**Summary and Conclusion**

Thrombolytic therapy, which commonly involves the administration of exogenous plasminogen activator, was developed as a result of an insight into the mechanism leading to acute myocardial infarction. A reduction in mortality and morbidity was observed as a result of the therapy but a significant failure rate was also detected due to the failure to achieve reperfusion or occurrence of reocclusion. The seriousness of the current scenario could be gauged by the fact that India alone is burdened with approximately 25% of the cardiovascular-related deaths and is projected to serve as a home to more than 50% of the patients with heart ailments worldwide. Myocardial ischemia-reperfusion injury is crucially involved in the pathogenesis of cardiovascular diseases.

A blood clot that stops blood flow in a coronary artery remains the main cause of many myocardial infarctions. It has also been well investigated that oxidative stress following ischaemic-reperfusion injury is a major apoptotic stimulus in many cardiac diseases. Under normal circumstances, the endogenous antioxidant systems neutralise the harmful effects of free radicals, but under pathophysiological conditions (such as hypoxia, ischemia, early reperfusion, etc), the amount of nascent oxygen free radicals and reactive oxygen intermediators (ROI) go beyond the capacity of endogenous antioxidants and oxidative stress develops. Several strategies are available as a contingent choice to thwart the harmful effects of myocardial ischemic injury, such as beta-blockers, angiotensin converting enzyme (ACE) inhibitors, antiplatelet agents, thrombolytics, calcium antagonist, nitrates and antioxidants.

Herbal drug industries are now a very fast growing sector in the international market because of the increasing realization of the health hazards associated with the indiscriminate use of modern medicines. The traditional medicine all over the world is revalued through an extensive research for their therapeutic application and the principle compounds present in them. There is a one-in-four chance that a drug used from any pharmacy has an active ingredient derived from a plant. It is a well established fact that fruit or plant extracts are a complex mixture of various components and in most cases, it is unclear if a single compound or mixture of compounds is responsible for the biological
effect. Similarly, it is not known if the combination of chemicals in a given plant would produce a superior effect to a single isolated compound of the plant.

There is an increase in trend towards the application of herbal medicines for the treatment of cardiovascular diseases. Moreover, the antithrombotic effect of several natural sources has been exploited but there is a paucity of information regarding thrombolytic efficiency. So an effective thrombolytic agent from a plant source that can conflict the shortcomings of the available drug remains the need of the hour. Bearing all this in mind, the present study entailed the evaluation of the thrombolytic potential of selected plant based thrombolyte.

The study was conducted in five different phases:

Phase I involved the screening of various plant based sources for thrombolytic property. The selected plant source was then screened for the phytochemical constituents present and cytotoxic nature against brine shrimp and normal healthy cell line (Vero).

Phase II included the antioxidant potential determination of the selected fruit, pomegranate, understanding the membrane stabilizing potential of the fruit and determining the minimum effective concentration for thrombolysis. A correlation analysis was performed between the percent thrombolysis and the serum cholesterol levels in the blood samples used for thrombolysis.

Phase III encompassed the active principle compound isolation and confirmation by various chromatographic techniques. A binding analysis was also performed for the isolated compound and a protein known to be involved in thrombolysis using Surface Plasmon Resonance (SPR).

Phase IV entailed the in vivo experiments with κ – carrageenan induced rat tail thrombus and analysis of various biochemical and pharmacological parameters related to thrombolysis.

Phase V comprised of the in silico characterization of active components present in the selected fruit with a few proteins known to be involved in thrombolysis. A binding affinity scoring was also performed in the ligand – protein complex using MMGB/SA.
In the first phase of the study, fifteen different plant based sources namely roots of *Zingiber officinale*, leaves and fruits of *Phyllanthus emblica*, seeds of *Sesamum indicum* and *Brassica juncea*, leaves of *Commiphora molmol*, water and endosperm of *Cocos nucifera*, leaves and pulp of *Carica papaya*, aril and rind of *Punica granatum* and *Garcinia mangostana* and pulp of *Ananas comosus* were screened for the thrombolytic efficiency. *Phyllanthus emblica* leaves portrayed least thrombolytic efficiency while *Garcinia mangostana* and *Punica granatum* revealed higher activity. Though mangosteen (*Garcinia mangostana*) wielded better clot lysing ability than *Punica granatum*, the latter was chosen for further studies owing to their cost effectiveness and availability.

*Punica granatum* is a nutrient dense fruit rich in numerous phytochemicals. There is strong evidence that extracts of all parts of the fruit elicit ameliorating therapeutical effect that target a wide array of diseases including cancer, Alzheimer’s disease, aging and AIDS. It is considered as a nutraceutical food. In line with the bioavailability studies, it is also understood that the bioactive components of the fruit pomegranate can be absorbed readily and then could exert biological activity.

Most of the biological activities are endorsed to the phytochemical components present in the fruit and understanding the chemical composition leads to the development of new medicine and hence pomegranate was checked for the various phytochemical constituents. Presence of alkaloids, carbohydrates, flavonoids, phenols, proteins and tannins were witnessed in the aril and rind of *Punica granatum*. Steroids were present in the aril of the fruit but absent in the rind, whereas quinones and free aminoacids were absent in both aril and rind of the fruit.

Plant extracts not only satisfy multiple biological activities but also possibly alleviate the toxicity of one or combinatorial phytocompounds present in them. As a result, the cytotoxic nature of the aril and rind extracts of *Punica granatum* was evaluated in two different systems namely brine shrimp (*Artemia salina*) and healthy kidney epithelial cell line (Vero). The results concluded that the IC\textsubscript{50} values were higher for the extracts and so the extracts do not pose any toxicity to biological systems.
In the second phase, the influence of the aril and rind of *Punica granatum* against the oxidative stress induced by the free radicals and the amount of antioxidants present in the fruits were assessed. Various concentrations of aril and rind extracts of *Punica granatum* ranging from 20-100 µg/ml were tested for the ability to scavenge a series of free radicals namely DPPH, nitric oxide, superoxide, hydroxyl and hydrogen peroxide. Among the five different *in vitro* models analysed, the fruit extract conferred better inhibition towards hydrogen peroxide radical generation.

The fruit was analysed for the level of enzymic antioxidants (catalase, peroxidase, polyphenol oxidase, glutathione peroxidase and superoxide dismutase) and non-enzymic antioxidants (vitamin C, vitamin E, tannins, polyphenols and flavonoids) present in them. The level of all the enzymic antioxidants was found to be higher in aril of *Punica granatum* when compared with the rind. There was a differential wax and wane in the levels of non-enzymic antioxidants among the aril and rind of *Punica granatum*. It was also observed that the level of polyphenols were higher than the other antioxidants analysed. This property is indicative of the health benefits of the fruit when consumed and could serve as a good source of antioxidants.

The extent of membrane protection rendered by the extracts against hypotonic solution induced and temperature induced damages were checked to know whether the extracts possess good membrane stabilizing activity and thereby offer significant protection of cell membrane. The samples showed good protection against both the damage and the effect was also as good as the standard acetyl salicylic acid used. Though the exact mechanism of action of membrane stabilization is not vivid, it could be possibly due to the inhibition of lysosomalous content release by the neutrophils at the site of inflammation.

A discernible increase in percent clot lysis by the aril and rind of pomegranate was perceived with the increase in concentration of the extracts. Thrombolytic activity of the extracts tested for thrombolysis at different concentrations followed a dose dependent increase, with the utmost activity at the highest concentration, confirming that the effect is unambiguous. The dose response curve tends to attain a plateau after a particular concentration and the minimum concentration for effective thrombolysis was found to be 10 mg/ml.

The effectiveness of the extract in lysing the blood clot of normal, diabetic and hypertensive participants were evaluated *in vitro* and the percent thrombolysis was
compared with the serum protein, cholesterol and HDL levels of the individual. The sample exhibited a differential, nonsignificant response for thrombolysis in participants validating that the mechanism of clot lysis by the extract is irrespective of the cause of formation of the clot and the level of cholesterol of the individual. The results of the second phase fortify the antioxidant and thrombolytic potential of the fruit that could be a promising source of remedy for many lifestyle related diseases.

**PHASE III**

Third phase involved the isolation and characterization of the active compounds in *Punica granatum* responsible for thrombolysis. Pomegranate contains hundreds of bioactive compounds and therefore requires a better understanding of the beneficial effects brought forth by each compound rather than the fruit as a whole. Flavonoids, a major phenolic compound is widely distributed in fruits and vegetables and is being proved to have a cardioprotective role.

The aqueous and ethanolic extracts of aril and rind of pomegranate were subjected to HPTLC with respect to flavonoids and it was found that aril possesses more flavonoids than rind. Presence of rutin, a flavonoid that has proven thrombolytic activity was also confirmed from the HPTLC profiling.

Flavonoid, rutin was isolated from the aril and was confirmed for its presence using UV absorption spectroscopy and HPLC. The amount of rutin was quantified using HPTLC technique. Later, when the isolated rutin and the commercially available rutin in the same concentration were checked for *in vitro* clot lysis, the percent activity was lower when compared with that of the whole fruit. Subsequently, when the concentration of the commercial rutin was increased, there was a steep rise in the clot lysing capacity that was equivalent to the whole fruit. This increase in clot lysis could be possible due to the synergistic effect of different phytochemicals present in the whole fruit.

Binding studies were performed using SPR for protein disulphide isomerase and rutin. PDI impedance results in failure of thrombus formation by preventing platelet accumulation and fibrin generation thereby validating it as a drug target for antithrombotic therapy. Rutin is known to selectively inhibit extracellular PDI and hence the binding efficiency of the isolated rutin and commercial rutin were compared using SPR.
The foremost consideration that limits the strength of rutin to be an effective target against PDI is the extensive metabolism of rutin in vivo. But most of the metabolites either have 3 – O – glycosidic linkages or a sulfhydryl group that are known to be active against PDI. Hence, rutin still remains the choice of interest for the treatment of thrombosis. Besides, rutin is just one isolated compound that is known to possess thrombolytic potential. Results of the present phase support the fact that there is a possibility for a synergistic effect of the different active compounds present in the selected fruit for effective thrombolysis. The efficiency of the extracts and the isolated rutin in animal models was checked in the next phase.

**PHASE IV**

In the fourth phase, the in vivo clot lysing ability of the extracts was checked in murine models. Prior to this, a toxicology screening (acute and subacute toxicity) was performed with a note on the body weight, organ weight, haematological parameters and biochemical indices. It was found that the extracts did not pose any threat to the animals tested and the LD$_{50}$ value was found to be greater than 5000 mg/kg.

Kappa carrageenan, a potent thrombogen was used to develop thrombus model in rats and thrombolysis was measured after subsequent treatment with respective extracts. After the experimental period, visual manifestation of thrombus, haematological parameters (RBC, haemoglobin, platelets, WBC, monocytes and neutrophils), coagulation time parameters (Bleeding time, clotting time, activated partial thromboplastin time and euglobulin clot lysis time), thrombotic factors (fibrinogen, D-dimer, C-Reactive protein, tissue plasminogen activator and creatine phosphokinase) and antioxidant status (catalase, superoxide dismutase, glutathione peroxidase, vitamin C, vitamin E and reduced glutathione) were analysed.

A visible clot formation was observed after the injection of kappa carrageenan and the infarct length was greatly reduced after the administration of standard drug streptokinase and the fruit extracts. The level of RBCs, platelets and haemoglobin increased at the onset of clot formation. Subsequently, when the clot was lysed with streptokinase, the level of platelets dramatically increased. This could be the likely reason for the reocclusion problem in thrombolytic therapy. In contrast, the increase in the level of platelets was lower when treated with fruit extracts when compared with that of the streptokinase. There was an increase in the levels of WBCs and neutrophils when a thrombus was formed and it fell towards normalcy when the treatment was initiated. In
the case of monocytes, the levels were within the normal range among all the groups that were analysed.

Bleeding time, clotting time and Activated Partial Thromboplastin Time (APTT) tend to decrease once the thrombus was formed and gradually increased during thrombolysis. APTT measures the overall coagulation pathway and hence longer the APTT, better the thrombolysis. On the contrary, euglobulin clot lysis time (ECLT) measures by and large the fibrinolytic pathway. Hence, shorter ECLT represents enhanced thrombolysis. The same trend was observed in the present study where the APTT tend to decrease and ECLT tend to increase at the onset of thrombolysis.

The change in the levels of D-dimer, fibrinogen and hs-CRP also confirmed the presence of clot and subsequently clot lysis in vivo. The levels of tissue plasminogen activator and creatine phosphokinase tend to slightly increase in animals when the clot was formed, which later peaked when administered with both fruit extracts and the standard drug streptokinase. Amount of extract required for thrombolysis in vivo was higher when compared to that of the requirement in vitro. This could be due to the fact that in vitro the clots are exposed to fruit extracts directly while in vivo it is subjected to bioavailability.

The antioxidant potential of the fruit in animal model was analysed with respect to enzymic and non-enzymic antioxidants. The oxidative stress is peaked when a thrombus is formed as well as during the reperfusion. There was an increase in all the antioxidants that were analysed in groups treated with the fruit extract when compared with that of the clot induced group and the animals treated with streptokinase.

The histopathological examination performed in liver, heart and tail of control and experimental rats confirmed that there was no toxic effect rendered by the extracts. Thus, the results of phase IV corroborate the thrombolytic efficiency of the fruit Punica granatum.

**PHASE V**

A further insight into the clot lysing efficiency of the fruit was brought about in the fifth phase where molecular docking studies were carried out. Five different flavonoids namely rutin, quercetin, quercetin sulphate, gallic acid and ellagic acid and clopidogrel, a standard drug were chosen as ligands while protein disulphide isomerase, PAI-1,
plasmin, TAFI and thrombin were chosen as protein targets. The ADME studies supported the bioavailability of all the ligands except rutin. Though, rutin has very poor bioavailability, the byproducts of rutin also favor thrombolysis. Hence rutin remains the choice of interest.

The *in silico* studies revealed excessive interactions of selected ligands with the proteins that favour thrombolysis. All the compounds were found to exhibit good docking with the selected proteins as confirmed by their docking score, glide score and hydrogen bonds. The binding energy scoring as determined by the MMGB/SA also supports the effectiveness of the chosen ligand to the target proteins. Synergistic effect proposed in the *in vitro* studies was confirmed from the results of the fifth phase because four other ligands namely quercetin, quercetin sulphate, gallic acid and ellagic acid also docked with the proteins. *Punica granatum* could impede both intrinsic and extrinsic pathway of coagulation because rutin is known to act on endothelial cell as well as platelet aggregation and fibrin generation.

To conclude, the outcome of the present study emphasizes the thrombolytic, antioxidant and membrane stabilizing activities of the selected fruit *Punica granatum*. The fruit extract was capable of lysing a clot regardless of the cause of clot formation and the cholesterol level. The superior thrombolytic activity of the whole fruit extract over the isolated compound, rutin was a momentous observation. The various bioactive components present in the fruit could be considered as a main player for this property.

The animal and docking studies sturdily reiterate the fact that the fruit *Punica granatum* possesses strong thrombolytic activity enabling it to be used as a novel candidate in herbal preparations to combat the shortcomings of the available thrombolytic drug and to act as an effective therapeutical agent for the treatment of diseases related to free radical damage. But further studies are required to pin point the precise mode of action of thrombolytic activity.

**SUGGESTIONS FOR FUTURE RESEARCH**

The end result of the present study has unfolded several prospects for future research. A few of them that can be carried over for active research are as given below:

- The thrombolytic activity of the fruit *Punica granatum* can be tested in real time animal models without any invasive techniques to have further insight into the molecular mechanism involved.
The effect of the extract on thrombolysis enhancing factors like Kruppel like factor 2 (KLF2) and β2 integrins can be studied.

The extent of reperfusion damage caused by the standard drug and the effectiveness of the pomegranate extract can be explored.

The synergistic effect of the different bioactive constituents can be deliberated through isobolograms with respect to ligand-binding assays and factorial designs in animal models.

The combinatorial effect of the extract and the standard drug streptokinase can be studied in vitro and in vivo.

The responsible compound/s can be purified further and can be compared for their differential effects caused by systemic, local and oral administration.

The efficiency of the various extracts of *Punica granatum* for thrombolysis can be studied.

("All that man needs for health & healing has been provided by God in nature, the challenge of science is to find it" – *Philippus Theophrastus*)