Chapter 7

Summary and conclusion
Fungal diseases caused by diverse fungal species are reported to be one of the fourth most common health problem faced by the human population. Among these, filamentous, pervasive and opportunistic fungi from *Aspergillus* species, are most abundant and the prime causative agent of infections like aspergillosis, rhinosinusitis otomycosis, and mycotic keratitis specifically in immunocompromised individuals. An extensive research has been done on the role of these factors in infections caused by species like *Aspergillus fumigatus* which is more prevalent in western countries. However, *Aspergillus flavus* is most abundant and the prime causative agent of infections like aspergillosis, rhinosinusitis and mycotic keratitis in India. During infection, fungus secretes a plethora of factors such as attachment molecules, hydrolytic enzymes, growth stimulants and mycotoxins. The pathways involved in the inflammatory reactions initiated by *Aspergillus fumigatus* has been studied extensively. But, the information on virulence factors involved in infection and pathogenesis of *A. flavus* and the epigenetic mechanism of mycotoxin mediated carcinogenicity is uncertain. Therefore, the present study entitled “Study on biochemical and functional properties of secretory components of *Aspergillus flavus*” is proposed to investigate the biochemical and functional properties of secretory components to find the possible virulence factor/s and aflatoxin-induced effect on epigenetic regulators. The salient findings of the present investigation are as follows:

**In silico and biochemical analysis of secretory components**

The work initiated with the *in silico* data analysis of secretory components of *A. flavus*. As a first step, the total proteome from the genome database was collected followed by the collection of secretome data from the FunSecKB database which uses SignalP (version 3.0 and 4.0, http://www.cbs.dtu.dk/services/SignalP/) to detect secretory proteins. Out of the total proteome (13485), 913 proteins were identified by *in silico* as a part of secretome, among which 508 proteins were *in silico* characterized. Further, we separated these proteins as enzymes and other proteins, among enzymes a group of hydrolases showed the abundance which includes proteases (15), lipases (28), chitinases (6) and other than enzymes, we also found two secreted lectins upon secretome analysis. Detailed analysis revealed that among the class of secreted proteases, serine proteases are...
more. ProtParam and phylogenetic analysis revealed that most of the proteins were thermodynamically stable and phylogenetically closely related to *A. oryzae* proteins. The biochemical analysis of the secretory protein components showed the activity of protease, chitinase, and lectin in secretion. Analysis of protease activity in presence of a different class of protease inhibitors confirmed the presence of serine protease in the secretome of *A. flavus* whereas analysis of non-protein component showed the presence of aflatoxin B₁.

### Purification and characterization of secretory protein components

The optimization, purification and characterization of protease (AFP), chitinase (AFC) and lectin (FFL) from *Aspergillus flavus* was performed using conventional chromatography methods. Firstly, the conditions for the production of protease, chitinase, and lectin were optimized and then purified by using FPLC chromatography method. The purified AFP digested the copolymerized casein in gel and exhibited optimum activity at pH 8.0 and temperature at 55°C. The thermal inactivation of AFP followed first-order kinetics with a half-life (t₁/₂) of 52, 20 and 14 min at 40, 50 and 60°C respectively. Inhibitor study suggested that AFP is a serine protease and its activity was inhibited in presence of aprotinin and PMSF. The AFC was purified to approx. 6 fold with an overall recovery of 26% having optimum activity at pH 5.0 and temperature 40°C. The FFL was purified to 11 fold with an overall recovery of 10% having an optimum activity at pH 7.4, temperature 35°C and was non-specific to all type blood groups. The activity of FFL was specifically inhibited by L-fucose (125 µM).

### *In vitro* effects of secretory protein components

The cell viability and proinflammatory effect of varying concentrations of purified AFP, AFC, FFL and the whole secretory protein components (SPC) were studied in multiple cell lines. It is evident from the past reports that secreted proteins of fungal species are implicated in the initiation of proinflammatory reactions. Our results demonstrated that the purified AFP and AFC had not shown any significant effect on the viability of cells whereas FFL and SPC showed a dose and time-dependent change in cell viability. An upregulation of IL-8 secretion was observed upon SPC and FFL treatment in L-132, U937 and PBMCs. The mRNA expression study upon stimulation with
SPC and FFL revealed that along with IL-8 other cytokines like TNFα, IL-6, IL-1α and IL-1β were also upregulated. The lectin-mediated upregulation of IL-8 was validated by inhibiting the FFL with different concentration of L-fucose. The upregulation of cytokine IL-8 was further shown to be associated with the activation of p38 and activator protein-1 (AP-1, c-Jun) transcription factor. Finally, inhibition of p38MAPK by a specific inhibitor study suggested the p38MAPK involvement in its activation, which in turn, transcriptionally activates the induction of IL-8 in response to the lectin.

**Cellular epigenetic alterations by aflatoxins**

The effect of AFB₁ on the epigenetic modulations was emphasized. Former reports have asserted mechanisms by which the mycotoxin/environmental toxicants leads to altered functions in host cells ensuing hyperproliferation and divergence from the regular signaling pathways. First, we analyzed the AFB₁ induced proliferation in a dose (10-1000 nM) and the time-dependent manner in multiple cell lines. We studied the expression level of multiple epigenetic regulators upon AFB₁ treatment. The exposure to AFB₁ resulted in an alteration in global DNA methylation and an upregulation in the expression of DNMT3a and 3b. The cells showed a remarkable decrease in the HAT activity and a significant increment in the HDAC activity along with an increment in the expression of a different class of HDACs at both gene and protein level. It also affected the upregulation of polycomb proteins like BMI-1 and EZH2 with an increased level of H2AK119Ub and H3K27me3. Further studies were carried in order to assess the effect of AFB₁ upon the expression pattern of protein arginine methyltransferase 5 (PRMT5) in human cell lines. The expression of PRMT5 and its binding partner MEP50 was induced upon aflatoxin B₁ treatment in a dose and time-dependent manner. Further global symmetric arginine dimethylation was also increased in the same manner. Knockdown study substantially proved the AFB₁ mediated upregulation of PRMT5. The pharmacological inhibitors of PKC isoforms studies revealed the role of PKCα in upregulation of PRMT5. The AFB₁ mediated expression of PRMT5 declined remarkably in presence of PKCα inhibitor, Go6976 whereas PKCδ inhibitor, rottlerin posed synergistic effect in the expression of PRMT5. Analysis of mitogen-activated protein kinase (MAPK) pathways showed an increase in the phosphorylation of ERK1/2 upon AFB₁ treatment. The MEK inhibitor
(U0126) and PKCα inhibitor (Go6976) decreased the AFB₁ mediated phosphorylation of ERK1/2 and expression of PRMT5 suggested the role of PKC/ERK signaling in AFB₁ induced upregulation of PRMT5.

The overall findings of the present study are summarized as:

- In silico and biochemical analysis revealed the presence of serine proteases/chitinases/lectin in the secretory component of A. flavus.

- Aflatoxin B₁ was detected by HPLC analysis in the secretome of A. flavus.

- A serine protease (AFP, 31 kDa), chitinase (AFC, 29 kDa) and fucose specific lectin (FFL, 36 kDa) were purified from secretory protein component of A. flavus.

- Dose-dependent induction in the IL-8 expression by SPC and FFL; whereas no significant effect in presence of AFP and AFC was observed. Induction in the IL-8 expression might be caused by the activation of p38MAPK-cJun.

- Low concentration of AFB₁ induced the proliferation in multiple cells and it brings alteration in the expression of multiple epigenetic regulators (PcGs, DNMTs, HDACs, and PRMT5).

- Aflatoxin B₁ leads to the overexpression of PRMT5 at gene and protein level along with an increase in symmetric dimethylation levels and it upregulation might be mediated by the PKCα-ERK1/2 pathway.

- The immunomodulatory effect of FFL further verified the role of lectin in immunomodulation and fungal pathogenesis.

- Our epigenetic study confirmed the AFB₁ mediated effect on multiple epigenetic regulators with tumorigenesis potential.
This is the first report which showed the AFB$_1$ mediated overexpression of PRMT5 that is reported to be overexpressed in several cancers.