Potential effects of xylopic acid on acetic acid-induced ulcerative colitis in rats

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ABSTRACT

Background/Aims: Xylopic acid (XA) has been reported to exhibit analgesic activity, alleviate neuropathic pain in rodents, and demonstrate anti-inflammatory effects. Intrarectal challenge of rats with acetic acid induces colitis that bears resemblance in terms of its pathogenesis, histopathology, and inflammatory profile to that in humans. Reactive oxygen species are implicated as the main driving force in this inflammatory bowel disease. This study aimed to investigate the anti-colitic potential of XA.

Materials and Methods: We investigated the effect of XA on body weight, disease activity, inflammatory cell infiltration, and generation of reactive oxygen species. Rats were treated with XA or sulphasalazine, challenged intrarectally with acetic acid with macroscopic and microscopic findings made after eight days.

Results: Administration of XA to rats with colitis resulted in an increase in body weight with significant ($p < 0.05$) improvement of the disease profile macroscopically. We observed decreased gross mucosal injury, reduced inflammation, and cellular proliferation with XA administration. Treatment with XA also resulted in decreased colonic epithelial expression of argyrophilic nucleolar organizer regions (AgNORs) with significant decrease ($p < 0.0001$) in the quantitative expression of AgNORs/nucleus ratio to levels comparable with non-colitic control. We also observed reduced proliferation of mucosal mast cell in the colonic segment of the rats treated with XA. Treatment with XA also significantly ($p < 0.0001$) increased the activity of SOD, CAT, and APx while it decreased the activity of MPO and MDA levels.

Conclusion: Xylopic acid possesses anti-colitic activity in rats induced with acetic acid colitis.

Keywords: Xylopic acid, ulcerative colitis, inflammation

INTRODUCTION

Xylopic acid (XA) is the principal constituent isolated from the dried fruit of *Xylopic aethiopica*, which is a known anti-inflammatory plant (1,2). It is a naturally occurring crystalline solid with a rigid tetracyclic skeleton that forms intermediates in the biosynthesis of plant growth hormones such as gibberellins (3). The anti-inflammatory activity of XA has been investigated. In a previous publication Osafo et al. (4), we established that the observed anti-inflammatory effect of XA in H_2S -induced paw edema was because of its intervention on the arachidonic acid pathway. XA also showed beneficial effects when tested against LPS-induced uveitis in Sprague Dawley rats (5). Its analgesic effects, possibly through inhibition of inflammatory pathways, have also been reported (6-9). This property makes XA a good candidate for screening against diseases of inflammatory origin including colitis. Colitis-induced with acetic acid is similar to the inflam-

matory bowel disease in humans in terms of pathogenesis, histopathological outlook, and the pro-inflammatory mediators involved (10,11). Administering a dilute solution of acetic acid by the rectal route produces a non-transmural inflammation marked by enhanced neutrophil intrusion into the intestinal tissue, extensive tissue necrosis of mucosal and submucosal layers, vascular dilation, edema, and submucosal ulceration. These notable signs associated with human colitis (12,13) can be assessed together as the disease activity index (DAI). Although the underlining cause of ulcerative colitis remains elusive, Thippeswamy et al. (14) reported that increased activity of myeloperoxidase (MPO) and level of malondialdehyde (MDA) coupled with the reduced activities of superoxide dismutase (SOD), ascorbate peroxidase (APx), and catalase (CAT) are prime biomarkers for colon damage. Paiva et al. (15) reported that an increased myeloperoxidase activity enhanced the levels of MDA, a product of lipid

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peroxidation. This initiates a cascade of free radical cycle, which worsens the inflammatory process and ends in cellular anti-oxidant exhaustion. Another notable feature of acetic acid-induced ulcerative colitis is the proliferation of inflammatory cells within the intestinal mucosa and submucosa width, which results in thickening of epithelial cells and the production of copious amounts of mucin. Studies by Muscara et al. (16) and Yu et al. (17) suggest that the thickening of epithelial cells could possibly trigger an increase in the rate of proliferation by liberating excess mucin in the colonic region. This results in an increased expression of the argyrophilic nucleolar organizer regions (AgNORs) in the colon tissues because of enhanced transcriptional and cellular activities (18). The NOR is located on the chromosome and is composed of tandem repeats of ribosomal genes (rDNA). This histopathological change in the nucleolar organizer region in colitis is evident, and estimates the extent of cellular activity and proliferation (19) used to define the level of colon injury, and also the benefits of a study drug (20). This study assessed the effects of XA isolated from *Xylopic acid* in acetic acid-induced ulcerative colitis.

A number of treatment options are available for the management of inflammatory bowel diseases in general, and ulcerative colitis specifically. Such therapies include the use of steroidal and non-steroidal anti-inflammatory agents as well as immune system suppressors. However, the search for newer and more efficacious agents with tolerable side effect profile continues. This has informed the investigation into the potential anti-colitic activity of XA.

MATERIALS AND METHODS

Materials

Extraction and purification of XA

XA was extracted from *Xylopic acid* according to the methods described by Ekong and Ogan (9) and Adosraku and Oppong Kyekyeku (21). Briefly, 1.37 kg of the dried fruits of *Xylopic acid* was pulverized using a heavy-duty blender (37BL85 240CB6, WARING Commercial, USA) and soaked with 5 L of petroleum ether (40°C-60°C) for three days in cylindrical jars. The petroleum ether extract was collected and concentrated at 50°C using a vacuum rotary evaporator (Rotavapor R-210, BUCHI, Switzerland). Five milliliters of ethyl acetate was added to the concentrate and allowed to stand for two days after which the XA crystals formed were washed with petroleum ether (40°C-60°C) and purified by recrystallization. For this briefly, 32 g of the impure XA was dissolved in 96% (v/v) ethanol, filtered while hot,

and crystals of XA were deposited in two days after the solution was left to cool. The yield of the purified XA was 1.47% (w/w). The purity of the XA was assessed using high performance liquid chromatography as described by Woode et al. (6). The isolated crystals were characterized, and the data obtained were consistent with findings by Adosraku and Oppong Kyekyeku (21) with 95% (w/w) purity. The pure compound when required was constituted as an emulsion using tween 80 and henceforth referred to as XA.

Experimental animals

Sprague Dawley rats (200-250 g) were purchased and kept in the Animal facility. Rats were humanely handled throughout the experimental period in accordance with standards that take into consideration regulatory requirements, including the Animal Welfare Regulations (USDA 1985; US Code, 42 USC § 289d) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS 2002). Also, studies on rodents were conducted with the approval of the Department Ethics Committee. Rodents were given enough time to get accustomed with their new environment after which they were randomly grouped. Rats (n=5) were housed in a polypropylene cage in a temperature-regulated housing (22±3°C) with access to pellet diet and water *ad libitum*. All rats used in the studies were sacrificed at the end of the studies.

Chemicals and reagents

Gelatin was procured from GME (Bruxelles, Belgium); sulphasalazine from Pfizer Inc (New York, USA); hydrogen peroxide was purchased from Bell's Healthcare (Cheshire, England); ascorbic acid from Holland and Barrett (Warwickshire, England); trichloroacetic acid was purchased from Amresco® (Solon, USA); liquid paraffin from KAMA Pharmaceutical Industries (Accra, Ghana); paraffin and bluing solution was purchased from IHC World LLC (Maryland, USA); ethyl alcohol from Fisher Scientific (Waltham, UK); hematoxylin and eosin Y was purchased from Abbey Color (Philadelphia, USA); acetic acid, xylene, picric acid, pyridine hydrochloride, formic acid, formaldehyde, thiobarbituric acid, EDTA, monopotassium dihydrogen phosphate, dipotassium monohydrogen phosphate, monosodium phosphate, disodium phosphate, silver nitrate, o-dianisidine dihydrochloride, toluidine blue, tris(hydroxymethyl) aminomethane, and pentobarbitone sodium were procured from Sigma-Aldrich Chemical Co (St Louis, USA).

Methods

Induction of colonic injury

Sprague Dawley rats (200-250 g) of either sex were unsystematically divided into nine groups (n=5) and treated for eight days as shown:

Group I (naïve control): normal saline (0.9% w/v) (2 mL kg⁻¹ p.o.)

Group II (disease control), normal saline (0.9% w/v) (2 mL kg⁻¹ p.o.)

Group III (positive control): sulphasalazine (500 mg kg⁻¹ p.o.),

Groups IV-VI (test groups): XA (10, 30, and 100 mg kg⁻¹ p.o.) respectively,

Colitis was induced in all groups except the naïve control group with 1 mL acetic acid (4.0% v/v) administered intra-rectally with a size 6 Ch/Fr pediatric catheter (Flexicare Medical Ltd, Mid Glamorgan, UK) on day 4. Experimental rats were subjected to the following:

Body weight determinations

Body weight changes were monitored over the eight days, and the effect of XA on overall body weight expressed was as area under the time course curve (AUC).

Hematological analysis

Blood samples were collected from the jugular vein of rats on the eighth day. A full blood count was done on the collected blood samples using hematology analyzer (BC-2800, Mindray, Shenzhen, China). The Biernacki reaction or erythrocyte sedimentation rate was done using the standard Westergren method.

Macroscopic colonic damage assessment

At the end of the eight-day period, colons were extirpated and examined for weight, consistency of the stool found within as well as gross macroscopic appearance and length, measured from 1 cm above the anus to the top of the cecum. The extent of the macroscopic damage was assessed as described by Kimball et al. (22) by determining the disease activity index (DAI), calculated as the sum of scores attributed to indices: stool condition, colon damage, colon weight change, and colon length shortening.

Microscopic colonic damage assessment

To evaluate microscopic colon damage by light microscopy, portions of the distal colon were immediately fixed in buffered formalin (10%), embedded in paraffin wax, cut into 3 µm transversal sections and stained with hema-

toxylin and eosin Y (H&E). In each specimen, six random fields of view were analyzed and the extent of the microscopic colonic damage assessed.

AgNOR staining

To examine the nucleolar organizer regions in the colon tissues, the method described by Nikicicz and Norback (23) was used. Briefly, the paraffin-embedded tissues were deparaffinized with xylene and serially dehydrated with increasing concentrations of ethanol. Sections of 6.0 µm were made with a microtome, washed with distilled water, immersed in one volume of staining solution consisting of 50% silver nitrate, 2.0% gelatin, and 1.0% formic acid, in the dark for 60 min and then rinsed in de-ionized water. The stained sections were dehydrated with ethanol, cleared with xylene, and mounted with distrene, plasticizer, and xylene on slides. The slides were examined under a light microscope (Leica ICC50 HD Jos. Hansen & Soehne GmbH, Hamburg, Germany). Brown or black dots within the nucleus or outside the nucleolus were quantified, and the data were expressed as the mean number of AgNORs/nucleus.

Mast cell proliferation

Full thickness segments of colon were fixed in Carnoy's fixative and stained with toluidine blue (1%, pH 2.3), and the overall morphology of colon was assessed. Mast cells were counted in coded sections at ×40 magnification using a micrometer grid (0.032 mm²). For each rat, 12 contiguous non-overlapping mucosal areas above the muscularis mucosae were evaluated.

Biochemical assay

Tissue samples of the -80°C stored extirpated colons were homogenized using a Potter-Elvehjem homogenizer (Ultra-Turrax T25, Janke & Kunkel IKA- Labortechnik, Staufen, Germany) on ice-cold Tris-HCl buffer (0.01 M, pH 7.4) to give a 10% homogenate used to assay for the activities of SOD, CAT, APx and myeloperoxidase, and MDA levels in duplicates.

Superoxide dismutase (SOD)

The SOD activity was measured by the method of Misra and Fridovich (24) which is based on the capacity of SOD to inhibit autooxidation of adrenaline to adrenochrome. Briefly, to a 500 µl tissue homogenate, 750 µL ethanol (96% v/v) and 150 µl chloroform (ice chilled) were added and centrifuged at 671 ×g for 20 min. To 500 µL of the supernatant, 500 µl EDTA (0.6 mM) and 1 mL carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was initiated by the addition of 50 µL adrenaline

(1.3 mM) and the increase in absorbance at 480 nm due to the adrenochrome formed was measured with a spectrophotometer. Percentage inhibition of auto oxidation of adrenaline was calculated from the formula:

One unit of SOD activity defined as the amount of protein causing 50% inhibition of the auto oxidation of adrenaline at 25°C was calculated using the formula:

Catalase activity (CAT)

Activity of CAT was measured using the method of Aebi (25). It was determined by measuring the decrease in hydrogen peroxide (H_2O_2) concentration at 240 nm at 20 s interval for 60 s. Medium consisted of 130 μL homogenate, 65 μL potassium phosphate buffer (50 mM, pH 7.0), and 65 μL H_2O_2 (10 mM). The blank consisted of 130 μL homogenate and 65 μL of potassium phosphate buffer (50 mM, pH 7.0) only. The concentration of H_2O_2 was calculated from the absorbance using the following expression:

where 39.4 $\text{mol}^{-1}\text{cm}^{-1}$ is the molar extinction coefficient for H_2O_2 . The CAT activity was expressed as U/mg protein. Ascorbate peroxidase (APx)

The APx activity was measured by monitoring the decrease in absorbance at 290 nm measured every 10 s for 60 s (extinction coefficient: 2.8 $\text{mM}^{-1}\text{cm}^{-1}$). The assay mixture contained 600 μL potassium buffer (50 mM, pH 7.0), 100 μL EDTA (0.1 mM), 100 μL H_2O_2 (1.25 mM), 100 μL (0.5 mM) ascorbic acid, and 100 μL homogenate. The reaction was initiated by adding H_2O_2 (26). The blank had all components other than the enzyme extract.

Myeloperoxidase (MPO)

The MPO activity was determined by a modified o-dianisidine method (27). The assay mixture contained 300 μL phosphate buffer (0.1 M, pH 6.0), 300 μL H_2O_2 (0.01 M), 500 μL freshly prepared o-dianisidine (0.02 M in deionized water) and 10 μL of the homogenate and made up to 3.0 ml with deionized water. The homogenate was added last, and the change in absorbance at 460 nm was measured every minute for 10 min. One unit of MPO was defined as that giving an increase in absorbance of 0.001 min^{-1} , and specific activity was expressed as U/mg protein.

MDA levels (lipid peroxidation)

Measurement was based on a method by Heath and Parker (28). Briefly, 3 mL mixture (1:1) of trichloroacetic acid (20%) and thiobarbituric acid (0.5%) was added to a 1 mL aliquot of the homogenate. The mixture was heated at 95°C for 30

min and then quickly cooled on an ice bath, centrifuged at 10,000 $\times g$ for 10 min, and absorbance of the supernatant read at 532 nm and 600 nm to correct for nonspecific absorbance. The concentration of MDA was calculated using MDA's extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$.

Statistical analysis

Data were presented as the mean $\pm$ SEM and one-way ANOVA followed by Dunnett's test when required. All graphs were plotted, and analyses were done using GraphPad Prism for Windows Version 5.01 (GraphPad, San Diego, CA, USA).

RESULTS

Effect of XA on rat body weight

The disease control group showed a steady decrease in the body weight over the course of the study (Figure 1a), with a significant total body weight loss when compared with the non-colitic naïve control group (Figure 1b). Treatment with sulphasalazine resulted in a steady increase in body weight of the rats (Figure 1a) albeit exhibiting no significant increase in total body weight when compared with the colitic control group (Figure 1b). Treating rats with XA at 10 mg kg^{-1} and 30 mg kg^{-1} similarly resulted in an increase in the body weight of the rats over the duration of the study (Figure 1a) but no significant change was observed in total body weight when compared with the colitic control. At the dose of 100 mg kg^{-1} , a significant increase was observed in the total body weight of the rats (Figure 1b).

Effect of XA on hematological parameters

Induction of colitis with acetic acid resulted in significant increases in the levels of white blood cells (WBC), hematocrit (HCT), and hemoglobin (HGB) compared to the naïve non-colitic rats (Table 1). However, the levels of lymphocytes (LYM), neutrophils (NEU), red blood cells (RBC), and platelets (PLT) significantly decreased compared to the naïve non-colitic rats (Table 1). Sulphasalazine-treated rats had significant increases in LYM, HGB, and RBC when compared with the colitic control rats (Table 1). Rats treated with XA showed decreased levels of HGB and HCT and an increase in PLT when compared with the colitic control rats. There were significantly decreased WBC and an increased LYM with 100 and 300 mg kg^{-1} of XA (Table 1).

Effect of XA on macroscopic colonic damage

In the naïve control rats, no observable macroscopic colon damage was observed (Figure 2a.i); but upon colitis

Figure 1. Effect of xylopic acid on rat body weight. Sprague Dawley rats were treated with either normal saline (10 mL kg⁻¹), sulphasalazine (500 mg kg⁻¹) or XA (10-100 mg kg⁻¹) p.o. for 8 days. Colitis was induced with intrarectal administration of 1 mL acetic acid (4 % v/v) in the test groups on day 4. Weight of animals were determined daily, and the percentage change in body weight calculated (Left panel). Total body weight measured during the study period was calculated as area under the time course curves, AUC (Right panel). Data is presented as Mean±SEM (n=5). *p<0.01, **p<0.001, nsP>0.05 when compared with colitic control, †p<0.01 when compared with non-colitic control.

Table 1. Effect of xylopic acid on blood parameters in acetic acid-induced ulcerative colitis in rats.

Treatment	WBC x 10 ³ /μL	LYM %	NEUT %	HGB g dL ⁻¹	RBC x 10 ⁶ /μL	HCT %	PLT x 10 ³ /μL
Naïve control	10.6±0.10 [#]	62.5±7.35	10.3±1.37	13.7±0.01	8.31±0.2 [#]	44.0±1.23	626±13.72 [#]
Acetic acid control	14.7±0.23 [*]	44.1±9.25 [*]	5.7±0.75 [*]	15.0±0.00 [*]	5.93±0.01 [*]	49.0±0.02 [*]	432±10.92 [*]
Sulphasalazine (mg kg ⁻¹)							
500	15.1±0.10 [*]	62.1±3.48 ^{**}	4.0±0.43 [*]	17.1±0.20 ^{**}	9.11±0.11 ^{**}	47.9±0.23 [*]	356±3.45 [*]
XA (mg kg ⁻¹)							
10	21.4±3.7 [*]	32.9±1.52 ^{**}	-	12.8±0.08 ^{**}	7.62±0.09 ^{**}	39.8±0.45 ^{**}	1683±13.75 ^{**}
30	11.6±0.25 ^{**}	51.3±2.34 ^{**}	5.1±1.12 [*]	12.2±0.15 ^{**}	7.06±0.25 ^{**}	38.8±0.13 ^{**}	834±10.92 ^{**}
100	4.4±0.32 ^{**}	52.5±1.98 ^{**}	5.4±0.57 [*]	9.8±0.21 ^{**}	5.08±0.29 [*]	31.5±0.35 ^{**}	559±7.35 ^{**}

Sprague Dawley rats were treated with either normal saline (10 mL kg⁻¹), sulphasalazine (500 mg kg⁻¹), or XA (10-100 mg kg⁻¹) p.o. for eight days. Colitis was induced with intrarectal administration of 1 mL acetic acid (4 % v/v) in the test groups on day 4. Animals were sacrificed on day 8, and blood samples collected for hematological analysis. Data are presented as mean±SEM (n=5). *p<0.0001 when compared with naïve control; #p<0.0001 when compared with acetic acid-induced colitic disease control.

induction, visible damage was observed to the colon coupled with increase in change in colon weight, colon shortening, and poor stool consistency (Figure 2a.ii). When XA was administered to the rats with colitis, a decrease was observed in extent of colon damage in rats. Enhanced stool consistency and improved colon length in the rats

were also observed at all doses of XA administered (Figure 2a.iv-vi) with similar observation in sulphasalazine-treated rats with colitis (Figure 2a.iii).

When the DAI was established, the naïve control rats were seen to show no significant DAI which was, however,

Figure 2. a, b. Effect of xylopic acid on macroscopic colonic damage and disease activity index (DAI) in acetic acid-induced colitic rats. Sprague Dawley rats were treated with either normal saline (10 mL kg⁻¹), sulphasalazine (500 mg kg⁻¹) or XA (10-100 mg kg⁻¹) p.o. for 8 days. Colitis was induced with intrarectal administration of 1 mL acetic acid (4 % v/v) in the test groups on day 4. Clinical features of extirpated colons on day 8 are presented (A) and quantified as the DAI (B). i - naïve control; ii - disease control; iii - sulphasalazine 500 mg kg⁻¹; iv - vi - 1-100 mg kg⁻¹ XA. Data is presented as Mean±SEM (n=5).

*p<0.01, **p<0.001 and ***p<0.0001 when compared with colitic control; ###p<0.0001 when compared with naïve non-colitic control. Micron bar represents 88.64 mm.

markedly (p<0.0001) increased upon colitis induction to a maximum score of 14.67±0.33 (Figure 2b). Treatment of rats with colitis with XA lowered the DAI to 11.00±1.00, 10.33±0.88, and 6.00±0.58 respectively at the administered doses of 10, 30, and 100 mg kg⁻¹ (Figure 2b).

Effect of XA on microscopic colonic damage

The microscopic evaluation of the colons in the non-colitic control rats showed no observable signs of inflammation, fibrosis, and necrosis with no infiltration of NEU (Figure 3a). The colitic control rat colons showed necrosis in the mucosa, with an observed presence of crypt abscesses, granulomatous inflammation with fibrosis, and massive thickening of the submucosa. The crypt of the colon in the acetic acid-induced colitic group was elongated with distortion, while exhibiting the loss of epithelial cells, ulceration, LYM infiltration, bowel wall thickening, and goblet cells depletion (Fig-

ure 3b). Treatment with sulphasalazine reduced the mucosal damage with reduced granulomatous inflammation and fibrosis (Figure 1c). The animals treated with XA showed a decreased gross mucosal injury caused by the acetic acid at 10, 30, and 100 mg kg⁻¹. Reduced granulomatous inflammation and cellular proliferation with XA treatment respectively were observed (Figure d-f).

Effect of XA on the AgNOR

The naïve control group presented with a low expression of AgNOR in cells since no colitis induction was observed in rats in that group (Figure 4a). The colitic control rats showed high AgNOR count (Figure 4b), while significantly reduced AgNOR count was observed in the sulphasalazine-treated rats (Figure 4c). In rats treated with XA, a decrease in epithelial AgNOR counts was observed at all dose levels (Figure 4d-f).

Figure 3. a-f. Effect of xylopic acid on microscopic colonic damage in acetic acid-induced colitic rats. Sprague Dawley rats were treated with either normal saline (10 mL kg^{-1}), sulphasalazine (500 mg kg^{-1}) or XA ($10\text{--}100 \text{ mg kg}^{-1}$) p.o. for 8 days. Colitis was induced with intrarectal administration of 1 mL acetic acid ($4\% \text{ v/v}$) in the test groups on day 4. Colons were extirpated on day 8. Representative micrographs are shown. Naïve control (a), acetic acid-induced colitic control (b), sulphasalazine 500 mg kg^{-1} (c), XA $10\text{--}100 \text{ mg kg}^{-1}$ (d-f). Region showing loss of cell architecture and inflammation indicated in the red oval region. Micron bar represents $100 \mu\text{m}$.

When the AgNOR/nucleus ratios were determined, it was realized not to be high in naïve control rats which was significantly elevated when colitis was induced in rats as seen in the colitic control rats (Figure 4g). Treatment with sulphasalazine reduced the AgNOR/nucleus ratio in colitis-induced rats, while significant ($p < 0.0001$) reduction was also observed at all dose levels of XA administered in the colitis-induced rats (Figure 4g).

Effect of XA on mast cell proliferation

There were a few and dispersed mast cells in the naïve control rats (Fig 5A). However, with colitis induction, an increased proliferation of mast cells was observed as seen in the colitic control rats (Figure 5b). Administration of sulphasalazine resulted in reduction of mast cell density (Figure 5c) with similar reduction in mast cell proliferation in the XA-treated rats at all doses administered (Figure 5d, e).

When mast cell proliferation was quantified, it was realized that a significant ($p < 0.0001$) increase was observed in the mean number of mast cells when colitis was induced. With an increase from 11 ± 0.49 cells in naïve control rats to 25 ± 0.99 cells in colitic control rats (Figure 5g). Upon sulphasalazine treatment, a reduction was observed in the cell number to 14.30 ± 0.65 with the administration

of XA to rats with colitis also significantly ($p < 0.0001$) decreasing the mast cell number to 17.10 ± 0.99 , 14.50 ± 0.56 , and 14.00 ± 1.07 at doses of 10 , 30 , and 100 mg kg^{-1} , respectively (Figure 5g).

Effect of XA on biochemical assay

In the SOD assay, the initial SOD expression decreased from $13.88 \pm 0.17 \times 10^2 \text{ U/mg protein}$ in the naïve control rats to $8.40 \pm 0.59 \times 10^2 \text{ U/mg protein}$ in the colitic control rats. Administration of XA increased the SOD expression significantly ($p < 0.0001$) at all administered doses ($10\text{--}100 \text{ mg kg}^{-1}$) to 14.29 ± 0.09 , 14.70 ± 0.19 , and $15.01 \pm 0.19 \times 10^2 \text{ U/mg protein}$, respectively. Also, sulphasalazine increased SOD expression to $13.73 \pm 0.14 \times 10^2 \text{ U/mg protein}$ (Figure 6a).

The CAT assay showed that colitic induction decreased the enzyme expression from $7.14 \pm 0.62 \times 10^6 \text{ U/mg protein}$ in naïve control rats to $1.67 \pm 0.79 \times 10^6 \text{ U/mg protein}$ in rats with colitis. However, XA treatment resulted in significant increase in CAT expression to 4.46 ± 0.84 and $5.17 \pm 0.39 \times 10^6 \text{ U/mg protein}$ at 30 and 100 mg kg^{-1} , respectively (Figure 6b). No significant increase was observed in the expression of CAT with administration of 10 mg kg^{-1} of XA when compared with the colitic control rats.

Figure 4. a-g. Effect of xylopic acid on the nucleolar organiser region in acetic acid-induced colitis. Sprague Dawley rats were treated with either normal saline (10 mL kg⁻¹), sulphasalazine (500 mg kg⁻¹) or XA (10-100 mg kg⁻¹) p.o. for 8 days. Colitis was induced with intrarectal administration of 1 mL acetic acid (4 %v/v) in the test groups on day 4. Colons were extirpated on day 8 and stained with silver nitrate. Representative micrographs are shown. Naïve control (a), acetic acid-induced colitic control (b), sulphasalazine 500 mg kg⁻¹ (c), XA 10-100 mg kg⁻¹ (d-f). Average count per 10 nuclei was determined at ×10 magnification (g). Data is presented as Mean±SEM (n=5). *p<0.0001 when compared with colitic control. #p<0.0001 when compared with non-colitic control. Regions with AgNOR proliferation indicated with black oval shapes. Micron bar represents 200 µm.

The APx expression significantly ($p<0.0001$) decreased from 274.0 ± 15.55 mM/mg protein in naïve control rats to 39.49 ± 8.46 mM/mg protein after colitis induction. With XA administration to the rats with colitis, a significant increase was observed in the APx expression to 247.5 ± 14.52 and 425.3 ± 7.78 mM/mg protein at 30 and 100 mg kg⁻¹, respectively (Figure 6c). At 10 mg kg⁻¹, XA

did not significantly elevate APx expression when compared with the colitic control rats.

The expression of MPO which was $31.86\pm0.61\times10^5$ U/mg protein in the naïve control rats, increased significantly ($p<0.05$) to $70.41\pm14.86\times10^5$ U/mg protein upon colitis induction. This however significantly decreased

to 13.36 ± 3.36 and $19.59 \pm 4.75 \times 10^5$ U/mg protein at 30 and 100 mg kg⁻¹ doses of XA, respectively (Figure 6d). Administration of 10 mg kg⁻¹ of XA, however, did not significantly decrease MPO expression when compared with the colitic control rats.

The MDA levels increased from 2539.0 ± 981.20 M/mg protein in naïve control rats to 6618.0 ± 557.0 M/mg

protein when colitis was induced. However, XA treatment lowered it to 2053.0 ± 968.20 , 2268.0 ± 987.3 , and 1214 ± 513.2 M/mg protein respectively at the administered doses (Figure 6e).

DISCUSSION

Intrarectal administration of acetic acid leads to increases in mucosal permeability, colon weight, and the numbers of

Figure 6. a-e. Effect of XA on biochemical assay in the colons of rats with acetic acid-induced colitis. Sprague Dawley rats were treated with either normal saline (10 mL kg⁻¹), sulphasalazine (500 mg kg⁻¹) or XA (10-100 mg kg⁻¹) p.o. for 8 days. Colitis was induced with intrarectal administration of 1 ml acetic acid (4% v/v) in the test groups on day 4. Colons were extirpated on day 8, homogenised and assayed for SOD (a), CAT (b), APx (c) MPO (d) and MDA (e). Data is presented as Mean±SEM (n=5). *p=0.01, **p≤ 0.001, ***P<0.0001, #p=0.012, ##p=0.0012, †p=0.003, nsp>0.05 when compared with colitic control, ap<0.0001, bp=0.00012, cp<0.0001, dp<0.05, zp=0.03 when compared with non-colitic control (normal saline) control.

activated inflammatory cells in the colonic tissues (29). The activated inflammatory cells (phagocytic leukocytes and NEU) produce excessive amounts of reactive oxygen species (ROS) (30). There is also the activation of macrophages and NEU in the intestinal mucosal tissues and lipid peroxidation (14,31). These processes culminate in the erosion, ulceration, and necrosis of the colonic tissues. An ideal anti-colitic compound should therefore have the potential to alleviate some of these processes to exert its effects.

XA ameliorated the macroscopic manifestations of the colitis-induced with acetic acid. XA improved stool consistency, colon damage, colon weight change, and colon length shortening, which resulted in reduced DAI in the

XA-treated groups compared with the disease control group. Again, microscopic analysis of the colons of the rats showed that XA healed the ulcers in the mucosal membrane and reduced the number of cellular infiltrates. The number of inflammatory cells into the colon tissue is of particular importance, since the activation and their eventual invasion of the colon tissues lead to the production of inflammatory mediators such as cytokines and ROS that cause the observed damage in ulcerative colitis. A reduction of these infiltrating cells by XA therefore is partly responsible for the observed beneficial effect.

The NOR has a peculiar property of staining black with silver nitrate solution to determine its concentration and

the number of AgNORs correlates with the rate of cell proliferation. An increase in the rate of cellular proliferation may be associated with colitis-induced neoplastic changes (18,32). Hence, staining of the argyrophilic non-histone proteins in the NOR is employed in the analyses of such changes in ulcerative colitis. Administration of XA at all doses used significantly reduced the number of AgNOR/nucleus compared to the colitis control. This observation signifies the inhibitory role of XA on the rate of cellular proliferation induced following the rectal administration of acetic acid. This result establishes the inhibitory role of XA on the proliferation of pro-inflammatory cells in the colon. However, the finding is not surprising since anti-apoptotic activities have been seen with other kaurane diterpenes including kaurenoic acid (33) and kamebakaurin (34).

The colon weight-to-length ratio is increased in ulcerative colitis as a result of edema of the colon tissues. The edema is contributed to directly by the breaks in the mucosae, and by the effects of histamine released from mast cells. Proliferation and accumulation of mast cells during chronic inflammatory processes occur as in ulcerative colitis (35,36). Upon stimulation, these mast cells degranulate and release a heterogeneous group of factors including histamine that promote the inflammatory response. Histamine is known to increase membrane permeability and promote edema formation. Treatment with XA reduced the accumulation of mast cell at the site of injury in agreement with the reduced mast cell proliferation seen in XA-treated groups. The observed inhibition of mast cell accumulation at injury site is consistent with earlier findings by Cavalcanti et al. (37) that identified kaurenoic acid, another kaurane diterpene, as an inhibitor of cell proliferation. This inhibitory effect probably accounts for the overall anti-inflammatory activities of XA.

In ulcerative colitis, there is depletion of CAT, SOD, and APx levels in colon tissues as a result of oxidative damage (38). CAT, SOD, and APx also play crucial roles as protective enzymes. CAT, which is localized in subcellular organelles of peroxisomes, catalyzes the conversion of hydrogen peroxide to water and oxygen (39). SOD is an enzyme shown to exert anti-inflammatory effects in inflammatory bowel diseases (40,41) by reducing lipid peroxidation, leukocyte rolling, and adhesion in colonic tissues (42). APx, an integral component of the glutathione-ascorbate cycle (43), removes peroxides by conjugation using ascorbate as substrate (44). Supplementation via XA treatment decreased lipid peroxidation thereby

significantly increasing activity of anti-oxidant enzymes. This resulted in reduced severity of inflammation hence exerting anti-inflammatory action. On the other hand, treatment with XA reduced the MPO activity and MDA levels. This is consistent with the reduction of inflammatory cell infiltrates in the colon tissues, as MPO is an index of NEU count. This means that by reducing the numbers of NEU in the tissues, XA reduced the activities of their granular content MPO that is important because it catalyzes the production of hypochlorous acid, a powerful oxidizing agent that contributes electrons needed for lipid peroxidation. It is not surprising therefore that XA administration also led to a reduction in the tissue levels of MDA, a by-product of the lipid peroxidation.

Blood tests, such as the complete blood count, have been proven important in the initial evaluation of ulcerative colitis (45). Mostly, inflammatory responses begin with infiltration of NEU and macrophages (46) with the activated macrophages producing a combination of broadly active inflammatory cytokines as well as cell adhesion molecules such as intracellular adhesion molecule 1, ICAM-1 (47). From this study, XA was found not to have significant impact on NEU infiltration though was realized to lower overall WBC proliferation hence contributing to its anti-inflammatory potential. This offers an advantage over conventional immunosuppressive drugs employed in the management in chronic inflammatory conditions such as ulcerative colitis.

Electron micrographs can be taken to analyze extensively the surface of the colon endothelium post-colitis to further understand the extent of tissue damage. Other markers of ulcerative colitis such as C-reactive protein, serum orosomucoid, and immunologic antibodies could be investigated.

In conclusion, our findings show that XA is effective in suppressing experimentally induced ulcerative colitis by increasing CAT, SOD, and APx activity while decreasing MDA levels and MPO activity *in vivo*. It also increased the body weight of rats with colitis, improved their stool consistency, and lowered the DAI. Again, XA lowers the elevated LYM and total white cell count associated with colitis and alleviates the colitis-mediated mucosal injury as well. Lastly, XA curtailed the mast cell proliferation and AgNOR/nucleus ratio, an indicator of its anti-inflammatory and anti-proliferative function on immune cells. Taken together, XA suppresses acetic acid-induced ulcerative colitis in rats.

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