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EVALUATION OF ANTI- THROMBOTIC ACTIVITY OF *CHRYSANTHEMUM INDICUM* FLOWERS- AN *IN VITRO* STUDY

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ABSTRACT

Cardiovascular diseases involving deep vein thrombosis, stroke, hypertension and heart attacks are the main causes of morbidity and mortality in world. Synthetic drugs used in such disorders leads to serious adverse effects. The discovery of the natural products in the treatment of these diseases reduce the side effects. Inhibiting the aggregation off platelets prevents the formation of thrombus. This is the most widely used mechanism to determine the potential efficacy of anti-thrombotic agents. So the aim of present study was to evaluate the *in vitro* anti-thrombotic activity of hydroalcoholic extract of *Chrysanthemum indicum* flowers. In this study was evaluated the anti-thrombotic activity of hydroalcoholic flower extract of *Chrysanthemum indicum* by using clotting time assay and $Cacl_2$ induced clotting time assay. The inhibitory activity of *Chrysanthemum indicum* flower extract was comparable with the standard drug. The percentage inhibition of *Chrysanthemum indicum* flower extract was attributed due to the presence of flavonoids, phenols, carotenoids. IC_{50} values of *Chrysanthemum indicum* flower extract by using clotting time assay and $Cacl_2$ induced assay were found to be 41.96 and 49.75 µg/ml respectively. Whereas the IC_{50} value of standard was found to be 24.36 µg/ml.

Keywords: *Chrysanthemum indicum* flower, anti-thrombotic activity, clotting time

INTRODUCTION

Thrombosis is a harmful pathological process that can result in a number of cardiovascular disorders, including myocardial infarction, stroke, ischemic heart disease, and pulmonary thromboembolism among worldwide, there are still significant rates of sickness and mortality. Thrombotic illnesses cause 10.0 million fatalities globally each year, and it is predicted that by 2030, they would be responsible for more than 23.6 million. The most frequent causes of death in affluent nations are acute venous and arterial thrombosis combined. The location and severity of the thrombosis affect this mortality. In-depth investigation has uncovered a variety of thrombus formation pathways. Thrombosis is the appearance of a blood clot (partial or complete blockage) in venous or arterial vessel walls, which limits blood flow normally and has therapeutic implications. Blood vessels allow blood to flow freely because of a complicated equilibrium maintained by blood cells (that includes platelets), plasma proteins, coagulation factors, inflammatory agents, cytokines, and the endothelium lining of arteries and veins. An imbalance with this physiological mechanism may lead to a higher chance of thrombosis rather than coagulopathy, which is a higher risk of bleeding. Under some clinical circumstances, patients may concurrently have an increased risk of thrombosis and haemorrhage **[1]**.

Numerous anti-thrombotic medications have been created, and they have proven essential in the prevention and treatment of thrombotic illnesses **[2]**. Currently, anti-thrombotic medications mostly consist of thrombolytic, anticoagulant, and antiplatelet medications. Anticoagulant and antiplatelet medications, which directly prevent thrombosis from forming, are the ones that are used in clinics the most frequently. However, the majority of them have some negative responses and unsatisfactory efficacy. A comprehensive analysis of a number of variables will determine the application and duration of anticoagulant or antiplatelet medication **[3]**.

Thrombosis is the term for a blood clot in blood vessels that limits blood flow. Acute venous and arterial thrombosis is the primary cause of death in industrialized countries. The location and severity of the thrombosis affect the death rate. Cerebrovascular accidents (CVAs) and myocardial infarctions are the main causes for the largest percentage of thrombosis-related fatalities in the US **[1]**.

Both veins and arteries can get thrombosis. It causes deep vein thrombosis and a pulmonary embolism, also known as venous thrombus embolism, in the veins.

Thrombosis in arteries most frequently results in myocardial infarction, ischemic stroke, and acute limb ischemia **[4]**.

Phytochemical substances found in herbal medicines are utilized to treat a wide range of illnesses. These medications are sometimes less effective than synthetic treatments, but they are nevertheless thought to be less harmful, have fewer adverse effects, and cost less money **[5, 6]**. In addition to curing illness, traditional medications have serious negative consequences on the body. Different techniques are used in laboratories to create synthetic medications, yet there have been reports of serious side effects **[6, 7]**.

The herb *Chrysanthemum indicum* has long been utilized for its antiphlogistic, blood tonic, detoxifying, and blood stasis dispersing properties. It is used to treat hypertension and gonorrhoea when combined with black pepper. *Chrysanthemum indicum* flowers have a significant therapeutic benefit. The flowers are used to cure a variety of conditions, including eczema, respiratory disorders, furuncles, hypertension and inflammation of the cervix, eyes and throat **[8]**.

The present study was undertaken to investigate the possible *in vitro* anti-thrombotic effect of hydroalcoholic extract of *Chrysanthemum indicum* flower.

MATERIALS AND METHODS

Collection of plant material:

The flowers of *Chrysanthemum indicum* L. were obtained from the local market. The collected flowers were washed with distilled water and shade dried for 4 to 6 days. Then the dried flowers were grin d in to fine powder. The flowers were authenticated by botanist of SV University, Tirupati.

Preparation of hydroalcoholic extract:

By soaking 50 g of *Chrysanthemum indicum* flower powder in 7:3 ratio of ethanol and water and allow them to stand with intermittent shaking for 3 days. The extract was filtered using muslin cloth and the final extract was collected and stored in refrigerator at 4^0 C. Phytochemical screening was performed by using this extract **[9]**.

Drugs and chemicals: Heparin and adenosine diphosphate were manufactured by Sisco Research Laboratories Pvt. Ltd. Ethanol (99.9%) was used for the process of hydroalcoholic extraction. Calcium chloride was used in $Cacl_2$ induced clotting time assay and it was manufactured by Thermo Fisher Scientific India Pvt. Ltd.

METHODS

***In vitro* Clotting time assay:**

1. The extract was tested for its inhibitory effects on platelet aggregation caused by thrombin, ADP, epinephrine, trypsin, bromelain, and papain. The extracted solution (40 µl) is added to rat platelets (100 µl) and left at room temperature for 5 min.

2. 20µL of thrombin/ADP/epinephrine are added, and the plate reader is used to measure the inhibition of blood clotting for 20 minutes at intervals of 30 seconds. The activity of extracted solution represented as a percentage of mean inhibition **[10]**.

$$\%\textbf{inhibition activity} = \textbf{Ac} - \textbf{As}/\ \textbf{Ac} \times \textbf{100}$$

Ac= Absorbance of control at 412 nm

As= Absorbance of sample at 412 nm

Heparin was used as standard.

***In vitro* Calcium chloride induced clotting time assay:**

1. This test allows the estimation of a 50% clotting time and its impact on fibrinolysis. The assay is carried out by mixing 100µL of human plasma with 40µL of the extracted solution.

2. The reaction was blended and allowed to incubate at room temperature for 5 minutes. 20µL of 0.16 M $Cacl_2$ is added to cause clotting, and the reaction is monitored with a microtitre plate reader at 412 nm for two hours at intervals of three minutes **[11]**.

$$\%\textbf{inhibition activity} = \textbf{Ac} - \textbf{As}/\ \textbf{Ac} \times \textbf{100}$$

Ac= Absorbance of control at 412 nm

As= Absorbance of sample at 412 nm.

RESULTS AND DISCUSSION

CLOTTING TIME ASSAY

The percentage inhibition of clotting time assay by the plant extract was estimated with Heparin as Standard. Hydroalcoholic flower extract of *Chrysanthemum indicum* exhibited 26.1% inhibition of clotting time at 12.5 µg/mL concentration and 76.9 % inhibition at 100 µg/mL concentration. The IC_{50} value was found to be 41.96 µg/mL. Heparin exhibited 33.8 % inhibition at 12.5 µg/mL and 98.46 % inhibition at 100 µg/mL concentration. IC_{50} value of Heparin was found to be 24.36 µg/mL shown in **Table 2 and Figure 1**.

CALCIUM CHLORIDE INDUCED CLOTTING TIME ASSAY

The percentage inhibition of $Cacl_2$ induced clotting time assay of the plant extract was estimated with Heparin as the standard drug. *Chrysanthemum indicum* exhibited 21.5 % inhibition of clotting time at 12.5 µg/mL concentration and 72.37% inhibition at 100 µg/mL concentration. The IC_{50} value of *Chrysanthemum indicum* was found to be 49.75 µg/mL. Heparin, exhibited 33.8% inhibition of clotting time at 12.5µg/mL and 98.46% inhibition at 100 µg/mL concentration. The IC_{50} value of Heparin was found to be 24.36 µg/mL shown in **Table 3 and Figure 2**.

Table 1: Preliminary Phytochemical screening

S. No.	Phytochemical Screening	RESULT
1.	Alkaloids	+VE
2.	Carbohydrates	+VE
3.	Phenols	+VE
4.	Cardiac glycosides	+VE
5.	Terpenoids	+VE
6.	Amino acids and proteins	-VE
7.	Saponins	+VE
8.	Flavanoids	+VE
9.	Mucilages and gums	+VE
10.	Anthraquinone glycosides	-VE

Table 2: % Inhibition of clotting time assay at various concentration

Sample	Conc (µg/ mL)	% clot inhibition	IC_{50} (µg/ mL)
Heparin	12. 5	33.8	24.36
	25	52.3	
	50	79	
	100	98.46	
Chrysanthemum indicum flower	12.5	26.1	41.96
	25	43	
	50	64.6	
	100	76.9	

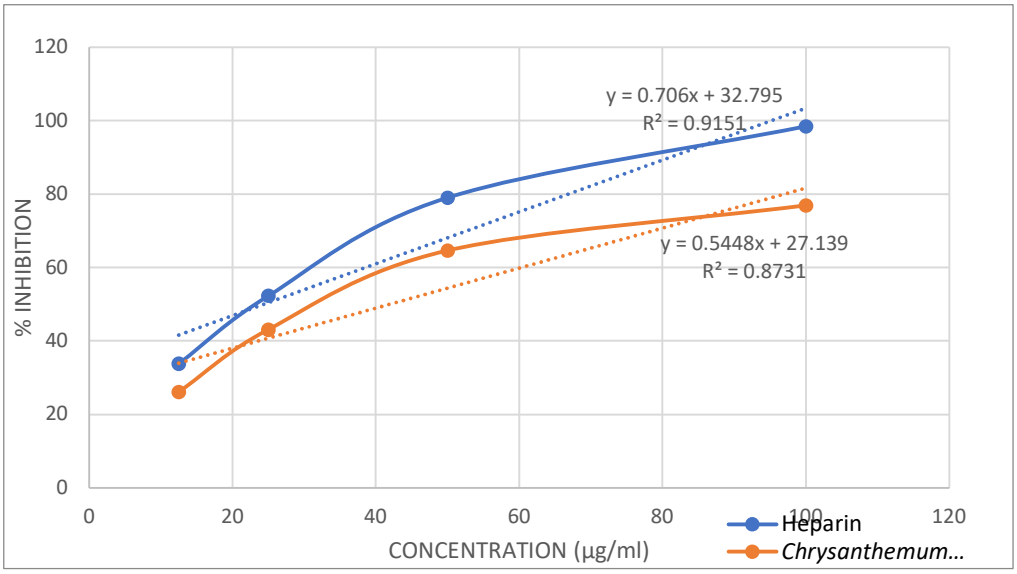

Figure 1: Percentage inhibition of clotting time assay against different concentrations of heparin and *Chrysanthemum indicum* L. the IC_{50} value of Heparin and the extract were 24.36 µg/mL and 41.96 µg/mL respectively

Table 3: % inhibition of $Cacl_2$ induced clotting time assay at various concentrations.

Sample	Conc (µg/ml)	% clot inhibition	IC_{50} (µg/ml)
Heparin	12.5	33.8	24.36
	25	52.3	
	50	79	
	100	98.46	
Chrysanthemum indicum flower	12.5	21.5	49.75
	25	38.4	
	50	61.5	
	100	72.3	

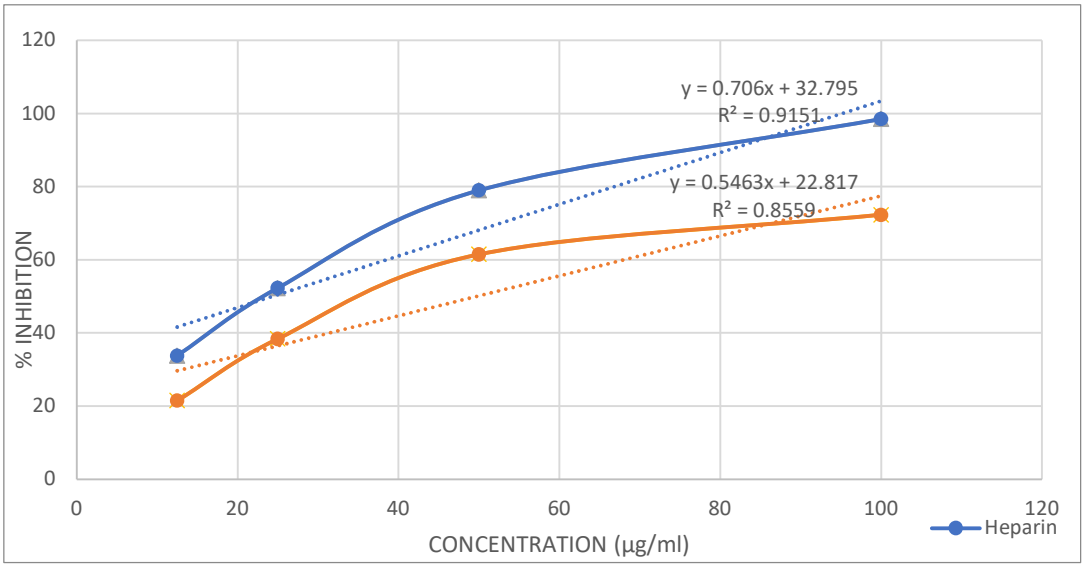

Figure 2: Percentage inhibition of $Cacl_2$ induced clotting time assay against different concentrations of heparin and *Chrysanthemum indicum L*. the IC_{50} value of Heparin and *Chrysanthemum indicum* were 24.36 µg/mL and 49.75 µg/mL respectively

DISCUSSION

Thrombosis is the term for a blood clot in blood vessels that limits blood flow. Acute venous and arterial thrombosis is the primary cause of death in industrialized countries. The location and severity of the thrombosis affect the death rate. Cerebrovascular accidents (CVAs) and myocardial infarctions are the main causes of for the largest percentage of thrombosis-related fatalities in India. Damage to the sinuses, endothelial damage, and coagulation abnormalities that produce an increase in blood viscosity decrease in blood flow are the causes of thrombosis. Heart attack, stroke, transient ischemic attack, and ischemia of the limbs and intestines can all result from thrombus **[12]**. The main reason for the development of thrombosis is hypoxia **[13]**. Therefore, the present study aim was to determine the anti-thrombotic activity of hydroalcoholic extract of *Chrysanthemum indicum* flowers by using clotting time and $Cacl_2$ induced clotting time assay.

Adenosine diphosphate (ADP) is one of the most significant hemostasis and thrombosis mediator **[14]**. Human purinergic G- protein coupled receptors (P2Y1 and P2Y12) two P2Y receptors, influence the impact of ADP on platelets **[15]**. Adenylate cyclase is inhibited by the Gi-coupled P2Y12 receptor and an increase in Ca^{+2} is mediated by the Gq-coupled P2Y1 receptor in the transduction of the ADP signal **[16, 17]**. Normal aggregation requires the simultaneous activation of the Gq and Gi pathways by ADP **[18, 19]**.

The complete activation of many coagulation factors, including coagulation factor XII, is attributed by the calcium ion, which is also involved in the regulation of

coagulation in the maintenance of hemostasis. It is known that some subtypes of protease-activated receptors allow thrombin, which is derived from prothrombin, to activate platelets in this manner. $Cacl_2$ stimulates a coagulation pathway, which in turn stimulates platelets indirectly **[20-22]**.

Hydroalcoholic extract of *Chrysanthemum indicum* flower is used to isolate flavonoids, carotenoids, anthocyanin, phenolic acids and essential oils. The active ingredients were most likely linarin, luteolin and chlorogenic acid.

Clotting time assay was performed by using hydroalcoholic extract of *chrysanthemum indicum* flower at different concentrations (12.5, 25, 50, 100 µg/mL). Hydroalcoholic extract of *chrysanthemum indicum* flower shown 76.9% of inhibition at 100µg/ml where as the standard drug (heparin) showed 98.46% of inhibition at 100µg/ml. IC_{50} (µg/mL) value of Hydroalcoholic extract of *Chrysanthemum indicum* flower was found to be 41.96 while that of heparin 24.36.

Estimation of Percentage inhibition of calcium chloride induced clotting time assay by using *chrysanthemum indicum* flower extract. $Cacl_2$ induced assay was carried out at different concentrations (12.5, 25, 50, 100µg/mL). Hydroalcoholic extract of *chrysanthemum indicum* flower shown 72.3% of inhibition at 100µg/ml where as the standard heparin **[23]** showed 98.46% of inhibition at 100µg/ml. IC_{50} (µg/mL) value of hydroalcoholic extract of *Chrysanthemum indicum* flower was found to be 49.75 while that of heparin 24.36.

The inhibitory activity of the hydroalcoholic extract of *chrysanthemum indicum* flower on clotting time assay and $Cacl_2$ induced clotting time assay was found to be dose dependent. The anti-thrombotic activity of hydroalcoholic extract of *chrysanthemum indicum* flower was attributed due to the presence of phenols, flavonoids, carotenoids.

It is well known that flavonoids and phenolic compounds have strong antioxidant properties **[24]**. Many mechanisms, including the scavenging of free radicals, the suppression of reactive oxygen species (ROS) production, and the inhibition of specific enzymes necessary for a normal redox balance, are responsible for these antioxidant abilities **[25]**. Both exogenously and intracellularly, ROS are produced as a result of regular metabolism or when cells are exposed to environmental changes **[26]** and Oxidative stress, which is defined as an imbalance between the production of ROS and defence mechanisms, plays a key role in the generation of cardiovascular diseases **[27, 28]**. Through stimulating smooth muscle cell proliferation and blocking endothelial nitric oxide synthase, oxidized low-density

lipoprotein (LDL) facilitates vasoconstriction and the advancement of platelet aggregation **[29]**.

Phenolic substances have anti-oxidant qualities that allow them to scavenge free radicals, inhibit enzymes that generate reactive oxygen species (ROS), and increase the activity of antioxidant enzymes in cardiac muscles, such as glutathione transferase, hemeoxygenase-1, and glutathione peroxidase.

CONCLUSION

The present study of *Chrysanthemum indicum* flower indicated that hydroalcoholic extract has significant potential in inhibiting the aggregation of platelets due to the presence of flavonoids and phenols. Further pharmacological studies were needed to find out the bioactive compounds responsible for the anti-thrombotic activity.

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