Evaluation of Modulatory Effect of Combination of *Spondias Pinnata* and Whey Preparation on Intestinal Antioxidants and Inflammatory Markers in Etoposide Induced Rat Model for Mucositis

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ABSTRACT

**Background:** Etoposide is an important chemotherapeutic agent that is used to treat a wide spectrum of human cancers. The efficacy of this drug is often limited due to severe side-effects such as mucositis, anorexia, myelosuppression, alopecia. At present there are no anti-mucositis agents without side effects. To combat these side effects of cancer treatment, innovative and specific nutritional interventions are needed. The experimental procedure included an oral treatment with combination of *Spondias pinnata* and whey preparation (100 mg/kg b.w) on etoposide induced mucositis.

**Methods:** Study was conducted at Central Research Laboratory, Kasturba Medical College. Rats were sacrificed by cervical dislocation and duodenum collected for estimation of biochemical parameters. Estimation was carried out for assessing the levels of TAO, GSH, LPO, NO and activity of MPO. Correlation analysis was performed by one-way Anova using graph pad prism to find the relation between control and test.

**Result:** The antioxidant (TAO and GSH) were found to be significantly high in the rats which received the combination compared to the individual *Spondias pinnata* and whey treated groups. Administration *Spondias pinnata* and whey in combination shows significant decrease in the TBARS, NO and MPO levels.

**Conclusion:** *Spondias pinnata* and whey preparation supplementation in combination have shown mucoprotective effects in rat model. Hence it can be considered as anti-mucositis therapeutic agents which can be safely administered during chemotherapy and can reverse mucositis.

**Key words:** Etoposide, Mucositis, Myeloperoxidase (MPO), Lipid peroxidation (LPO), Nitric Oxide (NO), *Spondias pinnata*, Whey preparation.

INTRODUCTION

Chemotherapy and radiotherapy are mainstay regimens for cancer treatments. However, both types of cancer treatment also affect normal cells, and their side effects on highly proliferative tissues have significant problems. One of these adverse effects is mucositis, a painful inflammation and ulceration of the mucous membrane lining the gastrointestinal tract (GIT). Mucositis can affect the entire mucosal lining of the GIT, but the oral and oropharyngeal mucosa are common sites.

Mucositis, the mucosal barrier injury, is one of the most devitalizing side effects of radiotherapy and chemotherapy treatment.1 It is characterized by both inflammation and cell loss in the epithelial barrier lining, especially the gastrointestinal tract.2,3 The side effects of chemotherapy can be traced to cause myelosuppression, alopecia and mucositis.4 Chemotherapy causing inflammation and ulceration of mucous membrane lining the digestive tract, which results in reduced food intake and drop in the pH levels and may cause treatment reduction or withdrawal.5 Intestine being an important organ required for absorption of nutrients, due to the side effects the patients likely to suffer from loss of appetite and may also have deficiencies of vitamins, minerals etc. Thus, innovative and integrated nutritional approaches are needed to target several of these altered processes.6,7 Extensive experimental outcomes reported that many of the agents including growth factors like palifermin, NSAIID, sucrafate, antifungal medicines etc. Drugs currently under investigation include keratinocyte growth factor, interleukin-1 and 11and TGF-b.8,9 Both the cytoprotector, amifostine and the pineal hormone; melatonin have been claimed to have some effects in the prevention of mucositis. It has denoted that the potential mechanism of c thalidomide action might include inhibition of cytokines such as interleukin-1, interleukin-6 and TNF-a, that are supposed to take part in the pathogenesis of oral mucositis.10 Some of the dietary means including

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both animal and plant products could relieve some of the complications of mucositis.15

*Spondias pinnata* (Anacardiaceae) is a deciduous tree distributed in India, Sri Lanka and South-East Asian countries. The gum exudates of the species have been found to contain acidic polysaccharides.16 A crude extract of *S. pinnata* has been reported to show antibacterial activity. In ethnomedicine, bark juice of *S. pinnata* is prescribed as a remedy for dysentery.18 and is shown to have antioxidant and free radical scavenging activity.19 and some anti-mucositis property.

Whey preparation derived from milk, is being touted as a functional food and can act as an antioxidant, antihypertensive, antioxidant, hypolipidemic, antiviral, antibacterial and chelating agent. Moreover, the whey preparation is an ethnic dietary component used during diarrhea. Many clinical trials have successfully been performed using whey in the treatment of cancer, HIV, hepatitis B, cardiovascular disease, osteoporosis, and as an antimicrobial agent also. Whey protein has also exhibited benefit in the arena of exercise modulatory effect of SP and WP on rat intestine with mucositis performance and enhancement.20 Here we tried to find the effect of such dietary component on mucositis.

**MATERIALS AND METHODS**

**Experimental design**

Adult albino female rats of Wistar strain were used for the study. Animals weighing about 220-250 g obtained from Central animal house, Kasturba Medical College, Mangalore, India, were the subjects of the study. Animals were acclimatized for a period of two-weeks and were then treated. They received standard pellet and water *ad libitum*. Rats were coded in groups of two per cage. Single dose of etoposide (60 mg/kg, i.p) was administered. *S. pinnata* extract and whey preparation was administered by orogastric gavage to the study group once in a day.

**Animal grouping (n=6)**

**Group 1** Normal control.

**Group 2** The rats received Etoposide alone (i.p) in a single dose of 60 mg per kg body weight.

**Group 3** The rats received Etoposide (i.p) followed by *Spondias pinnata* bark extract in a dose of 100mg per kg body weight, orally once in a day from 0h. to 72 h.

**Group 4** The rats received Etoposide (i.p) followed by whey preparation in a dose of 100mg per kg body weight orally once in a day from 0h. to 72 h.

**Group 5** The rats received Etoposide (i.p) followed by a combination of *S. pinnata* and whey preparation in a dose of 100mg per kg body weight orally once in a day from 0 h to 72 h.

The change in the body weight and food intake was monitored on daily basis. After 72 h, rats were sacrificed by cervical dislocation. The small intestine was dissected out, washed in ice-cold PBS, and blotted. The middle piece was used for assessment of biochemical parameters. Samples were immediately homogenized and stored at -20°C until analysis.

**Chemicals**

Chemicals and reagents were of HPLC or analytical grade procured from Sigma-Aldrich, USA. Etoposide (100 mg/ kg b w) was administered through the orogastric gavage to the study group once in a day. The rats received Etoposide (i.p) followed by whey preparation in a dose of 100mg per kg body weight orally once in a day from 0h. to 72 h.

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**Assay for lipid peroxidation (LPO)**

The lipid peroxidation products in the homogenate were measured through the estimation of Thiobarbituric acid reactive substances (TBARS) by the method described by.19 1ml of tissue homogenate was precipitated with 2.5ml of ice cold Trichloroacetic acid (TCA). The samples were centrifuged at 3000g for 10 min. To 2ml of this supernatant, 0.67%of Thiobarbituric acid (TBA) was added and kept in boiling water bath for 10 min and cooled. The pink chromogen that was developed was read immediately at 412nm using a Systronic-117 UV-Visible spectrophotometer. The values were expressed in ng/ml/g of wet tissue.

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**Estimation of reduced glutathione**

Tissue GSH concentration was estimated according to the method described by Ellman.20 One milliliter of supernatant was precipitated with 1 ml of metaphosphoric acid and cold digested at 4°C for 1 h. The samples were centrifuged at 1,200g for 15 min at 4°C. To 1ml of this supernatant, 2.7ml of phosphate buffer and 0.2ml of 5, 5’ dithio-bis-2-nitrobenzoic acid (DTNB) were added. The yellow color developed was read immediately at 412nm using a Systronic-117 UV-Visible spectrophotometer. The values were expressed in mg/gm of wet tissue.

**Estimation of nitric oxide**

Activity of nitric oxide was determined by the procedure of Green et al.21 The accumulation of nitrite in the supernatant is an indicator of production of nitric oxide (NO), which is produced due to oxidative stress occurring in the brain. Production of NO was determined by spectrophotometric assay with Griess reagent (0.1% N-1-Napththyl ethylene diamine dihydrochloride, 1% sulphanilamide and 2.5% phosphoric acid). Equal volume of homogenate and Griess reagent was be mixed, the mixture was incubated for 10 min at room temperature and absorbance was measured at 540nm. The concentration of nitrite on the supernatant was determined from standard curve and expressed in μg/g.

**Myeloperoxidase (MPO)**

Myeloperoxidase activity was measured spectrophotometrically,22 by using 4-AP (4-Amino Antipyriyne) as the hydrogen donor. Activity was measured as an increase in absorbance at 510nm/min. Test analysis – Add 100 microliter homogenate, 0.5 ml buffer, 0.5ml and 0.5 ml 4-AP. Immediately after the addition of 4-AP, read in a spectrophotometer at 1’ interval for 5 min. Control analysis-Add 100 microliters homogenates, 1.0ml buffer, and 0.5 ml 4-AP. Immediately after the addition of 4-AP, read in a spectrophotometer at 1’interval for 5 min. One unit of myeloperoxidase activity is defined as the amount of enzyme producing 1nmole of hydrogen peroxide per min. Modulatory effect of SP and WP on rat intestine with mucositis.

**RESULTS**

Rats which received etoposide alone (group 2) showed significant decrease (p <0.01) in the TAO levels compared to control groups (group 1 v/s group 2). Animals which received etoposide followed by *Spondias pinnata* (100 mg/ kg b w) showed significant increase (p <0.01) in TAO level when compared to animals exposed to etoposide alone (group 2 v/s group 5). Animals which received etoposide followed by whey (100mg/kg bw) and combination (100 mg/kg bw) showed significant increase...
in TAO level when compared to animals exposed to etoposide alone (p<0.05). (Figure 1)

Rats which received etoposide alone (group 2) showed a significant decrease (p<0.0001) in GSH levels compared to controls (group 1 v/s group 2). Animals which received etoposide followed by Spondias pinnata (100mg/kg bw), whey (100mg/kg bw) and combination showed significant increase (p<0.01) in GSH levels when compared to animals exposed to etoposide alone (group 2 v/s group 5). (Figure 2)

Animals exposed to chemotherapy showed significant increase in the TBARS level in duodenum compared to controls (p<0.0001). Animals exposed to chemotherapy followed by S pinnata bark extract and combination showed significant decrease in TBARS level compared to etoposide control. (Etoposide vs S pinnata and combination, p<0.0001) and there a decrease in TBARS levels in animals exposed to chemotherapy followed whey proteins (p<0.001). (Figure 3)

A significant increase in duodenal NO level was observed in rats exposed to etoposide compared with control group (p<0.01). Animals which received etoposide followed by S. pinnata (100 mg / kg body weight) and combination (100 mg/kg body weight) treatment showed a significant decrease in duodenal nitric oxide level (p<0.001), when compared to rats which received etoposide alone (group 2 v/s group 5) and in rats which received whey 100 mg /kg body weight. (Figure 4)

The MPO activity was significantly increased (p<0.0001) in animals exposed to etoposide alone compared to control group. Animals exposed to etoposide followed by S pinnata bark extract and combination showed significant decrease in MPO level in the duodenum compared to etoposide controls (p<0.0001). (Figure 5)

**DISCUSSION**

Mucositis is a foremost cause of dosage reduction and untimely interruption of treatment during cancer therapy, and thus significantly influences the survival of cancer patients. Patients with mucositis leads to severe
clinical symptoms, including pain resulting from ulceration, nausea, vomiting, heartburn, diarrhea, constipation, succeeding malnutrition and weight loss.\textsuperscript{1,2} Ulceration is concomitant with a threat of systemic infection. Therefore, mucositis is a major clinical and economic cargo that severely affects patient outcome and quality of life, in addition to increasing the risk of morbidity and mortality.\textsuperscript{2,3} However, currently the all available means of management of mucositis are largely limited to the control of its pain, oral hygiene, infection, bleeding, and malnutrition,\textsuperscript{4,11} but with some major side effects. Current study is planned to implicate some commonly used dietary means to check their impact on mucositis. The side-effects may also result in termination of therapy and hence there is a requirement for better and alternate adjuvant therapy. Literature survey shows that S pinnata bark extract contains large amounts of flavonoids, and phenolic compounds which exhibit high antioxidant and free radical scavenging activities.\textsuperscript{23}

Rats which received etoposide alone showed significant decrease in the TAO levels compared to control groups. Administration of whey preparation and combination following etoposide treatment have shown more significant increase in the TAO levels than treatment with S. pinnata. The cationic whey protein isolate has been shown to have antioxidant effect in model experimental systems, and so can be considered as a factor that can regulate the intensity of lipid oxidation.\textsuperscript{24} GSH is a major component of the intestinal antioxidant system. Several studies have confirmed the role of glutathione, as a powerful antioxidant, which is increased by dietary whey protein.\textsuperscript{25,26} Moreover, whey proteins are a source of cysteine, a precursor for the synthesis of glutathione (GSH) that plays a key role in endogenous cellular antioxidant defences.\textsuperscript{27} Whey preparation has been found to restore Glutathione levels in leukocytes, liver and cutaneous tissue in mice by suppressing hydroperoxide and ROS levels.\textsuperscript{28} Accumulation of GSSG by oxidative mechanisms within the cytosol may lead to cell apoptosis. S. pinnata intervention brings about the restoration of GSH levels in kidney and liver tissue. Increased free radical stress disturbs the membrane lipids by their oxidation, results in the formation of products such as malondialdehyde and TBARS and will worsen normal functioning of the cells.\textsuperscript{29} Beta sitosterol present in S. pinnata has been implicated in protecting glutathione by decreasing levels of cytokines.\textsuperscript{30}

The study showed significant decrease in GSH levels in rats exposed to chemotherapy compared to normal controls. Administration of Spondias pinnata (100 mg/kg b.w) or whey preparation (100 mg/kg b.w) following etoposide treatment showed increase in the GSH levels. However, the increase in GSH levels after the administration of combination of Spondias pinnata and whey preparation (100 mg each/kg b.w) following etoposide is much more than either administered individually, showing that the compounds present in them act synergistically in increasing GSH levels.

The level of Thiobarbituric acid reactive substances (TBARS) is a scale of lipid peroxidation. Augmented free radical stress disturbs the membrane lipids by their oxidation which results in the formation of products such as malondialdehyde and TBARS and will exacerbate normal functioning of the cells.\textsuperscript{30} Animals exposed to chemotherapy showed significant increase in TBARS level in the duodenum compared to control group. Animals exposed to chemotherapy followed by Spondias pinnata bark extract or whey preparation or combination showed a significant decrease in TBARS level in duodenum equated to etoposide control.

Nitric oxide (NO) is known as an endothelium derived relaxing factor.\textsuperscript{31} There are some evidences that injuries of the small intestine may be exacerbated significantly by elevated NO levels.\textsuperscript{32} Present study showed significant increase in nitric oxide levels in etoposide treated group compared to control. Administration of a combination of Spondias pinnata extract and Whey preparation showed a more significant reduction in the NO levels than with either Spondias pinnata extract or Whey preparation alone. 4-O-β-glucoside, a component of S. pinnata extract has been shown to inhibit nitric oxide synthase, thus decreasing nitric oxide levels.\textsuperscript{33,34} Whey protein and α-Lactalbumin may decrease nitric oxide production by inhibiting the production of mediating cytokines.\textsuperscript{35} MPO is an inflammatory marker and is found to intensify during tissue injury. An elevation in MPO activity was observed after etoposide treatment, which indicates the accumulation of neutrophils that might contribute to etoposide induced small intestinal damage.\textsuperscript{36} The levels of MPO in current study is significantly increased in rats that received etoposide alone when compared to normal control may be due to increased levels of free radicals generated during the development of mucositis. There was significant decrease in the level of MPO in the rats which received etoposide followed by Spondias pinnata, whey preparation and the combination. The complications of mucositis could be inhibited by modulating the underlying pathways provides an opportunity for the development of more targeted therapies and interventions. Thus the development of an effective intervention against chemotherapy related mucositis has high priority in oncological supportive care.

CONCLUSION

The study results demonstrates that combination of S.pinnata with whey preparation have better effect on the prevention/ recovery from mucositis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

GSH: Reduced Glutathione; TAO: Total Anti-Oxidants; MPO: Myeloperoxidase; SP: Spondias Pinnata; WP: Whey Preparation.

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SUMMARY

Effects of streptococcus thermophilus th-4 on intestinal mucositis


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