WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 6.805
Volume 5, Issue 6, 160-172. Research Article ISSN 2277– 7105

THROMBOLYTIC EFFECT OF *TREMA ORIENTALIS* AND PASS PREDICTION, MOLECULAR DOCKING, ADME/T PROPERTY ANALYSIS OF ITS ISOLATED COMPOUNDS.

Mohammad Shah Hafez Kabir[1*], Md. Saiful Islam Arman[2,3], Sumya Afroze[4], Md. Shohel Hossain[2], Fahmida Abdullah Tuly[2], Mosarrof Hossain[3], Md. Najem Uddin[3], Hosna Banu[5], Md. Mominur Rahman[1]

[1]Department of Pharmacy, International Islamic University Chittagong, Chittagong-4203, Bangladesh.

[2]Department of Pharmacy, Manarat International University, Dhaka, Bangladesh.

[3]Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh.

[4]Department of Microbiology, University of Chittagong, Chittagong-4331, Bangladesh.

[5]Department of Pharmacy, University of Khulna, Khulna-9208, Bangladesh.

Article Received on
01 April 2016,

Revised on 22 April 2016,
Accepted on 12 May 2016

DOI: 10.20959/wjpr20166-6319

***Corresponding Author**
Mohammad Shah Hafez Kabir
Department of Pharmacy, International Islamic University Chittagong, Chittagong-4203, Bangladesh.

ABSTRACT

The present study aims to investigate the thrombolytic effect of ethanol extract of *Tremas orientalis* leaves by *in vitro* clot lysis method and *in silico* PASS prediction and molecular docking used for eight phytoconstituents namely (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid isolated from *T. orientalis*, to identify whether these compounds interact with the responsible protein (tissue-type plasminogen activator). And also ADME/T properties of the phytoconstituents were analyzed using Qikprop 3.2 module. *In vitro* clot lysis model was used to observe the thrombolytic effect of the extract, where it exhibited 52.38 ± 2.45% clot lysis. In the PASS prediction for their thrombolytic activity of the isolated phytoconstituents, we found wide range of activity. A wide range of docking score found during molecular docking by CPI server. (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid showed the docking score -8.6, -7.4, -7.6, -7.2, 5.5, -8.6, -8.2, -6.1, respectively. Both *in silico* models showed similar value for the same

compound, because (-)-ampelopsin f showed high value in both *in silico* models. All the data support that (-)-ampelopsin f is the best compounds for thrombosis management, as it possessed higher value both in PASS prediction and Molecular docking. After (-)-ampelopsin f, N-(trans-p-coumaroyl) tyramine may be the second choice. So, (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine may be competitive candidate for promising thrombolytic agent. From the ADME profiles of (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine, it cleared that they might safe for human. Further *in vivo* investigation need to identify whether (-)-ampelopsin f, N-(trans-p-coumaroyl) tyramine and other compounds have thrombolytic effect or not.

KEYWORDS: *Trema orientalis*, PASS prediction, Molecular docking, ADME/T properties.

1 INTRODUCTION

Trema orientalis is an evergreen tree which belongs to the family Ulmaceae. It has been used extensively in various ways. *T. orientalis* is the same as *Celtis orientalis* Linn., *Celtis guineensis* Schum. and Thonn., *Trema bracteolate* Hochst Blume, *Sponia orientalis* Linn. Decne, and *Trema guineensis* (Schum. and Thonn.) Ficalho. Aside its uses in paper production and in the manufacturing of poles, it has been used for medicinal purposes including the treatment of respiratory, inflammatory, and helminthic diseases. Almost every part of the plant is used as medicine in various parts of Africa.[1][2] The generic name *Trema* is derived from a Greek word which means perforation or hole and alludes to pitted seeds of the tree, whereas the specific name *orientalis* is derived from the Latin word "*orientalis*" meaning eastern. The plant has common names such as pigeon wood, hop out, charcoal tree, Indian charcoal tree, Indian nettle tree, and gunpowder tree.[3] Phytoconstituents namely (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid isolated from *T. orientalis*.[4]

Thromboembolic diseases are serious and life threatening. Despite the availability of antithrombotic drugs for the prevention and treatment of arterial and venous thrombosis, thrombotic diseases continue to be a major cause of death and disability worldwide. Therefore, there remains a need for more effective therapies to combat these disorders.[5]

In the recent years, there has been a growing interest in using medicinal natural products in the prevention and treatment of many illnesses, including cardiovascular disorders. Day-by-

day the context, concept and methods of the uses of natural products in treatment of human have undergone remarkable changes. Such changes occurred due to the fact that natural medicine or traditional medicine made a revolutionary come-back with renewed strength and vigor to play a more significant role in the management of human health. Significant efforts have been concentrating towards the discovery and development of natural products from various plant and animal sources which have antiplatelet[6], anticoagulant, antithrombotic[7], and thrombolytic activity. Epidemiologic studies have provided evidence that foods with experimentally proved antithrombotic effect could reduce risk of thrombosis. Some plants or plant parts showing thrombolytic activity have also been reported.[8]

The aim of the present study to evaluate the thrombolytic effect of ethanol extract of *Trema orientalis* leaves and to predict whether the isolated compounds from *Trema orientalis* had thrombolytic effect, which was done by using two *in silico* tools PASS prediction and Molecular docking. And also ADME/T properties of the phytoconstituents were analyzed using Qikprop 3.2 module.

2 MATERIALS AND METHODS

2.1 Plant material

Fresh leaves of *Trema orientalis* were collected from Bandarban, Chittagong, Bangladesh in the month of September 2013. It was authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2 Preparation of Extract

The leaves were dried for a period of 10 days under shade and ground. The ground leaves (500 gm) were soaked in sufficient amount of ethanol for one week at room temperature with occasional shaking and stirring then the whole mixture was filtered and the filtrate thus obtained was concentrated using a rotary evaporator (Bibby RE200, Sterlin Ltd, UK) to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (about 5.5%). The extract prepared was for thrombolytic effect screening.

2.3 Drugs and chemicals

To the commercially available lyophilized streptokinase (SK) vial (Square Pharmaceuticals Ltd.) of 1500000 I.U., 5mL 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. All chemicals and reagents were of reagent grade.

2.4 Sample solution preparation

A 100 mg of the extract was suspended in 10 ml distilled water and the suspension was shaken vigorously on a vortex mixer. The suspension was kept overnight and decanted to remove the soluble supernatant, which was filtered through a 0.22-µm syringe filter. A 100 µl of this aqueous preparation was added to the Eppendorf tube tubes containing the clots to check thrombolytic activity.

2.5 *In vitro* Thrombolytic effect assay

Experiments for clot lysis were carried as reported earlier.[9] Briefly, 1.5 ml venous blood drawn from the healthy volunteers was distributed in three different pre weighed sterile Eppendorf tube (0.5 ml/tube) and incubated at 37°C for 45 min. 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 Eppendorf tube containing pre-weighed clot, 100 µl of aqueous solution of sample extract was added. As a positive control, 100 µl of SK and as a negative non-thrombolytic control, 100 µl of distilled water were separately added to the control tubes numbered. All the tubes were then incubated at 37°C for 90 min and observed for clot lysis. After incubation, 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 percentage of clot lysis. The experiment was repeated with the blood samples of the 12 volunteers.

2.6 *In silico* Prediction of activity spectra for substances (PASS)

Prediction of phytoconstituents namely (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, Epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid isolated from *Trema orientalis*[10] for thrombolytic activity was done with the help of computer program, PASS (Prediction of activity spectra for substances). Software estimates predicted activity spectrum of a compound as probable activity (P_a) and probable inactivity (P_i). The prediction of activity is based on structure-activity relationship analysis of the training set containing more than 205,000 compounds exhibiting more than 3750 kinds of biological activities. The values of P_a and P_i vary between 0.000 and 1.000. Only activities with $P_a > P_i$ are considered as possible for a particular compound. If $P_a > 0.7$, the probability of experimental pharmacological action is high and if $0.5 < P_a < 0.7$, probability of experimental pharmacological action is less. If the value of $P_a < 0.5$, the chance

of finding the activity experimentally is less, but it may indicate a chance of finding a new compound.[11-14]

2.7 *In silico* Molecular docking

All the structures of phytoconstituents need to upload in mol2 format with charges and hydrogens added. When a molecule submitted, The CPI server checks the format suitability and calculates the interaction profile of this drug towards all the targets in the database using DOCK6.[15, 16] Users can view the real-time progress online, and the page showing the current docking status of the uploaded drug will also be provided for bookmarking. It takes between 6 and 20 h to finish a one-molecule task and an email will be sent on completion.

The outputs comprise the two following major elements.

(i) Library drugs which share similar (or opposite) interaction profile with the user's molecule, ranked by the similarity (or disparity) with known indications and ADR information, suggesting the underlying new indication and ADR of the user's molecule.

(ii) The candidate off-targets that tend to interact with the user's molecule. The server will visualize the drug-protein interactions, with amino acid residues around 6A^{o} of the molecule highlighted.

2.8 ADME/T property analysis

Ligand based ADME/Toxicity prediction

The QikProp module of Schrodinger (Maestro, version 10.1) is a quick, accurate, easy-to-use absorption, distribution, metabolism, and excretion (ADME) prediction program design to produce certain descriptors related to ADME. It predicts both physicochemical significant descriptors and pharmacokinetically relevant properties. ADME properties determine drug-like activity of ligand molecules based on Lipinski's rule of five. ADME/T properties of the compound (DIM) was analyzed using Qikprop 3.2 module.[17]

2.9 Statistical analysis

The significance between % clot lysis by SK and drugs tested by Tukey test using the software SPSS, version 22.0 (SPSS for Windows, Version 22.0, IBM Corporation, New York, USA). Data are expressed as mean ± SEM. The mean difference between positive and negative control was considered significant at P values < 0.001.

3. RESULTS

3.1. *In Vitro* Thrombolytic effect

In thrombolytic effect assay, addition of 100µl streptokinase as positive control (30,000 I.U.) to the clots and subsequent incubation for 90 minutes at 37°C, showed 78.60±1.28 % lysis of clot. On the other hand, distilled water treated as negative control exhibited a negligible percentage of lysis of clot (7.20±1.95%). The mean difference in clot lysis percentage between positive and negative control was found statistically very significant (P<0.0001). Treatment of clots with extract provided the clot lysis 52.38 ± 2.45%. All the results presented in Table 1 and Figure 1.

Table 1: Clot lysis effect of ethanol extract of *Trema orientalis* leaves on human blood.

Drug/Extract	% of clot lysis
Negative control (water)	7.20±1.95
Positive control (streptokinase)	78.60±1.28 [a]
Ethanol extract of *Trema orientalis* leaves	52.38 ± 2.45 [a,b]

Values are mean ± SEM (n = 12); [a]P <0.001, Tukey test as compared to negative control, [b]P < 0.001, compared to positive control. Statistical representation of the effective clot lysis percentage by extract, positive thrombolytic control (streptokinase), and negative control (sterile distilled water) processed by Tukey test by using SPSS for windows, version 22.0.

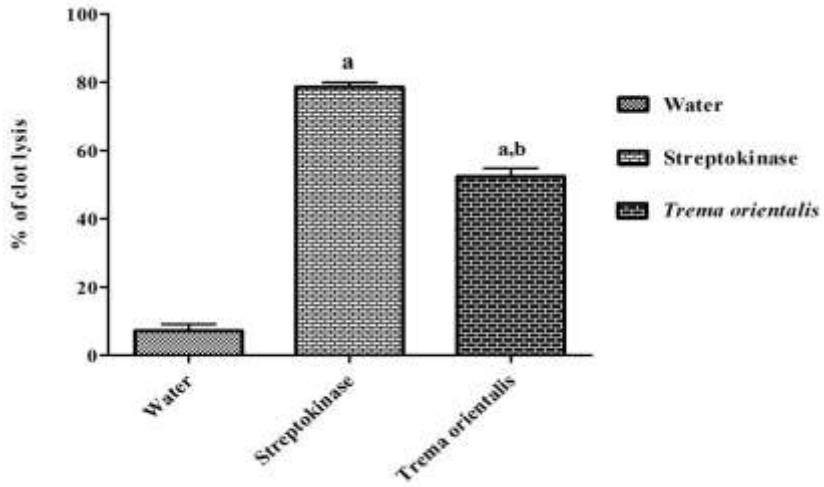

Figure 1: Clot lysis effect of Ethanol extract of *Trema orientalis* leaves on human blood.

Values are mean ± SEM (n = 12); [a]P <0.0001, Tukey test as compared to negative control (Water), [b]P < 0.001, compared to positive control (Streptokinase). Statistical representation of the effective clot lysis percentage by extract, positive thrombolytic control (streptokinase),

and negative control (sterile distilled water) processed by Tukey test by using SPSS for windows, version 22.0.

3.2 *In silico* PASS prediction

Eight phytoconstituents namely (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid were analyzed by the PASS for their thrombolytic activity and results were used in a flexible manner. All the compounds showed greater Pa than Pi (Table 2). (-)-ampelopsin f showed highest Pa for thrombolytic activity (Pa=0.692).

Table 2: PASS predictions of (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid for thrombolytic activity.

Phyto compounds	PASS predictions for thrombolytic activity	
	Pa	Pi
(-)-ampelopsin f	0.692	0.026
(+)-catechin	0.348	0.005
(+)-syringaresinol	0.163	0.128
(-)-epicatechin	0.348	0.005
hexacosanoic acid	0.621	0.013
N-(trans-p-coumaroyl) tyramine	0.600	0.073
simiarenone	0.244	0.151
trans-4-hydroxycinnamic acid	0.541	0.023

3.3 *In silico* Molecular docking

In the present study, molecular docking performed to identify the docking score of (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid towards tissue-type plasminogen activator (PDB code 1A5H), which is a protein involved in the breakdown of blood clots. A wide range of docking score found during molecular docking by CPI server. (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid showed the docking score -8.6, -7.4, -7.6, -7.2, 5.5, -8.6, -8.2, -6.1, respectively. All the results presented in Table 3 and Figure 2.

Table 3: Docking results with (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid in the tissue-type plasminogen activator.

Drug name	Docking Score
(-)-ampelopsin f	-8.6
(+)-catechin	-7.4
(+)-syringaresinol	-7.6
(-)-epicatechin	-7.2
hexacosanoic acid	-5.5
N-(trans-p-coumaroyl) tyramine	-8.6
simiarenone	-8.2
trans-4-hydroxycinnamic acid	-6.1

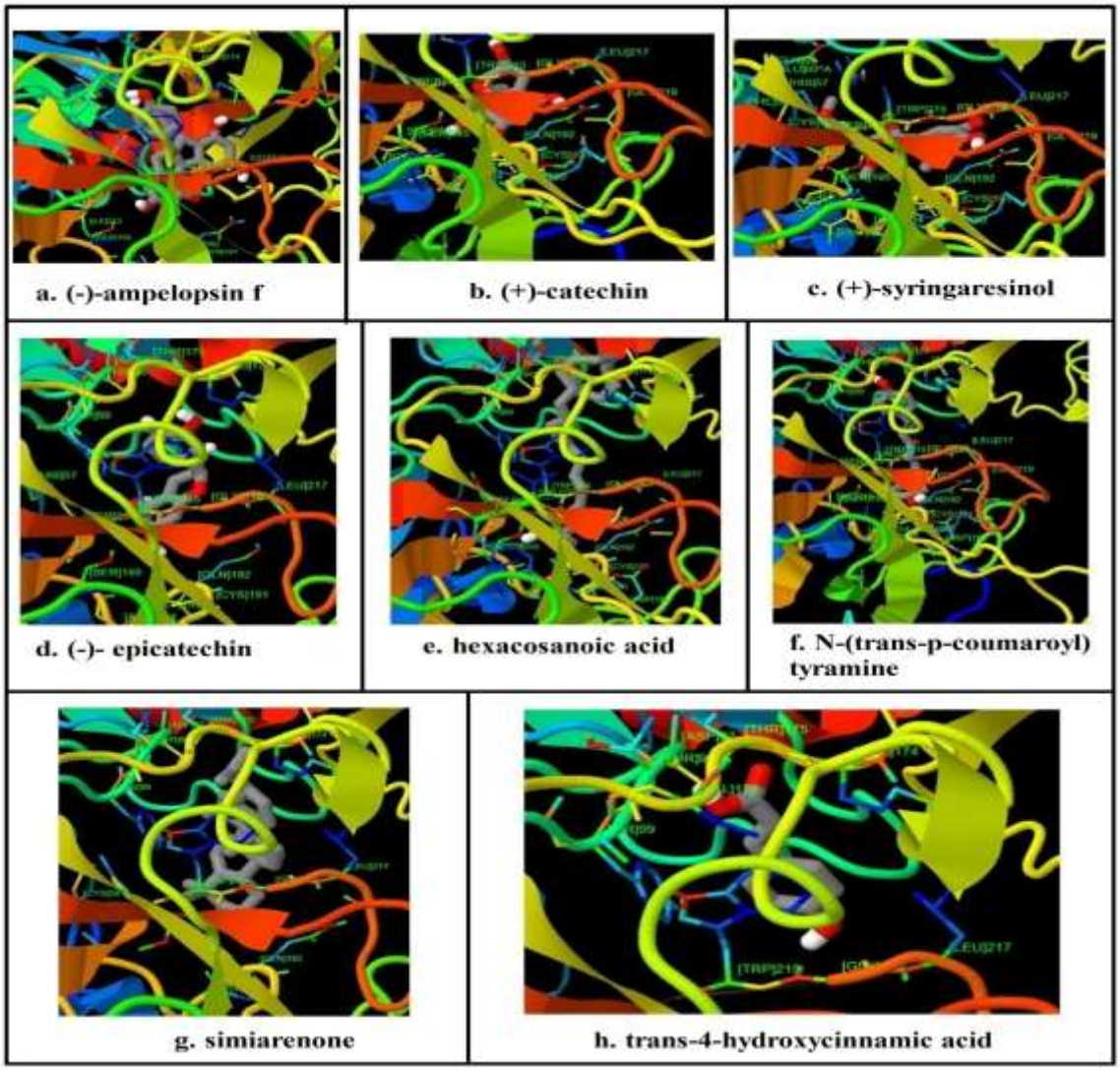

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Figure 2: Molecular docking analysis of (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid with tissue-type plasminogen activator complex obtained from docking.

3.4 ADME and Toxicity analysis

Ligand based ADME/Toxicity prediction

The drug-like activity of the ligand molecule was categorized using ADME properties by QikProp module of Schrodinger. The ADME property of the (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid were evaluated with QikProp module of Schrodinger, shown in Table 4. The selected properties are known to influence metabolism, cell permeation, and bioavailability. All the predicted properties of the isolated compounds were in the range for satisfying the Lipinski's rule of five to be considered as drug like potential, except hexacosanoic acid and simiarenone for LogP and simiarenone for Molar refractivity, where they showed unsatisfying value.

Table 4: ADME/T properties of (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid by QikProp.

Name of Molecules	MW[α]	HB donor[β]	HB acceptor[€]	LogP[¥]	Molar Refractivity[µ]
(-)-ampelopsin f	472.0	0	6	4.240149	125.808952
(+)-catechin	296.0	1	6	2.502560	73.979294
(+)-syringaresinol	434.0	2	8	4.775909	120.113571
(-)-epicatechin	296.0	0	6	2.673059	73.841995
hexacosanoic acid	373.0	0	2	5.876701	100.361984
N-(trans-p-coumaroyl) tyramine	283.0	2	3	2.217660	75.451485
simiarenone	436.0	1	1	8.180825	163.277817
trans-4-hydroxycinnamic acid	163.0	0	3	0.761330	37.521500

[α]Molecular weight (acceptable range: <500).

[β]Hydrogen bond donor (acceptable range: ≤5).

[€]Hydrogen bond acceptor (acceptable range: ≤10).

[¥]High lipophilicity (expressed as LogP, acceptable range: <5).

[µ]Molar refractivity should be between 40-130.

4 DISCUSSIONS

In our thrombolytic assay, the comparison of positive control with negative control clearly demonstrated that clot dissolution does not occur when water was added to the clot. When compared with the clot lysis percentage obtained through water, a well significant (*P* value < 0.001) thrombolytic activity was observed after treating the clots with the extract.

In order to accelerate the research for potent natural products, computer-aided drug discovery program PASS was used to predict the biological activity. PASS prediction tools were constructed using 20000 principal compounds[18] and about 4000 kinds of biological activity on the basis of structural formula with mean accuracy about 90%.[19] The result of prediction is presented as the list of activities with appropriate Pa and Pi ratio. Eight phytoconstituents namely (-)-ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid were analyzed by the PASS prediction for their thrombolytic activity and found wide range of activity. (-)-ampelopsin f was the best compound for thrombolytic effect from all the compounds, though it had bigger Pa value (0.692). According to Pa value, the compounds showed thrombolytic effect as following,

(-)-ampelopsin f > hexacosanoic acid > N-(trans-p-coumaroyl) tyramine > trans-4-hydroxycinnamic acid > (+)-catechin and (-)-epicatechin > simiarenone > (+)-syringaresinol.
In molecular docking study, ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid showed the docking score -8.6, -7.4, -7.6, -7.2, 5.5, -8.6, -8.2, -6.1, respectively, towards tissue-type plasminogen activator. From all these phyto compounds, (-)-ampelopsin f exhibited best docking score (-8.6), which also possessed maximum thrombolytic effect prediction from PASS prediction. And N-(trans-p-coumaroyl) tyramine also showed same docking score (-8.6) as (-)-ampelopsin f and got good prediction for thrombolytic effect in PASS prediction. From both *in silico* methods, (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine might be competitive candidate for promising thrombolytic agent compared with other experimental (*in silico*) compounds.

In our study, we also tried to explore out the ADME and toxicity profiles all of eight compounds, i.e., ampelopsin f, (+)-catechin, (+)-syringaresinol, (-)-epicatechin, hexacosanoic acid, N-(trans-p-coumaroyl) tyramine, simiarenone and trans-4-hydroxycinnamic acid. As described in Table 4, the ADME profile of all the compounds had wide range, but all were within the acceptable range except hexacosanoic acid and simiarenone for LogP and simiarenone for Molar refractivity, where they showed unsatisfying value. From the ADME profiles of (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine, it cleared that they might be safe for human.

5 CONCLUSION

From the study it was found that, Trema orientalis could be great source of new thrombolytic drugs. Both *in silico* models showed similar value for the same compound, because (-)-ampelopsin f showed high value in both *in silico* models. All the data support that (-)-ampelopsin f is the best compounds for thrombosis management, as it possessed higher value both in PASS prediction and Molecular docking. After (-)-ampelopsin f, N-(trans-p-coumaroyl) tyramine may be the second choice. So, (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine may be competitive candidate for promising thrombolytic agent. From the ADME profiles of (-)-ampelopsin f and N-(trans-p-coumaroyl) tyramine, it cleared that they might be safe for human. Further *in vivo* investigation need to identify whether (-)-ampelopsin f, N-(trans-p-coumaroyl) tyramine and other compounds have thrombolytic effect or not.

Conflict of interest statement

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to the authority of International Islamic University Chittagong, Bangladesh, for providing the facilities to conduct this research work. The authors thank GUSTO (A research group) for the financial support. The authors are thankful to all members of GUSTO (A research group) for their kind help in the experiments.

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