RESEARCH ARTICLE

Putative antioxidant property of *Annona squamosa* on acetic acid induced ulcerative colitis

V. Chinni Krishnaiah*[1,2], T. S. Mohamed Saleem[2], D. Sujatha[1,3], B. Pusphakumari[1], G.Anitha[1]

Department of Pharmacology, Sri Padmavathi School of Pharmacy, Tirupathi, India
Department of Pharmacology, Annamacharya College of Pharmacy, Rajampet-516126, India
Department of Pharmacology, National Institute of Pharmaceutical Education & Research, Hyderabad, India

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ABSTRACT

The purpose of the present study is to evaluate the protective role of ethanolic extract of *Annona squamosa* against acetic acid induced ulcerative colitis. Male Wistar rats received two different doses of extract (250 and 500 mg/kg bw p.o.), sulfasalazine, or vehicle for 3 consecutive days before induction of ulcerative colitis by intra-rectal acetic acid administration, and continued further for 7 days after the induction. The colonic mucosal injury was assessed by macroscopic scoring. Furthermore, the mucosal content of malondialdehyde (MDA) and endogenous antioxidants (GSH, SOD and catalase) activity were considered as parameters of the redox state. Acute inflammatory response was determined by measuring myeloperoxidase (MPO) from rat colon. Effect of *Annona squamosa* against ulcerative colitis was identified by decreasing mucosal epithelium damage, evident by decrease in macroscopical score, wet weight of the colon. All biochemical parameters were reverting by *Annona squamosa* treatment in ulcerated rats and the protective role of *Annona squamosa* was comparable with reference standard sulfasalazine. From this study it has been concluded that *Annona squamosa* attenuates acetic acid induced ulcerative colitis by reducing oxidative stress in rats.

Key words: Inflammatory bowel disease, Oxidative stress, *Annona squamosa*, Endogenous antioxidant, Rat colon

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) of the colon and rectum. The main etiological factors to cause UC have been reported, includes genetic, immunologic, and environment [1-2]. In recent research it was reported that oxidative stress also plays a major role in the development of UC. Generation of reactive oxygen species (ROS), hypochlorous acid and oxidant derivatives in inflamed mucosa, may be pathogenic in IBD [3-4. Various mechanisms has been proposed for the generation of ROS in inflamed mucosa like activated phagocytic leukocytes and neutrophils, capable of producing superoxide and cascade of various reactive species leading to a very reactive hydroxyl radical and peroxide. These products cause the impairment in cell membrane stability and death of the cells by lipid peroxidation in IBD [5-7. Several experimental and human studies proved that anti-oxidant supplementations like vitamin E, selenium and trimetazidine were found to be beneficial in UC models [8-10].

Existing drugs to treat UC shows effective and may exert maximum side effects. So, the search of new therapeutic agents with fewer side effects and also with putative antioxidant properties would be very useful [11]. The plant *Annona squamosa* (annonaceae) is commonly called as custard apple in English sharifa in Hindi [12]. Hepatoprotective activity of leaves of *Annona squamosa* documented by Saleem et al. [13-14]. The crushed leaves are sniffed to overcome hysteria & fainting spells. Traditionally the leaves were applied to ulcer and wounds [15](Pandey and Barve, 2011), the antiulcer activity of leaves of this plant also well documented [16](Mohamed Saleem et al., 2012). This research was aimed at investigating the protective role of leaves of *Annona squamosa* acetic acid induced UC in order to support or refute the claims by traditional herbalists in India.

Address for correspondence

Mr. V. Chinni Krishnaiah
Assistant Professor,
Department of Pharmacology
Annamacharya College of Pharmacy,
Rajampet-516126, India
E.mail: chinnipharmacy@gmail.com

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MATERIALS AND METHODS

Plant material

Annona squamosa was collected in and around Tiruchanoor in the month of June 2009 and identified by Dr. N. Yasodamma, Prof. of Botany, Sri Venkateswara University, Tirupathi. The coarsely powdered leaves were extracted with ethanol on a reflux water bath for 3 hr. The cycle was repeated for three times. The extract was concentrated on rotary flash evaporator to semi solid consistency and then dried over a water bath (yield – 200 g/kg).

Drugs and chemicals

Acetic acid and sulfasalazine were procured from Himedia Pvt.Ltd, India and all other chemicals and reagents used were of analytical grade, procured form SD fine chemicals Pvt. Ltd. India.

Animals

Male Wistar albino rats (150-200 g) were used for this study. The study was approved by the Ethics Committee for animal experimentation (1016/a/06/CPCSEA/003/2009),. The animals were obtained from the parent institute and kept in animal house in standard conditions. All animals were fasted 24 h prior to the experimental procedure.

Induction of ulcerative colitis

A polypropylene tube with 2 mm diameter was inserted through the rectum into the colon to a distance of 8 cm. A solution of two ml (3%, v/v) acetic acid (Merck , Germany) in saline was instilled. The rats were then maintained in a supine Trendelenburg position for 30 s to prevent early leakage of the intracolonic instillate [17](Millar et al., 1996).

Treatment protocol

Animals were divided into six groups (n = 6), Group 1: Normal control group received saline, Group 2: Colitis model received saline and subjected to rectal instillation of acetic acid after 3 days, Group3: received sulfasalazine (500 mg/kg) and subjected to rectal instillation of acetic acid after 3 days, Group 4 & 5: *Annona squamosa* treated group received 250 and 500 mg/kg bw p.o, respectively and subjected to rectal instillation of acetic acid after 3 days. All treatment regimens were continued for another 7 consecutive days. On the 11th day, rats were sacrificed under ether anesthesia and laparatomy was performed. Colonic segments were excised, freed of adherent adipose tissue, washed with saline, and were examined for macroscopic scoring and biochemical studies. Average wet weight of the colon was observed.

Macroscopic scoring

Mucosal damage was assessed macroscopically by the scoring system of Millar et al. [17], where 6 cm of colon extending proximally for 2 cm above the anal orifice were cut, weighed then split longitudinally. In each rat, the macroscopic injury of each ulcer was scored by an independent observer according to a scale ranging from 0 to 4 as follows: (0) no macroscopic changes, (1) mucosal erythema only, (2) mild mucosal edema, slight bleeding or small erosions, (3) moderate edema, bleeding ulcers or erosions, and (4) severe ulceration, erosions, edema and tissue necrosis. Intermediate values reflected intermediate appearances.

Biochemical parameters

Colons were scrapped and homogenized in chilled phosphate buffer (pH 7.4) using a homogenizer. The homogenates were centrifuged at 800 rpm for 5 min at 4°C (REMI C-24) to separate the molecular debris. The supernatant so obtained was centrifuged at 10,000 rpm for 20 min at 4°C (REMI CM-12) to get the post mitochondrial supernantant (PMS), which was used to assay the tissue myeloperoxidase (MPO) [18], lipid peroxidation (LPO) [19], reduced glutathione (GSH) [20], superoxide dismutase (SOD) [21], catalase (CAT) [22].

Statistical analysis

All values were expressed as Mean ± SEM. (n = 6 in each groups). One way ANOVA was applied to test for significance of biochemical data of the different groups. Significance is set at $p<0.001$.

RESULTS

Acute toxicity study

Annona squamosa was found to be safe, as no animal died at a single oral dose of 4g /kg. *Annona squamosa* didn't exhibit any gross behavioral changes at the single oral dose of 4g /kg.

Acetic acid induced ulcerative colitis model

Effect of *Annona squamosa* on activity index

Administration of acetic acid has slow down the animals, indicated by the slowness in the activity when compared to the normal animals. The animals treated with standard drug sulfasalazine (G-II) were observed to be more active when compared to the control animals (G-I). On administration of *Annona squamosa* at low dose (250 mg/kg) the activity was found to be slow as observed in the control group (G-I), while the activity was intermediate in case of animals treated with high dose of *Annona squamosa* (500 mg/kg). The results were presented in Table 1.

Effect of *Annona squamosa* on crypt abscesses formation

Administration of acetic acid caused severe crypt abscesses in the colon of the animal. The standard group treated with sulfasalazine (100 mg/kg), has shown absolutely no crypt abscesses formation, when compared to the control group (G-I). Whereas, the abscesses formation was moderate in the animals receiving low dose of *Annona squamosa* and mild when high dose of *Annona squamosa* was administered when compared to the control group(G-I). The results were presented in Table 1.

Effect of *Annona squamosa* on gross mucosal inflammation

A severe mucosal inflammation was observed in control group (G-I) when treated with acetic acid. The mucosal inflammation was very mild in the standard group (G- II) when treated with sulfasalazine. The gross mucosal inflammation was moderately severe upon administration of *Annona squamosa* at low dose (G-III), where as it was observed to be mild when treated with high dose of *Annona squamosa* (G-IV), on comparison with the control group (G-I) (Table-4). The results were presented in Table 1.

Effect of *Annona squamosa* on wet weight of the colon

The average wet weight of the colon was increased when treated with acetic acid (G-I), but the wet weight of the colon decreased upon administration of the standard drug sulfasalazine (G-II), when compared to the control (G-I). Animals treated with the high dose of *Annona squamosa* (G-IV) showed a significant decrease in the wet weight of the colon ($p < 0.01$) when compared to the control group (G-I). The effect of *Annona squamosa* at low dose on the wet weight of the colon was not significant on comparison with the control group. The results were presented in Table 2.

Effect of *Annona squamosa* on diarrhea

Administration of acetic acid causes severe diarrhea as observed in the control group (G-I). The standard group (G-II) treated with sulfasalazine has shown mild diarrhea when compared to the control group. On administration of *Annona squamosa* moderate diarrhea was observed with the low dose group (G-III) and mild diarrhea with the high dose group (G-IV) when compared to the severity of the control group (G-II). The results were presented in Table 2.

Effect of *Annona squamosa* on disease score

Acetic acid has induced severe disease score was observed in the control group animals (G-I). The group treated with standard drug sulfasalazine showed moderate score when compared to the control group (G-I). The groups treated with *Annona squamosa* has shown mild disease score in high dose and moderate disease score in low dose group (G-III) when compared to the control group (G-I) (Table-). The effect of *Annona squamosa* on different parameters of UC was observed to be dose dependent. The effect of *Annona squamosa* at the high dose was almost comparable with that of the standard. The results were presented in Table 2.

The colon in untreated animals was normal without any signs of inflammation as seen in (plate 1-a).

Administration of acetic acid caused severe mucosal inflammation and crypt abscesses formation (plate 1-b). Treatment with sulfasalzine has reduced mucosal inflammation and crypt abscesses formation (plate 1-c). The effect of *Annona squamosa* at low dose was not much promising, as traces of mucosal inflammation and crypt abscesses were noted (plate 1-d). *Annona squamosa* at high dose has shown similar action as that of sulfasalzine, as no mucosal inflammation and crypt abscesses could be observed (plate 1-e).

Effect of *Annona squamosa* on MPO activity

A significant increase in MPO activity was observed in disease control when compared to the normal. The MPO values were decreased significantly in groups treated with sulfasalazine and ethanolic extract of *Annona squamosa* at both the doses (250 mg/kg, 500 mg/kg), when compare to the control group respectively. The results were presented in Table 3.

Effect on SOD

There was a significant ($p < 0.05$) decrease in the SOD levels of the disease control group (G-II) when compared to the normal group (G-I). The standard and test groups (G-III and IV, V) showed a significant ($p < 0.001$) increase in the SOD levels when compared to the control groups (G-II) (Figure-2)

Effect on catalase

On administration of acetic acid a significant ($p < 0.001$) reduction in the catalase levels was observed in the control group (G-II) when compared to the normal group (G-I). On treatment with sulfasalazine and alcoholic extract of *Annona squamosa* at both the doses caused (100 mg/kg) a significant ($p < 0.001$, 0.05) increase in the enzyme levels when compared to the control group (G-II) (Figure-3).

Effect on reduced glutathione

Glutathione levels were observed to decrease significantly ($p < 0.001$) in control group (G-II) when compared to the normal group (G-I). The standard group and the test groups (G- III and IV, V) showed a significant ($p < 0.001$; 0.01) elevation in the enzymes levels when compared to control group (G-I) respectively (Figure-4).

Effect on lipid peroxidation

There was a significant ($p < 0.001$) increase in the malondialdehyde levels in the control group (G-II) when compared to the normal group (G-I). On the treatment with sulfasalzine (G-III), a significant ($p < 0.001$) decrease was observed when compared to the control group animals (G-II). Group IV and V receiving different doses of alcoholic extract of *Annona squamosa* showed a significant ($p < 0.001$) reduction in LPO when compared to the control group (G-II) (Figure-5)

DISCUSSION

Acetic acid induced colitis is an easily inducible model of IBD and the similarity of the inflammatory mediators phase bears some resemblance to acute human intestinal inflammation. The acetic acid induced colitis model has been extensively used by various authors as a model which causes severe diffuse distal colitis [23-25]. Several major causative factors in the initiation of human colitis such as enhanced vasopermeability, prolonged neutrophils infiltration and increased production of inflammatory mediators are also seen involved in this animal model of Inflammatory bowel disease. In the present study, moderate but persistent ulceration was observed in the control animals supporting acetic acid induced ulcerative colitis as one of the suitable model for evaluating useful drugs against UC.

In acetic acid induced ulcerative colitis model, epithelial or mucosal necrosis and transient inflammation and edema that variably extended in to the lamina propria, sub mucosa (or) external muscle layers, depending on the concentration and length of exposure of acetic acid mucosal and sub mucosal inflammation followed by epithelial injury is common and was associated with activation of arachidonic acid pathways.

Both the test groups treated with 250 mg and 500 mg/kg *A. squamosa* showed a significant reduction in crypt abscess formation and gross mucosal inflammation of the colon which can be due to the

evident improvement in the different scores like activity score, disease score and severity of diarrhea. Similar improvement in the parameters of the colitis was reported by the established drugs used against UC [26-27].

The wet weight of the inflamed colonic tissue is considered reliable and sensible indication of severity and extract of inflammatory response. The average wet weight of the test groups treated with 250 mg and 500 mg/kg of *Annona squamosa* showed significant reductions which were almost comparable to that of standard group implying its potential use in the treatment of UC.

Diarrhea forms one of the most important clinical manifestations of ulcerative colitis. The decrease in severity of diarrhea in test groups treated with *Annona squamosa* implies the protective activity of *Annona squamosa* against the inflammatory UC.

Myeloperoxidase is an important enzyme of neutrophils, related to oxidant burst for bacterial killing. The colonic MPO activity, an index of neutrophil activation and inflammation was increased in acetic acid treated animals. Activated neutrophils pass out of the circulation and enter the inflamed mucosa and submucosa of the large intestine during acute inflammation, leading to over production of reactive oxygen and nitrogen species, proteases, lactoferin and lipid mediators that can contribute to intestinal injury [28]. This increase in MPO activity was substantially reduced in rats treated with *Annona squamosa* at both the doses. This indicates anti-inflammatory effect of *Annona squamosa* in the prevention of acetic acid induced ulcerative colitis.

Reactive oxygen species, either directly or via the formation of lipid peroxidation products, may play a role in enhancing inflammation through the activation of stress kinases (c-Jun activated kinase, extracellular signal-regulated kinase, p38) and redox-sensitive transcription factors, such as NF-kB. This results in increased expression of a battery of distinct proinflammatory mediators [29].

ROS attack the cellular macromolecules, thus disrupting epithelial cell integrity and hindering mucosal recovery, especially in case of impaired endogenous defense systems [7]. In acetic acid induced model ROS formation may play a major role, as indicated by the elevation of LPO and decreasing of SOD, CAT and GSH, thus supporting the role of oxidative stress in ulcerative colitis.

It was observed from the present study, that there is a significant increase in the levels of protective SOD, CAT and GSH enzymes. This effect is followed by an reduction in LPO levels, indicating the antioxidant property of the *Annona squamosa.*

The presence of phytoconstituents like flavanoids and polyphenols may be responsible for the antioxidant activity of *Annona squamosa*, similar potential of *Annona squamosa* as good antioxidant source was reported earlier, but against *in vitro* scavenging of 1, 1-diphenyl-2-picryl hydrazyl, 2,2- azinobis- (3-ethyl benjothizoline-2-sulphonate and nitric oxide) [30](Baskar et al., 2006).

Our study once again justifies that potent antioxidants may be of use to treat UC and we can also suggest that protection offered by *Annona squamosa* against acetic acid induced UC might be due to its antioxidant property. Further studies to identify responsible active constituents and probable mechanism of action might be rewarding.

CONCLUSION

From this research it has been concluded that the protective role of ethanolic extract of *Annona squamosa* provide pharmacological support to folkloric, ethno-medical uses of this plant in the management of inflammatory GIT disorders. Therefore, *Annona squamosa* could be beneficial as a complementary agent in UC and offers an alternative approach to modulate the inflammatory process involved in this disease. Results from the current study can generate promising outcomes in other models of ulcerative colitis.

Table 1: Effect of *Annona squamosa* on acetic acid induced ulcerative colitis

S. No	Name of the group	Activity score	Crypt abscesses	Gross mucosal inflammation
1	Control (acetic acid 8%)	Slow (4/6)	Severe abscesses (6/6)	Severe (5/6)
2	Standard (acetic acid 8%+sulfasalazine100mg/kg)	Active (5/6)	No abscesses (0/6)	Mild (3/6)
3	Low dose (acetic acid 8%+ *A.squamosa* 250mg/kg)	Slow (4/6)	Moderate abscesses (4/6)	Moderate (4/6)
4	High dose (acetic acid 8%+ *Annona squamosa*500mg/kg)	Intermediate (5/6)	Mild abscesses (3/6)	Mild (4/6)

Table 2: Effect of *A. squamosa* on acetic acid induced ulcerative colitis

S. No	Name of the group	Disease score	Wet weight (g)	Diarrhea
1	Control (acetic acid 8%)	Severe (6/6)	1.01±0.02	Severe
2	Standard (acetic acid 8%+sulfasalazine 100mg/kg)	Moderate (4/6)	0.690±0.1**	Mild
3	Low dose (acetic acid 8%+ *A.squamosa*250mg/kg)	Moderate (5/6)	0.897±0.04*	Moderate
4	High dose (acetic acid 8%+ *A. squamosa* 500mg/kg)	Mild (4/6)	0.71±0.09**	Mild

All values are expressed as mean ± S.E.M.
**** =p < 0.05, when compared to the control.***
***** =p < 0.05, when compared to the control***

Table 3: Effect of *A. squamosa* on MPO activity in colon

S. No	Name of the Group	MPO activity
1	Normal (vehicle)	0.63±0.11
2	Control (acetic acid 8%)	0.91±0.09*
3	Standard (acetic acid 8% + sulfasalazine 100 mg/kg)	0.52±0.11***
4	Low dose (acetic acid 8%+ *A.squamosa*250mg/kg)	0.71±0.03**
5	High dose (acetic acid 8%+ *A. squamosa* 500mg/kg)	0.48±0.04***

* =p < 0.01, when compared to the normal
** =p < 0.05, when compared to the control
*** =p < 0.01, when compared to the control

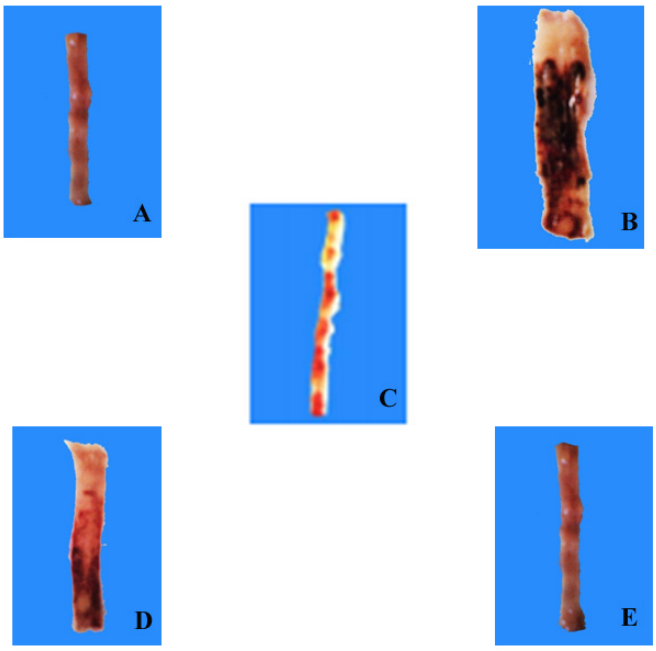
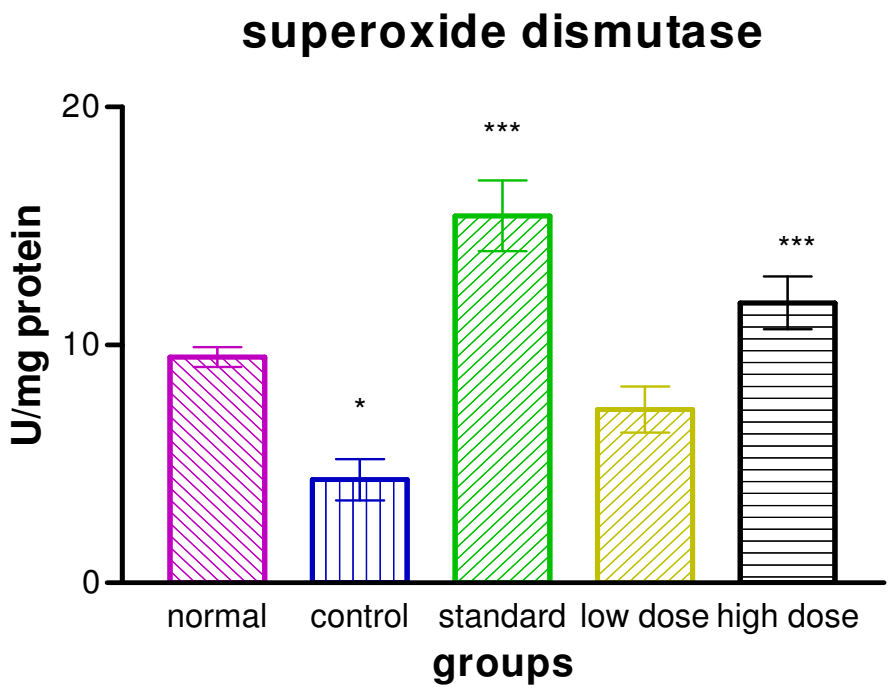


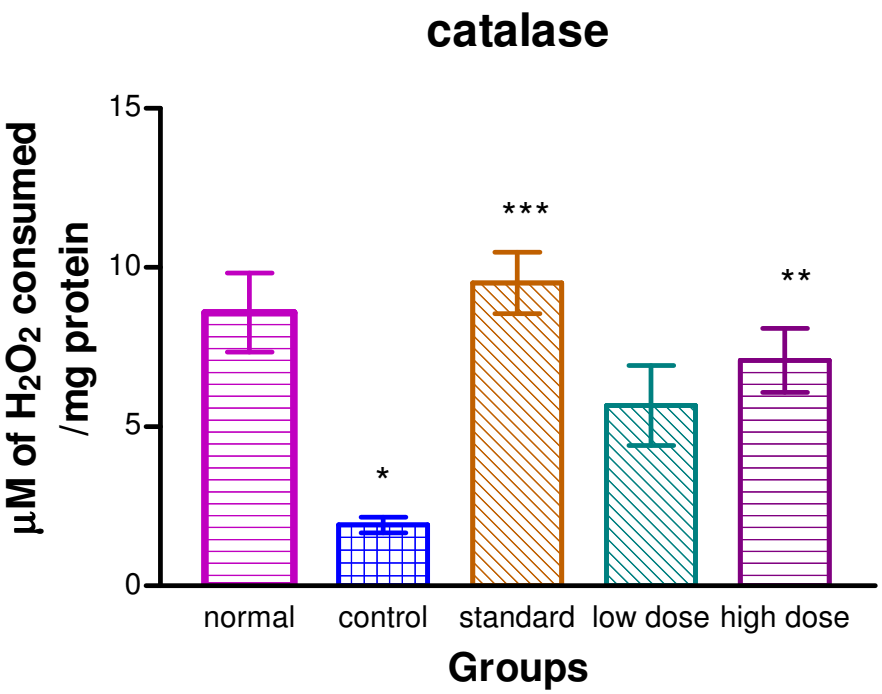
Figure 1: Macroscopical evaluation of rat colon. A) Normal colon, B) Colon treated with acetic acid alone, C) Colon treated with acetic acid and sulfasalazine, D) Colon treated with acetic acid and low dose of *Annona squamosa*, E) Colon treated with acetic acid and high dose of *Annona squamosa*

Figure 2: Effect of *A. squamosa* extract on SOD levels in colon



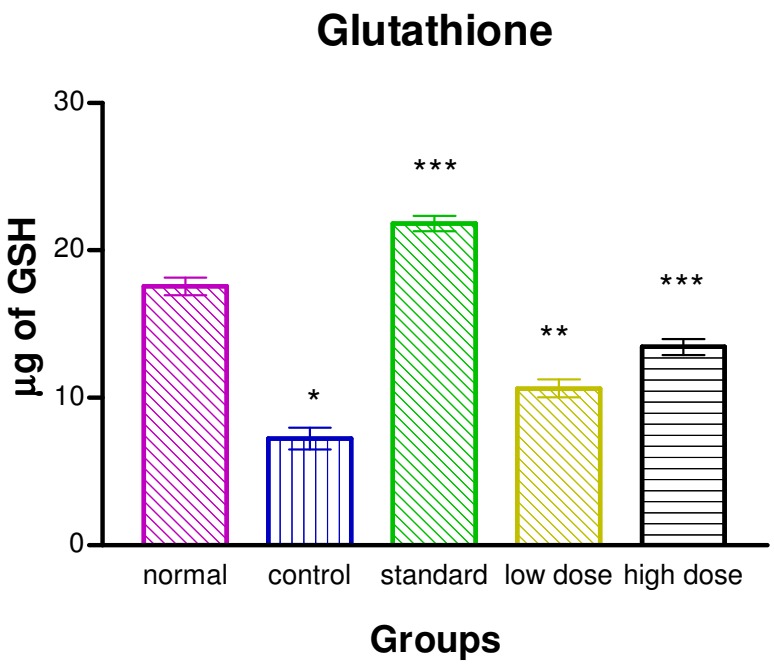
All values are expressed as mean ± S.E.M.
*** = *p* < *0.05* when compared to the normal**
***** = *p*< 0.001 when compared to control**

Figure 3: Effect of *A. squamosa* extract on CAT levels in colon



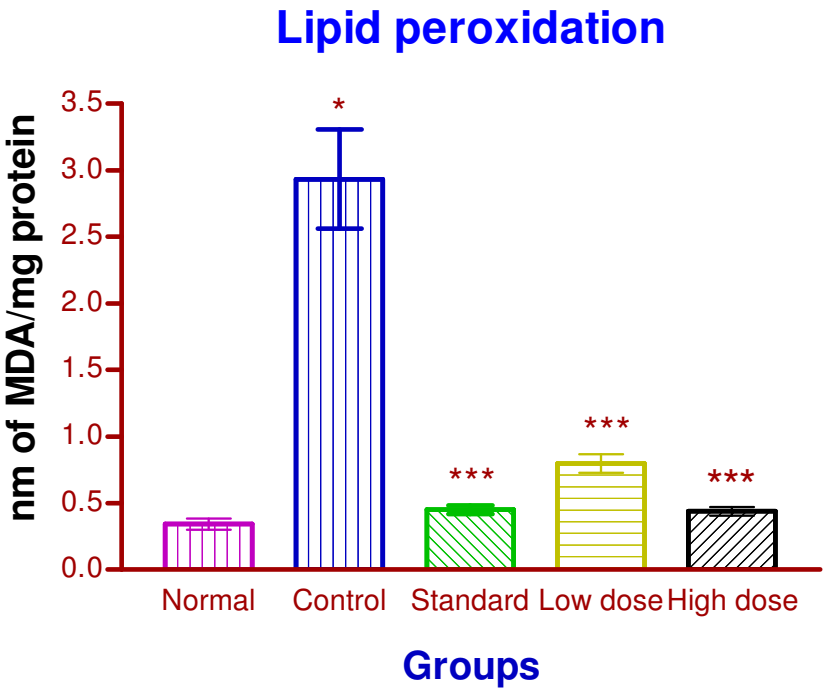
All values are expressed as mean ± S.E.M.
*** = *p* < 0.001 when compared to the normal**
*** = *p* < 0.05 when compared to the control**
***** = *p*< 0.001 when compared to control**

Figure 4: Effect of *A. squamosa* extract on GSH levels in colon



All values are expressed as mean ± S.E.M.
*** = *p* < 0.001 when compared to the normal**
**** = *p* < 0.01 when compared to the control**
***** = *p*< 0.001 when compared to control**

Figure 5: Effect of *A. squamosa* extract on LPO levels in colon



All values are expressed as mean ± S.E.M.
*** = *p* < 0.001 when compared to the normal**
***** = *p*< 0.001 when compared to control**

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