Asian Journal of Research and Reports in Gastroenterology

Volume 8, Issue 1, Page 6-26, 2025; Article no.AJRRGA.126786

Citrus limon Juice Promotes Healing of Acetic Acid-Induced Ulcerative Colitis by Enhancing Colonic Antioxidant Defense, Immune Response, and Microbial Balance in Cadmium Exposed Male Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/ajrrga/2025/v8i1157

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/126786

Original Research Article

Received: 13/09/2024
Accepted: 15/11/2024
Published: 20/01/2025

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Cite as: *Ige, Serah Funke, Oluwaseun Bayode Ademilua, Taiwo Adebayo Ajagbe, Regina Damilola Efunwole, Fiyinfoluwa Helen Ogundipe, Kilan Abdulroheem, Adeola Muslimah Yusuf, Favour Adekunle, Precious Obalalu, Oluwakemi Joseph, Rohimot Adetutu Ogunfuyi, and Joy Damilola Bolaji. 2025. "Citrus Limon Juice Promotes Healing of Acetic Acid-Induced Ulcerative Colitis by Enhancing Colonic Antioxidant Defense, Immune Response, and Microbial Balance in Cadmium Exposed Male Wistar Rats". Asian Journal of Research and Reports in Gastroenterology 8 (1):6-26. https://doi.org/10.9734/ajrrga/2025/v8i1157.*

ABSTRACT

Ulcerative colitis (UC) an inflammatory disease, causing damage to the colon lining is intensified by long term exposure to cadmium chloride ($CdCl_2$) resulting in altered barrier and microbial population. *Citrus limon* has various antioxidant, anti-inflammatory, and gut microbial modulating properties. Hence, the study aims to determine *Citrus limon* juice (CLJ) effect on colitis healing of cadmium exposed rats. Sixty-four male Wistar rats weighing 200g ± 20g were randomly divided into control group, $CdCl_2$ group, UC group, CLJ group, $CdCl_2$ + UC group, $CdCl_2$ + CLJ group, UC + CLJ group and $CdCl_2$ + UC + CLJ group. 5mg/kg of $CdCl_2$ and 7.5ml/kg of CLJ was administered orally for 25 days. Colitis was induced on day 16, 17 and 18 with 2% acetic acid. Compared to control group, colitis group elicited significant increase in colon weight, colonosomatic index, glutathione (GSH), 8-hydroxy-2-guanosine (8-OHdG), malonidialdehyde (MDA), myeloperoxidase (MPO), lymphocytes, *Escherichia. coli*, *Staphylococcus aureus*, fecal *Pseudomonas spp*, but decrease in body weight, red blood cell, hemoglobin, hematocrit, colonic *Klebsiella spp*, and moderate inflammatory cells infiltration. Also, $CdCl_2$ + UC elicited a significant increase in colon weight, colonosomatic index, superoxide-dismutase (SOD), 8-OHdG, MDA, lymphocytes, basophils, colonic *E. coli, Staphylococcus aureus*, *fecal Pseudomonas spp*, severe inflammatory cells infiltration and epithelial damage compared to control and UC groups. Treatment of $CdCl_2$ + UC with CLJ prevented epithelial damage, cells infiltration, decreased SOD, GSH, MPO, tumor necrosis factor (TNF-α), lymphocyte, neutrophil, monocyte, fecal *Pseudomonas spp*, increased colonic *Klebsiella spp*. *Citrus limon* juice ameliorated intensification and delayed healing of ulcerative colitis caused by cadmium exposure, by regulating colonic antioxidant-immune defense system and microbial population. These findings suggest that CLJ may serve as a potential therapeutic agent for mitigating the effect of cadmium chloride in the healing of ulcerative colitis.

Keywords: Citrus limon juice; ulcerative colitis; superoxide dismutase; glutathione; myeloperoxidase; hematology; microbial population; inflammatory cells.

1. INTRODUCTION

Ulcerative colitis is a chronic inflammatory disease affecting the colon, characterized by relapsing and remitting mucosal inflammation, starting in the rectum and extending to proximal segments of the colon (Torres et al., 2012; Høivik et al., 2013; Rowan et al., 2022). The colon which plays its primary role in the absorption of water, electrolytes, vitamins, formation and movement of feces toward the rectum for elimination is divided into four major segments; the cecum and ascending colon, transverse colon, descending colon, and sigmoid colon (Azzouz & Sharma, 2018; Ogobuiro et al., 2023).

Intestinal epithelium, the first line of protection against luminal bacteria and foreign toxicants providing mechanical barrier is composed of five distinct cells types mainly; the enterocytes, goblet cells, enteroendocrine cells, Paneth cells and microfold cells (Beisner et al., 2010). It is also a habitation to immune cells, such as dendritic cells, T cells, B cells, and macrophages, all working in close relation to maintain intestinal homeostasis (Beisner et al., 2010; Eri & Chieppa, 2013). These factors and their interactions with the gut microbiota is important for maintaining intestinal homeostasis and inflammation inhibition (Yoo et al., 2020).

However chronic and progressive immune disorders related to alterations in the composition of the microbiota (dysbiosis), immune response, genetic and environmental factors are all triggers for the beginning of ulcerative colitis and its risk factors include; family history, genetics, drugs, oral contraceptives, and environmental pollutants such as cigarette smoking (Rowan et al., 2022; Tomasello et al., 2014; Ananthakrishnan, 2015; Jiang et al., 2020).

Exposure to cadmium, one of the natural occurring environment pollutants derived from agricultural and industrial sources occurs via; intake of contaminated food, water, cigarette smoking and inhalation (Ige et al., 2020; Genchi et al., 2020). Its sources of contamination are related to its use in industries as a corrosive reagent, stabilizer in PVC products, color pigments, and Ni-Cd batteries (Genchi et al., 2020). Its absorption takes place mainly through the respiratory tract and to an extent via the gastro-intestinal tract (Genchi et al., 2020).

The gastrointestinal tract and the lungs are target organs for dietary intake and inhaled cadmium (Tinkov et al., 2018). Due to its subsequent ingestion and mucociliary clearance, a substantial inhaled proportion (60%) ends up in the digestive tract (Satarug, 2018). As an

exogenous pollutant, it can invade the gastrointestinal tract and influence the occurrence and development of gastrointestinal diseases (Yuan et al., 2016). Its exposure causes a major alteration in bacterial populations and their relative abundance in the gut followed by an increased lipopolysaccharide (LPS) production, resulting in changes in the metabolic activity of the intestinal microbiome (Kim et al., 2015; Tinkov et al., 2018). It induces oxidative stress and also interferes with the activity of antioxidant enzymes, such as catalase, manganese superoxide dismutase, and copper-zinc superoxide dismutase (Genchi et al., 2020).

In the intestine, it stimulates inflammatory response and cell damage resulting in increased gut permeability, bacterial translocation and increase mice susceptibility to infections (Joly Condette et al., 2014; Earley et al., 2015; Liu et al., 2020). It has been reported to intensify and delay the healing of ulcerative colitis in rats (Jiang et al., 2020; Adegoke et al., 2017).

However, Lemon (*Citrus limon*) fruit belonging to the rutaceae family is a very good potential treatment for ulcerative colitis due to its antioxidant, anti-inflammatory properties, as well as its modulating effect on the gut microbiota (Musumeci et al., 2020). Its chemical composition includes eriodictyol, limocitrin, rutin, isorhamnetin and the major component hesperidin (Abd-elrahman et al., 2019). Hesperidin is an inhibitor of histamine, a neurotransmitter implicated in allergic and inflammatory reactions (González-Molina et al., 2010). It is metabolized by the intestinal microbiota to their aglycones hesperetin and naringenin respectively (Sost et al., 2021). They are beneficial for the health through immune modulation, anti-inflammatory properties, and improved carbohydrate and lipid metabolism (Sost et al., 2021).

2. METHODOLOGY

Animal care and management: Sixty-four male Wistar rats weighing 200g ± 20g were used for this study. They were obtained and kept at the animal house of the Department of Physiology, Ladoke Akintola University of Technology (LAUTECH), Ogbomosho Oyo State. They were acclimatized for fourteen (Tinkov et al., 2018) days in a well-ventilated room 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.

Ethical certification: Ethical approval was obtained from the Faculty of Basic Medical Sciences ethical research committee. Ethical approval number: ERCFBMSLAUTECH:043/06/2024.

Lemon (*Citrus limon*) juice preparation: Fresh *Citrus limon* fruits were bought from Waso market Ogbomoso, Oyo State, Nigeria, rinsed with distilled water and its juice squeezed out using a manual grating machine. The lemon (*Citrus limon*) fruit juice was freshly prepared for each day of the administration.

Ulcerative colitis induction: Ulcerative colitis was induced according to the modified method of Ige et al. (2020). Rats was fasted for 24 hour and allowed free access to water. After which, rectal flushing was done using 1ml of distilled water followed by a single intra-rectal administration of 2% acetic acid (2mL/100g BW) to all colitis groups on the 16, 17 and 18 day of treatment. The rats were kept in Trendelenburg position for 50 seconds to prevent reflux.

Study design: The rats were randomly divided into eight groups of eight rats per group. Group one (control), group two (cadmium), group three (colitis), group four (*Citrus limon* juice), group five (cadmium + colitis), group six (cadmium + *Citrus limon* juice), group seven (colitis + *Citrus limon* juice) and group eight (cadmium + colitis + *Citrus limon* juice). Group 2,5,6 and 8 were administered 5mg/kg BW of CdCl orally for 25 days. Group 4,6,7, 8 were administered 7.5ml/kg BW of CLJ orally for 25 days. 2% Acetic acid was used to induced UC in Group 3,5,7 and 8 on the 16^{th}, 17^{th} and 18^{th} day of treatment. The whole treatment lasted for 25 days.

Animal sacrifice and organ collection: The rats were sacrificed on the twenty sixth (26th) day of administration, by cervical dislocation method. Blood averaging (1.5-2.0ml) was collected from the rats through cardiac puncture for hematological analysis. The distal colon of the rats measuring six (6) cm beginning from the proximal end of the rectum was excised and the fecal content collected for fecal microbial count analysis. The excised distal colon was rinsed in saline and divided into three portions (proximal, mid and distal). The distal portion of the colon was preserved in 0.25M sucrose solution maintained 4ºC for biochemical assays. The mid portion of the colon was preserved in 10% formalin for histological analysis, while the proximal region was reserved for intestinal microbiota count analysis.

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 according to the methokd of Alsharif et al., (2022). Changes in body weight, colonic weight, colonosomatic index, were assessed.

Assessment of antioxidant enzymes and oxidative markers in the colon: Colons were homogenized using a Potter-Elvehjem homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA-Labortechnik, Staufen, Germany) on ice-cold Tris-HCl buffer (0.01 M, pH 7.4) to give a 10% homogenate which was used for assays of Superoxide dismutase (SOD), Catalase, Reduced glutathione(GSH), 8-hydroxy-2-deoxyguanosine(8-OHdG), Malondialdehyde (MDA), Myeloperoxidase (MPO) level, Tumor Necrosis Factor (TNF-α) and nuclear factor kappa B cells (NF-kB) .

Activity of SOD and catalase in the colon was determined as described by Misra and Fridovich and Sinha respectively (Misra & Fridovich, 1972; Sinha, 1972). The method of Ansari et al*.* and Otu-Boakye et al*.* was followed for GSH and MDA respectively, (Ansari et al., 2021; Otu-Boakye et al., 2023). Myeloperoxidase**,** 8-OHdG levels**,** TNF-α assays were done using enzyme linked immunosorbent assay (ELISA) kits, as instructed by the manufacturer (eBIOSCIENCE, Bender MedSystems GmbH, Wien, Austria).

Analysis of intestinal and fecal microbiota using serial dilution method: The large intestines (distal colons) were removed and individual sections cut longitudinally. After removal of the intestinal fluids, the tissue samples were washed with sterilized buffered peptone water (LaB M) mixed with 20% glycerol (Merck Millipore) and then vortex mixed to break down bacterial clumps and to remove loosely attached bacteria. Samples were stored in 20% glycerol in sterilized buffered peptone water (LaB M) at −20 °C until microbiological analysis was performed (Nagy et al., 2016; Li et al., 2021).

Intestinal tissues and feces were separately homogenized with sterilized buffered peptone water (LaB M) and were subjected to serial dilutions using ¼ strength Ringer's solution (LaB M) the procedure of Yanni et al*.* was then followed for further examination, (Yanni et al., 2020).

Histological assessment of ulcerative colitis: The intensity of colonic tissues damage was assessed by staining with hematoxylin and eosin (H & E) stains and characterized according to Appleyard and Wallace. A histopathologist, who was unaware of the treatments, observed and characterized the histological tissues. Pictures were obtained using a digital camera (Olympus DP21) attached with a microscope (Otu-Boakye et al., 2023; Appleyard & Wallace, 1995).

Statistical analysis: Data were presented as Mean ± standard error of the mean (Mean ± SEM) and analyzed using graph pad prism 5, One-way analysis of variance (ANOVA). Tukey's post-hoc test was used for multiple comparisons. $P<0.05$ was considered statistically significant**.**

3. RESULTS

Effect of *Citrus limon* juice on body weight changes, colon weight, and colonosomatic index in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats: Rats induced with colitis showed a significant increased colon weight and colonosomatic index accompanied by a significant weight reduction. The exposure of these colitis rats to cadmium resulted in a further significant increase in colon weight and colonosomatic index when compared to cadmium group and control group. However, treatment of cadmium exposed colitis rats with *Citrus limon* juice resulted in a reduction in body weight.

Effect of *Citrus limon* juice on antioxidant enzymes in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats: Rats induced with colitis showed increase in GSH level when compared to control group, while cadmium exposed colitis rats showed a significant increase in SOD when compared to colitis group and control group. Treatment of cadmium exposed colitis rats with *Citrus limon* juice showed a significant reduction in SOD and GSH when compared to cadmium exposed colitis group and when compared to control group. No significant difference was seen in catalase level across all groups.

Effect of *Citrus limon* juice on oxidative stress markers in acetic acid-induced ulcerative colitis of cadmium exposed male wistar rats: Colitis group showed a significant increase in 8-OHdG, MDA and MPO when compared to control group. Cadmium exposed colitis rats showed a significant increase in 8-

OHdG when compared to cadmium. MDA was increased in all cadmium exposed rats when compared to control group. Treatment of colitis group with *Citrus limon* juice showed a significant reduction in MPO when compared to colitis group.

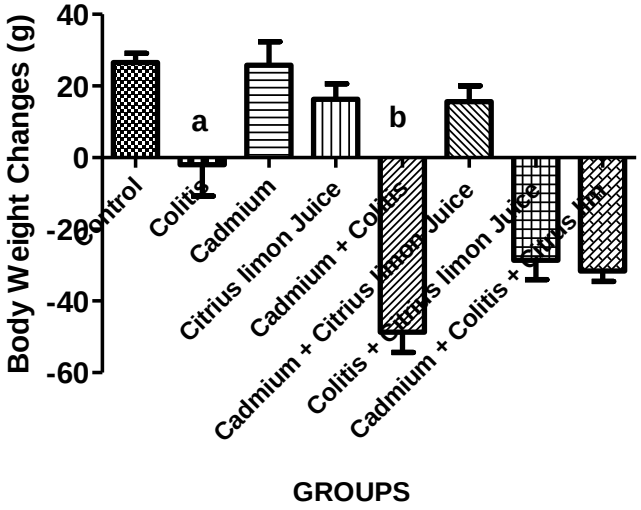


Fig. 1a. Effect of *Citrus limon* juice on body weight changes in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
b - Represents significant difference (P<0.05) when compared with colitis group

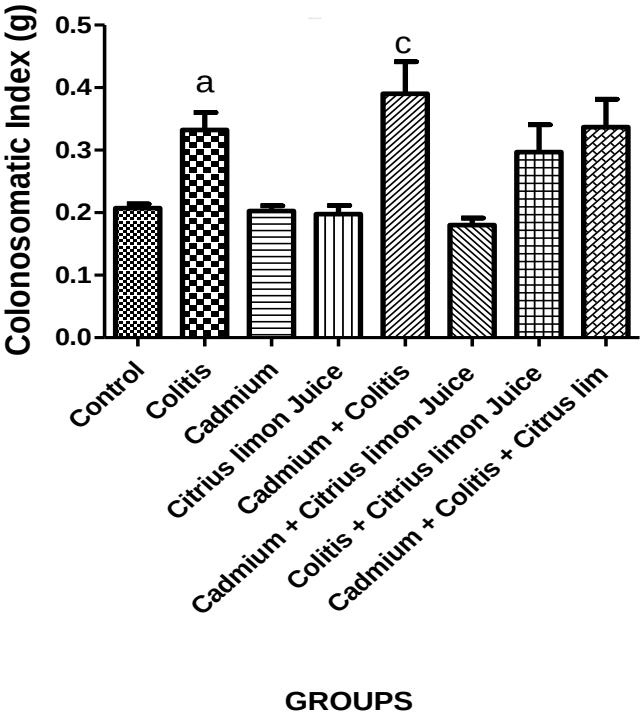


Fig. 1b. Effect of *Citrus limon* juice on colonosomatic index in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05when compared with control group
c - Represents significant difference (P<0.05) when compared with cadmium group

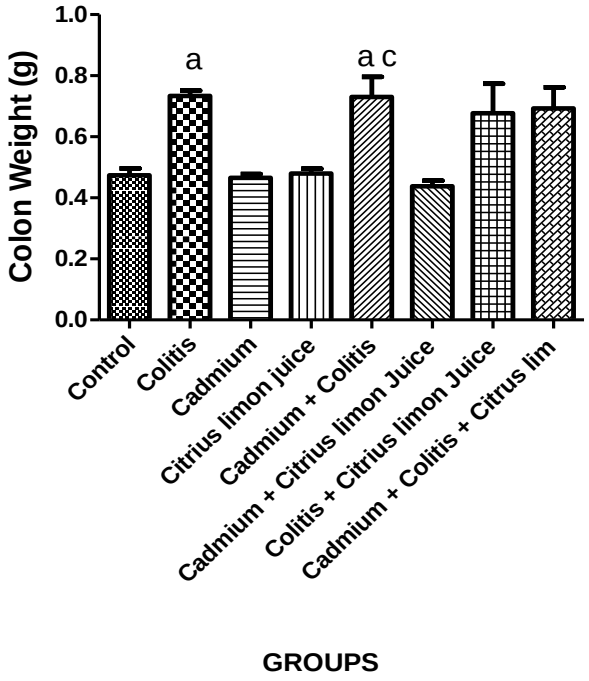


Fig. 1c. Effect of *Citrus limon* juice on colon weight in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
c - Represents significant difference (P<0.05) when compared with cadmium group

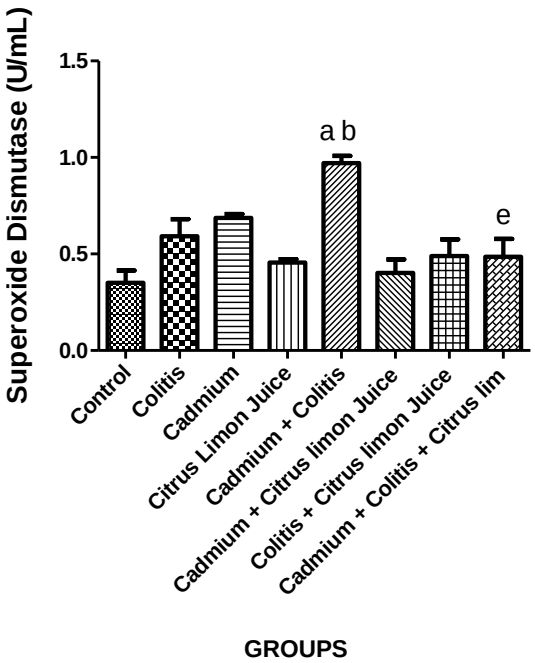


Fig. 2a. Effect of *Citrus limon* juice on superoxide dismutase levelin acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
b - Represents significant difference (P<0.05) when compared with colitis group
e - Represents significant difference (P<0.05) when compared with cadmium + colitis group

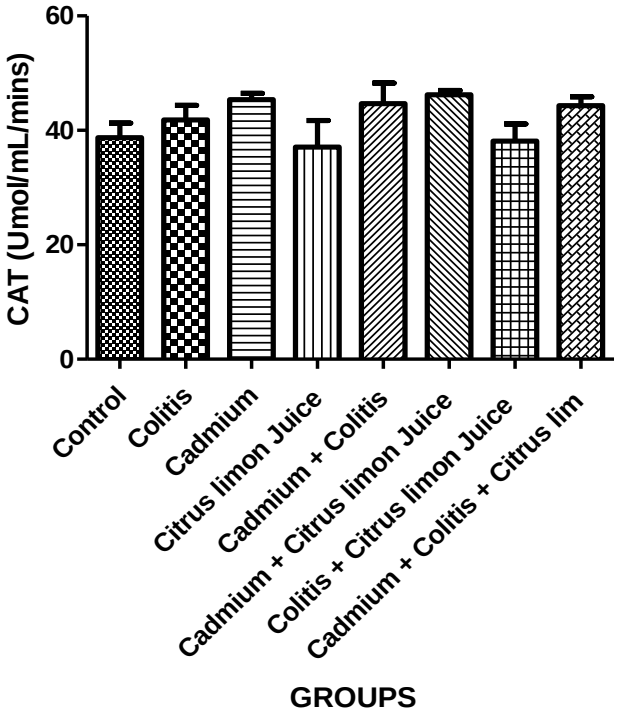


Fig. 2b. Effect of *Citrus limon* juice on superoxide dismutase levelin acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

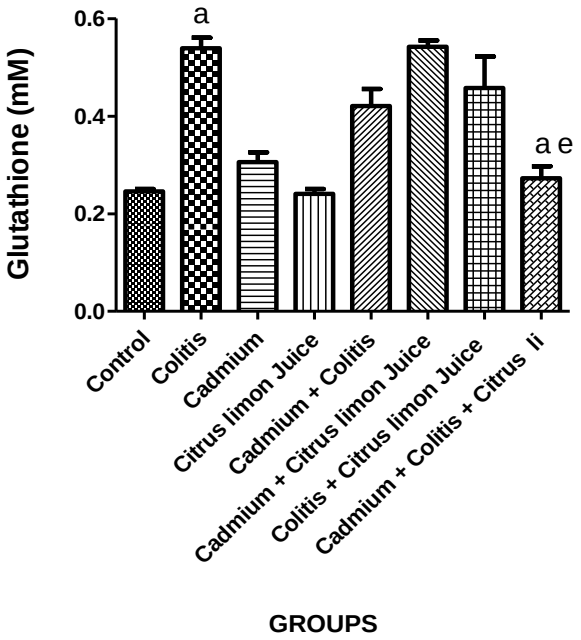


Fig. 2c. Effect of *Citrus limon* juice on reduced glutathione levelin acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
e - Represents significant difference (P<0.05) when compared with cadmium + colitis group

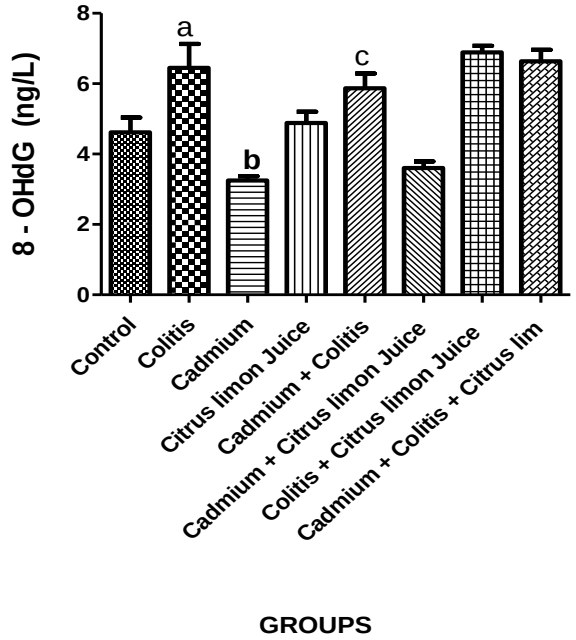


Fig. 3a. Effect of *Citrus limon* juice on 8-hydroxy-2-deoxyguanosinelevel in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
b - Represents significant difference (P<0.05) when compared with colitis group
c - Represents significant difference (P<0.05) when compared with cadmium group

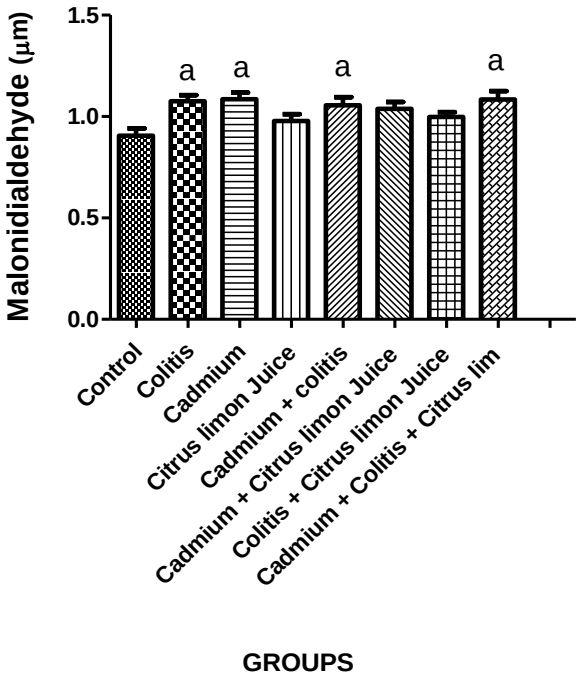


Fig. 3b. Effect of *Citrus limon* juice on malondialdehyde level in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group

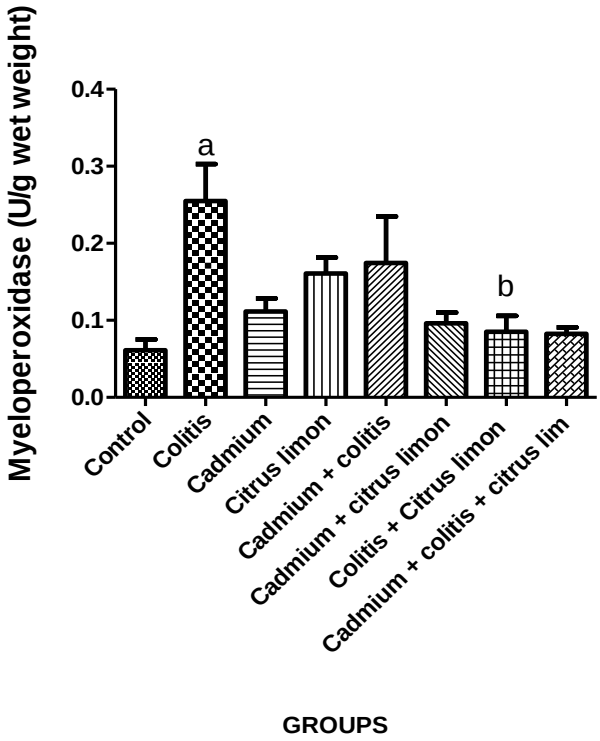


Fig. 3c. Effect of *Citrus limon* juice on myeloperoxidase level in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

a -Represents significant difference (P<0.05) when compared with control group
b - Represents significant difference (P<0.05) when compared with colitis group

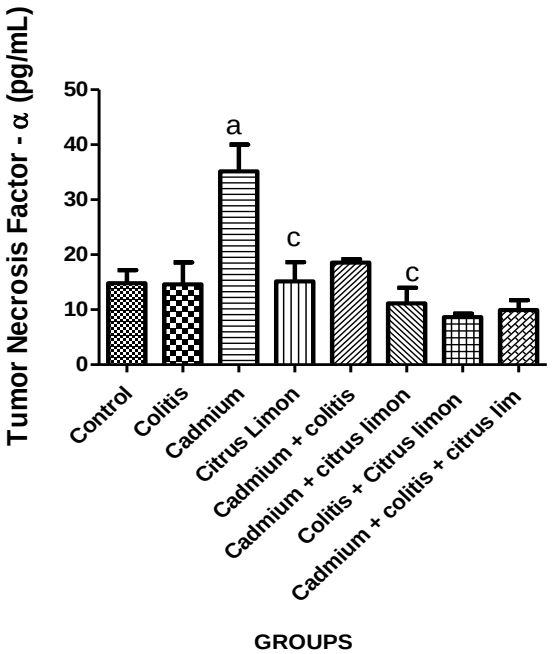


Fig. 4a. Effect of *Citrus limon* juice on tumor necrosis factor - alpha

a -Represents significant difference (P<0.05) from control group.
c - Represents significant difference (P<0.05) from cadmium group

Table 1. Effect of *Citrus limon* juice on Red blood cell (RBC) indices

Red Blood Cell (RBC) Indices	Control	Colitis	Cadmium	*Citrus limon* juice	Cadmium + Colitis	Cadmium + *Citrus limon* Juice	Colitis + *Citrus limon* Juice	Cadmium + Colitis + *Citrus limon* Juice
RBC	7.45 ± 0.10	5.95 ± 0.16[a]	7.09 ± 0.26	7.18 ± 0.01	6.5 ± 0.90	7.06 ± 0.24	6.6 ± 0.35	6.22 ± 0.25
RDW-CV	15.6 ± 0.69	15.8 ± 0.20	14.7 ± 0.86	16.2 ± 0.93	15.5 ± 0.72	14.75 ± 0.42	17.4 ± 0.61	17.2 ± 0.40
RDW-SD	41.1 ± 1.75	41.5 ± 1.27	40.3 ± 1.93	43.7 ± 0.32	39.9 ± 4.51	42.1 ± 2.45	47.2 ± 2.88	48.1 ± 3.28[e]
HCT	47.4 ± 0.96	37.6 ± 1.36[a]	49.5 ± 0.51	47.5 ± 3.11	41.8 ± 5.85	50.5 ± 2.48	44.6 ± 3.53	42 ± 1.19
HGB	14.6 ± 0.38	12.5 ± 0.52[a]	15.5 ± 0.78	13.8 ± 0.52	13.4 ± 1.25	15.2 ± 0.41	13.9 ± 0.98	13.4 ± 0.30

a -Represents significant difference (P<0.05) from control group
e - Represents significant difference (P<0.05) from cadmium + colitis group

Table 2. Effect of *Citrus limon* juice on white blood cell (WBC) indices

White Blood Cell (WBC) Indices	Control	Colitis	Cadmium	*Citrus limon*	Cadmium + Colitis	Cadmium + *Citrus limon* Juice	Colitis + *Citrus limon* Juice	Cadmium + Colitis + *Citrus limon* Juice
WBC Count	8.73 ± 1.08	5.63 ± 0.54	8.98 ± 1.78	10.7 ± 1.33	8.28 ± 0.78	12.5 ± 2.82	11 ± 1.34[b]	12.2 ± 2.72
Lymphocytes	69.3 ± 2.87	75.9 ± 6.24 [c]	56.3 ± 7.66	69.3 ± 11.3	79.1 ± 3.74 [c]	78.9 ± 2.63	69.3 ± 3.09	70.1 ± 5.85
Monocyte	1.65 ± 0.35	1.97 ± 0.35	1.3 ± 0.2	0.53 ± 0.2 [b]	2.5 ± 0.42	0.77 ± 0.53	0.55 ± 0.25[b]	3.9 ± 0.56
Neutrophil	17.8 ± 6.64	28 ± 4.45	33.8 ± 4.26 [a]	17.8 ± 7.25	24.4 ± 9.48	19.4 ± 3.78	7.66 ± 1.90[b]	15.6 ± 1.99
Basophil	0.53 ± 0.15	1.1 ± 0.1	0.9 ± 0.34	0.6 ± 0.17	1.86 ± 0.46 [a, c]	0.73 ± 0.30	0.56 ± 0.20	1.52 ± 0.43
Eosinophil	1.15 ± 0.96	0.43 ± 0.15	0.37 ± 0.17	1.13 ± 0.40	0.56 ± 0.15	2.25 ± 0.07 [c]	0.7 ± 0.17	1.1 ± 0.26

a -Represents significant difference (P<0.05) from control group.
b - Represents significant difference (P<0.05) from colitis group.
c - Represents significant difference (P<0.05) from cadmium group

Effect of *Citrus limon* juice on pro-inflammatory markers in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats: Colitis group showed no significant difference in TNF-α when compared to control group, however a significant increase was observed in cadmium group when compared to control group. No significant difference was observed in TNF-α level in Cadmium exposed colitis rats when compared to colitis group. However, cadmium + *Citrus limon* juice group showed a significant reduction in TNF-α when compared to cadmium group.

Effect of *Citrus limon* juice on Red blood cell (RBC) indices in acetic acid-induced ulcerative colitis cadmium exposed male Wistar rats: Colitis group showed a significant decrease in decrease red blood cell count, hemoglobin concentration and hematocrit level when compared to the control group while cadmium exposed colitis group showed a non-significant decrease when compared to colitis group. However, colitis + *Citrus limon* juice group and cadmium + colitis + *Citrus limon* showed no significant difference when compared to colitis and cadmium + colitis group.

Effect of *Citrus limon* juice on white blood cell (WBC) indices in acetic acid induced ulcerative colitis of cadmium exposed male Wistar rats: Colitis group showed a significant increase in lymphocyte when compared to cadmium group, while rats exposed to cadmium showed a significant increase in neutrophils when compared to control group. Cadmium exposed colitis rats showed a significant increase in lymphocytes and basophil level when compared to cadmium group. However, treatment of colitis rats with *Citrus limon* juice showed a significant increase in neutrophil, white blood cell count, and monocyte.

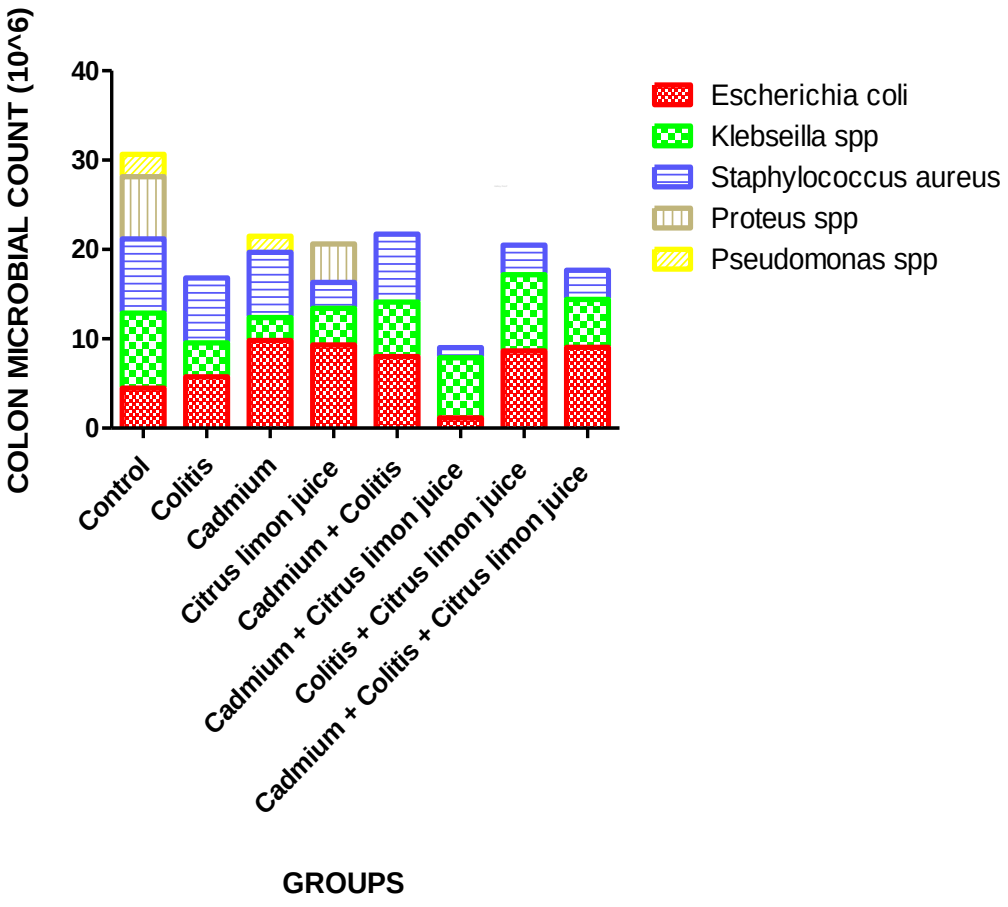


Fig. 5a. Effect of *Citrus limon* juice on colon microbial count in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

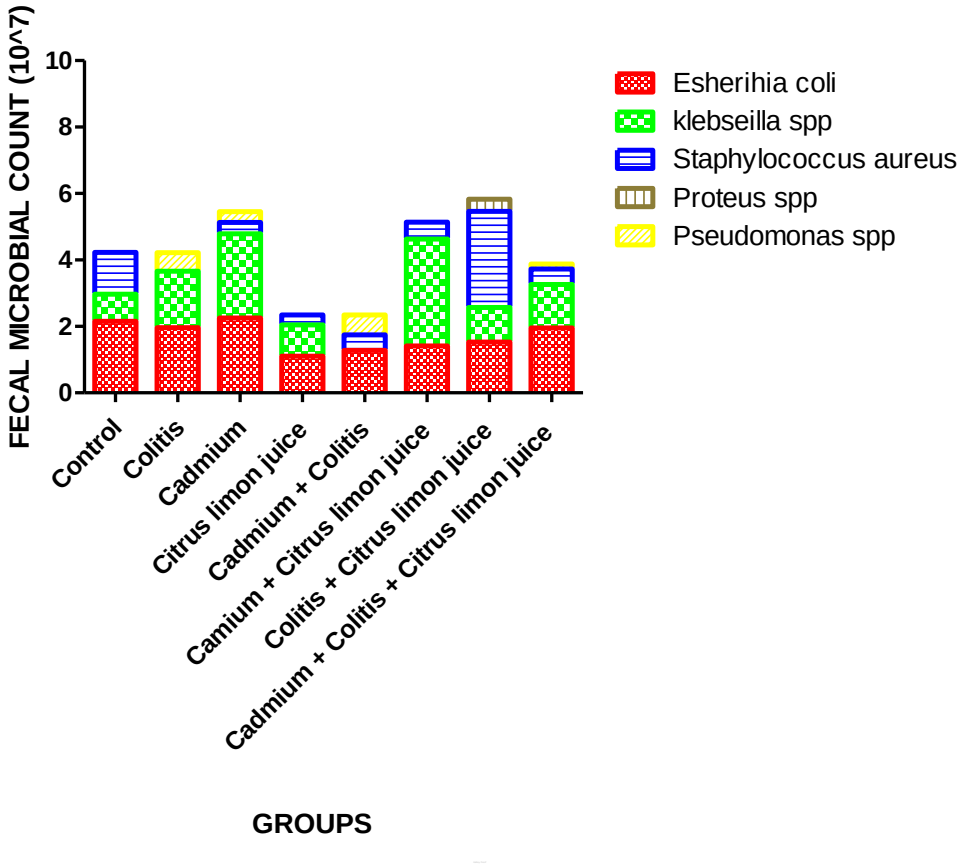
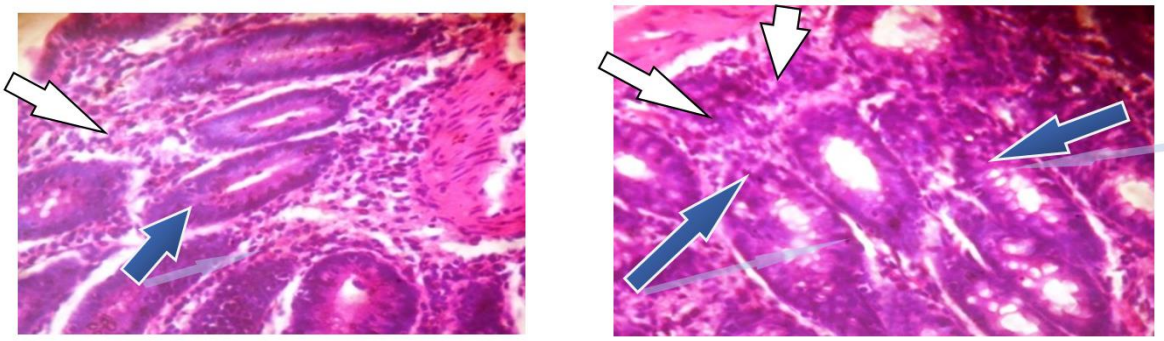


Fig. 5b. Effect of *Citrus limon* juice on fecal microbial count in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats

Effect of *Citrus limon* juice on colon and fecal microbial count in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats: Cadmium exposed colitis rats showed an increase in colonic *E. coli*, *Staphylococcus aureus* and a decrease in *Klebsiella spp*. However, their treatment of colitis with *Citrus limon* juice showed an increase in *Klebsiella spp* and a decrease in *Staphylococcus aureus* population. Cadmium exposed colitis rats showed an increase in fecal *Pseudomonas sp*. However, their treatment with *Citrus limon* juice showed a reduction in fecal *Pseudomonas spp.*

Effect of *Citrus limon* juice on colon histology in acetic acid-induced ulcerative colitis of cadmium exposed male Wistar rats:



Control **Colitis**

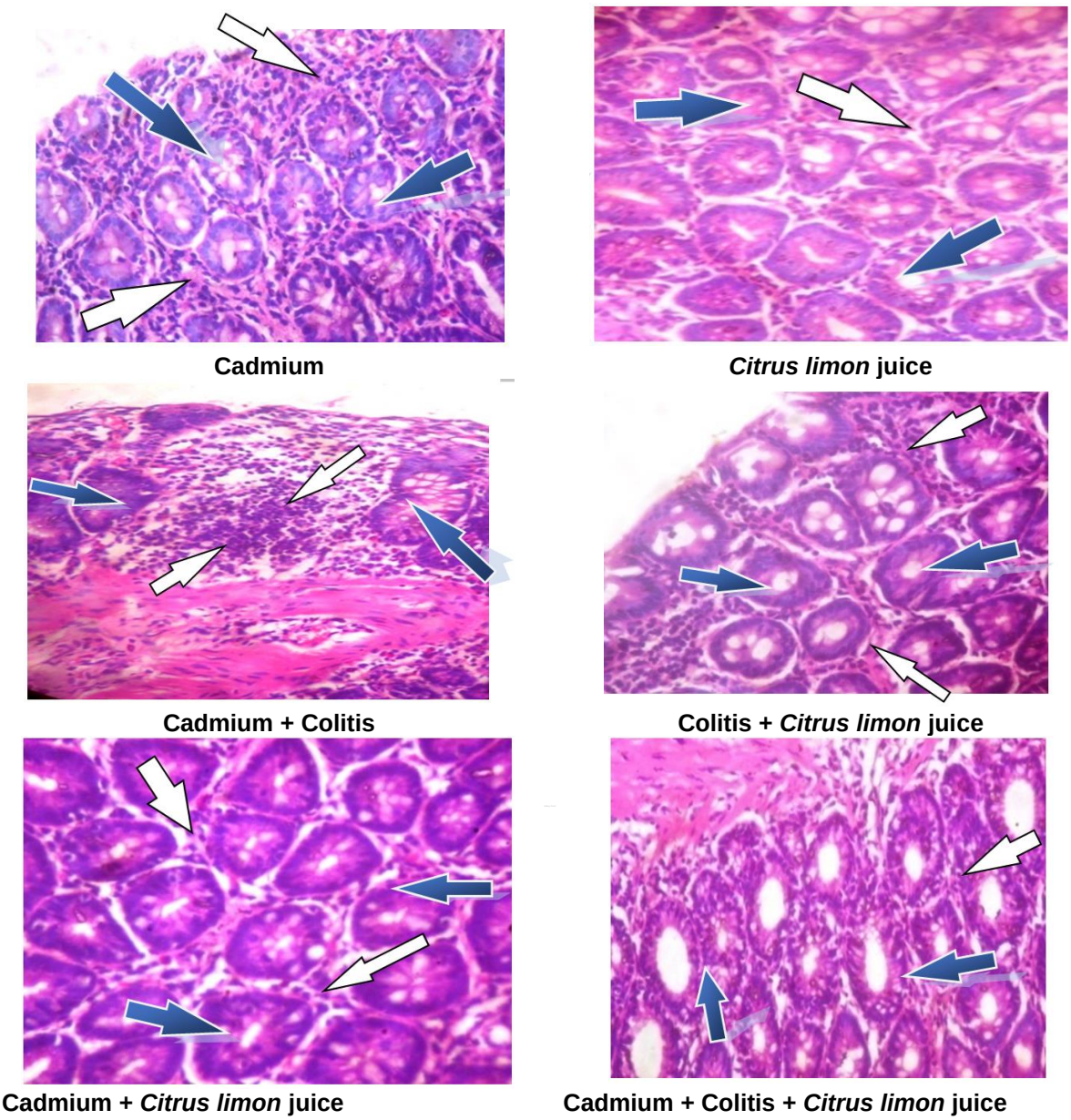


Fig. 6. Photomicrograph showing the effect of *Citrus limon* juice on acetic acid-induced ulcerative colitis in cadmium exposed male Wistar rats

Colon epithelial layer

Inflammatory cells infiltration of the lamina propria

4. DISCUSSION

Cadmium has been implicated in the delayed healing of ulcerative colitis through the excessive production of reactive oxygen species, depletion of antioxidants, and the loss of mucosa integrity (Jiang et al., 2020; Adegoke et al., 2017). This present study investigated the effect of *Citrus limon* juice on acetic acid-induced ulcerative colitis in cadmium chloride exposed male Wistar rats.

The findings of this study showed that colitis rats exposed to cadmium showed a significant decrease in body weight, increase in colon weight and colonosomatic index, while treatment with *Citrus limon* juice resulted in further weight loss. In agreement with previous studies, this observation is believed to be the result of mal-absorption, reduced food intake, loss of fluid through diarrhea and rectal bleeding followed by an increased lipid and protein degeneration by

cadmium (Adegoke et al., 2017; Ansari et al., 2021).

Citrus limon juice has been reported to have lipid-lowering activity (Lee et al., 2018). Consumption of its fruits or juices appears to correlate with ameliorated blood lipid profiles, survival in the elderly, lower risk of cancers, decreased blood pressure, diminished risk of heart maladies' occurrence, and treatment of obesity (Ramful-Baboolall et al., 2014).

The findings of this study showed that treatment of colitis rats and cadmium exposed colitis rats with *Citrus limon* juice showed a reduction in weight. The reduction according to previous studies, has been attributed to the role of *Citrus limon* in regulating lipid metabolism while inhibiting lipid accumulation around the abdominal organs (Lee et al., 2018; Wu et al., 2021).

Further findings in this study revealed that colitis rats showed a significant increase in glutathione level compared to control rats, and cadmium exposed colitis rats showed a significant increase in superoxide dismutase when compared to control and colitis rats. These antioxidant defense enzymes are the major antioxidants present to combat free radicals produced through oxidative stress, following a problem related to a disease, (Tavazzi et al., 2001). The increase level of superoxide dismutase observed in cadmium exposed rats, according to previous study is associated with a rapid antioxidant defense response to the presence of excessive free radicals generated through the action of cadmium, while higher level of reduced glutathione observed in colitis rats has been suggested to provide additional support to the antioxidant defense system response to free radicals (Rana et al., 2014; Rao et al., 2021).

Citrus limon due to its high flavonoid's compounds such as hesperidin and hesperetin have been reported to have high antioxidant activity that is not only limited to scavenging of free radicals but also plays a major role in augmenting and enhancing the antioxidant cellular defenses (Parhiz et al., 2015).

The findings of this study also demonstrated that treatment of cadmium exposed colitis rats with *Citrus limon* juice resulted in a significant reduction in glutathione level when compared to colitis rats, so also cadmium exposed colitis rats showed a significant increase in superoxide dismutase rats when compared to control and colitis however treatment with citrus juice caused reduction in SOD when compared with cadmium exposed colitis rats, therefore indicating the restoration of the antioxidant defense system to a near normal level. This result is in agreement with previous studies report on the modulating and regulatory potential of *Citrus limon* juice on the antioxidant defense system response to oxidative stress (Jaiswal et al., 2015).

Cadmium exerts its toxic effect as a xenobiotics by inhibiting the activity of complex II, and complex III of the electron transfer chain, the principal site of reactive oxygen species (ROS) production as a result of oxidative stress, resulting in ROS accumulation and the activation of sequence of events such as apoptosis (Genchi et al., 2020; Chatterjee et al., 2011). Oxidative stress biomarkers, are very relevant in the evaluation of disease state and progression (Marrocco et al., 2017).

The findings of this study revealed that colitis rats had a significant increase in MDA, OHdG and MPO when compared to control rats, while cadmium rats showed a significant reduction in OHdG when compared colitis rats. However, cadmium exposed colitis rats showed a significant increase in MDA when compared to control group and a significant increase in OHdG when compared to cadmium group rats. This is in agreement with previous studies that reported a significant increase in MDA, OHdG and MPO as a result of oxidative stress induction via cytotoxic oxygen metabolites stimulation and lipid peroxidation while the increase in MDA in cadmium exposed colitis rats may be suggested to be influenced by damage caused by cadmium mechanism of inflammation (Farjad & Momeni, 2018; Naini et al., 2021; Ahmed et al., 2023;).

Citrus limon juice has been reported to reduce lipid peroxidation and also protect DNA from damage through one of its component vitamin C (Zhao et al., 2022; Klimek-Szczykutowicz et al., 2020). Malondialdehyde is characterized by cross linking cellular macromolecules including proteins and DNA, and induces cellular damage (Zhang et al., 2016).

Findings of this study demonstrated that treatment of colitis rats with *Citrus limon* juice resulted in a significant reduction in MPO. This is in agreement with a previous study report on *Citrus limon* reducing the level of lipid peroxidation (Zhou et al., 2022). However,

treatment of cadmium exposed colitis rats with *Citrus limon* juice showed no significant difference in, OHdG and MDA when compared to control rats.

Cadmium has been reported to upregulate inflammatory cytokines in the colon (Jiang et al., 2020; Kaur & Goggolidou, 2020). It increases the level of inflammation in colitis rats through increased neutrophil/lymphocyte ratio thereby delaying the healing of colitis (Adegoke et al., 2017). It releases into the intestinal mucosa has been associated with intestinal epithelial barrier disruption (Barbara et al., 2021).

Findings of this study showed that rats induced with colitis showed no significant difference in TNF-α when compared to control rats, and this is been attributed to the natural onset of healing beginning few days after colitis induction (Jiang et al., 2020). However, a significant increase in TNF-α level was observed in cadmium exposed rats.

Citrus limon exhibit anti-inflammatory effects due to its high concentration of D-limonene, which play a major role in reducing cell migration, cytokine production (Amorim et al., 2016). Findings of this study revealed that, treatment of cadmium exposed rats with *Citrus limon* juice showed a significant reduction in TNF-α level when compared to cadmium rats. This is in agreement with a previous report on the regulatory activity of *Citrus limon* on TNF-α in human colon by some of its components; nomilin and D- limonene (Amorim et al., 2016; Zhou et al., 2022).

Cadmium has similar chemical composition to essential metals, such as iron, zinc, and calcium and can be taken up cells through ionic and molecular mimicry (Nair et al., 2013). It binds to the same protein in the blood and tissues such as albumin and metallothionein, and compete for uptake in the cells (Schaefer et al., 2020) Blood plays a major role in the transport of oxygen and nutrients (Azzouz & Sharma, 2018). Its binding to metallothionein has been reported to cause oxidative stress, bone metabolism disturbance through the displacement of calcium, reduce blood flow and nutrients uptake inhibition (Schaefer et al., 2020).

Findings from this study revealed that rats induced with colitis showed a significant reduction in red blood cell, hemoglobin and hematocrit when compared to control rats. Also, the exposure of colitis rats to cadmium didn't cause any significant changes in red blood cell, hemoglobin and hematocrit concentration when compared to colitis or control group. Therefore, it is believed that the reduction in red blood cell, hemoglobin and hematocrit are the consequences of blood loss from the gastrointestinal tract (Antunes et al., 2015).

Citrus limon has been suggested as a prevention of cardiovascular diseases, and may play a major role on hematological parameters due to its components such as vitamin c, flavonoids, iron and pyridoxine (Riaz et al., 2014). It was reported to cause a significant increase in red blood cell and hemoglobin concentration (Riaz et al., 2014). However, findings of this study showed that treatment of colitis rats, cadmium exposed rats and cadmium exposed colitis rats with *Citrus limon* juice didn't result in the restoration of the red blood cell count, hematocrit and hemoglobin concentration.

One of the ways the immune system provides defense against foreign particles and pathogenic organisms is through inflammation (Miles & Calder, 2021). Uncontrolled or excessive inflammation would cause damage to the host tissues damage and a resulting pathology (Miles & Calder, 2021). Cadmium has been reported to increase inflammation in rats during colitis through increased neutrophil or lymphocyte ratio (Adegoke et al., 2017.

The findings of this study showed a significant increase in lymphocytes in colitis group when compared to cadmium group and a significant increase in neutrophil in cadmium group when compared to control group. However, exposure of colitis rats to cadmium resulted in a significant increase in lymphocyte and basophil when compared to cadmium group and a significant increase in basophil when compared to control group. This, according to a previous study suggest a functional immune defense system rapid response to cadmium toxicity and severity of inflammation (Chapuy et al., 2014).

Citrus fruits exert their role in maintaining the integrity of the immunological barriers, and enhancing the functions of the immune cells such as natural killer cells, T-lymphocytes and B-cells through their components such as vitamin C and folate (Miles & Calder, 2021).

The findings of this study also demonstrated that treatment of colitis rats with *Citrus limon* juice

caused a significant decrease in neutrophil and monocyte level while an increase was observed in white blood cell count. The reduction in neutrophil and monocyte can be attributed to the flavonoid component of *Citrus limon*, inhibiting monocyte inflammation and neutrophil generation of superoxide radicals (Zielińska-Przyjemska & Ignatowicz, 2008; Ávila et al., 2021).

Thousands of bacteria species dwelling in the colon, in a state of "healthy" homeostasis, help with digestion, metabolism, and immune modulation (Wexler & Goodman, 2017; Amon & Sanderson, 2017). Disruption of this state, called dysbiosis, is believed to be associated with various consequences such as ulcerative colitis (Bidell et al., 2022). Exposure to cadmium induces a significant alteration in bacterial populations and their relative abundance in gut, accompanied by increased lipopolysaccharide (LPS) production, reflecting changed metabolic activity of the intestinal microbiome (Kim et al., 2015; Tinkov et al., 2018).

Findings of this study revealed an increase population of colonic *E. coli* and colonic *Staphylococcus aureus* and fecal *Pseudomonas spp* in cadmium exposed colitis rats relative to control and colitis group. This is in agreement with report from previous studies on increase population *of E. coli and Pseudomonas spp* due to indirect (immuno-compromised host) cadmium stimulation of these microbes to produce virulence factors that triggers gene expression, inflammatory response and intestinal barrier damage (Luciardi et al., 2021). *Pseudomonas spp* increased population has also been attributed to its versatile and high tolerance for cadmium (Hoff et al., 2020).

Extracts from *Citrus limon* fruits have been reported to have inhibitory and antibacterial activity against *Staphylococcus mutans*, Staphylococcus capitis, Pseudomonas fluorescens and Escherichia coli (d'Alessio et al., 2013; Otang & Afolayan, 2016).

Treatment of cadmium exposed colitis rats with *Citrus limon* juice resulted in an increase colonic *Klebsiella spp* and a decrease colonic *Staphylococcus aureus* and fecal Pseudomonas *spp*. This agrees with previous studies on *Citrus limon* effect in maintaining a healthy balance microbial population, by increasing colonic *Klebsiella spp* population which play a major role in preventing inflammation through *E. coli* clearance, while at the same time preventing colonic *Staphylococcus aureus* colonization through one of its component citric acid (Piewngam & Otto, 2024; Sost et al., 2021; Cabral et al., 2023).

Histological examination revealed that induction of colitis caused moderate infiltration of inflammatory cells and preservation of epithelial layer while their exposure to cadmium resulted in a severe destruction of epithelial layer and infiltration of inflammatory cells. However, their treatment with *citrus limon* juice showed a moderate preservation of epithelial layer and infiltration of inflammatory cells. According to previous studies, the moderate and severe epithelial layer destruction and inflammatory cells infiltration is suggested to be associated with T-cell abnormalities and increased macrophage inflammatory protein-2 expression by cadmium (Kaur & Goggolidou, 2020; Hameed et al., 2015). The preservation of the epithelial layer and the prevention of inflammatory cells infiltration by can be attributed to the ability of *Citrus limon* to enrich the mucus layer (Samanta et al., 2023).

5. CONCLUSION

Citrus limon juice neutralized the intensification and delayed healing effect of cadmium chloride on ulcerative colitis by modulating and regulating the antioxidant-immune defense system response, while maintaining a healthy microbial population.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

We are grateful to the technical staff of the Department of Physiology, Ladoke Akintola University of Teaching Hospital, Ogbomoso.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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