Original Article

Comparative Histological Study on the Protective and Therapeutic Role of Açai Berry Extract on Experimentally Induced Fundic Gastric Mucosal Injury in Adult Male Albino Rat

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ABSTRACT

Introduction: Gastric injury is considered a common global medical problem with different etiology. Açai berry, a fruit rich in antioxidant and anti-inflammatory compounds, is lately considered a promising protective and therapeutic agent.

Aim of the Work: To assess the probable protective and therapeutic effects of açai berry extract on gastric fundic mucosal injury, experimentally induced by diclofenac sodium.

Materials and Methods: 50 adult male albino rats were equally distributed into 5 groups; I (Control), II (Açai berry extract) that received açai extract orally (300 mg/kg) once per day for consecutive 14 days, III (Gastric injury) which received one dose of diclofenac sodium (100 mg/kg) orally, IV (Protected) that received açai extract for consecutive 14 days then one dose of diclofenac sodium and V (Treated) that was given diclofenac sodium once then given açai extract for consecutive 14 days. The fundic specimens were examined by light microscopy (H&E, PAS, caspase-3, PCNA) and scanning electron microscopy (SEM).

Results: Group III showed sloughed mucosal surface cells, areas of mucosal discontinuity, cytoplasmic vacuolation, inflammatory cellular infiltration and congested blood vessels. Absent PAS-reactivity and strong caspase-3 immunoreaction together with decreased PCNA immunoreaction were also reported. SEM of group III revealed honeycomb appearance with loss of some surface mucosal cells and cavitation of other surface cells. Wide gastric pits and deep craters were also observed. Group IV exhibited preserved normal histological structure of the fundic mucosa. Group V revealed marked improvement of the histological changes reported in group III.

Conclusion: Açai berry extract had beneficial effects in protection against and treatment of fundic gastric injury, however, these effects were more prominent in the protected than the treated groups.

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Key Words: Açai berry, caspase-3, gastric mucosa, PCNA, scanning electron microscopy.

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INTRODUCTION

Gastric injury is the most widespread disease of the digestive tract all over the world. It is an inflammatory condition of the gastric mucosa that could eventually result in gastric ulcer[1]. The latter is caused by suppression of the gastric defensive mechanisms and exacerbation of the aggressive factors and inflammatory mediators[2]. It could lead to many serious complications e.g. hemorrhage, perforation and pyloric obstruction[3]. Smoking, excessive alcohol intake, stress, Helicobacter pylori infection as well as excessive and/or chronic consumption of non-steroidal anti-inflammatory drugs (NSAIDs) are important contributing causes of gastric injury and ulcer[4].

NSAIDs are commonly utilized analgesics, however, they have serious adverse effects such as cardiotoxicity as well as hepatorenal and gastrointestinal toxicity[5]. Diclofenac sodium (DS) is a commonly used NSAID that was reported to cause serious gastric mucosal injuries[6]. Therefore, it is frequently used as a model for induction of gastric injury in laboratory animals[7].

Açai berry is a dark purple fruit, grown in the Amazon region with many nutritional advantages and potential medical properties. It is regarded as a superfruit as it has strong anti-inflammatory property and the highest antioxidant power among different types of berries[8,9]. Açai berry and its extract contain many bioactive compounds such as unsaturated fatty acids, phenolic acids, flavonoids (quercetin), anthocyanins, carotenoids and hydroxycinnamic acids[10]. Besides, they have high protein, vitamin and mineral content[11]. This content accounts for their numerous beneficial health effects including anti-hypertensive and antidiabetic effects besides their role in body weight reduction[12]. Hence, the aim of the current research was to study the possible protective versus therapeutic roles of açai berry extract on the experimentally

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induced fundic gastric mucosal injury by diclofenac sodium in adult male albino rat.

MATERIALS AND METHODS

Experimental animals

50 adult male albino rats with their weight ranging between 180 to 200 grams were used in this study. They were obtained from the Histology Department Animal House, Tanta Faculty of Medicine. Before stating the experiment, all rats were adapted, for 1 week, to the environment by being subjected to 12h light/dark cycle in well-ventilated cages and served laboratory diet and water. This research followed the safety precautions and requirements of the Research Ethics Committee of Tanta Faculty of Medicine, Egypt (34530/3/21).

Chemicals

Diclofenac sodium was purchased from the market as 50 mg tablets under the trade name "Voltaren", manufactured by Novartis Pharmaceuticals, Cairo, Egypt. Each tablet was crushed and dissolved in 0.5 ml distilled water, so that each 1 ml of the solution contained 100 mg of DS.

Açai berry extract was purchased from Natrol company, California, USA in the form of 600 mg pure powder gelatinous capsules. The content of each capsule was added to 2 ml normal saline, each 1ml of the resulting solution contained 300 mg of açai berry extract. The solution was freshly prepared daily.

Experimental design

The 50 rats were distributed into 5 equal groups:

Group I (Control group): was equally subdivided into:

A. Subgroup IA (Negative control): received no treatment.

B. Subgroup IB (Vehicle control): was given 1 ml of normal saline orally by orogastric tube.

Group II (Açai berry extract group): received 300 mg/kg of açai berry extract once per day orally by orogastric tube for 14 consecutive days[13,14].

Group III (Gastric injury group): received 100 mg/kg of diclofenac sodium once orally by orogastric tube to induce gastric injury[15].

Group IV (Açai berry extract protected group): was given açai berry extract for 14 consecutive days in the same dose and route as in group II, then received diclofenac sodium once.

Group V (Açai berry extract treated group): was given diclofenac sodium as in group III. One hour later, the rats were given açai berry extract in the same dose and route for 14 consecutive days as in group II.

Animal specimens

Finally, the rats received sodium phenobarbital via intraperitoneal injection (60 mg/kg) as anesthesia[16] then were sacrificed. From each rat, the stomach was extracted, opened along its greater curvature and washed in phosphate buffer saline (PBS). The fundic specimen was obtained and split into two parts; one part was fixed for 24 hours in 10% formal saline then processed for light microscopic studies. The other part was preserved in PB glutaraldehyde for 24 hours then processed for scanning electron microscopic examination.

Light microscopic studies

After fixation, dehydration by ascending alcohol grades and clearing by xylol were performed. Then, impregnation in soft paraffin and embedding in hard paraffin were done to form paraffin blocks. Finally, 5 µm-thick fundic sections were sliced[17], stained, examined using Olympus light microscope (Tokyo, Japan) and photographed using Olympus digital camera (DXC1850P, Tokyo, Japan) connected to the microscope at Histology Department, Tanta Faculty of Medicine.

Histological study

1- Hematoxylin and eosin stain (H&E)[17]

It was performed for evaluation of the normal histological features of fundic mucosa.

2- Periodic Acid Schiff (PAS) reagent[18]

It was done to reveal mucin distribution. PAS positive materials appeared deep red to magenta red in color.

Immunohistochemical study

The sections were deparaffinized, rehydrated and soaked in 0.3% H_2o_2 in methanol to prevent staining of the background. Heat-induced antigen recovery was then done. After washing in PBS, the slides were treated overnight with the primary antibodies, washed in PBS. The slides were then treated with the biotinylated goat secondary antibodies followed by the peroxidase-labelled streptavidin-biotin complex, each for 30 minutes. Finally, diaminobenzidine solution was applied for reaction visualization followed by hematoxylin counterstaining. Negative control sections were produced by same methodology without addition of the primary antibody[17,19].

1- Detection of Caspase-3

Caspase-3 is a pro-apoptotic protein expressed in both nuclei and cytoplasm and considered an indicator for apoptosis[20]. A polyclonal rabbit anti-caspase-3 antibody (Lab Vision Corporation, USA) was used as the primary antibody. Positive control is human tonsil. The positive reaction appeared as brown color[21].

2- Detection of proliferating cell nuclear antigen (PCNA)

PCNA is a nuclear marker that indicates cellular proliferation[22]. A mouse monoclonal anti-PCNA antibody (MS-106-R7 obtained from Lab Vision Company) was used as the primary antibody. Positive control is rat spleen. The proliferating nuclei appeared brown in color, whereas the non-proliferating nuclei appeared basophilic[22].

Scanning electron microscopic study

After fixation, the specimens were post fixed by being placed in PB osmium tetroxide for 2 hours. The specimens were then rinsed twice in PB for 15 minutes each and dehydrated using graded ethanol series then dried using Co2 critical point drier. Finally, the sections were mounted on aluminum stubs and gold-coated using gold sputter coater[23,24] to be examined and photographed with a scanning electron microscopy (JSM- 5500 LV; JEOL Ltd-Japan) at Electron Microscopic Unit of Tanta Faculty of Medicine.

Morphometric study

Ten random, distinct non-overlapping fields from each slide of the 5 groups were analyzed by the image-J analysis program (1.46 version software program, National Institute of Health, USA) for measurement of:

1. Mean thickness of the gastric fundic mucosa, in microns, in H&E stained sections at (x200).
2. Mean PAS color intensity in PAS-stained sections at (x400).
3. Mean caspase-3 color intensity in caspase-3 immuno-stained sections at (x400).
4. Mean number of PCNA-positive cells in PCNA immuno-stained sections at (x400).

Statistical analysis

Analysis of the morphometric study results was done via SPSS software version 13 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Tukey's test were performed to compare various groups with group I. The tests' outcomes were presented as mean ± standard deviation (SD) and referred to as significant if the probability value (*P*) measured <0.05 and highly significant if measured <0.001 and non-significant if measured >0.05[25].

RESULTS

All animals survived throughout the experiment. As regards light and scanning electron microscopic as well as morphometric studies of both subgroups of the control group (IA and IB), their results were similar with no statistical difference. Therefore, collectively, both subgroups were referred to as group I (control group) in the text as well as the tables and the figures. Meanwhile, group II (açai berry extract group) exhibited similar histological and immunohistochemical results, as well as statistically non-significant difference in its morphometric results, compared to group I.

Light microscopic results

Hematoxylin and eosin stained sections

Group I (Control group): Group I exhibited the normal histological features of gastric fundic mucosa that appeared thick and made up of surface epithelium, lamina propria (LP), and muscularis mucosa (MM), which separated it from the underlying submucosa. Many closely packed fundic glands were seen filling the lamina propria, at right angle to the surface and opening into it through narrow pits. These glands were made up of three parts; neck, body and base (Figure 1a). The mucosal surface was lined by tall columnar surface mucous cells showing oval elongated nuclei. The glands' neck contained low columnar mucous neck cells with flattened basally located nuclei and pale acidophilic cytoplasm (Figure 1b). Parietal cells, that appeared large with spherical centrally located nuclei and deeply acidophilic cytoplasm, were obvious in the body region. The basal region of the glands contained chief cells that appeared basophilic with basal spherical nuclei (Figure 1c).

Group III (Gastric injury group): This group showed areas of discontinuity taking the whole thickness of the fundic glands and reaching down to MM (Figure 2a). Apparent marked decrease in the fundic mucosal thickness together with exfoliation of some surface and glandular cells were reported in some sections (Figure 2b).

Some mucous neck cells exhibited deeply stained cytoplasm and pyknotic nuclei. Some parietal cells showed vacuolated cytoplasm, while some other parietal cells exhibited lightly acidophilic cytoplasm (Figure 2c). Destruction of the neck region of some glands with separation of their lining cells as well as presence of deeply basophilic chief cells with pyknotic nuclei were observed. Dilated congested blood capillaries in the LP and vacuolated smooth muscle cells of the MM were also noticed (Figure 2d). Some sections showed dilated gastric pits together with marked infiltration of the LP with mononuclear inflammatory cells. Focal discontinuity of the muscularis mucosa with its invasion by inflammatory cells and dilated congested blood vessels from the submucosa was noticed (Figure 2e).

Group IV (Açai berry extract protected group): Fundic mucosa of this group revealed apparent preservation of its normal histological features and thickness. The fundic glands were regular and perpendicularly arranged to the surface with narrow pits (Figure 3a). However, some sections revealed focal areas of minimal sloughing of the surface cells and the superficial parts of some fundic glands together with mild infiltration of the LP with mononuclear inflammatory cells (Figure 3b).

Group V (Açai berry extract treated group): Fundic mucosa of this group showed improvement of the histological changes reported in the gastric injury group III with restoration of its normal thickness and architecture (Figure 4a). However, in some sections, the fundic mucosa was still not completely recovered and revealed focal sloughing of some surface cells and the superficial parts of some glands. Some mucous neck cells appeared vacuolated. Besides, some cells lining the glands' body showed vacuolation of their cytoplasm (Figure 4b). In addition, cystic dilatation of few fundic glands were

observed in few sections (Figure 4c).

Periodic acid Schiff (PAS) stained sections

Group I (Control group): This group revealed strong PAS reaction that appeared as a thick magenta red layer coating the mucosal surface and extending downward inside the pits and the upper glandular region (Figure 5a).

Group III (Gastric injury group): Most sections of this group revealed absent PAS reactivity (Figure 5b). However, focal areas of PAS positive reactivity extending into the gastric pits were detected in some sections (Figure 5c).

Group IV (Açai berry extract protected group): This group revealed strong reaction over the surface and inside the pits and the upper glandular parts (Figure 5d).

Group V (Açai berry extract treated group): This group showed apparently moderate reaction over the mucosal surface and strong reaction inside the pits and the upper glandular parts (Figure 5e).

Immunohistochemical results

Caspase-3 immunostaining

Group I (Control group): Caspase-3 immunostained sections of group I exhibited weak positive brownish cytoplasmic immunoreaction in the glandular neck and basal parts (Figure 6a).

Group III (Gastric injury group): This group exhibited strong positive cytoplasmic and nuclear brownish immunoreaction of caspase-3 in most of the cells lining all glandular parts with the strongest expression in the neck region (Figure 6b).

Group IV (Açai berry extract protected group): This group revealed mild caspase-3 immunoreaction in cytoplasm and nuclei of some cells in the basal glands' parts besides few cells in their upper part (Figure 6c).

Group V (Açai berry extract treated group): Examined sections showed apparently moderate caspase-3 immunoreaction in the cytoplasm and nuclei of some cells in the basal part and few cells in the neck region (Figure 6d).

Proliferating cell nuclear antigen (PCNA) immunostaining

Group I (Control group): PCNA immunostained sections of group I exhibited strong positive brownish nuclear expression of PCNA in many cells at the neck, body and basal parts of the fundic glands but mainly in their upper parts (Figure 7a).

Group III (Gastric injury group): This group exhibited positive brownish nuclear PCNA expression in some cells scattered throughout the fundic glands (Figure 7b).

Group IV (Açai berry extract protected group): Strong positive nuclear PCNA immunoreaction was reported in many cells in all regions, mainly the upper regions, of the fundic glands (Figure 7c).

Group V (Açai berry extract treated group): This group revealed strong immunoreaction in many nuclei in all regions of the fundic glands but mainly in their upper parts (Figure 7d).

Scanning electron microscopic results

Group I (Control group): This group revealed normal fundic mucosal surface formed of many surface mucous cells that form rosettes around the openings of the gastric pits. The cells were polygonal dome-shaped with irregular upper surface and well-demarcated margins. In addition, mucus aggregations (globules or streaks) were randomly dispersed over the mucosal surface (Figures 8a,b).

Group III (Gastric injury group): Disturbed architecture of the fundic mucosal surface was recorded in this group. Wide irregular gastric pits and areas of mucosal discontinuity with sharp edges deeply extending into the mucosa were detected (Figure 9a). Focal areas of lost surface mucous cells were also evident. Many surface cells appeared flattened with rough upper surface and lost intercellular demarcations while some other cells showed cavitations on their surface. In addition, some mucus globules were observed (Figure 9b). Other sections showed complete loss of the surface cells together with presence of prominent wide pits, giving the denuded surface a honeycomb appearance (Figure 9c). In addition, deep craters were also noticed in certain regions (Figure 9d).

Group IV (Açai berry extract protected group): This group showed preserved general architecture of the fundic mucosal surface. Most surface mucous cells preserved their well-demarcated dome-shaped contour except for few cells that appeared swollen and detached (Figure 10a). In focal areas, few surface cells showed umbilication in the center of their surface. Mucus globules were also detected covering the surface cells (Figure 10b).

Group V (Açai berry extract treated group): This group revealed apparent improvement of the changes detected in the injury group III. Some cells were dome-shaped with well-defined boundaries. However, other cells showed flattening and irregularity of their surface around the gastric pits' openings giving a doughnut-like appearance. Some mucus globules were also seen (Figure 11a). On the other hand, other sections exhibited some cells with rough or eroded upper surface and lost intercellular demarcation (Figure 11b).

Morphometric results & statistical analysis

Mean thickness of gastric fundic mucosa in µm (Table I)

The gastric injury group III exhibited highly significant decrement in the mean fundic mucosal thickness in respect to group I. On the other hand, açai berry extract protected group IV exhibited non-significant difference in respect to group I while revealing highly significant increment in respect to group III. Meanwhile, açai berry extract treated

group V showed highly significant increment in its mean thickness in respect to group III but still showed highly significant decrement in respect to group I and a significant decrement in respect to group IV.

Mean color intensity of PAS reaction (Table II)

The gastric injury group III expressed highly significant decrement in PAS color intensity in respect to group I. Meanwhile, açai berry extract protected group IV showed a non-significant change in its color intensity in respect to group I besides highly significant raise in comparison with group III. In addition, açai berry extract treated group V exhibited highly significant raise in its mean PAS color intensity when compared with group III while showing highly significant intensity reduction when compared with both groups I and IV.

Mean color intensity of caspase-3 immunoreaction (Table III)

The gastric injury group III exhibited highly significant raise in caspase-3 color intensity as compared to control group I. Açai berry extract protected group IV expressed non-significant difference in respect to group I together with a highly significant decrement in respect with group III. Moreover, açai berry extract treated group V exhibited highly significant reduction in caspase-3 color intensity in respect to group III besides a significant increment compared with both groups I and IV.

Mean number of PCNA positive cells (Table IV)

The gastric injury group III exhibited highly significant decrement in PCNA positive cells' mean number in respect to group I. Meanwhile, açai berry extract protected group IV showed non-significant difference in respect to group I and highly significant raise when compared to group III. However, açai berry extract treated group V revealed highly significant increment in cells' mean number when compared with group III and highly significant decrease in

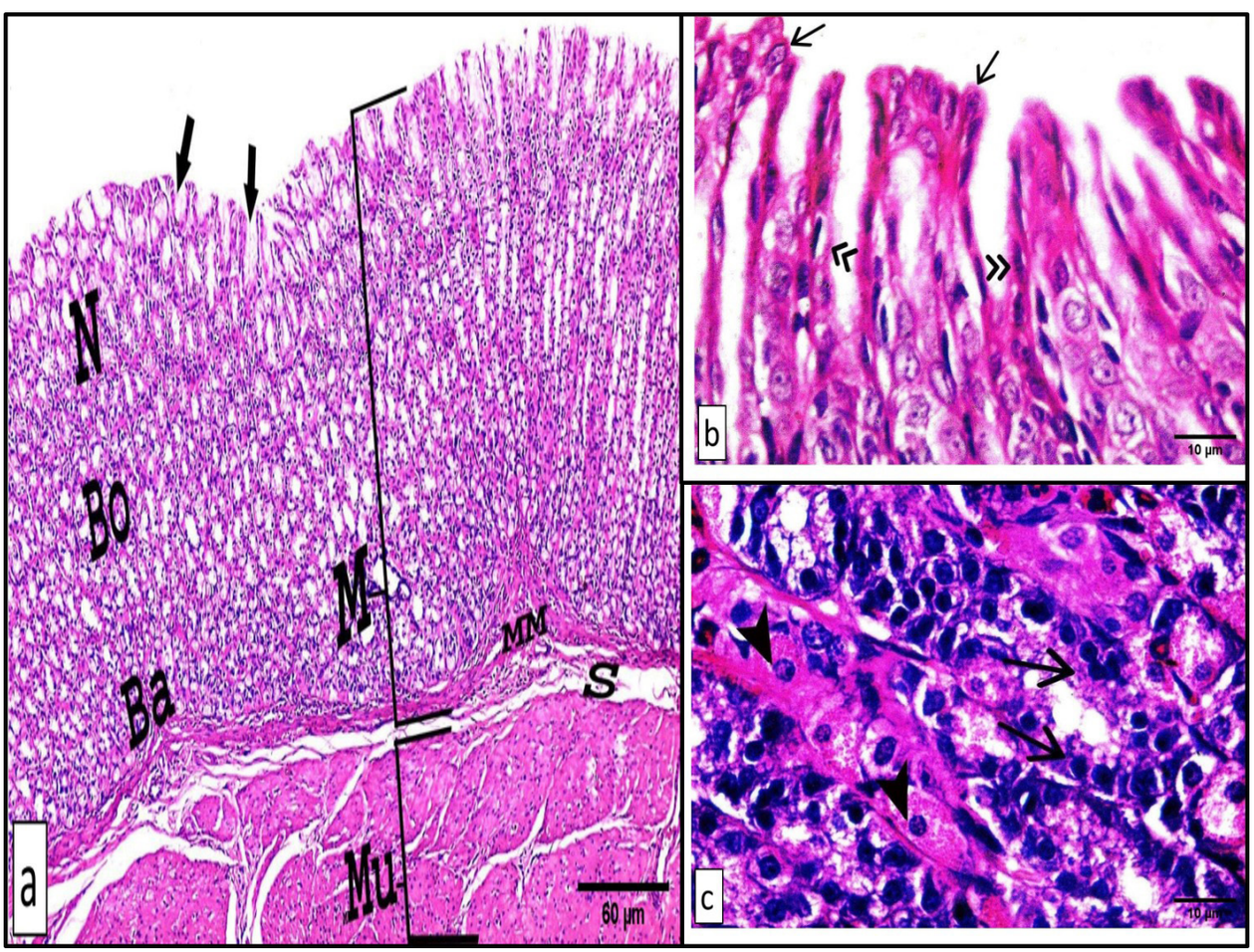

Fig. 1: H&E stained sections of group I: (a) Normal fundic mucosa (M). The fundic glands are at right angle to the surface, opening into it through narrow pits (thick arrow). The glands are formed of; neck (N), body (Bo) and base (Ba). Notice: muscularis mucosa (MM), submucosa (S) and muscularis externa (Mu). (H&E, X 200, scale bar = 60) (b) Tall columnar surface mucous cells with oval elongated nuclei (thin arrows). Mucous neck cells with pale acidophilic cytoplasm and basal flattened nuclei (double arrow heads) are also seen. (c) Parietal cells with deeply acidophilic cytoplasm and central round nuclei (arrow heads) and chief cells with basophilic cytoplasm and basal spherical nuclei (thin arrows). (H&E, X 1000, scale bar = 10)

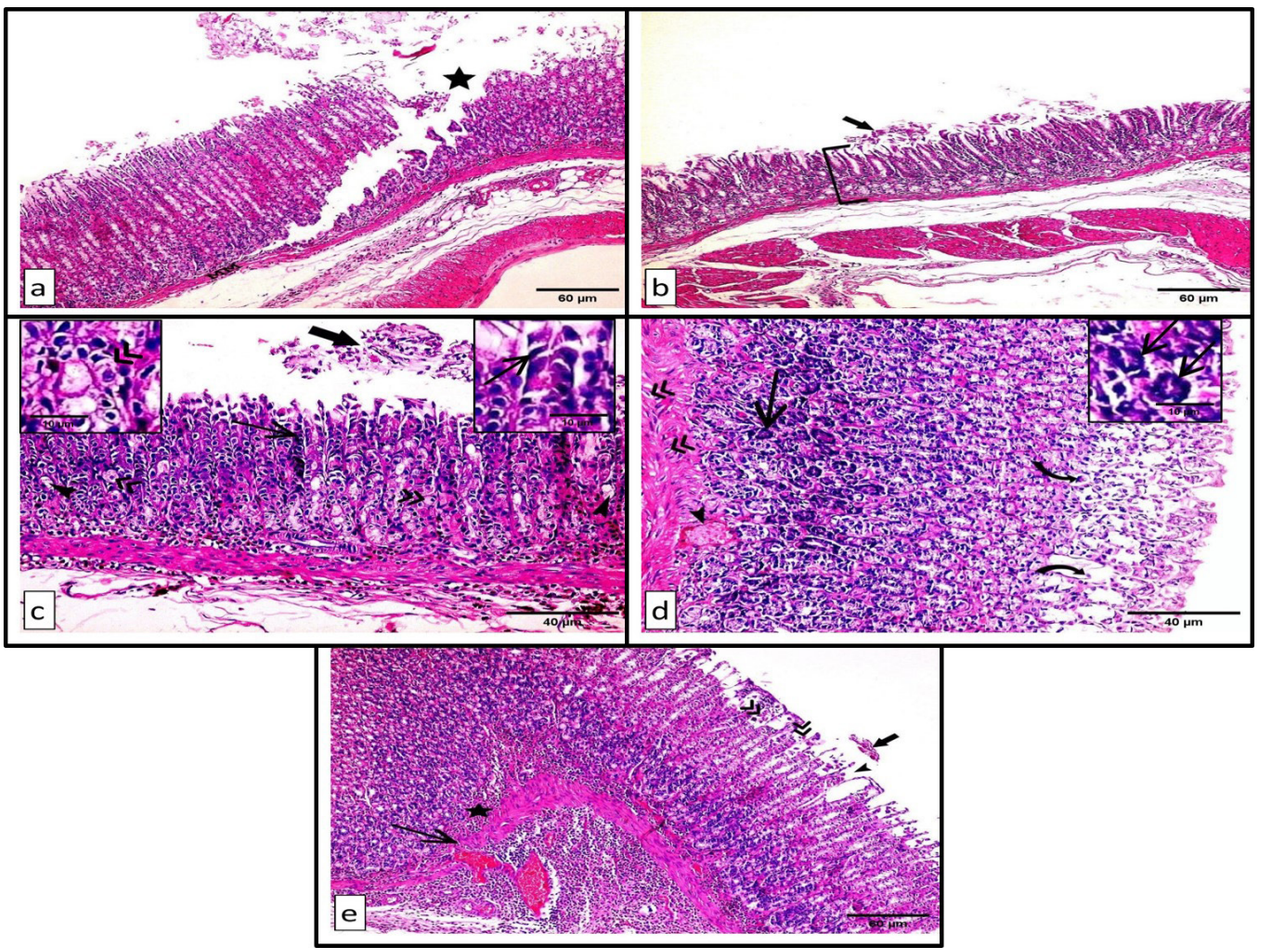

Fig. 2: H&E stained sections of group III: (a) Fundic mucosal discontinuity (star) reaching down to muscularis mucosa (MM). (b) Apparent decrease in mucosal thickness ([). Exfoliated cells (thick arrow) are also noticed. (H&E, X 200, scale bar = 60) (c) Mucous neck cells with deeply stained cytoplasm and pyknotic nuclei (Thin arrows). Some parietal cells are vacuolated (arrow heads) while others exhibited lightly acidophilic cytoplasm (double arrow heads). Exfoliated cells (thick arrow) are noticed. (d) Destructed neck region of the glands with separation of their lining cells (curved arrows) and deeply basophilic chief cells with pyknotic nuclei (thin arrows). Dilated congested blood capillaries (arrow head) in lamina propria and vacuolated smooth muscle cells of the muscularis mucosa (double arrow heads) are noticed. (H&E, X 400, scale bar 40, insets X1000, scale bar=10) (e) Dilated gastric pits (arrow head) and marked mononuclear cellular infiltration in lamina propria (star). Focal discontinuity of muscularis mucosa with its invasion by a congested blood vessel from the underlying submucosa is observed (thin arrow). Notice: Exfoliated cells (thick arrow) and loss of lining cells of neck region (double arrow heads). (H&E, X 200, scale bar = 60)

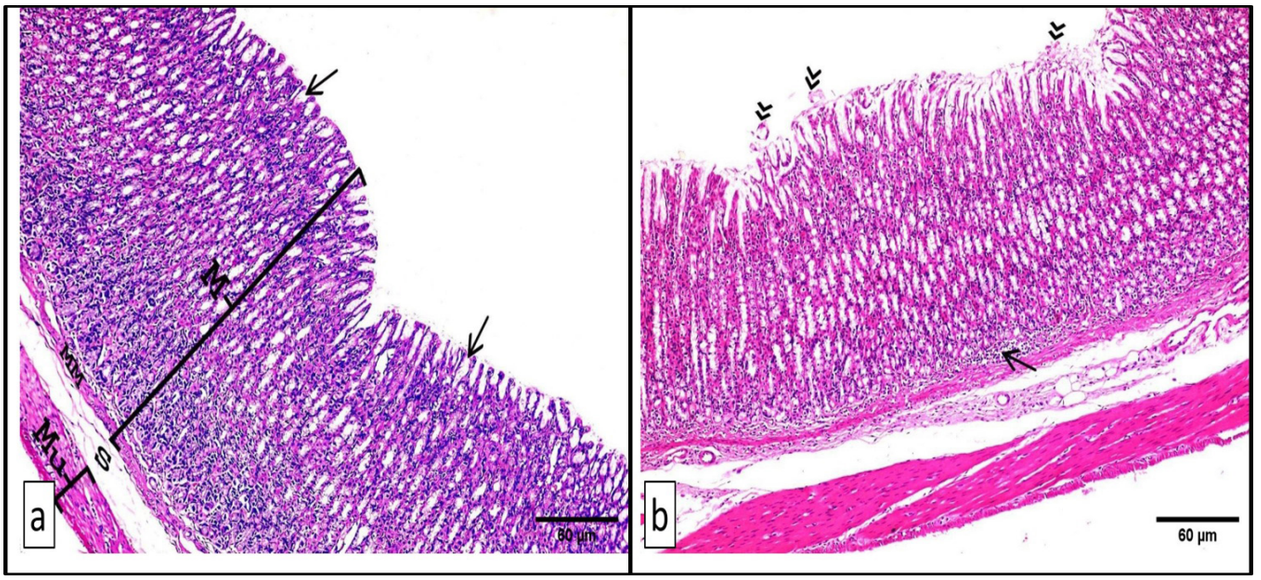

Fig. 3: H&E stained sections of group IV: (a) Apparently normal gastric fundic mucosa (M). The fundic glands are regular and perpendicular to the surface with narrow pits (thin arrows). Notice: muscularis mucosa (MM), submucosa (S) and muscularis externa (Mu). (b) Focal exfoliation of some surface cells and the superficial parts of some glands (double arrow heads). Notice: mononuclear cellular infiltration in the lamina propria (thin arrow). (H&E, X 200, scale bar = 60)

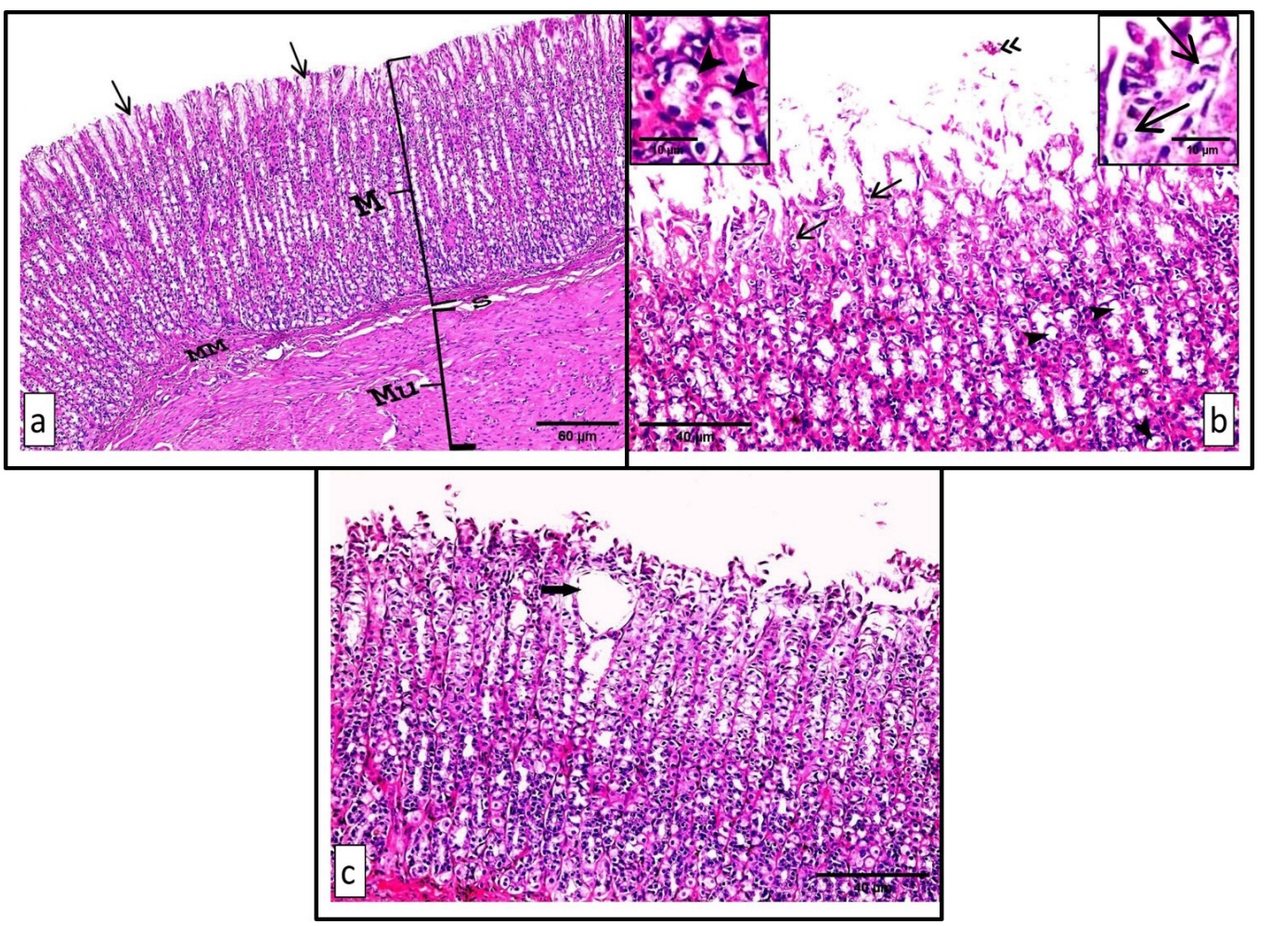

Fig. 4: H&E stained sections of group V: (a) Apparently normal fundic mucosa (M). The fundic glands are regular and perpendicular to the surface with narrow pits (thin arrows). Notice: muscularis mucosa (MM), submucosa (S) and muscularis externa (Mu). (H&E, X 200, scale bar = 60) (b) Focal exfoliation of surface cells and upper parts of the glands (double arrow heads). Vacuolated mucous neck cells (thin arrows) and vacuolated lining cells of the body of the glands (arrow heads) are seen. (c) cystic dilatation of a fundic gland (thick arrow). (H&E, X 400, scale bar = 40, inset X1000, scale bar=10)

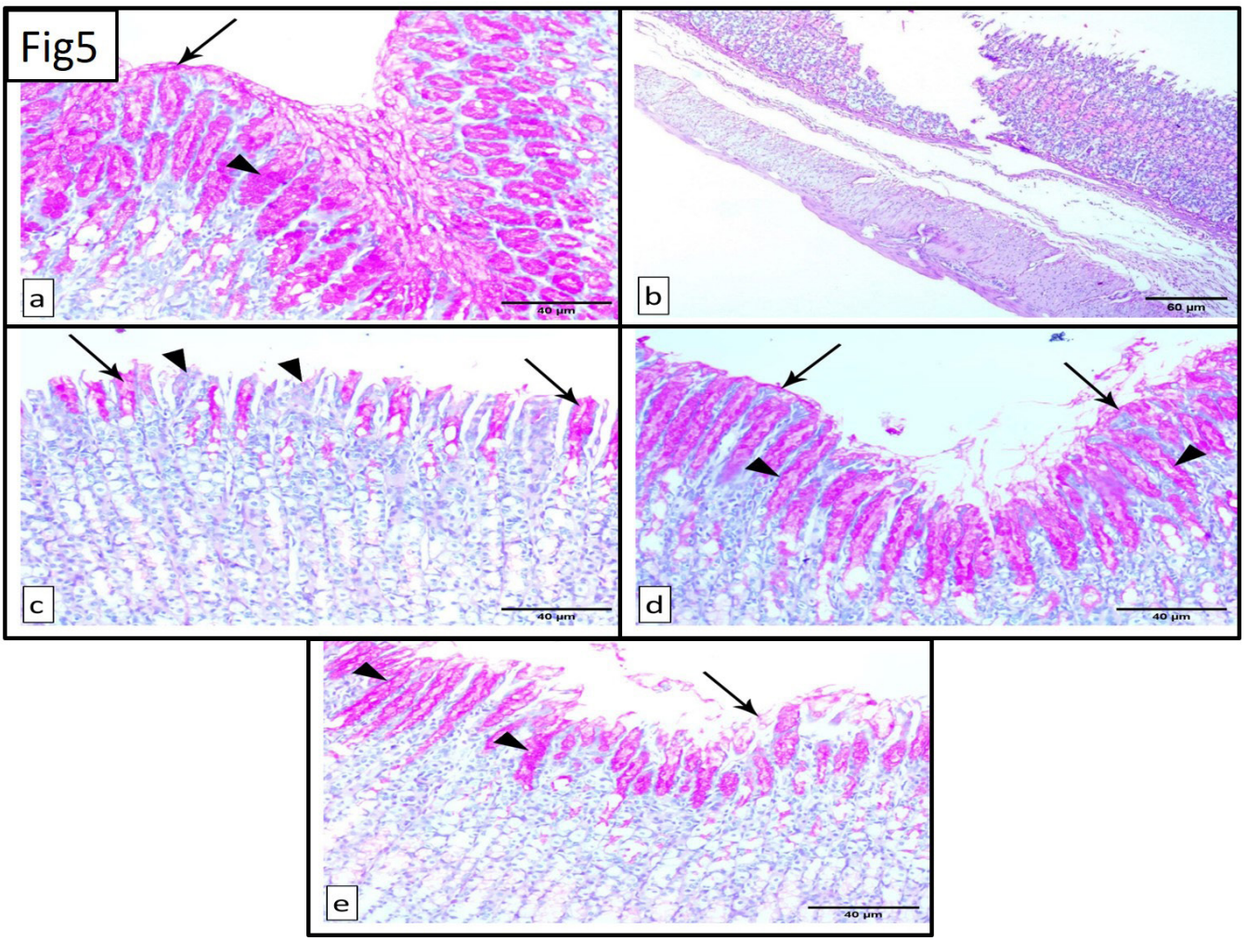

Fig. 5: PAS-stained sections: (a) Group I: Strong positive reaction over the mucosal surface (thin arrows) and into the gastric pits and upper glandular regions (arrow heads). (PAS, X 400, scale bar = 40) (b) Group III: Absent PAS reaction of the fundic mucosa. (PAS, X 200, scale bar = 60) (c) Group III: Focal reaction extending into the gastric pits (thin arrows). Notice areas with weak reaction (arrow heads). (d) Group IV: Strong reaction over the surface (thin arrows) extending into the pits and upper glandular regions (arrow heads). (e) Group V: Moderate reaction over the surface (thin arrow) and strong reaction in the pits and the upper glandular regions (arrow heads). (PAS, X 400, scale bar = 40)

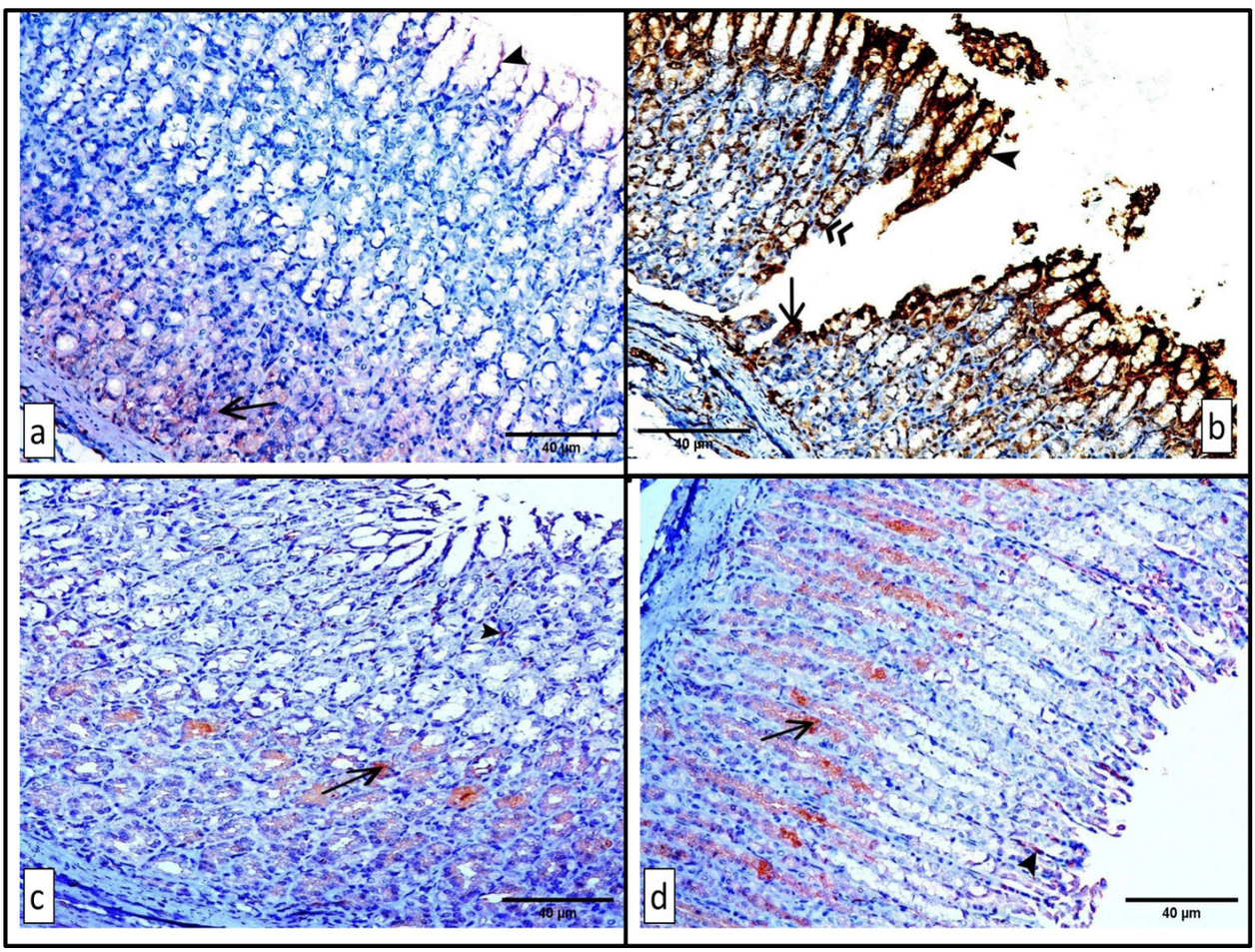

Fig. 6: Caspase-3-immunostained sections: (a) Group I: Minimal positive brownish cytoplasmic immunoreaction in few cells in the neck (arrow head) and basal glandular parts (thin arrow). (b) Group III: Strong positive immunoreaction in both cytoplasm and nuclei of most cells lining the neck (arrow head), body (double arrow head) and basal regions of the fundic glands (thin arrow). (c) Group IV: Mild positive immunoreaction in the cytoplasm and nuclei of some cells in the basal glandular parts (thin arrow) and few cells in the neck region (arrow head). (d) Group V: Moderate positive immunoreaction in the cytoplasm and nuclei of few cells in the neck (arrow head) and some cells in the basal parts (thin arrow) of the fundic glands. (Caspase-3 immunostaining, with counterstain hematoxylin, X400, scale bar = 40)

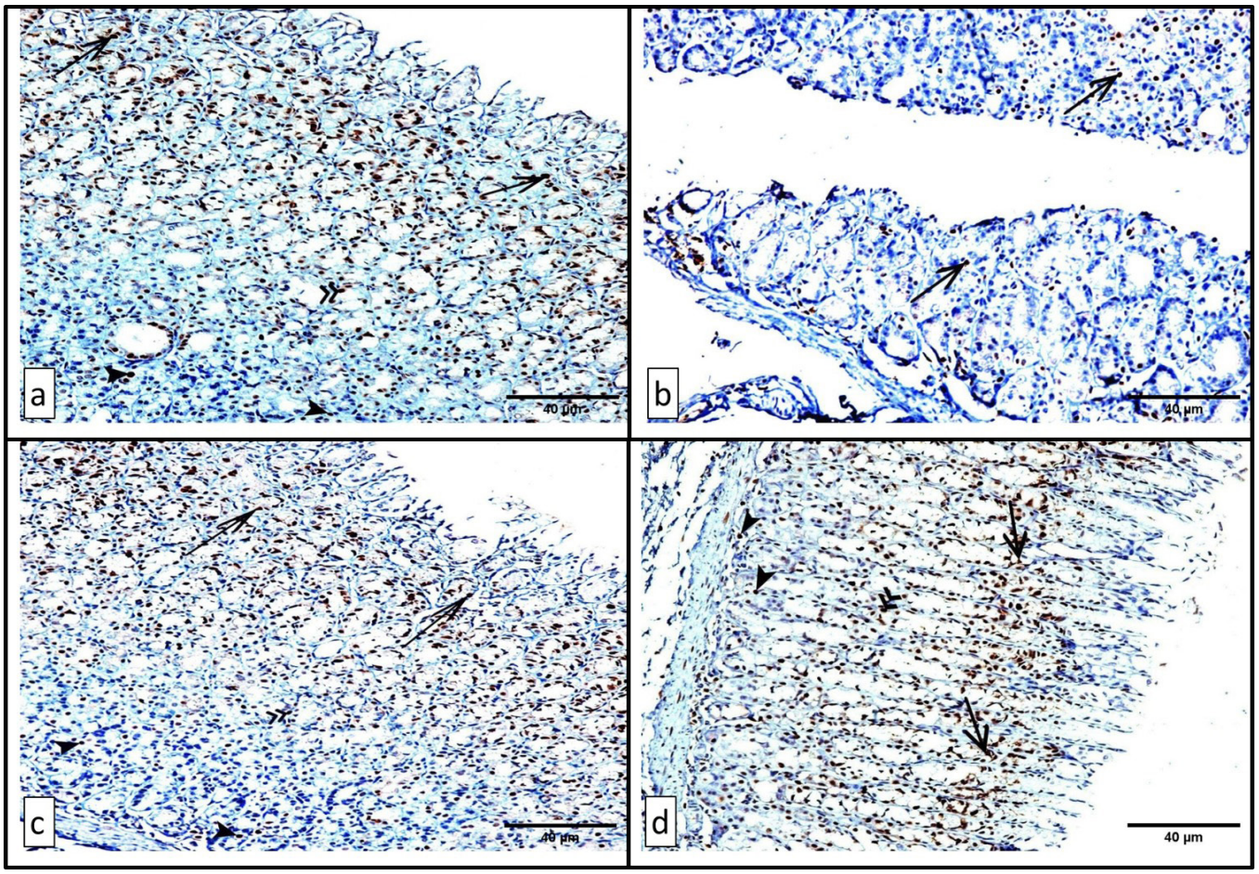

Fig. 7: PCNA-immunostained sections: (a) Group I: Strong nuclear brownish immunoreaction in many cells in the neck (thin arrows), body (double arrow head) and basal regions (arrow heads) of the fundic glands. (b) Group III: Positive nuclear reaction in some scattered cells at the fundic glands (thin arrows). (c) Group IV: Strong nuclear immunoreaction in most of the cells in the neck (thin arrows), body (double arrow head) and basal regions (arrow heads) of the fundic glands. (d) Group V: Strong nuclear immunoreaction in most of the cells in the neck (thin arrows), body (double arrow head) and basal regions (arrow heads) of the fundic glands. (PCNA immunostaining, with counterstain hematoxylin, Mag. x 400, scale bar = 40)

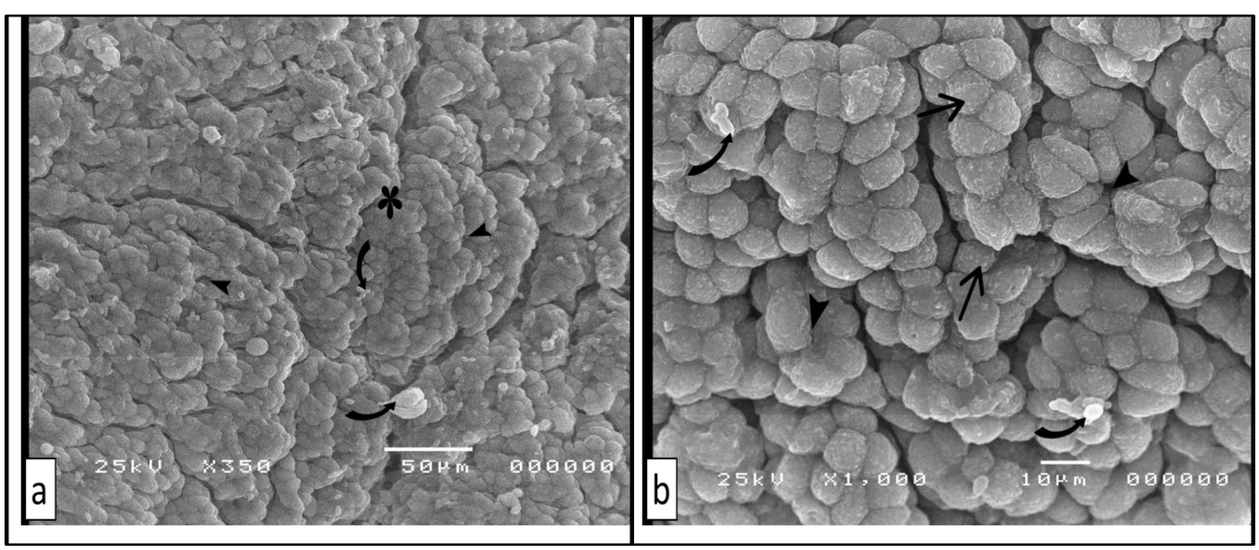

Fig. 8: SEM micrographs of group I (Control) showing: (a) Normal fundic mucosal surface. The pits (arrow heads) are surrounded by surface mucous cells (asterisk). Mucus aggregations are seen in the form of globules or streaks (curved arrows). (Mic. Mag. X 350) (b) Polygonal mucous cells (thin arrows) with well-demarcated margins and dome-shaped irregular surface are forming rosettes surrounding the pits openings (arrow heads). Mucus globules are seen (curved arrows). (Mic. Mag. X 1000)

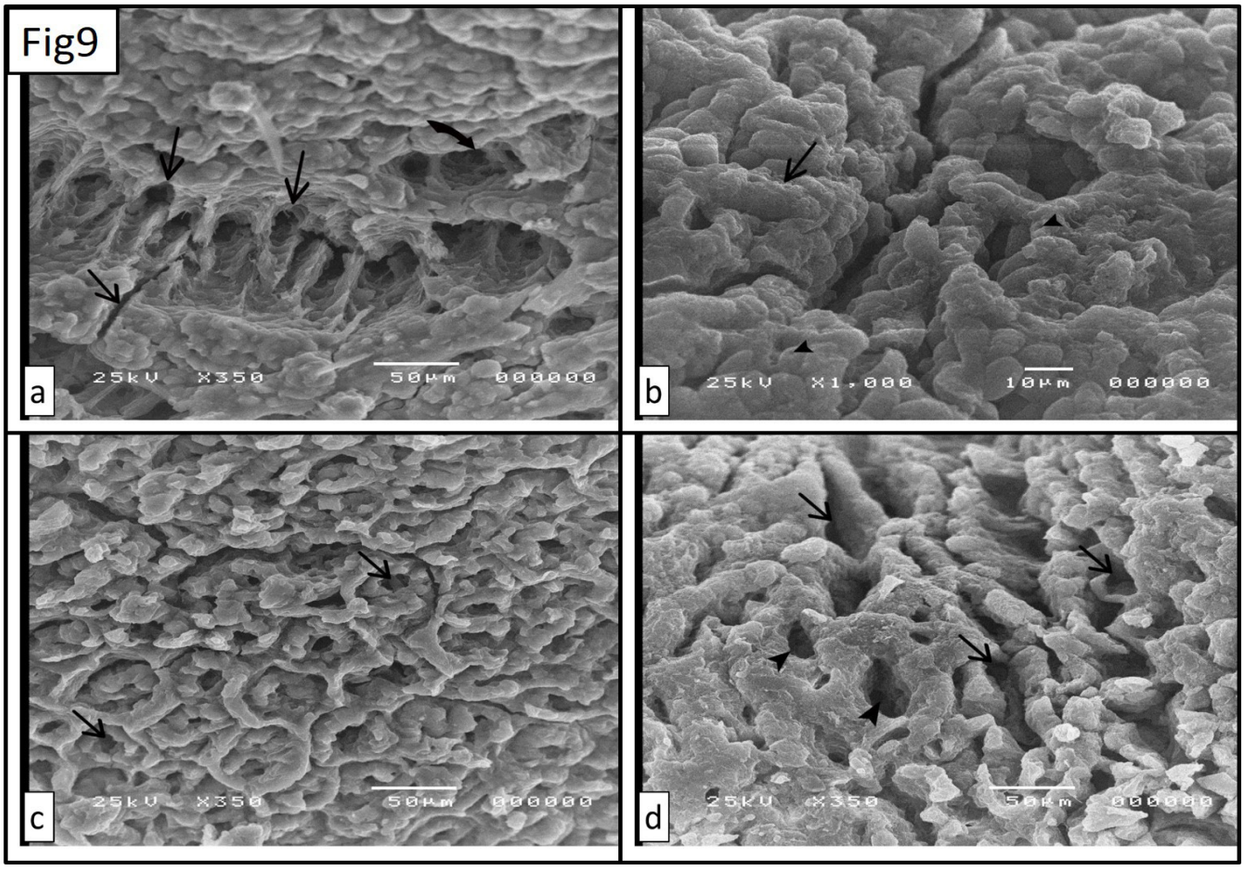

Fig. 9: SEM micrographs of group III showing: (a) Wide irregular gastric pits (curved arrow). Area of mucosal discontinuity with sharp edges deeply extending into the mucosa is seen (thin arrows). (Mic. Mag. X 350) (b) Rough surface and lost intercellular demarcation of some cells (thin arrow) and cavitation of the surface of other cells (arrow heads). (Mic. Mag. X 1000) (c) Complete loss of surface mucous cells with honeycomb appearance of wide gastric pits' openings (thin arrows). (Mic. Mag. X 350) (d) Deep craters (thin arrows). Notice: dilated gastric pits (arrow heads). (Mic. Mag. X 350)

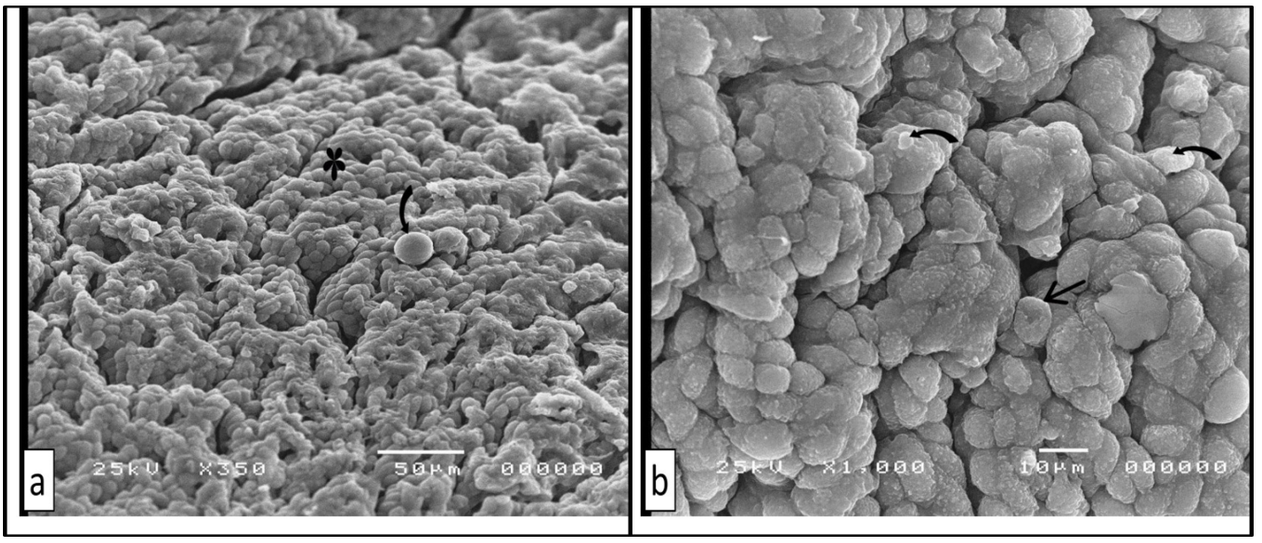

Fig. 10: SEM micrographs of group IV showing: (a) Apparently normal fundic mucosal surface architecture. Most surface mucous cells appear dome-shaped and well-demarcated (asterisk). One detached swollen cell is seen (curved arrow). (Mic. Mag. X 350) (b) A surface mucous cell (thin arrow) with umbilication in the center of its surface. Mucus globules (curved arrows) overlying the epithelial cells are observed. (Mic. Mag. X 1000)

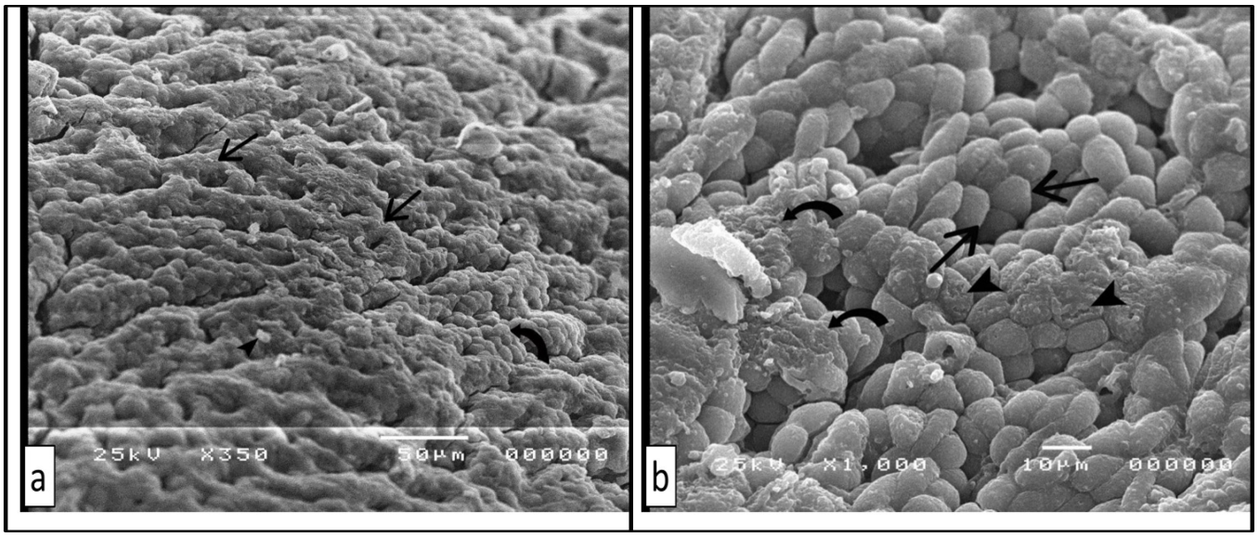

Fig. 11: SEM micrographs of group V showing: (a) Dome-shaped surface cells with well-defined boundaries (curved arrow). Other cells are flattened around gastric pits' openings giving doughnut-like appearance (thin arrows). Some mucus globules are seen (arrow head). (Mic. Mag. X 350) (b) Some surface cells with roughness of their surface and loss of demarcation in between them (curved arrows). Some cells with eroded surface (arrow heads) are observed. Other dome-shaped well-demarcated surface cells (thin arrows) are also seen. (Mic. Mag. X 1000)

Table I: Mean thickness of gastric fundic mucosa in µm

Groups	Thickness of gastric fundic mucosa						ANOVA	
	Range			Mean	±	SD	F	*P-value*
Group I	391.153	-	557.957	494.026	±	46.461		
Group II	382.855	-	551.266	492.093	±	47.876		
Group III	163.62	-	329.845	219.876	±	63.315	48.178	<0.001**
Group IV	359.695	-	539.129	475.727	±	57.476		
Group V	308.167	-	445.519	394.663	±	48.390		
				TUKEY'S Test				
	I			II		III		IV
II	1.000							
III	<0.001**			<0.001**				
IV	0.938			0.958		<0.001**		
V	0.001**			0.002**		<0.001**		0.011*

* means significant difference ($P < 0.05$) ** means highly significant difference ($P < 0.001$)

Table II: Mean color intensity of PAS reaction

Groups	Color intensity of PAS						ANOVA	
	Range			Mean	±	SD	F	*P-value*
Group I	53.01	-	56.024	54.657	±	1.316		
Group II	52.012	-	55.03	53.635	±	1.162		
Group III	10.02	-	23.04	20.677	±	4.013	294.826	<0.001**
Group IV	49.045	-	56.02	51.644	±	2.230		
Group V	41.001	-	49.045	43.145	±	3.168		
				TUKEY'S Test				
	I			II		III		IV
II	0.905							
III	<0.001**			<0.001**				
IV	0.092			0.443		<0.001**		
V	<0.001**			<0.001**		<0.001**		<0.001**

* means significant difference ($P < 0.05$) ** means highly significant difference ($P < 0.001$)

Table III: Mean color intensity of caspase-3 immunoreaction.

Groups	Color intensity of caspase-3 immunoreaction						ANOVA	
	Range			Mean	±	SD	F	*P-value*
Group I	0.4	-	2.2	1.219	±	0.635		
Group II	0.2	-	2.2	1.199	±	0.782		
Group III	28.03	-	92.13	68.596	±	28.339	50.725	<0.001**
Group IV	2.01	-	4.02	2.619	±	0.838		
Group V	14.03	-	27.01	19.438	±	4.827		
TUKEY'S Test								
	I			II		III		IV
II	1.000							
III	<0.001**			<0.001**				
IV	0.999			0.999		<0.001**		
V	0.022*			0.022*		<0.001**		0.041*

* means significant difference ($P < 0.05$) ** means highly significant difference ($P < 0.001$)

Table IV: Mean number of PCNA positive cells

Groups	Number of PCNA positive cells						ANOVA	
	Range			Mean	±	SD	F	*P-value*
Group I	78.02	-	84.12	79.881	±	2.099		
Group II	77.05	-	82.13	79.068	±	1.619		
Group III	8.02	-	15.75	11.660	±	3.066	795.417	<0.001**
Group IV	69	-	80.71	77.421	±	3.469		
Group V	60.42	-	77.75	64.056	±	4.976		
TUKEY'S Test								
	I			II		III		IV
II	0.980							
III	<0.001**			<0.001**				
IV	0.453			0.790		<0.001**		
V	<0.001**			<0.001**		<0.001**		<0.001**

* means significant difference ($P < 0.05$) ** means highly significant difference ($P < 0.001$)

respect to both groups I and IV.

DISCUSSION

Gastric injury is a common global health problem affecting more than half of the general population[26]. Diclofenac-induced gastric injury was reported to be more common in the fundus of the stomach[27] and was established as a standard animal model in several studies[7]. Açai berry is considered a superfood with several health benefits[28]. Therefore, this research was designed to assess the role of açai berry extract in both prevention and treatment of DS-induced fundic mucosal injury in adult male albino rat.

H&E stained group III sections (gastric injury group) exhibited areas of mucosal discontinuity. This came in agreement with Olivia *et al*.[29] who attributed it to imbalance between the injurious factors e.g. [enhanced secretion of gastric acid, inflammatory mediators and reactive oxygen species (ROS)] and the protective factors such as [bicarbonate, mucus and prostaglandins (PGs) secretion, cell regeneration and endogenous production of antioxidants], that eventually breaks the gastric mucosal defense mechanisms.

Prostaglandins are essential for gastric mucosal barrier integrity, defense mechanism and homeostasis via stimulating the epithelial cell regeneration, increasing the mucus production and improving the gastric blood flow besides lowering the gastric acid production and guarding against the oxidative stress damaging effects[30]. Elshopakey and Elazab,[31] explained DS-induced gastric injury to be due to inhibition of PGs formation in gastric mucosal cells by inhibiting cyclooxygenase-1 & -2 (Cox-I & -II) enzymes.

The previous researchers also illustrated that diclofenac markedly reduces the levels of the antioxidant enzymes as catalase, glutathione and superoxide dismutase. Simon & Prince,[32] added that diclofenac also stimulates the production of ROS, that together with the decreased antioxidant enzymes, contribute to the gastric mucosal injury and ulceration.

In group III of the current study, exfoliation of the surface and glandular cells was frequently observed. Nooh

and El-Saify,[33] attributed it to the lost integrity of the tight junctions as a result of the breakdown of the junctions' transmembrane proteins.

Group III also revealed a significant decrement of the fundic mucosal thickness compared to control group I. This coincided with Saleh and Mutlag,[34] who attributed it to the extensive sloughing of the fundic mucosal cells.

Pyknotic nuclei were observed in both mucous neck and chief cells of group III with deeply basophilic cytoplasm of the chief cells. Bjarnason *et al*.[35] attributed these changes to the direct mucosal cell injury caused by pepsin and lipase as a result of the reduced mucus and PGs production. Youssef,[22] considered these changes as signs of apoptosis that occur secondary to the diclofenac-induced mitochondrial damage and the raised ROS production that induces DNA damage, lipid peroxidation and protein modification that all eventually induce apoptosis.

Group III also showed cytoplasmic vacuolation of the parietal cells. A similar finding was observed by El-Mehi and El-Sherif[36] who explained it by the increased production of oxygen free radicals that increase the cell membrane permeability and, accordingly, the intracellular movement of electrolytes and water, with subsequent swelling of both the cells and their organelles, which are both visualized as cytoplasmic vacuolations.

Dilation of the gastric pits, reported in group III, coincided with the observations of Mohamed *et al*.[37] who attributed it to gastric glands hypersecretion after PGs inhibition.

Blood vessels' dilatation as well as congestion were reported in group III. Similar results were recorded by Zaghlool *et al*.[38] who attributed them to the increased degranulation of the mast cells, after loss of the PGs inhibitory effect, with the subsequent release of their mediators that eventually cause vasodilatation of the mucosal blood vessels.

Marked inflammatory cellular infiltration was recorded in the lamina propria of group III. This could be due to disruption of the gastric mucosal barrier that exposes the mucosa to gastric acid and enzymes with increased susceptibility to bacterial penetration. The later induces release of the chemotactic factors that attract many inflammatory cells[23].

Group III exhibited interrupted muscularis mucosa. This finding was recorded by Eleawa *et al*.[39] and Kengkoom *et al*.[40]. The later authors illustrated that the luminal gastric acid could penetrate deeply through the disrupted mucosal barrier and cause damage that reaches down to the muscularis mucosa.

On the other hand, H&E stained sections of both açai berry extract protected group IV and treated group V exhibited better results than group III, that were more prominent in group IV than in group V.

Group IV revealed preservation of the normal fundic mucosal architecture and thickness. However, exfoliation of some cells as well as mild inflammatory cellular infiltration were still observed in some sections of this group.

Meanwhile, group V revealed improvement of the histological changes reported in group III with restoration of the thickness and microscopic features of the regenerating gastric mucosa. However, in few sections, the fundic mucosa still revealed exfoliation of the superficial parts of the glands with cytoplasmic vacuolation of few cells lining the body regions besides cystic dilatation of few glands.

Oliveira *et al*.[41] stated that açai berry extract has strong antioxidant properties linked to its content of phenolic compounds, particularly quercetin, that effectively reduce the production of ROS and inhibit the lipid peroxidation, thus protecting the cellular proteins from the oxidative damage and also preventing the cellular damage caused by the free radicals.

Singh *et al*.[42] also demonstrated that açai berry extract has a high oxygen radical absorbance capacity (ORAC) value, the highest O2 scavenging capacity and the highest superoxide dismutase (SOD) enzyme level when compared to other fruits and vegetables. SOD is considered the main enzyme that neutralizes the ROS and prevents the gastric mucosal injury[43].

Recently, Kim *et al*.[44] proved that açai berry has a beneficial role in the gastric defense mechanism as it increases the epithelial barrier integrity through strengthening different tight junction proteins.

As regards its role in inflammation, açai berry extract was reported to effectively reduce the inflammation via suppressing the secretion of the pro-inflammatory cytokines and interleukins. It also contains a unique flavone called velutin that has the strongest anti-inflammatory properties of all other flavones[45]. Herve *et al*.[46] attributed the gastroprotective and anti-inflammatory activities of açai berry to the antihistaminic properties of its polyphenols and quercetin that decrease the degranulation of the gastric mast cells and so decrease the histamine levels, thus, reducing the inflammation.

Epicatechin, contained in açai berry, was documented to have local gastroprotective properties through increasing the secretion of alkaline mucus that in turn increases the thickness of the mucus barrier and neutralizes the acidic gastric juice[47]. Moreover, tannins, one of the components of açai berry, was also reported to cause protein precipitation at the site of the gastric ulcer, thus, providing a protective layer that increase the gastric mucosal opposition to proteolytic enzymes' action[48].

The cystic dilatation, occasionally observed in group V, came in agreement with Zdziarski *et al*.[49] who considered it to be a feature of ulcer healing.

Healing of the gastric ulcer is based on decreasing the inflammation and enhancing the cell migration,

epithelial regeneration and neovascularization[50]. Earlier, Kang and Kim,[51] stated that the antioxidant properties of the polyphenols, anthocyanin, proanthocyanidin and flavonoids, contained in açai berry extract, make it effective in the ulcer healing process and tissue regeneration. Anthocyanins also enhance the endothelial nitric oxide synthase enzyme levels that enhance nitric oxide (NO) production[52]. The latter regulates the mucosal blood flow, suppresses the neutrophils' adhesion and infiltration through the vascular endothelium and enhances the angiogenesis, epithelial cells proliferation and growth factors formation[53].

As regards PAS-stained sections, group III revealed lack of PAS reactivity except for few focal positive reactivity of minimal thickness with a highly significant decrement in its color intensity in comparison with control group I. Youssef,[22] illustrated that diclofenac markedly decreases glycoprotein biosynthesis secondary to inhibition of PGs synthesis as well as injury of both mucous surface and neck cells.

However, PAS-stained sections of açai berry extract protected group IV and treated group V exhibited microscopically and statistically better results than group III, that were more prominent in group IV. The latter revealed strong reaction over the mucosal surface and inside the pits and the upper glandular parts, that was non-significantly different from that of group I. Meanwhile, group V revealed moderate reaction that was statistically higher than group III but still statistically lower than group I. This could be attributed to açai's content of malvidin anthocyanidin as well as flavonoids and polyphenols which all stimulate PGE2 synthesis that in turn increases mucus and bicarbonate production in the gastric mucosa[54,55].

As regards caspase-3 immunostained sections, group III revealed strong caspase-3 expression in many cells lining the fundic glands but mainly in their upper and lower parts, with highly significant raise of its color intensity compared to control group I. Similar findings were reported by Simon and Prince,[32]. Diclofenac induces apoptosis by uncoupling of mitochondrial oxidative phosphorylation that reduces adenosine triphosphate (ATP) production. Consequently, mitochondrial cytochrome c is liberated into the cytosol resulting in ROS production, which activates the caspase-3, which in turn activates the endonuclease enzyme and causes DNA fragmentation and cell apoptosis[56].

Caspase-3 immunostained sections of açai berry extract groups IV and V exhibited better results than group III with the best result in group IV. The later revealed mild positive immunoreactivity mainly in the lower part of the glands that was non-significantly different from that of group I. Meanwhile, group V revealed moderate positive immunoreactivity in both lower and upper parts of the fundic glands with highly significant decrease in its color intensity in respect to group III, while being significantly higher than those of groups I and IV. These findings coincided with El Morsy *et al*.[57] who reported that açai decreased the tissue levels of caspase-3.

Cell proliferation is essential for the healing of gastric injuries. Stem cells are found at the gastric glands' isthmus and middle region. They are pluripotent cells that proliferate, and then the newly formed cells move either downward into the gland or upward into the pits and mucosal surface lining to replace the lost specialized cells[22].

Group III PCNA-stained sections exhibited weak expression in some scattered cells in the fundic glands with highly significant decrement in their mean number in comparison with the control group. These findings resembled those of Youssef,[22] who stated that diclofenac inhibits proliferation of gastric stem cells. Abd-Elhamid *et al*.[58] and Ali *et al*.[59] explained that NSAIDs inhibit cell proliferation and tissue regeneration by reducing the binding capacity of epidermal growth factor (EGF), a protein required for gastric cell proliferation and differentiation, to its receptors with subsequent inhibition of its signaling pathways.

PCNA-stained sections of açai berry extract protected group IV and treated group V exhibited better results in comparison with group III with the best results in group IV. The later revealed strong reaction in many cells in all regions of the fundic glands, mainly in their upper parts with non-significant change in their mean number from that of group I. Group V also revealed strong reaction with highly significant increase of the mean cells' number in respect to group III while showing highly significant decrement in respect to groups I and IV.

Kang *et al*.[60] reported that açai promotes cell proliferation besides decreasing cell programmed death. Fagundes *et al*.[54] illustrated that malvidin anthocyanidin, contained in açai berry extract, stimulates cell proliferation via increasing expression of epidermal growth factor (EGF) gene. Pini *et al*.[61] added that açai polyphenols increase expression of DNA repair genes that promote cell proliferation.

Scanning electron microscopy was performed to study the fundic mucosal surface. SEM results from group III confirmed the light microscopic results and revealed disturbed surface architecture with presence of areas of discontinuity or complete cellular loss with honeycomb appearance of the surface. Many cells were flattened with rough upper surface and lost intercellular demarcation while other cells revealed cavitations. In addition, deep craters were also noticed in certain regions.

The honeycomb appearance of the fundic mucosal surface could be explained according to Bashandy and Noya,[62] to be due to complete loss of the surface mucosal cells, exposing the gastric pits' openings.

Flattening of the surface cells came in line with Mohammed *et al*.[63] who noticed loss of dome-shaped appearance of the surface cells. This could be explained according to Saleh and Multag,[34] to be due to damage of the cellular cytoskeletal proteins secondary to exposure to excessive amounts of ROS.

Cells with rough upper surface and cavitations were found in group III. Da Fonseca *et al*.[64] reported similar changes and attributed them to degenerative changes in the epithelial cells. Tandoh *et al*.[65] illustrated that NSAIDS disrupt the structural integrity of the gastric epithelial cell membranes. This could cause hole-like defects in the cell membranes, referred to as cavitations, that eventually causes rupture of the cell membranes and cell lysis.

The deep craters observed in group III were also reported by Mohammed *et al*.[63] and El-Mehi and El-Sherif,[36] who explained them to be due to complete destruction of the gastric glands and extensive denudation of the lamina propria.

SEM results obtained from açai berry extract given groups (group IV and group V) also supported the light microscopic results. Group IV revealed preservation of the fundic mucosal surface architecture and presence of apparently normal surface mucosal cells. However, few cells appeared swollen and detached or showed umbilication of their surface.

The swollen cells could be explained according to El-Mehi and El-Sherif,[36] who confirmed that both low oxygen tension and depleted ATP inside the cells affect sodium-potassium pump causing sodium and fluid to shift into the cells causing their swelling.

Cells with umbilicated surface, noticed in group IV, were described by Bashandy and Noya,[62] and Mohammed *et al*.[63] as cells having a small depression in the center of their upper surface that appears as a result of an exocytosed mucus granule.

SEM results of group V revealed improvement of the changes detected in injury group III. Some cells appeared normal while others appeared flattened with doughnut like appearance. Some other cells showed roughness or erosions of their surface with loss of the intercellular demarcation. This doughnut-like appearance were in line with Bravo *et al*.[66] who illustrated that this occurs after flattening of the cells around gastric pits' openings.

Boutemine *et al*.[67] stated that the polyunsaturated fatty acids contained in açai berry, provide a source of the lipids responsible for cell membrane remedy. They added that linoleic acid is a precursor of PGs that decrease gastric acid production and protect the cells. Furthermore, Polegato *et al*.[68] documented that açai also improves mitochondrial function and ATP production. So, it effectively reduces cellular hypoxia and prevents cell damage.

CONCLUSION

Taken together from the previously discussed results, it could be concluded that açai berry extract remarkably protected against diclofenac-induced fundic gastric injury and also exerted a considerable effect in its treatment. However, açai protective effect was more prominent than its therapeutic effect. These beneficial effects of açai could be most probably via enhancing cell proliferation together with its anti-inflammatory, anti-apoptotic and previously reported antioxidant effects.

RECOMMENDATIONS

From the results of the current study, we recommend clinical trials for using açai berry extract as a concurrent treatment with diclofenac sodium to protect against their hazardous effects on gastric/fundic mucosa. We also recommend clinical trials for its use in treatment of diclofenac-induced gastric mucosal injury and ulcer.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية مقارنة على الدور الوقائى والعلاجى لمستخلص توت الأساي علي اصابة الغشاء المخاطى لقاع المعدة المستحدثة تجريبيا في ذكر الجرذ الأبيض البالغ

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المقدمة: تُعد إصابة المعدة مشكلة صحية عالمية مختلفة الأسباب. يعتبر توت الأساى فاكهة غنية بالمركبات مضادة للأكسدة والالتهابات والتى تعد مادة وقائية وعلاجية واعدة.

الهدف من العمل: تقييم الدور الوقائى والعلاجى المحتمل لمستخلص توت الأساى على إصابة الغشاء المخاطي بقاع المعدة المستحدثة تجريبيا بمضادات الالتهاب الغير ستيرودية.

المواد والطرق: أجريت هذه الدراسة على ٥٠ من ذكور الجرذان البيضاء البالغة ،تم تقسيمهم إلى خمس مجموعات؛ المجموعة الأولى (المجموعة الضابطة)، المجموعة الثانية (مجموعة مستخلص توت الأساى) التي تلقت مستخلص توت الأساى بجرعة ٣٠٠ مجم/كجم مرة واحدة يوميًا عن طريق الفم لمدة ١٤ يومًا متتابعين، المجموعة الثالثة (مجموعة إصابة المعدة) التي تلقت جرعة واحدة بالفم من الديكلوفيناك بجرعة ١٠٠ مجم/كجم، المجموعة الرابعة (المجموعة المحمية بمستخلص توت الأساى) التى تلقت مستخلص توت الأساى لمدة ١٤ يوما متتابعين ثم تلقت جرعة واحدة من الديكلوفيناك وأخيرًا المجموعة الخامسة (المجموعة المُعالجة بمستخلص توت الأساى) والتي تلقت الديكلوفيناك مرة واحدة ثم تلقت مستخلص توت الأساى لمدة ١٤ يومًا متتابعين. تم فحص عينات قاع المعدة بالميكروسكوب الالكترونى (بصبغات الهيماتوكسيلين والايوسين و حمض شيف الدورى بالاضافة الى الصبغات المناعية الكسباس-٣ ومستضد تكاثر نواة الخلية).

النتائج: أظهرت عينات المجموعة الثالثة المصبوغة بصبغتى الهيماتوكسيلين والإيوسين انفصال الخلايا السطحية ومناطق من انقطاع الغشاء المخاطى وتجاويف سيتوبلازمية وارتشاح خلوى وكذلك احتقان الأوعية الدموية. وأيضاً أظهرت سالبية تفاعل حمض شيف الدورى بينما أظهرت تفاعل مناعي قوي للكسباس-٣ وتفاعل مناعي ضعيف لمستضد تكاثر نواة الخلية. كشف الفحص بالمجهر الإلكترونى الماسح لهذه المجموعة فقد للخلايا المخاطية السطحية مع ظهور شكل قرص العسل الى جانب ظهوربعض الخلايا السطحية ذات سطح خشن مع تجاويف، واتساع فتحات (حُفر) الغدد المَعدية و وجود حُفرعميقة . بينما أظهرت المجموعة الرابعة حفاظًا على البِنية النسيجية الطبيعية للغشاء المخاطي لقاع المعدة وكشفت المجموعة الخامسة عن تحسن في التغيرات النسيجية المسجلة في المجموعة الثالثة.

الخلاصة: يمتلك مستخلص توت الأساى تأثيرات واعدة فى الوقاية من وعلاج إصابة المعدة بواسطة بمضادات الالتهاب الغير ستيرودية.