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PHYTOCHEMICAL SCREENING AND THROMBOLYTIC ACTIVITY OF AQUEOUS AND HYDRO-ALCOHOLIC EXTRACTS OF *ALLIUM SATIVUM* PEEL

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ABSTRACT

Introduction: During the study and development of novel thrombolytic agents, it is an important consideration to evaluate the efficacy of thrombolytic drugs, which are commonly prescribed in medical settings and are the standard treatments for myocardial infarction and ischemic stroke. But currently available therapy has drawbacks such as bleeding, shortness of breath etc. In recent years there has been a tremendous increase in demand for herbal drugs because of its efficacy and better therapeutic results. Allium sativum is also considered to be therapeutically important in the traditional system of medicine.

Method: To determine the Thrombolytic activity of Aqueous and Hydro-alcoholic extracts of *Allium sativum* Micro centrifuge tube method was performed.

Results And Discussion: The Aqueous and Hydro alcoholic extracts of Allium sativum peel were subjected to phytochemical screening. The result indicated that peel extract shows the presence of carbohydrate, flavonoid, saponins and phenols. Aqueous garlic peel extract (200 µg/ml, 400 µg/ml, 800 µg/ml) clot lysis, i.e. 13.66±0.01202, 16.33±0.00882, 19.33±0.00667 respectively, was obtained. When compared with the negative control (water) the mean clot lysis % of aqueous garlic peel extract, the difference was significant ($P < 0.001$ in all). When compared with the positive control (SK) the mean clot lysis % difference of all the two test i.e. aqueous, hydro alcoholic garlic peel extract was significant ($P < 0.001$ in all).After treatment

of clots with 100 µl of Hydro alcoholic garlic peel extract (200 µg/ml, 400 µg/ml, 800 µg/ml) clot lysis, i.e. 12.33±0.0833, 16.66±0.04387, 21±0.05774) respectively, was obtained.

Conclusion: In conclusion, the present study, using *In- vitro* experiments established the aqueous and hydro alcoholic extracts have moderate Thrombolytic activity.

Keywords: In – vitro, Thrombolytic activity, clot lysis

INTRODUCTION:

The accumulation of fibrin clot in blood vessels induces thrombosis, which leads to cardiovascular diseases. Thrombosis is an abnormal blood clot inside a blood vessel. The blood clots can detach from the vascular wall and travel in the blood. These free floating thrombi (now

Called emboli) can lodge anywhere in the cardiovascular system, including the lungs or brain (as in a thrombotic stroke) **[1]**. Fibrin is implicated in many heart attacks, since cardiac arrest usually occurs after a plaque's cap fractures, causing a blood clot to form over the fracture and

Block blood flow

Thrombolytic therapy has become a conventional treatment for myocardial infarction (AMI), yet at present, clinically prescribed thrombolytic drugs have problems such as delayed action and other side effects like bleeding, re occlusion etc. **[2]**. A variety of fibrinolytic enzymes, such as tissue plasminogen activator (t-PA), urokinase and streptokinase have been extensively used as thrombolytic agents **[3]**. Several investigations are presently being pursued to enhance the efficacy and specificity of fibrinolytic therapy. Fibrinolytic enzymes have attracted interest as thrombolytic agents because of their efficiency in the fibrinolytic process including plasmin activation **[4]**.

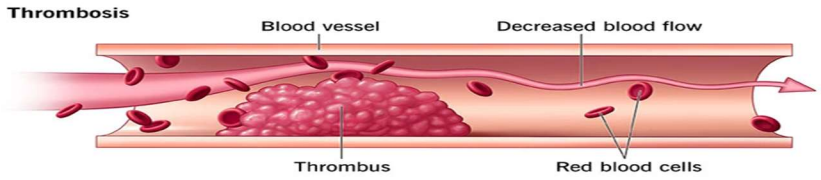

Figure 1: Thrombosis

TYPES OF THROMBOSIS

Arterial thrombosis:

It refers to a blood clot that blocks an artery. Arteries carry blood away from the heart to other parts of the body. Arterial blood clots can block blood flow to the heart and brain, often resulting in a heart attack or stroke **[5]**.

Venous thrombosis: Also known as venous thromboembolism or VTE refers to a blood clot in a vein. Veins carry blood to the heart

from other parts of the body. VTE is a condition that includes deep vein thrombosis (DVT) and pulmonary **[6-18]**.

MATERIALS AND METHODS:

PLANT COLLECTION AND IDENTIFICATION: The dried peel of *Allium sativium* was collected. The peel was shade dried and milled into coarse powder by a mechanical grinder.

PREPARATION OF PLANT EXTRACT:

The powered peel was extracted using ethanol and distilled water using maceration process in this process the powered drug is placed into the extractor with ethanol and water as hydro alcoholic solvent. After extraction the extract was concentrated by evaporation then it was kept in a refrigerator for thrombolytic activity.

EXTRACTION OF PLANT MATERIAL:

The whole peel of *Allium sativum* was collected from local market and authenticated. The peel was cleaned and shaded dried to remove moisture. The multiple solvents like ethanol and water as hydro alcoholic and aqueous extraction procedure were used to prepare extract by using maceration process. The extract was shade dried and weighed to find out the percentage of extract formed from the dried powder. About 20 mg of the powder was taken in beaker and suspended in 250 ml distilled water (DW) and 20mg of the powder was taken in beaker and suspended in 250ml of hydro alcoholic extract and the suspension was shaken vigorously.

MACERATION

This is an extraction procedure in which coarsely powdered drug material, either leaves or stem bark or root bark, is placed inside a container; the menstrum is poured on top until completely covered the drug material. The container is then closed and kept for at least three days. The content is stirred periodically, and if placed inside bottle it should be shaken time to time to ensure complete extraction. At the end of extraction, the micelle is separated from marc by filtration or decantation. Subsequently, the micelle is then separated from the menstrum by evaporation in an oven or on top of water bath. This method is convenient and very suitable for thermo labile plant material.

***IN –VITRO* MICRO CENTRIFUGE TUBE METHOD**

MICRO CENTRIFUGE TUBE METHOD:

Micro centrifuge tubes were taken and the empty weight of each tube was noted. Note it as W_1. 11 millilitres of venous blood was taken from healthy volunteers (n = 11) and transferred to different pre-weighed micro-centrifuge tube. The micro-centrifuge tubes were subjected to incubation at for 45 minutes **[19]**. After 45 min, the formation of clot

occurred and serum was completely separated from the tubes (carried out without disturbing the clot formed). The remaining clot of each tube was again weighed to calculate the weight of the clot [clot weight (W_3) = weight of clot containing tube (W_2) − weight of tube alone (W_1)] **[20]**. Each micro-centrifuge tube was labelled appropriately and 100 µg/ml, 200 µg/ml, 300 µg/ml, dilutions of extract was prepared by taking 2 mg/ml standard solution and those were added to the tubes accordingly **[21, 22]**. One hundred micro liters of streptokinase was used as a positive control whereas 100 µL of sterilized distilled water was distinctly added to the control tubes numbered as a negative non-thrombolytic control. After that, the tubes were incubated again at 37 °C for 90 min for observing clot lysis. Then, following the incubation, the obtained fluid was removed from the tubes. Those tubes were again weighed to observe the difference in weight after clot disruption and noted as (W_4). (W_5)= weight of tube after 90 minutes incubation (W_4)- weight of empty tube, finally the difference in weight of clot is determined (W_6)= weight of clot after 90 minutes incubation (W_3)- weight of initial clot(W_5) and the result was expressed as the percentage of clot lysis using the following equation:

% of clot lysis =W_3−W_6 weight of clot × 100% of clotlysis [23].

Here,

W_1=empty weight of micro centrifuge tube.

W_2= weight of tube containing clot.

W_3= weight of clot (W_2-W_1).

W_4= weight of tube after 90 minutes incubation.

W_5= weight of clot after 90 minutes incubation (W_4-W_1).

W_6= difference in clot weight before and after incubation (W_3-W_5)

Fig 2: Weighing of empty tube

Fig 3: Addition of blood to tube

Fig 4: Allowed to clot

Fig 5: Weighing tube after addition of blood

Fig 6: Addition of sample

EVALUATION OF IN-VITRO THROMBOLYTIC ACTIVITY:

MICROCENTRIFUGE METHOD: Both the extracts i.e. Aqueous and Hydro alcoholic extracts are tested for thrombolytic activity using micro centrifuge tubes **[24]**.

FOR AQUEOUS EXTRACT

Table 1: % clot lysis for aqueous extract

Concentration	W1	W2	W3	W4	W5	W6	%clot lysis	Average
Control(1)	0.79	1.30	0.51	1.28	0.49	0.02	2%	2%
Control(2)	0.77	1.32	0.55	1.31	0.54	0.01	1%	
Control(3)	0.79	1.31	0.52	1.28	0.49	0.03	3%	
Standard(1)	0.78	1.29	0.51	0.89	0.11	0.40	40%	41.33%
Standard(2)	0.78	1.28	0.50	0.85	0.07	0.43	43%	
Standard(3)	0.77	1.29	0.52	0.88	0.11	0.41	41%	
Test(200µg/ml)								
Dilution 1	0.77	1.26	0.49	1.10	0.33	0.16	16%	13.66%
Dilution 2	0.77	1.21	0.44	1.09	0.32	0.12	12%	
Dilution 3	0.77	1.25	0.48	1.12	0.35	0.13	13%	
Test (400µg/ml)								
Dilution 1	0.78	1.24	0.46	1.09	0.31	0.15	15%	16.33%
Dilution 2	0.79	1.27	0.48	1.11	0.32	0.16	16%	
Dilution 3	0.80	1.23	0.43	1.05	0.25	0.18	18%	
Test (800µg/ml)								
Dilution 1	0.79	1.31	0.52	1.12	0.33	0.19	19%	19.33%
Dilution2	0.77	1.30	0.53	1.09	0.32	0.21	21%	
Dilution3	0.78	1.29	0.51	1.11	0.33	1.18	18%	

Addition of 100 µl SK, the standard control to the clots along with 90 min of incubation at 37°C, showed 41.33±0.00882 clot lysis **[25]**. Clots when treated with 100 µl sterile DW (negative control) showed only negligible clot lysis (2.00% ±0.0333%). The mean difference in clot lysis percentage between positive and negative control was very significant ($P <$ 0.001). After treatment of clots with 100 µl of *Aqueous garlic peel extract* (*200 µg/ml, 400µg/ml, 800µg/ml)* clot lysis, i.e.13.66±0.01202, 16.33±0.00882, 19.33±0.00667 respectively, was obtained. When compared with the negative control (water) the mean clot lysis % of Aqueous garlic peel extract, the difference of was significant ($P < 0.001$ in all). When compared with the positive control (SK) the mean clot lysis % difference of all the three test i.e. aqueous garlic peel extract was significant ($P <$ 0.001 in all) **[26]**. Statistical representation of the effective clot lysis percentage of aqueous extract, positive thrombolytic control (SK), and negative control (sterile DW) is represented in the **Table 2**.

Table 2

Concentrations	Average	MEAN±SEM
Control(DW)	2%	2±0.00333
Standard(SK)	41.33%	41.33±0.00882**
Test(200µg/ml)	13.66%	13.66±0.01202**
Test(400µg/ml)	16.33%	16.33±0.00882**
Test(800µg/ml)	19.33%	19.33±0.00667**

****P<0.001The result is highly significant at<0.001 comparing against control, n=3.SEM: Standard Error of Mean. DW: distilled water, SK: Streptokinase**

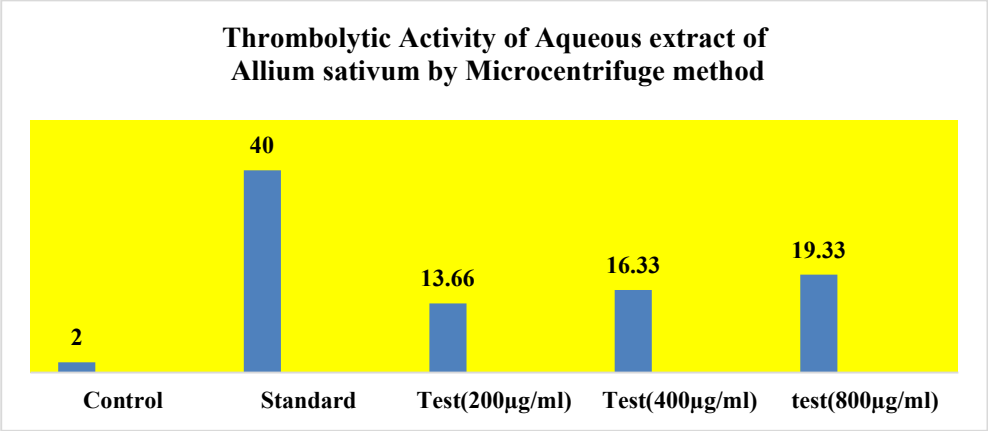

Figure 7: Clot lysis by SK, DW, and Aqueous garlic peel extract (*200µg/ml, 400µg/ml, 800µg/ml)*

FOR HYDRO- ALCOHOLIC EXTRACT

Table: 3% clot lysis for hydro-alcoholic extract

Control(1)	0.79	1.29	0.50	1.26	0.47	0.03	3%	**3%**
Control(2)	0.78	1.30	0.52	1.26	0.48	0.04	4%	
Control(3)	0.77	1.31	0.54	1.29	0.52	0.02	2%	
Standard(1)	0.78	1.32	0.54	0.90	0.10	0.44	44%	**41.33%**
Standard(2)	0.77	1.30	0.53	0.88	0.11	0.42	42%	
Standard(3)	0.79	1.31	0.52	0.89	0.10	0.42	42%	
Test(200µg/ml)								
Dilution 1	0.77	1.23	0.46	1.13	0.36	0.10	10%	**12.33$**
Dilution 2	0.77	1.28	0.51	1.15	0.38	0.13	13%	
Dilution 3	0.78	1.26	0.48	1.10	0.34	0.14	14%	
Test (400µg/ml)								
Dilution 1	0.77	1.12	0.48	1.09	0.31	0.17	17%	**16.66%**
Dilution 2	0.18	1.23	0.45	1.06	0.29	0.16	16%	
Dilution 3	0.77	1.24	0.47	1.07	0.30	0.17	17%	
Test (800µg/ml)								
Dilution 1	0.79	1.24	0.45	1.04	0.25	0.20	20%	**21%**
Dilution2	0.79	1.34	0.55	1.13	0.34	0.21	21%	
Dilution3	0.78	1.30	0.52	1.08	0.30	0.22	22%	

Table: 4

Concentrations	Average	MEAN±SEM	P
Control (DW)	3%	2±0.00333**	
Standard (SK)	42.66%	41.33±0.00882**	<0.001
Test (200µg/ml)	12.33%	12.33±0.0833**	<0.001
Test (400µg/ml)	16.66%	16.66±0.04387**	<0.001
Test (800µg/ml)	21%	21±0.05774**	<0.001

P<0.001 **the result is highly significant at<0.001 comparing against control, n=3.SEM: Standard Error of Mean. DW: distilled water, SK: Streptokinase

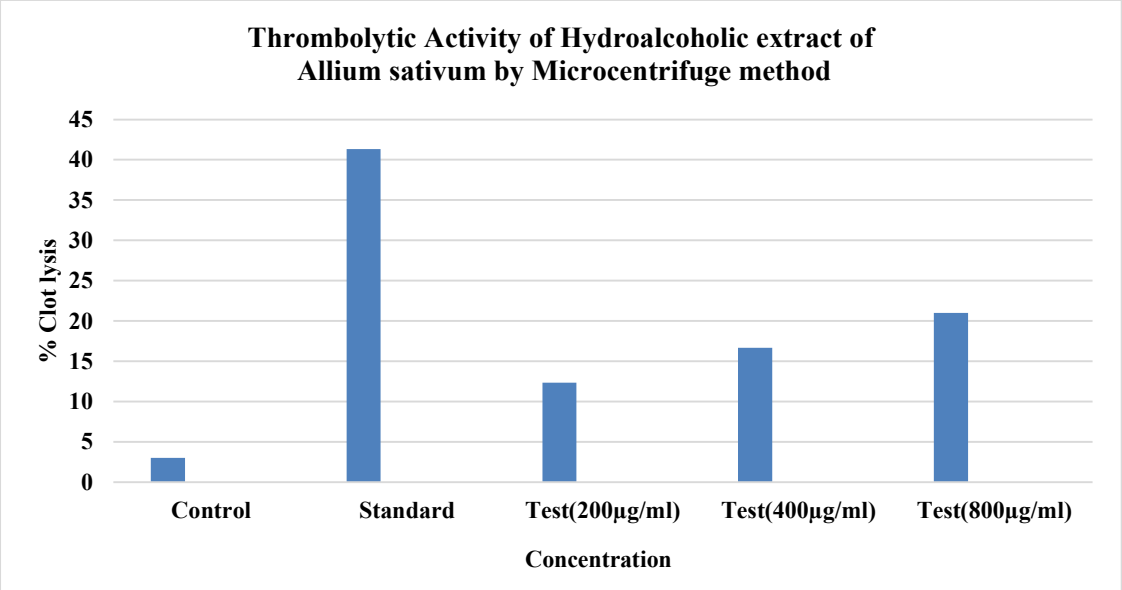

Figure 8: Clot lysis by SK, DW, and Aqueous garlic peel extract (*200µg/ml, 400µg/ml, and 800µg/ml)*

Addition of 100 µl SK, the standard control to the clots along with 90 min of incubation at 37°C, showed 41.33±0.00882 clot lysis. Clots when treated with 100 µl sterile DW (negative control) showed only negligible clot lysis (2.00% ±0.0333%) **[27]**. The mean difference in clot lysis percentage between positive and negative control was very significant ($P <$ 0.001). After treatment of clots with 100 µl of *Hydro alcoholic garlic peel extract* (*200 µg/ml, 400µg/ml, 800µg/ml)* clot lysis, i.e.12.33±0.0833, 16.66±0.04387, 21±0.05774) respectively, was obtained. When compared with the negative control (water) the mean clot lysis % of Aqueous garlic peel extract, the difference of was significant ($P < 0.001$ in all). When compared with the positive control (SK) the mean clot lysis % difference of all the three test i.e. aqueous garlic peel extract was significant ($P <$ 0.001 in all) **[28]**. Statistical representation of the effective clot lysis percentage of aqueous extract, positive thrombolytic control (SK), and negative control (sterile DW) is tabulated in **Table 4**.

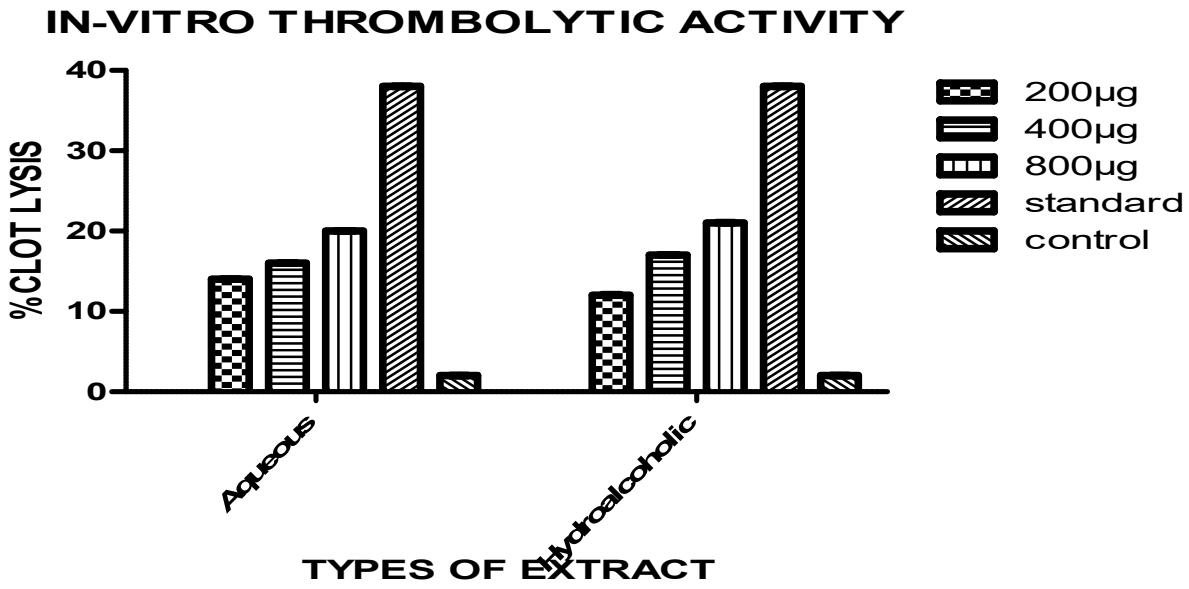

Figure 9: ***In-vitro*** **thrombolytic activities of aqueous and hydro alcoholic extracts compared with standard (streptokinase) and control (distilled water)**

STATISTICAL ANALYSIS:

The statistical analysis was carried out by using bonferroni test method using graph pad prism version 5.1. The results are expressed as MEAN± SEM., n=3.

DISCUSSION:

In case of thrombolytic test, it can be demonstrated that our findings may have significant implications in cardiovascular health. A widely used thrombolytic agent streptokinase acts by converting additional plasminogen to plasmin. But this agent has several adverse effects which encouraged the researcher to discover alternative agent **[29]**. Therefore, we tried to find out whether the garlic peel extract possesses clot lysis property or not. The comparative study between positive and negative control clearly showed that clot lysis did not occur when water was added to the clot. On the other hand, addition of different fractions of extract revealed a significant clot lysis. Among the two extracts, Hydro alcoholic extract showed highest clot lysis in both Micro centrifuge methods (21±0.05774) several studies reveal that flavanoids and saponins are responsible for clot lysis activity **[30]**. It is assumed that these phytochemicals exert their activity by disrupting the fibrinogen and fibrin in a clot that ultimately leads to fibrinolysis. As phytochemical analysis revealed that the crude extract contains flavonoids and saponins, it may predict that these phytochemicals may be responsible for its clot lysis activity.

The test model used is a newly developed,

validated, sensitive, reliable, and simple technique that can be performed with limited facilities available. Various research works are undertaken in quest of thrombolytic drugs. More site specificity with fewer side effects of thrombolytic drugs is desirable in any natural thrombolytic product. Herbal drugs can be a source to address this concern.

CONCLUSION

In the current study, *In –vitro* micro centrifuged method were used for the establishing the thrombolytic activity of aqueous and hydro alcoholic extract of *Allium satium* peel, which shown to have moderate thrombolytic activity then compared to standard. *Allium satium* peel was rich in quercetin, a flavonoid, ajoene, an organo sulfur compound, allicin a thiosulphate and some other steroids saponins which might be responsible for thrombolytic activity. Finally *Allium satium* peel caused thrombolysis by inhibiting platelet aggregation and suppressing fibrin clot formation in blood which have to be confirmed by other studies **[30]**.

FUTURE SCOPE

This is an important finding, which may have implications in cardio vascular health especially in atherothrombotic patients. This is only a preliminary study and to make the final statement about the potentiality of this herb as thrombolytic drugs may require further study. Studies may be undertaken to identify the chemical structure of the active ingredients of the peel extracts and to elucidate the exact mechanism of action and pharmacology by animal studies

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