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In vitro Anticoagulant Activity of Siddha Sastric Formulation Linga Mathirai compared with Low Molecular Weight Heparin

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ABSTRACT

Linga Mathirai (LM) is a *Siddha Sastric* formulation indicated for treating cardiovascular diseases. The present study aimed to evaluate the efficacy of LM against the formation of thrombus and platelet aggregation on compared with Low molecular weight Heparin. *In vitro* clot lysis and platelet aggregation assays were employed for the study on blood samples of rat. Whole blood samples and platelets obtained by centrifugation were used for clot lysis and aggregation assay respectively. Platelet aggregation was induced by agonists Thrombin and Adenosine diphosphate (ADP). The different concentrations of LM (100, 200, 500, 1000 µL) were prepared with the adjuvant Ginger decoction for test samples and 100 µL low molecular weight heparin (LMWH) was used for treating standard group. Against clot formation and platelet aggregation, LM did not exhibit significant efficacy on compared with normal saline (control) and heparin. However, LM at its higher concentration 1000 µL exhibited significant inhibitory activity on platelet aggregation induced by both Thrombin and ADP when compared with distilled water (control). Under this study, it is concluded that the test drug *Linga Mathirai* had comparable and slightly lesser efficacy than low molecular weight heparin.

INTRODUCTION

The formation of thrombus at the site of atherosclerotic plaque is a major cause for Myocardial Infarction, Unstable Angina and Stroke in the setting of Coronary and Cerebral artery diseases (Badimon *et al*., 2012). Fibrin, thrombin and platelets forms main component in thrombus (Vermylen *et al*., 1986). Currently available thrombolytic drugs have the ability to dissolve the blood clot but contraindicated in patients who have undergone surgery and patients having history of bleeding disorders, bleeding condition in gastro intestinal tract and hypertension etc. Thrombolytic drugs could not have the potency against platelet aggregation. For therapeutic administration, thrombolytic drugs are augmented with antiplatelet drugs (Umesh *et al*., 2014). These issues directed the interest of research towards the compounds derived from plants and natural sources having anticoagulant and antiplatelet activity. Certain studies have reported that bioactive compounds isolated from *Gingko biloba*, *Bacopa monnieri* Linn, *Lagenaria siceraria* and *Ananas comosus* inhibit the platelet aggregation and thrombin activity (Henry *et al*., 2009; Kojima *et al*., 1986; Matsuura, 2001; Pietri *et al*., 1997). Till now, there is a lacuna in the field of research on natural minerals in medicines. All the matters existing in the universe including human body is made up of five primordial elements viz., *Vinn* (Space), *Vali* (Air), *Thee* (Fire), *Neer* (Water) and *Mann* (Earth) according to *Pancha bootham* theory in *Siddha* system of Medicine. These five elements form the basis of body constitution, characteristics, tastes, diseases and their treatment. The body has seven basic physical constituents called *Udal Thathukkal viz., Saaram, Senneer, Oon, Kozhuppu, Enbu, Moolai* and *Sukkilam/Suronitham*.

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Each physical constituent is related with a part of the body. *Saaram* and *Senneer* are related with plasma and blood respectively which are made up of basic elements - water and fire (Jayaprakash *et al*., 2011).

According to the fundamentals of *Siddha*, *Lingam* a mercurial compound having fire element can be used in treating the diseases of blood vessels since it has the property in curing the diseases which affects the water element in the body (Thiyagarajan, 2008). *Lingam* based formulations has been used in the treatment of *Vatha* diseases, chronic inflammatory diseases such as arthritis, dermatitis, etc and hemiplegia. *Linga Mathirai* (LM) is a *Siddha Sastric* pill comprises of purified *Lingam* done by *Surukku* method (Cinnabar – Mercuric Sulphide) and purified *Vengaaram* (Borax) done by frying method mixed in equal proportions and triturated with breast milk. In the literature, 60 mg of LM is indicated for the management of *Uruthira Vaiyvu* along with the adjuvant Ginger juice or Ginger decoction (Abdhula, 1995). *Uruthira Vaiyvu* or *Thamaraga Noi* is a *Siddha* terminology for cardiovascular disease having cardinal features of shortness of breath, chest pain, weakness, fatigue and pedal oedema (Kuppusamy, 2004).

The present study was designed as a preliminary screening for the evaluation of anticoagulant and antiplatelet activity of *Linga Mathirai* and done by two assays such as clot lysis and platelet aggregation.

MATERIALS AND METHODS

Preparation of test sample

A sufficient quantity of *Linga Mathirai* was ground into fine powder in a mortar and stored in air tight sterile container. In humans, the mode of administration of LM is mentioned as two times daily, per oral suspended in 10 mL of Ginger decoction as adjuvant at 60 mg dosage. Ginger decoction was prepared by the following procedure. The dried rhizome of Ginger 10 g was coarsely powdered and taken in a 500 mL glass jar. To this 200 mL of water was added and heated mounting over the hot plate boiling down to 50 mL and filtered to get Ginger decoction. For each study, freshly prepared test sample was prepared by suspending powdered LM 60 mg in the adjuvant Ginger decoction 10 mL (6 mg LM in 1 mL decoction).

Animal procurement and maintenance

Sprague dawley rats of weighing 150 g to 200 g were purchased from Sri Venkateshwara Enterprises, Bengaluru, India and they were acclimatized in Animal house of Sairam Advanced Centre for Research Chennai, India at 21 - 23ºC after getting IAEC approval. Animal ethical guidelines of CPCSEA, Ministry of Animal Husbandry and Welfare, Govt. of India were strictly followed for the care and maintenance of procured animals. The animals were fed on standard rodent pellet and RO water was provided *ad libitum.* The animals were kept at overnight fasting before experimentation.

In vitro clot lysis activity

The study was performed by the basis of clot dissolution method (Prasad *et al*., 2006).

Materials

Micro pipettes 100 µL,`200 µL, 500 µL, 1000 µL, Eppendorf tubes 1.5 ml, Heparin – LMWX (Enoxaparin Sodium) 40 mg in 0.4 mL (Gland Pharma Ltd, Hyderabad), Normal Saline, LM test sample and 3 mL blood each from four Sprague dawley rats (A, B, C & D).

Procedure

Four healthy Sprague dawley rats were randomly selected and allotted as A, B, C & D. Six sterile Eppendorf tubes were weighed, noted (W1) and numbered as A1, A2, A3, A4, A5 and A6. 3 mL of fresh blood was withdrawn from the Sprague dawley rat 'A' through cardiac puncture immediately after sacrificed under CO_2 inhalation. 500 µL blood samples were added to A1-A6 tubes and incubated at 37˚C for 45 min. After 45 min, clot was formed and the serum was aspirated out without disturbing the clot. The tubes with clot were weighed again and noted as W2. Then the clot weight (W3) before treatment was determined by W2 – W1. Each tube with clot was treated as follows: A1 treated with 100 µL of Normal Saline (Control), A2 treated with 100 µL of Heparin (Standard) and A3 – A6 treated with 100 µL, 200 µL, 500 µL and 1000 µL test samples respectively. All the treated tubes were incubated for 90 min at 37˚C. After 90 min, clot lysis was observed and the supernatant was aspirated from the tubes without disturbing the clot and immediately weighed as W4 (Weight of the tube with clot after treatment).

The weight of the clot after treatment (W5) was calculated by W4 – W1 and percentage of clot lysis was determined by [(W3 – W5) / W3 x 100]. The same above procedure was carried out on the blood samples of B, C & D. The weight of the clot was observed in four blood samples before and after treatment of control, standard and different concentrations of test samples. The percentages of clot lysis were observed.

Platelet aggregation assay

The inhibitory activity of test sample LM at different concentrations was tested separately on Thrombin and Adenosine diphosphate (ADP) induced platelet aggregation (Born and Cross, 1963).

Materials

3.8% Sodium citrate, Adenosine diphosphate (ADP) – 5 mM, Thrombin 5 U/mL, Micro pipettes 100 µL,`200 µL, 500 µL, 1000 µL, Centrifuge tubes, Heparin – LMWX (Enoxaparin Sodium) 40 mg in 0.4 mL (Gland Pharma Ltd, Hyderabad), Washing buffer (pH: 6.5 & 7.4), LM test sample and 4.5 mL blood each from four Sprague dawley rats (A, B, C & D).

Procedure

A healthy Sprague dawley rat (A) was sacrificed under CO2 inhalation and immediately, 4.5 mL of blood was collected through cardiac puncture and transferred into a centrifuge tube containing 3.8% sodium citrate (9:1 v/v). The blood was centrifuged at 1200 rpm for 15 min and 2200 rpm for 3 min consecutively. The supernatant was collected and centrifuged at 3200 rpm for 15 min. The supernatant was discarded and 5 mL of washing buffer (pH: 6.5) was added to the sediment. The suspended sediment was centrifuged at 3000 rpm for 15 min and supernatant was discarded. The platelets were collected as sediment and again suspended in 0.5 ml of washing buffer (pH: 7.4) and stored in deep freezer at -80ºC. The stored platelets were used within 4 h for the study. The distilled water 100 µL (Control), Heparin 100 µL (Standard) and test samples LM at 100, 200, 500, 1000 µL were added to each 100 µL platelets (A1, A2, A3, A4, A5 & A6) and incubated at 37ºC for 5 min. With these, Thrombin/ADP 20 µL was added and again incubated at 37ºC for 5 min. The inhibition of platelet aggregation was measured at 412 nm using micro plate reader for 20 min at interval of 30 s. The above same procedure was repeated on the platelets obtained from other three rats (B, C & D). The percentage of formation of Platelet aggregation induced by Thrombin and ADP and inhibition of Platelet aggregation in four blood samples were observed.

Statistical analysis

All data were expressed as Mean ± Standard Error of Mean (SEM). All groups were compared among them for testing significance by one way ANOVA followed by Tukey Kramer Multiple Comparison test using GRAPH PAD INSTAT version 3 software programmes. Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION

Arteriosclerosis is the thickening, hardening and loss of elasticity of the walls of arteries. It is the major pathological cause for Coronary and Cerebral thrombosis leading to heart attack and stroke causing mortality and morbidity (Ernest and Virgina, 2010). Current researches recommend focusing on the development of thrombolytic drugs from natural sources and traditional system of medicines since undesirable adverse effects are caused by available thrombolytic drugs. For this present study, a *Siddha Sastric* formulation *Linga Mathirai* (LM) had been chosen considering as an effective drug for the treatment of inflammation related to blood vessels as cited in the literature.

Clot lysis activity of *Linga Mathirai* at different concentrations was done on blood samples of Sprague dawley rat and compared with the activity of Normal Saline as control and low molecular weight Heparin - Enoxaparin as standard. Enoxaparin is an anticoagulant drug which acts by inhibiting thrombin indirectly by binding to a protease inhibitor antithrombin III. Also, it inhibits platelet aggregation therefore serving as an effective control drug for *in vitro* studies (Janet, 2006). Enoxaparin has fibrinolytic activity through the stimulation of endothelial release of tissue plasminogen activator (t-PA) and inhibits coagulation activity by increasing the release of tissue factor pathway inhibitors (TFPI) (Wiegand *et al*., 2014). After incubation of 500 µL of blood samples at 37ºC for 45 min, clot weight was noted and recorded as clot weight prior to the treatment of test samples. On treating with test samples, again the blood samples were incubated at 37ºC for 90 min and clot weight was recorded as after treatment estimation. The blood samples treated with 100 µL Normal saline had shown reduction in mean clot weight to 0.23 g from 0.27 g the difference been 0.04 g. The blood samples treated with 100 µL Heparin had shown in reduction of mean clot weight to 0.19 g from 0.26 g and the difference was 0.07 g. The blood samples treated with 100 µL LM had shown in reduction of mean clot weight to 0.24 g from 0.29 g with the difference as 0.05 g. The blood samples treated with 200 µL LM had shown reduction in mean clot weight to 0.22 g from 0.27 g, the difference calculated as 0.05 g. The blood samples treated with 500 µL LM had shown reduction in mean clot weight to 0.24 g from 0.29 g and the difference was 0.05 g. The blood samples treated with 1000 µL LM had shown reduction of mean clot weight to 0.19 g from 0.25 g and the difference was 0.06 g. The mean percentage of clot lysis for normal saline and heparin was found 14.72% and 24.69% respectively. But the mean percentage of clot lytic activity of 1000 µL LM was found 24.12% and was not statistically significant when compared with control and standard. From the above results, we inferred that 100 µL Heparin and 1000 µL LM both had clot lysis activity better than other group but no statistical significance in difference was observed in between the six groups and it is represented in the figure 1 & 2.

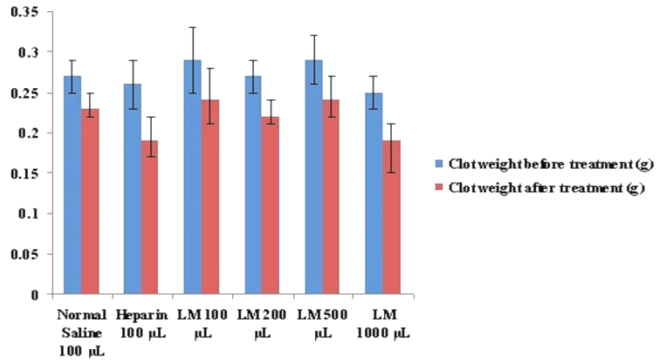

Fig. 1: Effect of the different concentrations of *Linga Mathirai* (LM) on the clot weight of rat blood samples compared to normal saline and low molecular weight heparin. Data are expressed as mean ± SEM, where n = 4. No significant differences are observed.

In many studies, Streptokinase has been used for treatment in the standard group and distilled water for treatment in normal group to determine the clot lysis activity of experimental drug. LMWH does not have much interference in inhibiting thrombin and fibrin formation on compared with other thrombolytic agents A study reported that on concomitant administration of heparin at its therapeutic dose with other thrombolytic agent - Tissue- type plasminogen activator (t-PA),

heparin did not inhibit thrombolysis as compared with only t-PA treated blood samples (Fry and Sobel, 1988). But another study reported that LMWH on subcutaneous administration, it is effective and safety on usage as adjunct to rt-PA thrombolytic treatment for acute infarction (Chamuleau SA *et al.*, 1998). From the present study, we inferred that normal saline has also some extent of inhibition against thrombin and fibrin formation. Hence in the further studies of platelet aggregation assay, distilled water was used for the treatment in normal group.

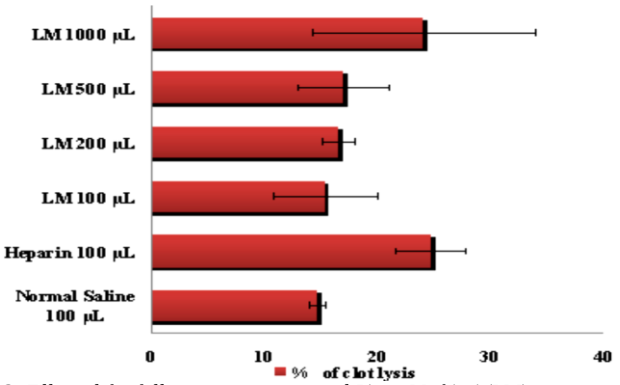

Fig. 2: Effect of the different concentrations of *Linga Mathirai* (LM) on percentage of clot lysis of rat blood samples compared to normal saline and low molecular weight heparin. Data are expressed as mean ± SEM, where n = 4. No significant differences are observed.

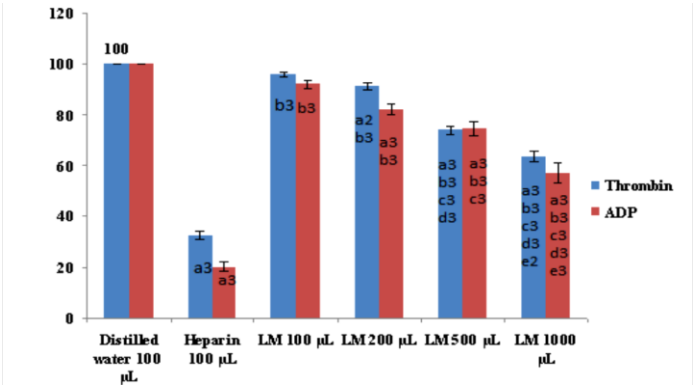

Fig. 3: Effect of the different concentrations of *Linga Mathirai* (LM) on percentage of aggregation formed on rat platelets induced by Thrombin and Adenosine diphosphate (ADP) compared to normal saline and low molecular weight heparin. Data are expressed as mean ± SEM, where n = 4; One way ANOVA: Tukey's multiple comparision test. ^{a3}p<0.001 significantly different compared with platelets treated with distilled water. ^{a2}p<0.01 significantly different compared with platelets treated with distilled water. ^{b3}p<0.001 significantly different compared with platelets treated with heparin. ^{c3}p<0.001 significantly different compared with platelets treated with 100 µL LM. ^{d3}p<0.001 significantly different compared with platelets treated with 200 µL LM. ^{e2}p<0.01 significantly different compared with platelets treated with 500 µL LM. ^{e3}p<0.001 significantly different compared with platelets treated with 500 µL LM.

Platelets play a vital role in hemostasis by interacting with activated plasma clotting factors at the site blood vessel injury and form a hemostatic plug (Harker and Mann, 1992). Platelet aggregation is the process of adhesion of platelets to each other at the site of blood vessel injury and cause thrombosis. Platelet aggregation is induced by number of agonists such as Adenosine diphosphate (ADP), Collagen, Thrombin, etc (Rumbaut and Thiagarajan, 2010). LMWH have lower reactivity to platelets but have anticoagulant activity in normal platelets rich plasma. It protects the platelets from aggregation by the formation of heparin-AT complex (Mousa, 2004). In this study, the antiplatelet aggregation of LM and LMWH was evaluated against thrombin and ADP induced platelet aggregation in blood samples of rat. The inhibitory activity of LM at different concentrations was tested on thrombin and ADP induced platelet aggregation. The results are presented in the figure 3, 4 & 5 as percentage of aggregation formation/inhibition ± S.E.M. Distilled water treated blood samples did not show any activity against thrombin and ADP activity. Heparin had the highest inhibitory activity on compared with other test groups. LM exhibited a dose dependent inhibitory activity on platelet aggregation induced by Thrombin and ADP. Among the different concentrations of LM, 1000 µL exhibited more efficacious inhibitory activity (P<0.001) of platelet aggregation and closely comparable with that of LMWH.

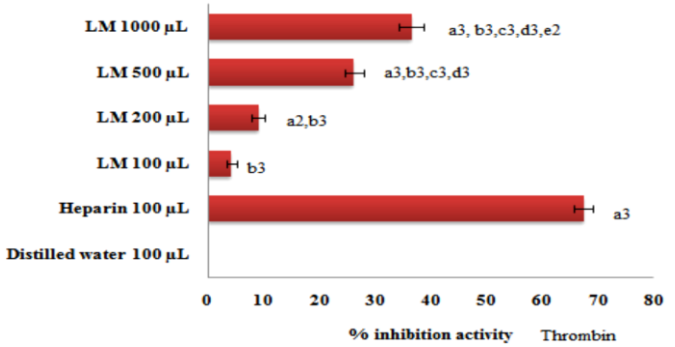

Fig. 4: Effect of the different concentrations of *Linga Mathirai* (LM) on percentage of inhibition of aggregation on rat platelets induced by Thrombin compared to normal saline and low molecular weight heparin. Data are expressed as mean ± SEM, where n = 4; One way ANOVA: Tukey's multiple comparision test. ^{a3}p<0.001 significantly different compared with platelets treated with distilled water. ^{a2}p<0.01 significantly different compared with platelets treated with distilled water. ^{b3}p<0.001 significantly different compared with platelets treated with heparin. ^{c3}p<0.001 significantly different compared with platelets treated with 100 µL LM. ^{d3}p<0.001 significantly different compared with platelets treated with 200 µL LM. ^{e2}p<0.01 significantly different compared with platelets treated with 500 µL LM.

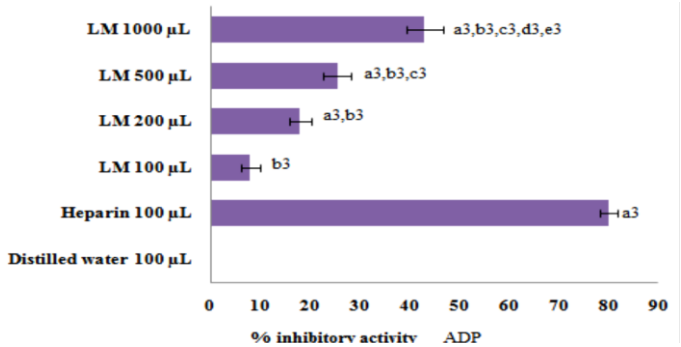

Fig. 5: Effect of the different concentrations of *Linga Mathirai* (LM) on percentage of inhibition of aggregation on rat platelets induced by Adenosine diphosphate (ADP) compared to normal saline and low molecular weight heparin. Data are expressed as mean ± SEM, where n = 4; One way ANOVA: Tukey's multiple comparision test. ^{a3}p<0.001 significantly different compared with platelets treated with distilled water. ^{b3}p<0.001 significantly different compared with platelets treated with heparin. ^{c3}p<0.001 significantly different compared with platelets treated with 100 µL LM. ^{d3}p<0.001 significantly different compared with platelets treated with 200 µL LM. ^{e3}p<0.001 significantly different compared with platelets treated with 500 µL LM.

CONCLUSION

From the results acquired from the above studies, LM demonstrated no statistically significant clot lytic property in different rat blood samples but the clot lysis activity is comparable with LMWH and also the study revealed that LM have marked antiplatelet aggregation property. It can be concluded that *Linga Mathirai* show better anticoagulant activity comparable to low molecular weight heparin and further *in vivo* studies have to be conducted on *Linga Mathirai*.

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