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Original paper

Therapeutic Potential of Silver Nanoparticles Biosynthesized Using Aqueous Extract of *Citrus Sinensis* Peel on Acetic Acid-Induced Ulcerative Colitis in Male Wistar Rats

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Abstract

Background and aim: Ulcerative colitis is an inflammatory disease, and antioxidants with anti-inflammatory effects of silver nanoparticles have been explored. This study investigates the therapeutic potential of silver nanoparticles bio-fabricated using aqueous extract of *Citrus sinensis* peel (CsPEx-AgNPs) on ulcerative colitis in male Wistar rats.

Materials and methods: Thirty adult male Wistar rats were assigned into; Non-Colitis Control, Colitis control, Colitis+CsPEx (*Citrus sinensis* Peel Extract, 250µg/Kg), Colitis+$AgNO_3$ (250µg/kg), Colitis+CsPEx-AgNPs (25µg/Kg) and Colitis+CsPEx-AgNPs (250µg/Kg) groups. Colitis was induced with a single intra-rectal administration of 6% acetic acid (1mL/100g B.W.), followed by oral administration of regimens in groups 3-6 for seven days. Eighth-day post-colitis induction, rats were sacrificed; blood and colon were collected for hematological, biochemical, and histopathological examination. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons.

Results: The colitis control group showed: reduced body weight, increased colonic weight and thickness, diarrhea and macroscopic scores, a significant decrease in packed cell volume, a significant increase in Platelet count, plateletcrit, red cell distribution width, depleted superoxide dismutase, glutathione, catalase levels and elevated concentrations of malondialdehyde, myeloperoxidase and tumor necrosis factor-α, mucosal ulceration, infiltration of inflammatory cells in the mucosa and submucosa. Treatment with CsPEx-AgNPs increased body weight, reduced colonic weight and thickness, diarrhea, and macroscopic scores; improved red blood cell and platelet indices; enhanced superoxide dismutase, glutathione, catalase levels and reduced the

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concentrations of malondialdehyde, myeloperoxidase and tumornecrosis factor-α; reduced cellular infiltrates and restored mucosal epithelial layer.
Conclusion: CsPEx-AgNPs have an anti-colitic effect and may provide therapeutic potential for the treatment of ulcerative colitis.
Keywords: *Ulcerative colitis, Inflammatory disease, Silver nanoparticle, Citrus sinensis peel, Antioxidant, Anti-inflammatory*

Introduction

The essential roles of the colon in large intestine physiology include proteolysis, transport of water and electrolytes, processing of indigestible food materials, and storage of faecal waste [1]. However, many disorders can impair the normal functions of the colon, one of which is ulcerative colitis. Ulcerative colitis (U.C.), being a form of Inflammatory Bowel Disease (IBD), is a chronic, remitting, and relapsing disease of the lower gastrointestinal tract with pathological processes affecting mainly the rectum and colon, which is characterized by recurrent mucosal inflammation [2].

The main clinical features of individuals with ulcerative colitis are abdominal pain, rectal bleeding, diarrhea, bloody stool, and excessive weight loss, which can be confirmed by laboratory, endoscopic and radiological examinations [3]. In animal model, acetic acid (A.A.)-induced U.C. is one of the methods for induction of U.C., which is aimed to mimic human intestinal inflammations through an increase in the generation of ulcers, inflammatory mediators, free radicals, and neutrophils infiltration as well as alterations in hematological parameters [4].

Although its etiology remains not fully understood, various studies have reported several factors that contribute to the pathogenesis of U.C. Ungaro et al. (2017) [5] identified epithelial barrier defects, defective immune responses, epigenetic and environmental factors as major causes of U.C. Furthermore, Shalkami et al. (2018) [4] added that genetic factors, predispositions, microbial infections, auto-immune reactions, and lifestyle habits such as smoking are also involved.

The pathophysiology of ulcerative colitis involves the recruitment patterns of lymphocytes, macrophages, cytokines, reactive oxygen, nitrogen metabolites, and other inflammatory mediators in local immune response [6], thereby creating a degenerative cycle that further aggravates tissue injury and alteration of colonic mucosal integrity [7]. Hence, because of these multifactorial causes, there is no reported curative therapy for ulcerative colitis despite all the available pharmacotherapeutics [8].

Using plants, fruit extracts, and medicinal herbs has shown potent anti-inflammatory actions in treating U.C. *Citrus sinensis* peel is a rich source of flavanones and polymethoxylated flavones not found in other plants. It is known for its anti-microbial and biomedical effects and ability to modulate critical cellular enzyme functions [9]. The unique physicochemical characteristics of noble-metallic nanoparticles such as gold, silver, and copper have been exploited in biomedical and pharmaceutical industries and other fields relating to human health and safety [10].

Owing to the unique properties of silver nanoparticles (AgNPs), many researchers have developed a keen interest in silver nanoparticles due to their enhanced activities, anti-microbial, antioxidant, anti-diabetic, anti-cancer, and anti-inflammatory effects, which has contributed to their continuous and extensive use in biomedical researches [11]. Flavonoids have gained much attention in the last few years due to the identification of major functional groups that provide reducing and capping abilities and their potency in the biosynthesis of Flavonoid-based AgNPs [12].

Adegoke et al. (2017) [13] reported the prevalence of U.C., especially in many developing and

industrialized countries, in which, similar to many long-term clinical conditions, U.C. is disruptive and damaging to the quality of life of patients due to its continuous and relapsing nature with a mortality rate higher than that of other digestive and bowel diseases. Several side effects associated with available conventional treatments necessitate an urgent need to develop alternative and novel approaches with more safety, efficacy, and enhanced therapeutic outcomes. This study aimed to investigate the therapeutic potential of silver nanoparticles bio-fabricated using an aqueous extract of *Citrus sinensis* peel in the treatment of acetic acid-induced ulcerative colitis in male Wistar rats.

Material and Methods

Plant and Chemical Collection

Sweet oranges (*Citrus sinensis*) were purchased from Odo-Oba market in Ogbomoso, Oyo State, Nigeria. All chemicals used were of analytical grade and purchased from Sigma Merck (South Africa). Distilled water was used throughout the experiments.

Preparation of Extract

Citrus sinensis was zested; peels were collected and cut into small pieces to increase the surface area and washed thoroughly with distilled water. The peels were air-dried at room temperature for two weeks. Aqueous extraction was done according to Niluxsshun et al. (2021) [9]. 4g of the dried peels was weighed and transferred into a bottle containing 40mL distilled water, mixed thoroughly, and boiled for 5 minutes. The extract obtained was allowed to cool and filtered using Whatmann no. 1 filter paper. Thus, the filtrate was designated as extract (CsPEx) and stored at 4ºC and the residue was discarded.

Synthesis of Silver Nanoparticles

Biosynthesis of silver nanoparticles using an aqueous extract of *Citrus sinensis* peel was performed according to previous methods [14], [9] with slight modification. 3mL of the extract was carefully added to 40mL of 1mM aqueous $AgNO_3$ solution and was exposed to sunlight for 30 minutes.

Characterization of Silver Nanoparticles (AgNPs)

The AgNPs synthesized were characterized using Ultraviolet-Visible Spectroscopy. The particles were subjected to optical measurements using a UV-visible spectrophotometer, CECIL CE model (B-UV 1800PC, China). A small aliquot was poured into a quartz cuvette, and the absorbance peak was determined by scanning the UV-vis spectra between 200-800nm using distilled water as blank. Further characterizations such as Fourier Transform Infrared (F.T.I.R.) spectroscopy and Transmission Electron Microscopy (T.E.M.) to determine the functional groups that contributed to the synthesis of AgNPs, as well as the size and shape of the particles, have been widely studied [9], [14], [15].

Animal Care and Management

Thirty adult male Wistar rats weighing 200 ± 20g were used for this study. They were purchased from the animal house of the Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso, and acclimatized for four weeks before use. The animals were healthy and housed in plastic cages containing wood shavings as bedding materials. They were housed and maintained at a constant room temperature under a 12-h light/dark cycle and given a balanced ration of feed and water of good quality source *ad libitum*.

Experimental Design

Rats were randomized and grouped into six groups, each consisting of five animals; Group 1: Rats were not induced with colitis and allowed free access to food and water; Group 2: Rats were induced with colitis and treated with normal saline; Group 3: Rats were induced with colitis and treated with CsPEx (250µg/Kg); Group 4: Rats were induced with colitis and treated with $AgNO_3$

(250µg/Kg); Group 5: Rats were induced with colitis and treated with CsPEx-AgNPs (25µg/Kg); Group 6: Rats were induced with colitis and treated with CsPEx-AgNPs (250µg/Kg).
Treatment regimens were administered via oral gavage once daily for seven days, starting from 24 hours after induction of colitis.

Induction of Colitis

Colitis was induced, according to Ige and Adekola (2021) [16]. Rats were fasted for 24 hours, without beddings, but allowed free access to water. After 24 hours of fasting, a single intra-rectal administration of 6% acetic acid (1mL/100g body weight) was administered in groups 2-6; the rats were kept in Trendelenburg position for 55 seconds to prevent reflux, followed by rectal flushing with 1mL of distilled water.

Assessment of Disease Activity Index of Ulcerative Colitis

The disease activity index (DAI) of ulcerative colitis was assessed as the basis for clinical assessment of intestinal inflammation and quantified as described by Ige et al. (2020) [17]. Changes in body weight, stool consistency (diarrhea score), colonic weight, colon thickness, colonosomatic index, and macroscopic scores were assessed.

Determination of Body Weight Change

The change in body weight of rats was calculated using the formula below;

$$\text{Body weight change} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

Stool Consistency (Diarrhea Score)

Diarrhea scoring method of Ige et al. (2020) [17] was adopted in this study to assess stool consistency. 0=Normal, 1=Loose stool without blood, 2=Visible blood, 3=Loose stool with visible blood, 4=Diarrhea with blood

Determination of Colonic Weight

A laparotomy was performed, and colonic segments (distal 6cm in length) were rapidly excised and gently cleaned of fecal contents. Blood stains and tissues around the excised colons were carefully cleaned to avoid stretch. The colon was opened along the mesenteric border and washed with cold saline. Colon specimen weights were measured using a sensitive scale.

Determination of Colonosomatic Index

Colonosomatic Index was calculated using the formula below;

$$\text{Colonosomatic index} = \frac{\text{Colon weight (g)}}{\text{Final body weight (g)}} \times 100$$

Determination of Colon Thickness

Colon thickness was measured in millimeters using a meter rule.

Assessment of Colonic Injury (Macroscopic Scoring)

Colon segments were placed on a plain sheet to visually inspect ulcerogenic features and the extent of mucosal damage or lesion. The colon was placed on a white paper with the inner layer exposed, and the mucosa was examined using a magnifying lens and quantified according to the method of Ige et al. (2020) [17]; 0=No damage, 1=Localized hyperemia with no ulcer, 2= Linear ulcers with no inflammation, 3=Linear ulcers with inflammation at one site, 4=More site of ulcers, the size of ulcer <1cm, 5=More site of ulcers, the size of ulcer >1cm.

Blood Collection and Hematological Analysis

Blood was collected via cardiac puncture using a 5mL syringe into anticoagulant bottles (Ethylenediaminetetraacetic acid, EDTA). Blood samples were analyzed using Mindray Auto-Haemolyzer BC-5300 (Shenzhen Mindray Bio-Medical Electronics, Co., L.T.D., China).

Tissue Preparation

Colon segments of rats were divided into two parts, and the first colon samples were kept in 0.25M

sucrose solution maintained 4ºC for biochemical assays. The second colon samples were preserved in 10% formalin for histopathological investigation. All samples were kept in different organ bottles under standard experimental conditions.

Determination of Biochemical Content of Colon Samples

Levels of antioxidant and pro-inflammatory markers of ulcerative colitis were analyzed in the colon samples. Superoxide Dismutase (S.O.D.), Tumor Necrosis Factor-alpha (TNF-α), and colonic total protein levels were assessed using Enzyme-Linked Immunosorbent Assay (ELISA) kit (Elabscience Biotechnology Inc. U.S.A.) following the manufacturer's instructions and as described by Magnani et al. (2000) and Ige et al. (2020) [17], [18].

Histopathological Examination

Segments of animal colon samples were fixed in neutral buffered 10% formalin for histopathological examinations according to the method of Avwioro (2010) [19]. They were further processed by passing through various reagents and graded alcohol, embedded in paraffin wax, dried, and stained with Haematoxylin and Eosin.

Statistical Analysis

Data obtained was analyzed using Graphpad prism (version 5.0). Results were expressed as Mean ± standard error of mean (S.E.M.). Data obtained from various groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey post-test. $P<0.05$ was considered to be statistically significant.

Results

Biosynthesis of CsPEx-AgNPs

Colour change from colourless to reddish brown was observed at five minutes of exposure to sunlight, which darkened with an increase in time at the end of thirty minutes, indicating the formation of silver nanoparticles (AgNPs), as shown in figure 1.

UV-Visible Spectroscopy

The UV-visible spectra of *Citrus sinensis* peel extract and its nanoparticles displayed optical absorption band peaks at 424nm and 440nm, respectively, confirming the synthesis of CsPEx-AgNPs, as shown in Figure 2.

Effect of CsPEx-AgNPs on Body Weight Change

There was a significant decrease in body weight in the Colitis control group compared to the Non-Colitis group, eighth-day post-colitis induction. However, there was a significant decrease in the body weights of rats in the Colitis+CsPEx and Colitis+$AgNO_3$ groups when compared with the Non-Colitis group. In contrast, there was a significantly increased weight change in the group treated with CsPEx-AgNPs (25µg/kg) and CsPEx-AgNPs (250µg/kg) when compared with the Colitis control group, as shown in Table 1.

Figure 1. Extract preparation and biosynthesis of silver nanoparticles.
C: *Citrus sinensis* peel aqueous extract; D: $AgNO_3$; D^+: *Citrus sinensis* peel aqueous extract mediated silver nanoparticles.

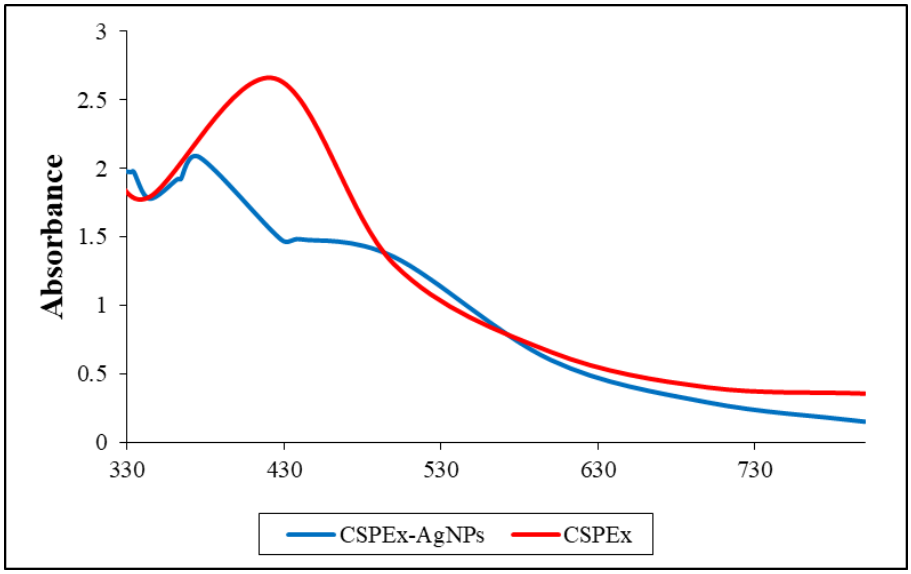

Figure 2. UV-visible spectroscopy of CsPEx and CsPEx-AgNPs.

Table 1. Body weight change on day 8 post colitis induction.

Treatments	Initial BW(g)	Final BW (g)	WC (g)	%WC
Non-Colitis Control	191.80±6.59	209.40±4.66	17.60±2.01	9.36±1.38
Colitis Control	205.20±5.93	189.60±5.94	-15.60±0.60[a]	-7.63±0.37[a]
Colitis+CsPEx (250µg/kg)	214.00±10.33	213.00±13.30	-1.00±3.47[a]	-0.73±1.68[a]
Colitis+$AgNO_3$(250µg/kg)	218.80±11.20	215.80±11.41	-3.00±3.39[a]	-1.38±1.52[a]
Colitis+CsPEx-AgNPs (25µg/kg)	207.40±9.97	207.80±11.72	0.40±4.20[ab]	0.12±1.97[ab]
Colitis+CsPEx-AgNPs (250µg/kg)	213.00±10.44	219.00±11.24	6.00±4.20[b]	2.85±2.14[b]

Number of rats = 5. Values are expressed as Mean±SEM. [a] shows $P<0.05$ vs non-colitis control. [b] shows $P<0.05$ vs colitis control.

Effect of CsPEx-AgNPs on Diarrhea Score, Colon Weight, and Colonosomatic Index

Table 2 shows the diarrhea score across all groups on the eighth day post colitis induction. There

was a significant increase in diarrhea score in the Colitis control group compared to the Non-Colitis group. Diarrhea scores in Colitis+CsPEx, Colitis+$AgNO_3$, and Colitis+CsPEx (250µg/kg) groups showed a significant decrease compared with the Colitis control group.
Non-significant increase in colonic weights was observed in Colitis control group (0.70±0.02) when compared with Non-Colitis control (0.57±0.03), Colitis+CsPEx (0.61±0.06), Colitis+$AgNO_3$ (0.62±0.05), Colitis+CsPEx-AgNPs (25µg/kg) (0.59±0.04) and Colitis+CsPEx-AgNPs (250µg/kg) (0.58±0.02) groups as shown in Table 2. There was a significant increase in the colon-to-body weight ratios (colonosomatic index) in the Colitis control group (0.36±0.02) when compared with Non-Colitis control (0.27±0.01). However, there were a significant decrease in colonosomatic index in Colitis+$AgNO_3$ and CsPEx-AgNPs (250µg/kg) groups compared with the Colitis control group.

Table 2. Effects of CsPEx-AgNPs on diarrhea score, colon weight and colonosomatic index.

Treatments	Diarrhea Score	Colon Weight (g)	Colonosomatic Index (%)
Non-Colitis Control	0.00±0.00	0.57±0.03	0.27±0.01
Colitis Control	1.60±0.40[a]	0.70±0.02	0.36±0.02[a]
Colitis+CsPEx (250µg/kg)	0.40±0.24[b]	0.61±0.06	0.29±0.03
Colitis+$AgNO_3$(250µg/kg)	0.40±0.24[b]	0.62±0.05	0.28±0.01[b]
Colitis+CsPEx-AgNPs (25µg/kg)	0.60±0.24	0.59±0.04	0.29±0.02
Colitis+CsPEx-AgNPs (250µg/kg)	0.20±0.20[b]	0.58±0.02	0.27±0.01[b]

Number of rats = 5. Values are expressed as Mean±SEM. [a] shows $P<0.05$ vs non-colitis control. [b] shows $P<0.05$ vs colitis control.

Effect of CsPEx-AgNPs on Colon Thickness
Figure 3 shows the colon thickness of rats across all groups. There was a significant increase in colon thickness in Colitis control, Colitis+CsPEx, and Colitis+$AgNO_3$ groups compared to the Non-Colitis group. There was no significant difference in the colon thickness in groups treated with 25 µg/kg and250µg/kg CsPEx-AgNPs compared with the Non-Colitis group. Compared with Colitis control, there was a significant decrease in the colon thickness of groups treated with both dosages of CsPEx-AgNPs. However, CsPEx-AgNPs (250µg/kg) group showed a significant decrease compared to other treatments.
Effect of CsPEx-AgNPs on Macroscopic Score
There was a significant increase in the macroscopic scores of Colitis control, Colitis+CsPEx, and Colitis+$AgNO_3$ groups compared to the Non-Colitis group. There was a significant decrease in the macroscopic scores of groups treated with both concentrations of CsPEx-AgNPs compared with Colitis control. However, there was no significant difference in the macroscopic scores of rats in 25 µg/kg and 250µg/kg CsPEx-AgNPs groups when compared with the Non-Colitis group, and both showed a significant decrease when compared with other treatment groups, as shown in Figure 4.

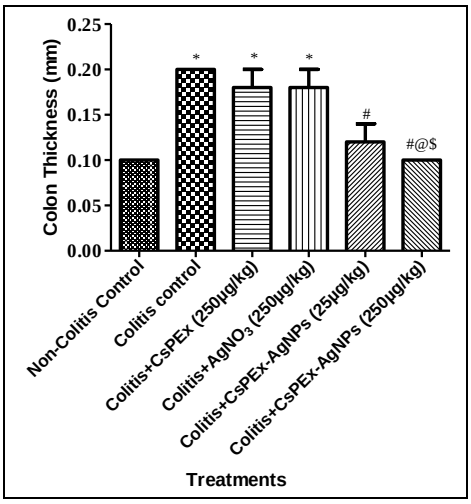

Figure 3. Effect of CsPEx-AgNPs on colon thickness.

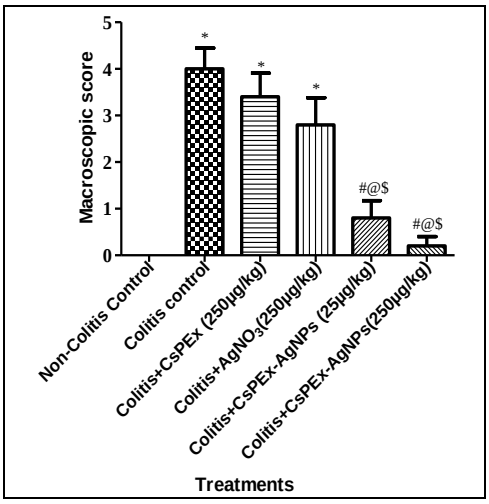

Figure 4. Effect of CsPEx-AgNPs on macroscopic score.

Number of rats = 5. Values are expressed as Mean±SEM* shows P< 0.05 vs non-colitis control. # shows P< 0.05 vs colitis control. @ shows P< 0.05 vs colitis+CsPEx. $ shows P< 0.05 vs colitis+$AgNO_3$.

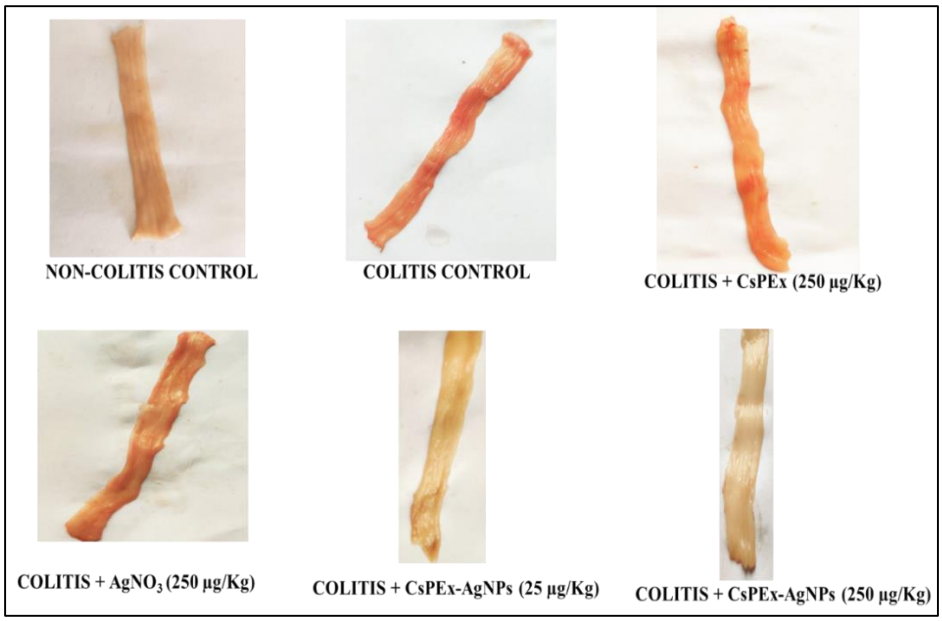

Figure 5. Macroscopic images of rats colon.

Macroscopic images showed that colonic section of the Non-colitis control group appeared normal. Colitis control group showed presence of chronic ulcer and inflammation. Colitis+CsPEx (250µg/kg) showed ulcer and moderate inflammation. Colitis+$AgNO_3$ (250µg/kg) appeared inflamed with signs of ulcer. Colonic section in 25µg/kg and 250µg/kg CsPEx-AgNPs appeared normal with absence of ulcer and oedema.

Effect of CsPEx-AgNPs on Red Blood Cell Indices

There was no significant difference in Red blood cell counts (RBC), Hemoglobin concentration (HGB), Mean cell volume (MCV), Mean cell hemoglobin (MCH), Mean cell hemoglobin concentration (MCHC), and Red cell distribution – coefficient of variation (RDW-CV) across all groups. There was a significant decrease in Packed Cell Volume (PCV) and a significant increase in Red cell distribution – standard deviation (RDW-SD) in the Colitis control group when

compared with the Non-colitis control group; however, CsPEx-AgNPs treated groups at both dosages showed a significant increase in PCV and a significant decrease in RDW-SD when compared with the Colitis control group but no significant difference when compared with Non-Colitis control group, as shown in Table 4.

Effect of CsPEx-AgNPs on White Blood Cell and Differential Count

No significant difference in White blood cell, lymphocyte, and neutrophil counts was observed across all groups, as shown in Table 4.

Effect of CsPEx-AgNPs on Platelet Indices

Table 4 shows a significant increase in Platelet count (PLT) and Plateletcrit (PCT) in the Colitis control group compared with the Non-colitis control. In contrast, a significant decrease in PLT was observed in the CsPEx-AgNPs groups at both dosages, and a significant decrease in PCT counts in CsPEx-AgNPs (250µg/kg) treated group only when compared with the Colitis control group. Also, there was no significant difference in Mean platelet volume (MPV), Platelet distribution width (PDW), and Platelet large cell ratio (PLCR) across all groups at day eight post-colitis induction.

Table 4. Effect of CsPEx-AgNPs on hematological parameters.

	Non-Colitis Control	Colitis Control	Colitis+CsPEx (250µg/kg)	Colitis+$AgNO_3$ (250µg/kg)	Colitis+CsPEx -AgNPs (25µg/kg)	Colitis+CsPEx -AgNPs (250µg/kg)
RBC(x10^6/µl)	8.78±0.16	7.94±0.11	8.12±0.25	8.08±0.27	8.52±0.15	8.65±0.37
HGB (g/dl)	12.22±0.12	10.74±0.85	11.12±0.46	10.88±0.07	11.40±0.25	12.06±0.53
PCV (%)	49.56±0.73	43.84±0.55[a]	46.15±0.78	46.48±0.81	48.38±0.33[b]	49.48±1.08[b]
MCV (fl)	56.46±0.40	54.92±0.50	55.28±1.33	55.34±0.54	55.60±1.27	55.82±0.44
MCH (pg)	13.96 ± 0.33	13.20±0.91	13.72±0.26	13.38±0.20	13.76±0.25	13.92±0.21
MCHC (g/dl)	25.12 ± 0.36	23.98±1.60	24.66±0.38	24.82±0.24	24.00±0.48	24.86±0.38
WBC(x10^6/µl)	12.25±1.45	14.22±2.53	12.36±1.34	9.84±1.23	9.52±0.89	9.20±1.21
LYM(x10^3/µl)	8.56±0.25	8.93±0.70	7.83±1.07	7.10±0.73	5.64±0.95	5.50±0.43
NEU (x10^3/µl)	2.28±0.44	3.38±0.62	2.63±0.42	2.66±0.30	2.30±0.28	2.13±0.32
PLT(×10^3/µl)	812.50±16.48	1072.67±32.96[a]	950.67±29.83[a]	979.33±14.68[a]	926.25±14.06[b]	910.75±31.39[b]
PCT(%)	0.65±0.04	0.84±0.04[a]	0.78±0.02	0.80±0.03	0.73±0.01	0.69±0.03[b]
MPV(fl)	7.68±0.24	7.54±0.27	7.64±0.22	7.66±0.17	7.80±0.25	8.04±0.16
PDW(fl)	10.30±0.95	9.64±0.66	9.68±0.43	10.22±0.43	10.33±0.80	10.80±0.38
PLCR(%)	13.48±1.20	11.42±1.60	11.84±1.52	11.54±1.40	12.10±0.57	12.14±2.06
RDW-SD (fl)	29.94±0.26	35.38±0.76[a]	33.08±1.12	32.68±0.69	31.88±0.54[b]	30.68±0.57[b]
RDW-CV(%)	15.56±0.86	17.58±0.67	16.88±0.47	16.42±0.66	16.12±0.34	16.16±0.47

Number of rats = 5. Values are expressed as Mean±SEM. [a] shows $P<0.05$ vs non-colitis control, [b] shows $P<0.05$ vs colitis control.

Effect of CsPEx-AgNPs on Superoxide Dismutase (SOD) Activity in Colitis

As shown in Figure 6, there was no significant difference in colonic SOD level in the Colitis control group compared with the Non-Colitis group at Day 8 post-colitis induction. However, compared with the Colitis control group, there was a significant increase in Colonic SOD levels in

groups treated with 25 µg/kg and250µg/kg CsPEx-AgNPs. There was a significant increase in the SOD levels of CsPEx-AgNPs (250µg/Kg) compared with other treatment groups.

Effect of CSPEx-AgNPs on Glutathione (GSH) Activity in Colitis

There was no significant difference in the glutathione (GSH) concentration of the Colitis control group compared with the Non-colitis control group. There was a significant increase in the glutathione (GSH) concentration of CSPEx-AgNPs (250µg/kg) treated group compared with the Colitis control group. There was no significant difference in the glutathione (GSH) concentration of Colitis + CSPEx (250µg/kg) group, Colitis + $AgNO_3$ (250µg/kg) and Colitis+CSPEx-AgNPs (25µg/kg) group when compared with colitis control group as shown in Figure 7.

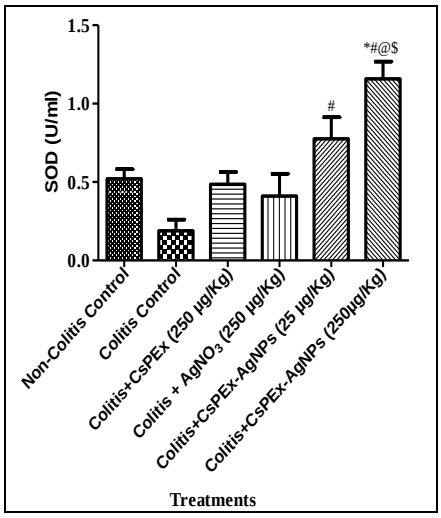

Figure 6. Effect of CsPEx-AgNPs on SOD level.

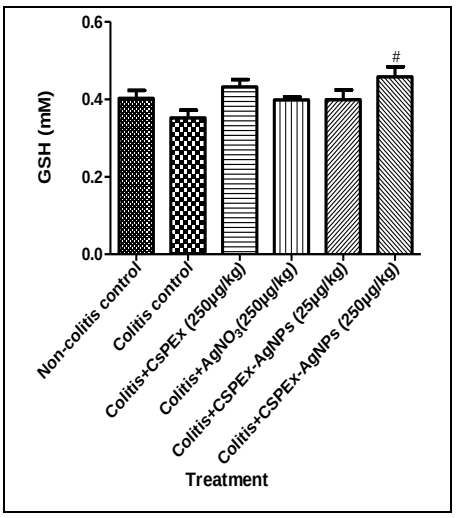

Figure 7. Effect of CsPEx-AgNPs on GSH level.

Number of rats = 5. Values are expressed as Mean±SEM. * shows P< 0.05 vs non-colitis control, # shows P< 0.05 vs colitis control, @ shows P< 0.05 vs colitis+CsPEx, $ shows P< 0.05 vs colitis+$AgNO_3$.

Effects of CsPEx-AgNPs on Catalase (CAT) Activity in Colitis

Figure 8 shows no significant difference in catalase activity in the Colitis control group compared with the Non-colitis control. Compared with the Colitis control group, there was a significant increase in catalase activities in the Colitis+CsPEx-AgNPs (250µg/kg) group. No significant difference in catalase activities in other treatment groups compared to the Colitis control group.

Effect of CSPEx-AgNPs on Myeloperoxidase (MPO) Concentration in colitis

There was a significant increase in MPO concentration in the Colitis control group compared to the Non-colitis control group. There was a significant decrease in MPO concentration in Colitis+CSPEx-AgNPs (25µg/kg) and Colitis+CSPEx-AgNPs (25µg/kg) groups when compared with Colitis control group. A significant decrease in MPO concentration was observed in the Colitis+CSPEx (250µg/kg) group compared to other treatment groups, as shown in Figure 9.

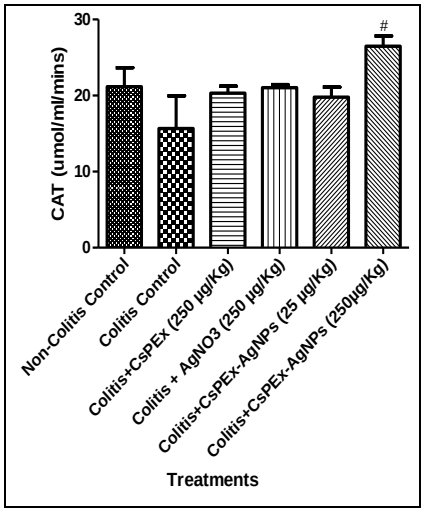

Figure 8. Effect of CsPEx-AgNPs on catalase level.

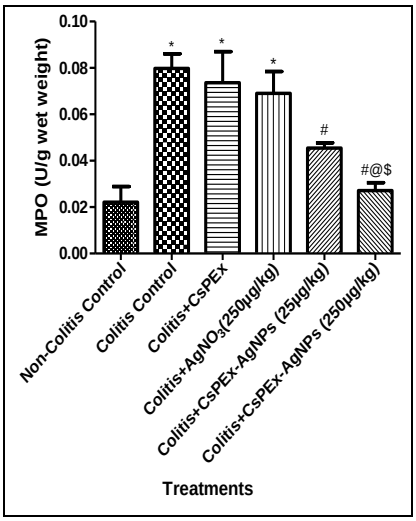

Figure 9. Effect of CsPEx-AgNPs on MPO concentration.

Number of rats = 5. Values are expressed as Mean±SEM. * shows P< 0.05 vs non-colitis control, # shows P< 0.05 vs colitis control, @ shows P< 0.05 vs colitis+CsPEx, $ shows P< 0.05 vs colitis+$AgNO_3$.

Effects of CsPEx-AgNPson Malondialdehyde (MDA) Concentration

Figure 10 shows no significant difference in malondialdehyde (MDA) concentrations in the Colitis control group compared with the Non-colitis control group. There was no significant difference in MDA concentrations in Colitis + CsPEx-AgNPs (25µg/kg) group and CsPEx-AgNPs (250µg/kg) group compared with Colitis control group.

Effect of CsPEx-AgNPs on Tumor Necrosis Factor-alpha (TNF-α) Activity

There was a significant increase in colonic TNF-α levels in the Colitis control group compared to the Non-Colitis control group. There was a significant decrease in colonic TNF-α level inCsPEx-AgNPs 250µg/kg group compared with the Colitis group. There was no significant difference in colonic TNF-α level in Colitis+CsPEx, Colitis+$AgNO_3$, and CsPEx-AgNPs (25 µg/kg) groups when compared with Colitis control, as shown in Figure 11.

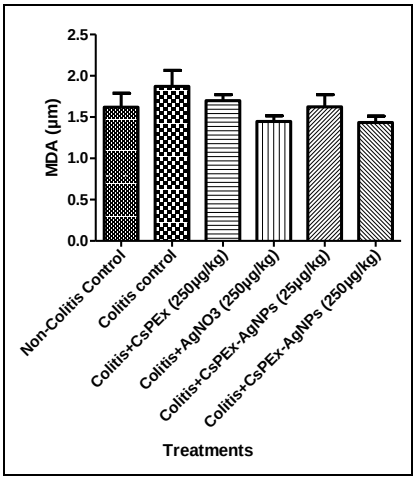

Figure 10. Effect of CsPEx-AgNPs on MDA concentration.

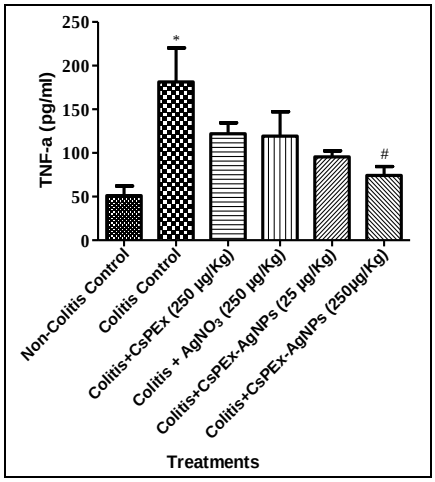

Figure 11. Effect of CsPEx-AgNPs on TNF-α level.

Number of rats = 5. Values are expressed as Mean±SEM. * shows P< 0.05 vs non-colitis control, # shows P< 0.05 vs colitis control.

Histopathological Images of Colonic Tissues

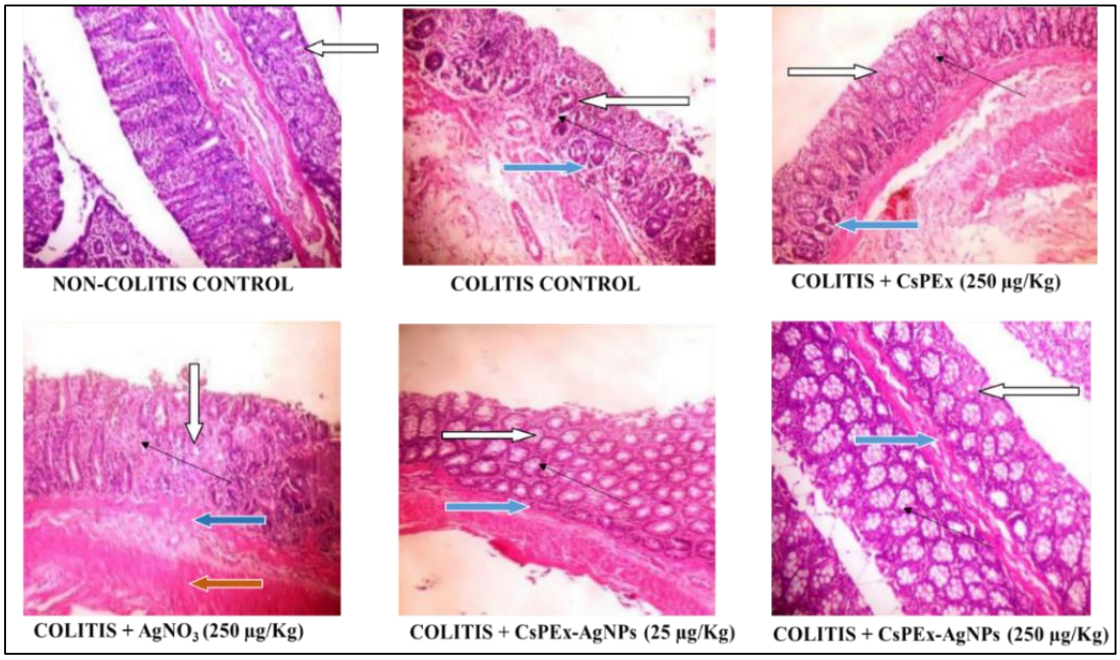

Figure 10. Photomicrograph of colonic sections stained by H&E (x100).
Non-Colitis control: shows preserved mucosal epithelial layer; the sub-mucosal layer appears normal. **Colitis control:** shows moderate mucosal ulceration (white arrow), lamina propria of the mucosal layer shows severe to chronic infiltration of inflammatory cells (slender arrow) and the submucosal layer appears moderately inflamed (blue arrow). **Colitis+CsPEx (250µg/Kg):** shows moderate mucosal ulceration (white arrow), the lamina propria of the mucosal layer shows severe infiltration of inflammatory cells (slender arrow), and the submucosal layer appears mildly inflamed (blue arrow). **Colitis+$AgNO_3$ (250µg/Kg):** shows fairly preserved mucosal epithelial layer (white arrow), lamina propria of the mucosal layer shows severe infiltration of inflammatory cells, which include lymphocytes and polymorphs (slender arrow), while the submucosal layer (blue arrow) and circular muscle (red arrow) appear normal. **Colitis+CsPEx-AgNPs (25µg/Kg):** shows moderately sloughed mucosal epithelial layer (white arrow), lamina propria of the mucosal layer shows mild to moderate infiltration of inflammatory cells, which include lymphocytes and polymorphs (slender arrow) and the submucosal layer appear normal (blue arrow). **Colitis+CsPEx-AgNPs (250µg/Kg):** well-preserved mucosal epithelial layer (white arrow); however, the lamina propria of the mucosal layer shows mild infiltration of inflammatory cells, which include lymphocytes and polymorphs (slender arrow) while the submucosal layer appears normal (blue arrow).

Discussion

This study investigates the therapeutic potential of CsPEx-AgNPs at doses of 25µg/Kg and 250µg/Kg in ulcerative colitis. AgNPs were biosynthesized using an aqueous peel extract of *Citrus sinensis* as a reducing and capping agent. The colour change after 30 minutes of exposure to sunlight confirmed the synthesis of AgNPs (CsPEx-AgNPs), as reported by previous studies [11], [20], [21]. The characterization of CsPEx-AgNPs using a UV-visible spectrophotometer displayed an optical absorbance peak at 440nm due to the surface plasmon resonance. Hence, this corroborates the previous studies by Kaviya et al. (2011) [14] and Niluxsshun et al. (2021) [9] that reported 445nm and 430-450nm, respectively, in which the biomoities such as flavonoids, alkaloids, coumarins, and phenolics with their functional groups were shown to be involved in reducing the silver salt to Ag0 via Fourier Transmission Infrared spectroscopy. Furthermore, the authors revealed that Transmission Electron Microscopy showed a particle size between 10-70 nm with spherical, triangular, and rod shapes.

The disease activity index of animals after colitis induction revealed weight loss and increased diarrhea, which are the hallmarks of IBD. The reduction in body weights of colitic rats observed in this study could result from malnutrition, increased protein catabolism arising from reduced food intake, food aversion, and rapid loss of body fluid due to loose stool and diarrhea [16]. In this study, rats treated with CsPEx-AgNPs showed body weight gain and reduced diarrhea, which was more prominent in the group treated with 250µg/Kg. Hence, it can be inferred that CsPEx-AgNPs attenuated protein catabolism in muscles and enhanced fluid absorption in the colon, thus preventing diarrhea.

The induction of colitis significantly increased colonic weight and thickness and induced significant ulceration and inflammation, as revealed by macroscopic evaluation, which may be a result of colonic epithelial oedema, ulceration, lesion, and necrosis with infiltration of the damaged colon with neutrophils and macrophages, which are considered indicative of inflammatory conditions [22]. Treatments with CsPEx-AgNPs at both dosages, especially at 250µg/Kg, were effective in significantly decreasing colonic thickness and improving the morphological damages induced by colitis owing to its heavy distribution and adherence in intestinal tissue. Hence, this result agrees with a similar investigation by Abdallah et al. (2020) [23], who reported the anti-ulcerogenic effects of silver nanoparticles using *Acasia salina* extract in acetic acid-induced colitis.

Red cell count and red blood cell-related parameters also indicate ulcerative colitis. Findings in this study showed that at the end of the treatment days, there was no significant difference in RBC count, HGB count, MCV, and MCHC of the Colitis control group, but a significant decrease in PCV which may occur as a result of anemia due to blood loss during ulcerative colitis [16], [24], [25]. Furthermore, Red Cell Distribution Widths (RDWs), a known sensitive indicator of iron deficiency anaemia, have also been used as a marker of ulcerative colitis. This study showed that induction of colitis significantly increased Red Cell Distribution Width (RDW) in Colitis control, which may be due to intestinal bleeding and iron malabsorption resulting from inflammation and ulceration, a prevalent and frequent complication in ulcerative colitis [26]. Hence, CsPEx-AgNPs at both dosages significantly reduced RDW and increased PCV in treated rats, thereby preventing persistent anaemia, maintaining red blood cell lifespan, and favoured erythropoiesis [27].

The effects of CsPEx-AgNPs on White blood cell, neutrophil, and lymphocyte counts of colitis rats were examined in this study, as they represent the first line of defense during inflammation and infections [28]. In this study, no significant difference in WBC, neutrophil, and lymphocyte counts was observed across all groups at the end of treatment days compared with Non-Colitis and Colitis control groups, which suggests that a decline in disease progression may occur, especially in colitis, after some days before the onset of relapse.

Platelet indices are considered markers of ulcerative colitis due to the bi-directional interaction between inflammation and haemostasis, thus, have been implicated in the pathogenesis of UC. In this study, the induction of ulcerative colitis caused a significant increase in PLT and PCT counts. However, no significant difference was observed in MPV, PDW, and PLCR in the Colitis control group compared with the Non-Colitis control group at the end of treatment days. These alterations indicate that activation of thrombocytes enhances mucosal inflammation as hosts' defense in both infectious and non-infectious inflammatory states to local activation of the haemostatic system, which is essential for the formation of platelet and that large metabolically active blood platelets take part in the inflammatory process in the colon [29], [30]. However, treatment with CsPEx-AgNPs ameliorated platelet indices as evidenced by a significant decrease in PLT and PCT, in line with a similar study by Polińska et al. (2011) [31], but no significant difference in MPV, PDW,

PLCR, which indicates that CsPEx-AgNPs could be helpful in the prevention of thrombocytosis, hypercoagulation and microvascular thrombosis [16]. Overall, CsPEx-AgNPs at these dosages did not induce haematotoxicity nor cause harmful hematological alterations in the treated rats, as against deleterious effects at higher dosages reported in previous studies [28], [32], [33].

In this study, induction of ulcerative colitis resulted in disruption and depletion of the antioxidant system in the Colitis control group, leading to non-significant reduction SOD, GSH, and CAT activities, which is in agreement with a previous study by Rana et al. (2014) [34], Baldo and Serrano (2017) [35], Olamilosoye et al. (2018) [25] and Ige and Adio (2020) [36]. They attributed decreased antioxidant enzymes in colitis to increase oxidative stress mediated by high levels of free radicals associated with Nitric oxide, Superoxide anions, and Hydrogen peroxide (H2O2). However, CsPEx-AgNPs at both dosages, especially at 250µg/Kg, significantly increased SOD, GSH, and CAT activities compared with the Colitis control group. This result agrees with previous studies by Mohan et al. (2014) [37], Mahmoud et al. (2017) [38], and Kaur et al. (2020) [39] that revealed enhanced antioxidant activity of silver nanoparticles. According to Owusu et al. (2020) [40], increased antioxidant level, in response to disease activity activated by colitis, is essential to inhibit the production of reactive oxygen species (ROS), protects the cells against damage by mediating dismutation of superoxide anion, preventing lipid peroxidation, leukocyte rolling and adhesion in colonic tissues as well as suppressing proinflammatory cytokines involved in oxidative stress and tissue damage.

The effect of CsPEx-AgNPs on inflammatory markers was also examined in this study. It was revealed that there was a significant increase in colonic MPO concentration and TNF-α activity as well as a non-significant increase in MDA concentration in the Colitis control group; hence, this corroborates the finding as observed by Hagar et al. (2007) [6] and Khan et al. (2016) [7] in which elevated levels of these inflammatory markers in colitis results in activating the production of other cytokines including adhesion molecules and arachidonic acid metabolites, thereby leading to excessive gut inflammation that augments colonic tissue damage. The significant decrease observed in colonic TNF-α and MPO concentrations in CsPEx-AgNPs treated group, especially at 250µg/Kg, attested to its anti-inflammatory effect, which may be due to its ability to inhibit lipid peroxidation, NF-κB production, cyclooxygenase and lipoxygenase pathways, leucocyte and prostaglandin productions and epithelial permeability, thereby preventing tissue against further injury. Hence, consistent with these results, Bhol and Schechter (2005) [41], Siczek et al. (2017) [42], and Asgharzadeh et al. (2021) [3] reported similar potent anti-inflammatory functions of silver nanoparticles via suppressing the expression of inflammatory markers.

Histopathological examination also confirmed biochemical findings. At day eight post colitis induction, colons of the rats in the Colitis control group showed mucosal ulceration, chronic infiltration of inflammatory cells in the lamina propria of the mucosal layer, and inflamed submucosal layer. However, CsPEx-AgNPs treated group showed a reduced number of cellular infiltrates, a well-preserved epithelial layer, and a typical appearance of both the submucosal layer and circular muscle. Osafo et al. (2019) [43] explained that the activation, eventual invasion, and distribution of inflammatory cells in colon tissues lead to the production of inflammatory mediators, such as cytokines and ROS, that cause the observed morphological damage in ulcerative colitis. Reduction of these infiltrating cells by CsPEx-AgNPs, therefore, is partly responsible for the observed beneficial effect and was found to have the ability to increase the regenerative capacity of damaged tissues, confirming previous studies by Kaur et al. (2020) [39] and Krajewska et al. (2020) [8].

Conclusion

In conclusion, this study showed that Silver nanoparticles bio-fabricated using an aqueous extract of *Citrus sinensis* peel has an anti-colitic effect and may provide therapeutic potential in the treatment of ulcerative colitis by lowering Disease Activity Index, ameliorating hematological parameters, enhancing antioxidant enzymes, suppressing inflammatory markers, reducing the number of cellulars infiltrates and restoring colonic mucosal epithelial integrity. Hence, using silver nanoparticles appears to be a promising approach that may be considered for treating ulcerative colitis.

Ethics approval

This study, handling, and use of animals were in line with the guidelines of the National Institute of Health (NIH) for the use of laboratory rats (NIH publication No. 8523, revised 1985). All experiments were examined and approved by the Ethical Committee, Faculty of Basic Medical Science, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.

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Conflict of interests

The authors have no competing interests to declare relevant to this article's content.

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