Original Article

The anti-ulcerative colitis effects of *Annona squamosa* Linn. leaf aqueous extract in experimental animal model

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Abstract: This study aimed to evaluate the anti-inflammatory effects of *Annona squamosa* (*A. squamosa*) leaf aqueous extract against acetic acid induced colitis in rats with a trial to explore its use for the treatment of colon inflammation. Sprague Dawley rats weighing 180-200 g were used in this study. Treatment with *A. squamosa* extract at dose 300 mg/kg for 4 weeks counteracted acetic acid induced ulcerative colitis by a significant decrease (P<0.05) of colonic tissue of malondialdehyde (MDA) and significant increases of catalase (CAT), glutathione (GSH) and glutathione peroxidase (Gpx) compared to ulcerative colitis control group. Furthermore, induction of oxidative stress was observed in the colonic tissue through the levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) which significant increase in colonic tissue DNA by acetic acid. Moreover AA induced significant increase in serum interleukin-10 (IL10), tumor necrosis factor-α (TNF-α), transforming growth factor (TGF 1β), and C reactive protein (CRP) as compared to the control group. On the contrary, our results showed AA induced significant decrease of vascular endothelial growth factor (VEGF) and thyroid hormones triiodothyronin and thyroxin (T3 & T4) in installed group with AA as compared to control which significantly improved after treatment with *A. squamosa* leaf extract. Histopathological observation in our study confirmed the biochemical study. Thus, therapeutic method offer a sign to analyze further the effectiveness of *A. squamosa* as a unique agent for alleviating colitis.

Keywords: *Annona squamosa*, natural remedies, ulcerative colitis, free radicals

Introduction

Ulcerative colitis (UC), Crohn’s disease (CD) are common diseases affecting large intestine and characterized by an inflammatory process with remissions and exacerbations. These disorders represent the inflammatory bowel disease (IBDS) [1]. In pediatric age group the disease presents by both intestinal and extra intestinal manifestations serious complications occur like anemia and stunted growth. Ulcerative Colitis activity index (PUCAI) was established to assess the activity of the disease and therapeutic response of children [2].

Inflammation happens as a natural defense mechanism of the mammalian tissues against injuries in order to eliminate or restrict the spread of the injurious agents. A large number of medicinal plants throughout the world are attributed with having various medicinal properties as they contain different classes of phytochemicals [3].

Many studies prove that every part of *A. squamosa* possess medicinal property [4]. Folkloric record reported the use of *Annona squamosa* as an insecticidal, an antitumor agent, anti-diabetic, antioxidant, anti-lipidemic and anti-inflammatory agent which has been characterized due to the presence of the cyclic peptides. In addition, the crushed leaves were inhaled to overcome the hysteria and fainting spells, and they were also applied on the ulcers and wounds [5]. All parts of this plant (leaf, bark, shoot and roots) have various compounds of medicinal importance and hence were used in different kinds of health problems. The ulcer is a major health hazard both in terms of morbidity and mortality. Some results have observed that leaves of *Annona* species exhibit antioxidant activity in different *in vitro* models due to the
presence of flavonoids like rutin and hyperoside [6, 7].

The altered oxidant/antioxidant status in inflamed colon has received attention in both human and animals. Evidence suggests that reactive oxygen species (ROS) are produced in excess in the inflamed mucosa and may be pathogenic in inflammatory ulcerative disease [8]. The main sources of ROS in inflamed mucosa are activated phagocytic leucocytes and neutrophils, capable of producing superoxide and cascade of various reactive species leading to very reactive hydroxyl radicals and peroxide. These products cause impairment in cell membrane stability and death of cells by lipid peroxidation in inflammatory ulcerative disease [9]. These intermediate products of oxygen metabolism (superoxide, hydroxyl radicals and \( \text{H}_2\text{O}_2 \)) are controlled by various cellular defense mechanisms comprising enzymatic (superoxide dismutase and catalase) and non-enzymatic (GSH) scavenger component.

Protection versus free radicals can be achieved by preventing their formation, by blocking the chain reactions, or by repairing the oxidative damaged biomolecules [10]. The present study focuses on the inhibition of ulcerative colitis by A. squamosa leaf extract which might make it, enable to be a source of developing novel anti-ulcer medicinal product from nature. As well as, to achieve better control clinical and laboratory manifestations of the disease using a natural product to minimize the use of medications and to avoid their side effects especially with the long term use in pediatric age group.

Materials and methods

Preparation of plant extract

Leaves of A. squamosa were collected during December 2013 from the medicinal garden of Ghabor Farm. These were washed with water and 50 g of fresh leaves (kept at 25°C for 5 days in absence of sunlight) were extracted in 1 L of boiling water for 2 h and concentrated the volume by boiling in a water bath. The brown extract thus obtained was cooled, filtered using Whatman No. 1 filter paper, and centrifuged at 10,000 rpm at 25°C. The supernatant was concentrated up to 100 ml on rotary vapor under reduced pressure. The lyophilized concentrated crude extract was used for the study [11].

Determination of DPPH radical scavenging activity

A 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method was performed as described by Koleva et al. [12]. Approximately 10 μL of different concentrations (15-500 μg/mL) of test sample solutions were added to 190 μL DPPH (150 μM) in ethanol solution. The solutions were vortexed and incubated for 20 minutes at 37°C. The solvent alone acted as a blank. The decrease in absorbance of test mixtures (due to quenching of DPPH free radicals) was determined as being 517 nm, and the percentage of inhibition was calculated. Ascorbic acid was used as a standard. The IC\(_{50}\) values were determined as the concentration of the test mixture that gave 50% reduction in absorbance from that of the control blank.

Determination of total antioxidant activity (TTA)

The total antioxidant activity was evaluated using the method described by Prieto et al. [13]. An aliquot of sample solution/vitamin E (equivalent to 500 mg) was combined with reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The samples were then cooled at room temperature, and the absorbance was measured at 695 nm against the blank in a spectrophotometer. The total antioxidant activity was expressed as gram equivalents of vitamin E.

Induction of experimental colitis

Twenty four hour fasted rats were lightly anesthetized with diethyl ether inhalation. Colitis was induced by 4% acetic acid through a 1 ml sterile syringe coupled to a No. 6 polyethylene catheter, externally lubricated with lidocaine gel. The catheter was introduced anally to a distance of 7 cm, in accordance with the standard method of this laboratory [14, 15]. The animals in the control group received an endoanal infusion of 1 ml normal saline solution.

Experimental design

Twenty-eight adult male (12 weeks old) albino rats, weighing 180-200 g, were used in the
present study. They were housed in stainless steel cages, maintained at room temperature and provided with water and standard feed ad libitum.

After an adaptation period of one week, the rats were randomly divided into four equal groups (eight rats each). Group 1 (G1) sham group; without colitis induction and treated with extract vehicle (normal saline), GII AA group: treated with 4% AA colitis induction which continued for 3 consecutive days, GIII extract groups treated with *A squamosa* extract orally (300 mg/kg) and GIV, rats were treated *A squamosa* extract (300 mg/kg) following by AA treatment.

**Sampling**

The proximal 5 cm of the dissected colon specimen was used for biochemical analysis of tissue catalase (CAT) according to Beers and Sizer [16]. Glutathione peroxidase (GPx) activity was determined by using a colorimetric kit (BioVision, USA.). The serum samples were processed in a single batch using a commercially available sandwich VEGF ELISA detection kit (R&D Systems, Minneapolis, MN) according to manufacturer instructions. The colonic samples were minced and homogenized using a polytron homogenizer. The supernatant was obtained by centrifuging at 3000 rpm for 20 minutes. Serum 8-hydroxy-deoxyguanosine (8-OHDG), reduced glutathione-GSH [17] and Malondialdehyde (MDA) level MDA was estimated by Satoh [18]. 75 mg of Thiobarbituric acid (TBA) was dissolved in 15% TCA, to this 2.08 ml of 0.2N HCl was added, the volume was made up to 100 ml using 15% TCA. 3.0 ml of this reagent was added to 0.75 mL of serum of the rats. The test tubes were kept in a boiling water bath for 15 minutes. They were cooled and centrifuged for 10 minutes at 10000 rpm. Absorbance of the supernatant was read against the blank at 535 nm. The results were expressed in nmol/mL of serum. Interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and transforming growth factor beta (TGF 1β) test kits were obtained from R&D (USA). C-reactive protein (CRP) levels were determined using ELISA commercial kit (Quantikine M murine; R&D Systems, Minneapolis, MN). Serum levels of C-reactive protein (CRP) were quantified by a highly sensitive ELISA kit (Life Diagnostics, West Chester, UK). Besides, serum activities of total T3 & T4 by radioimmunoassay kit purchased from (Diagnostic Product Corporation (DPC), 96th St., Los Angeles).

**Ethic statement**

The study was conducted accordance with the guidelines set by the European Economic Community (EEC) regulations (Revised Directive 86/609/EEC) and approved by the Ethical Committee at National Center for Radiation Research, Egyptian Atomic Energy Authority, Cairo, Egypt (NCRR-EAEA).

**Histological examination**

Autopsy samples were taken from the aorta of rats in different groups and fixed in 10% formal saline for twenty four hours. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 degrees in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by slide microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin & eosin stain for examination through the light electric microscope [19].

**Immunohistochemical study: proliferating cell nuclear antigen (PCNA)**

Colonic tissue was immediately placed in 4% paraformaldehyde solution for histological studies. Evaluation of epithelial cell proliferation was performed by immunohistochemistry using a mouse monoclonal anti-PCNA antibody (Santa Cruz Corporation, USA). PCNA index were recorded by the image analyzer (Leica Q500 MC program) in the Histology Department, Faculty of Medicine, Ain Shams University.

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\text{PCNA index} = \frac{\text{PCNA positive nuclei/crypts}}{\text{Total number of nuclei/crypts}} \times 100
\]

Total number of nuclei/crypts.

**Statistical analysis**

The data analysis was carried out with SPSS Inc. software (version 22). One-way ANOVA was used to study a significant difference between means of the experimental groups with a significance level of $P<0.05$. Tukey-Kramer’s test was used to compare the significance among
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Table 1. Effect of *Annona squamosa* leaf aqueous extract on antioxidants, MDA, IL10, TNF and CRP in control and experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (I)</th>
<th>Acute colitis (II)</th>
<th><em>A. squamosa</em> (III)</th>
<th>Treatment (IV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (U/mg)</td>
<td>65.49±4.04*</td>
<td>38.14±5.16*</td>
<td>68.18±4.86*</td>
<td>59.26±4.65*</td>
<td>0.003*</td>
</tr>
<tr>
<td>GSH (nmol/mg)</td>
<td>0.67±0.11*</td>
<td>0.23±0.07*</td>
<td>0.59±0.08*</td>
<td>0.55±0.06*</td>
<td>0.014*</td>
</tr>
<tr>
<td>GPx (U/mg)</td>
<td>61.50±4.27</td>
<td>29.81±3.33*</td>
<td>58.42±4.92*</td>
<td>72.09±3.46*</td>
<td>0.000*</td>
</tr>
<tr>
<td>MDA (nmol/mg)</td>
<td>12.82±1.39*</td>
<td>72.84±5.68*</td>
<td>15.28±1.68*</td>
<td>21.7±3.13*</td>
<td>0.000*</td>
</tr>
<tr>
<td>IL10 (pg/ml)</td>
<td>455.0±18.60*</td>
<td>816.17±14.27*</td>
<td>478.7±12.55*</td>
<td>458.47±11.87*</td>
<td>0.000*</td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>8.22±0.86*</td>
<td>36.70±2.67*</td>
<td>10.0±0.82</td>
<td>14.32±0.98*</td>
<td>0.000*</td>
</tr>
<tr>
<td>CRP (ng/ml)</td>
<td>3.59±0.31*</td>
<td>6.56±0.35*</td>
<td>3.79±0.12*</td>
<td>4.66±0.82*</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SE; a, b, ab & c: Statistically significant from control or ulcerative colitis group, respectively at P<0.05 using one-way ANOVA followed by Tukey-Kramer as a post-hoc test. *F (P<0.05).

Table 2. Effect of *Annona squamosa* leaf aqueous extract on TDF-1, 8-OHDG and VEGF in control and experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (I)</th>
<th>Acute colitis (II)</th>
<th><em>A. squamosa</em> (III)</th>
<th>Treatment (IV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β (pg/ml)</td>
<td>37.25±2.50*</td>
<td>58.45±2.63*</td>
<td>40.50±2.90*</td>
<td>44.36±3.53*</td>
<td>0.000*</td>
</tr>
<tr>
<td>8OHDG (pg/ml)</td>
<td>12.46±1.13*</td>
<td>30.47±3.84*</td>
<td>14.75±1.25*</td>
<td>19.00±1.39*</td>
<td>0.000*</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>3.75±0.48</td>
<td>1.33±0.05*</td>
<td>4.50±0.33*</td>
<td>10.47±0.66*</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SE; a, b & c: Statistically significant from control or ulcerative colitis group, respectively at P<0.05 using one-way ANOVA followed by Tukey–Kramer as a post-hoc test. *F (P<0.05).

Table 3. Effect of *Annona squamosa* leaf aqueous extract on thyroid hormones in control and experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control (I)</th>
<th>Acute colitis (II)</th>
<th><em>A. squamosa</em> (III)</th>
<th>Treatment (IV)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 (ng/dl)</td>
<td>55.75±4.33*</td>
<td>34.25±3.42*</td>
<td>53.50±4.37*</td>
<td>51.00±3.58*</td>
<td>0.000*</td>
</tr>
<tr>
<td>T4 (µg/dl)</td>
<td>4.64±0.47</td>
<td>2.75±0.25*</td>
<td>4.02±0.44*</td>
<td>3.97±0.36*</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SE; a, b & c: Statistically significant from control or ulcerative colitis group, respectively at P<0.05 using one-way ANOVA followed by Tukey-Kramer as a post-hoc test. *F (P<0.05).

The results pertaining to the free radical scavenging activity of the two different extracts, along with the standard reference vitamin C. The model of stable DPPH free radicals can be used to evaluate the antioxidant activity in a relatively short time. The concentration of the sample necessary to reduce the initial concentration of DPPH by 50% (IC<sub>50</sub>) under the experimental conditions was determined. A lower value of IC<sub>50</sub> indicates higher antioxidant activity. The aqueous extract showed comparable levels of free radical scavenging activity with an IC<sub>50</sub> value of (157.2 µg/mL).

**Total antioxidant**

The total antioxidant activity of aqueous extracts was 189 µg, against the standard α-tocopherol.

**In vivo results**

The results of the present study showed a significant increase (P<0.05) in the content of MDA and significant decrease (P<0.05) in the activities of CAT, Gpx and level of GSH in the colon tissues of a group of rats injected with acetic acid (colitic group) as compared with the normal control group as shown in Table 1. The elevation of MDA level was significantly decreased and the activities of CAT, Gpx and level of GSH were significantly (P<0.05) improved in a group of rats injected with acetic acid and treated with aqueous extract of *Annona squamosa* with F values.
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Figure 1. A. Photomicrograph of a section of colon of a control rat showing closely packed simple tubular colonic crypts extending down to muscularis mucosa (mu). The lamina propria appears filling the space between the colonic crypts and contains mononuclear cells. B. Photomicrograph of a section of the colon in group II (colitis). Some crypts show dilated lumen in their basal parts (↑). The sub-mucosal layer showed massive number of focal inflammatory cells infiltration. C. Annona squamosa group showing the luminal surface is lined by simple columnar cells with basal oval nuclei and apical brush border. Goblet cells predominate in the glands and show basal nuclei. D. The group of rats injected with AA and treated with A. squamosa leaves extract. Goblet cells formation was noticed in lining mucosal epithelium. E, F. Immunohistochemical staining for PCNA in control colon. G. Immunohistochemical staining for PCNA in GII (Annona squamosa) only. I, J. Immunohistochemical staining for PCNA in GIV (AA+ Annona squamosa).

Table 4. PCNA index in control and experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>No</th>
<th>Mean ± SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>8.96±0.71b</td>
<td>0.012</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>38.51±2.84a</td>
<td>0.002</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>10.40±1.33b</td>
<td>0.003</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>13.0±0.87a</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SE; a, b: Statistically significant from control or ulcerative colitis group, respectively at P<0.05 using one-way ANOVA followed by Tukey-Kramer as a post-hoc test. P<0.05.

As shown in Table 2 which revealed significant elevation (P<0.05) in the concentrations of serum interleukin-10 (IL10), tumor necrosis factor-α (TNF-α), transforming growth factor (TGF 1β), C reactive protein (CRP) and 8-hydroxy-2’-deoxyguanosine (8-OHDG) in a group of rats injected with acetic acid as compared to the normal control group. It was noticed that the elevation in the concentration of these parameters significantly (P<0.05) decreased in the group of rats treated with extract of Annona squamosa as compared with the colitic rats group. Our results showed AA induced significant decrease of vascular endothelial growth factor (VEGF) which significantly (P<0.05) increased in the group of rats treated with extract of Annona squamosa. On the other hand the levels of thyroid hormones triiodothyronine and thyroxin (T3 & T4) in an installed group with AA showed a significant decrease (P<0.05) as compared to control which significantly improved after treatment with A. squamosa leaves extract as shown in Table 3.

Histopathological findings

There was no histopathological alteration, and the normal histological structure of the mucosa and sub-mucosa were recorded in the normal control group and the group administrated with A. squamosa leaves extract only as shown in Figure 1A, 1B. But in a group of rats injected with AA and induced colitis the sub-mucosal layer showed massive numbers of focal inflammatory cells infiltration (Figure 1C). On the contrary, the group of rats injected with AA and treated with A. squamosa leaves extract showed significant Protective effects on colon where Goblet cells formation were noticed in the lining mucosal epithelium (Figure 1D).

Immunohistochemistry for PCNA

Our results demonstrated that PCNA was positively stained in cellular nucleus of colonic glands; particular in the lower third of the crypts (Figure 1E, 1F). Immunohistochemical staining for PCNA showed that PCNA labeled nuclei nearly reached up to the surface in many crypts in GII (Figure 1G). Moreover, the PCNA index showed significant increase compared to group I (Table 4). Positively stained nuclei for PCNA remained at the lower third of the crypts in GIII. The PCNA index showed non-significant increase compared to control group (Figure 1H; Table 4). By PCNA, positively stained nuclei were located in the lower third of the crypts. Besides, damaged cells have strongly positive PCNA immunohistochemical stain with irregular in the shape of crypt. By PCNA, positively stained nuclei were located in the lower third of the crypts in GIV (Figure 1I, 1J). However, they appeared significant decrease compared to group II (Table 4). The percentage of PCNA-positive cells of colon mucosa in normal control group were 8.96±0.71, whereas the percentage of PCNA-positive cells in AA control group were 38.51 ± 2.84, A squamosa-treated group was 13.0±1.58 (Table 4). Our results revealed that cell proliferation in colonic glands was increased in rats with experimental colitis, as determined by the biomarker, PCNA.
Discussion

Many factors have been caused in the events of ulcerative colitis, such as neutrophil infiltration and the over-production of proinflammatory mediators, including cytokines, and reactive oxygen species (ROS). The tissue injury produced by neutrophils and macrophages has been attributed to their ability to release ROS, nitrogen metabolites, cytotoxic proteins, lytic enzymes and cytokines as well as their disrupting effects on the epithelial integrity [20].

Acetic acid induced colitis model is similar to human ulcerative colitis in terms of histological features. It affects the distal colon portion and induces non-transmural inflammation, massive necrosis of mucosal and sub-mucosal layers, mucosal edema, neutrophil infiltration of the mucosa and sub-mucosal ulceration [21].

This study reported that the technique resulted in ulcerative colitis as was evident from the acetic acid administration. *Annona squamosa* Linn is a multi-target tree with edible fruits and is a source of the medicinal & industrial products. *Annona squamosa* Linn is used as an antioxidant, anti-diabetics, hepato-protective, cytotoxic activity, gene toxicity, anti-tumor activity, anti-lice agent. It is related to contain alkaloids, carbohydrates, stable oils, tannins & phenolic compounds. Earlier works on the phytochemistry of *Annona squamosa* leaf, reported the presence of alkaloids, falvonoids, phenols, saponins, glycosides in water, methanol, chloroform, and petroleum ether extracts [22, 23].

The results of our study showed a significant increase (P<0.05) in the content of MDA and significant decrease (P<0.05) in the activities of CAT, Gpx and level of GSH in the colon tissues of a group of rats injected with acetic acid suggesting that AA may aggravate oxidative and inflammatory injury in rats induced colitis. In this study, *A squamosa* decreased oxidative damage compared with ulcerative colitis. There are multiple evidences suggesting the role of oxygen free radicals in colitis. CAT, GSH and GPx are an important antioxidant enzyme which may be used for the free radical destruction and may be prevented by the free radical species the results are consistent with William and Juneja [24] who hypothesize that aqueous extract of *A. squamosus* may cause the alleviation of ROS generation through its effect on these antioxidants in colon. This study also provides an indication to further explore the antioxidative effects of *A. squamosa* species for alleviating chronic colitis. *Annona squamosa* L (Annonaceae family), having high content of nutritional compounds such as vitamin C, thiamine, niacin, niacinamide, dietary fiber, potassium and iron in considerably significant amounts [25].

Compared with those of the control group, the levels of IL-10, TNF-a, CRP and the TGF-1B in the colonic tissues were significantly increased in the group of rats installed with AA, implying that AA may aggravate colonic damage by triggering the release of inflammatory factors in rats with colitis, the results in accordance with [26]. These inflammatory mediators significantly improved after treatment with an extract of *Annona squamosa*, this extract possesses centrally and peripherally mediated analgesic properties. The central analgesic action may be mediated via inhibition of central pain receptors, while the peripheral analgesic effect may be mediated through inhibition of cycloxygenase and/or lipoxygenase (and other inflammatory mediators). This hypothesis is in consonance with those of Wagner et al. [27] who postulated that acetic acid writhing and hot plate test methods are useful techniques for the evaluation of centrally and peripherally acting analgesic drugs, respectively.

*Annona squamosa* leaves was found to possess significant analgesic and anti-inflammatory activity which is quite comparable to Pentozocine and Aceclofenac there by leading to believe in a mixed central and peripheral mechanism for its action [28]. Furthermore a detailed investigation on the extract is underway to determine the phytoconstituents that are responsible for these activities as well as to define the exact mechanism of action of the herbal drug.

The results indicate the leaf extract of *Annona squamosa* L enhance the plasma levels of thyroid hormones and there is evidence that leaf extract of this plant has thyroid inhibitory and antiperoxidative in nature [29] which also effective in proper functioning of carbohydrate as well as lipid metabolism [30].

PCNA had been used in the past in an attempt to measure the proliferative activity ofodontost
genic epithelium cysts [28]. At late S phase, PCNA is outstanding within the nucleoli. PCNA is seen to be powerfully associated within the nuclear regions wherever DNA synthesis is happening [31]. In the present study, PCNA expression in the lower third of the crypts of colon was significantly increased in GII. This suggests that the highest proliferative activity is in the suprabasal cell layers [31, 32]. This could also reflect a unique epithelial differentiation process, in which the basal cells assume some characteristics of preameloblasts, which indicate that it might have entered to some extent towards ameloblast differentiation [33]. The appearance of differentiated cell in the basal layer probably accounts for the fact that the major proliferation compartment is suprabasally. Immunohistochemical study detected decrease in the number of PCNA positive in group (IV) which indicated a decrease in the cell division in the crypt region. While, there was increase in the number of PCNA positive nuclei in group (II). The term of proliferative antigens is correlated with various degrees of epithelial dysplasia and inflammatory changes in biopsy specimens from patients with long-standing ulcerative colitis [34]. In the present work, A. squamosa at either treatment level significantly reduced the increase in the percentages of PCNA-positive cells seen in rats treated with AA.

The products of lipid peroxidation, particularly the cytotoxic aldehydes like malondialdehyde (MDA), are important because they can also induce damage DNA [35]. A highly active hydroxyl radical can break the DNA threads creating (8-OHdG). Thus, this DNA damage may compromise gene expression [36]. Moreover, the 8-OHdG adduct was determined as a marker of oxidative DNA harm because this lesion is a frequently found adduct in mutated oncogenes and tumor suppressor genes [37]. Thus, our observation of the increased oxidative DNA damage in the colon is highly significant, and could lead to serious consequences, if unrepaired.

Histological examination showed that mucosa and submucosa were the main sites of colonic injury, with abundant inflammatory cell infiltration, local erosion and ulcer formation compared with those in the control group, while a group of rats treated with leaf extracts of Annona squamosa Goblet cells formation were noticed in the lining mucosal epithelium these cells are mucus comprises an important barrier that prevents bacteria and other inflammatory agents from invading the mucosa [38]. Goblet cells are reduced in number and size in ulcerative colitis. Recent studies in mouse models of colitis focus the significance of the role of mucin in preserving the integrity of protective mucus barriers which breakdown can result in colitis [39].

Medical treatment of ulcerative colitis in pediatric population includes anti-inflammatory therapy, corticosteroids and immunomodulatory agents.

Although medications can achieve a good laboratory and clinical control yet, there are limiting factors to their use due to the adverse effects. Corticosteroids are effective in the acute exacerbation but on the long term side effects appear like osteoporosis, compression fractures, weight gain, cushingoid appearance restlessness as well as personality changes and emotional liability [40]. Immunomodulatory agents as purine are useful in treatment of ulcerative colitis yet they have serious draw backs as pancreatitis, hepatitis and bone marrow suppression. Patients should be monitored for leukopenia. Treatment of fulminant cases includes cyclosporine tacrolimus and infliximab are nephrotoxic causing irreversible renal damage and insufficiency. Tacrolimus although it is a potent inhibitor of T-lymphocytes but, still it is nephrotoxic, causes bone marrow suppression and neurological symptoms [41].

In children elective colectomy is indicated in severe cases with growth retardation and malnutrition when dysplasia is detected which may causes malignancy [42]. So, the need for a naturally occurring anti-ulcer medicinal product as Annona squamosal leaves extract in an important issue especially in pediatric patients to avoid the adverse effect of anti-inflammatory, immunomodulatory drugs and steroids on the long term.

Conclusion

Ulcerative colitis is a chronic and inflammatory disease caused by the inflammation and sores in the lining of large intestine. Annona squamosa leaves extract have potential anti-ulcer activity and can be used as anti-ulcer medici-
nal product from nature. However, inhibition of ulceration events by A. squamosa is suggestive of an important balance between antioxidant enzymes and anti-inflammatory cytokine products.

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**Disclosure of conflict of interest**

None.

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