CHAPTER I

GENERAL INTRODUCTION
Platelets: Role in Normal Physiology and Pathophysiology

Platelets are anuclear discoid-shaped moderately refractile, colourless blood cells of diameter 2-4 µm. They originate from the cytoplasm of bone marrow megakaryocytes, regulated by the hormone thrombopoietin. A single megakaryocyte can give rise to 5000-10,000 platelets. The normal range of platelets in circulation of human beings is 100,000–200,000/µL, making platelets the second most numerous cells in the blood. Approximately $10^{11}$ platelets are produced everyday by a healthy human adult. Platelets have a lifespan of is 7-10 days. The most well-established function of platelets is their role as pivotal cell mediators of haemostasis, thrombosis and wound-healing in response to injury (Smyth et al., 2009). Upon injury to vascular endothelium, constituents of the endothelium such as, collagen, fibrinogen, von Willebrand factor (vWF) become exposed and are recognized by platelets, leading to their interaction with specific platelet receptors. This initiates platelet adhesion leading to platelet activation and stimulation of intracellular signalling cascades resulting in platelet aggregation and haemostatic plug formation to stop bleeding and initiate wound repair (Packham, 1994). However, disorders of platelet number and function result in a multitude of bleeding and thrombotic diseases. A shortfall in platelet production and function may lead to unrepressed bleeding. On the other hand, upregulated platelet activation may result in arterial thrombosis, which may block the capillaries resulting in catastrophic events like stroke, myocardial infarction (MI), pulmonary embolism or the blockage of blood vessels to other parts of the body including the extremities of the limbs (Ferroni et al., 2012). An aberration or disease of the platelets is called thrombocytopathy, which could be either a decreased platelet count (thrombocytopenia), a decline in the normal functioning of platelets (thrombasthenia), or an elevated platelet count (thrombocytosis) (Huebsch and
Harker, 1981). Altered platelet functions are also the root cause of the pathophysiology of multifactorial diseases including, atherosclerosis, MI, coronary heart disease (CHD) and other cardiovascular diseases (CVDs), cerebrovascular disease and stroke, cancer, malaria and arthritis (Gregg and Goldschmidt-Clermont, 2003; Ombrello et al., 2010).

Moreover, platelets release a whole host of biologically active substances like growth factors such as, platelet-derived growth factor (PDGF), a powerful chemotactic agent, and tumour growth factor beta (TGFβ), which induces the deposition of extracellular matrix. These growth factors play a substantial role in the repair and regeneration of connective tissues. Other growth factors that are involved in wound healing like, fibroblast growth factor, insulin-like growth factor 1, platelet-derived epidermal growth factor, and vascular endothelial growth factor are also released by platelets (Huebsch and Harker, 1981; Gawaz et al., 2005; Vieira-de-Abreu et al., 2011). Platelets also release pro-inflammatory molecules that arbitrate inflammatory and immune reactions. Apart from promoting coagulation, platelets are key mediators in fibrinolysis and angiogenesis as well. Thus, platelet biology is today a growing field of research due to its central role in human health and disease (Ferroni et al., 2012). Although platelets are anuclear, they exhibit few characteristics of nucleated cells, such as protein synthesis (to a limited extent) and undergo apoptosis, a process of programmed cell death. Mitochondria play a vital role in mediating these processes in platelets (Leytin et al., 2009). Apoptosis, for several years, was thought to be an innate property of nucleated cells; but now it has been established that platelets too possess all the required machinery to undergo apoptotic cell death, clearly suggesting that nucleus is not necessary for carrying out the process. Even though the normal lifespan of platelets is around a week, they may undergo an early
apoptosis and sometimes at an exaggerated rate both *in vivo* and *in vitro*, in response to various

**Table 1.1**

<table>
<thead>
<tr>
<th>Natural agonists</th>
<th>Thrombin, Collagen, ADP, Epinephrine, Arachidonic acid</th>
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</thead>
<tbody>
<tr>
<td>Artificial agonists</td>
<td>Ionomycin, Calcium ionophore A23187, Potassium ionophore U46619, Valinomycin, Thromboxane analogue</td>
</tr>
<tr>
<td>Physical factors</td>
<td>Temperature stress when stored <em>in vitro</em> at 37 °C, Hyperthermia, Hypothermia, Platelet senescence after 5 days of standard banking conditions at 22 °C, Shear stress</td>
</tr>
<tr>
<td>Chemical compounds</td>
<td>Hydrogen peroxide, Dibucaine and Tetracaine (local anesthetics), Cisplatin (chemotherapy drug), Rotenone (broad spectrum pesticide), Calmodulin antagonists - N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide, Tamoxifen and Trifluoperazine, BH3 mimetics such as, ABT-737, Methoxy-antimycin, Aspirin, Vancomycin, Balhimycin</td>
</tr>
<tr>
<td>Other naturally derived compounds</td>
<td>βγ-CAT (a non-lens beta-gamma-crystallin and trefoil factor complex isolated from toad <em>Bombina maxima</em> skin secretions), Peptidoglycan from <em>Staphylococcus aureus</em> 113, Gossypol, Andrographolide</td>
</tr>
<tr>
<td>Pathological conditions</td>
<td>Uremia, Diabetes, CVDs, Bernard Soulier syndrome, Kawasaki disease, Dengue, Malaria, <em>Helicobacter pylori</em> infection, Thrombotic thrombocytopenic purpura.</td>
</tr>
</tbody>
</table>

**Table 1.1: Inducers of platelet apoptosis**
stimuli such as, pathological shear stress, platelet storage, hyperthermia, and physiological agonists or chemical compounds (Leytin et al., 2009; Wadhawan et al., 2004; Wang et al., 2010a; Leytin et al., 2006; Lin et al., 2009a) (Table 1).

Generally, among nucleated cells two types of signal transduction pathways initiate apoptosis: the receptor-mediated and the mitochondria-linked apoptotic pathways. However, in platelets intrinsic pathway of apoptosis is well-documented and death ligand-mediated extrinsic pathway remains to be clearly elucidated (Leytin, 2012). The intrinsic apoptotic program directs the lifespan of platelets. There is a dynamic interplay between the pro-survival and proapoptotic genes of the Bcl2 family and the precise balance in their expression dictates the survival and death of platelets. The dietary and therapeutic components in the circulatory system may interfere with this delicate balance and may either prolong platelet survival or decrease their lifespan. Nonetheless, platelets are genetically programmed to die via apoptosis, whatsoever. This is due to the fact that platelets besides containing mitochondria, express a range of mRNAs and proteins (death receptors, Bcl2 and caspase families) associated with apoptosis. These proteins and organelles derived from their nucleated parent cells (megakaryocytes), endow platelets with the apoptotic machinery. Thus, even normal platelets upon reaching the end of their lifespan undergo apoptosis (Li et al., 2001).

**Effect of Conventional Therapeutic Drugs on Platelets:**

Therapeutic drugs have been the life saviours in the modern era, as they are successfully used to treat many ailments that were deemed incurable in the ancient times. However, many of these drugs are reported to cause serious and sometimes life-threatening side-effects. Since most of the drugs enter the circulation, the blood
cells seem to be an obvious target of offshoot reactions. Drugs can stimulate an entire range of hematologic alterations including thrombocytopenia, (Mintzer et al., 2009). The normal range of platelet count in humans is 150,000-450,000/µL of blood and when the count drops to 100,000 platelets/µL of blood, the condition is termed as thrombocytopenia (Candemir et al., 2012). Although, drug-induced thrombocytopenia (DITP) develops 1 to 2 weeks after commencing a new drug, antithrombotic agents such as, abciximab, tirofiban, and eptifibatide can cause thrombocytopenia instantly as they can block fibrinogen binding to platelet GP IIb-IIIa. There are reports suggesting that more than 200 drugs cause immune thrombocytopenia. These include commonly used drugs such as, antibiotics (Rifampin, Sulfamethoxazol, Vancomycin, Sulfanomides, Linezolid etc.), anticonvulsants (carbamazepine, phenytoin, valproic acid), RGD mimetic agents (eptifibatide and tirofiban), anti-inflammatory drugs (Acetaminophen, Diclofenac, Quinine), antineoplastics (Interferon-α), cardioprotective (Eptifibatide, Quinidine, Tirofiban), antihypertensive (Methyldopa), as well as antidiabetic drugs (Chlorpropamide). Due to the extensive use of these drugs and rather high incidence of DITP, it potentially weighs down the beneficial effects and on the other hand it further increases the disease burden (George and Aster, 2009; Reese et al., 2010; Mintzer et al., 2009). Apart from immune-mediated thrombocytopenia, drug-induced drop in platelet count may also occur due to elevated rate of platelet apoptosis. The fact that drugs induce platelet apoptosis is a very recent knowledge. Currently, vancomycin and balhimycin (antibiotics), cisplatin (anti-cancer drug) aspirin (anti-platelet agent) and dibucaine (a local anaesthetic) are reported to stimulate platelet apoptosis.
Platelets and Phytochemicals

Platelets are very sensitive to various external and internal stimuli and are a vulnerable target for the reactive oxygen species (ROS) generated in the endothelium during oxidative stress (Straface et al., 2010). It has been reported that oxidative stress is a major contributing factor for the pathophysiology of several diseases including CVDs (Vassalle et al., 2012). Therefore, it seems plausible to inter-relate oxidative stress, altered platelet functions and CVDs, going by the fact that accelerated platelet activation and aggregation eventually lead to the development of ischemia/reperfusion, atherothrombosis and MI. Consequently, many of the current treatment strategies for CVDs include antiplatelet drugs which target the various events of platelet activation and aggregation (Jneid and Bhatt, 2003). Moreover, the role of platelet-derived microparticles (MPs) in the progression of CVDs cannot be ignored. In the present medical scenario, the available antiplatelet therapies are accompanied by several harmful side effects including severe internal bleeding owing to thrombocytopenia (Donovan et al., 2010). Also, many of the conventional drugs also reported to target platelets (as discussed in the above section). Hence, people are turning towards plant-based therapeutics nowadays due to the fact that many of them are potent antioxidants. Plant-derived bioactive molecules are known worldwide for their restorative and health benefitting potentialities. They are the active principles of many folk and traditional medicinal formulations, which have been in use for several centuries. For example, the fruits and flowers of the *Crataegus oxycantha* & *monogyna* (common Hawthorn) have been traditionally exploited as cardiac tonic. The bark of the *Terminalia arjuna* tree and *Inula racemosa* (also known as Pushkarmoola) have been used in the traditional Ayurvedic system of medicine to treat cardiac problems. *Astragalus membranaceus* is a cardioactive and
immunostimulatory Chinese herb used in the Oriental system of medicine (Miller, 1998). However, the traditional medicines received their due attention from the scientific community only from the past half a century or so. Now they are being isolated in their pure form, characterized and successfully implicated in the treatment of a plethora of diseases. They also have become the choice of treatment lately for scores of oxidative stress-induced ailments including CVDs with a notion that they lack any side effects (Wallace, 2011). However, there have been few recent reports regarding the side effects of phytochemicals especially their proapoptotic effects on platelets (Lin et al., 2009b) (Table 1.2).

Table 1.2

<table>
<thead>
<tr>
<th>Name of the Compound</th>
<th>Structure</th>
<th>Effect on Platelets</th>
<th>Other Biological Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol (3,5,4'-trihydroxy-trans-stilbene)</td>
<td><img src="image" alt="Resveratrol Structure" /> Molecular weight- 228.24</td>
<td>Proapoptotic (5-25 μM) Inhibits platelet aggregation (0.15-0.25 μM)</td>
<td>Antioxidant, Cardioprotective, Chemopreventive, Anti-inflammatory, Antimutagenic and Antiviral.</td>
</tr>
<tr>
<td>Thymoquinone (2-Isopropyl-5-methylbenzo-1,4-quinone)</td>
<td><img src="image" alt="Thymoquinone Structure" /> Molecular weight- 164.2</td>
<td>Proapoptotic (≥5 μM)</td>
<td>Anti-oxidant, Anti-cancer, Cardioprotective, Hepatoprotective, Renoprotective, Analgesic and Anti-convulsant.</td>
</tr>
</tbody>
</table>
### Table 1.2: Differential action of phytochemicals on platelet functions

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Molecular Weight</th>
<th>Proapoptotic (µM)</th>
<th>Anticancer, Antioxidant and Hepatoprotective. Used to treat upper respiratory tract infections, diarrhea, rheumatoid arthritis, and laryngitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gossypol</strong></td>
<td>518.46</td>
<td>(25 µM)</td>
<td>Antimalarial and Chemopreventive.</td>
</tr>
<tr>
<td>(2,2′-bis-(Formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Andrographolide</strong></td>
<td>350.45</td>
<td>(25-100 µM)</td>
<td>Anti-apoptotic. Inhibits platelet aggregation (0-100 µM)</td>
</tr>
<tr>
<td>(3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylene-1-naphthalenyl]ethyldiene]dihydro-4-hydroxy-2(3H)-furanone)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cinnamamonnin-B1</strong></td>
<td>864.75</td>
<td>Anti-apoptotic.</td>
<td>Anti-apoptotic. Inhibits platelet aggregation (0-100 µM)</td>
</tr>
<tr>
<td>(Epicatechin-(4β→8,2β→O→7)-epicatechin-(4α→β)-epicatechin)</td>
<td></td>
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</tbody>
</table>

Molecular Weight: 518.46
Mechanism of Apoptosis

Programmed cell death is a distinct genetic and biochemical pathway essential to metazoans for successful embryonic development and maintenance of normal tissue homeostasis (Gluecksmann, 1951). Apoptosis, the most common pathway of programmed cell death, is a controlled mode of cellular self-destruction. The term ‘apoptosis’ which means "falling off or dropping off of leaves from trees." in Greek, was first introduced by Kerr, Wyllie and Currie in 1972. It is a physiologic strategy adopted by all cell types for development and morphogenesis, for controlling their population, differentiation, proliferation, regulation and function of the immune system, and for removal of aged, defective and harmful cells (Meier et al., 2000).

Morphological Modifications

Several characteristic morphological changes are associated with an apoptotic cell, of which cell shrinkage and pyknosis are the early signs (Kerr et al., 1972). Cell shrinkage is characterized by reduction in cell size, deformation, dense cytoplasm and tightly packed organelles, and the cell loses its contact with neighbouring cells. Pyknosis is a result of condensation and marginalization (at the nuclear membrane) of chromatin, and chromosomal DNA fragmentation. This is followed by plasma membrane blebbing, karyorrhexis (fragmentation of the nucleus) and separation of cell fragments into compact membrane-enclosed structures called apoptotic bodies that contain cytosol, condensed chromatin, and organelles, during a process called “budding”. These bodies are consequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes (Kurosaka et al., 2003). There is basically no inflammatory response involved in the processes of apoptosis and elimination of apoptotic cells, due to the fact that apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue.
Moreover, the cells are phagocytosed immediately by adjacent cells and these engulfing cells too do not release antiinflammatory cytokines (Savill and Fadok, 2000; Elmore, 2007).

**Biochemical Events**

The aforesaid morphological changes are a result of certain biochemical events, especially the activation of proteolytic enzymes which ultimately lead to DNA fragmentation as well as, the cleavage of a whole host of cytosolic structural proteins, which maintain the integrity and shape of the cell (Hengartner, 2000). Cell death receptors, Bcl2 and related proteins, and the caspases are the three major families of proteins involved in arbitrating apoptotic signalling, regulation and execution (Cory and Adams, 2002).

The tumour suppressor p53 takes centre stage as a marker gene in apoptosis, which controls transcription of various genes involved in apoptosis. It can activate its downstream target genes in the extrinsic and intrinsic pathways through transcription-dependent mechanisms in a specific sequence to induce apoptosis (Lim and Park, 2009).

**Death Receptors**

Death receptors are the receptors on the cell surface, which can transmit apoptotic signals instigated by precise ligands including Fas ligand, tumour necrosis factor alpha (TNFα) and TRAIL. They play an essential role in apoptosis as they activate the cascade of caspases within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore, very rapid (Ashkenazi and Dixit, 1998; Suliman et al., 2001).
The Bcl2 Proteins

The Bcl2 proteins are a family of proteins that are essential for the apoptotic response. Up to 30 members of this family have been identified of which some are pro-survival members and others proapoptotic members. In addition, there are a number of other pro-survival proteins such as, Bcl-XL, Bcl-w, A1, and Mcl-1. The proapoptotic group of Bcl2 members can be divided into two subgroups: the Bax-subfamily consists of Bax, Bak, and Bok; the BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, BIK, BNIP3, and Spike). The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic Bcl2 proteins. When there is an excess of proapoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of antiapoptotic proteins the cells will tend to be more resistant (Cory and Adams, 2002).

Caspases

Caspases are a group of cysteine-dependent aspartate-directed proteases that are activated in the initial stages of apoptosis and cleave proteins at aspartic acid residues. Different caspases have different specificities involving recognition of neighbouring amino acids. They are expressed as pro-caspases (inactive zymogen form) in most cells, and can be cleaved to form active enzymes following the induction of apoptosis. Once activated, they activate other pro-caspases, initiating a proteolytic cascade. Some pro-caspases are also able to aggregate and autoactivate. The resulting protease cascade magnifies the apoptotic signalling pathway, thus leading to rapid cell death. The activated caspases mostly work in an irreversible manner towards cell death. Caspases have been broadly classified into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5) (Creagh and Martin, 2001).
Expression of Cell Surface Markers

Expression of cell surface markers such as phosphatidylserine (PS) is a key biochemical feature resulting from the flipping of the inward-facing PS of the cell’s lipid bilayer to expression on the outer surface of plasma membrane. The cell membrane is characterized by asymmetric distribution of phospholipids in the lipid bilayer, with PS and phosphatidylethanolamine (PE)—the anionic phospholipids—found in the inner leaflet, and phosphatidylcholine (PC) on the outer leaflet. Generally, this asymmetry is dynamically sustained by translocase, an ATP-dependent enzyme. However, during apoptosis, either downregulation of translocase or activation of other enzymes such as scramblase leads to the PS translocation. Phagocytic cells recognize this “eat me” signal culminating in the phagocytic elimination of the dying cells (Israels and Israels, 1999).

Inducers of Apoptosis

Apoptosis can be triggered by a multitude of stimuli which may initiate extracellularly (extrinsic signals) or intracellularly (intrinsic signals). The Extracellular/extrinsic inducers include toxins, hormones, lack of a "survival" signal (which inhibits apoptosis) such as, a growth factors, nitric oxide (NO) or cytokines. These signals have to traverse the cell membrane to elicit a response. The intracellular/intrinsic apoptotic signalling may originate in response to oxidative stress, glucocorticoids, hyperthermia, ionizing radiation, starvation, viral infection, hypoxia, chemotherapeutic agents and increased intracellular calcium (Ca$^{2+}$) concentration (Elmore, 2007). These signals damage the cell membrane and prompt the release of intracellular apoptotic signals by a damaged cell. A number of cellular components, such as poly ADP ribose polymerase, may also help regulate apoptosis.
The pathways of apoptosis are extremely intricate and consist of an energy-dependent cascade of molecular events. The apoptotic pathways are broadly categorized into three main pathways: the extrinsic or death receptor pathway, the intrinsic or mitochondria-dependent pathway and the perforin/granzyme-dependent pathway.

**The Extrinsic Pathway**

It involves transmembrane receptor-mediated interactions that are set off by the activated death receptors belonging to the TNF receptor gene superfamily (such as TNFR1, FasR, and the TRAIL receptors DR3, DR4 and DR5) binding to the corresponding death ligands (TNF α, FasL, Apo3L, Apo2L and Apo2L). All the members of the TNF receptor family possess a common cytoplasmic domain of around 80 amino acids referred to as “death domain” (Ashkenazi and Dixit, 1998), which plays an important role in transmitting the death signal from the cell surface to the intracellular signalling pathways. Following the ligation, the cytoplasmic part of the death receptor containing the death domain (DD) recruits adapter molecules like FADD or TRADD, to form the death inducing signalling complex (DISC). The DISC in turn sequesters procaspase 8 leading to its autocatalytic activation and release of the active caspase 8, which subsequently activates downstream effector caspases leading to the cleavage of specific substrates culminating in death of the cell (Suliman et al., 2001).

**The Intrinsic Pathway**

The intrinsic pathway on the other hand, is initiated by the non-receptor-mediated intrinsic stimuli that elicit intracellular signals which directly target the apoptotic machinery within the cell, among which the mitochondria are critical targets. The apoptotic stimuli cause changes in the inner mitochondrial membrane
leading to the opening of the mitochondrial permeability transition pore (MPTP), loss of the mitochondrial transmembrane potential ($\Delta \Psi m$) and release of apoptogenic factors such as cytochrome c from the mitochondrial intermembrane space to the cytosol [Garrido et al., 2006]. In the presence of cytochrome c and dATP, the Apaf1 protein forms the apoptosome. The clustering of procaspase 9 to the apoptosome leads to the activation of the former by dimerization. The activated caspase 9 in turn activates the effector caspases. A family of proteins called Bcl2 plays a pivotal function in the control and regulation of mitochondrial events by affecting the mitochondrial membrane permeability. The Bcl2 family proteins can be either proapoptotic (e.g., Bcl10, Bax, Bak, Bid, Bad, Bim, Bik, Blk) or antiapoptotic (Bcl2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG) (Cory and Adams, 2002).

In addition to Fas signalling, cytotoxic T lymphocytes also exert their cytotoxic effects on tumour cells and virus-infected cells via a unique pathway involving the granzyme B/perforin system. Granzyme B (GrB, a serine protease) and perforin (a molecule capable of forming pores in intracellular membranes) are taken up by the target cell, wherein GrB directly triggers the activation of caspases of the target cell and thereby induces apoptosis (Elmore et al., 2001; Danial and Korsmeyer, 2004).

**Execution Pathway**

The extrinsic and intrinsic pathways converge at the execution phase, wherein the execution caspases 3, 6 and 7 activate cytoplasmic endonucleases and proteases, which degrade nuclear material and cytoskeletal proteins respectively. The activation of execution caspases results in the cleavage of several substrates such as poly (ADP-ribose) polymerase (PARP), cytokeratins and other cytoskeletal and plasma membrane proteins, and nuclear protein NuMA. These events are eventually
responsible for the morphological and biochemical changes in the apoptotic cell (Lim and Park et al., 2009). Caspase 3 cleaves gelsolin into smaller fragments, which in turn, cleave actin filaments. This leads to disruption of the cytoskeleton, intracellular transport, cell division and signal transduction (Danial and Korsmeyer, 2004).

**Programmed Cell Death in Platelets**

Despite lacking a nucleus, platelets have been shown to undergo the process of programmed cell death via apoptosis. Platelet apoptosis through the intrinsic mitochondria-mediated pathway has been clearly elucidated. Platelets exhibit all the conventional events of the pathway excluding the nuclear events (Fig. 1.1). Although apoptosis was discovered in nucleated cells 40 years ago, it took two more decades to discover the same in platelets, when for the first time it was reported that platelets exhibit apoptosis-like events when stimulated with ionomycin (Leytin, 2012). Later on, platelet apoptosis was shown to be induced by various chemical agonists such as, thrombin, collagen, ADP, arachidonic acid, epinephrine, calcium ionophore – A23187 and dibucaine.

Several studies have implicated oxidative stress to be a key culprit in the mediation of apoptosis in platelets and therefore, an exaggerated rate of platelet apoptosis can be observed in various oxidative stress-induced pathological conditions such as hyperlipidemia, Kawasaki disease, Bernard-Soulier syndrome, altered cardiac functions, type-2 diabetes and chronic uremia (Gregg and Goldschmidt-Clermont, 2003; Straface et al., 2010; Sener et al., 2005; Rand et al., 2010; Cohen et al., 2002; Bonomini et al., 2004). Further, apoptosis of mature platelets has been shown to be the major reason behind reduced platelet count in drug-induced as well as, immune thrombocytopenia. The most recent report regarding drug-induced thrombocytopenia
was regarding cisplatin, a widely used anti-tumour drug (Zhang et al., 2012). It was demonstrated that the thrombocytopenia was a result of apoptosis of platelets via the activation of ERK signalling pathway by the drug, thus shedding light on the involvement of possible molecular signalling pathways in platelet apoptosis. Li et al. (2000) showed that platelets express several caspases (caspase 1, 2, 3, 4, 6, 8 and 9), some at the mRNA level and some at the protein level; they also express mRNA for death receptors (DR3, DR4, DR5, TRAIL, p55 an RIP) and Bcl2 family protein (Bcl-X, Bfl1, Bad, Bak, Bax, and Mcl1). The study further provided strong evidence that platelets do possess an apoptotic mechanism for their clearance. The study also demonstrated that platelets develop lesions during storage, thus losing their viability, which was reportedly due to the release of bioactive substances such as interleukins (IL-1β, -3, -6 and -8), TNF α and TGF β from platelets and/ WBCs. It was further shown that stored platelets undergo apoptosis via caspase 3 activation and subsequent cleavage of its substrate gelsolin. In contrast, Wadhawan et al. (2004) demonstrated a caspase 3-independent apoptotic mechanism in aged platelets under in vitro conditions, which involves calpain-mediated proteolysis of specific substrates, which, along with their degradation products, and procaspase 3, move to platelet cytoskeleton in the absence of simultaneous rise in F-actin. This study suggested a significant role of cytoskeleton in the signalling processes associated with platelet senescence. Yet another study by Li et al. (2010) demonstrated the importance of platelet surface glycoprotein (GP) Ibα and vWF interaction in the induction of apoptosis in human platelets. This interaction was until then known to be involved in platelet adhesion and aggregation. It was also showed that this interaction occurs during pathological shear stress on platelets, and that the association of intracellular signalling protein 4-3-3ζ with the cytoplasmic domain of GPIbα is critical for apoptotic signalling.
Thereby, the study claims that identifying the agents which can block these interactions could be developed into an ideal class of compounds, which would be able to effectively prevent apoptosis during platelet storage or thrombocytopenia. Furthermore, pathogenesis of haemorrhage in fever or hyperthermia-related diseases such as dengue or heat stroke was demonstrated to be a consequence of increased rate of platelet apoptosis with a concomitant reduction in agonist-induced platelet aggregation. Hyperthermia induces the classical mitochondria-mediated apoptotic events along with the shedding of extracellular domain of GPIbα (Wang et al., 2010a). This observation suggests the GPIbα-vWF interaction-independent mechanism, which is mediated by calpain. Therefore, calpain activators such as dibucaine are very effective inducers of platelet apoptosis and at the same time cause platelet dysfunction. The study attributes the importance of calpain-mediated platelet apoptosis in thrombotic thrombocytopenic purpura (TTP) (Zhang et al., 2011). Nonetheless, apoptosis is not the only mechanism of platelet clearance as demonstrated by Brown et al. (2000). The study provided in vitro evidence that effete platelets can go through a caspase-independent constitutive cell death program involving scavenger receptor-mediated recognition and clearance by phagocytes.

Although, the mRNA level expression of the markers of extrinsic apoptotic pathway such as death ligand TRAIL, death receptors (TNFR1, DR3, DR4 and DR5) and adaptor proteins (TRADD and RIP) has been shown in stored platelet concentrates, and caspase 8 activation in resveratrol-treated platelets, there is no solid evidence yet to ascertain the possibility of extrinsic death receptor-mediated apoptosis in platelets. The mechanism has not been exactly elucidated (Lin et al., 2009b; Leytin et al., 2012; Li et al., 2000).
Fig. 1.1 Schematic illustration of the general mechanism of intrinsic/mitochondria-mediated and extrinsic/death ligand-mediated apoptotic pathways in platelets

Apart from apoptosis, there are reports of platelets undergoing death through the necrotic pathway too. Necrosis is elicited by cell insults such as excessive ROS production, calcium overload or ATP depletion. When potentially induced with collagen and thrombin, platelets exhibit the phenotypic characteristics of necrotic cells, including bioenergetic failure of the cell, elevated toxic levels of cytosolic Ca\(^{2+}\) and rapid loss of plasma membrane integrity. The distinguishing features of necrotic platelets include activation of non-apoptotic proteases like calpain and lysosomal
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cathepsin D, excessive generation of ROS, cell and organelle swelling and cytolysis. Cyclophilin D, a mitochondrial inner membrane protein plays a crucial role in the necrotic cell death by catalyzing the formation of MPTP. Necrotic platelets are not only procoagulant in nature but also elicit inflammatory responses (Jackson and Schoenwaelder, 2010).

Determination of Platelet Apoptosis

Endogenously Generated H$_2$O$_2$

Human platelets produce and release ROS, such as H$_2$O$_2$, under physiological stimulation and in pathological situations, such as diabetes and ischemia/reperfusion. H$_2$O$_2$, in turn, evokes cytochrome c release, caspase 3 and 9 activation and PS exposure. Homovanillic acid (HVA) is a specific H$_2$O$_2$-sensitive fluorescent probe successfully used for detecting H$_2$O$_2$ production in mitochondria. HVA reacts with H$_2$O$_2$ to form a fluorescent adduct. CMH2DCFDA is an ROS-sensitive fluorescent probe that can be used to monitor ROS production in living cells. CMH2DCFDA oxidation yields a fluorescent adduct dichlorofluorescein (DCF). Fluorescence is recorded using a fluorescence spectrophotometer (Barja, 2002; Lopez et al., 2007).

Mitochondrial Inner Membrane Potential Depolarization

The proton electrochemical potential gradient spanning the inner mitochondrial membrane features $\Delta \Psi m$ as a chief constituent. It is an essential factor for evaluation of mitochondrial function. The disintegration of $\Delta \Psi m$ one of the key facets of apoptosis and therefore, fluorescent potentiometric dyes redistributed according to transmembrane electrical potential can be regarded as appropriate tools for observing $\Delta \Psi m$ alterations. $3',3'$-dihexyloxycarbocyanine iodide (DiOC$_{6}(3)$) and $5',6',6'$-tetrachloro-1,1',3,3'-tetracyethylbenzimidazolylcarbocyanine iodide (JC-1) are the commonly used probes for flow cytometric determination of $\Delta \Psi m$ depolarization.
in platelets. These probes are cationic and cell-penetrating, and the ΔΨ\textsubscript{m} drives the manner in which they accumulate in the mitochondrial matrix. In apoptotic platelets marked by ΔΨ\textsubscript{m} depolarization, there is a characteristic decrease in the fluorescence of DiOC6(3)-stained platelets, indicating increase in the percentage of depolarized cells. While, JC-1 accumulates in mitochondria as red fluorescent aggregates at high membrane potentials, whereas, it exists as a green fluorescent monomeric form at low membrane potential. Agonist-induced changes in ΔΨ\textsubscript{m} are quantified as the integral of the decrease in JC-1 fluorescence ratio (Kroemer and Reed, 2000; Salvioli et al., 1997). Depolarization of ΔΨ\textsubscript{m} in platelets has been also detected by rosamine and rhodamine dyes, chloromethyl-X-rosamine (CMXRos) and tetramethylrhodamine methyl ester (TMRM).

**Bcl2 Protein Family Expression**

The integrity of outer mitochondrial membrane (OMM) is regulated by pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl2) members of Bcl2 family proteins. In apoptotic cells, the equilibrium between Bcl2 regulatory proteins budges in the pro-apoptotic direction. Proapoptotic Bcl2 proteins interact with the OMM, leading to increased membrane permeability and release of apoptogenic factors. Staining of fixed permeabilized platelets with anti-Bax and anti-Bak antibodies demonstrates the increased binding of these antibodies to proapoptotic Bax and Bak proteins following agonist treatment (Leytin, 2012).

**Cytochrome c Release from Mitochondria**

Cytochrome c release from mitochondrial intermembrane space is a key event in the intrinsic pathway of apoptosis mediated by OMM permeabilization. Cytochrome c release results in the formation of apoptosome in the cytosol. The apoptosome in turn recruits and activates caspase 9, an initiator caspase of the
intrinsic pathway (Kroemer et al., 2007). Cytochrome c release in platelets can be determined by subcellular fractionation followed by Western blotting.

**Caspase Activation, a Cytosolic Marker of Platelet Apoptosis**

The cysteine proteases, caspases, are mostly located in the cytosol although they are also found in other cellular compartments. Activation of caspases, which involves cleavage of inactive zymogen procaspase precursors, is one of the key manifestations of apoptosis. Activated caspases instigate, propagate and augment apoptotic signalling. Substrate cleavage is measured with a fluorescence spectrophotometer. The activities of caspases 3 and 9 are calculated from the cleavage of the respective specific fluorogenic substrates (AC-DEVD-AMC/FAMDEVD-FMK for caspase 3 and AC-LEHD-AFC for caspase 9) (Amor et al., 2006).

**Augmentation of Cytosolic Calcium**

Elevated cytosolic Ca\(^{2+}\) concentration is often seen in apoptotic platelets. In many cases intracellular Ca\(^{2+}\) concentration is essential for \(\Delta \Psi_m\) depolarization, caspase activation, and PS exposure. Intracellular Ca\(^{2+}\) is detected with the Ca\(^{2+}\)-sensitive fluorochromes such as, Fluo-3-acetoxymethyl ester (Fluo-3AM) or Fura-2-acetoxymethyl ester (Fura-2AM) by flow cytometric or spectrofluorimetric analyses. Further, the dependence of the apoptotic pathway on cytosolic Ca\(^{2+}\) is examined using a membrane-permeable Ca\(^{2+}\) chelator BAPTA-AM (Asai et al., 2008).

**Mitochondrial Permeability Transition Pore Formation**

A crucial event in mitochondria-mediated apoptosis is the formation of MPTP, which extends across the inner and outer mitochondrial membranes. MPTP is responsible for \(\Delta \Psi_m\) dissipation, permeabilization of the membranes and subsequent release of proapoptotic proteins from mitochondria to the cytosol. CyclosporinA (CsA) is a well-known inhibitor of MPTP formation. Pre-treatment of platelets with
CsA inhibits caspase 3 activation in agonist-treated platelets. Moreover, it also strongly inhibits other manifestations of apoptosis such as $\Delta \Psi_m$ depolarization, shedding of MPs, and platelet shrinkage (Leytin, 2009).

**Phosphatidylserine Exposure**

During apoptosis, PS is translocated from the inner to the outer leaflet of plasma membrane. Externalization of PS is another basic feature of apoptosis and can be detected by flow cytometry using PE- or FITC-conjugated annexin V probe (Rosado et al., 2006).

**Platelet Apoptotic Markers at the Whole-Cell Level**

Similar to nuclear cells, in anucleate platelets the apoptotic changes on whole-cell level include MP formation and cell shrinkage. Since both platelets and platelet-derived MPs contain on their surface the most abundant platelet surface receptor GPIIbIIIa, integrin αIIbβ3, breakdown of platelets to MPs has been shown as αIIbβ3-positive events in the MP gate, using FITC-conjugated antibody to αIIbβ3. For analyzing the effects of hydrodynamic rheological forces on platelet apoptosis, platelets are exposed to different shear stresses, ranging from physiologic arterial and arterioles levels (10–44 dynes/cm$^2$) to pathologic high levels (117–388 dynes/cm$^2$) as occurs in stenotic vessels. Shear stresses induce generation of platelet MPs. Maximal effect of shear forces on MP formation is observed at shear ‘dose’ of 204 dynes/cm$^2$ (Kroll et al., 1996).

**Platelet Shrinkage**

As a result of shear-induced shedding of MPs, ‘residual’ platelets have a reduced cell volume. High shear stress-induced platelet shrinkage can be examined using forward light scatter (FSC) flow cytometric histograms, since FSC light signal intensity has been shown to correlate with cell size. Treatment of platelets with
pathologic high shear stresses of 204 and 388 dynes/cm² significantly decreases the mean FSC characteristics of platelet population, indicating decrease of the mean platelet volume. Both MP formation and platelet shrinkage are also highly expressed after treatment of platelets with A23187 (Leytin et al., 2009).

**Morphological Changes in Apoptotic Platelets**

Transmission and scanning electron microscopy present detailed information on platelet structure, including morphology of apoptotic platelets. The morphological changes in apoptotic platelets comprise plasma membrane blebbing, filopod extension, cell shrinkage and MP shedding. In washed human platelets aged *in vitro* in the absence of plasma at an apoptosis-supporting temperature of 37 °C, granule fusion with the platelet surface membrane and cytoplasmic condensation are detected by transmission electron microscopy, similar to late stages of apoptosis reported in nucleated cells (Brown et al., 2000).

**Upstream and Downstream Platelet Apoptotic Markers**

Since platelets do not contain a nucleus and nuclear DNA, some classic apoptosis markers, such as, chromatin condensation and fragmentation, and nuclear break-up, are not applicable to revealing platelet apoptosis. Nonetheless, the intrinsic mitochondria-dependent pathway of apoptosis is well-characterized in platelets. Two major upstream markers of platelet apoptosis are $\Delta \Psi m$ depolarization, expression and mitochondrial translocation of proapoptotic (Bax and Bak) and antiapoptotic (Bcl2) members of Bcl2 protein family (Gyulkhandanyan et al., 2012). The latter are evaluated in two cellular compartments, the cytosol and at the outer mitochondrial membrane (Leytin et al., 2008). Other upstream markers are the expression, activation and mitochondrial translocation of Bid, Bcl-XL, Bcl-w, Mcl-1 proteins of Bcl2 family, of mitochondrial release of cytochrome c to the cytosol and caspase 9
activation (Leytin, 2012). MPTP formation is also an upstream marker of platelet apoptosis, and inhibition with CsA allows the study of the role of MPTP formation in the control of downstream apoptotic events such as, caspase 3 activation (Leytin et al., 2009). Downstream markers of platelet apoptosis are executioner caspase 3 in the cytosolic cell compartment, platelet shrinkage, and PS exposure on the platelet plasma membrane. Shedding of platelet MPs is also a downstream marker of platelet apoptosis at the whole-cell level, analyzed by FSC-anti-GPIIbIIIa dot (Leytin et al., 2009). Alternatively, scanning electron microscopy can be used for visualizing morphological manifestations of platelet apoptosis at the whole-cell level and plasma membrane, including platelet shrinkage, MP formation, blebbing of plasma membrane, and extrusion of filopods (Leytin et al., 2004). Transmission electron microscopy reveals intracellular manifestations of platelet apoptosis such as, cytoplasmic condensation, and fusion of platelet granules with the plasma membrane (Brown et al., 2000).

**Identification, Characterization and Quantification of Platelet Apoptosis in the Lab**

The Nomenclature Committee on Cell Death (NCCD) 2009, has formulated recommendations for determination of apoptosis in nucleated cells. The NCCD advocates researchers to quantify apoptosis with more than one assay, whenever possible, so that the probability of artefacts is nullified. Besides, it also suggests the use of more descriptive expressions such as, ‘percent cells with a $\Delta \Psi m$', ‘percent cleaved caspase 3 positive’ etc. instead of general terms like ‘percent apoptosis’ (Kroemer et al., 2009). Keeping up with the recommendations of NCCD, Gyulkhandanyan et al., (2012) have described the following methodology for the assessment of platelet apoptosis. Firstly, the characteristic upstream and downstream
markers of platelet apoptosis as described in the previous sections have to be simultaneously and quantitatively determined. This is a commonly used integrated approach followed by researchers studying platelet apoptosis. Secondly, the apoptotic platelets have to be identified by comparing with positive and negative controls. Healthy donor platelets treated with suitable diluent buffer might be used as negative control, while strong agonist (A23187 or thrombin)-treated platelets might be used as positive control. The advantage of these agonists as positive controls is their ability to induce wide range of apoptotic events such as, ΔΨm depolarization, MPTP formation, Bax and Bak expression, caspase 9 and 3 activation, gelsolin and moesin cleavage, PS exposure, platelet shrinkage and MP formation. Therefore, to confirm apoptosis in platelets, the authors propose the use of several apoptosis markers simultaneously, carrying out the experiments in parallel with positive and negative controls, and comparing the level of each specific apoptotic response with that in the positive and negative control platelets to establish the range and degree of apoptotic responses. Platelets are regarded as apoptotic if the extent of specific apoptotic response is statistically higher than that in respective negative control population. For each of tested apoptosis markers, the scaling of the effect is set by comparing with positive control considered 100%. Such a kind of measurement permits quantitative depiction of the level of various apoptotic responses in platelets under the influence of different chemical and physical agents, in scrutinizing platelet apoptosis in human diseases and animal models, and in estimating the effect of pro- and anti- apoptotic drugs.

**Mechanism of Platelet Aggregation**

Platelet aggregation is the clumping together of platelets in the blood and is a part of the sequence of events leading to the formation of a thrombus (clot). Platelet adhesion, activation, and aggregation play an essential role in thrombosis and
haemostasis. At the site of wound or injury, the platelets play an active role in plug formation to stop bleeding from the damaged blood vessels. Platelets undergo aggregation with the help of fibrinogen and vWF, which act as connecting agents. The GPIIb/IIIa is a Ca$^{2+}$-dependent receptor, which is the most abundant receptor involved in aggregation, plays a crucial role in platelet aggregation. The ligands for GPIIb/IIIa are fibrinogen, fibronectin, vitronectin, thrombospondin, and vWF. Other receptors which also participate in platelet aggregation include GPIb-V-IX complex (vWF) and GPVI (collagen) (Gralnick et al., 1985; De Marco et al., 1985; Weiss et al., 1989; Shattil et al., 1998).

The subendothelial collagen is exposed during injury to blood vessel. The exposed collagen plays a key role in the recruitment of circulating platelets and thus stimulates their activation in thrombus growth (Savage et al., 2001; Calvo et al., 2007). Initially, platelets are recruited to collagen indirectly via the by high affinity binding of platelet GPIb-V-IX to collagen bound vWF. This eventually aids in the direct binding of platelets to collagen via other major collagen receptors such as integrin α2β1 and GPVI resulting in platelet activation (Calvioli et al., 2007; Watson, 1999). In addition, the high shear conditions caused by stenosed arteries may also lead to the binding of GPIb-V-IX receptor to vWF in plasma, resulting in intracellular signalling cascade for platelet activation (Kroll et al., 1996). Further, platelet activation leads to conformational change of fibrinogen receptor and integrin αIIbβ3 (GpIIb–IIIa), resulting in the binding of fibrinogen through its Arg–Gly–Asp (RGD) sequence. The fibrinogen binding ultimately paves way for platelet aggregation and release of platelet agonists including ADP and thromboxane A2, and α2 receptor-activation, which further exacerbates aggregation and thrombus formation (Watson and Gibbins et al., 1998). Aggregation and adhesion of platelets thus results in the
formation of platelet plug. Myosin and actin filaments in platelets contract during aggregation, a process that further strengthens the plug. Platelet aggregation is inhibited by inflammatory products like PGI2 and PGD2 and is augmented by exogenous administration of anabolic steroids (Savage et al., 2001)

**Platelet-Derived Microparticle Generation**

MPs are minute membrane fractions consisting of phospholipid bilayer reorganized with PS exposed on the outer leaflet along with transmembrane proteins and receptors. MPs were first described in 1967 when Wolf reported platelet membrane fragments in human plasma, and he coined the name ‘platelet dust’ for these fragments. This ‘dust’ contained vesicles, smaller than 0.1 mm in diameter, which promoted coagulation. In past decades it has become obvious that several cell types can release MPs. They have different origins - platelets, monocytes, endothelial cells, red blood cells, granulocytes, cardiomyocytes, podocytes, vascular smooth muscle cells, cancer and progenitor cell populations (Morel et al., 2011; Antoniak et al. 2009; Burger et al, 2013; Rautou et al., 2011; Zahra et al. 2011; Pirro et al., 2008) (Table 1.3). Platelet MPs (PMPs) are derived from activated or apoptotic platelets and account for more than 80% of circulating MPs.

The bilayer encloses cytosolic components such as enzymes, transcription factors, and mRNA derived from their parent cells and express antigens such as, GPIbα-IX-V, P-selectin and integrins (Freyssinet, 2003; Ahn et al., 2004). Various stimuli that can evoke activation or apoptosis, such as, cytokines (IL-6), thrombin, collagen, noradrenalin and A23187, endotoxins, soluble CD40 ligand, erythropoietin, hypoxia and shear stress stimulate the cell membranes to release PMPs
(Nomura et al., 2000; Terrisse et al., 2010; Takano et al., 2004; Tschuor et al., 2008; Prasad et al., 2003; Morel et al., 2011).

Table 1.3

<table>
<thead>
<tr>
<th>Type of MP</th>
<th>Normal Concentration in Circulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-derived MPs</td>
<td>$237 \times 10^6/L$</td>
</tr>
<tr>
<td>Endothelial cell-derived MPs</td>
<td>$64 \times 10^6/L$</td>
</tr>
<tr>
<td>Monocyte-derived MPs</td>
<td>$23 \times 10^6/L$</td>
</tr>
<tr>
<td>RBC-derived MPs</td>
<td>$26 \times 10^6/L$</td>
</tr>
<tr>
<td>Neutrophil-derived MPs</td>
<td>$24 \times 10^6/L$</td>
</tr>
</tbody>
</table>

Table 1.3: Normal range microparticles in circulation derived from different types of cells

Two types of cellular processes that lead to the formation of MPs have been recognized: cell activation and apoptosis. Platelets are activated by agonists such as, thrombin, A23187, ADP + collagen, or shear stress (VanWijk et al., 2003). The activated cells begin to shed MPs within minutes of agonist treatment, in time- and Ca$^{2+}$- dependent manner. The primary indication of cell activation is the increase in cytosolic Ca$^{2+}$ concentration particularly at the site of vesiculation. Consequently, the elevated Ca$^{2+}$ levels activate kinases and calpain, and inhibit phosphatases (Coleman et al., 2001). MP formation involves the collapse of the membrane skeleton, thus disrupting the cell membrane and structural stability of the cell. One of the components of membrane skeleton, talin is degraded by calpain, and is considered as one of the pathways through which the augmented intracellular Ca$^{2+}$ concentration facilitates MP formation. PMP formation is also associated with GP IIb–IIIa complex. GP IIb–IIIa in its active form functions as a fibrinogen receptor on the platelet surface, binding amino acid sequence RGD of fibrinogen molecule and thus assists the release of MPs (VanWijk et al., 2003). In case of the apoptotic pathway, the
dynamic membrane blebbing of cells plays a major role in the shedding of MPs (Mallat and Tedgui, 2001). These blebs may vary from MPs formed by cell activation in size, lipid and protein composition and pathophysiological consequences. Actin-myosin cytoskeletal structures generate the necessary contractile force that impels the membrane blebbing. Apoptotic membrane blebbing also depends on activation of the Rho-associated kinase ROCK I, which amplifies actin-myosin force generation and links actin-myosin filaments to the plasma membrane. During apoptosis, caspases cleave the zymogen form of ROCK I leading to its activation. Thus, MP formation during apoptosis results from ROCK I activity and the resulting disruption of the membrane skeleton structure (VanWijk et al., 2003). The shed PMPs express GpIb (CD42b), platelet endothelium adhesion molecule (PECAM-1; CD31), integrin aIIb\beta3 (GpIIb-IIIa), P-selectin (CD62P), CD 63, CD41a, and CD 61. CD 42 is not expressed on PMPs; however, it is always present on intact platelets, and therefore serves as a useful marker for platelet contamination in flow studies (Piccin et al., 2007).

PMPs are not just by-products of cellular processes, but are dynamically involved in the normal physiology and pathophysiology (Fig. 1.2). PMPs play a critical role in coagulation and haemostasis due to their thrombogenic nature. They can instigate coagulation due to the presence of coagulation factor-binding sites on their phospholipid surfaces (Owens and Mackman, 2011). They reportedly regulate inflammation, influence vascular functions and apoptosis and may also contribute to cell proliferation and differentiation (Mause and Weber, 2010). Nevertheless, an increase in the number of circulating PMPs results in a large number of diseases. Elevation in procoagulant PMP levels result in thrombotic disorders such as, acute coronary syndrome, MI and stroke (Preston et al., 2003). Raised PMP levels is also a characteristic feature of disease conditions including tumour progression,
hypertension, obstructive sleep apnea, sepsis, diabetes mellitus and venous thrombosis (Italiano et al., 2010; Pisetsky, 2011; Knijff-Dutmer et al., 2002; Boilard et al., 2010). Moreover, PMPs exert a pro-inflammatory effect which is evident with the rise in serum IL-6 levels, a phenomenon observed in obesity and metabolic syndrome (Nomura et al., 2011). They also elicit apoptosis in endothelial and vascular smooth muscle cells leading to vasoconstriction and endothelial dysfunction, development of thrombosis and atherosclerosis. Thus, increased levels of plasma PMPs can be regarded as a reliable marker for CVDs (Li et al., 2009).

**Microparticles and Coagulation**

Activated platelets and circulating PMPs offer active surface area with procoagulant aminophospholipids, which aids the assembly of the specific enzymes of the coagulation cascade. Activated MPs display negatively charged PS at their surface, which interact with blood coagulation factors in circulation and permit the local concentrations essential to reach optimal thrombin generation resulting in efficient haemostasis (Lentz, 2003). PS increases the procoagulant activity of tissue factor (TF). TF and PS are both exposed on the surface of MP and are thought to be the main triggers of coagulation cascade. TF plays a crucial role in the onset of blood coagulation because *in vivo* coagulation is set off when TF binds factor VIIa and catalyzes its activation (Morel et al., 2006, Wiiger and Prydz, 2007). TF circulates in plasma, mostly on monocyte/macrophage-derived MPs that can interact with activated platelets through a mechanism involving P-selectin GP ligand-1 (PSGL-1) on MPs and P-selectin on platelets (Falati et al., 2003). MPs can also contribute to the development of platelet- and fibrin-rich thrombi at sites of vascular injury, through the recruitment of cells and the accumulation of TF (Hrachovinová et al., 2003). Several studies suggest that MP-mediated coagulation might be clinically significant,
for example, association between the number of circulating MPs and the risk of thromboembolic complications has repeatedly been demonstrated (Puddu et al., 2010).

**Microparticles in Haemostasis and Thrombosis**

PMPs seem to take part in the process of haemostasis. Patients with bleeding disorders like Castaman's defect and Scott's syndrome display a deficiency in the ability to generate PMPs along with bleeding tendency (Owens and Mackman, 2011). The influence of PMPs in vascular cell activation has been demonstrated. PMPs interact with phospholipase A₂ leading to the release of arachidonic acid, which is in turn metabolized to TxA₂ in platelets (Barry et al., 1997). This paves way for the transactivation of platelets and endothelial cells and supports the interaction between monocytes and endothelial cells. It has been shown that most of the circulatory MPs in healthy individuals are CD41+ and that these MPs endorse small quantities of thrombin generation, despite the presence of inhibitory antibodies to TF and FVII. It is assumed that this low level generation of thrombin in healthy individuals may result in protein C activation and consequently possess anticoagulant effect (Berckmans et al, 2001). Since PMPs are highly procoagulant, it has been proposed that they may facilitate the pathogenesis of arterial thrombotic disease (Italiano et al., 2010).

**Crosstalk between Microparticles and Target Cells**

PMPs upon binding to cells can alter their functional properties. For instance, PMPs bind haematopoietic progenitors and stimulate their engraftment, or their binding to neutrophils evokes an increase in both CD11b expression and phagocytic activity in a dose-dependent mode. The reports puts forward that besides providing platelet factors, PMPs might also have other probable functions: in particular, as activators and mediators of neutrophil-induced ischemic injury, thrombosis, and
inflammation. It has also been demonstrated that mobilized peripheral-blood (mPB) CD341 cells express a considerably elevated level of GP IIb/IIIa (CD41 antigen). Therefore, it has been hypothesized that the presence of the CD41 antigen on mPB CD341 cells is due to the binding of PMPs on their surfaces (Janowska-Wieczorek et al., 2001). PMPs also bind to the subendothelial matrix acting as a substrate for further binding of platelets. Such an interaction may have a significant function in the platelet adhesion to the endothelial injury site (Merten et al., 1999). Moreover, PMPs provide a catalytic surface and thus hasten coagulation. They can bind to neutrophils to arbitrate interactions among leukocytes, and elevated levels of PMPs may intensify leukocyte-mediated tissue injury in thrombotic and inflammatory disorders. Thus, PMPs can bind to neutrophils, resulting in their aggregation and phagocytic properties (Li et al, 2009). PMPs are also thought to transfer biological information between cells by functioning as vectors of signal molecules. Although PMPs act on haematopoietic and blood cells, majority of the information exchange by PMPs occurs at the endothelium and contributes to the (patho) physiologic effects of MPs. Thus, PMPs can influence vasodilation, and the antiadhesive and antithrombotic properties of the vascular wall. Furthermore, they may regulate vascular permeability and the proliferation of smooth muscle cells. They mediate adhesion of monocytes to endothelial cells (ECs) by inducing adhesion-molecule exposure, provoking the production, endurance, adhesion, and chemotaxis of haematopoietic cells, and increasing the engraftment of hematopoietic stem cells (Janowska-Wieczorek et al., 2001). It has been suggested that cytoskeleton may function as a link to signalling pathways so that the GPIIb/IIIa complex can perform its role (Li et al, 2009). PMPs are apparently used as a pathway by cells to exchange information in addition to the transduction linked to the activation of receptors or transporters. PMPs isolated from
cardiac arrest patients, severely affected endothelial function in rat aortas by influencing the pathway of endothelial NO transduction (Boulanger et al., 2001). In contradiction, it has been observed that PMPs affect ECs by protecting them from apoptosis and by inducing the proliferation and formation of tubule-like structures. On the other hand, PMPs can cause damage to ECs via eliciting inflammatory response and diminishing endothelium-dependent vessel dilation (VanWijk et al., 2003).

The PMP-induced proliferation, chemotaxis, and tube formation of ECs are reported to be mediated via the pertussis toxin-sensitive G protein, ERK, and the PI3-kinase pathway (Kim et al., 2004). Pertussis toxin, which is a G-protein inhibitor, obstructs the effects of PMPs on GPIIb/IIIa. As a result, the G proteins that control the activity of adrenergic receptors may be involved in coupling agonist interaction to the receptor function of GPIIb/IIIa. The PI3-kinase plays a critical role in mediating EC survival, proliferation, cytoskeletal reorganization, and cellular motility, which are all essential for vessel growth. The stimulation of PI3-kinase is mediated by angiogenesis-related cytokines such as, vascular endothelial growth factor and basic fibroblast growth factor (Li et al, 2009). PMPs bind to the endothelium, leukocytes, and the submatrix of a vascular wall, and are possibly ingested by leukocytic and smooth-cell phagocytes in order to acquire the GPIIb/IIIa. It has also been demonstrated the preventive effect of GPIIb/IIIa inhibitor drugs such as abciximab, eptifibatide, and tirofiban on NF-κB activation through acquired GPIIb/IIIa receptors. It has been suggested that these drugs may have novel implications in anti-inflammatory treatment protocols (Salanova et al., 2007). Thus, the beneficial effect of GPIIb/IIIa antagonists as a consequence of their effect on PMPs through platelet-derived GPIIb/IIIa receptors has been highlighted in the study. The presence and subsequent effect of PMPs and their receptors in human cells have scope for further
research. If GPIIb/IIIa receptor antagonists do produce a direct and discernible effect on ECs, smooth-muscle cells, and leukocytes through PMP pathway, they have a great therapeutic potential for treatment of acute coronary syndrome (Li et al, 2009).

**Fig. 1.2**

![Schematic representation showing the release of microparticles from platelets and their pathophysiological effects](image)

**Role of Microparticles in Cardiovascular Diseases and Diabetes Mellitus**

Acute coronary syndrome is initiated by the erosion and rupture of an atherosclerotic plaque. When the plaque ruptures the subendothelial protein matrix is instantaneously disturbed, allowing platelet-adhesion molecules such as vWF factor and collagen to interact with circulating platelets. Platelets adhere to collagen and vWF factor at the site of injury via specific GP receptors. This leads to platelet activation, shape change and release of storage granules containing platelet agonists such as, ADP and TxA2, and a shape change of platelet fibrinogen receptor GPIIb/IIIa. Besides, already-activated platelets and the released PMPs provide a new prothrombotic interface for fibrin, blood cells, and a growing thrombus. As a consequence, there is thrombus growth and narrowing of the vessel. The elevated
shear stress due to vascular narrowing, sustain this process by supporting further platelet activation and PMP release. Ultimately, an occlusive thrombus forms and patients go through grievous events (Li et al., 2009). Platelet activation induced by agonists such as collagen or thrombin, leads to several events such as, shape change, secretion, aggregation, specific platelet protein phosphorylation, PS exposure on the extracellular face of the platelet membrane, and release of PMPs that are rich in procoagulant activity (Knijff-Dutmer et al., 2002; Li et al., 2009). The PMPs contain receptors (GPIIb/IIIa, Ib, Ia, and IIa) for platelet–subendothelium attachment and P-selectin, which is associated with platelet–leukocyte interactions and inflammatory response (Li et al., 2009). Several studies have evidenced that circulating MPs might serve as potential prognostic marker for atherosclerotic vascular disease. PMPs that express P-selectin and CD63 on their surface are a sign of platelet activation in peripheral arterial disease and MI. A recent study demonstrated enhanced levels of PMPs in survivors of MI. They have shown that a there is a considerable connection of large PMPs with plasma thrombin antithrombin complexes and soluble CD40 ligand (sCD40L) in patients with MI. Further, it has also been showed that interior and exterior diameters of carotid artery correlate inversely with MPs derived from platelets, endothelial cells, and leukocytes (Italiano et al., 2010). It has been observed that diabetic patients develop and platelet hyperaggregability along with increased levels of PMPs. The PMPs play a significant role in further exacerbating the clotting process, leading to hypercoagulability. Besides, PMPs promote the expression of adhesion molecules by monocytes and endothelial cells and therefore, it appears that they might even participate in the development or progression of atherosclerosis in diabetics (Nomura et al., 2011).
Microparticles and Arthritis

Boilard et al. (2010) explored the involvement of platelets in the autoimmune disease rheumatoid arthritis (RA) and demonstrated the presence of PMPs in the synovial fluid from patients with RA. Further, the authors showed the proinflammatory nature of PMPs via the elicitation of cytokine responses (IL-1) from synovial fibroblasts (Boilard et al., 2010). RA is an incapacitating systemic inflammatory disease which affects 1% population of the world. It mainly afflicts the synovial joints by evoking inflammation that is characterized by immune cell recruitment and blood vessel dilation. It has been reported that platelets accumulate in the joints of RA patients and that elevated numbers of PMPs are found in the synovial fluid of patients. It has been proved that PMPs indeed augment RA. Pharmacologic and genetic studies have revealed that collagen receptor GP-VI and its associated gamma chain of the Fc receptor play a key role in eliciting PMP production in arthritis pathophysiology. Further, fibroblast-like cells lining the joint cavity trigger the shedding of PMP. Consequently, the released PMPs interact with and activate fibroblast-like synoviocytes (FLS), which are important effector cells that mediate both immune activation and joint destruction. The stimulated the FLS bring about inflammatory cytokine responses via IL-1. Thereby, the IL-1 packaged into the PMPs seems to play a principal role in amplifying inflammation. Furthermore, elevated level of PMPs in RA patients hints at their possible role in the progression of CVDs. RA patients are more susceptible to cardiovascular mortality, suggesting its association with the degree of inflammation. These reports put forward that PMPs might be the culprits involved in the inflammatory and thromboembolic processes in RA patients (Knijff-Dutmer et al., 2002).
Microparticles and Cancer

The involvement of high PMP levels in cancer metastasis cannot be ignored because it is linked to belligerent tumours and low clinical outcome (Helley et al., 2009). Several studies have demonstrated that PMPs have the capacity to induce angiogenesis and are involved in the metastasis of cancer (Kim et al., 2004). They promote the proliferation, survival, migration, and tube formation of human umbilical vein endothelial cells. Thus, PMPs are thought to promote the formation of new blood vessels during tumor growth. This effect is arbitrated by the combined action of VEGF, FGF-2, and a lipid factor (Brill et al., 2005). In addition, intra-myocardial injection of PMPs raised the amount of new capillaries formed in the heart muscle in the background of ischemia. Though the contribution of PMPs in cancer development is not clearly known; the fact that some tumour cells are capable of activating platelets and inducing platelet aggregation is well-documented. Thus, it is thought that the PMP concentration at the sites of tumours may be unusually high. In gastric cancer, PMP levels are better forecasters of metastasis than plasma levels of IL-6, RANTES, and VEGF (Kim et al., 2003). PMPs can also trigger the secretion of MMP-2 in prostate cancer cells, promoting tumour invasiveness by their easy passage through collagen, a major component of the extracellular matrix (Dashevsky et al., 2009). Thereby, the generation of PMPs over an extended period can be lethal, and it is imperative to assess their count not only during disease conditions but also during chemotherapy.

Microparticles and Infectious Diseases

PMPs might serve as vectors that convey receptors to recipient cells, leaving them disposed to infection. Thus, PMPs have a possible role to play in infectious diseases. For instance, it has been demonstrated that PMPs and megakaryocyte-
derived MPs transfer the chemokine coreceptor CXCR4, which is critical for the entry of X4 HIV strains, to the surface of CXCR4 negative cells. Thus, the recipient cells are at risk of contracting the infection. Recently, yet another study demonstrated that high levels of in HIV-infected patients than controls. Thus, increased levels of MPs in patients may be a mechanism that leads to the spreading of infection (Italiano et al., 2010).

**Role of Microparticles in Thromboembolism**

Thromboembolism (TE) causes serious life-threatening complications in cancer patients. It has been observed that about 10% of all cancer patients tend to develop arterial or venous thromboembolic events during their course of disease. Chemotherapy is considered as a significant contributor to the thromboembolic events (Lechner and Weltermann, 2008). Recent studies have proposed the probable link between the prothrombotic state in cancer patients and circulating TF-exposing MPs (Tesselaar et al., 2007). It has also been reported that cancer patients undergoing chemotherapy have a three times higher risk than those without chemotherapy of developing venous TE (Sallah et al., 2002). It has been observed that TE is a major reason for death in cancer patients under outpatient chemotherapy (Khorana et al., 2007). It has been hypothesized that chemotherapy elicits an increased MPs shedding from activated platelets, apoptotic endothelial cells, or tumour cells. These MPs will then enter the circulation rapidly and bind to injured endothelial cells. Thus, the elevated levels of MPs on the surface of endothelium, particularly TF-bearing MPs, might initiate the process coagulation activation resulting in thrombus formation (Lechner and Weltermann, 2008). It has been observed that there is higher possibility of thrombosis between the first and subsequent cycles of chemotherapy, which suggests that chemotherapy-induced TE might be due to the cytotoxic effects on
normal host cells (Khorana et al., 2008). However, further investigation is required to appraise the effect of endothelial damage, circulating MPs, and TF on chemotherapy-associated hypercoagulability (Lechner and Weltermann, 2008).

Phytochemicals Exhibiting Pro-Apoptotic Effects on Platelets

Owing to the severe adverse reactions of chemotherapeutics, a lot of research is going on in the field of plant-based therapeutics. The bioactive molecules extracted from plants are known all over the world for their healing and health benefitting efficacies. The folk, traditional and oriental medicinal formulations which have been in use for several centuries contain phytochemicals as the active principles. The scientific community took note of these plant-based medicines from the past half a century or so and have been the focus of intensive research. Most of the phytochemicals have innate antioxidant potency and their apparent non-toxic nature have made them an alternative mode of treatment lately for a number of oxidative stress-associated diseases. However, recently there have been few reports regarding the adverse effects of phytochemicals especially their proapoptotic effects on platelets (Lin et al 2009b; Thushara et al., 2013a). Resveratrol, thymoquinone, gossypol and andrographolide are the hitherto reported phytochemicals that stimulate platelet apoptosis.

Resveratrol

Resveratrol (3,5,4’-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural polyphenol predominantly present in high concentration in the grape skin and therefore a considerable amount of it is present in wines, in particular red wines. Plant formulations containing resveratrol, have been used in traditional Indian and Chinese medicines for centuries, and recommended for CVDs, inflammation, diabetes, microbial infections and disorders of the gastrointestinal tract. It was first isolated
from the roots of the white hellebore (*Veratrum grandiflorum*) and the Japanese knotweed (*Polygonum cuspidatum*). Later on there were numerous reports on the beneficial effects of resveratrol, including its inhibitory activity on arachidonate metabolism through the interactions with 5-lipoxygenase (LOX) and cyclooxygenase (COX) pathways in WBCs. But it was not until 1992 that resveratrol came into the limelight when the cardioprotective effects of red wine attracted a lot of attention which was attributed to the presence of resveratrol (Timmers et al., 2012). Resveratrol has been met with universal success with several studies identifying its multiple direct targets including COX, peroxisome proliferator-activated receptor (PPAR) and endothelial nitric oxide synthase (eNOS). The proteins that are thought to be involved in resveratrol-mediated effects on a plethora of disease pathophysiologys include AMP-activated protein kinase (AMPK), silent mating type information regulation-2 homolog-1 (SIRT1), N-ribosyldihydronicotinamide: quinone oxidoreductase (NQO2), NFE2-related factor-2 (Nrf2) and NF-κB (Nakata et al., 2012). As such, the wide array of affected proteins heightens the likelihood of off-target harmful effects and evokes the need for evaluation of toxicity of resveratrol in the cases of either prolonged usage or over-dosage. Since its cardioprotective activity has been validated by several studies, it is quite obvious that one of its targets has to be platelets. It has been shown that trans-resveratrol inhibits the activation of blood platelets under *in vitro* conditions, besides exhibiting antioxidative effects on the generation of ROS and lipid peroxidation in resting and stimulated platelets.

Under *in vitro* conditions, resveratrol was found to inhibit ADP-, collagen- and thrombin-induced aggregation of platelets from healthy human subjects. In addition, it was also demonstrated that oral administration of resveratrol effectively brought down hyperlipidemia-mediated platelet aggregation in hypercholesterolemic
rabbits (Wang et al., 2009a). Further, Shen et al. (2007) demonstrated that resveratrol at lower concentration (0.15-0.25 µM) completely inhibited collagen-induced platelet activation. This work was further advanced by Lin et al. (2009b), wherein they sought to investigate the effects of resveratrol at higher concentrations on platelets. In the study it was found that resveratrol concentrations above 5 µM triggered platelet apoptosis. The underlying mechanisms that influenced such an effect were further characterized and it was found that resveratrol dose-dependently dissipated ΔΨm with a maximum of 70% efficacy at 25 µM. Thus, it was clear that resveratrol induced apoptosis in platelets via the intrinsic pathway. Furthermore, resveratrol (5-25 µM) mediated all the classical events associated with the pathway, including translocation of proapoptotic Bcl2 family protein Bax from the cytosol to mitochondria, release of cytochrome c from the mitochondria into the cytosol, activation of caspases 9 and 3, cleavage of gelsolin (substrate for caspase 3) and actin (substrate for cleaved gelsolin) and expression of surface marker PS. However, it did not stimulate the expression of p-selectin, thus ruling out the possibility of platelet activation. There are few reports regarding the biphasic effect of antioxidants wherein they behave as pro-oxidants at higher concentrations. Resveratrol, however did not exhibit any pro-oxidant activity even at the high concentration of 25 µM, which was validated by fact that it did not cause the generation of hydroxyl radicals, indicating that it changed ΔΨm by mechanisms other than ROS generation. Further, the study also explored the involvement of extrinsic pathway in resveratrol-mediated death of platelets. Resveratrol induced the activation of caspase 8 in a time- and dose-dependent manner. It also triggered the cleavage of Bid (the downstream signal of active caspase 8) into tBid (truncated Bid). In the presence of caspase 8 inhibitor (z-IETD-fmk), it failed to induce activation of caspases 9 and 3, which suggests that they are
downstream signals of activated caspase 8. On the other hand, resveratrol-induced depolarization of ΔΨm was reversed only to some extent in the presence of z-IETD-fmk. It was also able to directly trigger both ΔΨm depolarization and cytochrome c release, which implies that resveratrol can directly activate apoptosis through the mitochondria-mediated intrinsic apoptotic pathway in platelets. The effect of a higher concentration of resveratrol on platelet apoptosis can be summarized that it probably involves two pathways: the mitochondrial and the extrinsic apoptotic pathways. The most likely mechanism is that resveratrol stimulates caspase 8 activation and cleavage of Bid into tBid, resulting in Bax translocation into mitochondria and release of cytochrome c from mitochondria. On the other hand, resveratrol may even directly target mitochondria by triggering ΔΨm depolarization eventually leading to the release of cytochrome c. The intrinsic and extrinsic pathways converge to a common execution phase, which is set off via the initiation of proteolytic cascade by activated caspases 9 and 3, followed by cytoskeleton remodelling, cell shrinkage, and expression of apoptotic markers (PS) culminating in the cell death (Fig. 1.3). The study signifies two aspects: because of its proapoptotic effects resveratrol can be exploited as a drug to dissolve pathological clotting by exacerbating platelet destruction in the case of diseases associated with thrombotic conditions; conversely it is extremely important to check the dosage of resveratrol and monitor the platelet count in a patient taking it as a therapeutic drug.

In a recent study, Mohan et al. (2006) have reported the events occurring in the upstream of mitochondria in resveratrol-induced apoptosis in human colon adenocarcinoma cells. The study highlights the activation of caspase 2 upstream of mitochondria, which is independent of antioxidant and NF-κB inhibitory properties of resveratrol. The activated caspase initiates the downstream mitochondrial events
associated with apoptosis. Caspase 8 activation was shown to be independent of death receptor pathway, which together with caspase 2 mediates the mitochondrial translocation of Bid. The important role played by Bax and Bak in resveratrol mediated apoptosis and the possibility of Bax/Bak-independent cell death mediated by caspase 8 or 2 was also demonstrated the study. Extending this work in resveratrol-mediated platelet apoptosis would shed more light on the caspase 8 mediated apoptotic events.

**Thymoquinone**

Thymoquinone (2-Isopropyl-5-methylbenzo-1,4-quinone), a homologue of ubiquinone, is a phytochemical isolated from the plant *Nigella sativa* (black cumin) and it is one of the most abundant and active ingredients of its seeds (cumin seeds). It is well known for its antioxidant, cardioprotective, hepatoprotective, renoprotective, analgesic and anticonvulsant properties (Badary et al., 2003; Gali-Muhtasib et al., 2004; Roepke et al., 2007). Besides, the seeds have been used in many traditional systems of medicines to treat bronchial asthma, headache, infections, obesity, back pain, hypertension and illnesses related to the gastrointestinal tract. Badary et al. (2003) have claimed thymoquinone (TQ) to be a powerful superoxide anion scavenger and thus it can be articulated that it is because this property that TQ is so effective in combating many of the oxidative stress-induced diseases including cancer. Incidentally, it has been shown to be a promising growth-inhibitory and proapoptotic drug against multi-drug-resistant neoplastic cells, reportedly with negligible toxicity to normal cells. It executes its proapoptotic effects on cancer cells via its inhibitory effects on cell cycle. It has also been shown that TQ induces apoptosis through p53-dependent pathways as well as through the p53-independent activation of caspases 8, 9 and 3 (Gali-Muhtasib et al., 2004; Roepke et al., 2007).
Most of the studies pertaining to the anti-cancer effects of TQ on a wide variety of cancer cell lines claimed it to be relatively non-toxic on the respective normal cells. However, none reported its effects on platelets despite the known fact that they are easily susceptible to therapeutic agents. Towhid et al. (2011) for the first time investigated the toxicity of TQ on platelets. It was proved that TQ at a concentration ≥5 µM indeed induced various events of apoptosis in platelets such as PS scrambling which in turn was due to the activation of caspase 3. TQ also augmented cytosolic Ca\(^{2+}\) activity and further it was shown that the absence of Ca\(^{2+}\) impaired TQ-induced caspase activity to some extent. In addition, it was also demonstrated that TQ at a concentration of ≥ 10 µM induced ceramide formation and depolarization of ΔΨ\(m\). These events resembled the classical mitochondrial pathway of apoptosis. Towhid and coworkers further probed the involvement of intracellular signalling pathways in the TQ-mediated platelet apoptosis. It was observed that treatment of TQ-pretreated platelets with PI3K inhibitor wortmannin resulted in the impairment of PS externalization, cytosolic Ca\(^{2+}\) and caspase 3 activities and also inhibited the depolarization of ΔΨ\(m\). The results confirmed the significant involvement of PI3K in TQ-triggered platelet apoptosis, which is contradictory to the fact that the PI3K/Akt pathway exhibits antiapoptotic effect in nucleated cells (Duronio, 2008). However, in platelets PI3K is associated with integrin signalling-mediated platelet activation (Niu et al., 2012). It was further shown that PI3K was activated by G-protein coupled receptors (GPCR) by means of GPCR blocker pertussis toxin (PTX), which abrogated PS exposure, and activation of caspase 3 in TQ-pre-treated platelets. Thus, it can be summed up that TQ triggers the apoptotic cell death program in platelets via the GPCR-activated PI3K pathway involving a concurrent increase in intracellular Ca\(^{2+}\), loss of mitochondrial membrane integrity, ceramide formation, caspase 3 activation,
cell shrinkage and PS exposure (Fig. 1.3). Moreover, the TQ concentrations required to induce platelet apoptosis are comparable to those used to induce anti-tumour effects on cancer cell lines. Nevertheless, it remains to be proven whether the \textit{in vivo} TQ dosage 30-60 μmol/kg/day can give rise to an optimum plasma concentration that can really trigger platelet apoptosis. This aspect needs further research and highlights the importance of secondary complications of therapeutic drugs arising from impaired platelet functions and reduction of platelet count.

\textbf{Fig. 1.3}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{The proposed mechanisms for resveratrol and thymoquinone induced platelet apoptosis. Resveratrol activates both caspase 9 (intrinsic pathway) by directly affecting $\Delta \Psi m$ and caspase 8 (extrinsic pathway). Both the pathways ultimately result in activation of effector caspase 3 leading to cell death. Thymoquinone induces intrinsic apoptosis via intrinsic mechanism through GPCR-activated PI3K pathway.}
\end{figure}
Gossypol

Gossypol \([\text{C}(30)\text{H}(30)\text{O}(8)]\) is a polyphenolic compound isolated from cottonseeds (genus *Gossypium*). It is a yellow pigment and is chemically reactive due to the presence of six phenolic hydroxyl groups and two aldehydic groups. It has the capacity to permeate cells and inhibit several dehydrogenase enzymes. Moreover, it can form Schiff base, and undergo ozonolysis, oxidation, and methylation reactions resulting in the formation of gossypol derivatives. Gossypol as well as its derivatives has been widely investigated for a multitude of biological activities such as, antifertility, antivirus, anticancer, antioxidant, antitrypanosomal, antimicrobial, and antimalarial activities. *In vitro* studies have shown that gossypol is lethal to RBCs as it induces cell death and thus leads to hemolytic anemia (Wang et al., 2009b). Recently, Dale and Friese (2006) have demonstrated the platelet proapoptotic effects of gossypol. It is a BH3 mimetic i.e., a molecule that can instigate apoptosis by causing Bax release. It is being widely studied for its anticancer efficacies. Further, gossypol at a concentration of 25 µM also stimulates the release of PMPs. This report further emphasizes the need to exercise caution regarding the side effects of drugs on platelets.

Andrographolide

Andrographolide is a labdane diterpenoid and is the most active and essential constituent of the medicinal plant *Andrographis paniculata* (Coon and Ernst, 2004). It was traditionally used as an herbal medicine to prevent and treat upper respiratory tract infections, diarrhoea, RA, and laryngitis in Asia and Scandinavia (Coon and Ernst, 2004; Poolsup et al., 2004). Of late, studies have demonstrated that it exhibits anticancer, antioxidant and hepatoprotective properties (Negi et al., 2008;
Bao et al., 2009; Lin et al., 2009c). It also reportedly suppressed v-Src transformation and sensitized cancer cells to TRAIL-induced apoptosis and arrested cell cycle. It was previously shown that andrographolide might be involved in increasing cyclic GMP/PKG, followed by inhibition of the p38 MAPK/HO-NF-kB-ERK2 cascade in collagen-stimulated platelets (Lien et al., 2013). Though, andrographolide inhibits platelet activation, its influence on platelet apoptosis was not known and the same was investigated be Lien et al. (2013). It was found that andrographolide (100 µM) markedly stimulated platelet apoptosis. Upon examining the cellular events associated with andrographolide-induced platelet apoptosis, it was found that, it triggered changes in ΔΨm, activated caspases 3 and 8, and stimulated the cleavage of Bid, the downstream of caspase 8, into tBid. The significant finding of this study was that besides its antiplatelet activity, andrographolide might limit platelet lifespan by initiating the caspase 8-dependent extrinsic apoptotic pathway.

**Phytochemicals that Exert Protective Effects on Platelet Apoptosis**

Despite numerous studies that have demonstrated the wide array of protective efficacies of phytochemicals, there is a dearth in reports on the platelet anti-apoptotic efficacy. To the best of our knowledge, as of now, there is only one report suggesting the platelet anti-apoptotic effect viz. cinnamtannin B1.

**Cinnamtannin B1**

Cinnamtannin B1 (CTB) is a condensed-trimeric-tannin belonging to the type-A proanthocyanidins found in many species such as *Laurus nobilis* L., *Cinnamomum verum*, *Cinnamomum zeylanicum*, *Vaccinium vitis-idaea* and *Parameria laevigata*. Proanthocyanidins have reportedly been shown to possess numerous health beneficial properties, including cardioprotective, antiatherogenic and antineoplastic effects, probably due to their powerful antioxidant nature (Jayaprakasha et al., 2006).
Proanthocyanidins including CTB have long been reported to exhibit anticancer property via selective proapoptotic action in a wide variety of cancer cell lines while exerting antiapoptotic effects on healthy cells. Besides, CTB also exhibits inhibitory effects on platelet aggregation, thus it can be considered to be a cardioprotective compound sans secondary complications such as thrombocytopenia which is usually associated with other antiplatelet drugs (Lopez et al., 2008). This is due to the fact that CTB shows potent antiapoptotic effect on platelets by protecting them from oxidative stress. In a pioneering work by Bouaziz et al. (2007), the effects of CTB on platelets were explored. CTB was till then known to induce apoptosis in a variety of tumour cell lines, however its effects on platelet physiology were not scrutinized. Bouaziz and coworkers isolated CTB from bay wood extract (*Laurus nobilis* L.) and assessed its effects on the various parameters of platelet apoptosis. CTB was able to curtail thrombin-evoked activation of caspase 3 and 9 as well as their translocation to the cytoskeleton. CTB also inhibited thrombin-induced PS externalization. Going by a previous study that showed thrombin-induced platelet apoptosis was triggered by endogenous generation of *H₂O₂*, the efficacy of CTB on scavenging the thrombin-generated *H₂O₂* was assessed. CTB not only inhibited the thrombin-induced endogenous generation of *H₂O₂* but also inhibited *H₂O₂*-induced activation of caspases and PS exposure (Fig. 1.4). The concentration of CTB used was similar to those used to induce apoptosis in cancer cells. Therefore, the underlying mechanisms through which CTB exerts differential effects on normal cells and tumour cells needs to be further delineated.
Fig. 1.4 The possible protective actions of cinnamtannin B1 (CTB) against platelet apoptosis. CTB inhibit endogenous $H_2O_2$ generation, intracellular $Ca^{2+}$ mobilization and $\Delta \Psi_m$ depolarization thereby preventing the activation of caspases.

**Concluding Remarks:**

Platelets play a pivotal role in human health and disease. Dysfunctional platelets are involved in the pathophysiology of many diseases including CVDs, diabetes mellitus and thrombocytopenia. They are extremely sensitive to apoptotic stimuli such as oxidative stress, *in vitro* storage conditions, certain therapeutic drugs and dietary constituents. Being anucleate, mitochondria take the centre stage in mediating the various events of platelet apoptosis. When the rate of platelet apoptosis exceeds the normal physiological rate, there can be two major consequences: release
of a huge number of MPs into the circulation and reduced platelet count or thrombocytopenia. PMPs are involved in thrombus formation and initiation of atherosclerotic plaque formation. In the case of CVD, cancer and diabetic patients, generation of MPs further worsens the disease condition. On the other hand, reduced platelet count leads to bleeding disorders and failure of blood clot. Phytochemicals are being increasingly used in the treatment approach for various diseases including CVDs and cancer mainly due to their exceptional antioxidant properties. Although they are considered to be very safe with not much secondary complications, recent reports on their toxicity on platelets have made us to think otherwise. Despite being good antioxidants with various beneficial properties, thymoquinone and resveratrol are shown to exert proapoptotic effects on platelets. Thereby there is an absolute need for a proper screening of phytochemicals for their adverse effects on platelets. There is also a risk of exacerbated production of MPs, which can nullify the beneficial effects of drug and may also aggravate the already existing disease condition. More research needs to be done in this field to delineate the different mechanisms through which phytochemicals could exert proapoptotic effects. Moreover, the ideal dosage should be carefully worked out so that their plasma concentrations do not exceed the ones required to elicit toxic effects on platelets. Alternately, the proapoptotic phytochemicals could be supplemented with phytochemicals which protect platelets from apoptosis. This approach could not only nullify the toxic effects on platelets but also enhance the quality of treatment due to their combined action. In this context mention can be made of the phytochemical cinnamtannin B1, which apart from its various beneficial properties also possesses remarkable antiapoptotic effects on platelets. There is a need for identifying such phytochemicals which can be safely used in treatment regimen without the fear of toxicity to platelets. They can also be
combined with other therapeutic drugs to further boost the effectiveness of the treatment. Generation of MPs is another crucial aspect that needs to be taken care of. As of now, there are no convenient methods to monitor or count the number of MPs in the circulation. Hence controlling the abnormal rate of platelet activation and apoptosis seems to be an effective approach to check the production of MPs and platelet-protective phytochemicals seem to be a safe bet.