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Asperegillusawamori Attenuates Colon Inflammation in Ulcerative Colitis in Rats.

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Abstract: *Inflammatory bowel diseases (IBD), is a chronic idiopathic inflammatory condition affecting gastrointestinal tract that includes ulcerative colitis (UC) and Crohn's disease (CD) and resulted in many hazards for patients. Aspergillus awamori is a fungus that has long been used for food processing earlier and has been reported that Aspergillus provides beneficial effects on a host's health by affecting the host's intestinal microflora especially on a reduction in the risk of gastrointestinal disease. Therefore, identifying new antioxidants, such as A. awamori, for UC treatment has recently attracted much attention worldwide. Thus, we aimed to investigate the potential alleviating effects of AsperegillusAwamori(AS. Awamori)on acetic acid ‑induced ulcerative colitis in rats. Post-treatment withAsperegillusAwamori (50 mg/kg b.w.) attenuated the severity of colitis as evidenced by decrease macroscopic damage, histopathological findings and leukocyte migration. ASP. Awamorialso suppressed the inflammatory response via attenuation of tumornecrosis factor-α (TNF-α), IL-Iβ and also via inhibition of oxidative stress as suppression of lipid peroxides and nitric oxide (NO), superoxide dismutase (SOD) and glutathione peroxidase (GPx). These findings highlight the beneficial effects of AS. Awamoriin IBDtreatment via its role in modulation of colonic inflammation, and oxidative stress.*

Key words: *IBD; ulcerative colitis, acetic acid, Aspergillus awamori, TNF-α, SOD,GPx*

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I. Introduction

Ulcerative colitis is multiple disorders mediated immunologically that are together referred to as inflammatory bowel diseases (IBD). It affects primarily the mucosal lining of the colon and rectum **(Podolsky, 2002)**. Reasons of IBD remain unclear, whereas environmental factors, in combination with genetic factors **(Fiocchi, 1998; Loftus Jr, 2004)** and altered immune response driven by microbial factors in the enteric environment **(Korzenik and Podolsky, 2006)** are proposed to be involved in its pathological events. For instance, the mucosal immune system is reported as the main mediator of intestinal inflammation and injury, with cytokines playing a central role in initiating inflammation **(Ardizzone and Bianchi Porro, 2005; Nakamura *et al.*, 2006).**

Activation of the intestinal immunity ends with the production of proinflammatory cytokines, such as tumor necrosis factor (TNF-a), interleukin-1b (IL-1b) and leukotrienes (LT) 7.Crucially, infiltration of inflammatory cells, besides the overproduction of pro inflammatory cytokines eventually gives rise to ulcerative mucosal disruption**(Dionne *et al.*, 1997)**.The nitric oxide (NO) system and cyclooxygenase (COX-2) have been shown to modulate many events in the gastrointestinal tract. Several studies are reporting that bothinducible nitric oxide synthase (iNOS) and COX-2 are clearly upregulated after the stimulation of host cells with bacteria or inflammatory cytokines, such as TNF-a and IL-1, indicating their role in process of the underlying ulcerative pathogenesis **(Nussler and Billiar, 1993; Simon, 1999)**.

It has been reported that Aspergillus provides beneficial effects on a host's health by affecting the host's intestinal microflora. Their beneficial effects on human health, including the alleviation of lactose intolerance, immunomodulation, hypocholesterolemic effects, and a reduction in the risk of gastrointestinal disease have been demonstrated previously**(Ljungh and Wadstrom, 2006; Delcenserie *et al.*, 2008)**. *Aspergillus awamori* (*A. awamori*), a variant of *Aspergillus niger*, is a fungus that has long been used for food processing in Japan**(Bigelis and Lasure, 1987)**. *A. awamori* is also known to produce enzymes that enhance carbohydrates and proteins digestion **(Gracia *et al.*, 2003)**. Furthermore, it was reported that unsaturated fatty acid levels are increased, while saturated fatty acid levels are decreased in skeletal muscle after

As. awamori feeding **(Saleh *et al.*, 2012)**. Polyunsaturated fatty acids reduce the risk of cardiovascular diseases by reducing blood lipids levels and platelet reactivity and aggregation, indicating that A. awamori feeding may be effective in reducing the risk of lifestyle-related diseases in humans. Here, we report that A. awamori modifies the plasma lipids profile and changes the liver fatty acids composition in rats with no harmful effects.At present available therapies for ulcerative colitis are only effective in ameliorating the disease symptoms, while having many associated disadvantages **(Sartor, 2004)**. To date, however, the possible modulatory role of aspergillus in colon inflammation has not been yet verified; hence, we aimed in the current investigation to evaluate and compare the possible modulating effect of aspergillus on acetic acid induced ulcerative colitis model in rats.

II. Materials And Methods

Experimental animals

Twenty-Five (25) male,12-16 weeks old agealbino rats with average body weight(150-170 g) were used in this study. The rats were obtained from Laboratory Animals research Center, National Central Institute; Dokki, Egypt. Animals were housed in separate metal cages (five animals per cage), fresh and clean drinking water was supplied,fed on consistent ration through course of experiment. Rats were kept at constant environmental and nutritional conditions throughout the period of experiment. The animals were left 7 days for acclimatization before the beginning of the experiment.All efforts were made to minimize animal pain or suffering during experimentation.

Chemical and drugs

All chemicals were of analytical grade and obtained from standard commercial suppliers. The chemicals and drugs used in the present study are shown in (**Table. 1**)**.**

Name	Form\concentration	Amount	Suppliers
Glacial acetic acid	5% solution	1 ml per rat	*El Nasr Pharmaceutical Chemicals Co* (ADWIC), Egypt
Aspergillus awamori	Black powder	25-100 mg/kg.bw	Gift from Dr.Ahmd Saleh, Faculty of Agriculture, Kafrelsheikh University

Antioxidants Chemicals ofMalondialdehyde (MDA), Nitric oxide (NO), Glutathione Peroxidase (GPx), and Superoxide Dismutase (SOD) kits were purchased from Biodiagnostic Co., Giza, Egypt. Acetic acid was obtained from El-Nasr Chemical Co. (Cairo, Egypt).

Experimental design

The 25 rats were randomly divided into three main groups as follows (Table.2);

- **Group1 (G1, normal control negative group):**composed of 5 rats, received saline orally for 8 daysfrom the start of experiment until the end of the experimentwith a single rectal instillation of saline on day 8th from the beginning of the experiment.
- **Group 2 (G2, acetic acid group):**included 10 rats, given a single dose of 5%acetic acid (1 ml/rat) intra-rectally at day 8th from the start of the experiment and euthanized at day 16th (the end of the experiment).
- **Group 3 (treated group):**Composed of 10 rats**,** injected by acetic acid 5% at day 8th from the experiment then treated by50 mg / kg aspergillus awamori daily from day8thuntil day 16th (the end of the experiment).

Table (2) showing experimental design and treatment of each group

Groups	N	Treatment		
		1st 8daysintra gastric	Intra rectal at day 8th	Oral (from day 9$^{th)}$
G1 Control –ve	5	Saline	Saline	-
G2 Control +ve	10	Saline	1ml acetic acid 5%	-
G3	10	Saline	1ml acetic acid 5%	Asp,50mg/kg.bw

Induction of colitis by acetic acid

The animals were fasted for 24 h with free access towater before induction of colitis. Colitis was induced in rats by intra-rectal (IR) administration of 1ml, of acetic acid 5% in normal salinesolution under ether anesthesia. A soft 6F polypropylene catheter 2mm diameter lubricated with jelly was inserted 6–8 cm via the anal canal into the colon. Two milliliter of AA 5% in normal saline, was slowly infused into the distal colon, and rats were maintained in a head‑down position (a supine Trendelenburg position) for 30 sec to limit the expulsion of the solution for 30 s to limit the expulsion of the solution **(Kandhare *et al.*, 2012)**.

Hematological analysis: The hematological analysis was determined by using fully automated hematology analyzer, Exigo®, Boule Medical AB., Sweden, at Faculty of Veterinary Medicine, Damanhur University. The following parameters were measured, Red blood cell count (RBCs; 10^6/µL), Hemoglobin (Hb; gm/dl), Packed cell volume (PCV; %), Hematocrit (HCT;), MCV, MCH, MCHC, Red cell distribution width (RDW; fl), total White blood cell count (WBCs count; 10^3/µL), Neutrophils count (Neu; 10^3 /µl) Lymphocytic count (Lym; 10^3/µL), Monocytes count (Mon; 10^3/µL), Basophils count (Baso; 10^3/µL), Esinophils (Esino; 10^3/µL), and Platelet count (PL; 10^3/µL).

Biochemical analyses: Colonic tissues were cut, weighted and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 rpm for 15 minutes at 4°C then the resultant supernatant were used for the determination of the following parameters: Nitric oxide (NO), L-malondyaldhyde (L-MDA), super oxide dismutase (SOD), and glutathione peroxidase (GPx).Total NO was determined by measuring its stable metabolites based on the method of Miranda et al.**(Miranda *et al.*, 2001)**. Lipid peroxide levels, expressed as malondialdehyde (MDA), was carried out according to the thiobarbituric acid assay of Buege and Aust**(Buege and Aust, 1978)**.GPx activity was determined according to the method of Paglia and Valentine **(Paglia and Valentine, 1967)**. SOD activity was assayed according to the method of Marklund and Marklund**(Marklund and Marklund, 1974)**.

Molecular analysis: Real time PCR (qPCR) was used to detect the relative gene expression of the pro-inflammatory cytokinesIL1β and TNFαin colon tissue that reflects the changes in transcription levels of these gene in acetic acid-induced ulcerative colitis rats in the *Aspergillus awamori*treated group in comparison to control-ve and control +vegroups. To conduct real time PCR, we first isolated total RNA from colon tissues of all rat groups. After extraction, the quality and concentration/ of total RNA were assessed by Nanodrop which revealed presence of pure RNA with a considerable higher concentration (ranged from 1400 to 2350 ng/µl).The isolated total RNA was reverse transcribed into cDNA which was used as a template for qPCR. Throughout the whole real time PCR experiment, the housekeeping gene encoding *β-actin* was used as an internal reference for normalization and data was expressed as mean ± standard error of mean (SEM). The expression level of the target genes in control rat (G1) was considered the base line.

Histopathological examination: Specimens from colon tissue were fixed in 10 % neutral formalin for twenty four hours. The fixed specimens were washed in tap water and then processed in ascending grades of ethyl alcohol for dehydration, cleared in xylene and paraffin embedded Sections of 4 -5 µ thickness were obtained and then stained with Harris hematoxylin and eosin (H & E), mounted in Canada balsam, dried and examined with light microscope**(Bancroft and Gamble, 2008)**.

Statistical analysis: All data are expressed as mean ± standard error (SEM) of eight rats per experimental group. Statistical analysis was performed using GraphPad Prism 5 statistical software (San Diego, CA, USA). Parametric one-way analysis of variance (ANOVA) followed by the Tukey–Kramer multiple comparisons test was used to compare the mean values of quantitative variables among the groups. The minimal level of significance was identified at $p < 0.05$.Significance of non-parametric data used for analyzing the macroscopical tests (score of bloody diarrhea and lesion score) was achieved using GraphPad Prism software version 5 (Graph Pad Software Inc., San Diego, CA) and was evaluated by the Kruskal–Wallis test [non-parametric ANOVA] followed by Dunn's multiple comparisons test, $p < 0.05$ 15.

Results

1. Clinical signs:

This disease is clinically presented with abdominal pain, diarrhea, and sometimes accompanied by mucus, blood, or pus secretion.

2. Gross pathology and histopathological findings:

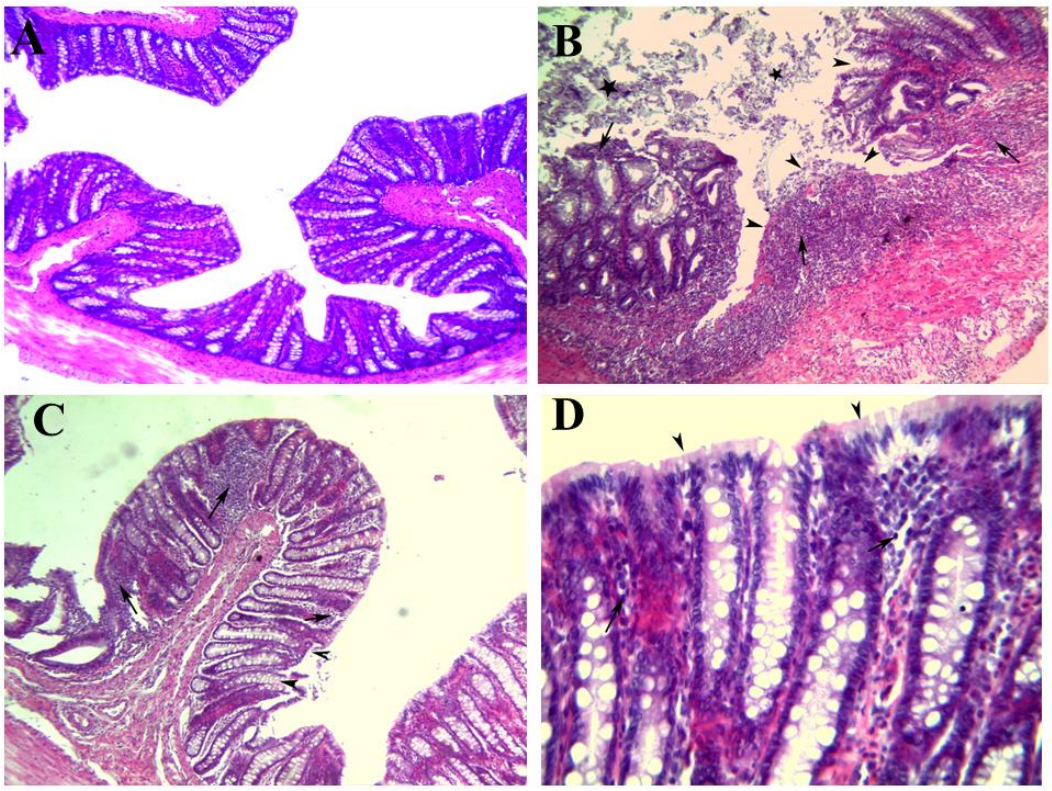
The Acetic acid control rats showed elevated macroscopic lesionsin the form of colon hemorrhage and ulceration. Ratsadministrated Aspergillus Awormi extract after induction of colitis, showed mild to moderate macroscopic changes. In the normal control group there was no visible damage.

Intra-rectal installation of 1 mL (5%) acetic acid in ratsinduced a severe inflammatory reaction as evidenced bythe gross macroscopic ulceration.

Normal control group, showing normal mucosal epithelium of tall columnar epithelial cells with goblet cells (Fig.1A). In the AA group, the colon morphological studies showed that AA induced moderate to serious damage to the mucous glands with inflammatory cell infiltration,widely eroded mucosa with ulcerations, and necrosis associated with edema (Fig.1B).

Treatment of acetic acid induced colitis with the*Aspergillus awamori*(50 mg) resulted in a moderate amelioration ofinflammation. Epithelial mucosa was intact in 50% of examined animals andslightly eroded inthe

others. Moderate to mild inflammatory cells infiltration were seen in mucosal and submucosal layer with mild edema and goblet cell loss (Fig.1C,D)



(FIG.1)(**A**) Control rats receiving saline rectally showed normal architecture of mucosa with intact epithelial surface, submucosa and muscularis layer, H&E X 100.(B)Rats receiving acetic acid rectally characterized by severe colitis described in heavy infiltration of mucosal and submucosal layer by leukocytes (arrows) with sloughing of epithelial mucosa (arrowhead) represented in (black stars), H&E X 100. (C-D) Treated group received (50 mg/kg BW) of AsperegillusAwamori revealed epithelial mucosal preservation with slight epithelia sloughing (arrowhead) and moderate inflammatory cell invasion of epithelial mucosa (arrows). H&E X100. (D)Colon of rats received (50 mg/kg BW) of AsperegillusAwamorirevealed mucosal preservation (arrowhead), diminished inflammatory cell invasion (arrows) with intact goblet cell (arrowhead) in (D), H&E X400.

3. Haematological result:

The results of hematological examination as demonstrated in table 3, showed significant decrease in Hb % content, RBCs count,HCT (PCV%) andMCHC and a significant increase in MCV and MCH of acetic acid group(G2) as compared to the control group (G1) (Table.3) However, no significant difference in RDW was noticed between G2 and G1.Acetic acid group (G2)showed a significant increase in total leukocyte (WBCs) and neutrophils count, and a significant decrease in lymphocyte countas compared to the control group (G1). Also Acetic acid-induced colitis group(G2)exhibited a significant increase in platelets countand MPVas compared to the control group (G1). In contrast, *Aspergillus awamori*– treated groups, showed a significant improvement in plateletscount and MPV (Table.3,4).

Table 3: Levels of the different parameters of hemogram affectedin *Aspergillus awamori* (ASP)treated group in comparison to the c-ve and c+ve group.

Group	Hb (g/dl)	RBCs (10^6/µl)	HCT (%)	MCV (fl)	MCH (fl)	MCHC (fl)
G1	14.2±0.54 ^{a}	5.6±0.19 ^{a}	44.8±1.03 ^{a}	70.48±1.65 ^{c}	23.82±0.76 ^{c}	33.1±0.87 ^{a}
G2	10.15±0.41 ^{c}	3.85±0.13 ^{c}	34.95±1.11 ^{d}	87.50±1.70 ^{a}	29.90±0.60 ^{a}	30.14±0.80 ^{b}
G3	13.18±0.42 ^{a}	5.3±0.12 ^{a}	40.08±1.05^{bc}	69.26±1.63 ^{c}	22.06±0.61 ^{c}	33.44±0.69 ^{a}

Data were statistically analyzed as mean ± SEM (n = 5).

- Columns carrying different superscript letters are significantly different at p≤0.05.

Table 4: Effects of Aspergillus (ASP) on blood leukocyticdifferentiationand platelets count from rats with acetic-acid-induced ulcerative colitis.

Items	G1	G2	G
Total leukocytes(10^3/µl)	7.58±0.33^{d}	13.95±0.42^{a}	10.9±0.44^{b}
Neutrophil count (10^3 /µl)	48.2±1.10^{d}	60.5±1.45^{a}	53.00±1.12^{c}
Lymphocytic count (10^3 /µl)	40.2±0.90^{a}	36±0.82^{b}	33.6±0.76^{c}
Monocytes(10^3 /µl)	9±0.43	10±0.50	10±0.56
Basophil(10^3 /µl)	1.4±0.24	1±0.12	1.6±0.11
Eosinophil(10^3 /µl)	1.2±0.22	1±0.21	0.8±0.10
Platelets count(10^3 /µl)	340.4±9.34^{c}	445.5±10.90^{a}	380±9.91^{b}
MPV (fl)	6.3±0.30^{b}	8.1±0.37^{a}	5.7±0.28^{bc}

Data were statistically analyzed as mean ± SEM (n = 5). Columns carrying different superscript letters are significantly different at p ≤ 0.05.

4. Oxidative stress and antioxidant activity result:

In the current research, Administration of acetic acid(G2)(model colitis) resulted in a significant elevation in colon MDA and NO levels and a significant decline in colon SOD, GPX levels in comparison with the control group (G1) **(Table 5).** In contrast, administration of *Aspergillus awamori*(G3) resulted in a significant reduction in colon MDA,NO levels and a significant elevation in SOD, GPX levels.

Table 5: Effectof *Aspergillus awamori* on the activities of SOD, GPX, and NO and MDA levels in colon.Mean values with different superscript letters in the same column are significantly different at (P≤0.05). Data are presented as (Mean ± SEM), SEM = Standard error of mean.

Group	MDA nmol /g tissue	GPx U/g tissue	SOD U/g tissue	NO umol /g tissue
G1	0.14±0.01^{e}	47.27±0.81^{a}	6.82±0.28^{a}	27.72±0.81^{d}
G2	0.87±0.04^{a}	19.18±0.52^{d}	2.14±0.14^{d}	56.34±1.2^{a}
G3	0.41±0.02^{c}	36.38±0.59^{bc}	4.92±0.2^{bc}	36.04±0.6^{c}

5. Molecular analysis results:

The qPCR data revealed a significant (P≤0.05) up regulation in the expression level of *TNFα and IL-b* gene in colon tissues of acetic acid-induced ulcerative colitis rats(G2)as compared to the control(G1) group (Table 6).This expression was significantly down regulated following administration of *Aspergillus awamori*with lowest expression at dose (50 mg/kg bw).

Table.6. Level of TNFα and IL-brelative gene expression in colon tissues in comparison to acetic acid-induced ulcerative colitis in rats.

Criteria	**Mean Fold change of TNFα**	**Mean Fold change of IL1b**
G1	1.00^{f} ±0.07	1.00^{g} ±0.06
G2	12.21^{a} ±0.46	14.72^{a} ±0.55
G3	2.41^{e} ±0.2	3.61^{e} ±0.24

Means within the same column carrying different superscript letters are significantly different (P≤ 0.05).

Discussion

Inflammatory bowel disease (IBD), is a chronic inflammatory disorder of the intestinal tract, resulting in abdominal pain, bloody diarrhea, mucous in stool, weight loss and sometimes leads to colorectal cancer**(Podolsky, 2002)**and jeopardize the quality of life of patients suffering from these disorders**(Fiocchi, 1998; Baumgart and Carding, 2007)**. Multifactorial causes were contributed to IBD pathogenesis such as genetic, immune and environmental factors**(Podolsky, 2002; Baumgart and Carding, 2007)**.

Currently, the clinical management of IBD is based on using anti‑inflammatory agents such as corticosteroids, aminosalicylates, and immunosuppressants. These drugs are known for having serious adverse effects **(Wang and Fu, 2005)**.

Therefore, for disease control and prevention, extensive studies on natural remedies particularly using those with antioxidant properties, have been conducted **(Molodecky and Kaplan, 2010)**.*Aspergillus awamori* is a fungus that has long been used for food processing in Japan. The products processed by *A. awamori* are given GRAS (Generally Recognized as Safe) status by the U.S. Food and Drug Administration **(Bigelis and Lasure, 1987)**. It has been reported that Aspergillus provides beneficial effects on a host's health by affecting the host's

intestinal microflora. Their beneficial effects on human health, especially on a reduction in the risk of gastrointestinal disease have been demonstrated previously **(Ljungh and Wadstrom, 2006; Delcenserie *et al.*, 2008)**. However, the exact mechanism by which *A. awamori* exerts its effect remains to be fully elucidated. Therefore, identifying new antioxidants, such as *A. awamori*, for UC treatment has recently attracted much attention worldwide and for that in this study was aimed to evaluate the potential ameliorative (therapeutic) effect of *Aspergillus awamori*on acetic acid‑induced colitis in rats to investigate IBD conditions in animal models as this model of colitis induction is reproducible, rapid, and have many characteristics resembling to human UC is known as one of the most common methods **(Low *et al.*, 2013)**. The macroscopic damage parameters of the colon after acetic acid treatment revealed increased colonic weight, mucosal hyperemia, edema, erosion, and ulceration in control groups and increased weight of colonic tissue due to inflammatory response, which is indicative of severity and extent of the disease **(Somani *et al.*, 2014)**. No changes were observed in normal saline group suggesting that handling and surgical procedure had no interference with experimental outputs.

Intra rectal administration of acetic acid resulted in inflammation characterized by increased neutrophil infiltration into the intestinal tissue, massive necrosis of mucosal and submucosal layers, vascular dilation, edema and submucosal ulceration. This is line with similar observation that are noteworthy features of human colitis **(Randhawa *et al.*, 2014)**.The histologic picture of inflammatory bowel disease due to acetic acid is marked by the presence of inflammatory cells as lymphocytes, neutrophils, and histiocytes infiltrating the mucosal and submucosal layer of the intestine. This enhanced inflammatory infiltration is accompanied by extensive mucosal injury, including disruption of extracellular matrix, edema, epithelial cell necrosis, and ultimately erosion and ulceration **(Guagnozzi and Lucendo, 2014)**.We found a significant difference in the severity of inflammation between acetic acid colitis and aspergillusawormitreatedgroups. The Aspergillus awormitreated group with thedose of 50 mg/kg did not impose macroscopically visible lesions and produced very mild histological colonic injures and significantly diminished the severity of macroscopic and microscopic injuries.

In the current study, Aspergillus *awamori* attenuated leukocyte influx to inflamed colon as revealed by histopathology. The mitigation of leukocyte influx may account for the beneficial effects of Aspergillus *awamori*against colon injury and is most likely mediated via the observed inhibition of TNF-α and oxidative stress in colonic mucosa **(Mizushima *et al.*, 2009)**. As in the current study, enhanced oxidative stress was verified by a significant elevation in colon MDA and NO level with concomitant decline in SOD&GPx activities in ulcerative colitis. These observations are in line with previous studies **(Witaicenis *et al.*, 2012; Nagib *et al.*, 2013)**.In these studies, the effective treatment mitigated the expression of TNF-α and IL-β genes at both mRNA and protein levels **(Zhang *et al.*, 2006; Gillberg *et al.*, 2013)**. Previously, decrease in the mRNA levels of proinflammatory genes have been regarded as an early sign of suppressed inflammatory signaling**(Gillberg *et al.*, 2013)** as the downstream of iNOS and TNF-α is regarded as an advantage in the management of IBD**(Liu and Wang, 2011)**.

From these results, it may be concluded that the extract of ***Aspergillus awamori*** was more effective in attenuating macroscopic and microscopic findings of inflammation and tissue damage induced in rats by intracolonic administration of acetic acid as shown by inhibition of TNF-α and IL-1β. All of these may suggest the possibility of developing AS.awarmi as a safe and potent anti-inflammatory and antioxidant substance in the fight against IBD.

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