8. Discussion

Etoposide is one of the most commonly used chemotherapeutic agents in clinical oncological practice. However, side effects are prevalent and result from the inhibition of rapidly dividing cells including gastrointestinal mucosal cells, bone marrow, haemopoietic cells (264). Clinical studies have shown that etoposide induces mucositis, myelosuppression, nausea, emesis and diarrhoea associated with histopathological changes in small intestine(264, 265). However, the association between mucositis and the intestinal inflammatory network remains unclear. The present study demonstrated the role of inflammatory cytokines such as TNF-α & IL-6 and histological changes in rat models of etoposide induced mucositis. This study also showed changes in enzyme systems such as sucrase, sodium potassium ATPase and apoptosis which reflects the extent of DNA damage, suggesting that these components may play a crucial role in the development of etoposide-induced mucositis.

8.1 Food, water intake and body weight

In the present study animals exposed to chemotherapy showed a significant reduction in the water and food intake which eventually resulted in reduced body weight compared to normal control. Administration of Spondias pinnata (post or pre & post, both the doses) and whey preparation (post or pre & post, 100 & 200mg/kg b.w) effectively increased food and water intake resulting in increased body weight of the animal.

In one study on lung cancer patients who were on chemotherapy, it has been reported that calorie intake was significantly lower (approximately 300kcal/day) resulting in weight-loss compared to patients who were not on chemotherapy (266). The decreased nutrient intake resulting in weight loss can be attributed to a variety of factors including alterations in taste and smell; chemotherapy induced anorexia, nausea, vomiting, diarrhoea, stomatitis, mucositis and dysphagia (267). Anorexia may occur due to physiological alterations in metabolism during chemotherapy and it can even hasten the course of cachexia, a progressive wasting syndrome characterised by weakness and marked weight loss (268, 269).

The study reported that the cause for improved food and water intake after administration of Spondias pinnata and whey appear to be three-fold. Firstly,
Discussion

*Spondias pinnata* and whey preparation help to repair/restore the epithelial cell, and it may be involved in healing of the mucosal layer which was confirmed by histological study. Secondly, they decrease the extent of duodenal apoptosis due to chemotherapy thus preventing mucosal breakdown and crypt damage. Thirdly, *Spondias pinnata* and whey preparation decreased the levels of TNF-α and IL-6 that had increased after etoposide treatment, resulting in reduced inflammation of duodenum.

8.2 Histological study

The present study showed an extensive mucosal damage resulting in villi destruction in small intestine of rats exposed to chemotherapy. Treatment with *Spondias pinnata* and whey preparation (100 & 200mg/kg b.w) following chemotherapy showed a decrease in the extent of cell destruction in small intestine. Similar effect was observed in animals which received *Spondias pinnata* and whey preparation before and after etoposide administration (Fig 9 & 18).

Mitchell has shown maximum apoptosis in small intestine after chemotherapy which resulted in decreased number of rapidly dividing stem cells of duodenum (270). Results from both human and animal studies also showed crypt hypoplasia in small intestine after chemotherapy (243-248, 270). Trier demonstrated an increased villi destruction 3–48 hours after methotrexate treatment in human subjects (245). Ijiri and Potten showed that all cytotoxic drugs in animals cause both apoptosis and villi damage due to their effect on fast dividing cells (243, 244).

8.3 *Spondias pinnata*, whey preparation and inflammatory cytokines

Current study showed that administration of etoposide increases the level of inflammatory cytokines (IL-6 and TNF-α). Treatment with *Spondias pinnata* showed a significant decrease the levels of both inflammatory cytokines when compared to chemotherapy group. Post treatment with *Spondias pinnata* showed better results when compared with pre & post treatment. Previous studies implicated the role of pro-inflammatory cytokines in the pathogenesis of chemotherapy-induced mucositis (57, 65, 202). Tissue injury causes the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) which are involved in many aspects of inflammation, including cell migration, edema development, fever and hyperalgesia. The TNF-α produced, in turn releases the
prostaglandins via cyclooxygenase pathway which ultimately result in inflammation (271, 272).

24-methylene cycloartenone a component of *Spondias pinnata*, is known to prevent chemotherapy induced intestinal epithelial damage (273). The protective effect against the intestinal damage could be due to the regulation of inflammatory cytokine levels in intestine.

It has been shown that whey preparation has some anti-inflammatory properties (130). An earlier report showed that whey preparation administered to hamsters demonstrated its ability to protect against 5-fluourouracil induced oral mucositis (274). Study by Chen Y showed that blockage of TGF-β signaling by specific inhibitors (Metelimumab and Lerdelimumab) of the TGF-β receptor I significantly reduced the production of TNF-α and other inflammatory cytokines such as IL-6 and IL-1 in human monocyte-derived macrophages (275). In the current study, post treatment with whey (100 & 200mg/kg b.w) showed a significant decrease in TNF-α and IL-6 levels when compared to pre and post chemotherapy.

Figure 10: Primary damage response after chemotherapy: Signalling occurs from chemotherapy and/or radiation therapy and ROS causes DNA damage and subsequent cell death in the epithelium of the mucosa. They also activate various transcription factors, leading to increased production of inflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-6, ultimately apoptosis (276) (Erowele,G. and Ndefo, U, 2009)


8.4 *Spondias pinnata* & whey preparation and apoptosis

Chemotherapeutic agents are known to cause DNA damage leading to apoptosis (277). Present study showed increased rate of apoptosis due to etoposide compared to control and was consistent with earlier report (278). Rats treated with *Spondias pinnata* showed decreased rate of apoptosis indicating an active restoration of mucosal damage. Better effect was observed in the rats which received 200mg/kg b.w of *Spondias pinnata* following chemotherapy than in rats which received 100mg/kg b.w of *Spondias pinnata*. Similar effect was observed in rats treated with *Spondias pinnata* (100mg/kg b.w & 200mg/kg b.w) before and after chemotherapy. Previous study showed that epithelial-derived IL-1β as the cause of mucositis onset, inducing mucosal barrier breach. Epithelial IL-1β is induced by DNA damage via an NF-kB–dependent mechanism resulting in cell apoptosis (279). Similar to the previous data (21), the present study also showed that chemotherapy-induced mucositis occurred by direct injury to basal epithelial cells and cells in the underlying tissue, which may be a consequences of DNA strand break. *Spondias pinnata* acts as therapeutic potential and is known to reduce tissue injury and prevent oxidative stress-mediated diseases such as cancer, atherosclerosis, diabetes and ageing (120) and it contains sterols such as 24-methylene cycloartanone, stigmastadienol and β-sitosterol which repair the tissue injury. Apoptosis and cell proliferation are regulated by JNKs pathway. The activation of JNKs leads to cell proliferation or apoptosis dependent on the type of stimuli involved in such activation. Extrinsic signalling pathway initiated by death receptors such as TNF-α, TRAIL (TNF-related apoptosis-inducing ligand) and FAS-ligand, bind to cell surface receptor (component of JNK-STAT system). These signals to the nucleus resulting in increased DNA transcription and expression of genes involved in apoptosis (280). Previous studies showed that β-sitosterol brings about dephosphorylation of the activated JAK and/or STAT and thus reducing inflammation (281, 282). In the present study anti apoptotic effect of *Spondias pinnata* may be due to inhibition of JNK pathway.

Whey is known to induce anti-inflammatory effect in the intestine of newborns (283). The present study showed that there was a decrease in apoptosis in the mucosa of rats treated with whey preparation indicating an active restoration of mucosal damage. Better effect (16.9%) was observed in the rats which received 200mg/kg b.w of whey.
before and after chemotherapy than in rats which received 100mg/kg b.w of whey preparation. Similar effect was observed in rats exposed to chemotherapy followed by whey preparation (100mg/kg b.w & 200mg/kg b.w). Earlier data showed that rats fed with whey protein prevents the DNA damage in breast carcinoma. The possible mechanism could be through tumor suppressor (Tp53 or p53) gene (284). The tumor suppressor Tp53 is considered to be one of the most important molecular players in the pathogenesis of all types of cancers. Loss of Tp53 function underlies decreased genomic stability and is associated with defect in DNA damage repair and loss of cell cycle control (285, 286). The present study showed whey has beneficial effects through decreasing the cell apoptosis which combated the chemotherapy induced mucosal toxicity in small intestine.

An earlier study showed that whey protein hydrolase (WHP) altered the transcript levels for Phosphatase and tensin homolog (PTEN), Tp53 and amphiregulin (AREG) in histologically normal mammary glands of tumor-bearing rats induced by N-Methyl-N-Nitrosourea (287). Further the transcript levels of suppressor gene activity of p53 reduced in these tissues were attenuated by dietary WPH, suggesting its role in DNA damage repair and tumor-protective effects.
Figure 11: The events in p53 activation by chemotherapy via DNA damage.

8.5 Chemotherapy and oxidative stress

8.5.1 Spondias pinnata & whey preparation and reduced glutathione

The generation of oxygen radicals is normally balanced by the presence of adequate endogenous antioxidant defence mechanisms (288). Oxidative stress has been implicated in the pathology of mucositis due to chemotherapy (289). Impaired mucositis healing occurs as a consequence of excessive ROS production.

Cysteine, which contains thiol (sulphhydryl) group, combines with glycine and glutamate form GSH. It acts as a major reductant in the cells. Through direct conjugation, GSH detoxifies a host of endogenous and exogenous toxins including toxic metals, petroleum distillates, lipid peroxides, bilirubin and prostaglandins (290). Thus, it serves as an active reducing agent in preventing tissue damage. Glutathione is
an indicator of a person’s overall health and wellbeing (291). When reduced glutathione levels are low, the person is in poor health or is fighting off an infection/disease. Oral administration of GSH showed beneficial effect on oxaliplatin-induced peripheral neuropathy (292). Further glutamate is precursor of GSH, may prevent neurotoxicity of paclitaxel, cisplatin, bortezomib and lenolidamide. Gaurav et al. demonstrated the beneficial effects of GSH in the reduction of gastrointestinal toxicity of irinotecan and 5-FU-induced mucositis and stomatitis (293). Present study showed significant decrease in GSH levels in rats exposed to chemotherapy compared to normal controls. This may be due to depletion of intracellular GSH consequent to chemotherapy. Administration of *Spondias pinnata* (100mg/kg b.w) following etoposide treatment increased the GSH levels. Treatment with *Spondias pinnata* (100 & 200mg/kg b.w) before and after etoposide restored the decreased glutathione levels induced by chemotherapy.

One of the previous studies showed that treatment with glutathione decreases cytokine activity in alcoholic liver disease. Pena et al. suggested monocytes and Kupffer cells of liver produce cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL-8) and IL-6 in response to an endotoxin like lipopolysaccharide [LPS]. This cytokine production is regulated by the oxidative stress-sensitive transcription factor NF-kB. Supplementation of glutathione (GSH) prodrugs such as oxathizolidine-4-carboxylic acid (OTZ) can inhibit activation of NF-kB and subsequent cytokine production in monocytes and Kupffer cells in vitro (294). OTZ is metabolized intracellularly by 5-oxo-L-prolinase to a compound that spontaneously decarboxylates to L-cysteine (295). Current study showed that administration of *spondias pinnata* enhances cellular glutathione by decreasing the levels of cytokines (TNF-α and IL-6) resulting in protective action against chemotherapy induced mucositis. This could be because β-sitosterol is one of the component *Spondias pinnata* (113). Marta et al. demonstrated that β-sitosterol might protect GSH from oxidation induced by phorbol 12-myristate 13-acetate (PMA) in RAW 264.7 macrophages. Further, the effects of β-sitosterol on antioxidant enzymes depend on the estrogen/phosphatidylinositol 3-kinase pathway (296). The estrogen receptors are expressed by macrophages (297). Beta sitosterol bind to estrogen receptor (298) with the enhancement in the GSH levels. The effects of beta sitosterol on antioxidant enzymes seem to be mediated by estrogen receptor activation.
The results of the present study showed that whey preparation (100 & 200mg/kg b.w) favourably influences synthesis of GSH. Administration of whey (100 & 200mg/kg b.w) before and after chemotherapy showed significant increase in the GSH levels compared to chemotherapy.

Many studies have confirmed the role of glutathione, a powerful antioxidant, which is increased by dietary whey protein (299, 300). WP is able to reduce the effects of oxygen radicals and lipid peroxidation by increasing the activity of the antioxidant glutathione, thus stimulating epithelisation and the proliferation of fibroblasts. WP has been found to significantly suppress hydroperoxide and ROS levels in leukocytes, liver and cutaneous tissues in mice by restoring the antioxidant glutathione (301). However, the exact mechanism by which whey protein achieves this is not fully understood.

Practitioners use whey protein along with chemotherapy, as a source of cysteine to increase intracellular glutathione levels which eventually protects normal tissue against the deleterious effects of chemotherapy (302, 303). The possible action of whey preparation seen in the present study may be through restoration of GSH levels.

8.6 Spondias pinnata & whey preparation and nitric oxide

Nitric oxide (NO), initially identified as an endothelial-derived relaxing factor (182), has been implicated in the pathogenesis of chemotherapy-related tissue damage (189). Nitric oxide is a highly reactive free radical that acts as a mediator of inflammatory responses (304). Present study showed significant increase in nitric oxide level after etoposide treatment compared to normal control. This indicates the role of reactive nitrogen species in etoposide induced intestinal damage. Treatment with Spondias pinnata (100mg/kg b.w) following etoposide showed significant reduction in the NO production. However, treatment with Spondias pinnata (200mg/kg b.w) before and after etoposide showed better effect than post treatment alone. A previous study showed that nitric oxide synthase (NOS) activity was more in rat duodenal tissue after the intravenous administration of 5-FU (305). One study on cardiopulmonary bypass reported that the excess production of cytokines such as TNF-alpha and IL-1 can cause the induction of NO synthase and the release of large amounts of NO that may cause tissue injury (306). Inhibition of NOS activity by 4-O-β-glucoside (307), one of
the components of *Spondias pinnata* (113), results in decrease the production of NO eventually reduces the mucosal injury in duodenum.

Administration of whey significantly reduced NO levels in all the groups except which received 200mg/kg b.w following chemotherapy.

The small intestine is a sensitive organ to chemotherapeutic drugs, which causes barrier dysfunction and enhances bacterial translocation (308). There is some evidence that injuries of the small intestine may be exacerbated significantly by elevated NO levels (309). NO rapidly reacts with superoxide anion to form peroxynitrite anion (ONOO\(^{-}\)), a highly reactive oxidizing agent which causes tissue damage (310). The beneficial effect of inhibition of NO production is through interference in the inflammatory mediator cascades by NF-κB transcriptional activation in a variety of cells, including monocytes and epithelial cells (311, 312). NF-κB is a DNA binding factor that is essential for the activation of several inflammatory mediators, e.g., tumor necrosis factor, IL-8, IL-1b, IL-2, and IL-6. Whey or αLA may have a latent effect to terminate aforesaid steps and protect against the chemotherapy induced mucosal damage (313). However, on this point, further experiment would be needed to explore the exact mechanism.

**8.7 *Spondias pinnata* & whey preparation and myeloperoxidase (MPO)**

During reperfusion injury, besides their direct damaging effect on the tissues, reactive oxygen species also trigger the local accumulation of leukocytes in the tissues and thus aggravate tissue injury indirectly through activation of neutrophils. The activated neutrophils secrete myeloperoxidase and proteases (314). In the present study an elevation in MPO activity observed after etoposide treatment, indicates that accumulation of neutrophils may contribute to etoposide induced small intestinal damage (315). However, none of the doses of *Spondias pinnata* (post or before and after chemotherapy) were able to restore/reverse the increased MPO activity, indicating that compounds of *Spondias pinnata* may not have any action on MPO activity.

Administration of whey decreases the activity of MPO in all the study groups.

It has been proposed that the gut barrier failure and the subsequent translocation of the enteric bacteria and endotoxin into the lumen, is a major contributing factor in the
development of mucositis (316, 317). The systemic inflammatory response to chemotherapy may induce the gut-associated lymphoid tissue to produce and liberate pro-inflammatory cytokines which stimulate the production of myeloperoxidase (318). Because lactoferrin attenuates the production of cytokines it may lower activity of myeloperoxidase and inhibit the development of mucositis in vivo (319, 320). Consistent with above report, present study also showed a decrease in the MPO levels after administration of whey preparation.

8.8 Spondias pinnata & whey preparation and cyclooxygenase (COX)

COX is an intracellular enzyme that catalyses the rate-limiting step in the synthesis of prostaglandins, a potent group of autocrine and paracrine lipid mediators that are involved in many normal cellular and pathophysiological processes (321). Inflammation causes the induction of cyclooxygenase-2 (Cox-2) (322). Present study showed increased COX-2 levels after chemotherapy compared to normal control. Treatment with Spondias pinnata (100 & 200mg/kg b.w) following etoposide showed significant reduction in COX-2 levels which indicates the protective action against mucositis. Administration of Spondias pinnata (in both doses 100 & 200mg/kg b.w) before and after etoposide showed significant decrease in COX-2 levels when compared to chemotherapy group. Cycloartenol, a phytosterol, has been attributed with anti-inflammatory property (323). It has been shown to be present in Spondias pinnata bark extract. The proposed mechanism is that cycloartenol may mimic the action of NSAIDs- decrease the production of prostaglandins by inhibiting the COX activity (324). This may be one of the reasons for Spondias pinnata showing beneficial effect towards chemotherapy induced mucositis.

Present study showed administration of whey (100 & 200mg/kg b.w) either following etoposide or both before and after etoposide showed significant reduction in the COX levels when compared to chemotherapy group. This finding showed that whey inhibited COX activity. Present result suggests that whey possibly reduces the gastrointestinal side-effects by inhibiting the induction of cyclooxygenase-2 in gastric cells. Alpha lactalbumin (α LA), β-lactoglobulin (βLG) and lactoferrin (LF) have been shown to be components of whey preparation (134). The decrease in COX levels could be due to inhibition of activity of COX by αLA through suppression of cytokines release (325). This suggests that inhibition of release of inflammatory
cytokines by αLA may contribute to its gastroprotective actions. It has also been reported that LF reduces inflammation in various animal models (326).

8.9 Spondias pinnata & whey preparation and Na+, K+-ATPase

The Na⁺, K⁺-ATPase localized in the basolateral membrane of intestinal absorptive cells or enterocytes plays a major role in nutrient transport in the small intestine by transferring K⁺ ions into and Na⁺ out of the cell (327). Present study showed a reduction in Na⁺, K⁺-ATPase activity which may be due to duodenal cell destruction by chemotherapy. Administration of Spondias pinnata (200mg/kg b.w) before and after etoposide showed significant increase Na⁺, K⁺-ATPase activity but not with only post treatment in both the doses. Within the enterocyte, ionic homeostasis is maintained by active extrusion of Na⁺ from the cell by the Na⁺, K⁺-ATPase or sodium pump. The activity of the Na pump may be coupled to other crucial functions of the cell, such as regulation of cell volume, nerve and muscle excitability, pH regulation, uptake of carbohydrates, amino acids and vitamins. The pathophysiology of chemotherapeutic drug induced mucositis is thought to be allied with disturbance of ionic homeostasis (328). An earlier study has shown that Na⁺, K⁺-ATPase activity is decreased in patients with haematological malignancies and GI malignancy who underwent chemotherapy (329). Present study showed that Spondias pinnata treatment restores the decreased Na⁺, K⁺-ATPase activity in rat duodenum, suggesting the potential effect of Spondias pinnata in restoring membrane functions as evidenced by regeneration of duodenal cells, supported by histological observation.

In the current study, administration of whey in both doses (100 & 200mg/kg b.w), post chemotherapy and also in pre and post chemotherapy group which received 100mg/kg b.w increased the level of Na⁺, K⁺-ATPase. A previous study showed that Na⁺, K⁺-ATPase activity inhibition can lead to disruption of mitochondrial energy metabolism in animal model (330). An altered ionic homeostasis has been reported in situations where Na⁺, K⁺-ATPase activity was reduced, such as under oxidative stress (331). This study showed elevation of suppressed Na⁺, K⁺-ATPase activity in rat duodenum.
8.10 *Spondias pinnata* & whey preparation and sucrase enzyme

Previous studies (2, 332, 333) showed that methotrexate induced mucositis causes villus blunting and crypt disruption leading to decreased sucrase activity in rat.

Present study showed a decrease in the sucrase activity after administration of etoposide compared to normal control. Rats treated with the *Spondias pinnata* (100 & 200mg/kg b.w) following chemotherapy showed significant increase in the sucrase activity when compared to etoposide treated group. Pre and post treatment with *Spondias pinnata* (200mg/kg b.w) also showed significant increases in the sucrase activity when compared to chemotherapy group.

Present study showed treatment with whey (100 & 200mg/kg b.w) following chemotherapy as well as administration of whey (100 & 200mg/kg b.w) before and after chemotherapy showed significant increase in sucrase activity when compared to etoposide control. Previous study showed that chemotherapy damages epithelial cells of mucosa that had digestive enzymes embedded in their microvilli (334). The decrease in the sucrase enzyme activity will affect the breakdown of sucrose in the small intestine (335). Present study showed restoration of sucrase enzyme by whey preparation administrated, suggesting a potential role of whey in rapid regeneration of damaged small intestinal mucosal cell.