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The Comparative Evaluation of the Effects of *Hibiscus Sabdariffa* on Inflammatory Cytokines, Duodenal Antioxidants and Histoarchitecture in Wistar Rats

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ABSTRACT

Background: *Hibiscus sabdariffa* (HS) is used as a food flavoring agent and an herbal beverage in different parts of the world. There is a need to ascertain the safety of administration of HS in different body organs. This study investigated the comparative effect of HS in the expression levels of inflammatory cytokines, duodenal antioxidant status, and histopathology in Wistar rats.

Materials and Methods: The study subjects included 30 Wistar rats (15 males and 15 females) with an average weight of 204 g and 147 g, respectively, and were randomly divided into three groups (n=5), A–C for each sex. Group A was given no treatment and served as the normal control group. Groups B and C received oral administration of 200 mg/kg and 400 mg/kg of Methanolic extract of *Hibiscus sabdariffa* (MEHS), respectively. The extract was administered once a day for 21 days using oral cannula. The animals were fasted overnight on the last day of MEHS administration and anesthetized using chloroform. Blood was collected from the animals for the interleukins assay procedure. The animals were sacrificed after blood collection, and the duodenum was harvested for histological processing and oxidative status determination.

KEYWORDS:
antioxidants; cytokines; duodenum; *Hibiscus sabdariffa;* histopathology inflammation; small intestine, Roselle

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Results: The male glutathione level was significantly increased in Group B and decreased in Group C, respectively ($p=0.039$) when compared with that of the control group; while the female duodenum showed no significant difference ($p>0.05$) in the levels of Superoxide Dismutase (SOD), Glutathione (GSH), and Catalase (CAT) across all test groups, when compared with the control. There was no significant difference ($p>0.05$) in the expression of inflammatory cytokines across all treated groups when compared with that of the control. The duodenal histological section of male rats in Groups B and Group C showed signs of inflammation and mucosal erosion.
Conclusions: MEHS demonstrated antioxidative potential at a low dose in male duodenum with attending tissue toxicity, but showed inflammatory and toxic effects at a higher dose in male rats. However, the tissue inflammatory effects of MEHS failed to induce oxidation nor the production of the inflammatory cytokines – interleukin 1α and interleukin 4. MEHS may not be safe for the male duodenum.

INTRODUCTION

Hibiscus sabdariffa (HS) is an herbaceous shrub that belongs to the *Malvaceae* family. It is commonly called Roselle in English, Karkadeh in Arabic,[1] Yakuwa, or Zoborodo in Hausa, Isapa in Yoruba, and Okworo ozo in Igbo language. The plant is used as a flavoring agent in food industries, and its aqueous extract is consumed as an herbal beverage in different parts of the world[2]. HS contains many vital nutrients and active compounds including alkaloids, tannins, saponins, glycosides, phenols, flavonoids, iron, copper, calcium, magnesium, and manganese.[3] Extracts of HS have been shown to lower blood pressure,[4] reduce inflammation,[5] and offer protection against oxidative stress.[6,7] Studies have shown HS to promote the absorption of iron in the duodenum,[8] increase intestinal mass,[9] as well as induce apoptosis in human gastric carcinoma.[10]

Inflammation is a protective mechanism employed by the immune system to combat infectious and potentially harmful agents.[11] During inflammation, activated immune cells secrete inflammatory cytokines which stimulate the production of reactive oxygen species (ROS) and/or antioxidant depletion.[12] While acute inflammation protects the body against invading tissue pathogens, chronic inflammation causes tissue damage mainly by destroying essential cell and tissue macromolecules.[13] In the duodenum, inflammatory-mediated epithelial damage by cytotoxic intraepithelial T-lymphocytes is the central mechanism of celiac disease-induced duodenitis.[14] Furthermore, inflammatory responses and damage have been linked to the pathogenesis of various chronic intestinal diseases including peptic duodenitis, Inflammatory Bowel Disease, and autoimmune enteropathy.[15] The anti-inflammatory and free-scavenging properties of HS have been demonstrated by previous studies.[5,7,16] Polyphenol, an active constituent of HS has been shown to decrease adipokines secretion, ROS generation, and the accumulation of triglyceride in hypertrophic adipocytes.[17] Despite the wide claims of the beneficial effects of HS in different body tissues, it is necessary to understand the oxidative and inflammatory responses in normal tissues following its administration as a way to ascertain its safety or toxicity potential. In this study, the effects of HS on the expression of inflammatory cytokines, duodenal antioxidants, and histoarchitecture were evaluated in Wistar rats.

MATERIALS AND METHODS

STUDY SETTING

This experimental study was carried out in the research laboratory of the Department of Anatomy, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, College of Health Science, Nnewi Campus, Anambra State, Nigeria and lasted for about 3 months.

PLANT COLLECTION, IDENTIFICATION, AND EXTRACTION

The dried aerial part of HS was procured from the local market at Nnewi, Anambra state, Nigeria. The botanical identification and authentication were carried out in the Department of Pharmacognosy and Traditional Medicine, College of Pharmacy, Nnamdi Azikiwe University, Agulu Campus, Anambra State, Nigeria with identification number PCG/1474/A/031. The plant calyces were shade-dried and ground. 1000 g of powdered plant sample was used for methanolic extraction as described by Okafor and colleagues.[18] The filtrate (extract) was then stored in the refrigerator at 4°C. The extract was made up to solution at varying doses per ml on each day of administration and given according to body weight and group treatment doses.

ANIMAL PROCUREMENT, CARE, AND HANDLING

The study subjects included 30 Wistar rats (15 males and 15 females) which were procured from the animal house of College of Health Sciences, Nnamdi Azikiwe University, Okofia, Nnewi Campus, Nigeria and acclimatized for 2 weeks (to exclude any intercurrent infection) under standard housing condition (ventilated room with 12/12-hour light/dark cycle at 24 ± 2°C). The rats were fed ad libitum with water and standard rat chow throughout the experimental period. Animal health status was monitored throughout the experiment according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines.[19]

EXPERIMENTAL DESIGN

The male and female rat groups with an average weight of 204 g and 147 g, respectively, were randomly divided into three groups (n = 5), A–C. Group A was given no treatment and served as the normal control group. Groups B and C received oral administration of 200 mg/kg and 400 mg/kg of Methanolic extract of *Hibiscus sabdariffa* (MEHS), respectively. A solution of 1 g per 20 ml distilled water was constituted with MEPO on each day of administration. The needed concentration for each animal was determined and taken from this stock and the remnant of the constituted extract was discarded after each day of administration. The extract was administered once a day for 21 days using oral cannula. The water consumption and feeding pattern of the rats before and after the administration periods were observed along with the physical and behavioral changes in response to the administration. The resource equation for sample size calculation for animal studies was used to determine the sample size for this study[20] while the extract administration doses were chosen based on cues from a previous study.[6]

ANIMAL SACRIFICE AND SAMPLE COLLECTION

The animals were fasted overnight on the last day of MEHS administration and anesthetized using chloroform. For the interleukins assay procedure, 2-ml of blood each was collected from the animals by ocular puncture using capillary tubes into a plain tube. The animals were sacrificed after blood collection, and the small intestine was harvested, and the duodenal part was excised and divided into two parts. One was fixed in a 10% formal saline for histological processing and analysis. The second part was homogenized and used for oxidative status analysis.

BIOCHEMICAL ANALYSIS OF OXIDATION

Superoxide Dismutase (SOD), Glutathione (GSH), and Catalase (CAT) were quantified in the ovary to determine the oxidative status, using the ovarian tissue homogenate as described in our earlier study.[21]

TISSUE PROCESSING

The tissue samples were trimmed down to a size of about 3 mm x 3 mm thick for an easy study of sections under the microscope and fixed in 10% formalin. After fixation, dehydration of the fixed tissues was done in ascending grades of alcohol 50%, 70%, 95%, and 100%, and cleared in xylene. Staining was done with hematoxylin and eosin (H&E) and mounted using Dibutylphthalate Polystyrene Xylene (DPX), after which, the sections were viewed under the light microscope. Photomicrographs of these sections were obtained using the Leica DM 750 digital microscope computer software.

ASSAY PROCEDURE FOR INFLAMMATORY CYTOKINES

Two inflammatory cytokines were assessed in this study: Interleukin 1α and Interleukin 4. The blood was allowed to clot for 10–20 min at room temperature and centrifuged at 2000–3000 rpm for 20 min; 40μl of sample was added to sample wells and then 10μl anti-pro-IL1α antibody (for Interleukin 1α assay) or anti-STX1A antibody (for Interleukin 4 assay) to sample wells, then 50μl streptavidin-HRP was added to sample wells and standard wells (not blank control well) and mixed well. The plate was covered with a sealer and incubated for 60 min at 37°C. The sealer was removed and the plate was washed five times with wash buffer. Further, 50μl of substrate solution A was added to each well and then 50μl of substrate solution B to each well. Plate covered with a new sealer was incubated for 10 min at 37°C in the dark. 50μl of stop solution was added to each well, the blue color changed into yellow immediately. The optical density (OD value) of each well was determined immediately using a microplate reader set to 450 nm within 10 min after adding the stop solution. All the reagents were brought to room temperature before use.

STATISTICAL ANALYSIS

The data were analyzed using IBM statistical package for social science (SPSS) for Windows, version 23 (IBM Corporation, Armonk, New York, USA). One-way analysis of variance (ANOVA) was used to test for significance in changes seen in the variables across groups. Tables and figures were used for the representation of data, and values were considered significant at $p < 0.05$. All the animals in each group were included in the analysis.

RESULTS

THE EFFECT OF MEHS ON THE ANTIOXIDANT LEVELS IN THE DUODENUM

In the male duodenum, GSH level was significantly increased ($p < 0.05$) in Group B, but showed a significant decrease ($p < 0.05$) in Group C, when both were compared to that of the control group (Group A), unlike the female duodenal antioxidants which remained unchanged compared with that of the control. SOD and CAT showed no significant changes ($p > 0.05$) in Groups B and C when compared with that of Group A for both male and female rat groups (Table 1).

THE EFFECT OF MEHS ON INFLAMMATORY CYTOKINES

No significant change was observed in the serum inflammatory cytokines across all treated groups when compared with that of the control ($p > 0.05$; Table 2).

THE HISTOPATHOLOGICAL EFFECT OF MEHS IN THE DUODENUM OF MALE AND FEMALE WISTAR RATS

Plates 1–6 (Figure 1) show the histological sections of male and female rat duodenum at different doses of MEHS. Plates 1–3 represent sections of the male rats, while Plates 4–6 represent sections of the female rats. Plate 1 (Group A) photomicrography shows a viable muscularis propria wall with mucosal lining cells. Plate 2 (Group B) photomicrography shows the infiltrate

Table 1 The effect of Hibiscus sabdariffa (HS) on the antioxidant levels in the duodenum of the small intestine

Antioxidants	Groups	Male rats (Mean ± SD)	p	Female rats (Mean ± SD)	p
SOD	A	13.38±1.45	0.728	8.43±3.99	0.135
	B	12.52±1.81		19.05±0.21	
	C	10.60±5.37		10.60±5.37	
GSH	A	11.70±0.76	0.039*	34.08±8.22	0.470
	B	18.94±0.98		7.08±7.49	
	C	8.59±3.63		8.59±3.63	
CAT	A	82.06±6.62	0.132	58.08±22.39	0.890
	B	67.66±6.75		61.89±3107	
	C	77.49±8.86		47.66±35.03	

Data were analyzed using One-way Analysis of Variance (ANOVA). Values were expressed as mean ± Standard deviation, and data were considered significant at *p < 0.05. SD: Standard deviation; SOD: Superoxide Dismutase; GSH: Glutathione; CAT: Catalase.

Table 2 The effect of Hibiscus sabdariffa (HS) on the interleukins 1α and 4 in male and female Wistar rats

Inflammatory cytokines	Groups	Male rats (Mean ± SD)	p	Female rats (Mean ± SD)	p
Interleukin 1α	A	18.50±3.79	0.263	18.50±3.79	0.263
	B	14.50±0.58		14.50±0.58	
	C	19.25±5.97		19.25±5.97	
Interleukin 4	A	666.75±88.85	0.998	666.75±88.85	0.998
	B	663.75±93.67		663.75±93.67	
	C	668.50±116.80		668.50±116.79	

Data were analyzed using One-way Analysis of Variance (ANOVA). Values were expressed as mean ± Standard deviation, and data were considered significant at *p < 0.05. SD: Standard deviation.

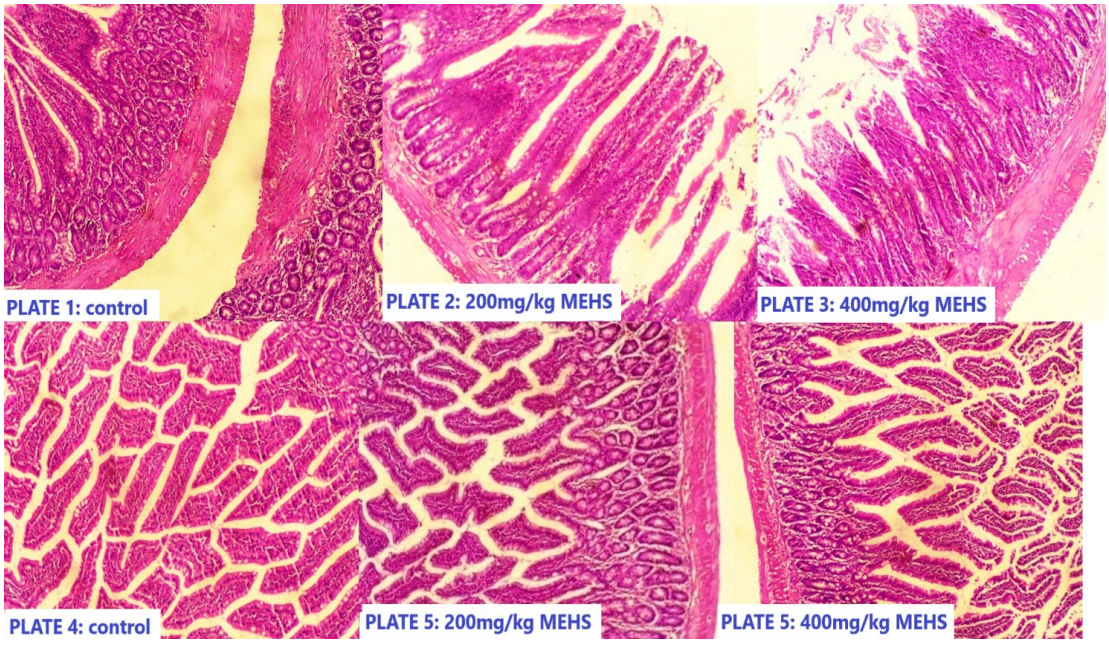

Figure 1 (Plates 1–6): Plates 1–3 show the histological section of the duodenum of a male rat at different doses of MEHS, while Plates 4–6 shows the histological section of the duodenum of a female rat at different doses of MEHS. Plate 1 represents the control and received only distilled water. Plate 2 received 200 mg/kg MEHS, while Plate 3 received 400 mg/kg MEHS. Plate 4 represents the control and received only distilled water. Plate 5 received 200 mg/kg MEHS, while Plate 6 received 400 mg/kg MEHS. All staining was done with H&E and photomicrography was taken at x200.

of inflammatory cells, with signs of mucosal erosion. Plate 3 (Group C) shows mucosal glands displayed on a lamina propria with infiltrates of inflammatory cells. Plate 4 (Group A) photomicrograph shows normal muscularis propria wall and mucosal lining cells. Plate 5 (Group B) photomicrography shows a normal muscularis propria wall and mucosal lining cells, with no sign of abnormality. Plate 6 (Group C) photomicrography shows a viable muscularis propria wall and mucosal lining cells with no sign of abnormality is seen. All staining was done with H&E and photomicrography was taken at x200.

DISCUSSION

Inflammation involves a complex cascade of immune response where immune cells migrate to sites of tissue damage to destroy potentially harmful agents.[11] Although acute inflammation provides rapid protection against endogenous and exogenous antigens, infectious agents, and toxic materials; chronic or persistent inflammation is often destructive.[11,13] Chronic inflammation is characterized by prolonged and pathological tissue infiltration by activated immune cells which ultimately causes tissue destruction.[25] Oxidative stress induction is one of the main mechanisms of cellular and tissue damage during chronic inflammation.[12] Oxidative stress is indicated by a marked increase in highly reactive radicals, and/or depletion of tissue antioxidants, and is associated with the pathogenesis of numerous chronic inflammatory diseases.[13] This present study assessed the effect of MEHS on the expression of inflammatory cytokines, duodenal antioxidants, and the duodenal histopathology of male and female adult Wistar rats.

MEHS demonstrated a limited antioxidative potentials in the male duodenum. Male rats treated with 200 mg/kg MEHS showed a significant increase ($p=0.039$) in the duodenal glutathione (GSH) level when compared with that of the control, although SOD and CAT levels showed no significant changes (Table 1). GSH, an endogenous, non-enzymatic antioxidant involved in the detoxification and neutralization of reactive radicals, is a very important and versatile antioxidant in the body;[26] the observed GSH increase in this study indicates an improved tissue antioxidant capacity by MEHS. Previous studies have reported the antioxidative and free-scavenging effects of HS;[7,16,27] the result from this present study is in alignment with these previous findings. High-dose of MEHS demonstrated signs of toxicity in the male duodenum, as reflected by the significantly reduced levels of GSH observed in Group C when compared with that of the control ($p=0.039$; Table 1). A decreased tissue GSH level significantly weakens the body antioxidant defense system and exposes the body to oxidative damage.[26] The depleted GSH level in the male duodenum appeared to be due to inflammatory-mediated oxidative tissue damage as histopathological findings showed intense infiltrates of inflammatory cells, with signs of mucosal erosion in the duodenum of rats treated with 200 and 400 mg/kg MEHS when compared with that of the control (Figure 1). It is important to note that despite the GSH level increase caused by 200 mg/kg MEHS in male rats, it did not translate to the safety of MEHS in the male duodenum as shown by the presence of inflammatory cells (Figure 1). This may be the hint that there may be another mechanism of MEHS-induced duodenal inflammatory response other than tissue oxidation. However, female rats treated with 200 mg/kg and 400 mg/kg MEHS showed no significant change in the duodenal GSH, SOD, and CAT levels when compared with that of the control (Table 1) as well maintained the normal histoarchitecture in the duodenum.

Inflammatory cytokines – small proteins released by activated immune cells during inflammation – are key drivers of the inflammatory processes.[28] Acute inflammation as seen in the duodenum of male rats treated with MEHS is associated with increased production of inflammatory cytokines which modulate the immune response and facilitate the process of tissue healing after damage.[29] Interestingly, however, despite the observed inflammatory cell infiltration and consequent mucosal erosion in the male duodenum, no significant changes were seen in the expression level of the evaluated inflammatory cytokines – interleukin 1α and interleukin 4, when the MEHS-treated groups were compared with that of the control (Table 2). This outcome is quite unexpected and could be explained by the possibility of an immune cell induction of other pro-inflammatory cytokines different from those evaluated in this present study.

In summary, MEHS increased the level of tissue GSH but also caused histological damage at a dose of 200 mg/kg. At a higher dose of 400 mg/kg, MEHS caused a significant reduction in the level of GSH and induced duodenal toxicity. Despite the observed alteration in the duodenal GSH and tissue damage, different doses of MEHS did not cause any change or effect in the expression of the inflammatory cytokines – interleukin 1α and interleukin 4. It should be noted that the observed effects of MEHS were seen only in the male rats, and not in the female rats, as MEHS did not cause any significant alteration in all the evaluated parameters of the female rats when compared with that of the control. The underlying reason or mechanism for this selective effect of MEHS in the male duodenal GSH level and histology, but not in the female is currently unclear, and beyond the scope of this present study. It is not clear whether these findings could be translated to other primates or human biology, as more specific and chronic study may be needed to get a clear understanding of the trajectory of the claims posited by the outcomes. However, the evidence submitted in this study could stand as a preliminary evidence to the potentials for other related primates, including humans.

CONCLUSIONS

MEHS demonstrated antioxidative potential at a low dose but showed inflammatory and toxic effects at a higher dose in male rats. However, the duodenal inflammatory effects of MEHS failed to induce the production of the inflammatory cytokines – interleukin 1α and interleukin 4. MEHS appears to be safe in the duodenum of female rats than in male rats. Further

studies need to be carried out to understand the inflammatory mechanism of MEHS-induced toxicity, as well as isolate active compounds that could be responsible for such effects.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICAL STATEMENT

This study was approved by the Research Ethics Committee of Anatomy Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nigeria. The experimental procedures of this study complied with ARRIVE guidelines,[22] National Institutes of Health (NIH) guidelines,[23] and National Health Research Ethics Committee of Nigeria (NHREC) guidelines for the care and use of laboratory animals.[24] Animal health status was monitored throughout the experiment according to the federation of European Laboratory Animal Science Associations (FELASA) guidelines.[19] No informed consent was required for this study.

CONSENT FOR PUBLICATION

N/A

AVAILABILITY OF DATA AND MATERIAL

The data used for this study can be accessed upon written request to the corresponding author.

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The authors received no funding for this study.

AUTHORS' CONTRIBUTION

IAO, CEC, IFE, and IGI conceived the study. CEC, IFE, and IGI executed the study and it was supervised by IAO. USO, JON, AND IAO performed the laboratory analysis, while SA and IAO did the statistical analysis and wrote the first draft of the manuscript. All the authors read and approved the final draft of the manuscript.

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