Fisetin Ameolirates the Azoxymethane and Dextran Sodium Sulfate Induced Colitis Associated Colorectal Cancer

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ABSTRACT
Colitis associated colorectal cancer (CAC) is the subtype of colorectal cancer (CRC), CRC is the third most cause of morbidity worldwide. Chronic inflammation caused by oxidative stress is the main risk factor for CAC establishment. Fisetin (3,3',4',7 tetrahydroxy flavones) is a dietary flavonoid with major health benefits. This study is aimed to investigate the anti-proliferative effect of Fisetin against azoxymethane (AOM) and Dextran sodium sulfate (DSS) induced CAC. Male Balb/c mice received intraperitoneal injection of AOM (10mg/kg) on day 1, then 2% DSS in drinking water for 7 days followed by drinking water for 14 days and this cycle repeated twice. Mice induced with AOM/DSS received Fisetin (20mg/kg) during the experimental period. On the day 62, animals were sacrificed and the colon was processed for biochemical and histopathological examinations. Diagnostic tumor marker 5’ nucleotidase and γ glutamyl transeptidase levels were decreased by fisetin treatment. Enzymatic and non-enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, vitamin C and vitamin E depletion due to AOM/DSS induction were brought near to normal by Fisetin. Administration of fisetin showed a significant increase in the protein expression levels of bax, caspase-3 and decreased expression level of bcl-2 compared with the control group. These results demonstrate that fisetin exhibits chemopreventive and therapeutic effect against CAC.

Keywords: Colitis associated colorectal cancer, Azoxymethane, Dextran sodium sulfate, Fisetin.

INTRODUCTION
Colitis associated colorectal cancer (CAC) is the subtype of colorectal cancer, which is associated with inflammatory bowel disease such as Ulcerative colitis and Crohn’s disease. Colorectal cancer (CRC) is the third most cause of morbidity and second most cause for mortality worldwide. According to the data of International Agency for Research on cancer (IARC), the incidence of the CRC had increased in Asian pacific region to that of Western countries. Chronic inflammation is a major factor involved in the development and establishment of CRC. Chronic inflammation may occurs due to various factors such as epithelial injury, pathogenic agents such as bacterial infection, dietary factors (high meat and refined sugars creating carbonyl threat at GI) and chemical insults leading to increased proliferation of malignant cell and carcinogenesis. In chronic inflammatory conditions, reactive oxygen species (ROS) such as superoxide anion free radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (HO$^-$), are produced at high levels and accumulated to cause oxidative stress (OS). Excessive production of free radicals beyond the antioxidant capacity of cell leads to proteins carbonylation, lipid peroxidation and DNA mutation. Thus, CAC is a multistage process occurs as stepwise manner-initiation of inflammation, dysplasia formation, adenoma and then lead to carcinoma. Chronic inflammation due to OS, even affects the key molecules of cellular pathway such NF-kB and STAT 3 (Signal transducers and activators of transcription). Many varieties of CRC rodent models are available as tool for the investigation of development and pathogenesis of CRC. AOM/DSS carcinogen induced CAC rodent model is widely used model which exactly mimics the recapitulate aberrant crypt foci-adenoma-carcinoma sequence that occurs in human CRC. The combination of tumorigenic AOM with inflammatory agent DSS for induction of CRC in rodents has proven to dramatically shorten the latency time of 30 weeks to less than 10weeks. AOM is the oxide of azoxymethane through N-oxidation and hydroxylation forms methylazoxymethanol (MAM) to induce DNA-reactive adducts. MAM formed can alkylate macromolecules in the liver and colon, and operate the addition of methyl groups at the O6 or N7 position of guanine in the DNA molecule. Methylation at the O6-position of guanine has been shown to be the primary promutagenic lesion produced by AOM. MAM formed is decomposed to form methylidyazonium ion which methylate cellular components and elicits oxidative stress inturn promotes tumor in the colon. Macroscopically, the AOM/DSS model resulted in 100% incidence of colonic tumors and hyperplasia, which were most frequently observed in the middle and distal colon.
Despite the clinical advances in conventional chemotherapy for CRC, resistance towards the chemotherapy is a biggest challenge in medicinal field while treating the CRC patients. Therefore, there is an urge to find new and effective chemopreventive drugs from plant source against CRC. Growing interests are now being focused on the natural compounds to exert their anticarcinogenic action by modulating LPO and antioxidant status\textsuperscript{11}. Phenolic compounds act as powerful chemopreventive agents by counteracting carcinogen-induced oxidative stress\textsuperscript{12}. Fisetin is a flavanol, subgroup of flavonoid present in many fruits and vegetables mostly in strawberries, apples, persimmons, kiwis, mango, cucumber and onions\textsuperscript{13}. It is evident from previous reports, that fisetin possess various properties such as anti-oxidant activities, anti-toxicity, antihyperlipidaemic, and neuroprotective properties\textsuperscript{14}. In the present study, we intend to focus on the antioxidant, anti-inflammatory, apoptosis inducing
property of fisetin against AOM/DSS induced colitis associated colorectal cancer in mice.

MATERIALS AND METHODS

Chemicals

Fisetin, azoxymethane, dextran sodium sulfate was purchased from Sigma Aldrich Chemicals Company, USA and all other chemicals used in the study were of analytical grade obtained from SISCO research laboratory (SRL), India.

Animals

Male balb/c mice weighing approximately 15-20 grams were purchased from Laboratory Animal Maintenance Unit, Tamilnadu Animal Science and Veterinary University, Madavaram, India. The animals were maintained under standard conditions of humidity, temperature (25 ± 2 °C) and light (12 h light/12 h dark). They were fed with standard rat pelleted diet (Hindustan Lever Ltd., Bangalore) and they had free access to water. The experiments were performed according to the guidelines of IAEC. All the efforts were taken to reduce the animal suffering.

Experimental design

The animals were divided into four groups (n = 6 per group). Balb/c mice in group 1 served as control and received 0.9% saline. Group 2 mice were administered a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, 2% DSS in the drinking water was administered to the mice in groups 2 for 7 and 14 days, followed by normal drinking water for recovery period. A total of three cycles of 2% DSS were performed. Group 3 mice was induced with AOM/DSS as that of group 2 and co-treated with 20mg/kg body weight fisetin throughout the study period. Group 4 animals received the same dose of fisetin as in Group 3.

The experiment was terminated at the end of day 62 and all the animals were killed by cervical decapitation. The colon was removed and opened longitudinally, then washed in the cold saline and homogenized in 0.1 M Tris-HCl buffer (pH 7.4), using a tissue homogenizer with a teflon pestle at 4°C. The resultant tissue homogenate was used for biochemical assays.

Evaluation of Biochemical parameters

The amount of protein in the colonic tissue homogenate was estimated by the method of Lowry method using BSA as standard13. The activities of colorectal cancer marker enzymes such as 5’Nucleotidase, γ-glutamyl transpeptidase and Cathepsin D were evaluated in colonic tissue. ALP and LDH was estimated. Antioxidant enzymes such as superoxide dismutase (SOD), Catalase (CAT), GPx, and GR were evaluated. To analyze lipid peroxidation in colon cancer MPO is assessed. Non-antioxidant enzymes such as vitamin C and Vitamin E were analyzed.

Assay of tumor markers

5’-Nucleotidase enzyme activity was estimated by the method of Fimi16 by calculating the nanomoles of inorganic phosphorus liberated per minute per milligram protein in tissue. The phosphorus liberated in the supernatant was estimated by the method of Fiske and Subbarow17. γ-Glutamyl transpeptidase in the colon tissues was assayed according to the method described by Rosalki and Ran18. The enzyme activity in tissue expressed as micromoles of p-nitroanilide formed per minute per milligram protein. Cathepsin-D in the tissues was estimated by the method of Sapolsky19 enzyme activity was expressed in nanomoles of tyrosine liberated per minute per milligram of protein.

Enzymic antioxidants

The superoxide dismutase (SOD) assay was determined according to the method of Misra and Fridovich20, based on 50% inhibition of epinephrine auto-oxidation at 480nm. The catalase (CAT) activity was determined by the method of Claiborne21, based on the value expressing CAT activity, micromoles of hydrogen peroxide decomposed per min/mg of protein at 240nm. Glutathione peroxidase (Gpx) were measured according to Rotruck22 and Glutathione reductase (GR) was analysed by the method of Staal23, Reduced Glutathione (GSH) was determined by Ellman24. Vitamin C and Vitamin E was determined in the colonic tissue homogenate according to the method of Omaye25 and Desai26.

Histopathological study

The colonic tissue were fixed in 10% buffered formalin solution, routinely processed and embedded in paraffin. Tissue sections of 3 micrometer thickness were stained with hematoxylin and eosin using Humason protocol. The
(A) Effect of Fisetin on SOD activity against AOM/DSS induced mice

(B) Effect of Fisetin on catalase activity against AOM/DSS induced mice

(C) Effect of Vitamin C against AOM/DSS induced mice

(D) Effect of Vitamin E against AOM/DSS induced mice

Figure 3 Effect of Fisetin on enzymic and non-enzymic antioxidant levels against AOM/DSS induced mice
slides were then evaluated under light microscope (Motic, Hongkong).

**Protein extraction and Western Blotting**

Colonic tissues of control and experimental groups of mice were homogenized in 135mM NaCl, 20mMTris, 2mM EDTA and 1mM PMSF (pH 7.4). The homogenates were centrifuged (10,000rpm for 15mins at 4°C) and the protein content of the supernatant was determined. Aliquots of supernatant (30µg total protein) were boiled for 5min in sample buffer (0.2M Tris-Hcl buffer, 10% glycerol, 2% SDS, 0.02% β-mercaptoethanol). Equal amounts of protein from each of the samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gel and transferred electrophoretically to a nitrocellulose membrane (Amersham Biosciences, NJ). The membrane was blocked with 5% BSA in Tris-Tween buffered saline at room temperature for 1 hr. The membrane was then incubated with respective primary antibodies BAX, Bcl-2, Caspase 3, STAT 3 (Stat 3 antibody were kindly gifted by Dr. Tapas Kundu, JNCASR, Bangalore) overnight at 4°C. The membrane was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 hr. Protein antibody complexes were detected by the addition of diaminobenzidine (DAB) as a substrate.

**Statistical analysis**

![Figure 4](image-url)
All the data were analyzed using SPSS/10 Student Software. Hypothesis testing methods included one-way analysis of variance (ANOVA). The values are expressed as mean ± S.D, P value of less than 0.05 was considered to indicate statistical significance.

RESULTS
Animals taken for the experiment survived throughout the period of experiment. Figure 1A, 1B and 1C shows changes of body weight, spleen weight and colon length of control and experimental groups of animals. Colon weight was increased in AOM/DSS induced group, due to the mucosal thickening occurred during CAC when compared to the control group. The colon length was shortened compared to the control group due to the chronic inflammation. Therefore, the colon weight/length ratio was high in AOM/DSS induced group when compared to the control group due to the treatment with Fisetin the ration is significantly lowered (p<0.05). Spleen weight is increased in the AOM/DSS induced group, treatment with Fisetin reduced the spleen weight when compared to control group.

Effect of Fisetin on marker enzymes
Tumor markers (5’ND, γ-GT, CD) and pathophysiological enzymes (ALP and LDH) levels were increased in the AOM/DSS induced mice when compared with the compared to control group (table 1), whereas fisetin significantly reduced these marker levels as compared to AOM/DSS induced mice due to its anti-oxidative effect. The level of tumor marker enzyme elevation shows the increased activities of 5’ND, γ-GT, CD. Subsequently treatment with fisetin reduced these levels of marker enzymes. There was no significant
changes were noted between fisetin alone treated group with the control group.

Units: 5′Nucleotidase- nmol of pi liberated, γ-Glutamyl transferase-nmol of p-nitroaniline formed/min/mg of protein, Cathepsin D-nmol of tyrosine liberated/min/mg of protein, Alkaline phosphatase-µmol of p-nitrophenol liberated/min/mg of protein, Lactate dehydrogenase-µmol of pyruvate liberated/min/mg of protein. Values are expressed as mean ± SD for six mice in each group.

Effect of fisetin on lipid peroxidation
MDA is a by-product of lipid peroxidation found elevated in colon cancer. Figure 2 shows the significant (p<0.05) increase in the MDA level in AOM/DSS induced group. On treatment with fisetin the level of MDA has been reduced considerably. The control and fisetin alone treated group showed no significant change in the MDA level.

Fisetin reduced the level of MDA in AOM/DSS induced mice. Values are expressed as mean ± SD for six mice in each group. *Control vs AOM/DSS Induced, †AOM/DSS vs Fisetin treated,*Indicates the statistical significance at P < 0.05, Ns Non-significant.

Effect of fisetin on biochemical parameters enzymatic and non-enzymatic antioxidants
The effect of fisetin on colonic SOD and CAT activity are shown in figure 3A and 3B. The activity of SOD and CAT were significantly decreased (p<0.05) in the AOM/DSS induced group when compared to the control mice. On treatment with fisetin the activity was brought near to normal (p<0.05) when compared to the AOM/DSS induced mice. Figure 3C and 3D represents the levels of non-enzymic antioxidants (Vitamins C and E) in colonic tissue of mice. The levels of non-enzymic antioxidants were decreased (p<0.05) significantly in AOM/DSS induced group when compared to the control group. Fisetin treatment significantly increased their levels when compared to the control group. No significance was found between control and fisetin alone treated group.

Fisetin increased the activity/levels of colonic enzymic and non-enzymic antioxidants in AOM/DSS induced CAC, (A) Effect of Fisetin on superoxide dismutase, (B) Effect of Fisetin on Catalase, (C) Effect of Fisetin on Vitamin C and (D) Effect of Fisetin on Vitamin E. Values are expressed as mean ± SD for six mice in each group. *Control vs AOM/DSS Induced, †AOM/DSS vs Fisetin treated,*Indicates the statistical significance at P < 0.05, Ns Non-significant.

Effect of fisetin on GSH a and GSH dependent enzymes in AOM/DSS induced CAC
The effect of fisetin against colonic GSH and GSH dependent enzymes in CAC is shown in figure 4A, 4B and 4C. The activity of GPx, GR and GSH were decreased (p<0.05) in the AOM/DSS induced group when compared to the control group. The treatment with fisetin increased (p<0.05) the levels of GPx, GR and GSH when compared with the AOM/DSS induced group. No significant was found in control group and fisetin alone treated group.

Fisetin increased the activity/levels of GSH and GSH dependent enzymes in AOM/DSS induced CAC, (A) Effect of Fisetin on glutathione peroxidase, (B) Effect of Fisetin on glutathione reductase, (C) Effect of Fisetin on glutathione reductase (C) Effect of Fisetin on glutathione peroxidase, (D) Effect of Fisetin on glutathione reductase

Table 1: Effect of Fisetin on the levels of marker enzyme

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>AOM+DSS</th>
<th>AOM+DSS+Fisetin</th>
<th>Fisetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′Nucleotidase</td>
<td>6.08±0.07</td>
<td>10.49±1.3</td>
<td>6.02±0.17</td>
<td>7.04±0.09</td>
</tr>
<tr>
<td>γ-Glutamyl transferase</td>
<td>8.09±0.21</td>
<td>12.23±0.13</td>
<td>9.45±0.16</td>
<td>8.98±0.27</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>15.98±0.98</td>
<td>20.87±0.56</td>
<td>14.56±0.78</td>
<td>15.0±0.12</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>55.03±4.5</td>
<td>80.90±6.5</td>
<td>74.23±5.8</td>
<td>57.34±3.9</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>29.66±0.23</td>
<td>57.66±0.26</td>
<td>27.66±0.76</td>
<td>32±0.34</td>
</tr>
</tbody>
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Figure 6: Effect of Fisetin on protein expression of Bax, Bcl-2, Caspase 3, STAT-3 and β-actin in AOM/DSS induced CAC mice

![Image of protein expression](image-url)

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glutathione. Values are expressed as mean ± SD for six mice in each group. *Control vs AOM/DSS Induced, bAOM/DSS vs Fisetin treated, *Indicates the statistical significance at P < 0.05. Non-significant.

Figure 5 shows the histological alterations in the control and experimental mice. Control and fisetin alone treated colonic tissue of mice exhibited normal intact crypt architecture. AOM/DSS induced colonic tissue of mice showed the hyperdysplasia lesion, inflammatory cells infiltration and crypt distortion, whereas treatment with fisetin reduced the dysplastic lesions, inflammatory cell infiltration and crypt restored the normal colonic architecture.

Figure 5 Photographical representation of histological changes in AOM/DSS induced colonic tissue. The images from H&E staining showed no dysplasia/tumor lesions in control group figure 5a. However, in AOM/DSS induced colonic tissue inflammatory cell infiltration, hyperdysplastic lesions and cell polymorphism figure 5b, treatment with fisetin reduced the infiltration and dysplastic lesions and colonic architecture appears near to normal figure 5c and figure 5d fisetin alone treated group the colonic crypt architecture is same to that of normal. Magnification X200.

Figure 6 shows immunoblot analysis of Bcl-2, BAX, Caspase-3, STAT-3 and β-actin. The expression of Bcl-2 and STAT-3 increased in the AOM/DSS induced group whereas treatment with fisetin significantly decreased the anti-apoptotic Bcl-2 and STAT-3. The expression of BAX, Caspase-3 significantly reduced in the AOM/DSS induced group whereas treatment with fisetin increased the pro-apoptotic BAX and caspase-3 protein expression. These results of this study indicate fisetin induces apoptosis.

Effects of Fisetin on Bcl-2, Bax, caspase 3, STAT-3 and β-actin. Western blot analysis of Bcl-2, Bax, caspase 3, STAT-3 and β-actin. The method of Western blot analysis was clearly illustrated in the Materials and Methods section. AOM/DSS-induced mice showed an increase in Bcl-2, STAT-3 and decreased expression of bax and caspase 3 in lane 2. Administration of Fisetin enhanced the expression of Bax and caspase 3 and decreased Bcl-xl and STAT-3 in lanes 3 and 4.

DISCUSSION

Colorectal cancer is the third most common cancer worldwide. The incidence of the colorectal cancer leaped during last decades in Asian countries. Colitis associated colorectal cancer occurs as sequential event from initiation, dysplasia, hyperdysplasia, adenocarcinoma and carcinoma 

The main objective of this study is to evaluate the potential inhibitory effects of fisetin in an AOM/DSS model of colon carcinogenesis. During CAC symptoms such as body weight loss, colon length reduction and spleen enlargement developed in AOM/DSS induced mice, treatment with fisetin significantly increased the well being of mice. 5'ND and γ-GT levels are tumour markers indicating the colon functionality. 5'ND upregulation exhibits the tumour promotion in various stages of human melanoma. γ-GT is a membrane bound enzyme and the marker of oxidative stress. In colorectal inflammation the levels of γ-GT found to be elevated. Cathepsin D is an aspartic lysosomal endopeptidase is required for the regulation of cell growth and tissue homeostasis of colon epithelium. Overexpression of cathepsin D exemplifies cancerous condition and such increase indicates the progression of colorectal cancer. In the AOM/DSS induced group the level of cathepsin D is increased when compared to control group, whereas treatment with the fisetin reduced the level of cathepsin D. Oxidative stress occurs as an imbalance of the generation and elimination of reactive oxygen and nitrogen species. Increased oxidative stress is the key feature of chronic inflammation. In the status of oxidative stress, excessive reactive oxygen species (ROS) and reactive nitrogen species (RNS) causes cell and tissue damage, exaggerates inflammation thereby leading to carcinogenesis. Increase in ROS causes lipid peroxidation that produces byproducts such as MDA and conjugated dienes, in specific MDA is a mutagenic agent contributing to CRC development. MDA is the end by-product during lipid peroxidation, fisetin reduces the level of MDA inaccordance to the previous findings.

Antioxidant defense mechanism has been developed to balance between production and scavenging of ROS for gastrointestinal tract (GI) homeostasis. As the GI tract been a constant site of pro-oxidant generation due to plethora of microbes, food ingredients and interactions between immune cells. Reduction in the anti-oxidant capacity is the first asymptomatic phase of the disease seen in the AOM/DSS induced animal model. Though intestinal cells has enormous enzymatic and non-enzymatic antioxidants including SOD, CAT, GPx, GR, GSH, Vitamin C and E to scavenge the ROS but excessive ROS generation depletes the antioxidant defense system as well as promotes excessive generation of free radicals through lipid peroxidation. SOD, CAT and GPx are primary line of cellular defense against ROS, where SOD catalyse the reaction of O2− reduction to H2O2. CAT/ GPx converts the H2O2 to water, GPx has higher affinity towards H2O2 than catalase. Glutathione (GSH) is oxidized to oxidized form of glutathione by the enzyme GPx, whereas GR catalyzes the reduction of oxidized glutathione to GSH, using NADPH as a reducer. In this study fisetin increased the levels of antioxidants suggesting fisetin improves the first line of cellular antioxidant defense system against oxidative stress.

GSH is a key intracellular thiol composed of glutamic acid, cysteine and glycine which protects cells from the ROS and RONS cellular damage. A recent study showed the decreased level of glutathione in the AOM/DSS induced CRC model. In the present study, fisetin administration significantly increased the activity of GPx, GR thereby raising up the activity of glutathione against AOM/DSS induced colitis associated colorectal cancer. Vitamin E is a lipid soluble antioxidant acts as strong free radical scavengers, thereby inhibiting lipid peroxidation.
Vitamin C is a water soluble antioxidant that scavenges reactive oxygen metabolites forming during carcinogenesis metabolism, thus protecting genetic material from initiation and promotion stages of carcinogenesis. Apoptosis is a programmed cell death and understanding its mechanism of action will aid the harnessing of this process for use in tumor diagnosis and therapy. Apoptosis occurs through intrinsic and extrinsic pathways. The intrinsic pathway of apoptosis process is controlled by the Bcl-2 family of proteins including anti-apoptotic Bcl-2 and pro-apoptotic BAX family respectively. Homeostasis of colonic epithelium in the intestinal crypt will be maintained by balancing between cell proliferation and apoptosis function and tumor formation. Thus, Bcl-2/Bax ratio appears to best viable in assessing the overall inclination of a cell to undergo apoptosis. Apoptosis results from the activation of caspase which acts as aspartate specific proteases. Caspases form a proteolytic network within the cell, whereby activates upstream initiator caspases 9 and in turn caspase 9 activates downstream caspase 3. Caspase 3 is an effector caspases, it plays an important role in both death pathways and cleaves a wide range of cellular substrates, including structural proteins and DNA repair enzymes, as well as its regarded as most downstream enzyme in the apoptotic process due to its location in the protease cascade pathway. Thus, fisetin administration resulted in overexpression of pro-apoptotic BAX, Caspase-3 along with downregulation of Bcl-2, which exhibits the cytotoxicity inducer potential. Hence, fisetin induces apoptosis by modulating the expressions of Bcl, Bax and caspase-3.

CONCLUSION
This study elucidate the mechanism by which fisetin increases the anti-oxidant capacity of cell through enzymatic and non-enzymatic antioxidant enzymes, proteins studies suggest that fisetin induces the apoptosis, through increased exression of Bax and active caspase 3 along with decrease in Bcl-2 protein levels. Fisetin induces apoptosis which is evident through declination of STAT-3 protein level. Thus, inhibition of STAT-3 shows a promising strategy to eradicate CAC, further studies needed to found out the relationship between cytokine involvement and tumor formation in CAC.

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REFERENCES