institute of Pharmaceutical Sciences for Women, Vijayawada, Andhra Pradesh, India

Abstract

Aim: The aim of the study was to carry out total phenolic content determination followed by *in vitro* thrombolytic studies of aqueous and alcoholic extracts of *Cassia siamea* L., extracts and pyrogallol. **Materials and Methods:** The plant leaf aqueous and alcoholic extractive values, preliminary phytochemical screening followed by total phenolic content was determined. Further, the plant extracts and one of the plant active constituents' pyrogallol were studied for *in vitro* thrombolytic activity using streptokinase (SK) as standard in human venous blood. **Results and Discussion:** The extractive values were found to be 15.0 and 13.2 w/w for aqueous and alcoholic extracts. The plant extracts showed the presence of phenolics and flavonoids. The total phenolic components present in aqueous and alcoholic extracts were found to be 3 mg/g and 0.3 mg/g equivalent of pyrogallol. The aqueous extract contains more amount of phenolic substances than alcoholic extract. The percentage clot lysis was found to be 2.25% in standard SK, 14.50% in aqueous extract, 18% in the ethanolic extract, and 17.75% in pyrogallol, respectively. The ethanolic extract exhibited better thrombolytic activity than aqueous extract. Pyrogallol also exhibited equivalent thrombolytic activity compared to standard. **Conclusion:** The results indicate that ethanolic extract exhibited thrombolytic activity which may be due to hydrolysable tannin pyrogallol. Further studies are required to isolate the responsible component from the ethanolic extract and pyrogallol can be studied for mechanism of action studies.

Key words: Antithrombolytic, *Cassia siamea,* ethanolic extract, pyrogallol

INTRODUCTION

Primary bioassay screens are most important for the initial screening of plants for bioactive principles and are often the first step in drug development.[1] Cardiovascular disease caused by blood clot (thrombus) formation is one among the most severe diseases which are increasing at an alarming rate in recent years.[2] Thrombolytic agents are used to dissolve the clot and in the management of thrombosis in patients.[3] Thrombolytic agents such as tissue plasminogen activator, urokinase, and streptokinase (SK)[4] are used all over the world for the treatment,[5] but their use is associated with hyper risk of hemorrhage,[6] anaphylactic reaction, and lacks specificity. Remarkable efforts have been made toward the discovery and development of natural constituents from various plant and animal sources which have antiplatelet,[7-8] anticoagulant,[9-10] antithrombotic,[11] and thrombolytic activity.[12-14] The plant under study *Cassia siamea L.,* commonly known as kassod tree, has been used traditionally for various activities such as malaria, antihepatotoxic,[15-17] typhoid, anti-anemic[18] scabies,[19] an antidote for snake, and scorpion bites.[20] The plant was found to contain various chemicals such as chromone alkaloids[21]), anthraquinone glycosides,[22] flavonoids,[23], and gallic acid,[24] Phenols were studied to be responsible components to elicit antithrombotic activity.[21,25] There were no literature reports available on the plant species

Address for correspondence:
Mrs. M. Vani, Department of Pharmacognosy and Phytochemistry, Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu, Vijayawada - 521 108, Andhra Pradesh, India. Phone: +91-9704625782.
E-mail: vanimamillapalli@yahoo.co.in

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for evaluation of the thrombolytic activity. Hence, the present study has been undertaken to carry out *in vitro* thrombolytic activity, total phenolic content of the leaf aqueous, and alcoholic extracts of the plant *C. siamea*.

MATERIALS AND METHODS

Collection and Authentication of the Medicinal Plant

The plant material leaves *C. siamea* were collected in the month of November during the evening from the grounds of Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu, coordinates 16°32′45″N 80°34′12″E of Vijayawada rural region, Krishna district, Andhra Pradesh, India. The herbarium was prepared and the sample was identified and authenticated by Dr. P. Satya Narayana Raju, plant taxonomist, Department of Botany and Microbiology, Acharya Nagarjuna University, Guntur (Dt), Andhra Pradesh, India. A voucher specimen was deposited for future reference in the Department of Pharmacognosy, Vijaya Institute of Pharmaceutical Sciences for Women, Enikepadu. The photographs of the plant and leaves are depicted in Figures 1 and 2.

Chemicals and Equipment

Dried leaf powder of Soxhlet apparatus (JSGW), distilled water, ethanol (Merck), vacuum pump (Biotech), aqueous extract, ethanolic extract, 0.2 N Folin–Ciocalteu reagent, 2% sodium carbonate (Finar), pyrogallol (Sigma-Aldrich), centrifuge (Lab India), incubator (Biotech), ultraviolet spectrophotometer (Lab India), human venous blood, microcentrifuge (REMI- RM12C), incubator (Biotech), and lyophilized SK (Samarth life sciences, Goregaon, India) was used.

Extraction and Preliminary Phytochemical Screening

The leaves were dried and powdered. The powder was subjected to Soxhlet extraction using water and alcohol as solvents. The photographs of dried leaves and leaf powder, extraction using Soxhlet apparatus, obtained extracts are depicted in Figure 3, and extractive values are presented in Table 1. The aqueous and ethanolic extracts were screened for the phytochemical constituents according to the standard methods.[26] The results are given in Table 2.

Quantitative Determination of Phenolics

This was determined using Folin–Ciocalteu method, as described by Olajire and Azeez. The extract (0.5 ml) was added to 10 ml deionized distilled water and 2.5 ml of 0.2 N Folin–Ciocalteu phenol reagent. The mixture was left undisturbed at room temperature for 5 min and then 2 ml of 2% sodium carbonate was added. The absorbance of the resulting solution was read at 780 nm and repeated 3 times.[27] Pyrogallol was used as a standard for the calibration curve. This was done in triplicate [Table 3, Figure 4].

Figure 1: *Cassia siamea* plant and branches with flowers and pods

Figure 2: *Cassia siamea* flowering twig and leaves

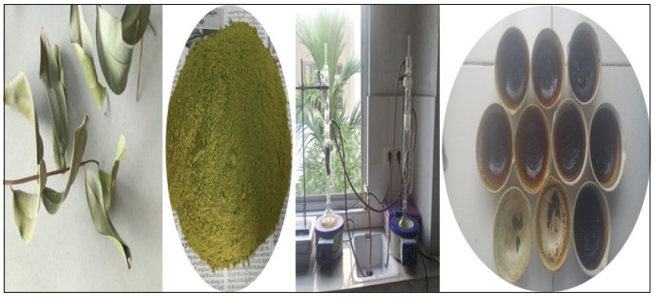

Figure 3: *Cassia siamea* dried leaves, powder, extraction, extracts

$$C = c.V/m$$

C – Total phenolic compounds mg/gm of plant extract
c – The concentration of standard established from the calibration curve mg/ml
V – The volume of extract in ml
m – The weight of pure plant extract

In vitro Thrombolytic Activity Determination

The clot lysis was carried out as per the method reported by Prasad *et al*.[28] In brief, 2.5 ml of venous blood drawn from healthy volunteers was distributed in five different pre-weighed sterile microcentrifuge tube (0.5 ml/tube) and incubated at 37°C for 45 min [Figure 5]. After clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight (Clot weight = Weight of clot containing tube – Weight of tube alone).

To each microcentrifuge tube containing pre-weighed clot, 100 µl of different extracts is to be added. To the commercially available lyophilized SK vial (Lupiflo, Lupin Limited, Mumbai, India), 2.5 ml of PBS was added and thoroughly mixed. This suspension was used as a stock from which 100 µl was added to the microcentrifuge tube as a positive control. Pyrogallol was also tested for the detection of thrombolytic activity. For negative control, 100 µl of distilled water was added. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, the 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 the percentage of clot lysis. The experiment was repeated 4 times with the blood samples [Table 4, Figure 6] of 4 volunteers.[28]

Table 1: Extractive values of aqueous and ethanolic extracts of *C. siamea*

Yield of AQLCS (%w/w)	Yield of ELCS (%w/w)
15.0	13.2

AQLCS: Aqueous extract of leaves of *C. siamea*, ELCS: Ethanolic extract of leaves of *C. siamea*. *C. siamea*: *Cassia siamea*

Table 2: Qualitative preliminary phytochemical screening of aqueous and alcoholic extracts of *C. siamea*

Phytochemical	AQLCS	ELCS
Alkaloids	+	+
Glycosides	+	+
Hydrolyzable tannins (Phenolics)	+	+
Flavonoids	+	+
Triterpenes	+	+
Steroids	+	+

AQLCS: Aqueous extract of leaves of *C. siamea*, ELCS: Ethanolic extract of leaves of *C. siamea*. *C. siamea*: *Cassia siamea*

Table 3: Quantitative phytochemical screening of aqueous and alcoholic extracts of leaves of *C. siamea*

Phytochemical	AQLCS	ELCS
Phenolics (mg/gm)	3±0.03	0.3±0.02

Values represented mean±SD. of three parallel measurements. AQLCS: Aqueous extract of leaves of *C. siamea*, ELCS: Ethanolic extract of leaves of *C. siamea*. SD: Standard deviation. *C. siamea*: *Cassia siamea*

RESULTS AND DISCUSSION

Extraction and Preliminary Phytochemical Screening

The percentage yield of extractive value was found to be 15% w/w for aqueous extract and 13.2 % w/w for ethanolic extract [Table 1] of *C. siamea*. The qualitative preliminary phytochemical screening of aqueous and alcoholic leaf extracts of *C. siamea* revealed the presence [Table 2] of alkaloids,

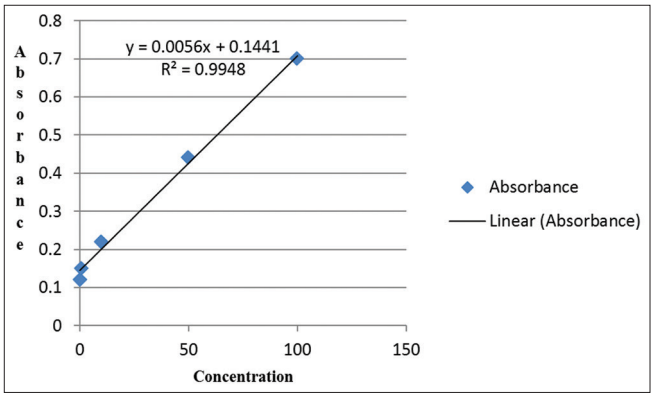

Figure 4: Standard calibration of pyrogallol for phenolics

Figure 5: Determination of thrombolytic activity of *Cassia siamea* extracts

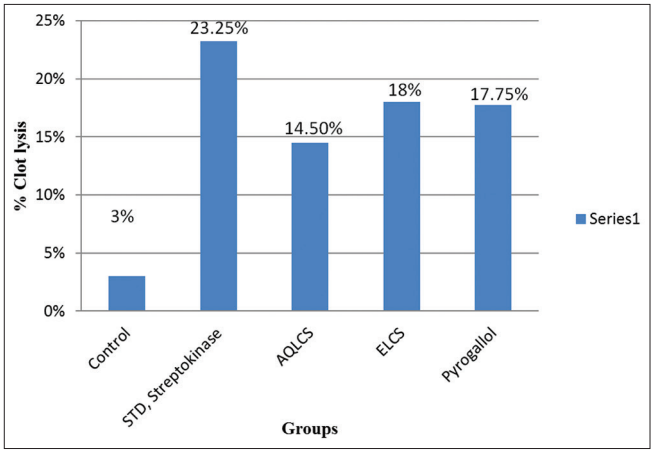

Figure 6: Determination of thrombolytic activity for leaf extracts of *C. siamea*. AQLCS – Aqueous extract of leaves of *C. siamea*, ELCS – Ethanolic extract of leaves of *C. siamea*

Table 4: Determination of percentage clot lysis of aqueous, ethanolic extracts of *C. siamea*

Groups	% Clot lysis (Mean±SEM)
Control	3±0.40
STD, SK	23.25±1.65****
AQLCS	14.5±1.89***
ELCS	18±2.04****
Pyrogallol	17.75±1.43****

The values were expressed as mean±SEM, n=4, at*** P<0.0005; ****p<0.0001 when compared with control by one-way ANOVA, Dunnett's test. AQLCS: Aqueous extract of leaves of *C. siamea,* ELCS: Ethanolic extract of leaves of *C.siamea,* STD: Standard, SEM: Standard error mean, ANOVA: Analysis of variance. *C. siamea*: *Cassia siamea*, SK: Streptokinase

glycosides, and hydrolyzable tannins, which gave blue color with ferric chloride indicating the presence of gallic acid, ellagic acid (phenolic acids), flavonoids, triterpenes, and steroids, respectively.

Quantitative Determination of Phenolics

The total phenolic content of AQLCS was found to be 3 mg/g equivalent of pyrogallol and 0.3 mg/g equivalent of pyrogallol for ELCS, respectively [Table 3, Figure 4]. The results indicate that aqueous extract contains more amount of phenolic substance than alcoholic extract.

Evaluation of Thrombolytic Activity

The results indicate that plant extracts possess thrombolytic activity when compared to the control group (The clot lysis 3% mean ± SEM, n= 4). The standard group which received SK exhibited clot lysis 23.25% at $P < 0.0001$ level of significance. Aqueous extract of the plant showed clot lysis 14.5% at $P < 0.0005$, ethanolic extract showed 18% clot lysis at $P < 0.0001$ and pyrogallol exhibited 17.75% clot lysis at $P < 0.0001$ levels of significance, respectively.

In vitro, phenolic content was determined for aqueous and alcoholic extracts of the plant using pyrogallol as standard [Table 3, Figure 4]. Qualitative preliminary phytochemical screening indicated the presence of hydrolyzable tannins, gallic acid, or ellagic acid [Table 2]. Dry distillation of gallic acid and ellagic acid would give pyrogallol.[29] The total phenolic content of aqueous extract (3 mg/g) was found to be more than ethanolic extract (0.3 mg/g) equivalent to pyrogallol [Table 3]. Pyrogallol exhibited 17.75% of clot lysis. However, ethanolic extract of the plant showed more clot lysis when compared to aqueous extract [Table 4, Figure 6].

Basing on the above observations, it was understood that other phytochemical components may be responsible, which showed better thrombolytic activity present in the ethanolic extract. Hydrolyzable tannins may not be responsible for the therapeutic effect present in the plant extracts. Further research can be carried out to isolate and find out the responsible components present in the ethanolic extract along with hydrolyzable tannins.

CONCLUSION

The observations from the findings of the experiment have shown that aqueous, ethanolic plant extracts of *C. siamea* and compound pyrogallol clearly exhibited thrombolytic activity. Further work is necessary to isolate the active components responsible as well as to carry out *in vivo* thrombolytic studies of the plant extracts of *C. siamea* and pyrogallol.

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