Chapter 5

Discussion
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5.1. Preamble

Amongst the various factors known to cause gastric ulcer, widespread use of NSAIDs is the foremost. The NSAID-induced stomach ulceration ranks fourth amongst the diseases causing morbidity and mortality. Oxidative stress due to various factors including use of NSAIDs is known to contribute to induction, progression and delayed healing of gastric ulceration. The NSAID-induced gastropathy continues to be of concern to both clinical practitioner and researchers. Thus, exploration of natural, non-toxic antioxidants, in from of herbal extracts/ fractions/constituents might provide inexpensive and non-toxic anti-ulcer formulations. Many of the prophylactic and ulcer-healing action of drugs (Biswa\textit{et al.}, 2003) and formulations (Me\textit{Alindon et al.}, 1996) is attributed to their antioxidant property. At the same time search for new NSAIDs, devoid of gastrotoxicity is an active area of drug development research (Gasco\textit{et al.}, 2004; Coruzzi\textit{et al.}, 2007; Halen\textit{et al.}, 2009).

In the present effort, the healing activity of i) an antioxidant-enriched fraction (K-7) of the methanol extract of \textit{P. kurrora}, and ii) mal C, a spice-derived diarylnonanoid with proven \textit{in vitro} antioxidant activity (Patro\textit{et al.}, 2005) against indomethacin-induced acute stomach ulceration in mice was assessed, and the action of the drugs was rationalized in terms of established biochemical mechanisms. Clinically, indomethacin has limited use, but is still extensively used in obstetrics to delay uterine contractions and in the neonatal unit to facilitate patent ductus arteriosus closure. The present studies were carried out using indomethacin since data from human studies suggest that indomethacin has greater ulcerogenic potential than ibuprofen and other NSAIDs (Henry\textit{et al.}, 1993; Garcia-Rodríguez and Jick, 1994).

In the latter part, the inflammatory activity of mal C and the signaling pathway therein, was established using several established acute inflammatory \textit{in vivo} and cellular models.

5.2. Mal C and K-7 heals indomethacin-induced gastric ulcer in mice, without showing any toxicity

Histopathological examinations of the stomachs of normal, ulcerated untreated and drugs (mal C and K-7)-treated mice revealed marked damage to the gastric mucosa with elongated haemorrhagic lesions confined to the glandular portion, within 6 h of
indomethacin (18 mg/kg) administration. Maximum subjective inflammatory score (IS) was observed on the 3rd day of ulceration (Figs. 4.4 and 4.5). Even without treatment, the ulcer craters receded (auto healing) partially, confirming the acute nature of ulceration. But the process was slow and evident only between 5th to 7th days after ulcer induction, when the ulcer craters receded partially. The extent of auto healing was only ~55%, compared to the peak ulceration.

Mice treated with mal C, K-7 and Omez showed significantly faster and better healing within three days. Under optimized treatment regime, the effects of mal C (10 mg/kg), and Omez (3 mg/kg) were comparable, and slightly better than that of K-7 (15 mg/kg). On the 7th day of ulceration, the natural recovery itself was substantial and the beneficial effect of the test samples was not important. Overall, both K-7 and mal C (at all doses) showed maximum efficacy on the 3rd day of treatment, mal C being more effective irrespective of the treatment regime (dose and treatment period). The qualitative histopathological assessment also correlated well with these data.

Given that toxicity is a key impediment in drug development, the possible toxic effects of the test drugs on mice were evaluated, which revealed them to be non-toxic even up to a dose of 500 mg/kg. There were no gross behavioral changes in the mice, while the functioning and histopathology of their vital organs like liver and kidney were also normal (Fig. 4.2 and Table 4.2). The non-toxicity of the drugs to a normal cell line (mouse macrophage RAW 264.7 cells) was also confirmed by the MTT assay.

Overall, the doses of these test drugs are comparable to those of classical ulcer healers like Omez, cimetidine, sucralfate etc. (Bauer et al., 1986). In view of the favourable acute toxicity data even at a considerably higher dose in the mouse model, mal C and K-7 promise to be safe anti-ulcer drugs at the optimized doses. The data with a normal cell line also supports this. Between the two test samples, the non-toxicity of mal C was expected considering that it is the constituent of a spice, freely consumed in India and other Asian countries.

5.3. The ulcer-healing action of mal C and K-7 involves modulation of the antioxidant defence system

Tissue damage during gastric ulceration is always associated with excess generation of free radicals leading to oxidative stress. This is reflected in the loss or impairment of
protein synthesis (El-Missiry et al., 2001; Szabo et al., 1985) and damage to key biomolecules such as lipids and DNAs. Inflammatory reactions induced by indomethacin are a significant source of reactive oxygen species (ROS) (Miura et al., 2002). Experimental evidence indicates that imbalances in the production and removal of ROS play a crucial role in gastric erosions and injury due to indomethacin (Takeuchi et al., 1991; Yoshikawa et al., 1993; Das et al., 1997; Kwiecien et al., 2002). Indomethacin induced gastropathy is also known to decrease the glutathione peroxidase (GPx) activity and aggravate the injury due to accelerated accumulation of \( \text{H}_2\text{O}_2 \) and lipid peroxidation (LPO) products. Furthermore, excessive peroxidation causes increased consumption of glutathione, hindering the recycle of antioxidants like vitamin E and vitamin C.

The oxidative stress is often assessed by measuring individual markers such as LPO, protein carbonyl, deactivation of antioxidant enzymes etc. However, the antioxidant capacity of plasma represents a better index of the body's total systemic antioxidant defence comprising of the activity of enzymes, such as superoxide dismutase and the selenium-containing GPx, as well as nonenzymatic antioxidants (radical scavengers and chelating agents) (Ghiselli et al., 2000). Thus, the measure of total antioxidant status (TAS) provides proper assessment of the cumulative action of the antioxidants present in plasma and body fluids, and their synergistic interaction. Hence, the oxidative stress was assessed from the plasma TAS values of normal, ulcerated and treated mice.

The results (Fig. 4.6) revealed that the indomethacin-induced stomach ulceration was accompanied with a severe oxidative stress in gastric tissue leading to significant reduction in the plasma TAS. The test samples improved the parameter markedly following treatment for three days. The chosen drugs provided a suppression of the oxidative damages, compared to that observed in natural recovery. The relative antioxidant capacities of the drugs was mal C > Omez > K-7.

A distinct biological feature of inflammation is the activation of inflammatory cells such as macrophages and neutrophils, which are responsible for the generation of ROS and proinflammatory mediators. Neutrophil infiltration into gastric mucosal tissues is a critical process in the gastric pathogenesis and has been shown to be closely related to the development of various gastric mucosal lesions (Yoshida et al., 1995; Ohta and Nishida, 2001). Neutrophil infiltration is an early event and precedes the appearance of ulceration.
Infiltration of inflammatory cells such as neutrophils and mononuclear cells play a key role in the recurrence of gastric ulcers. MPO and NOS are widely used as an index of neutrophil infiltration in various experimental gastric injuries (Takeuchi et al., 1998; Khattab et al., 2001). The MPO activity at the site of inflammation is frequently increased in ulcerated condition and reduced with the healing process (Souza et al., 2004).

The present results showed extensive neutrophil infiltration in the gastric mucosa due to ulceration, associated with increased MPO activity (Figs. 4.7a and 4.7b). True to their antioxidant and ulcer-healing capacities, mal C and K-7 reduced both these parameters, mal C being far potent. The effect of Omez was, however, less and did not match with its excellent anti-ulcer property. This may be due to other operative mechanisms in its healing action (Ng et al., 2008). Factors such as control of intragastric pH (Goldstein et al., 2007) and stimulation of epithelial cell proliferation through increased serum gastrin level (Takeuchi et al., 2003) are attributed to its healing property.

Given the complexity of the ulcer-healing process the anti-oxidative effect of the drugs may, at best has a secondary role in it. Various other biochemical and immunological parameters play major roles in the indomethacin-induced ulceration. Hence, the efficacy of the drugs, K-7 and mal C in controlling some of these aspects were also investigated in the present studies to rationalize their biological action.

5.4. Mal C and K-7 alters the cyclooxygenase (COX) pathway

The NSAIDs exert both their therapeutic and toxic effects mainly through inhibiting COX and decreasing the levels of circulating PGs, especially PGE2 (Halter et al., 2001) at the gastric mucosa, causing gastric ulceration and also exacerbating pre-existing gastric ulcers in rodents and humans (Wallace, 1997). Indomethacin reduces PGE2 production via inhibition of the both the cyclooxygenases (COXs), COX-1 and COX-2. Decrease in the level of PGE2 has been reported to prevent the PG-mediated angiogenesis (Dormond et al., 2001) that is essential for ulcer healing. Exogenous PGE or its analogues are the drugs of choice in attenuating and healing the indomethacin-induced gastric mucosal damages.

In the present study, a drastic reduction in the expressions of COX-1 and COX-2 was evident from the western blots (Figs. 4.8a and 4.8b). This, in turn, reduced the mucosal PGE2 synthesis significantly (Fig. 4.9). The results are consistent with some earlier reports with indomethacin (Nygard et al., 1994; Tarnawski et al., 1990). The chosen drugs (mal C
and K-7) could augment the mucosal PGE$_2$ level by enhancing the COX-1 and COX-2 expressions. The higher capacity of mal C to augment COX-1 might explain its superiority as an anti-ulcer agent, since the COX-1-derived PG is considered beneficial for healing. The enhanced PGE$_2$ synthesis might account for their ability to inhibit the neutrophil-mediated free radicals generation (Gryglewski et al., 1987). The effect of Omez in regulating the expressions of COX isoforms and production of PGE$_2$ were significantly less. PGE$_2$ also regulates gastrointestinal mucosal homeostasis through its influence on various functions and mediators.

5.5. The healing action of mal C and K-7 is mediated by modulation of nitrogen-metabolizing enzymes

Recent evidence suggests that NSAIDs affect a wide variety of cellular processes independent of COX inhibition, accounting for at least some of the anti-inflammatory and immunomodulatory properties of these agents (Tegeder et al., 2001). Amongst the various other factors, the nitrogen-metabolizing enzymes are also key contributors in host immune defense mechanisms and wound healing (Jenkinson et al., 1996; Satriano 2003; Tarnawski and Jones, 2003). In acute inflammatory responses, such as wound healing, heat stroke and glomerulonephritis, arginine has been implicated as an important regulator of diverse pathways including generation of polyamines and the cytostatic free radical molecule, NO (Bogdan, 2001). The arginine pathway plays a vital role in wound healing since L-arginine becomes an essential amino acid after wounding with almost undetectable levels in the wound milieu (Caldwell et al., 1991). Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing cell proliferation and collagen synthesis as well as breaking strength (Witte and Barbul, 2002).

Metabolism of arginine that can be catalyzed by arginase and NOS, plays a vital role in gastric ulceration and its healing. Upregulation of arginase increases the level of polyamines, which play a significant role in wound healing. The regulatory role of arginase in acute intestinal inflammation and tissue repair has been demonstrated (Bernard et al., 2001; Satriano, 2004; Gobert et al., 2004). On the other hand, catabolism of L-arginine by NOS produces NO, which can play dual roles in gastric mucosal defense and injury. NOSs exist as constitutive (cNOS), and inducible isoforms (iNOS). The low concentration of NO, produced by the endothelial NOS (eNOS), one of the cNOS isoforms helps in wound
healing by increasing blood flow (Whittle, 1994) and angiogenesis (Ziche et al., 1994; Ma and Wallace, 2000) in the damaged gastric mucosa. However, the enhanced generation of NO by the iNOS may contribute to the pathogenesis of various gastroduodenal disorders including peptic ulcer (Jaiswal et al., 2001; Souza et al., 2004). Thus, the temporal switch between i-NOS and arginase activities in vivo decides ulceration and healing (Modolell et al., 1995; Shearer et al., 1997).

It was found that ulceration downregulated the mucosal arginase activity (Table 4.3), while increasing the iNOS expression (Fig. 4.10) significantly. This suggested a shift of the arginine metabolism towards the NO/iNOS pathway during ulceration. The elevated expressions of iNOS accounted for the increased total NOS activity as well as serum nitrite level due to ulceration (Table. 4.3). The augmented neutrophil counts due to the indomethacin treatment, as observed in this study, would generate more iNOS-derived NO, and superoxide radicals enhancing oxidative mucosal damage. On the other hand, the importance of eNOS and eNOS-derived NO in regulating microvascular structure during acute inflammation has been demonstrated using eNOS deficient mice (Luo et al., 2003). Because indomethacin administration reduced the eNOS expression, it would aggravate ulceration and delay its healing.

Treatment with mal C and K-7, especially the former restored the tissue arginase activity almost to normalcy. Omez also increased the enzyme activity, but to a lesser extent. The upregulation of arginase by the test drugs would enhance the polyamines synthesis, and thereby assist the healing (Gobert et al., 2004). Both mal C and K-7 reversed the indomethacin-induced alteration of eNOS and iNOS expressions. However, while mal C showed stronger effect on iNOS, K-7 augmented eNOS enormously. As expected, Omez had significantly less effect on the expressions of the enzymes. The effects of the test samples on the reduction of total NOS activity and NO level were consistent with these results. It is noteworthy that the effects were reflected both in the serum and tissue levels, mal C showing supremacy over K-7 in all the parameters, except iNOS expression.

The improved arginase activity and favorable eNOS/iNOS ratio, caused by treatment with mal C may also be a key contributing factor in its efficient ulcer-healing. In agreement with these results, it was found that the non-selective NOS inhibitor, L-NAME inhibited the ulcer healing activity of mal C (Fig. 4.11). On the other hand, the iNOS inhibitor, L-NIL did not have any effect. Taken together these results suggested that the
eNOS-derived NO contributed maximum to the ulcer healing property of mal C, although a role for neuronal NOS-derived NO cannot be excluded. In contrast, despite showing less effect on modulating eNOS/iNOS expressions and NO production, Omez provided excellent healing.

There is increasing evidence that endogenous NO acts in concert with endogenous PGs in the maintenance of gastric mucosal integrity (Ko and Cho, 1999). In particular, NO has been shown to be fundamental for the activity of COX (Salvemini et al., 1993), and COX enzymes represent endogenous targets for modulating the effects of NO in the gastric mucosa (Cuzzocrea and Salvemini, 2007). More interestingly, it has been shown that NO released from constitutive NOS activates COX-1 (Cuzzocrea and Salvemini, 2007) and that iNOS selectively activates synthesis of COX-2-derived PGE2 (Kim et al., 2005). Furthermore, inhibition of NOS also reduced PGs (Salvemini et al., 1993). Although the crosstalk between COX and NOS is not clear, it is suggested that ghrelin, by releasing small amounts of endogenous NO, ensures maintenance of COX-1 activity, providing gastroprotective effect. By inhibiting COX-1, indomethacin may interfere with the positive interaction between NO-COX-1 and hence attenuates ghrelin-induced gastroprotection.

5.6. Mal C and K-7 switch arginase/NOS pathway by altering cytokine balance

The indomethacin-induced gastropathy is attributed to the increased expression of pro-inflammatory cytokines (Yoshikawa et al., 1993; Brzozowski et al., 2001), which correlates with the extent of ulceration. Even the cross-talk amongst NOS/NO and arginase/polyamine is guided by the cytokine profile of the host (Jenkinson et al., 1996; Satriano, 2004). There are reports suggestive of an intense reciprocal regulation of NOS and arginase activities in vivo, depending on the cytokine profile of the host (Shearer et al., 1997; Modollel et al., 1995).

After trauma, the Th1/Th2 imbalance with Th2 predominance is reflected by an increase of the arginase inducing cytokines such as IL-4, IL-10, and TGF-β (Modolell et al., 1995). However, little is known on the interplay of cytokines and the NO synthesis pathway during indomethacin-induced gastric ulceration. In view of this, the immune response due to ulceration, and its modulation by mal C, K-7 and Omez was monitored. This enabled us to associate the inflammatory response with a better prognosis.
Indomethacin administration raised the levels of the pro-inflammatory cytokines (TNF-α, IL1-β, and IL-6) while reducing the anti-inflammatory cytokines (IL-4, IL-10 and TGF-β), thereby creating a cytokine imbalance. Increased TNF-α, and IL-1β is known to increase iNOS activity by promoting binding of NF-κB to the iNOS promoter and blocking their expressions attenuates the induction of iNOS expression (Shearer et al., 1997; Titheradge, 1999).

We selected IL-1β since it also modulates ulcer healing via the COX-2 pathway. Blocking the expression of TNF-α, and IL-1β might attenuate the induction of iNOS expression and NO generation (Titheradge, 1999; Chatterjee et al., 2006). Likewise, IL-4 that remains under the influence of NO, controls the expression of growth factors, and production of the proinflammatory cytokines such as TNF-α. Our result of decreased IL-4 level due to ulceration is consistent with an earlier report (Slomiany et al., 1999). The anti-inflammatory cytokine IL-10 also plays a key role in inflammation. After its initial depletion by NSAIDs, its concentration rises slowly to counter-regulate the production of the pro-inflammatory cytokines. Given that IL-10 is stimulated by PGE2, we also observed reduction of IL-10 on indomethacin administration, as reported earlier (Rhind et al., 2002).

Treatment with mal C and K-7 reversed the imbalance by reducing the Th1 cytokines drastically, and restoring the levels of IL-4, IL-10, and TGF-β to near normalcy, at both tissue and serum levels. Regarding the cytokine modulation also, the relative efficacy of the test samples was mal C > K-7 > Omez. Possibly, the increased PGE2 synthesis due to administration of mal C and K-7 increased the IL-10 (Banerjee et al., 2008). The upregulation of the anti-inflammatory cytokines by the test samples is likely to inhibit the stimulatory effect of indomethacin on the level of pro-inflammatory cytokine release in blood and gastric mucosa.

Upregulation of the Th2 cytokines like IL-4, IL-10, and TGF-β by mal C and K-7 would also induce the arginase activity (Modollel et al., 1995), as observed in the studies. Moreover, the enhanced IL-4 level due to the treatment with the test samples would trigger the TGF-β–SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair by collagen deposition. The immunosuppressive Th2 cytokine, TGF-β has a direct role in stimulating epithelial restitution (Kaviratne et al., 2004). Besides suppressing the IFN-γ-induced iNOS gene expression and thereby generation of excess NO,
it also increases arginase activity during inflammatory processes (Shearer *et al.*, 1997; Mitani *et al.*, 2005). The altered arginase activity and iNOS expressions, observed during ulceration, and drugs-treatment are consistent with their respective effects in modulating the mucosal TGF-β status. Consistent with a previous report (Kaviratne *et al.*, 2008), the effect of Omez on the cytokines levels was much less. This was also consistent with its lesser effect in regulating the arginase and NOS activities, compared to mal C and K-7.

The regulatory T cells and Th2 cytokines often collaborate to suppress the Th1 response. Perhaps even more importantly, they strongly promote the mechanism of wound healing. This feature of the Th1 / Th2 paradigm might explain the frequent bimodal nature of immune responses in general (Abbas *et al.*, 1996). However, the role of cytokine imbalance in gastropathy has not been properly documented. The present results highlighted that the balance of the pro and anti-inflammatory cytokines could also play a significant role in NSAID-induced gastric mucosal injury.

Overall, our results revealed an intricate relation between the modulation of cytokine balance by mal C and K-7 and their ability to alter the COX as well as NOS/arginase pathway in providing better healing for stomach ulceration. A combination of all these events might be contributing to tilt the balance between the inflammatory and repair mechanisms towards the latter. Next, we examined the key aspect of would healing, *i.e.*, angiogenesis that is essential for the restoration of microvascular network in the mucosa and thus crucial for oxygen and nutrient supply.

### 5.7 Mal C augments pro- vs anti-angiogenic factors for better ulcer healing

In spite of the established role of free radicals and cytokines in the aetio-pathology of gastric ulceration, ulcer-healing remains a complex process involving angiogenesis and cell proliferation. It involves filling the mucosal defect with proliferating and migrating epithelial and connective tissue cells. At the ulcer margin, epithelial cells proliferate and migrate onto the granulation tissue to cover (reepithelialize) the ulcer and also invade granulation tissue to reconstruct glandular structures within the ulcer scar. The reepithelialization and reconstruction of glandular structures is controlled by growth factors: trefoil peptides, EGF, HGF, bFGF and PDGF; and locally produced cytokines by regenerating cells in an orderly fashion and integrated manner to ensure the quality of mucosal restoration. Angiogenesis, which is a pivotal process in various types of wound
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healing, is regulated by the balance between the designated proangiogenic growth factors (VEGF) and the antiangiogenic factors (such as endostatin). An impaired angiogenesis and wound healing results due to the imbalance in these factors, which is often caused by deregularization of the PG, NO and cytokine generation (Wallace and Granger, 1996).

The growth factors are produced by different cells to perform specific functions at the required time. Within 48-72 h of ulceration, granulation tissue that consists of proliferating connective tissue cells develops at the ulcer base. Migration of fibroblasts into the granulation tissue and their proliferation are triggered by growth factors: TGF-β, PDGF, EGF, FGF and cytokines: TNFα and IL-1, resulting in angiogenesis. Granulation tissue supplies connective tissue cells (synthesizing extracellular matrix) for restoring the lamina propria and microvessels for the restoration of the microvasculature within ulcer scar. Sequential analysis of gene expression during gastric ulcer healing revealed that genes such as EGF-R, c-fos, c-jun etc. (within 30 min – 2 h) are involved in early response, while EGF, bFGF, PDGF and VEGF (activated within 6 h – 2 days) provide the intermediate response. In addition, late response genes (activated within 14 days) are also responsible for angiogenesis.

Delay of angiogenesis in gastric ulcer by COX inhibitors such as NSAIDS has been demonstrated by clinical and other experimental data (Halter et al., 2001). The antiulcerogenic potential of the chosen drugs could be attributed to their divergent ability to tilt the balance between pro- vs anti-angiogenic factors. The earlier results of the investigation established the superior healing capacity of mal C against indomethacin-induced gastric ulceration. Also in contrast to K-7, mal C is an isolated pure compound, which would ensure its authenticity. Hence the present angiogenic studies were confined to mal C only.

For this, emphasis was given on VEGF, PDGF, and bFGF, and endostatin, as representative pro- and antiangiogenic factors respectively. VEGF is a highly specific mitogen for vascular endothelial cells promoting endothelial proliferation and migration (Achen and Stacker, 1998) resulting in a accelerated ulcer healing process (Szabo and Vincze, 2000). VEGF is stored in α-granules within the platelet (Wartiovaara et al., 1998). Serum VEGF levels previously have been shown to correlate with the numbers of circulating platelets (Verheul et al., 1997). Given that indomethacin inhibits ADP-induced
platelet aggregation (Jin and Kunapuli, 1998) and the resulting α-granule release (Pengo et al., 1986), it is predictable that VEGF release from platelets would be reduced after indomethacin treatment. bFGF is a potent angiogenic factor and induces endothelial cell replication, migration and extracellular proteolysis (Szabo et al. 1991). It may promote angiogenesis both by a direct effect on endothelial cells and indirectly by the upregulation of VEGF in endothelial cells (Stavri et al. 1995). VEGF and bFGF act synergistically in the induction of angiogenesis both in vitro (Pepper et al. 1991) and in vivo (Mattern et al. 1997). Also, induction of bFGF induced angiogenesis is partly dependent on the activation of VEGF (Tille et al. 2001). In contrast, endostatin, the most potent inhibitor of angiogenesis (O’Reilly et al., 1997) acts via inhibition of endothelial cell growth and migration, apoptosis promotion, and antagonization of VEGF.

PDGF regulates a diverse array of cellular processes including cell migration, proliferation, transformation, and apoptosis (Martinet Y et al., 1986). It has been suggested to play critical roles in wound healing and also in disorders such as neoplasia and atherosclerosis. PDGF-stimulated cell proliferation is mediated via ligand-dependent dimerization and phosphorylation of PDGFR-α and/or PDGFR-β, which results in the activation of Ras and the downstream MAP kinase signaling pathway to promote cell proliferation (Heldin CH et al., 2002).

Increased expression of bFGF has been noted in granulation tissue of gastric (Satoh et al., 1992) and duodenal ulcers (Folkman et al., 1991) in rats and a neutralising anti-bFGF antibody delayed healing of gastric ulcers in the same rat model (Satoh et al., 1992). In an open, pilot study, administration of bFGF to patients with NSAID-associated gastric ulcers showed a 44% healing rate and a mean 89% decrease in area of unhealed gastric ulcers at four weeks (Hull et al., 1995).

During the current investigation, it was observed that the indomethacin-induced gastric ulceration was associated with a significant reduction in VEGF level and an increase in the endostatin level in serum (Table 4.4). The fundamental angiogenesis regulator, VEGF binds to at least two specific receptors; VEGF-R1 or Flt-1 and VEGF-R2 or Flk-1/KDR, expressed mainly on endothelial cells. These receptors initiates phosphorylation of numerous cytosolic proteins involved in signal transduction that triggers endothelial cell proliferation, migration and microvascular tube formation (Ferrara, 1999). The down-
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regulation of the expressions of VEGF and Flt-1 in the gastric tissue was observed much latter than the ulcer initiation, but that of Flk-1 started within 8 h of ulcer induction. Along with this, reduced circulating level of PDGF (Fig. 4.16), and reduced expressions of PDGF and bFGF (Fig. 4.17) were also observed in the ulcerated mice. All these clearly established impaired ulcer healing by indomethacin. Interestingly, the expressions of all the pro-angiogenic proteins were practically non-existent at peak ulceration.

Treatment with the mal C reversed these parameters at serum and tissue levels. Consistent with these results the number of microvessels in granulation tissues that were markedly reduced due to ulceration was also improved by mal C (Table 4.4). The reduction in the number of microvessels on indomethacin administration reflected inhibition of angiogenesis, resulting in delayed ulcer healing in mice. Since mal C restored most of the angiogenic parameters to their respective normal values, it accelerated the ulcer healing.

5.8. Mal C inhibits leukocyte-endothelial cell adhesion in healing ulcer

Like in all inflammation, the pathophysiology of NSAID induced gastropathy is governed by leucocytes-endothelial cell interactions (Wallace et al., 1990). The intricate process follows a large number of sequential activities, coordinated by recruitment of different mediators like the selectins and CAMs at different time points. Following the primary insult, macrophages and mast cells are stimulated to release mediators, such as histamine, ROS, PA?, leukotrienes, and cytokines. The engagement of the mediators with their receptors on endothelial cells results in the rapid mobilization of P-selectin from its preformed pool in Weibel-Palade bodies to the cell surface. Within minutes there is an increased recruitment of rolling leukocytes in postcapillary venules that allows for an enhanced exposure of the previously circulating cells to other mediators, liberated from the inflamed tissue. This is followed by shedding of L-selectin on leukocytes and a corresponding increase in the expression and activation of β2-integrins on leukocytes. The newly expressed and/or activated CD11/CD18 can then bind to its counter-receptor ICAM-1, which is constitutively expressed on endothelial cells. The adhesive interactions enable recruitment of firmly adherent and emigrating leukocytes, and also promote the homophilic adhesion and emigration of leukocytes via PECAM-1. Eventually via specific signaling pathways, the nuclear transcription factors, responsible for the biosynthesis of endothelial
cell adhesion molecules are activated. Consequently, within a few hours after the initial insult, there is a profound increase in the density of virtually all endothelial CAMs to sustain inflammation at a higher level and for a longer duration (Panés et al., 1999).

Maximum concentrations of soluble L- and E-selectins (Fig. 4.18a and b) levels, within 8 h of indomethacin administration suggested significant shedding of L-selectin that is required for progression of inflammation by leukocyte-endothelial cell adhesion. Thereafter, since these are not required, their concentrations would reduce, as observed in the investigation. On the other hand, initially the concentration of P-selectin would be more at the inflamed tissue to trigger the chain of events. Hence the soluble P-selectin was built only at 36 h. Regarding the soluble ICAM-1 and VCAM-1, both these modulators at the same time, but at the initial time point the serum ICAM-1 level was less. This reflected a greater role of tissue ICAM-1 in the indomethacin-mediated ulceration. Apparently, up to 8 h, the tissue ICAM-1 expression was high and shedding was low, resulting in a more gradual increase in the level of soluble ICAM-1. These results grossly correlated with the earlier report where significant increases of gastric mucosal surface expression of P-selectin and ICAM-1 has been shown in an established rat model of NSAID induced gastropathy (Morise et al., 1998).

The anti-inflammatory property was reflected from the fact that treatment with mal C brought all these inflammatory modulators at least partially. Wallace et al. (1990) have shown that NSAID induced gastric mucosal injuries are significantly reduced by immunoneutralisation of CD18, intercellular adhesion molecule 1 (ICAM-1), P-selectin, and to a lesser extent E-selectin. The present data is consistent with this.

Several antihistamines and mast cell stabilizers have been developed to antagonize the actions of inflammatory mediators and are widely used in the treatment of inflammation. However, there is no evidence that they directly inhibit leukocyte-endothelial cell adhesion as a primary mode of action. The present results clearly revealed that mal C reduces a large number of key inflammatory modulators to prevent leukocyte-endothelial cell interaction. Amongst other factors discussed above, this also is responsible for its healing activity against NSAID-induced gastric ulceration.
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ANTI-INFLAMMATORY PROPERTY OF MAL C
Inflammation is a complex stereotypical response of the body to cell damage and vascularized tissues, and is the oldest defense mechanism that is controlled by cytokines, products of the plasma enzyme systems, lipid mediators released from different cells, and vasoactive mediators released from mast cells, basophils and platelets (Ross et al., 2002). However, overproduction of these inflammatory mediators leads to many diseases such as rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis and cancer. Pharmacological options for the treatment of inflammatory diseases are associated with severe side effects, and therefore the search for less toxic, yet equally efficacious compounds is an area of intense research (Mckellar et al., 2007). Although several drugs, including the NSAIDs are extensively used as anti-inflammatory agents, most of these have side effects in the gastrointestinal tract and other body systems (Lee and Burckart, 1998; Makarov, 2001).

In the previous section the overall non-toxicity, in particular to GI tract of mal C was clearly demonstrated. These observations revealed that in contrast to the conventional NSAIDs, mal C may be a potentially better agent to control anti-inflammation. Hence, in this study, this aspect was investigated using several established acute inflammatory in vivo and cellular models, and the molecular mechanism underlying its action was established with the LPS-induced mouse macrophage RAW 264.7 cell line as the inflammatory model.

5.9. Mal C acts as a superior anti-inflammatory agent in mice

Increased vascular permeability is considered as one of the essential features of acute inflammatory responses (Fujii et al., 2000; Wada et al., 2000). LPS-induced animal models are used to examine the effects of different medicines and food on systemic inflammation since LPS stimulation augments the inflammatory response resulting in septic shock (Oberbeck et al., 2003). Therefore, the anti-inflammatory property of mal C was examined by using LPS as the stimulator for systemic inflammation in mice and assessing the protective effect of mal C, if any on the vascular permeability.

Topical administration of LPS in mouse acts mainly on the vascular endothelial cells to elevate cutaneous vascular permeability which is facilitated by many inflammatory mediators like TNF-α, IL-1α, histamine, NO and PGs. It was found that topical administration of LPS on mouse skin led to increased vascular permeability that was associated with enhanced levels of plasma nitrite and the pro-inflammatory cytokines,
TNF-α and IL-1β. Pre-treatment of mice with mal C dose-dependently reduced the cutaneous vascular permeability as well as the nitrite level in the LPS-challenged mice, confirming its anti-inflammatory effect in vivo. The induced internal tolerance on vascular permeability by mal C is probably through its direct effect on the endothelial cells. The results established the optimum dose for mal C as 10 mg/kg. At the same dose, mal C also reduced the LPS-induced stimulated release of TNF-α and IL-1β in mice in a time dependent manner. The potency of mal C (10 mg/kg) was similar to that of the positive controls, Omez (5 mg/kg) and dexamethasone (50 mg/kg).

5.10. Mal C provides immediate defense against inflammatory agents

To explore the mechanism underlying this anti-inflammatory action, we examined the effect of mal C on inflammation related macrophage function. Macrophages, ubiquitously distributed in tissues are the key players in inflammation and provide an immediate defense against foreign elements prior to leukocyte migration (Zhang and Mosser, 2008). Endotoxins such as LPS induce pro-inflammatory mediators and cytokines that contribute to the sequence of events in activated macrophage cells during the inflammatory process (Laskin and Pendino, 1995). It was previously shown that stimulation of macrophages by LPS results in the expression of iNOS, which catalyzes the production of NO from L-arginine (Nussler and Billiar, 1993; Xie et al., 1994). NO acts as an intracellular messenger and regulates cellular functions such as vasorelaxation and inflammation, while its overproduction in macrophages, predominantly via upregulation of iNOS contributes to numerous pathological processes, including the induction of tissue injury in inflammation (Bogdan, 2001). Physiologically, high-output NO can provoke deleterious consequences including tissue injury and septic shock, due to the generation of reactive radicals like peroxynitrite. In addition, macrophages also release PGs which play a major role as mediators in inflammatory processes. Between the two COX isozymes, COX-2 is induced by several stimuli, and is responsible for the production of large amounts of PG at the inflammatory site (Lee et al., 1992). Hence, the effect of mal C on the LPS-stimulated production of NO and PGE₂ and the expression of iNOS, COX-2 was investigated.

Pre-treatment of macrophage cells with mal C inhibited the LPS-induced nitrite and PGE₂ production in a dose dependent manner. The inhibitory effect of mal C for nitrite was
not due to cell damage since it was not cytotoxic (up to 75 µM), as revealed by the MTT assay. However, while post-administration of the specific iNOS inhibitor, 1400W could suppress the augmented iNOS activity in the LPS-treated cells, mal C had no appreciable effect. This confirmed that mal C did not inhibit the iNOS activity, although the western blot analysis revealed that it reduced the iNOS expression concentration dependently. Taken together, these results suggested a possible upstream inhibition of iNOS by mal C.

To confirm this, the ability of mal C to inhibit the transcriptional activation of the iNOS gene was examined. For this, the iNOS-transfected RAW 264.7 cells were prepared and the iNOS promoter activity in them was examined. The results confirmed that mal C blocked the LPS-induced iNOS promoter activity in the transfected cells. In separate experiments, mal C was also found to down regulate the expression of the important pro-inflammatory enzyme, COX-2 and also PGE$_2$ level that was barely detectable in the unstimulated as well as only mal C-treated cells. The down regulation of PGE$_2$ by mal C may be due to its ability to block the COX-2 expression in the LPS-treated cells.

5.11. Mal C exerts anti-inflammatory property by altering the cytokines balance

To explore the mechanism underlying this anti-inflammatory action, the effect of mal C on inflammation related macrophage function was subsequently examined. Adoptive immune responses of T cells, dendritic cells, and B cells contribute immensely in the pathogenesis of inflammation by producing cytokines and chemokines. Macrophages play a key role in the specific and non-specific immune responses during inflammatory processes by producing many pro-inflammatory molecules including cytokines. The cytokines are principal factors in the process of macrophage activation, aggravation of inflammatory diseases. They mediate the tissue responses in different stages of inflammation (Laskin and Pendino, 1995).

Excessive production of TNF-α plays key role in amplifying inflammation by triggering a cascade of cytokines, responsible for increased mobilization of macrophages at the site of inflammation. It also increases vascular permeability through the activation of NF-κB, activator protein-1 (AP-1) and other transcription factors (Kips et al., 1993). In rheumatoid arthritis and inflammatory bowel disease, humanized blocking of monoclonal antibody to TNF-α and its soluble receptors have produced remarkable clinical responses,
even in patients who are relatively unresponsive to steroids (Markham et al., 2000). Further, the pro-inflammatory cytokines such as TNF-α, and IL-1β induce the expressions of iNOS and COX-2, resulting in the production of NO and PGE₂, respectively. The study revealed significant LPS-mediated augmentation of TNF-α, and IL-1β in mice as well as the RAW cells that correlated very well with increased expressions of iNOS and COX-2, and their activities (as revealed from the NO and PGE₂ levels). Expectedly, mal C treatment reversed the changes in all these parameters. These suggested that the amelioration of TNF-α by mal C at both in vivo and in vitro conditions can be harnessed for its use as an effective TNF blocker.

The pro-inflammatory cytokine, IL-12 is comprised of two disulfide-linked subunits designated as p35 and p40. Elevated iNOS production during pathological disease state is associated with upregulation of IL-12p40 level (Pahan et al., 2001), which also contributes to septic shock and autoimmune diseases (LaSala et al., 2005). On the other hand, IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of many inflammatory proteins, including cytokines (TNF-α, GM-CSF, IL-5, chemokines) and inflammatory enzymes (iNOS) that are over-expressed in inflammation (Pretolani and Goldman, 1997). The down regulation of IL-12p40, in conjunction with increased expression of IL-10 by pre-treatment of the LPS-stimulated RAW cells with mal C explained its ability to reduce various inflammatory modulators (NO, iNOS and COX-2), and thereby inflammation.

5.12. Mal C acts as an antioxidant and inhibits NF-κB activation in inflammation

Nuclear factor κB (NF-κB) plays a central role in the regulation of many immune and inflammatory processes including sepsis (Tak and Firestein, 2001; Aupperle et al., 2001). Therefore, regulation of NF-κB activation represents an opportunity for the development of novel therapeutics for the inflammatory diseases. Many anti-inflammatory drugs, such as glucocorticoids, NSAIDs, and other immunosuppressants, which act as inhibitors of the NF-κB pathway and suppress the expression of different inflammatory genes, are used for the treatment of various inflammatory diseases (Lee and Burckart, 1998).

The inflammatory responses of LPS in macrophages include the initial induction of ROS, which leads to the activation of MAPKs and NF-κB and induction of iNOS protein
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expression and NO production. Stimulation of macrophages with LPS generates ROS via the activation of a membrane-bound NADPH oxidase which plays important role in propagating inflammation through secretion of pro-inflammatory molecules (Forman and Torres, 2001) and by regulating signal transduction pathways. The antioxidant chemicals inhibit NF-κB activation and suppress NF-κB dependent (Torres and Forman, 2003) gene expressions. Since, mal C suppressed the LPS-induced augmentation of these parameters in the RAW 264.7 cells, we examined the effects of LPS alone and in conjunction with mal C on production of ROS. This and all subsequent experiments were carried out using mal C at its IC50 value (~10 μM). The intracellular ROS levels can be conveniently assayed from the fluorescence enhancement of the cell permeable oxidation sensitive probe, DCFH-DA (de Arriba et al., 2006). The protocol used in the studies confirmed generation of excessive ROS in the LPS-treated RAW 264.7 cells. Mal C showed impressive intracellular ROS scavenging activity in RAW 264.7 cells, suggesting a possible antioxidant mechanism for its anti-inflammatory property.

At inflammatory sites, pro-inflammatory cytokines such as TNF-α and IL-1β are produced by immune-activated macrophages, which exert various actions through the activation of the NF-κB signal pathway (Tak and Firestein, 2001; Aupperle et al., 2001). The murine iNOS promoter contains an NF-κB-binding element at -85 bp serving as a major regulatory factor for iNOS expression and NO production (Xie et al., 1994). To confirm that the inhibition of the expression of iNOS and COX-2 by mal C is influenced by the NF-κB signaling pathway, its effect on the NF-κB expressions in cytoplasm and nucleus, as well as the NF-κB-DNA binding activity in the LPS-treated macrophages were examined.

Our western blot showed significant translocation of the NF-κB p65 subunit to the nucleus in the LPS-treated cells compared to the control cells. Mal C blocked the translocation by inhibiting phosphorylation of I-κB-α without affecting its basal level. In addition, it also reduced the DNA binding activity of the p65 subunit in the promoter regions of the inflammatory modulators. Although NF-κB-inducing kinase (NIK) cannot directly phosphorylate IkBα in vitro (Fischer et al., 1999), it appears to be a functionally important subunit for the NF-κB pathway, because mutated NIK inhibited NF-κB activation in TNF-α or LPS-stimulated monocytes more effectively than mutated IKKα or
IKKβ (Lee et al., 2003). Both NIK and Akt have been implicated in the canonical NF-κB pathway by converging on IKK to promote NF-κB activation at two sites: IKKα at threonine 23 (Akt-mediated) and IKKαβ at serines 176/177 (NIK mediated) (Ozes et al., 1999; Hu et al., 2005). NIK can also directly activate IKKαβ via phosphorylation at serines 180/181. Therefore, these events promote the phosphorylation and proteolytic degradation of IκB. Because mal C did not show any effect on the phospho form of Akt (data not shown), it might be regulating the canonical NF-κB activation by inhibiting the NIK activation. Thus, mal C inhibits the inflammatory gene products, iNOS and COX-2 by controlling ROS generation, thereby inactivating the NF-κB pathway.

5.13. Mal C also inhibits MAPKs activation in inflammation

There is compelling evidence supporting that MAPKs regulates NF-κB activation by multiple mechanisms (Carter et al., 1999). At least three families of MAP kinases (ERK, JNK, and p38 MAPK), present in mammalian cells play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokines and stress (Johnson and Lapadat, 2002). Studies have demonstrated the implication of MAPKs in LPS-induced iNOS expression (Kim et al., 2004) and NF-κB activation (Carter et al., 1999). Cell stimulation induces a signaling cascade that leads to the activation of MAPKs via phosphorylation of both tyrosine and threonine residues (Kyriakis and Avruch, 1996). This, in turn, induces a conformational change exposing the active site for substrate binding. Also, the specific MAPKs inhibitors suppress the expression of the iNOS gene. LPS-induced activation of MAPKs transduces signals to activate the transcription of NF-κB-mediated proinflammatory cytokines which play a critical role in the regulation of cell growth and differentiation as well as the control of cellular responses. Hence, the role of MAPKs has been studied intensively.

It was found that incubation of RAW 264.7 cells with LPS led to activation of all the three families of MAPKs, and treatment with mal C inhibited the LPS-induced phosphorylation of p38 and JNK, but not ERK 1/2. The results with p38-, and JNK-, and ERK 1/2-specific inhibitors as well as NF-κB-specific inhibitor confirmed that the increase in nitrite and PG levels due to the LPS treatment were mediated through the MAPK and NF-κB pathways, and mal C blocked these.
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JNK has been reported to be an important mediator of inflammatory processes induced by LPS (Davis, 2000) and regulates AP-1 transcription factor activity and the production of inflammatory cytokines (Dong et al., 2002). A major substrate for JNK kinase is c-Jun, a central component of the AP-1 heterodimer. Mal C also suppressed the expression of phospho-c-jun suggesting the involvement of AP-1 transcription factor in the process. AP-1 is composed of members of the Jun (c-jun, junB, and junD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) families, but the most common are the c-jun/c-Fos heterodimers that bind to the TPA-response elements (AP-1 sites) in the promoter of various genes. A c-jun component of AP-1 rather than the c-Fos is part of the complex at AP-1 sites in the RAW 264.7 cells (Martin and Martin, 2005). Thus, the reduction in the nuclear translocation of c-jun by mal C, at the same concentration provided a clear evidence of inhibition of AP-1 activation, and thereby of JNK.

The role of NF-kB in regulating COX-2 expression is ambiguous. Studies to analyze the function of cis-acting elements such as, CREB, C/EBPb, and NFkB using mutant COX-2 promoters has clearly demonstrated that NF-kB is much less important in inducing COX-2 expression in the LPS-stimulated macrophages compared with the other two elements (Kang et al., 2006). Besides inhibiting the NF-κB signal pathway, mal C also blocked phosphorylation-dependent activation of JNK (responsible for C/EBPb activation) in the LPS-stimulated macrophages, indicating that it specifically inhibits both NF-κB activation as well as C/EBPb activation.

A substantial body of data indicates that NF-kB activation is modulated by MAP kinase/ERK kinase kinase-1 (MEKK1), a kinase upstream of MAP kinases (Reiser et al., 1998; Wang Richmond, 2001; Bonvin et al., 2002). However, more recently, another protein suggested to play a role in TLR signaling is double-stranded RNA (dsRNA)-activated serine/threonine kinase R (PKR), a well-characterized component of the antiviral response (Cabanski et al. 2008). In response to bacterial LPS, PKR was found to be involved in the activation of JNK, but not p38 or ERK1/2 pathways of MAPKs and of transcription factors such as NF-κB or signal transducer and activator of transcription-1 (STAT1). Thus, the LPS-mediated activation of TLR-4 and subsequently of NF-κB may not necessarily require MAPK activation.
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MAP kinases are known to regulate NF-kB activation by multiple mechanisms. Accumulating evidence indicates that NF-kB activation is modulated by ERK as well as p38 MAP kinases. Between these two kinases, mal C inhibited the p38 MAP kinase only. Thus, its effect on NF-kB activation would be regulated if at all, via p38 MAP kinase. However, the present data revealed that although mal C strongly down-regulated JNK phosphorylation, its capacity to inhibit p38 phosphorylation was much less. This suggested that mal C may be inhibiting the LPS-induced NF-κB activation directly with minor contribution from the MAPK pathway. Based on this evidence, it is tempting to propose that the mal C-mediated NF-κB regulation may involve the PKR pathway. The predominant effect of mal C on the JNK pathway is consistent with this hypothesis. Previous studies have demonstrated that PKR-mediated activation of MAPKs in response to inflammatory stimuli in various cell types (Goh et al., 2000). Recently, LPS has also been reported to activate PKR in mouse tissue macrophages (Hsu et al., 2004).


LPS-induced activation of macrophages is mainly mediated through transmembrane signaling TLR 4 and results in a potent inflammatory response characterized by the release of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12 as well as other mediators such as iNOS and COX-2.

The activated TLR4, in turn, activates several intracellular signaling pathways through recruitment of adaptor proteins such as MyD88 and TRIF (Iwasaki and Medzhitov, 2004), involved in the IKK-NF-κB and MAP kinase pathways. These signaling pathways activate a variety of transcription factors. Since mal C showed profound effect on the inflammatory gene products of the NF-κB pathway, its role in LPS-medicated NF-κB activation in the RAW cells was examined. The receptor complex composed of TLR4 and MD-2 constitutes a molecular antenna that recognizes and signals mammalian cells for the presence of LPS. Stimulation of TLR4 facilitates the activation of the MyD88-dependent and MyD88-independent pathways, which have already been described in chapter 2 (Fig. 2.6). The MyD88-dependent pathway is involved in the early phase of NF-κB activation and MAP kinase pathways (Iwasaki and Medzhitov, 2004). The promoter regions of the pro-inflammatory mediators contain several binding sites for transcriptional factors, such as NF-κB (p50/p65) and activator protein-1, AP-1 (c-Fos/c-Jun), causing inflammation.
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Although inhibition of downstream molecules such as cytokines, growth factors, interleukins, and NO, regulated by NF-κB has been extensively studied, upstream signal transduction pathway leading to the inhibition of NF-κB is poorly understood. Because NF-κB is induced through various pathways, identifying upstream signalling molecules shall prove a better target for managing inflammation. Our studies revealed that mal C inhibited the MyD88-dependent pathway, since it downregulated the expression of MyD88, resulting in reduction in the phosphorylation IRAK-4 and TRAF6 in the LPS-treated cells. This would reduce NF-κB activation. The mechanism of action of mal C can be summarized as shown in Fig. 5.1.

Fig. 5.1. Possible molecular mechanism of the anti-inflammatory activity of mal C.
5.15. Mal C is a safe and effective anti-inflammatory Agent

Despite their extensive clinical use, the NSAIDs are far from being ideal as anti-inflammatory agents. Several of these, including the NSAIDs are critically involved in the pathogenesis of various diseases, and have side effects in the GI tract and other body systems (Lee and Burckart, 1998; Makarov, 2001). Recently, TNF-α-neutralizing antibody and IL-1 receptor antagonist have been shown to provide sustained clinical benefits (Firestein et al., 1992). However, these biological agents also have adverse effects including serious infection (Slifman et al., 2003). Therefore, new and safe immunosuppressive drugs without side effects are needed to manage inflammatory disorders in vitro and in vivo. It was found that mal C blocked the expression of NF-κB dependent pro-inflammatory genes, iNOS, COX-2, TNF-α and IL-1β, in LPS-stimulated RAW 264.7 macrophages cells, through suppressing the phosphorylation of I-κBα and the activation of NF-κB, and by inhibiting the p38 and JNK pathways, which, in turn, inhibits c-jun phosphorylation. The inhibition of the LPS-mediated NF-κB activation by mal C is due to its ability to block the MyD88-dependent pathway of TLR4 signaling. It also reduced vascular permeability and LPS-mediated inflammation in mice. The above findings, in conjunction with its non-toxicity to normal animals and cells, even up to a high dose of 500 mg/kg suggested mal C as a potential new phytochemical for the prevention of inflammatory diseases. However, further work especially with its biodistribution, pharmacokinetics and pharmacodynamics is warranted for this purpose. Based on this evidence, mal C is proposed to be a promising new phytochemical agent for prevention of inflammatory diseases.