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Protective potentials of *Annona muricata* fruit pulp on etoposide-induced gastrointestinal toxicity in Wistar rats

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Abstract:

BACKGROUND: Mucositis, one of the devastating consequences of chemotherapy and also limits the efficacy of the treatment. At present, there are no antimucositic agents without side effects. Hence, there is a need for better adjuvant therapy using plant or food sources. Here, we have made an attempt to study the effect of *Annona muricata* (AM) fruit pulp on etoposide-induced mucositis.

MATERIALS AND METHODS: The study was conducted at Central Research Laboratory, Kasturba Medical College, Mangalore. The effect of AM fruit pulp (100 mg and 200 mg/kg body weight) on etoposide-induced mucositis was studied in Wistar rats ($n = 36$) in comparison with normal and AM controls. Intestinal tissue was collected for histology and estimation of total antioxidants (TAO), glutathione (GSH), myeloperoxidase (MPO), and nitric oxide (NO) levels along with histological changes were studied. Statistical analysis was performed by one-way analysis of variance.

RESULTS: TAO and GSH levels were found to be significantly high in the rats which received 200 mg of AM/kg body weight than 100 mg of AM/kg body weight when compared with etoposide control. The levels of inflammatory markers - MPO and NO - were found to be decreased ($P < 0.001$) in the animals received 200 mg/kg body weight of AM in comparison with etoposide group and lower dosage of AM pulp. Histology of intestine also showed a protective effect of AM (200 mg/kg body weight) against etoposide toxicity.

CONCLUSION: The results show that AM fruit pulp has the capacity to act as antimucositic agent and also reduced inflammation.

Keywords:

Annona muricata, etoposide, mucositis, myleoperoxidase, nitric oxide

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Introduction

Chemotherapy and radiotherapy are extremely effective in the curing of several forms of cancer, but can also cause damage to healthy tissues, which may then lead to apoptosis and altered cellular functions. Hence, these treatment modalities have various side-effects and could be one of the underlined causes for increase in the mortality.[1-5]

Etoposide, most commonly recommended chemotherapeutic agent that primarily targets the enzyme topoisomerase II, and leads to a permanent break in DNA strand.[6] Due to its capability to prevent polymerization of tubulin, it acts as antimitotic agent.[7,8] It has been shown that its mode of action is through the formation of free radicals and thus shows varied effects on different organs. The efficacy of etoposide is often limited by severe side effects and toxic sequelae, such as bone marrow suppression, mucositis, fatigue, anorexia, myelosuppression,

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acneiform eruptions, low blood pressure, erythema, and alopecia.[9]

Mucositis is one of the most common limiting factors that prevent further dose escalation during therapy,[9,10] lessens the efficiency of therapy, patient survival, and rising health-care costs. The gastrointestinal tract is the most vulnerable organ and is prone to destruction leading to malabsorption and cachexia.[11] When cells are exposed to chemotherapeutic agents, reactive radical species especially ROS are released which causes damages to nucleic acids, proteins, and lipids. These free radicals in turn induce cytokine alterations in the cellular metabolic processes.[11-13] To overcome from this free radical-induced alterations in cell metabolism, the antioxidant system comes into battle. At present, there are no effective antimucositic agents without side effects. There is an unmet need for mucositis remedies that reduce or prevent the severity and duration of symptoms, so that patient's chemotherapy or radiation regimen can be maintained or intensified. Therefore, there is real desire to have natural potential agent that will help to overcome the hurdles faced by the patients during the therapy.

Plants remain to be the mainstay in the treatment of many diseases. They have fewer side effects than other conventional drugs. Plants used as food and in traditional medicine are more likely to yield antioxidants and pharmacologically active compounds.[5-8] In the present work, we have attempted to study one such plant source, i.e., *Annona muricata* (AM) fruit pulp. AM a member of *Annonaceae*, commonly called soursop is a broadleaf flowering, evergreen tree has a widespread pantropical distribution that contributes to the wider economic growth of some of the tropical countries such as Cuba, Central America, Southeast Asia, and Pacific.[14] Soursop fruit is an edible fruit with its juicy, creamy, and slightly sweet taste.[15] Studies on chemical composition of fruit pulp, leaves, and seeds of this species have reported the presence of a great number of alkaloids, phenols, and acetogenins. The biological effects of these identified components are primarily because of their action against cancer cells due to their inhibitory effects against the mitochondrial complex I (mitochondrial NADH: ubiquinone oxidoreductase).[16,17] Several researches have demonstrated the activities including anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic, analgesic hypotensive, anti-inflammatory, and immune-enhancing effects.[18] With the above knowledge, we have attempted to study its effect on rat mucositis model for antimucositic effect.

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 used after obtaining the ethical clearance (File No: KMC/MNG/IAEC/04-2017).

Animals were acclimatized for 2 weeks before the study and were then treated. They received standard pellet and water *ad libitum*. Rats were coded in groups of two per cage. Mucositis was induced by intraperitoneal administration of 60 mg/kg body weight of etoposide in a single dose. A volume of 1 ml of aqueous solution of AM fruit pulp 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 100 mg/kg body weight AM fruit pulp in 1 ml distilled water, orally once in a day
- Group 4 – The rats received 200 mg/kg body weight AM fruit pulp in 1 ml distilled water, orally once in a day
- Group 5 – The rats received etoposide (i.p.) followed by AM *fruit* extract in a dose of 100 mg/kg body weight, orally once in a day from 0 to 72 h
- Group 6 – The rats received etoposide (i.p.) followed by AM fruit extract in a dose of 200 mg per kg body weight orally once in a day from 0 to 72 h.

The change in the body weight and food intake was monitored on a daily basis. After 72 h, rats were sacrificed by overdose of ketamine. The small intestine was dissected out, washed in ice-cold phosphate buffer saline, and blotted. For histological assessment, jejunum was fixed in formalin (10%). The middle piece was used for the assessment of biochemical parameters. Samples were immediately homogenized and stored at −20°C until analysis.

Chemicals

Chemicals and reagents were of High Performance liquid chromatography or analytical grade procured from Sri Durga Laboratories, Mangalore, India.

Biochemical parameters

Estimation of total antioxidants

The total antioxidants (TAO) level was estimated according to the method described by Koracevic *et al*.[19] Each sample had its own control in which Fe-ethylenediaminetetraacetic acid mixture, hydrogen peroxide, and sodium benzoate were added after 20% acetic acid. For each series of analysis, a negative control was prepared, except that of the sample, homogenate, was replaced with 0.1 M Sodium phosphate buffer, pH 7.4. Uric acid (1 Mm/L) was used as standard. The

reaction mixture was incubated at 37°C for 60 min, then 20% Acetic acid and 0.8% thiobarbituric acid were added and incubated for 10 min at 100°C, then cooled in ice bath. The absorbance was measured at 532 nm. The TAO level is expresses as μmol/L.

Estimation of reduced glutathione

Tissue glutathione (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 1200 g for 15 min at 4°C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer, and 0.2 ml of 5, 5′ dithiobis-2-nitrobenzoic acid were added. The yellow color developed was read immediately at 412 nm using a Systronic-117 UV-Visible spectrophotometer. The values were expressed as mg/gm. tissue.

Myeloperoxidase

Myeloperoxidase (MPO) activity was measured by spectrophotometric method[21] using 4-Aminoantipyrine (4-AAP) as the hydrogen donor. The activity was expressed as an increase in absorbance at 510 nm/min. A mixture containing 100 microliter homogenate, 0.5 ml of buffer, 0.5 ml hydrogen peroxide, and 0.5 ml 4-AAP. Immediately after the addition of 4-AAP, OD was recorded at 1′ interval for 5 min. Control analysis - Add 100 microliters homogenates, 1.0 ml buffer, and 0.5 ml 4-AAP. Immediately after the addition of 4-AAP, read in a spectrophotometer at 1′interval for 5 min. One unit of MPO activity is defined as the amount of enzyme-producing 1 nmole of hydrogen peroxide per min.

Estimation of nitric oxide

The activity of nitric oxide (NO) was determined by the procedure of Menaka *et al*.[22] The accumulation of nitrite in the supernatant is an indicator of production of NO, which is produced due to oxidative stress. The activity of NO was determined by spectrophotometric assay with Griess reagent (0.1% N-1- Naphthyl ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid). Equal volume of homogenate and Griess reagent was mixed, the mixture was incubated for 10 min at room temperature, and absorbance was measured at 450 nm. The concentration of nitrite on the supernatant was determined from standard curve and expressed in μg/g tissue.

Histology

Small intestine was evaluated for histological variations in different groups with H and E stain. Segments were observed under light microscope (10X) in all the study groups (control, etoposide + 100, and 200 mg/kg body weight AM).

Statistical analysis

The analysis was carried out using Graph Pad Prism 7.2, (SanDiego , Canada) and the data were presented as the mean ± standard error of the mean. Statistical analysis for multiple comparisons was performed by One-way analysis of variance with Bonferroni's corrections. Data which is not following normal distribution were analyzed separately using nonparametric test (Kruskal–Wallis test) to compare all the groups. Individual comparisons between the groups were performed by Mann–Whitney test. $P \leq 0.05$ was considered statistically significant.

Results

Rats which received etoposide alone (Group 2) showed a significant decrease ($P < 0.01$) in the TAO levels compared to control groups (Group 1 vs. Group 2). Animals which received etoposide followed by AM (100 mg/kg b w) showed significant increase ($P < 0.01$) in TAO level when compared to animals exposed to etoposide alone (Group 2 vs. group 5). Animals which received etoposide followed by AM (200 mg/kg body weight) 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 ($P < 0.001$) in GSH levels compared to controls (Group 1 vs. Group 2) [Figure 2]. Animals which received etoposide followed by AM (100 mg/kg body weight) and (200 mg/kg body weight) showed significant increase ($P < 0.01$) in GSH levels when compared to animals exposed to etoposide alone (Group 2 vs. Group 5).

The MPO activity was significantly increased ($P < 0.001$) in animals exposed to etoposide alone (Group 2) compared to controls (Group 1 vs. Group 2) and to all other groups [Figure 3].

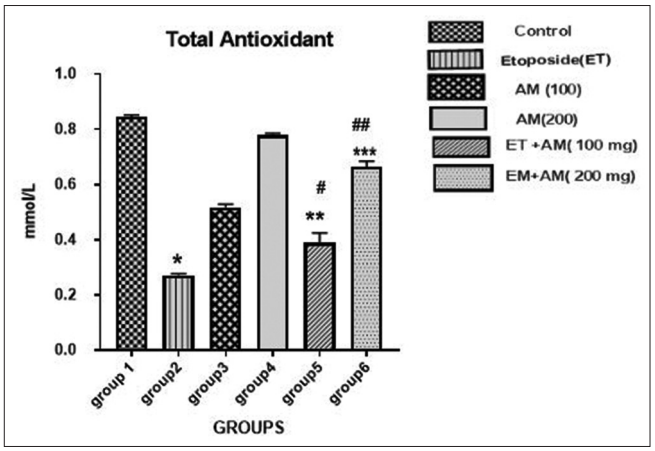

Figure 1: Total antioxidant level (mmol/L) in rat duodenal tissue. Analysis of variance significance (Bonferroni's test, each bar represents mean ± standard error of the mean, n = 6). Group 1 versus Group 2, Group 3, $^{*}P < 0.0001$, Group 5 $^{**}P < 0.001$, and Group 6$^{***}P < 0.01$ Group 2 versus Group 5 and Group 6 $^{\#}P < 0.01$, $^{\#\#}P < 0.05$

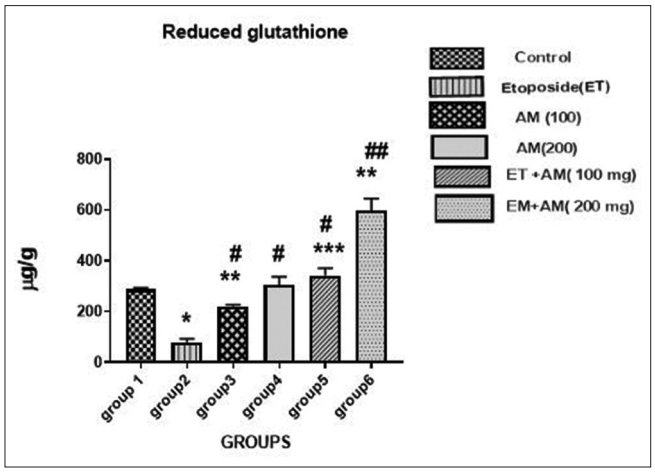

Figure 2: Duodenal GSH level (µg/g tissue) in rats. Analysis of variance significance (Bonferroni's test, each bar represents mean ± standard error of the mean, $n = 6$). Group 1 versus Group 2, Group 3, Group 5, and Group 6, $^{*}P < 0.0001$, $^{**}P < 0.001$, $^{***}P < 0.01$ Group 2 versus Group 5 and Group 6 $^{\#}P < 0.01$, $^{\#\#}P < 0.05$ Group 2 versus Group 3, Group 4, Group 5, and Group 6, $^{\#}P < 0.001$, $^{\#\#}P < 0.01$

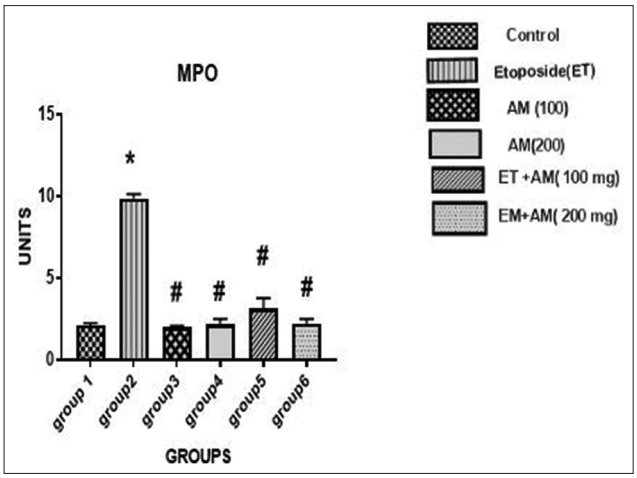

Figure 3: The estimation of MPO activity in rats (1U = 1 nmol of H_2O_2 into water/min) analysis of variance significance (Bonferroni's test, each bar represents mean ± standard error of the mean, $n = 6$). Group 1 versus Group 2, $^{*}P < 0.0001$ Group 2 versus Group 3, Group 4, Group 5, and Group 6 $^{\#}P < 0.0001$

AM could prevent the changes in MPO levels due to etoposide administration and remain in the normal levels. MPO activity remained unaltered in animals exposed to AM treatment (Group 5 and Group 6) alone compared to normal controls (Group 1).

A significant increase in duodenal NO level was observed in the rats exposed to etoposide compared with the control group (Group 1 vs. Group 2 $P < 0.0001$. Animals which received etoposide followed by AM fruit pulp (200 mg/kg body weight) showed a significant decrease in duodenal NO level compared to etoposide control rats ($P < 0.001$, Group 2 vs. Group 3, 4, 5, and 6). However, NO level remained unaltered in rats which received 100 mg/kg body weight of AM (Group 3 and Group 4) in comparison with normal controls [Figure 4].

Histology of the jejunum

On treatment with etoposide, a significant disruption of the villi in jejunum was observed compared to control rats. The administration of etoposide followed by AM (100 and 200 mg/kg body weight) minimized the destruction of villi compared to etoposide-treated group [Figure 5].

Discussion

Cancer is a most debilitating problem increases the mortality rate worldwide. Because of the multifactorial causes of cancer development eventually needs careful therapeutic strategies to overcome its consequences during disease, therapy, and recovery phases. Majority of medical research work is to overcome the hitches of cancer development, treatment, and post-treatment consequences.[1,23] The main goal lines of therapy for patients with recurrent cancer are to improve quality of life and extend survival. Conventional therapies for the treatment include surgery, chemotherapy, gene therapy, and radiotherapy. One of the previous studies has been reported that AM is found be useful in the treatment of different types of cancer due to the presence of several polyphenolic compounds that exhibit free-radical scavenging effect.[16] Among the different groups of AM, fruit and squamosa are found to be the best due to high concentration of thiol groups in it.[17]

The administration of cytotoxic substances into the system results in the production of free radicals, which causes damage to the cells, tissues, and blood vessels. The action of etoposide resulting in the accumulation of single- or double-strand DNA breaks, inhibition of replication and transcription of DNA, and apoptotic cell death. Enzymatic reaction on Etoposide can result in the formation of phenoxyl radical at C-4′ position of the E ring, and a commonly formed C4′-radical leads to double-strand breaks under aerobic conditions. Double-strand breaks are widely accepted to be the most toxic form of DNA damage.[24]

Antioxidants break the free radical chain of reactions by giving away their own electrons to feed free radicals, without becoming free radicals themselves.[16] Antioxidants are thus useful in protecting cells from such oxidative damage. GSH protects the tissue against oxidative processes; hence, it is called as body's master antioxidant. Complementary strand hydrogen atom abstraction by the peroxyl radical is efficiently quenched by thiols. Its action during detoxification process remains significant in the maintenance of intracellular redox balance. Reduction in the GSH levels is appreciated in the rats exposed to etoposide in contrast with

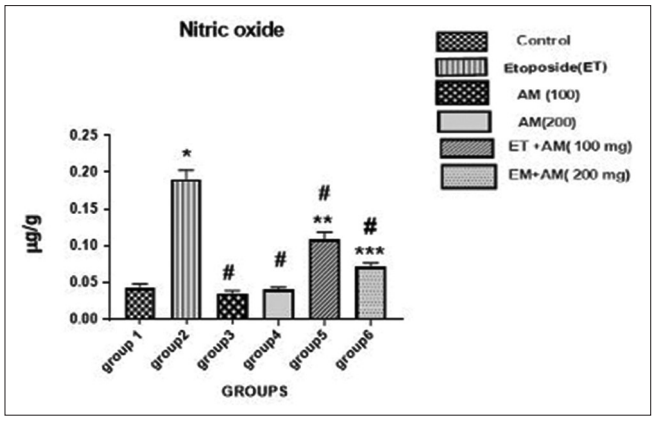

Figure 4: Duodenal nitric oxide level (µg/g of tissue) in rats. Analysis of variance significance (Bonferroni's test, each bar represents mean ± standard error of the mean, $n = 6$) Group 1 versus Group 2, 5 and 6, $^{*}P < 0.0001$, $^{**}P < 0.001$, $^{***}P < 0.01$ Group 2 versus Group 3, 4, 5 and 6, $^{\#}P < 0.001$

normal control is principally by severe oxidative stress induced by chemotherapy. GSH directly quenches reactive hydroxyl groups, other oxygen-centered free radicals and thus reduces the damage caused by free radical in the system. In the previous study by Reddy *et al*. it has shown significant positive change with oral administration of *Spondias pinnata* bark extract.[25,26] The current study with oral administration of AM fruit pulp in etoposide-induced rat showed significant rise in GSH levels. The GSH level was found drastically low in the rats which received etoposide alone when compared to normal controls. The TAO level also showed parallel results with GSH. There was significant fall in the TAO levels in the rats received chemotherapy alone when compared to normal controls. However, a significant rise in the TAO levels in rats received etoposide followed by AM reveals the antioxidant properties of AM. Among the 2 doses, 200 mg/kg body weight showed a better effect than 100 mg/kg body weight.

MPO an essential maker of inflammation released into extracellular medium during various inflammatory conditions.[19] The activation of various inflammatory markers during infection increases vascular permeability resulting in influx of various cytokines at the site. This cascade of the inflammatory process also increases the infiltration of neutrophils. MPO is a peroxidase, acts on hydrogen peroxide, a highly reactive oxygen species formed during the neutrophil's respiratory burst produces hypochlorous acid (HOCl).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. The levels of MPO in the current study are 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 or may be a natural reaction

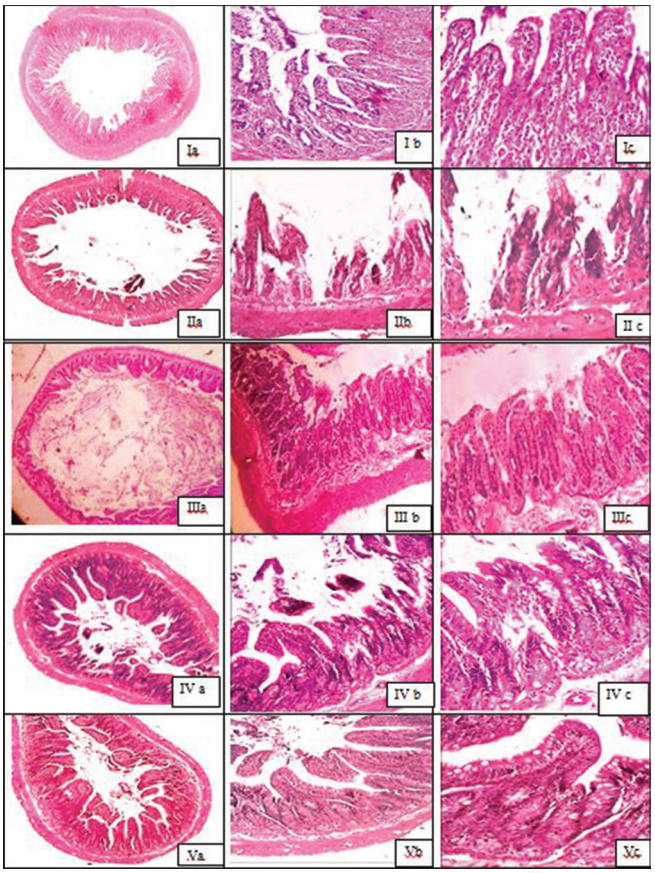

Figure 5: Histology: Histology (5 µ sections) of jejunum with the magnification of (10×). I - Normal control (a = 2×, b = 10× and c = 20×), II - Etoposide control (a = 2×, b = 10× and c = 20×), III - *Annona muricata* control (a = 2×, b = 10× and c = 20×), IV – Etoposide + *Annona muricata* 100 mg/kg body weight (a = 2×, b = 10× and c = 20×) and V Etoposide + *Annona muricata* 200 mg/kg body weight (a = 2×, b = 10× and c = 20×)

elicited against the toxic effect of the chemotherapeutic drug. The administration of AM fruit pulp could decrease the extent of inflammation to significant levels is evident by decrease in the MPO levels.

NO, a product of L-arginine by the action of enzyme NO synthase, acts as messenger molecule and is linked with inflammation and oxidative stress. It has both cytoprotective and cytotoxic effects which have been noted in various types of pathophysiological conditions, including the digestive system.[27] It has been reported that NO levels are increased due to unregulated iNOS activity during inflammation.[28] The levels of intestinal tissue NO were found to increase in etoposide treated group. Compared with etoposide-controls, animals that received etoposide and AM, the level of NO was found to be low. The study substantiates the protective effect of GSH against NO formation/levels in the intestinal tissue as observed by an inverse relationship between GSH and NO levels during the study.[29]

The cell damage due to NO and its derivative from iNOS is influenced by the availability of the cellular

GSH. One of the recent studies on murine macrophage cell lines demonstrated that NO and GSH substantiated the influence of GSH redox system on NO, while pre-treatment with N-acetyl-cysteine was protective.[30]

Studies by Adefegha SA.*et al*.,[31] has been reported that AM possesses analgesic and anti-inflammatory activities because of the interaction between immune response and opioid system as well as inhibition of NO generation. In the current study, the rats which were exposed to etoposide treatment exhibited a widespread mucosal damage by the destruction of the villus in small intestine.

In the present study, intestinal MPO level was markedly increased in etoposide-treated rats, and a protective effect was observed as a result of concurrent administration of AM with etoposide. The increase of this inflammatory marker is concomitant with the changes seen in the intestinal histology also. Component analysis of soursop fruit pulp revealed the presence of about 16 phenolic compounds. The major phenolic compounds in soursop fruit include cinnamic acid derivatives, p-coumaric acids, and several other minor compounds. It has been reported that soursop fruit extract showed the abilities of OH radical scavenging and Fe^{2+} chelation. These properties could be the basis of the observed protective/anti-inflammatory effect of AM fruit pulp in the present study. Thus, it can be proposed that AM may have potent anti-inflammatory effects in etoposide-induced mucositis. Considering the other findings of the study together, it can be concluded that AM could prevent chemotherapy-induced injury and histological perturbations through enhancement antioxidant defense system and suppression of oxidative stress.

However, extensive study is required to ensure the protective effect as an adjuvant during chemotherapy, even under cancer conditions since the present study is focused only on normal group.

Conclusion

The present study has demonstrated that AM fruit pulp administration has shown mucoprotective effects in our rat model. Therefore AM fruit pulp can be considered as antimucositic agent that can be administered during chemotherapy to reverse mucositis.

Limitations

Since the study was done under normal conditions further its applications have to be substantiated in the rat model for cancer.

Future plan

The study has to be done in large group with different doses and time of exposure.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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