IJMPR, 2026, 10(11), 1-8 ISSN: 2319-5878

INTERNATIONAL JOURNAL OF MODERN PHARMACEUTICAL RESEARCH

www.ijmpronline.com

Research Article

Impact Factor: 7.533

Coden USA: IJMPR3

EVALUATION OF THROMBOLYTIC ACTIVITY OF THE ROOT EXTRACT OF *Hortonia anguistifolia* AND SEED OIL OF *Calophyllum inophyllum*

Lakshitha D.A.A.[1]*, Siriwardhena M.A.[1], Wijayabandara M.D.J.[1], Pathirana W.[2]

[1]Department of Pharmacy and Pharmaceutical Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.
[2]Formerly at Department of Pharmacology and Pharmacy, Faculty of Medicine, University of Colombo, Kynsey Road, Colombo 08, Sri Lanka.

Article Received on: 24/09/2026 **Article Revised on: 14/10/2026** **Article Published on: 01/11/2026**

***Corresponding Author**

Lakshitha D.A.A.
Department of Pharmacy and Pharmaceutical Sciences, Faculty of Allied Health Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka.
https://doi.org/10.5281/zenodo.23795920

How to cite this Article: Lakshitha D.A.A.1*, Siriwardhena M.A.1, Wijayabandara M.D.J.1, Pathirana W.2. (2026). Evaluation of Thrombolytic Activity of The Root Extract of Hortonia Anguistifolia And Seed Oil of Calophyllum Inophyllum. International Journal of Modern Pharmaceutical Research, 10(11), 1–8.

ABSTRACT

Several important compounds have been isolated from different parts of *the Hortonia anguistifolia* plant. *Calophyllum inophyllum* seed oil has been used as a treatment for blackish discoloration of the skin following contusions, suggesting thrombolytic activity. The thrombolytic activities of the root extract of the plant *Hortonia anguistifolia*, endemic to Sri Lanka, and of *C.inophyllum* seed oil are to be investigated. *H.anguistifolia* root methanol extract was prepared by maceration. *C.inophyllum* oil was extracted from seeds by expression. Concentration series were prepared for both extracts. Thrombolytic activity was determined using the clot lysis method. Activities were expressed as a percentage of activity at the given concentration. Streptokinase was used as the positive control for the clot lysis assay. The highest percentage of clot lysis of 32.92±2.135% for *H. anguistifolia* root extract was observed at 10 mg/ml concentration. The highest percentages of clot lysis (52.08±0.09 % and 49.15±0.33 %) for two oil samples of *C.inophyllum* were observed at 700 µl/ml concentration for both samples. The results of this study revealed the potential thrombolytic effects of *H.anguistifolia* root methanol extract and *C. inophyllum* seed oil. Further investigations on these thrombolytic agents for the isolation of bioactive constituents and finding underlying clot lysis mechanisms are suggested.

KEYWORDS: *Hortonia anguistifolia*, *Calophyllum inophyllum*, Calophyllolide, Thrombolytic activity, Streptokinase.

INTRODUCTION

Blood is a connective tissue that flows through vertebrate arteries and veins smoothly, but if a clot or thrombus blocks the flow of blood, it results in thrombosis, which can be serious and even cause death. Diseases arising from clots in blood vessels include pulmonary emboli, deep vein thrombosis, ischemic stroke and myocardial infarction, which are the major causes of mortality and morbidity.[1] Other than surgical interventions to remove or bypass the blockade or the regeneration of collateral vessels to provide a new blood supply, the only treatment available is the administration of thrombolytic agents to dissolve the blood clot. Thrombolytic agents include tissue plasminogen activator (t-PA), urokinase (UK) and streptokinase (SK), which are used all over the world.[2] Given the limited number of thrombolytic agents currently in use, the present study assumes much significance.

The coagulation mechanism is a complex series of cascading reactions involving the development of enzymes from their precursor (Zymogens, Procoagulants, Proenzymes). Most of the substances that are necessary for coagulation are present in an inert form and must be converted to an activated state. As one enzyme is formed, it then becomes available to convert the next zymogen to its activated form of enzyme (serine protease). This process continues until a clot consisting of fibrin meshwork is formed. In addition to the zymogens, protein co-factors, surface membrane phospholipids and calcium ions play an active role in the final development of the fibrin clot or the thrombus.[3] The clot formation cascade is widely available in the literature.[4]

Hortonia anguistifolia

The genus *Hortonia* is endemic to Sri Lanka and belongs to the family Monimiaceae. Members of this family are herbs or shrubs and rarely climbers. This family comprises about 39 genera and 440 species, which are widely spread in the Southern Hemisphere, mainly in the tropical and subtropical regions of USA.[5]

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The latest revision of the family Monimiaceae by Prof. M. D. Dassanayake in Flora of Ceylon, lists three distinct species, *Hortonia angustifolia, floribunda* and *ovalifolia*.[6]

Although three species of *H. angustifolia* are found in a range of environments from sea level to the mountain regions, there have been no reports of the medicinal use of these plants in Sri Lanka.[7] Possibly for the same reason, no Sinhala name could be identified for the plant. When literally translated, an appropriate Sinhala name may be suggested as "*Udyana angili pathra* tree".

Fig. 1: *Hortonia angustifolia* leaf (A) and branches (B).

Hortonia angustifolia tree grows to about 5 m in height and has twigs standing horizontal or somewhat drooping. Leaf morphology has been used as the primary character for keying the three species. (Figure 1)

H. angustifolia is characterized by having leaves 8-14 x 2-6 cm, narrowly lanceolate to narrowly elliptic, attenuate at apex, long-acuminate or caudate, 3-veined from the base; with major veins parallel to the margin, lamina flat or bent down at apex. The flower is about 10 mm in diameter, with 4-5 sepals, pale yellow, and flowers from July to November. Fruit of 3-8 drupelets, about 12 mm long, dark purplish crimson, with a seed about 10mm long.

H. angustifolia is distributed in the moist lowlands in Sri Lanka, at elevations of about 700m at the edges of streams and in secondary wet forests and along roadsides.[8] *H. anguistifolia* plants have been found in Pittabeddara (84–137m), Kotapola (140m), Kanneliya (165m), and Deniyaya (420m) areas of Sri Lanka.[9]

Calophyllum inophyllum

C. inophyllum (Clusiaceae) is a large evergreen tree native to southern coastal India, East Africa, Malaysia, and Australia. It is a low branching and slow growing tree with a broad and irregular crown. It usually reaches 8 to 20 m in height. The tree supports a dense canopy of glossy, elliptical leaves (Figure 2). The very fragrant white flowers are 25 mm across and occur in racemose or paniculate inflorescences consisting of 4 to 15 flowers.

Its different organs, leaves, bark, fruits, and seeds were used in traditional medicine to cure many diseases, but its nuts, containing oil, were mostly used for skin care. *Domba* oil is applied to areas of skin with dark discoloration following contusions, indicating the possible thrombolytic effect.

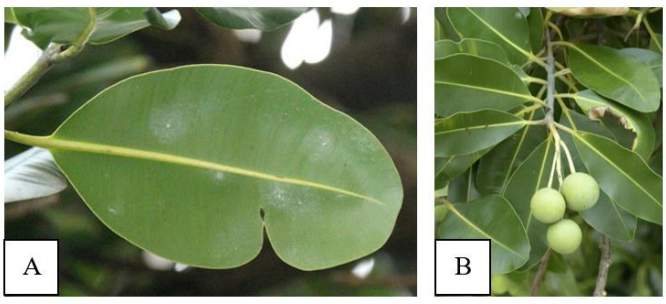

Fig. 2: *Calophyllum inophyllum* leaf (A), branches and fruits (B).

A review article on the genus Calophyllum has comprehensively reviewed its ethnobotanical uses, phytochemistry and pharmacology.[10] It lists over a dozen medicinal uses for different parts of the plants belonging to the Genus. There are hundreds of bioactive molecules, among which 38 are coumarins. Only one reference on *C. inophyllum* is found on anticoagulation activity related to the coumarin calophyllolide which is closely related to thrombolysis activity. The article does not specify if this coumarin was found in the seed oil.

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MATERIALS AND METHODS

The following reagents and instruments were used in the study. An enzyme vial containing Streptokinase 1500000 I.U., Cadila Pharmaceuticals, India, was obtained from the Medical Supplies Division, Ministry of Health, Sri Lanka. An electronic weighing scale, CAS - MWP 600 (Department of Pharmacy and Pharmaceutical Sciences, University of Sri Jayewardenepura, Sri Lanka).

Authenticated *H. anguistifolia* root samples were collected from the Kalawana area. *Domba* seeds were collected from Thorakolayaya in Hambantota District which is in the dry zone and the University of Sri Jayewardenepura premises, located in the wet zone. Authentication of *C. inophyllum* was done by Bandaranaike Memorial Ayurvedic Research Institute, Navinna.

Preparation of *H. anguistifolia* root methanol extract

Powdered root sample of *H. anguistifolia*, of which 100 g was introduced into a stoppered flask. Then 100% methanol was added until one inch above the powder level. The flask was stirred frequently and then allowed to stand overnight. This procedure was repeated for three days. Then the solvent was separated and the residue evaporated using a vacuum rotary evaporator.

The methanol extract and residue after evaporation is shown in Figure 3. The resultant residue was weighed and the percentage yield was determined. The residue was stored in an airtight container until further use.

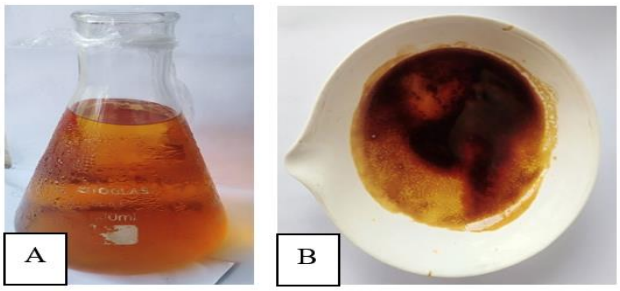

Fig. 3: *H. anguistifolia* root methanol extract (A) and residue after evaporating the solvent (B).

C. inophyllum seed oil extraction

C. inophyllum seeds were collected from two different areas in Sri Lanka. Seeds collected from Thorakolayaya in Hambantota District, Sri Lanka, were used to produce sample 01 (S 01) and sample 02 (S 02) was extracted from seeds collected from the University of Sri Jayewardenepura, Nugegoda, Sri Lanka.

A combined process of rendering with heat and pressing was employed for the small - scale extraction process. Ripe fruits of *C. inophyllum* were sun-dried for one to two weeks. Dried nuts were deshelled to expose the kernel. They were cleaned, sliced, and dried in the oven at 40^0C for 4-5 days.

When they were dry enough to easily crack between two fingers, they were ground in a mortar and pestle until a brown colored paste was formed. The paste was placed in a steamer and steamed for 30 minutes, then transferred into a cloth pouch while it was hot. The pouch was introduced into the apparatus with two horizontal pressing bars so that the pouch hangs below the two bars. The pouch was lifted, and two bars pressed successively until the entire pouch was pressed, extracting the oil (Figure 4). The extracted greenish-brown *C. inophyllum* oil was stored in an airtight glass container.

Fig. 4: Steps followed in *C. inophyllum* seed oil extraction on a small scale, starting from kernel to separation of oil. *A: Dried nuts were deshelled to expose the kernel. B: Sliced kernels were dried. C: Dried kernels were ground. D:*

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Brown colored paste. E: The paste was steamed. F: The pouch containing the paste was introduced to the apparatus. G: Oil was extracted by expression. H: The separated greenish-brown oil.

Sample preparation for *in vitro* clot analysis method

Dilution series were prepared with *H. anguistifolia* dried root methanolic extract and the two *C. inophyllum* oil extracts to evaluate *in vitro* thrombolytic activity by clot lysis method.

The dilution series of *H. anguistifolia* root extract was prepared by dissolving 100 mg of dried extract in 10 ml of normal saline to obtain a 10 mg/ml stock solution. It was then diluted to obtain the dilution series of 1mg/ml, 2mg/ml, 4mg/ml, 6mg/ml, 8mg/ml and 10mg/ml.

The dilution series of two *C. inophyllum* oil extracts were prepared as 0.1ml/ml, 0.2ml/ml, 0.3ml/ml, 0.4ml/ml, 0.5ml/ml, 0.6ml/ml, and 0.7ml/ml using liquid paraffin as the solvent.

A volume of 100 µl of each dilution of both the plant materials was used for the clot lysis test. Therefore, the amount of dried root extract and seed oil extracts responsible for the lysis reaction is $1/10^{th}$ of the amounts shown in the above dilution series. Therefore the $1/10^{th}$ dilution series are 0.1mg/100µl, 0.2mg/100µl, 0.4mg/100µl, 0.6mg/100µl, 0.8mg/100µl and 1mg/100µl for the root extract of *H. anguistifolia* and 10µl/100µl, 20µl/100µl, 30µl/100µl, 40µl/100µl, 50µl/100µl, 60µl/100µl and 70µl/100µl for both the seed oil extracts.

Clot lysis analysis

We carried out the clot lysis experiment as reported in a previous publication. [9] Sheep blood was used for this analysis. It was obtained from the Medical Research Institute, Danister De Silva Mawatha (Baseline Road), Colombo 08, Sri Lanka.

Measured 500µl of sheep blood and was transferred to each of the weighed sterile microcentrifuge tubes and incubated at 37 °C for 45 minutes. After clot formation, the separated serum was removed. Tubes were again weighed to determine the clot weight. To each microcentrifuge tube containing the pre-weighed clot, 100µl of each dilution from the root extract of *H. angustifolia* and two *C. inophyllum* seed oil dilution series were added. Tests were conducted in duplicate. The *C. inophyllum* oil dilutions in paraffin oil were shaken gently to homogenize.

Lyophilized streptokinase vial (1500000 IU) was reconstituted with 5 mL normal saline. As a positive control 100µl of Streptokinase containing 30000 IU and, as a negative control, 100µl of the respective solvents, normal saline and liquid paraffin, were added to the control tubes as required in duplicate. All the tubes were incubated at 37 °C for 90 minutes and observed for clot lysis (Figure 5). After incubation, the fluid separated was removed and the tubes were again weighed to observe the difference in weight after clot lysis. The difference in weight before and after clot lysis was expressed as a percentage of clot lysis. The experiment was performed in duplicate and the mean value was taken.

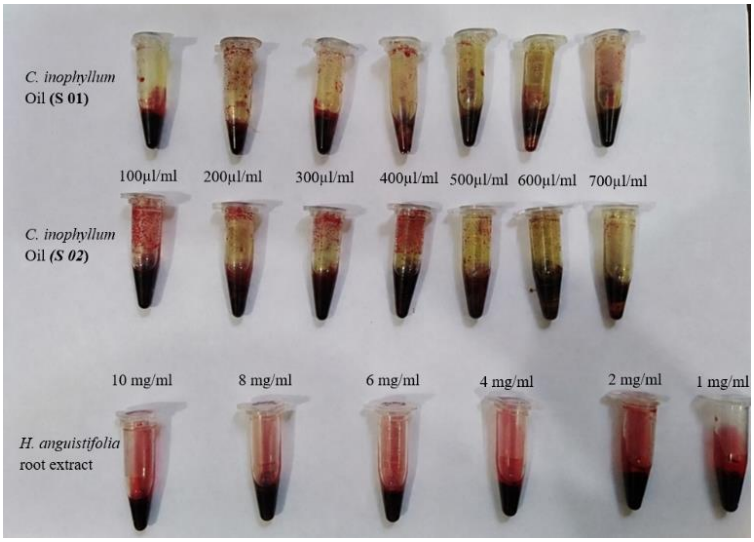

Fig. 5: **Microtubes set up containing plant extracts and isolated blood clots.**

RESULTS

The yield of *H. anguistifolia* root methanol extract.

The methanol extract of 100 g of *H. anguistifolia* root gave a yield of 3.55 % w/w.

Results of *in vitro* thrombolytic activity of the root of *H. anguistifolia*

The results of concentration-dependent *in vitro* thrombolytic activity of the root of *H. anguistifolia* extract using the clot lysis method and *P values* for each concentration of *H. anguistifolia* extract when compared

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with the positive control (streptokinase 30000 IU) and negative control (Normal saline) are shown in Table 1.

Concentration-dependent thrombolytic activity in sterile microcentrifuge tubes with dilution test series of *H. angustifolia* is illustrated in Figure 6.

Table 1: Clot lysis between *H. anguistifolia* root extracts, positive and negative controls, along with the significant difference in percentage.

Concentration (mg/ml)	% Of clot lysis of H. *anguistifolia* root extracts.	*P values* when compared with the positive control (Streptokinase)	*P values* when compared with the negative control (Normal saline)
1	4.05±0.759	0.000	1.000
2	19.26 ± 0.305	0.000	0.000
4	20.04± 0.085	0.000	0.000
6	21.67± 0.345	0.000	0.000
8	26.83 ± 0.095	0.000	0.000
10	32.92 ± 2.135	0.000	0.000
Positive (30 000 IU streptokinase)	62.52 ± 0.316	-	0.000
Negative (Normal saline)	3.94 ± 0.952	0.000	-

Significant *in vitro* thrombolytic activity of extract was statistically evaluated using analysis of variance (ANOVA). A significance level 0.05 was used.

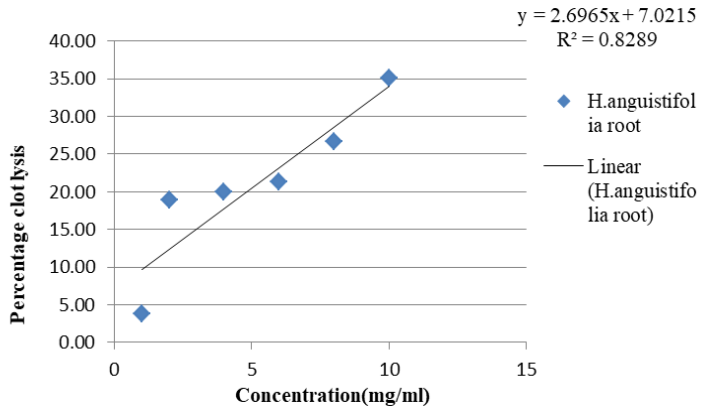

Fig. 6: Concentration-dependent thrombolytic activity of *H. anguistifolia.*

Determination of *in vitro* thrombolytic activity of the two *C. inophyllum* oil.

The results of concentration-dependent *in vitro* thrombolytic activity of *C. inophyllum* oil samples using clot lysis method and *P values* for each concentration of *C. inophyllum* oil when compared with positive control (streptokinase 30000 IU) and negative control (Liquid paraffin) are shown in Table 2. Concentration-dependent thrombolytic activity of two oil samples is illustrated in Figures 7 and 8.

Table 2: Clot lysis between the two *C. inophyllum* samples (S 01, S 02), positive and negative controls, along with the significant difference in percentage.

Concentration (µl/ml)	% Of clot lysis *C. inophyllum* oil		*P values* when compared with the positive control (Streptokinase)		*P values* when compared with the negative control (liquid paraffin)	
	S 01	S 02	S 01	S 02	S 01	S 02
100	3.74±0.15	5.49±0.79	0.000	0.000	0.996	1.000
200	8.41 ± 1.27	16.35 ± 0.19	0.000	0.000	0.666	0.000
300	13.00± 1.55	35.91± 2.52	0.000	0.000	0.003	0.000
400	13.98± 2.35	37.49± 0.86	0.000	0.000	0.001	0.000
500	34.28± 0.73	41.22± 0.13	0.000	0.000	0.000	0.000
600	41.10± 0.37	41.89± 0.06	0.000	0.000	0.000	0.000
700	52.08±0.09	49.15±0.325	0.000	0.000	0.000	0.000
Positive (30000IU streptokinase)	62.52 ± 0.31	62.52 ±0.316	-	-	0.000	0.000
Negative (Liquid paraffin)	3.94 ± 0.95	3.94 ± 0.952	0.000	0.000	-	-

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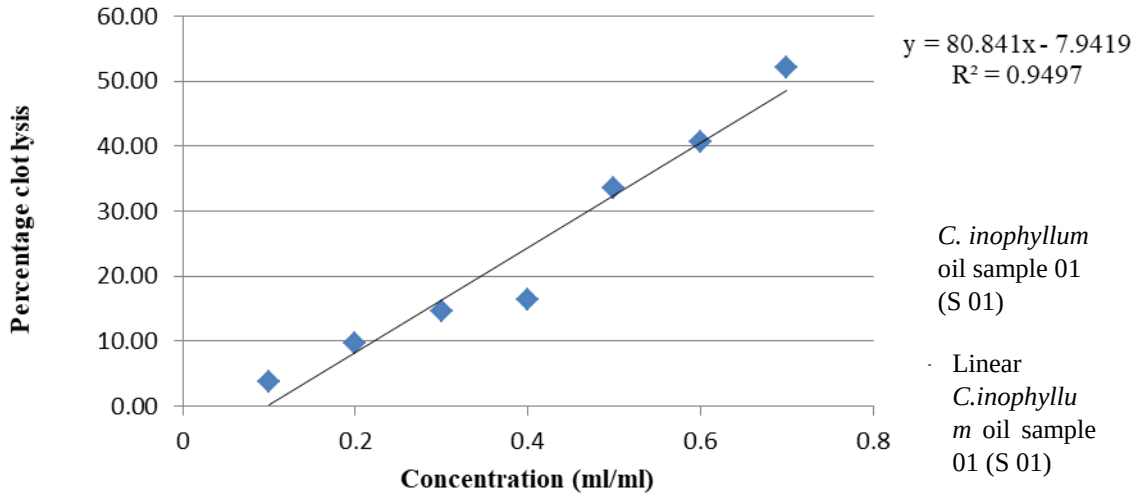

Fig. 7: Concentration-dependent thrombolytic activity of *C. inophyllum* oil sample 01(S 01).

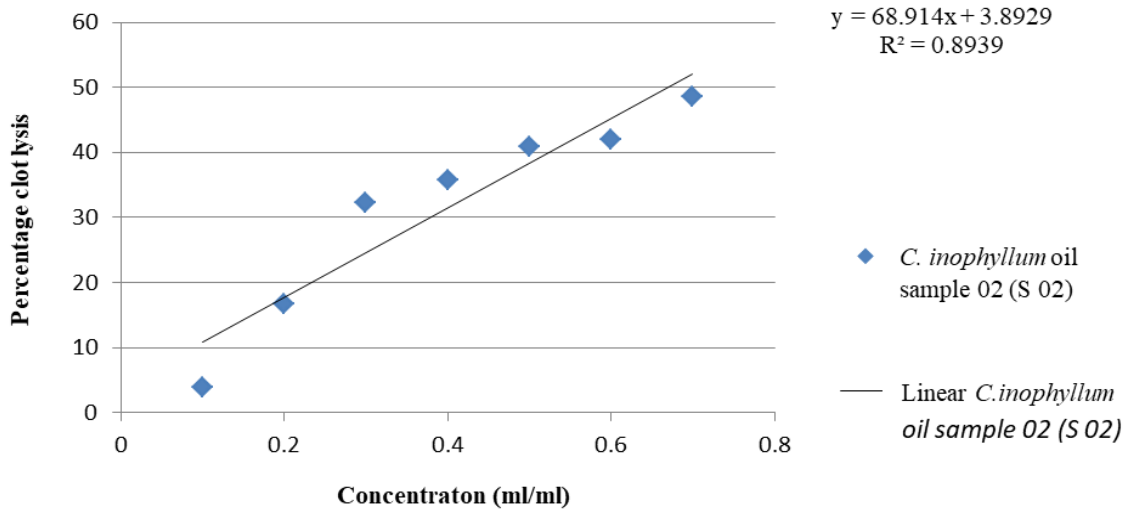

Fig. 8: Concentration-dependent thrombolytic activity of *C. inophyllum* oil sample 02 (S 02).

P values for each concentration of the extract when compared with the positive control, Streptokinase 30000 IU and the negative control, liquid paraffin were determined. Except in lowest concentration in serial dilutions against liquid paraffin with a *p* value of 1, all other p values were 0.0. Significance of *in vitro* thrombolytic activity of the greenish brown *C. inophyllum* oil was statistically evaluated using analysis of variance (ANOVA). A significance level 0.05 was used.

DISCUSSION

The study was carried out to evaluate the *in vitro* thrombolytic activity of the root of the endemic plant *H. anguistifolia* and *C. inophyllum* seed oil. Considering the erythrocyte volume of 45% in blood, in the 500 µl of blood transferred to microcentrifuge tubes, a thrombus of 225 µl weighing approximately 225 µg is formed. This is the weight of thrombus that is being subjected to lysis with the 100 µl of the dilution series.

The highest percentage of clot lysis for *H. anguistifolia* root extract was observed as 32.92 ± 2.135 % at 10mg/ml concentration. This means that only 1 mg of the root extract is responsible for clot lysis. Streptokinase 30 000 IU was used as the positive control, which has a 62.52 ± 0.316% clot lysis ability and normal saline was used as the negative control with a 3.94 ± 0.952% clot lysis. There is a significant difference ($P<0.05$) between *H. anguistifolia* root extract and normal saline (negative control) at higher concentrations than 1mg/ml. It was shown that *H. anguistifolia* root extract has high thrombolytic activity when compared with normal saline at high concentrations. In addition, there is a significant

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difference (P<0.05) between *H. anguistifolia* root extract and streptokinase. It was shown that *H. anguistifolia* root extract has low thrombolytic activity when compared with 30 000 IU streptokinase. Examination of the Figure 6 curve and its parameters is an indication of the robustness of the thrombolysis analytical procedure. The minimal deviation between replicates and the highly predictable linear response underscores the assay's reliability.

In this study, thrombolytic activity for *C. inophyllum* oil has been performed for two oil samples (S 01, S 02). The highest percentage of clot lysis for the *C. inophyllum* oil samples 01 and 02 was observed as 52.08± 0.095 % and 49.15±0.325% respectively, at 700µl/ml concentration for both samples. Liquid paraffin was used as a negative control with a clot lysis activity of 3.94 ± 0.95%. There is a significant difference (P<0.05) between *C. inophyllum* oil sample 01(S 01) and liquid paraffin (negative control) at higher concentrations than 200µl/ml. There is a significant difference (P<0.05) between *C. inophyllum* oil sample 02 (S 02) and liquid paraffin (negative control) at higher concentrations than 100µl/ml. It was shown that both *C. inophyllum* oil samples have higher thrombolytic activity than the negative control at high concentrations. There is a significant difference (P<0.05) between both *C. inophyllum* oil samples and streptokinase. It was shown that both *C. inophyllum* oil samples have low thrombolytic activity when compared with 30000IU streptokinase. Examination of the curves in Figures 7 and 8 and their parameters are an indication of the robustness of the thrombolysis analytical procedure. The strong linearity observed in both samples, evidenced by R^2 values approaching 1, confirms a consistent dose-response. This underscores the assay's reliability in predicting clot lysis efficacy across varying concentrations.

CONCLUSION

The study revealed the positive thrombolytic effects of *H. anguistifolia* root extract and both samples of *C. inophyllum* seed oil. Further investigations are needed to understand more about the pharmacological activities of these plant extracts and their mechanisms.

When *C. inophyllum* oil is dissolved in liquid paraffin, it may resist the release of seed oil on a thrombus that is in an aqueous environment. Table 2 shows that the oil becomes considerably effective at the concentration level of 500µl/ml which is a 50% concentration. The maximum effect at a concentration of 700µl/ml shows that when more of the oil comes into contact with the thrombus, the lysis is greater. This points to the fact that rubbing the oil on blackish discoloration of skin following contusions is justified. An emulsion of the oil in normal saline with a natural emulsifier such as lecithin may result in improved thrombolytic activity as greater oil penetration into the thrombus is possible. [11]

The results also indicate that *C. inophyllum* seed oils from both the dry and wet zones have similar thrombolytic effects. Therefore, the seeds can be pooled together for oil extraction.

According to the results, *H. anguistifolia* root extract and *C. inophyllum* seed oil have positive results for thrombolytic activity. Although no reports are available on systemic administration of the *C. inophyllum* seed oil, it can be recommended that the priority should be to investigate the systemic thrombolytic activity of this oil for wider, more critical applications, provided it is non-toxic when taken internally.

Since the strength of 1mg/100µl brings about maximum thrombolytic activity of *H. anguistifolia* (33%), which is about half that of streptokinase 30000 IU/100µl (62.5%), it has good prospects in clinical use for thrombosis cases in the future. Two *C. inophyllum* oil samples appear more active in this regard (Table 1). Much work has to be done on the toxicities of both these plant-based extracts.

ACKNOWLEDGEMENTS

We wish to express our gratitude towards academic and non-academic staff of the Bachelor of Pharmacy Degree Program and the Research Laboratory, Department of Medical Laboratory Sciences of both, the Sri Jayewardenepura University and Bandaranayke Memorial Ayurvedic Research Institute, Navinna for providing with laboratory facilities and advice in various ways to complete the project.

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