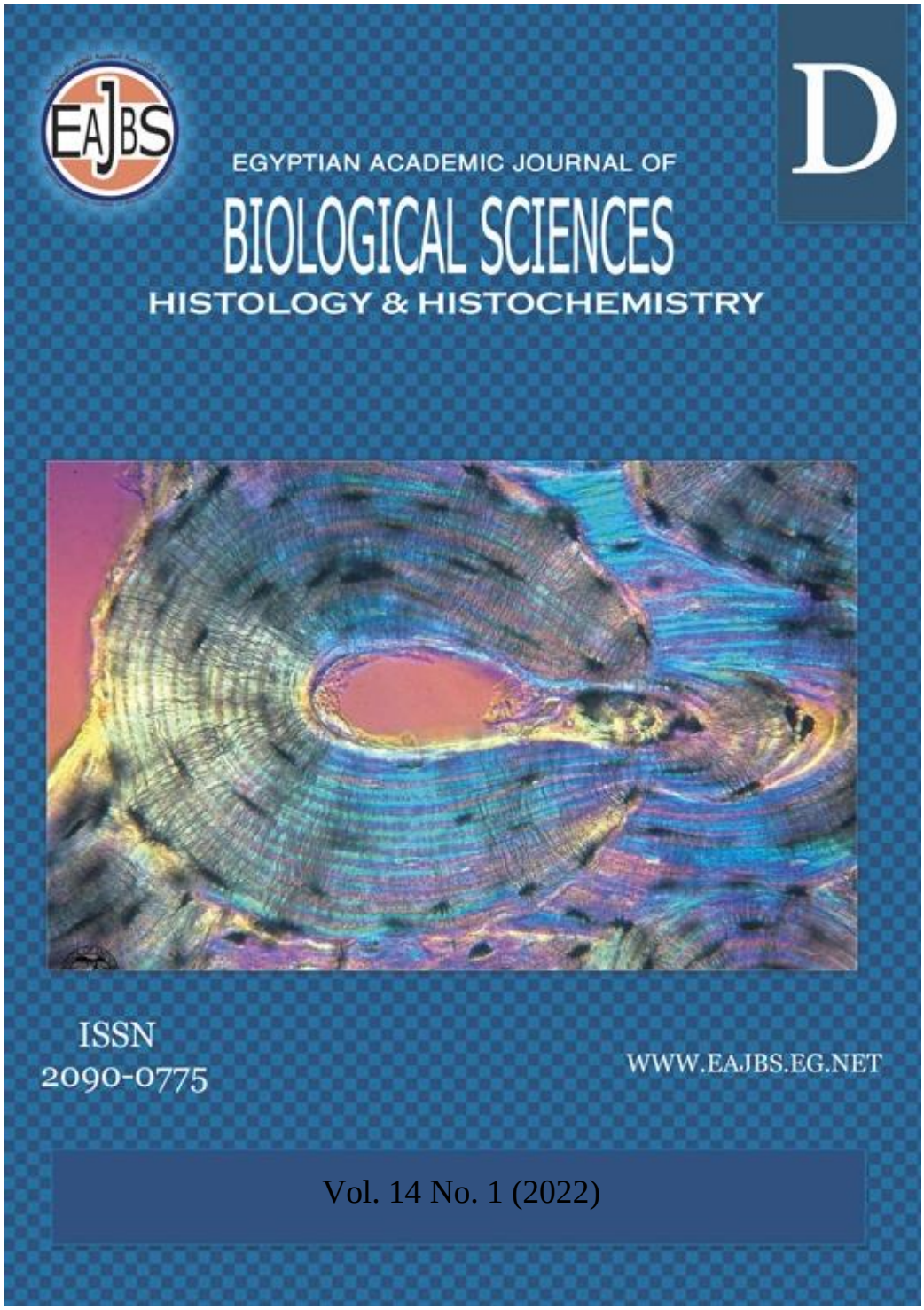

Citation: Egypt. *Acad. J. Biolog. Sci. (D-Histology and histochemistry) Vol.14(1) pp27-41(2021)*
DOI: 10.21608/EAJBSD.2022.216580

Egypt. Acad. J. Biolog. Sci., 14(1): 27-41 (2022)

Egyptian Academic Journal of Biological Sciences
D. Histology & Histochemistry
ISSN 2090 – 0775
http://eajbsd.journals.ekb.eg

Therapeutic Effect of Amygdalin on Acetic Acid-induced Colitis in Rats: Histopathological and Immunohistochemical Study

Yasser M. Elbastawisy[1, 3] and Hazem Abdelhamid Mohamed[2, 3]

1. Anatomy & Embryology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
2. Anatomy & Embryology Department, Faculty of Medicine, Assiut University, Assiut, Egypt.
3. Anatomy & Embryology Department, Faculty of Medicine, Taibah University, Madinah, Saudi Arabia.

E.Mail: yasserbast@yahoo.com

ARTICLE INFO
Article History
Received:25/12/2022
Accepted:19/1/2022
Available:22/1/2022

***Keywords*:**
Amygdalin, ulcerative colitis, iNOS, and COX-2.

ABSTRACT

Ulcerative colitis is a progressive disabling inflammatory bowel disease characterized by idiopathic, repetitive, and diffuse inflammation of the mucosa of the colon and the rectum. The pathogenesis of UC includes continuous inflammation of the colonic lamina propria, with damage of the mucosal barrier and infiltration with inflammatory factors. The underlying etiology of UC is still unclear. In this study, thirty adult male albino rats (150-200 g) were divided into 3 equal groups; group I (control), group II (subjected to intra-colonic instillation of 5% acetic acid for 3 consecutive days), and group III (received acetic acid as in group II followed by intraperitoneal injection of Amygdalin once a week for 3 weeks). Rats were euthanized and colonic samples were prepared and stained with Hematoxylin & eosin, alcian blue, and immunohistochemical staining using iNOS & COX-2. Colonic mucosa of group II showed mucosal ulceration, hemorrhage, distorted crypts, absent goblet cells, mononuclear lymphocytic infiltration, and a marked increase in both iNOS & COX-2 immunoreactivity in comparison with group I. On the other hand, colonic mucosa of group III exhibited regeneration of the surface epithelium & goblet cells together with a decline in both iNOS & COX-2 immunoreactivity in comparison to group II. In conclusion, amygdalin is a promising therapeutic agent in managing UC.

INTRODUCTION

Inflammatory bowel disease (IBD) is a progressive disabling inflammatory condition affecting the gastrointestinal tract comprising two main subtypes; ulcerative colitis (UC) which is limited to the colonic mucosa and Crohn's disease (CD), which may affect any part of the gastrointestinal tract from the mouth to the anus (K Ko & K Auyeung, 2014). UC is the most common form of IBD all over the world characterized by idiopathic, repetitive, and diffuse inflammation of the mucosa of the colon and rectum (Feuerstein & Cheifetz, 2014). It presents with abdominal pain, diarrhea, mucopurulent bloody stools, and weight loss and may proceed, in severe cases, to carcinoma of the colon (Vejzovic, Bramhagen, Idvall, & Wennick, 2018).

IBD may affect people at any age but, is observed mostly in those aged 20-40 years old with considerable consequences on social capability and lifestyle (Knights, Lassen, & Xavier, 2013).

The pathogenesis of IBD includes continuous inflammation of the colonic lamina propria, with damage of mucosal barrier and infiltration with inflammatory factors (Koch *et al.*, 2000). Though there is recent progress in understanding the pathogenesis of IBD, the underlying etiology is still unclear. Genetic susceptibility, many environmental and immunological factors seem to play a role together with intestinal microflora and autoimmune reactions (Ozturk *et al.*, 2015). The pathogenesis of IBD is significantly increased by the production of cellular reactive oxygen species (ROS) which in turn lead to an increase in the inflammation process (Cetinkaya *et al.*, 2005). Recently oxidative stress proved to play a role in the pathophysiology of IBD (Alzoghaibi, 2013). Patients with IBD showed a significant increase in several inflammatory markers like TNF-α, malondialdehyde (MDA), and pentraxin-3 together in association with an increase in IBD inflammation which can be easily observed histologically (Wirtz *et al.*, 2017). The production of a cascade of free radicals and an increase in lipid peroxidation leads to reduction of the cellular antioxidant capacity, resulting from the secretion of many inflammatory mediators from migrated granulocytes in the inflamed mucosa which finally reduces the cellular antioxidant capacity with subsequent colonic inflammation and the progression of the disease (Cetinkaya *et al.*, 2005). Moreover, studies on mucosal biopsies confirmed that oxidative stress increased and the antioxidant defense system significantly decreased in patients with IBD (Dutta *et al.*, 2019).

IBD can be produced experimentally by several methods; Dextran sulfate sodium and 2,4,6-trinitrobenzene sulfonic acid solution are used to induce a picture that resembles Crohn's disease, whereas acetic acid and oxazolone models are closer to ulcerative colitis (Sharifi, Vahedi, Nedjat, Rafiei, & Hosseinzadeh-Attar, 2019).

As the IBD is characterized by the secretion of a lot of inflammatory mediators in tissues. So, the treatment strategies focus on managing inflammation by identifying good inflammation-reducing agents such as vitamin D (Harbord *et al.*, 2017; Panés *et al.*, 2016), inhibiting disease progress as a systemic and topical steroid, 5-aminosalicylate compounds, and immunosuppressive agents (Jauregui-Amezaga *et al.*, 2016). Clinical trials are currently assessing the application of stem cell transplantation (Andrew & Messaris, 2016). Despite all these trials, about one-third of the patients with UC is still in need of surgical removal of the whole colon (Chung *et al.*, 2010; Uddin *et al.*, 2014).

Recently, multiple studies are carried out to explore the therapeutic potentials of natural medicinal ingredients because of their safety, effectiveness, fewer side effects, and economic efficiency (Bai et al., 2019; Jaswal, Palanivelu, Ramalingam, & reports, 2018). Extracts from some raw herbal drugs have a variety of components that can induce various biological activities. Some of these extracts were effective in treating inflammatory reactions (Hwang *et al.*, 2008).

Amygdalin (Vit B17), is an aromatic cyanogenic compound that occurs naturally in the seeds of apples, pits of apricots and peaches, and bitter almonds (Guo, Wu, Sheng, Yang, & Tan, 2013). It is a bioactive compound that has long been utilized for its useful medicinal properties, as it has been reported to exert anti-inflammatory (Jaswal *et al.*, 2018), anti-fibrotic (He *et al.*, 2020), anticancer (Chang et al.,

2006), antioxidant and immunomodulatory effects (Elsaed, 2019). Amygdalin has other favorable effects as it degrades into hydrocyanic acid which is an anticancerous and analgesic agent at the same time (Halenar *et al.,* 2017). Amygdalin by itself is non-toxic but it can be converted by some enzymes into poisonous substances, so it has to be used with caution (de Santana Souza *et al.,* 2017). Despite the available information on the in vivo and in vitro antioxidant activities of amygdalin being limited together with lack of approval by the Food and Drug Administration and absence of positive clinical trials investigating the anticancer effects of amygdalin, this compound continues to be manufactured and used as an anticancer therapy at alternative (holistic) cancer treatment clinics found in some parts of Europe and Mexico (Guo *et al.,* 2013).

To our knowledge, no available data about the use of amygdalin in the treatment of IBD so, this study was designed to investigate the possible curative effect of amygdalin on acetic acid-induced colitis in rats.

MATERIALS AND METHODS

Animals:

Thirty adult male albino rats (150-200 g) were obtained from the Animal Breeding Unit of Taibah University (KSA) and were kept under constant conditions; 12:12 light/dark cycle and a room temperature of 28^{o} C. They were allowed free access to the standard rat chow diet and water ad libitum and food intake. Rats were acclimated to the environment for 2 weeks before the beginning of the experiment. National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) were observed.

Chemicals and Administration:

Rats were randomly allocated into three equal groups (n=10). Group, I rat served as control and were subjected to intracolonic 0.9% saline enema. Group II and III animals were experimental groups. Rats of group II received 5% acetic acid (Dar Nadeen Pharmaceutical Chemicals Company, KSA) by intra-colonic instillation of 1 ml/rat/day for 3 consecutive days, using a soft pediatric catheter of external diameter 2 mm, inserted in the colon 8 cm proximal to the anal opening. Rats were sustained in a supine Trendelenburg position for 30 seconds to prevent the leakage of the instilled solution (Kiernan, 2015). Animals of Group III received 5% acetic acid as in group II followed by intraperitoneal injection of Amygdalin solution (P. A6005, Millipore-Sigma) 5 mg/kg once a week for 3 weeks starting three days after acetic acid installation (Gonçalves, Araújo, & Di Santo, 2018).

Sampling and Tissue Preparation:

At the end of the experiment, all rats were euthanized by intraperitoneal ketamine 50 mg/kg and xylazine 10 mg/kg. colon samples from all animals were fixed in 10% neutral buffered formalin for 24 hours and processed for the usual tissue handling for paraffin blocks preparation. Sections of 3-4 µm thickness were stained with the following stains:

1. Hematoxylin & eosin (H&E) stain.
2. Alcian blue stain.
3. Immunohistochemical staining using the following primary antibodies:
   a. Inducible nitric oxide synthase (iNOS): It has an important role in the pathogenesis of intestinal inflammation. Expression of colonic iNOS correlates with the activity of UC. Normal expression of iNOS could be detected in neutrophils, smooth muscle, and sparse distribution within epithelial cells.
   b. Cyclooxygenase enzyme-2 (COX-2): is a proinflammatory mediator. Its activity reflects the severity of colonic inflammation. Normally, COX-2 expression could be seen in the surface of epithelial cells and

mononuclear cells of lamina propria.

Sections were incubated with either anti iNOS antibody or anti-COX-2 antibody.

Both of them were rabbit polyclonal antibodies (Lab Vision Corporation Laboratories). The used technique was avidin-biotin-peroxidase complex. The detection system histostain SP kit was used (LAB-SA system, Zymed Laboratories Inc, SF, USA). Counterstaining of nuclei was performed using Mayer's hematoxylin stain. Positive iNOS and COX-2 immunoreactivity appear as brown cytoplasmic deposits (Barnes, Kappelman, & ACG, 2018).

Tissue Examination:

The stained sections were examined under the light microscope (Nikon Eclipse E600W, Japan) and the represented areas were photographed.

Image & Statistical Analysis:

The result images were analyzed using ImageJ software (1.52a, NIH, USA) with a specific built-in routine for area fraction measurement and strain quantification. One slide was prepared from each rat and five random fields from the colonic mucosa were analyzed. The data were expressed as mean ± SD. Comparison of the findings and their significance in normally distributed data was done by t-test. In abnormally distributed data Mann-Whitney U test was used. P values< 0.05 were regarded as statistically significant and P < 0.01 was regarded as highly significant.

RESULTS

Haematoxylin and Eosin-Stained Sections:

Sections of colonic mucosa of the control rats showed numerous tightly packed crypts occupying the whole thickness of the lamina propria resting on muscularis mucosa. The surface epithelium and crypt lining are formed mainly of simple columnar absorptive cells with intact brush-border together with goblet cells. The connective tissue lamina propria also showed normally resident immune cells mainly lymphocytes (Figs. 1A & 1B).

Colonic mucosa of group II demonstrated areas of ulcerated epithelium with detached apical parts of epithelial cells and interrupted basement membrane together with distorted dilated crypts with nearly absent goblet cells. The lamina propria demonstrated heavy mononuclear lymphocytic infiltration and congested blood vessels in addition to extravasated RBCs. The muscularis mucosa appears disrupted (Figs. 1C & 1D).

Colonic mucosa of group III exhibited intact continuous surface epithelium (regeneration of the surface epithelium) and preserved crypts lined by normal columnar absorptive cells and a few goblet cells. The lamina propria showed congested blood vessels and extravasated RBCs and intact muscularis mucosa (Figs. 1E & 1F).

Alcian Blue-Stained Sections:

Examinations of sections of the control group demonstrated numerous deeply stained goblet cells lining the crypts and intact film of mucus on the surface epithelium (Fig. 2A).

Examination of sections in the mucosa of group II showed ulcerated areas with nearly absent goblet cells and mucous in addition to a reduction in the intensity of Alcian blue stain (Fig. 2B).

Colonic mucosa of group III revealed few goblet cells and reduced intensity of Alcian blue stain (Fig. 2C).

Immunohistochemical Results:

iNOS Immunostained Sections:

Examination of the mucosa of the colon of group I exhibited weak brown cytoplasmic positive immunoreaction in the epithelium and few cells in the lamina propria (Fig. 3A), while colonic mucosa of group II exhibited a marked increase in iNOS positive immunoreactivity in both epithelial cells and cells of lamina propria in relation to the control group (Fig. 3B). On the other hand, sections in the colonic mucosa of group III demonstrated a relative decrease in

positive immunoreaction in comparison to groups II (Fig. 3C).

COX-2 Immunostained Sections:

Examination of sections in the colon of the control group demonstrated scanty brown cytoplasmic positive immunoreaction in both surface epithelium and the lamina propria (Fig. 4A). Whereas sections of group II showed a marked increase in COX-2 positive immunoreactivity in the epithelial cells and cells of lamina propria in comparison with the control group (Fig. 4B). While colonic mucosa of group III exhibited a relative decline in COX-2 immunopositive cells in comparison to group II (Fig. 4C).

Morphometric Results:

Groups II revealed a significant decrease in the values ($p<0.05$) for the mean area % of mucin versus the control group in alcian blue-stained sections. On the other hand, group III revealed a significant increase in the mean area % of mucin in comparison with groups II (Fig. 2D). Regarding iNOS immunoreactivity, group II showed a significant increase in the area % ($P<0.05$) when compared with the control group while group III exhibited a significant decrease ($P<0.05$) in comparison with groups II. (Fig. 3D). As regards mean area % of COX-2 immunoreactivity, group II exhibited a significant increase ($p<0.05$) when compared with the control group whereas group III revealed a significant decrease ($P<0.05$) versus groups II (Fig. 4D).

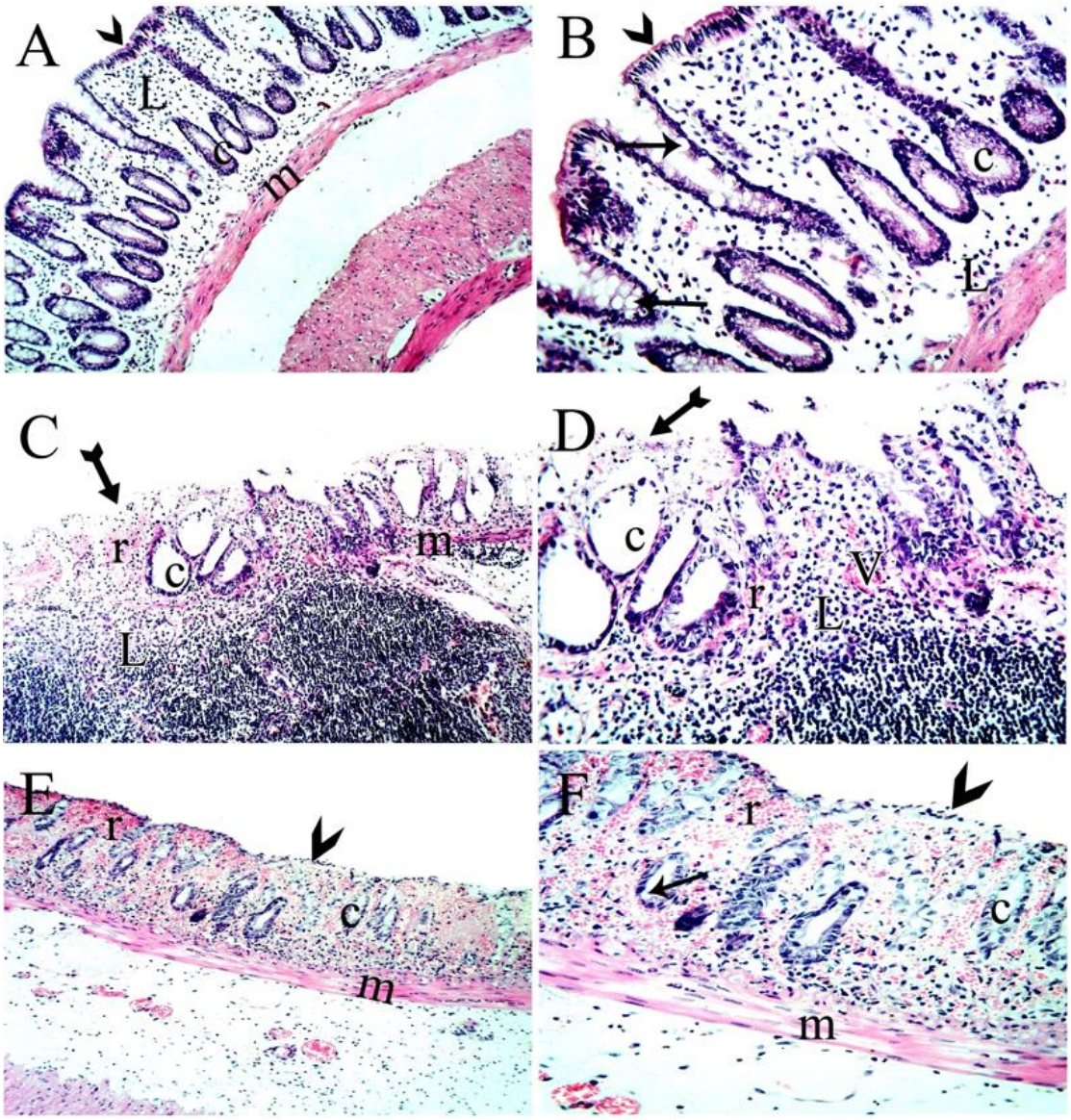

Fig. 1: (A&B); Photomicrograph of a paraffin section of colonic mucosa of the control group showing columnar absorptive epithelium with intact brush border (arrowhead) and goblet cells (arrow). The lamina propria rests on muscularis mucosa (m) and contains tightly packed crypts (C) and numerous lymphocytes (L). **(C&D);** group II showing area of the ulcerated epithelium (tailed arrow) and distorted dilated crypts (C) with nearly absent goblet cells. The lamina propria shows heavy mononuclear lymphocytic infiltration (L), congested blood vessels (V) together with extravasated RBCs (r). Muscularis mucosa (m) appears interrupted. **(E&F);** group III showing re-epithelialization (arrowhead), relatively few preserved crypts (C) with some normal columnar absorptive cells and few goblet cells (arrow), intact muscularis mucosa (m). There is apparent congested vasculature and extravasated RBCs (r). [Hx&E (**A, C & E** X 100) & (**B, D & F** X200)]

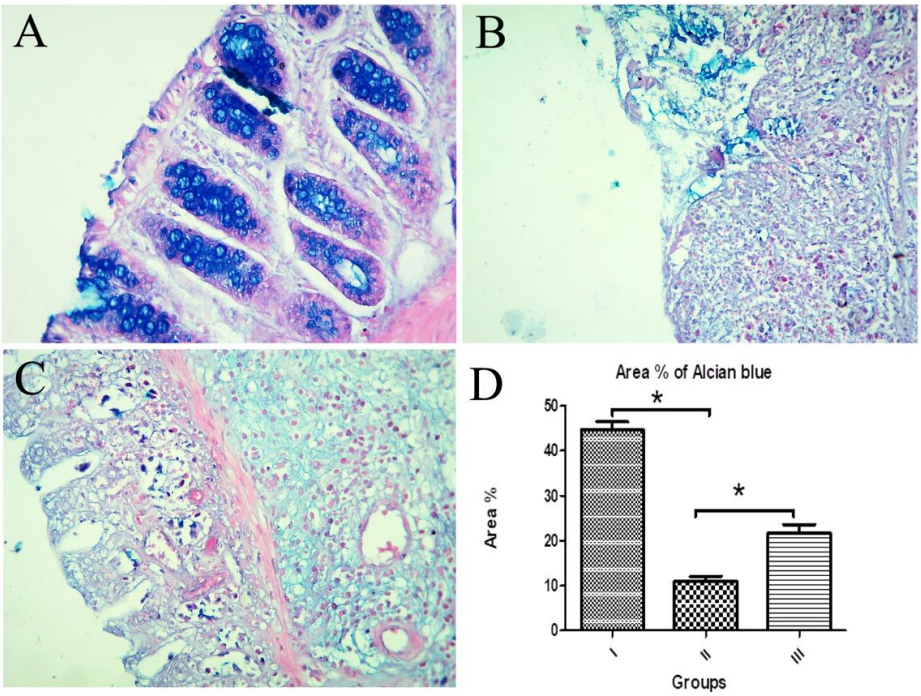

Fig. 2: (A); Photomicrograph of a paraffin section of colonic mucosa of the control group stained for demonstration of mucin. It shows numerous deeply stained goblet cells lining the crypts and intact film of mucus on the surface epithelium. **(B);** group II showing ulcerated area, nearly absent goblet cells and mucous in addition to a reduction in the intensity of the stain. (**C);** group III showing reappearance of goblet cells and few mucous in the lumen of the crypts [Alcian blue X200]. **(D);** Diagram of the mean mucin-stained area percentage of the 3 animal groups. * Indicates a significant difference (P<0.005).

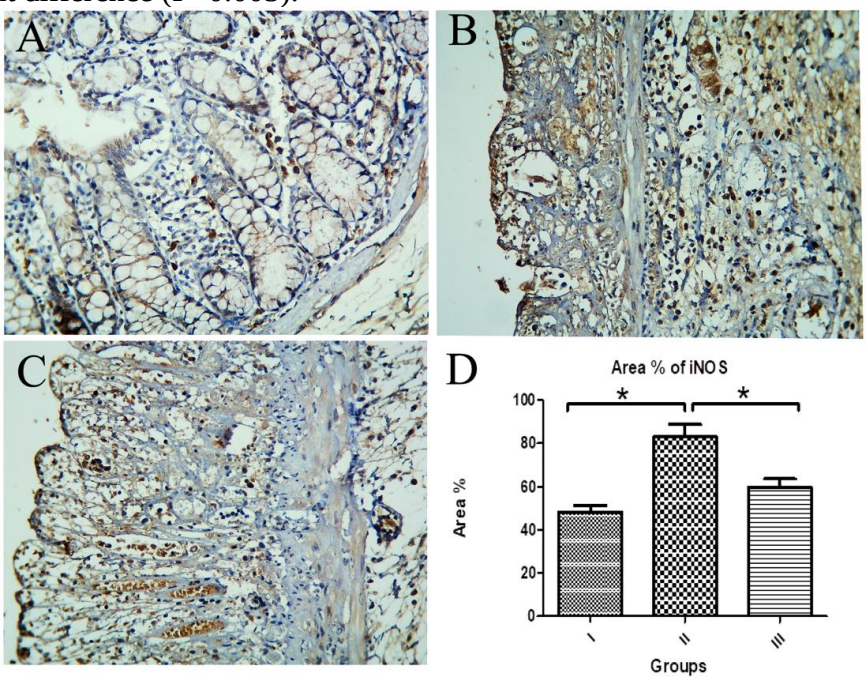

Fig. 3: (A); A paraffin section of the control rat colonic mucosa treated for demonstration of iNOS immunoreactivity. It shows weak brown cytoplasmic positive immunoreaction in the epithelium and few cells in the lamina propria. **(B);** group II exhibited a marked increase in iNOS positive immunoreactivity. **(C);** group III showed a decrease in iNOS positive immunoreactivity in relation to group II [iNOS immunostaining x 200]. **(D);** Diagram of the mean iNOS stained area percentage of the 3 animal groups. * Indicates a significant difference (P<0.005).

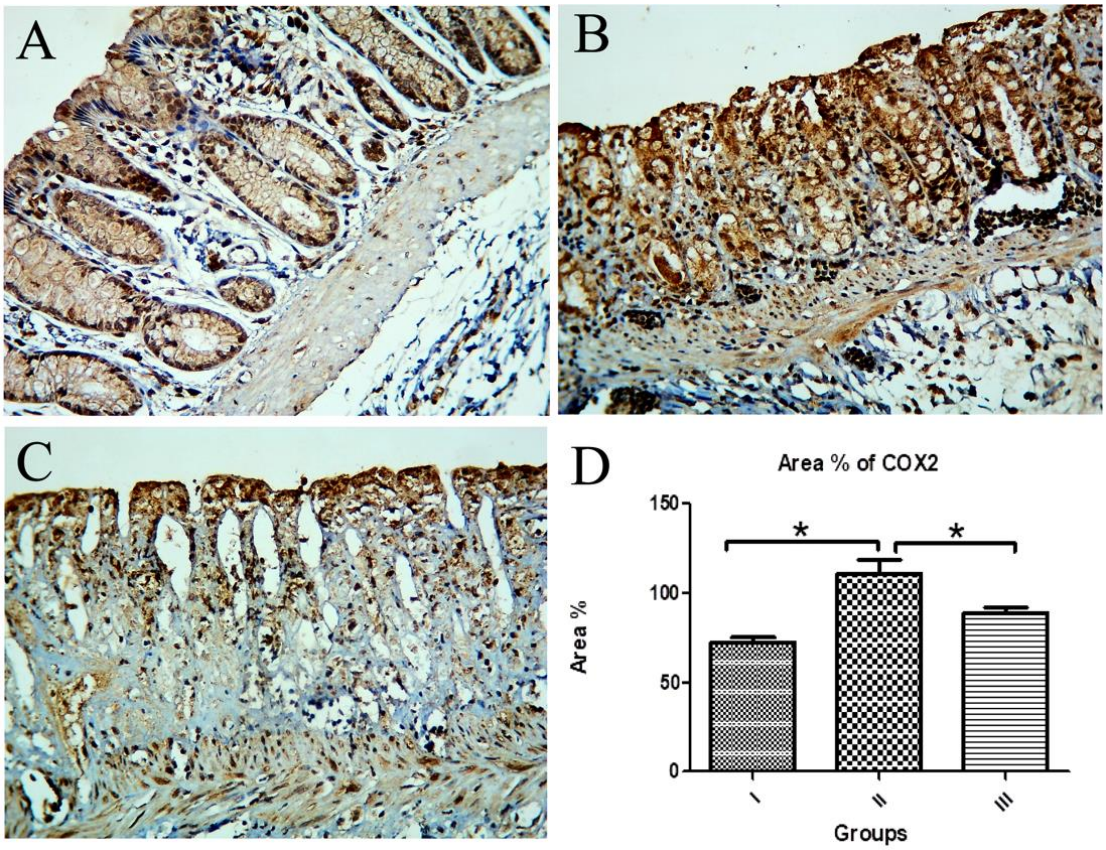

Fig. 4: (A); A paraffin section of the control rat colonic mucosa treated for demonstration of COX-2 immunoreactivity. It shows scanty brown cytoplasmic positive immunoreaction in both surface epithelium and cells of lamina propria. **(B);** group II demonstrated a marked increase in COX-2 positive immunoreactivity in the epithelial cells and cells of lamina propria. **(C);** group III exhibited a decline in COX-2 immunopositive cells in comparison to group II [NOX-2 immunostaining x 200]. **(D);** Diagram of the mean COX2 stained area percentage of the 3 animal groups. * Indicates a significant difference (P<0.005).

DISCUSSION

UC is an idiopathic IBD that is characterized by repeated episodes of acute inflammation together with ulceration and bleeding of colonic mucosa. The pathogenesis of UC is still unclearly understood and multiple trials were conducted to find out the underlying mechanisms (Karakoyun *et al.*, 2018 and Tanideh *et al.*, 2016). In this study, acetic acid-induced colitis is used as a model to mimic human UC in its pathological features, pathogenesis, and inflammatory mediators (Karakoyun *et al.*, 2018).

In the present study, acetic acid administration resulted in ulceration of the colonic surface epithelium with a detachment of apical parts of the epithelial cells and loss of the basement membrane together with distorted dilated crypts and nearly absent goblet cells. The lamina propria demonstrated heavy mononuclear lymphocytic infiltration, congested blood vessels, and extravasated RBCs together with interruption of the muscularis mucosa. Several studies have demonstrated histopathological changes like our results. Sen *et al.* (2017) demonstrated severe epithelial damage, loss of crypts, and inflammatory cell infiltration in the colon of acetic acid-treated rats. Also, Bademci *et al.* (2020) demonstrated hyperemia, hemorrhage, and necrotic areas in the colonic mucosa of acetic acid-treated rats. Moreover, Abdel Mohsen & Ahmed (2019) and Xue *et al.* (2020), and Xue *et al.* (2020) reported similar findings in addition to

the loss of the goblet cell. In the current investigation, depletion of goblet cells was confirmed by sections stained by alcian blue that also showed a reduction in the intensity of alcian blue stain in colonic sections of group II. This was consistent with the findings of Zhang *et al.* (2017) and Kasinathan *et al.* (2018), who described depletion of goblet cells in experimentally induced UC in alcian blue-stained sections. Abdel Mohsen & Ahmed (2019) explained goblet cell depletion as being part of tissue destruction that occurs during the inflammatory process.

Al-Rejaie *et al.* (2013) explained the acetic acid-induced pathological changes by the enhancement of oxidation and lipid peroxidation and free radicals' accumulation in colonic mucosa which in turn provokes tissue destruction with a subsequent increase in reactive oxygen species (ROS) and nitric oxide synthase (NOS). Others (Colares *et al.,* 2016, Moura *et al.,* 2016 and Xue *et al.,* 2020) attributed ulcer development to these free radicals which trigger damage of lipid membrane leading to tissue impairment and production of localized inflammation with overproduction of inflammatory cytokines ending in loss of mucosal integrity and ulceration.

The inflammatory process is confirmed in the current investigation by iNOS and COX-2 immune study. The colonic mucosa of group II showed increased expression of both iNOS and COX-2 in immune-stained sections when compared to the control group. These findings are in accordance with Suluvoy *et al.* (2017) who claimed that enzymes like iNOS and COX-2 are the major enzymes upregulated during inflammation. Similar findings were described by Bezerra *et al.* (2017) who ascribed iNOS and COX-2 overexpression in acetic acid-induced colitis in rodents. Pastrelo *et al.* (2017) attributed iNOS and COX-2 upregulation in colon specimens during UC to the amplification and exacerbation of the inflammatory process. Also, Jalalabadi *et al.* (2018) postulated that COX-2 is involved in causing inflammation and apoptosis while Araujo *et al.* (2017) claimed that iNOS is implicated in the pathogenesis of colitis as it can synthesize large levels of nitric oxide that could interact with free radicals forming more toxic compounds leading to tissue damage.

Several studies aimed to find therapies capable of managing UC however, most symptoms of this disease couldn't be completely eradicated. The effective full management of this disease is still a far goal. Exploring safe and effective therapeutic drugs is still a hot topic in research as regards this disease.

Amygdalin was proven to induce an anti-inflammatory effect by counteracting the inflammatory mediators produced in inflammatory diseases with close pathogenicity to UC (Shin *et al.,* 2003). The current investigation used amygdalin as a curative agent for rats with acetic acid-induced UC. In this study, the use of amygdalin in group III rats promoted regeneration of the surface epithelium, preserved the crypts together with intact muscularis mucosa. However, the lamina propria still show congested blood vessels and extravasated RBCs. Similar findings were ascribed by Bademci *et al.* (2020) who reported histological improvement of the colonic inflammation induced by acetic acid in rats after treatment with vitamin D. They attributed this improvement to the effective anti-inflammatory and antioxidant properties of vitamin D. Moreover, Wang et al. (2019) reported that methane-rich saline could alleviate tissue damage, control inflammation, inhibit oxidative stress and reduce apoptosis in acetic acid-induced UC. They claimed that methane-rich saline prevented acetic acid-induced colitis by blocking the TLR-4/NF-κB/MAPKs signaling pathway and improved the

anti-inflammatory response by promoting the IL-10/JAK1/STAT3 signaling pathway.

In the current investigation amygdalin markedly reduced both iNOS and COX-2 immunoreactivity in both epithelial cells and cells of lamina propria of colonic mucosa of rats with acetic acid-induced UC. In support of our findings, Wang et al. (2020) reported that amygdalin decreases IL-6, TNFα and increases IL-10 expression. They also suggested that amygdalin plays an anti-inflammatory role via MAPKs, AP-1, and NF- κB P65 signaling pathways.

In the present study, amygdalin-induced regeneration of goblet cells appeared in sections stained with alcian blue. This was in agreement with Yakovenko *et al.* (2016) and Jang *et al.* (2018) who described healed colonic mucosa, increased goblet cells, and mucin discharged by goblet cells which forms a thick mucus layer overlying the mucosa after treatment by rebamipide. Helal *et al.* (2020) attributed the ability of rebamipide to treat ulcerative colitis to the decreased expression of TNF-α reaction in colonic tissue.

In conclusion, amygdalin was capable of reversing the histopathological changes of acetic acid-induced colitis by suppressing the inflammatory cytokines (iNOS and COX-2) expression. So, amygdalin could be considered a promising therapeutic agent in managing UC. Further investigations are recommended to determine the optimum amygdalin dose and the duration needed to reach its best curative effect in UC.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of Interest: The author declares no conflict of interest.

Acknowledgments: We are grateful to all behavior laboratory technicians who helped me in this research.

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ARABIC SUMMARY

التأثير العلاجي للأميجدالين على التهاب القولون المحدث بحامض الخليك في الجرذان: دراسة هستوباثولوجية ومناعية هستوكيميائية

ياسر محمد محمود البسطويسي [1،3] ، حازم عبدالحميد محمد [2،3]

1. قسم التشريح و علم الأجنة – كلية الطب – جامعة المنصورة – مصر.
2. قسم التشريح و علم الأجنة – كلية الطب – جامعة أسيوط – مصر.
3. قسم التشريح و علم الأجنة – كلية الطب – جامعة طيبة – المدينة المنورة – المملكة العربية السعودية.

يعتبر التهاب القولون التقرحي عبارة عن التهاب متزايد في الأمعاء يتميز بكونه متكرر ومنتشر في الغشاء المخاطي للقولون والمستقيم ، ويشتمل تطور المرض على التهاب مستمر في صفيحة القولون المخصوصة مع تلف الحاجز المخاطي وكذلك التخلل بعوامل التهابية ، و لا تزال الأسباب الكامنة وراء التهاب القولون التقرحي غير واضحة حتى الآن.

في هذه التجربة تم استخدام ثلاثين ذكرا من الجرزان البالغة بعد تقسيمهم إلى ثلاث مجموعات متساوية ، المجموعة الأولى كانت المجموعة الضابطة ، المجموعة الثانية تم اعطائها حامض الخليك عن طريق حقنة شرجية لمدة 3 أيام متتالية أما المجموعة الثالثة فقد تم اعطائها حامض الخليك كما في المجموعة الثانية متبوعة بالحقن داخل الصفاق لمحلول الأميجدالين مرة واحدة في الأسبوع لمدة 3 أسابيع ، ثم تم أخذ عينات من القولون و صبغها بصبغات الهيماتوكسيلين والأيوسين ، والألشيان الأزرق ، والصبغات المناعية الهستوكيميائية ضد مصنع اكسيد النيترات المستحث وانزيم الأكسده الحلقية 2 ، و قد أظهر الغشاء المخاطي للقولون في المجموعة الثانية تقرحًا ونزيفًا وتشوها في الجريبات و غياب الخلايا المكونة للمخاط و أيضا التخلل بخلايا التهابية و زيادة ملحوظة في النشاط المناعي لمصنع اكسيد النيترات المستحث وانزيم الأكسده الحلقية 2 مقارنة بالمجموعة الأولى ، كما ظهر تحسن ملحوظ في هذه العلامات في المجموعة الثالثة مقارنة بالمجموعة الثانية ، و علي ذلك يمكن أن يكون الأميجدالين عاملًا واعدًا في علاج التهاب القولون التقرحي.