Sepsis is the tenth leading cause of death. Most of sepsis cases (40 percent) are fatal, and in most cases, the resulting multi organ failure and not the basic underlying infection, is the primary or main cause of death. Sepsis describes a complex clinical syndrome that develops when initial, appropriate host response to an infection becomes amplified and then dysregulated. Sepsis can be caused by infection with Gram-negative bacteria, Gram-positive bacteria, fungi, or viruses. Sepsis may also occur in the absence of detectable bacterial invasion, which include, microbial toxins (exo or endo), particularly Gram-negative bacterial endotoxin (LPS) and endogenous cytokine production have been implicated as mediators and initiators (Hardaway, 2000; Wheeler and Bernard, 1999).

Many features of local and systemic inflammation can be mimicked by administration of LPS i.e., endotoxemia. LPS is capable of selective activation of a class of macrophages through an elaborate series of events (Aderem and Ulevitch, 2000; Martins et al., 2006; Wright et al., 1990). Toll like receptors (TLRs) initiate a cascade of intracellular signaling activating various kinases e.g. PKC, MAPK, SRC resulting in the activation of pro-inflammatory transcription factors such as activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), and interferon response factor-3 (IRF-3), leading to the production of pro-inflammatory chemokines, cytokines and nitric oxide (Beutler, 2004).

Cytokines are key elements in the inflammatory response that characterizes sepsis and septic shock. Two types of cytokines are released: proinflammatory (which include early such as tumor necrosis factor-α [TNF-α], IL-1β and IL-8 and late HMGB1) and anti-inflammatory (such as IL-10). TNF-α is one of the most important cytokines involved in the pathophysiology of sepsis and is released early in the process of sepsis. TNF-α induced tissue injury is largely mediated through neutrophils that respond by producing elastase, superoxide ion, sPLA2, hydrogen peroxide, platelet-activating factor (PAF), thromboxane A2 and leukotriene B4,. In addition, TNF-α amplifies inflammatory cascades in an autocrine and paracrine manner by activating macrophages/monocytes to secrete other pro-inflammatory cytokines (Calandra et al., 2002; Herbertson et al., 1995). IL-1β stimulates the synthesis and release of prostaglandins, elastases, and collagenases. It promotes transendothelial migration of neutrophils, and activates endothelial microvascular cells, which respond
by releasing PAF and IL-8 (both of which are powerful neutrophil-stimulating agents) (Curfs et al., 1997). TNF and other proinflammatory cytokines (like IFN-γ) stimulate macrophages to release HMGB1 (Ivanov et al., 2007; Wang et al., 1999). HMGB1 is also released by macrophages when stimulated directly by exogenous bacterial products (such as endotoxin or CpG-DNA) (Rendon-Mitchell et al., 2003; Tang et al., 2007). HMGB1 is now perceived to be a major mediator of prolonged and sustained inflammation in sepsis, since it is released about 16hrs after early cytokines and its level remain high for several days (Chen et al., 2004; Jiang et al., 2007; Tang et al., 2005). Animals exposed to high levels of recombinant HMGB1 develop a sickness syndrome characterized by piloerection, decreased mobility, increased somnolence, weight loss and fever similar to sepsis syndrome.

Despite the extraordinary developments in understanding the immunopathology and pathobiology of sepsis, therapeutic advances have been drastically slow. Currently, available strategies for the management of sepsis patients include: rapid identification of causative organisms; timely patient identification and diagnosis; improved ventilatory techniques (low-pressure ventilation); appropriate, timely antimicrobial therapy; appropriate (goal-directed) haemodynamic support; targeted pharmacological therapies (recombinant activated protein C) immunological therapy and glycaemic control (intensive insulin therapy); effective supportive therapies (prophylaxis against stress ulcers, administration of anticoagulants and dialysis); appropriate nutrition; and patient management by highly qualified clinicians, techniques and nursing staff (Bollaert et al., 1998; Bone et al., 1992; Briegel et al., 1994; Ibrahim et al., 2008; Martins et al., 2006; Natanson et al., 1998; Rivers et al., 2001; Wheeler and Bernard, 1999; Zhang et al., 1995). These strategies have helped to reduce the support failing organs, incidence of infections, and prevent complications (Rivers et al., 2001). But these measures require whole-hearted involvement of the entire healthcare team and the provision of strong support in achieving these objectives cannot be stressed enough. So presently the focus is to find better drugs to combat disease and curb it at initial stages.

Development of drotrecogin alfa (activated), which is a recombinant version of activated protein C (APC), for sepsis therapy created big hopes. It was developed by Eli Lilly and Company and is marketed under the brand name Xigris. Activated
protein C has profound anti-apoptotic & anti-inflammatory properties, in addition to its anticoagulant activity. However APC was found to have its own complications in ADDRESS (Administration of Drotrecogin alfa (activated) in Early Stage Severe Sepsis) trial.

A series of antilipopolysaccharide treatment strategies for sepsis have been done for more than 20 years, including antiendotoxin antibodies, lipid A (harboring the LPS biological activity) antagonists, polymixinB, extracorporeal endotoxin absorber, bactericidal /permeability-increasing protein, cathelicidins, limulus antilipopolysaccharide factor and lactoferrin. These therapeutic agents have demonstrated efficacy in animal research, however, numerous attempts to neutralize LPS in clinical trials in septic patients have proven ineffective (Nahra and Dellinger, 2008). TLR4 antagonists (like E5564 and TAK-242) were also developed to curb sepsis menace, however they too provided no respite (Leon et al., 2008).

HMGB1 levels in serum remains high days after actual insult giving a wide therapeutic window for therapy compared to other cytokines. It is speculated that agents capable of decreasing HMGB1 release might prove as potential candidates for sepsis therapy (Sappington et al., 2003). This study was undertaken to identify novel compound targeting HMGB1 and further elucidating its role in sepsis/ endotoxemia.

Since HMGB1 is released in both processes i.e., apoptosis and necrosis (Raucci et al., 2007; Tang et al., 2010) so we needed to assess non toxic doses of test compounds for macrophage cells. Any kind of stress would change the distribution of cellular HMGB1. Non toxic doses of all the test compounds were used keeping this in mind same is depicted in the results under viability assay (Fig 3.1).

Screening of test compounds

Based on HMGB1 release it would be cumbersome to screen many compound so we screened compounds on the basis of their nitric oxide (NO) scavenging potential in activated macrophages. During the course of sepsis, increased amounts of nitric oxide levels are produced, and elevated levels of nitric oxide metabolites in patients with sepsis have been correlated with LPS or endotoxin levels and with organ-failure scores (Gomez-Jimenez et al., 1995; Groeneveld et al., 1996).
Statins are reported to greatly reduce the leucocytes migration and leucocytes recruitment induced by lipopolysaccharide (LPS) (Diomede et al., 2001; Pruefer et al., 2002). Statins reduce leucocytes adhesion to endothelium by down-regulating surface expression of endothelial cell adhesion molecule (ECAMs): P-selectin, CD11b, and CD18 (Weber et al., 1997; Yoshida et al., 2001). Statins have also been seen to affect the production of many acute phase reactants, such as TNF-α, IL-8, IL-6, monocyte chemoattractant protein-1 (MCP–1), and C-reactive protein (CRP) (Albert et al., 2001; Arnaud et al., 2005; Musial et al., 2001). Many statins are reported to have beneficial role in sepsis, we studied effect of rosvastatin (a synthetic statin) in endotoxin induced inflammation in RAW 264.7 macrophages cells. Rosuvastatin did not showed any significant downregulation on LPS induced NO in RAW 264.7 cells (Fig 3.3).

Crocus extracts (Crocus sativus L. (Iridaceae) are reported to process anti-inflammatory and immunomodulatory actions (Hosseinzadeh and Sadeghnia, 2005; Kianbakht and Ghazavi, 2011). One of the main components of crocus extracts is safranal which is shown to have high antioxidant and free radical scavenging activity (Hosseinzadeh and Sadeghnia, 2005). Safranal did not show any effect on LPS induced NO generation, which was confirmed by iNOS expression (Fig 3.4).

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is an anthraquinone derivative from the rhizome of Rheum palmatum, an herb widely used as a laxative in traditional Chinese medicine (Shi et al., 2001). It has been reported that emodin possesses a variety of biological activities, such as vasorelaxative (Huang et al., 1991), immunosuppressive (Kuo et al., 2001), hepatoprotective (Lin et al., 1996), and anti-tumor activity (Shi et al., 2001; Chang et al., 1996). Treatment of RAW 264.7 macrophages with emodin (20 μg/ml) inhibited the expression of a panel of inflammatory-associated genes, including TNF-α, iNOS, interleukin-10 (IL-10), cytosolic inhibitor of κB (IκB)α, IκB kinase (IKK)-α and IKK-γ, and the nuclear translocation of nuclear factor-κB (NF-κB) (Li et al., 2005). Our study showed significant inhibition of LPS induced NO by emodin (Fig 3.4). Since effect of emodin on HMGB1 in macrophages were reported during the course of study, so we did not pursue its use further (Chen et al., 2010).
Effect of psychosine on inflammatory mediators in RAW 264.7 cells and peritoneal macrophages

Galactosyl sphingosine (Psychosine) is abnormally increased in brain in a disease known as Krabbe disease. Psychosine accumulation is believed to be the primary cause of the rapid degeneration of the myelin-forming cells and consequent demyelination that is seen in this disease (Miyatake and Suzuki, 1972). The mechanisms by which psychosine mediates cell death is unclear yet. In Krabbe disease the microglial cells are activated which become phagocytic and secrete a variety of cytokines, including the proinflammatory cytokines, TNF-α, interleukin 1-beta and interferon-gamma. These cytokines perpetuate and augment microgliosis as well as induce glial cells and astrocytes to become hypertrophic and undergo reactive astrocytosis (Merrill and Benveniste, 1996). The role of psychosine outside neuronal system is poorly studied. In addition effect of psychosine on different cell types varies considerably, however effect on RAW 264.7 cells has not been seen yet.

We observed that psychosine showed different response in RAW 264.7 cells and primary peritoneal macrophages. The results were in concordance with earlier reports of psychosine showing different response in different cells depending upon origin of cell line (glial cell line vs primary mixed gial cells) (Bashir and Haq, 2011). The primary peritoneal macrophages is mixed in nature including primarily macrophages and specific B cell subsets, thus probably explaining the variation in response of psychosine in two.

In LPS stimulated RAW 264.7 cells, psychosine showed decrease in NO and TNF-α level but no change in levels of HMGB1 was seen. On the other side, in activated peritoneal macrophages, psychosine showed a significant increase in LPS-induced NO, TNF-α, along with HMGB1. The psychosine induced significant release of HMGB1 levels in primary peritoneal macrophages even in the absence of LPS. It has already been reported that psychosine potentiates the LPS-induced expression of iNOS and the production of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) production in rat primary astrocytes (Giri et al., 2002). Psychosine is said to cause apoptotic mediated death of oligodendrocytes (Haq et al., 2003; Zaka and Wenger, 2004). As HMGB1 is released in apoptotic death and also mediates the inflammatory response.
response in later stages. Thus increase in HMGB1 release by psychosine might be one of mediators of psychosine induced lethality. However we used low doses of psychosine which had no effect on RAW 264.7 cell viability (Fig 3.1) so it is proposed that psychosine mediated HMGB1 release in peritoneal macrophages is not because of apoptosis or necrosis but a different pathway may be involved. Based on the known literature, it can be proposed that, psychosine at low levels may result in activation of cells releasing HMGB1 which surge inflammation and later mediate inflammation and apoptosis (Galbiati et al., 2007; Galbiati et al., 2009). However, further studies need to check the actual role of HMGB1 in Krabbe disease.

**Effect of aloe-emodin on inflammatory mediators in RAW 264.7 cells and peritoneal macrophages**

Aloe-emodin (AE), a hydroxyanthraquinone naturally present in the leaves of Aloe vera (Dutta et al., 2007), has antiviral, antimicrobial and hepatoprotective activities (Arosio et al., 2000; Eshun and He, 2004) and anticancer activity in neuroectodermal tumors (Pecere et al., 2000), lung squamous cell carcinoma (Lee, 2001), hepatoma cells (Kuo et al., 2002) and in glial cell line (Acevedo-Duncan et al., 2004). Studies have shown aloe-emodin as antioxidant under various stress. However aloe-emodin role in LPS induced inflammation has not yet been studied.

During this study we found aloe-emodin decreased LPS induced NO production and iNOS expression dose dependently (Fig 3.7). Similar dose dependent effect was seen on LPS induced extra-cellular release of HMGB1 in RAW 264.7 macrophages and primary peritoneal macrophage cells (Fig 3.13 & 3.14). On LPS stimulation HMGB1 is first translocated from nucleus to cytoplasm and then outside the cells. Aloe-emodin was seen to partially decrease the nuclear to cytoplasmic translocation of HMGB1 (Fig 3.15).

The pro-inflammatory cytokines i.e., TNF-α, IL-1β are released much early than HMGB1 and mediate early inflammatory damage on stimulation with LPS. The drugs which inhibit pro-inflammatory cytokines both early cytokines (TNF, IL-1) and late cytokines (HMGB1) are seen as good candidate for sepsis/endotoxemia therapy. The aloe-emodin treatment inhibited both TNF-α, IL-1β in dose dependent manner in RAW 264.7 cells with 15µM decreasing the levels most (Fig 3.17 & 3.18). However
in peritoneal macrophages the pattern of inhibition of TNF-α and IL-1β is different. Aloe-emodin inhibited the TNF dose dependently in peritoneal macrophages, but IL-1β inhibition showed different pattern with maximum inhibition at 10µM.

In response to oxidative stress, macrophages also induce heme-oxygenase 1 (HO-1), which has protective effect in innate immune cells and exerts anti-inflammatory effects that limit the damaging consequences of inflammation and immunity (Otterbein et al., 2003; Wagener et al., 2003). Aloe-emodin increased the LPS induced HO-1 expression in RAW 264.7 cells (Fig 3.19). Recent studies proved a close relationship between HO-1 and HMGB1 in inflammatory conditions (Tsoyi et al., 2009). HO-1 has been reported to down regulate HMGB1 release via carbon monoxide in activated macrophages (Tsoyi et al., 2009). Up-regulation of HO-1 is seen to protect mice from the lethal effect of LPS- and CLP-induced sepsis, paralleled by a decrease in the systemic levels of HMGB1. Thus HO-1 may be involved in aloe-emodin mediated HMGB1 inhibition.

Drugs inhibiting HMGB1 are seen as good targets for sepsis therapy. Efficacy of aloe-emodin against sepsis was tested in mice model of endotoxemia. It was observed that aloe-emodin protected the mice in both sub-lethal and lethal model of endotoxemia. The survival was seen more in mice administered with aloe-emodin at the dosage of 0.9µg/mouse (Fig 3.20).

Proinflammatory cytokines are major players in sepsis lethality and among the cytokines involved in endotoxic shock, TNF-α appears to play a central role. Indeed, increased serum TNF-α levels appear during endotoxemia and TNF-α injection induces shock, tissue damage, and death (Tracey et al., 1986). During endotoxic shock, TNFα shows a large spectrum of harmful effects. These effects include increase in procoagulant activity of vascular endothelial cells, activation of macrophages and neutrophils, and increase in combination with IFNγ in the expression of adherent molecules resulting in increased neutrophil/monoocyte adherence to endothelial cells and tissue infiltration. Aloe-emodin decreased the levels of proinflammatory cytokines i.e., TNF-α and IL-1β (Fig 3.21 &3.22). The pattern of inhibition however varies in case of TNF-α and IL-1β indicating pleiotropic
Discussion

actions of aloe-emodin. Aloe-emodin treatment at both high and low doses showed no effect on IL-6 levels in endotoxemic mice (Fig 3.23).

TNFα in early stages and HMGB1 in later stages mediates neutrophil and macrophage accumulation which damages the organs, thereby causing multi-organ dysfunction syndrome (MODS). Treatment with aloe-emodin protected endotoxemic mice from organ dysfunction as seen by serum biochemistries (LFT and KFT) checked 18hrs after LPS administration (Fig 3.25 & 3.27). Protective effect of aloe-emodin was also observed in histopathology study of liver, kidney and lung sections analysed by H&E staining. Treatment with aloe-emodin shows improved organ histology compared to endotoxemic mice (Fig 3.24, 3.26 & 3.28). Histopathology showed significant decrease in neutrophil infiltration in lung tissue sections compared to endotoxemic mice. Neutrophil infiltration and high level of cytokine in lungs leads to acute lung injury (ALI), which is an early characteristic of multiple organ dysfunction and is responsible for high mortality and poor prognosis in patients with sepsis [Ware, 2000]. In this present study, the levels of both TNF and neutrophils increased in endotoxemic mice even after 18hrs of LPS administration. Aloe-emodin was observed to inhibit both release of TNF and levels of neutrophils in lung tissues compared to endotoxemic mice (Fig 3.29 & 3.30).

This study can serve as a model to evaluate the mechanism of aloe-emodin mediating inhibition of HMGB1 and its effect in other septic models like cecal ligation and puncture (CLP).

Thus, the results of present study clearly reveal that the aloe-emodin has potential to ameliorate HMGB1 release under inflammatory stress and also to decrease the level of proinflammatory cytokines and oxidative stress markers. Aloe-emodin showed good immunotherapeutic potential in LPS induced endotoxemic murine model of sepsis. The present study provides the basis for the future evaluation of aloe-emodin as a potential adjunct to the already existing sepsis therapy.