Synopsis

Blood coagulation is a process that occurs normally in an animal during bleeding, to prevent the loss of blood from the body and is assisted by two key players -blood platelets and coagulation factors particularly thrombin. When this process becomes extensive, it leads to thrombosis, a condition which can lead to severities such as cardiac failure, brain hemorrhage, renal failure and deep vein thrombosis. Anti-thrombotic drugs which are administered to combat thrombosis can be classified into three categories - anticoagulants, antiplatelet drugs (also known as blood thinners) and fibrinolytic drugs. The anticoagulant and antiplatelet drugs constitute the anti-thrombotic drugs which ensure the prevention of thrombosis whereas fibrinolytic drugs are administered after the thrombotic plaque is formed in order to dissolve the clot. The present day human society has been plagued by cardiovascular disease (also known as coronary heart disease) due to sedentary lifestyle and unhealthy food habits. The major fear in case of this disease is the blockage of the coronary artery by the atherosclerotic plaque, which then results in decreased blood supply to the cardiac tissue. When the plaque completely blocks the artery, owing to the blood pressure, the atherosclerotic plaque ruptures resulting in the formation of a thrombus (blood clot), which cuts off the blood supply to the cardiac tissue and can finally lead to a cardiac arrest. Therefore, anti-thrombotic drugs play a pivotal role in combating heart disease. Amongst these, the administration of anticoagulant and antiplatelet drugs is highly crucial to prevent the formation of the thrombus in patients suffering from heart disease. There have been many drugs in the market at present but they can cause side effects such as unwanted bleeding, allergy and non-specific reactions. Also, the current need is to ensure that the anti-thrombotic drugs administered do not hamper haemostasis, although they prevent blood coagulation. Hence, there is a constant need to discover newer anti-thrombotic drugs from unexplored microbial sources. This thesis describes the identification of compounds from endophytic fungi that inhibit thrombin and platelet aggregation.

We have screened forty eight endophytic fungi isolated from the plants *Datura metel* and *Cassia fistula* for thrombin inhibitory activity. Two isolates from *D. metel* -Dm 3.3, Dm 14.2 and three isolates from *C. fistula* - C2, C3, C4 tested positive for thrombin inhibitory activity. When each of these five endophytic fungal isolates was further tested for their potential to inhibit thrombin and platelet aggregation separately; Dm 3.3, C2 and C3 showed potent anti-

thrombotic properties. The present thesis work describes the identification of anti-thrombotic metabolites of two of these three endophytic fungal isolates namely - Dm 3.3 and C2.

Based on ITS sequence analysis, Dm 3.3 was identified taxonomically to be *Colletotrichum gloeosporioides*. The hexane extract of endophytic fungal isolate, *C. gloeosporioides* showed higher inhibition of purified thrombin (Sigma thrombin) and plasma thrombin as well as the potential to inhibit platelet aggregation induced by ADP, when compared to the ethyl acetate extract of the fungus. When the hexane extract of *C. gloeosporioides* was analysed by GC-MS, two major metabolite peaks were identified by the NIST library data -retinoic acid and 6β-hydroxytestosterone. There have been several reports on the presence of retinal in fungi such as *Mucor mucedo* and have suggested its role as an intermediate in the biosynthesis of the morphogen, trisporic acid (a retinoid derivative) with a vital role in the sexual reproductive phase of fungi in inducing the formation of zygophores (gametes) from the mycelium. There have also been reports which speculate that the homologs of the enzyme RALDH (retinaldehyde dehydrogenase) in fungi could possibly convert retinal to retinoic acid. The presence of steroids in fungi has been extensively reported to play a key role to induce meiosis in the antheridials and oogonium of the fungus –*Saprolegnia ferax*. Therefore, both retinoid compounds and steroids play a crucial role in the sporulation of fungi, thus justifying the presence of the two metabolites, retinoic acid and 6β-hydroxytestosterone, in *C. gloeosporioides*.

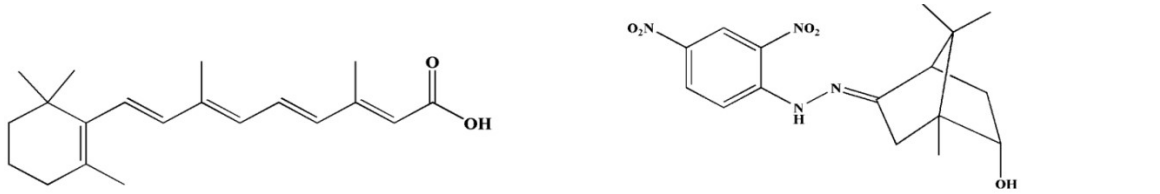
Since, we had observed two major metabolite peaks in the hexane extract of *C. gloeosporioides* which corresponded to retinoic acid and 6β-hydroxytestosterone, we investigated the anti-thrombotic properties of both the standards - retinoic acid and 6β-hydroxytestosterone. We observed that retinoic acid, but not 6β-hydroxytestosterone, showed potent inhibition of both thrombin and platelet aggregation. The three forms of vitamin A namely - retinoic acid, retinal and retinol, showed potent inhibition of thrombin and platelet aggregation. Retinoic acid showed the maximum inhibition of thrombin (IC_{50}values: Sigma thrombin – 67µg/ml; plasma thrombin – 49µg/ml), while retinol showed the maximum inhibition of platelet aggregation (97% inhibition at 120µg/ml). Therefore, we can infer that retinoic acid to be the active anti-thrombotic secondary metabolite present in *C. gloeosporioides*.

In parallel to the work on the anti-thrombotic properties of the *D. metel* endophytic fungus –*C. gloeosporioides* (Dm 3.3), we have also investigated the thrombin and platelet aggregation inhibitory metabolites of C2, the endophytic fungus isolated from the plant *Cassia fistula*. The molecular taxonomic method of ITS PCR enabled us to identify the endophytic fungus C2 as

Penicillium sclerotiorum. The ethyl acetate extract of the mycelium of *Penicillium sclerotiorum* when grown in potato dextrose broth showed the highest thrombin inhibitory activity. Hence, this extract was subjected to a bio-assay guided column chromatographic fractionation using silica gel column. Three fraction pools were obtained, each of which was found to possess a unique anti-thrombotic active principle (AP). Active principle 1 (AP1) was purified from fraction pool I by preparative TLC, while active principle 2 (AP2) was purified from fraction pool II by column chromatography (sephadex LH20 column followed by silica gel column) and preparative TLC. The compound AP1 showed inhibition of both Sigma thrombin (IC_{50} value – 58µg/ml) and plasma thrombin (IC_{50} value – 89µg/ml) as well as the inhibition of platelet aggregation (70% inhibition at 120µg/ml). AP2 showed inhibition of Sigma thrombin (IC_{50} value – 104µg/ml) and plasma thrombin (IC_{50} value – 107µg/ml) as well as the inhibition of platelet aggregation (41% inhibition at 120µg/ml). Amongst these metabolites, AP1 showed higher anti-thrombotic potential as compared to AP2. Thus, AP1 and AP2 are the two active metabolites purified from *P. sclerotiorum* which show potent anti-thrombotic properties.

The structure elucidation of the active principles – AP1 and AP2 purified from the *Cassia fistula* endophytic fungus, *Penicillium sclerotiorum*, was performed using state-of-the art analytical techniques such as IR spectroscopy, UV spectroscopy, LC-MS, NMR spectroscopy and micro-analysis. The mass of AP1 was analysed by mass spectrometry to be **415 m/z** and the structure characterization of this metabolite is awaited. The structure of AP2 was elucidated to be 5-(2,4-dinitrophenyl)hydrazono-2-bornanol, which has a mass of **390 m/z** (as analysed by mass spectrometry) and belongs to the class of monoterpenoid derivatives called camphenes.

Thus, the thesis work describes the identification of anti-thrombotic compounds from two endophytic fungi – *Colletotrichum gloeosporioides* and *Penicillium sclerotiorum*. Since these compounds have a microbial origin, there is a potential advantage to scale up their production by exploiting the tools of media optimization and environmental stress application.



Retinoic acid **5-(2,4-dinitrophenyl)hydrazono-2-bornanol**

Structure of the anti-thrombotic secondary metabolites which were identified in this study