Thrombosis, a critical event in the arterial diseases is associated with myocardial infarction and stroke. Venous thromboembolic disorders account for considerable amount of morbidity and mortality and is the second leading cause of death in patients with cancer. The use of novel techniques has advanced the insight of the molecular and cellular basis of thrombus formation, through mouse models (Furie and Furie, 2008). An intense research activity in the antithrombotic field was devoted to compounds showing antiaggregatory potency during the last three decades of the 20th century. Among the huge number of synthetic molecules tested, only very few of them found a clinical use (Dupin et al., 2002).

Intravenous tPA infusion is the fastest way to initiate thrombolytic therapy; however, poor recovery can be expected in up to 50% of patients (Alexandrov et al., 2001). Tissue plasminogen activator delivery to the thrombus is dependent on the residual flow to and around the arterial obstruction when given intravenously (Alexandrov, 2004). Though antithrombotic agents such as warfarin, aspirin, clopidogrel and heparin have proven efficacy and are widely prescribed in the prevention and treatment of many cardiovascular and cerebrovascular diseases, a significant disadvantage of the use of these therapies is an increased incidence of major hemorrhagic occurrences (Bond et al., 2005).

The herbal drugs have been used throughout the world and have received greater attention in recent times, because of their diverse nature of curing diseases, safety and well tolerated remedies compared to conventional drugs. The prevalence of drug interaction is substantial but unknown (Tyagi et al., 2010). Several herbs contain pharmacologically active components that are responsible for the biological activity (Bent, 2008). Herbs and their components possessing antithrombotic activity has been reported before; however herbs that could be used for thrombolysis has to be explored further (Prasad et al., 2007).

Hence, to the possible extent sincere efforts have been made to collect relevant literature of the study. After thorough reviewing of all possible sources, it was observed that very few studies have been conducted earlier on certain dimensions of the present study. Information on the thrombolytic activity of the fruit *Punica granatum* is still very
scanty in literature. The review of literature pertaining to the present research entitled “Evaluation of the Thrombolytic Potential of *Punica granatum* by *in vitro, in vivo and in silico* Approaches” is discussed under the following headings.

2.1 Blood Constituents

2.2 Clot – Characteristics and Formation of clot
   - 2.2.1 Virchow’s Triad
   - 2.2.2 Characteristics of a clot
   - 2.2.3 Mechanism of Thrombosis

2.3 Thrombolysis and Fibrinolysis
   - 2.3.1 Basic concepts
   - 2.3.2 Components of Fibrinolytic system
   - 2.3.3 Thrombolytics

2.4 Free radicals

2.5 Antioxidants

2.6 *In silico* approach

2.7 Selected Fruit – *Punica granatum*

### 2.1 BLOOD CONSTITUENTS

The term ‘Blood’ was coined by William Harvey. Approximately 8% of an adult's body weight is made up of blood with a mean temperature of 38°C and a pH of 7.35 – 7.45 making it slightly basic. Whole blood is about 4.5 – 5.5 times as viscous as water, indicating that it is more resistant to flow than water. This viscosity is vital to the function of blood because if blood flows too easily or with too much resistance, it can strain the heart and lead to severe cardiovascular problems. Blood has three main functions: transport, protection and regulation.

Blood is classified as a connective tissue and consists of two main components:

- a. Plasma, which is a clear extracellular fluid
- b. Formed elements, which are made up of the blood cells and platelets.

#### a. Plasma

It is a mixture of **proteins, enzymes, nutrients, wastes, hormones and gases**. The specific composition and function of its components are as follows:
Proteins: These are the most abundant substances in plasma by weight and play a part in a variety of roles including clotting, defense and transport. There are three major categories of plasma proteins namely albumins, globulins and fibrinogens.

CONSTITUENTS OF BLOOD

Aminoacids: These are formed from the breakdown of tissue proteins or from the digestion of digested proteins.

Nitrogenous waste: Being toxic end products of the breakdown of substances in the body, these are usually cleared from the bloodstream and are excreted by the kidneys.

Nutrients: Those absorbed by the digestive tract are transported in the blood plasma. These include glucose, aminoacids, fats, cholesterol, vitamins and minerals.

Gases: Some oxygen and carbon dioxide are transported by plasma. Plasma also contains a substantial amount of dissolved nitrogen.

b. Formed Elements:

The formed elements are so named because they are enclosed in a plasma membrane and have a definite structure and shape. All formed elements are cells except for the platelets, which are tiny fragments of bone marrow cells. Formed elements are: erythrocytes, also known as red blood cells, leukocytes, also known white blood cells and platelets (Saladin, 2012).
2.2 CLOT – CHARACTERISTIC AND FORMATION OF CLOT

2.2.1 Virchow’s triad

The Chinese physician Huang described the process of pathologic hemostasis as early as 2650 BC (Anning, 1957). But it was the contribution of the triad of factors by the German pathologist Rudolph Virchow that led to the development of thrombosis (Virchow, 1856). The three factors included abnormalities of blood vessel wall, blood constituents and blood flow, which have become known collectively as “Virchow’s triad”. Virchow’s triad remains the best overall explanation of the participants in the pathogenesis of thrombosis even after 150 years.

A current update of the three factors accounts for deviations in the endothelium and endocardium (“abnormalities of vessel wall”); in platelets and the coagulative and fibrinolytic pathways (“abnormalities in blood constituents”); and in hemorheology and turbulence at bifurcations, large vessels burdened by irregular atheroma and stenotic regions (“abnormalities in blood flow”) (Chung and Lip, 2003).
Evaluation of the Thrombolytic Potential of *Punica granatum* by *in vitro*, *in vivo* and *in silico* Approaches
2.2.2 Characteristics of a clot

Within the vascular system, the fluidity of the blood is dependent on a delicate system of checks and balances between platelets, proteins and myriad factors that either promote or inhibit clot formation. Key to keeping blood flowing smoothly is an intact, undamaged intima - the blood vessel's interior layer, which is made up of endothelial cells that secrete a number of factors to inhibit platelets from becoming “sticky” or activated and thus triggering the coagulation cascade. The circulating elements, numerous proteins and coagulation factors (designated by roman numerals) have an effect on coagulation. A meshwork of red blood cells, platelets and fibrin strands unite to form a clot or thrombus, but there are subtle differences depending on where in the vascular system clotting occurs.

In arteries, the primary clotting mechanism depends on platelets, which adhere to the wall of the damaged blood vessel and seal the site of bleeding. It appears as a white clump, so a thrombus rich in platelets is called a “white clot.” Antiplatelet medications are usually prescribed for treatment of thrombi that occur in arteries.

In veins, the primary clotting mechanism depends on the thrombin system. This system consists of several proteins that, once activated, engage in a cascade of chemical reactions that ultimately produce a substance called fibrin. Strands of fibrin form a web that snares red blood cells with platelets and a “red clot” forms. Anticoagulants, which work to impair various aspects of the coagulation cascade, are indicated for prevention and treatment of thrombi that occur in veins (Finkel et al., 2009).

2.2.3 Mechanism of Thrombosis

Basically, there are three major functions of blood constituents that get activated: Formation of fibrin coagulum by the coagulation cascade, Platelets adherence and aggregation and Fibrinolysis.

An injury to the blood vessel and the endothelial lining may initiate the major event of thrombotic process (Slauson and Cooper, 2002). A normal intact endothelium does not favor adherence of platelets, white blood cells and fibrin but in contrast, the deeper parts of the vessel wall have considerable thrombogenic properties. When a vessel is injured, a sequence of intertwining reactions is initiated with the injured vessel, platelets and coagulation factors resulting in vasoconstriction and the formation of a coagulum consisting of platelets and fibrin. A mere passive layer is formed by the
endothelial cells between the blood and the blood vessel. Although a passive layer, it performs vital functions in hemostasis, managing tissue fluid and leukocyte movement into the vessel wall and in regulating the vascular tone (Blann, 2003). A damaged endothelium that releases high levels of prothrombotic von Willebrand factor and loss of thrombomodulin tend to promote thrombosis (Ramot and Nyska, 2010).

Coagulation cascade

The coagulation cascade is a stepwise series of reactions that occur along two pathways, which results in the formation of a fibrin mesh clot:

- The intrinsic clotting pathway is activated when blood comes into contact with a damaged lining of the blood vessel (endothelium).

- The extrinsic clotting pathway is triggered when damaged vascular tissue releases tissue factor, which causes adherence, activation and aggregation of platelets. Both pathways merge at a common juncture, called the final common pathway. It’s at this point that factor X is activated, which then causes prothrombin to be converted into thrombin, leading to the conversion of fibrinogen to fibrin. Blood clots that develop in the arteries can cause heart attack, stroke and severe leg pain and difficulty in walking.

Blood clots in the veins or venous system can cause DVT in the pelvic, leg and upper extremity veins. When these DVTs break off and travel through the bloodstream to the heart and then to the lung blood vessels, they cause acute pulmonary embolism (McCarron, 2010).

Risk Factors for Embolism:

Surgery, increasing age, cancer (active or occult), cancer therapy (chemotherapy, angiogenesis inhibitors, radiotherapy), previous VTE, venous compression (tumor, hematoma, arterial abnormality), increasing age, obesity, pregnancy and the postpartum period, selective estrogen receptor modulators, erythropoiesis – stimulating agents, myeloproliferative disorders, paroxysmal nocturnal hemoglobinuria, central venous catheterization, inherited or acquired thrombophilia, trauma, immobility, acute medical illness, inflammatory bowel disease, nephrotic syndrome, estrogen – containing oral contraceptives or hormonal replacement therapy (Geerts et al., 2008).
Evaluation of the Thrombolytic Potential of *Punica granatum* by *in vitro*, *in vivo* and *in silico* Approaches
2.3 THROMBOLYSIS AND FIBRINOLYSIS

2.3.1 Basic concepts

The process when a plasmin cleaves fibrin at specific locations into smaller water soluble products is termed as fibrinolysis. The thrombi contain inactive plasminogen and in order to start the fibrinolysis the plasminogen activators, like tPA need to enter into the clot. A free diffusion of tPA is possible owing to its size but is hindered by the higher affinity of the enzyme towards the fibrin. The tPA from the bloodstream binds to the surface of the fibrin, thereby increasing its concentration to several folds and the fibrinolysis is initiated. In the presence of fibrin the tPA activates plasminogen, the generated plasmin digests the fibrin and the digestion results in the exposure of new binding sites on fibrin, which leads to the accumulation of plasminogen molecules on the surface of the clot (Marder, 2011).

The fibrins are cleaved by the plasmin crosswise that form thick bundles. Under these circumstances, fibrinolysis proceeds in the outer thin layer of fibrin and later, dissolution takes place layer by layer. The speed of the fibrinolysis is influenced by the structure of the fibrin network, the size of the pores and by the other fibrin – occluded components, which can interact with the fibrinolytic enzymes. Other important factor is the flow rate of blood, what affects the diffusion of the fibrinolytic enzymes and the removal of the degradation products from the surface of the clot (Collet et al., 2000).

CLOT FORMATION AND LYSIS IN BLOOD VESSEL

Under physiological conditions, both coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, inhibitors, cofactors and receptors. Molecular links between these systems permit localized, timely removal
of ongoing or acutely induced fibrin deposits. These co-ordinated molecular events insure blood fluidity while preventing blood loss (Chapin and Hajjar, 2015).

2.3.2 Components of the fibrinolytic system

<table>
<thead>
<tr>
<th>Zymogen</th>
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<tr>
<td>- Plasminogen (N-terminal glutamic acid and lysine variants)</td>
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<table>
<thead>
<tr>
<th>Plasminogen activators</th>
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<tbody>
<tr>
<td>- Tissue plasminogen activator (tPA)</td>
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<tr>
<td>- Urokinase (uPA)</td>
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<table>
<thead>
<tr>
<th>Inhibitors</th>
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<tbody>
<tr>
<td>- Plasmin inhibitors</td>
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<tr>
<td>α2-plasmin inhibitor (α2-PI)</td>
</tr>
<tr>
<td>α2-macroglobulin (α2-MG)</td>
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</tbody>
</table>

Protease nexin
- Plasminogen activator inhibitors
- Plasminogen activator inhibitor-1 and -2 (PAI-1, PAI-2)
- C1-esterase inhibitor
- Protease nexin

<table>
<thead>
<tr>
<th>Attenuator</th>
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<tbody>
<tr>
<td>- Thrombin-activatable fibrinolysis inhibitor (TAFI)</td>
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<tr>
<th>Major receptors</th>
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<tbody>
<tr>
<td>- Activating:</td>
</tr>
<tr>
<td>Annexin 2</td>
</tr>
<tr>
<td>αMβ2 integrin</td>
</tr>
<tr>
<td>Urokinase receptor (uPAR)</td>
</tr>
<tr>
<td>- Clearance:</td>
</tr>
<tr>
<td>Low-density lipoprotein receptor-related protein (LRP)</td>
</tr>
<tr>
<td>Mannose receptor</td>
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</tbody>
</table>
The zymogen plasminogen is converted to the active serine protease, plasmin, through the action primarily of two-chain tissue plasminogen activator (tc-tPA) or two-chain urokinase (tc-uPA). These activators are secreted as single-chain (sc-tPA and scuPA) forms from endothelial cells and from renal epithelium, monocyte/macrophages, or endothelial cells respectively.

Both tPA and uPA can be inhibited by plasminogen activator inhibitor-1 (PAI), while plasmin is inhibited by its major inhibitor, α 2-plasmin inhibitor (α 2-PI) and to a lesser extent by α 2-macroglobulin (α 2-MG). Once plasmin is generated, it converts single chain tPA and uPA to double chain forms. It is then rapidly inhibited unless it remains bound to fibrin or to its cell surface receptors. Inhibitors are indicated by red boxes (Cesarman-Maus and Hajjar, 2005).
(A) tPA and plasminogen (PLG), bind fibrin through lysine residues (K). This trimolecular assembly greatly enhances plasmin (PN) generation, which results in further exposure of carboxy-terminal lysines and ultimately, in fibrin degradation. Fibrin-associated plasmin and tPA are protected from their major inhibitors, α2-plasmin inhibitor (α2-PI) and plasminogen activator inhibitor-1 (PAI-1) respectively. Thrombin-activatable fibrinolysis inhibitor (TAFIa), a plasma carboxypeptidase, cleaves lysine residues and attenuates fibrin dissolution by decreasing the fibrin-binding sites (K) for fibrinolytic enzymes. Urokinase (uPA) acts independently of fibrin. (B) Annexin 2 is present on the endothelial cell surface as a heterotetramer with the S100 family protein p11. Annexin 2 binds both PLG and tPA, serving as a cofactor for plasmin generation and protecting plasmin from circulating inhibitors, such as α2-PI. (C) Integrin αMβ2 on leucocytes binds both PLG and uPA, serving as a cofactor for plasmin generation. uPA receptor (uPAR) may also bind uPA (Kwon et al., 2005).

2.3.3 Thrombolitics

Acute myocardial infarction, stroke and venous thromboembolism that comprises cardiovascular diseases, can lead not only to sudden death but also to long-term
disability at a large cost to society. The recognition that thrombosis within the infarct related coronary artery plays a major role in the pathogenesis of myocardial infarction or stroke and the observation that early administration of thrombolytic agents results in recanalization of occluded coronary arteries has provided the basis for the development of thrombolytic therapy in acute myocardial infarction (Collen and Lijnen, 1991).

Thrombolytic compounds were first used for myocardial infarction in 1959 and were designed to: (1) establish patterns of efficacy and safety for thrombolytic agents; and (2) define the real impact of early thrombolytic therapy on mortality (Green, 2000).

Treatment of arterial clots may include:

i. Clot busters (Thrombolytic agents) like streptokinase

ii. Aspirin and clopidogrel (oral antiplatelet agents)

iii. Intravenous antiplatelet agents

iv. Heparin (a blood thinner and anticoagulant)

v. Tissue plasminogen activators

vi. Ultrasound therapy.

In addition to medications, special interventional catheters may be used to remove or compress these arterial clots (Goldhaber and Grasso – Correnti, 2002).

**Streptokinase** derived from *Streptococci* bacteria has reportedly been used to treat myocardial infarction since 1959. Intracoronary or intravenous streptokinase therapy is available. Intravenous therapy is more common because of limited facilities for intracoronary therapy and other difficulties that arise from its use. In addition to increasing the conversion of plasminogen to plasmin, streptokinase also decreases plasma and blood viscosity, decreases erythrocyte aggregation, decreases blood pressure, decreases vascular resistance and possibly alters platelet function. These additional functions of streptokinase may help to prevent reocclusion. It is an effective thrombolytic agent for a clot that is formed recently.

Since it is isolated from bacteria, it poses the problem of antigenicity after repeated use. This response decreases streptokinase effectiveness and possibly causes allergic reactions. In contrast to activase and heparin, streptokinase is usually supplemented with aspirin (Iyengar and Godbole, 2011).
MECHANISM OF ACTION OF STREPTOKINASE

Low molecular weight heparin with its smaller size, results in a more predictable anticoagulation response because of its availability and decreased binding to proteins unlike tPA. It is absorbed slowly and has a long half life. They act by interfering with factor X, thus preventing the growth and propagation of formed thrombi (Weyland, 2009).

Aspirin is an effective antiplatelet which prevents platelet aggregation and synthesis of thromboxane A2 in platelets. Thromboxane A2 that stimulates the aggregation of platelets also promotes vascular constriction. The effect of aspirin is very long that it is seen for the complete ten day life span of the platelets. This is normally given in combination with streptokinase. Aspirin prevents further clot formation and eventually lyses the clot formed also (An et al., 2011).
**ACTION OF ASPIRIN ON THROMBUS**

- Plaque
- RBCs
- Thrombus
- Aspirin

**Tissue plasminogen activators (tPA)** - Tissue plasminogen activators, originally called fibrinokinase were discovered in 1947 as a substance that is produced within the body that has the ability to degrade plasmin. In the early 1980s, the first purified human form of tPA was obtained in small amounts from uterine tissue.

**MECHANISM OF ACTION OF tPA**

(www.nature.com)

The enzymatic activity of tPA is due to the presence of serine protease domain while plasma clearance is dependent on residues of all the other domains. It is a weak plasminogen activator in the absence of fibrin. Reteplase (rPA), a recombinant tPA developed in the beginning of the 1990s, is produced in *Escherichia coli* cells by multiplication of its genetic sequence. It has shown to decrease the risk of intracranial bleeding compared to tPA. It has an advantage of its size and meanwhile high affinity towards fibrin remains the demerit of tPA (Wander and Chhabra, 2012).

**Ultrasound (US)** – The use of ultrasound is a promising endeavor to enhance thrombolysis. Since thrombolytic treatment is accompanied by the risk of bleeding, the
use of US-enhanced fibrinolysis paved way to increase the efficacy of the therapy. A combination of US-enhanced thrombolytic treatment along with the use of thrombolytic agents has been shown to further enhance fibrinolysis (Tsivgoulis et al., 2010).

**ULTRASOUND AND THROMBOLYSIS**

<table>
<thead>
<tr>
<th>Without Ultrasonic Energy</th>
<th>With Ultrasonic Energy</th>
<th>With Ultrasonic Energy And Thrombolytic</th>
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<tbody>
<tr>
<td>When a clot forms, plasminogen receptor sites are embedded deep within the fibrin. For the clot to be dissolved, lytic agents must access the receptor sites. The tightly wound fibrin strands inhibit the drug from penetrating, limiting access to receptor sites on the interior of the clot.</td>
<td>An endovascular device is placed directly into the thrombus, where the micro-transducers transmit high frequency, low power sound waves. These waves cause the fibrin strands to thin, exposing the plasminogen receptors. This makes the thrombus more permeable for better lysis.</td>
<td>The Intelligent Drug Delivery Cathete delivers the thrombolytic, while the non-cavitational pressure waves created by the ultrasound force the drug deep into the clot. This limits the amount of lytic that escapes downstream.</td>
</tr>
</tbody>
</table>

(www.ekoscorp.com)

There are several new avenues that are opened up in the development of the mechanical delivery of plasminogen activators to thrombus and the mechanical management of thrombus itself. One such development is the new class of pharmacologic agents that directly lyse clots without requiring a plasminogen activator. These pharmacological agents act rapidly than the conventional plasminogen activators and are neutralized soon after they enter systemic circulation. Alfimeprase and plasmin are two such direct fibrinolytic agents that are not plasminogen dependent and are not inactivated by PAI-1 (Comerota and Gravett, 2008).
2.4 FREE RADICALS

The unpaired electrons make the free radical species very unstable and therefore they behave quite reactive with other molecules and they try to pair the electron/s and generate a more stable compound (Jesberger and Richardson, 1991). The most dangerous free radicals are the atomic and molecular varieties of oxygen commonly known as Reactive Oxygen Species (ROS) which includes not only the oxygen radicals (Ø and OH) but also some non-radical derivatives of oxygen, including hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl) and ozone (O₃) (Bhuiyan et al., 2009).

By and large, the free radicals attack a nearby stable molecule, “stealing” its electron. And the “attacked” molecule becomes a free radical by itself and a series of events is initiated. These events can be so lethal that it can result in the disruption of the cell itself. Free radicals are continuously formed as normal byproducts of oxygen metabolism during oxidative phosphorylation in mitochondria. Hence, mitochondria remain the main source of free radicals (Fahn and Cohen, 1992).

Free radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by the antioxidative defense system; however, oxidative stress is generated when equilibrium favors free radical generation as a result of a depletion of the antioxidant levels (Shyur et al., 2005).

Increasing evidence has been emerging in the last few decades relating oxidative stress to a variety of pathological conditions including diabetes, drug or xenobiotic toxicity, cancer and treatment, inflammation, post ischaemic stress and injury, arthritis and cardiovascular disease. Free radicals are also known to spoil foods and degrade materials such as rubber, gasoline and lubricating oils (Ahsan et al., 2010).
MAJOR ROS – GENERATING REACTIONS IN THE CELL

1. \( \text{O}_2 + \text{e}^- \rightarrow \cdot \text{O}_2^- \) Superoxide is generated, e.g. in respiratory chain due to univalent reduction of \( \text{O}_2 \)

2. \( 2\text{O}_2 + \text{NADPH} \rightarrow 2\cdot\text{O}_2^- + \text{NADP} + \text{H}^+ \)

During respiratory burst in immune cells

3. \( \cdot\text{O}_2^- + \cdot\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \)

4. \( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \cdot\text{OH} \)

Fenton reaction

5. \( \cdot\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \cdot\text{OH} + \cdot\text{OH} \)

Haber-Weiss reaction

6. \( \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 \)

Hypoxanthine-xanthine-uric acid reaction

7. Lipid peroxidation: a self – perpetuating chain reaction

\[ \text{ROOH + Metal}^{(s)} \rightarrow \text{ROO}^- + \text{Metal}^{(s-1)} + \text{H}^+ \]

\[ \text{X}^- + \text{RH} \rightarrow \text{R}^- + \text{XH} \]

\[ \text{R}^- + \text{O}_2 \rightarrow \text{ROO}^- \]

\[ \text{ROO}^- + \text{RH} \rightarrow \text{ROOH} + \text{R}^-, \text{etc} \]
**FREE RADICALS AND DISEASES**

Free radicals are known to be the sole cause for several diseases. Once formed, they start a chain of reaction and the menace comes from the damage when they interact with cellular components such as DNA, cell membrane etc (Vithya et al., 2012).

**In ischemia / reperfusion injury**

Scientists have suggested that oxygen radicals can arbitrate myocardial tissue injury during ischaemia and in particular, during reperfusion. Neutrophils are seen accumulated, producing an inflammatory response that is believed to be responsible, in part, for the extension of tissue injury associated with reperfusion. It has revealed that the inhibition of neutrophil accumulation and adhesion is associated with decreased infarct size. This strongly suggests that myocardial cells at risk region undergo irreversible changes upon reperfusion and accumulation of neutrophils (Saeed, 2005).

**In Diabetes mellitus**

Oxidative stress plays a major role in the pathogenesis and development of complications of both types of diabetes. However, the exact mechanism by which oxidative stress could contribute to and accelerate the development of complications in diabetes mellitus is only partly known and remains to be explained. Hyperglycemia is considered a double edged sword, where in one hand, it induces free radicals; on the other hand, it impairs the endogenous antioxidant defense system in patients with diabetes. Biomarkers of increased oxidative stress, as measured by indices of lipid peroxidation and protein oxidation, increase in both T1DM and T2DM (Matough et al., 2012).

**In Cardiovascular disease**

The role of oxidative stress and free radicals in cardiovascular disease has been elucidated by the scientists based on the "free radical theory" of disease. Reactive oxygen species and oxidative stress are important features of cardiovascular diseases including atherosclerosis, hypertension and congestive heart failure. However, comprehensive strategies with antioxidants to ameliorate cardiovascular disease have not generally yielded favorable results. Thus assuming a general antioxidant strategy that it will yield specific effects on cardiovascular disease will be overly simplistic. Indeed, there are several sources of reactive oxygen species that are known to be active in the cardiovascular system (Sugamura and Keaney, 2011).
FREE RADICALS AND DISEASES

In Obesity

Obesity is a chronic disease of multifactorial origin and can be defined as an increase in the accumulation of body fat. Adipose tissue is considered as an important factor involved in obesity because it is not only a triglyceride storage organ, but studies have shown their role as a producer of bioactive adipokines. These in turn induces ROS production thereby generating an oxidative stress. Adipose tissue is the organ that secretes adipokines which in turn generates ROS. Hence it is considered an independent factor for the generation of systemic oxidative stress.

There are several mechanisms by which obesity produces oxidative stress, for instance, the mitochondrial and peroxisomal oxidation of fatty acids. Secondly overconsumption of oxygen that generates free radicals in the mitochondrial respiratory chain that is found coupled with oxidative phosphorylation in mitochondria. Lipid-rich diets are also capable of generating ROS because they can alter oxygen metabolism (Fernández-Sánchez et al., 2011).

In Cancer therapy

The cell damage-inducing properties of ROS have been well utilized in the treatment of cancer. Many chemotherapeutic agents including cisplatin, doxorubicin,
adriamycin, bleomycin, mitomycin C and ionizing radiation are known to induce cell death by generating ROS. Photodynamic therapy performed with a photosensitizer (usually a haematoporphyrin derivative) and a light source has been shown to act by generation of ROS (Datta et al., 2000).

2.5 ANTIOXIDANTS

The free radicals are normally scavenged by the in vivo antioxidants present which are insufficient most of the times. This deficit in endogenous antioxidants results in an incomplete removal of the free radicals thereby generating an imbalance. Consequently, there is a requirement for dietary antioxidants to counteract the excess free radicals (Heravi et al., 2013).

The standard endogenous antioxidants encompasses enzymic (catalase, glutathione reductase and superoxide dismutase), non-enzymic (glutathione, coenzyme Q etc) and exogenous factors (β-carotene, vitamin C, vitamin E and selenium). The exogenous factors are normally supplemented through a diet and these molecules scavenge both reactive oxygen and reactive nitrogen species (Hussein, 2011).

Mitochondrial defense system quenches the highly toxic oxygen radical with the aid of the enzyme systems in mitochondria namely catalase, superoxide dismutase, glutathione peroxidase and glutathione-S-transferase. These enzymes detoxify the ROS generated during electron transport chain and lipid peroxidation and thereby regulating the inner membrane permeability (Flora, 2009).

The plants develop defense systems through the formation of different antioxidants when they are exposed to active oxygen. Many aromatic and spice plants contain compounds that possess confirmed strong antioxidative components. It was found that the phytoconstituents present in the plant namely alkaloids, flavonoids, carotenoids, polyphenolics, coumarins, tannins, essential oil etc are responsible for the biological activity. The essential oils derived from aromatic plants not only serve as fragrance and flavor agents but also as dietary antioxidant expected to prevent several diseases caused by free radicals (Pramod et al., 2011).
### Prevention of free radical generation

<table>
<thead>
<tr>
<th></th>
<th>Coupling of ROS generating systems</th>
<th>To prevent leakage of $O_2$ radicals to the environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Metal chelators ferritin, transferrin, caeruloplasmin and other metallothioneins</td>
<td>Sequestering of metals to prevent generation of hydroxyl radical</td>
</tr>
<tr>
<td>3</td>
<td>Melanin</td>
<td>Prevents UV radiation damage</td>
</tr>
<tr>
<td>4</td>
<td>Target modification, e.g. stable modification of LDL by dehydroascorbate</td>
<td>Imparts resistance to metal ion induced oxidation</td>
</tr>
</tbody>
</table>

### Interception of free radicals generated

**Non-enzymatic**

|   | 
|---|---|
| 1 | $\alpha$-tocopherol (vitamin E) | Intercepts free radicals to harmless end products before they can cause cellular damage |
| 2 | Ascorbate (vitamin C) |
| 3 | GSH (Glutathione) |
| 4 | $\beta$-carotene |
| 5 | Bilirubin |

**Enzymatic**

|   | 
|---|---|
| 1 | Superoxide dismutase (SOD) | Converts superoxide to hydrogen peroxide |
|   | $\cdot O_2 + \cdot O_2 + 2H$ $\rightarrow$ $H_2O_2 + O_2$ |
| 2 | Catalase (CAT) | Breaks down $H_2O_2$ to $H_2O$ and oxygen ($O_2$). Uses a second molecule of $H_2O_2$ as electron acceptor |
|   | $2H_2O_2$ $\rightarrow$ $2H_2O + O_2$ |
| 3 | Glutathione peroxidase | Removes $H_2O_2$ and other hydroperoxide using an organic substrate as electron acceptor |
|   | $H_2O_2 + AH_2$ $\rightarrow$ $2H_2O + A$ |

Plant extracts with antioxidant activity are traditionally used to strengthen the natural immune defenses. The antioxidant defense machinery protects plants against oxidative stress damages. Plants possess efficient enzymatic (superoxide dismutase, ...
catalase, peroxidase, glutathione peroxidase etc.) and non-enzymatic defense systems (ascorbic acid, tocopherol, alkaloids, phenols etc) (Gill and Tuteja, 2010).

**ANTIOXIDANTS SYSTEM**

![Antioxidants System Diagram](www.news-medical.net)

**2.6 In silico approach**

Molecular docking is a key computational tool in structural and molecular biology that predicts the binding site location and conformation of a compound when bound to a protein. The goal of ligand—protein docking is to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. Docking can be used to perform virtual screening on large libraries of compounds, rank the results and propose structural hypotheses of how the ligands inhibit the target, which is invaluable in lead optimization (Morris and Lim-Wilby, 2008).

In a structure based drug design, scoring is one of the most important components but the prediction of this protein – ligand interactions still remains a challenge. Once the mode of binding of a ligand is understood, scientist could explore the molecular mechanisms involved and design a drug for more efficiency (Huang *et al.*, 2010). Three dimensional structures of the receptor and the ligand/s are the two inputs that are fed for a docking process. Most commonly, it is the ligand that is flexible while the receptor is retained rigid and stationary (Zhu, 2012).
2.7 SELECTED FRUIT – *Punica granatum*

Pomegranate (*P. granatum*), is a fruit bearing deciduous tree, native of the Himalayas in northern India, but it has been cultivated and naturalized throughout the Middle East, the entire European Mediterranean region, the drier parts of southeast Asia, northern and tropical Africa and to some extent the United States. The pomegranate juice contributes to about 30% while the seed contribute to 3% of the fruit weight (Viladomiu et al., 2013). The red colour of the fruit extract is due to its numerous anthocyanins (such as delphinidin, cyanidin and pelargonidin). It is also rich in hydrolysable tannins (such as punicalin, pedunculagin, punicalagin, gallagic and ellagic acid esters of glucose). All these phytochemicals accounts for 92% of the antioxidant activity of the whole fruit (Nema et al., 2013).

Pomegranate is one of the oldest known drugs as it is mentioned in the Ebers papyrus of Egypt written in about 1550 BC (Moneim, 2012). Pomegranate has been considered as a “healing food“ owing to its vast advantageous biological effects against several diseases since ancient times (Viuda-Martos et al., 2010). It has been widely used in the preparations in Ayurvedic medicines (as an antiparasitic agent, a “blood tonic” and to heal aphthae, diarrhea and ulcers) and Unani system of medicine (for diabetes) (Prakash and Prakash, 2011).

Extracts of all parts of the fruit appear to have therapeutic properties. Pomegranate juice is a polyphenol rich juice with high antioxidant capacity. The tannins present in the pomegranate juice have been shown to be effective against atherogenesis, oxidative stress, cancer, inflammation and atherosclerosis (Basu and Penugonda, 2009).

Dried fruit peel is used for diarrhea and to treat respiratory and urinary tract infections. It has been reported to wield diverse pharmacological functions as good source of antioxidants, with good antifertility, cytotoxic, hepatoprotective and hypoglycemic activity. They contain substantial amounts of polyphenols namely ellagic acid, ellagittannins and gallic acid (Ahmed and Ali, 2010).
BIOACTIVE EFFECTS OF POMEGRANATE CONSTITUENTS

Pomegranate is rather an extraordinary, albeit mysterious (and messy), fruit with a complete medicinal power contained within its juice, peel and seeds. Lansky, a prominent researcher on the medicinal properties of pomegranate, cautions against focusing on ellagic acid standardization to the exclusion of other therapeutically important pomegranate constituents (Jurenka, 2008).

Presently, there are several anticoagulants, antiplatelets and thrombolytics available for the treatment of thrombus disorders. While the thrombolytics dissolve the existing clots, the anticoagulants and antiplatelets prevents the clot formation but the possibility of reocclusion remains after treatment also. In this regard a single molecule that could both dissolve the clot and prevent the formation of new clots would be useful in the treatment of thrombosis (Singh et al., 2011).

Thus *P. granatum* could be an efficient and inexpensive therapeutic agent for the treatment and prevention of thromboembolic diseases. After thorough reviewing of all possible sources, it was observed that there is no scientific data in the literature on the effect of *P. granatum* on clot lysis or in the treatment of thrombosis. Hence, the experimental design for the evaluation of the thrombolytic potential of *Punica granatum* by *in vitro, in vivo* and *in silico* approaches was presented in the following chapter.