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Artemisinin Attenuates Inflammation in Rats with Ulcerative Colitis Through Inhibition of Inflammatory Biomarkers, Oxidative Stress and Adhesion Molecules

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Abstract

Background Ulcerative colitis is a chronic refractory inflammatory disease affecting the colon. Several drugs have been developed for it, nevertheless, there are limitations in the therapy due to the inadequate responses and significant undesirable effects. Therefore, novel safer drugs with more therapeutic efficacy are needed.

Objective To investigate the potential anti-inflammatory effects and histological outcome of artemisinin in acetic acid-induced ulcerative colitis in rats.

Methods Rats with colitis were received either artemisinin 100 mg/kg or sulfasalazine 100 mg/kg orally for 7 days. Macroscopical and microscopical assessment, the measurement of the colonic cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin-4 (IL-4)), myeloperoxidase (MPO), and E-Selectin.

Results Both macroscopical lesion area and histological colonic damage induced by acetic acid were significantly reduced by artemisinin and sulfasalazine accompanied by attenuation of the elevated colonic TNF-α, IL-4, MPO activity and E-Selectin.

Conclusion Artemisinin had an effective role in experimental colitis in rats through anti-inflammatory and antioxidant actions.

Keywords Acetic acid, artemisinin, oxidative stress, E-Selectin, ulcerative colitis, IL-4

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List of abbreviations: AA = Acetic acid, ANOVA = Analysis of variance, CD = Cluster of differentiation, DAI= Disease activity index, DMSO = Dimethyl Sulphoxide, E = Eosin, H = Hematoxylin, ICAM-1 = Inter cellular adhesion molecule-1, IHC = Immunohistochemistry, IL=Interleukin, MAPK = Mitogen-activated protein kinase, MPO = Myeloperoxidase, NF-Kb = Nuclear factor kappa B, ROS = Reactive oxygen species, STAT = Signal transducer and activator of transcription, SPSS = Statistical package for social sciences, TNF-α = Tumor necrosis factor-alpha, UC = Ulcerative colitis, VCAM-1= Vascular cell adhesion molecule

Introduction

Ulcerative colitis (UC) is a chronic refractory inflammatory disease affecting the colon and its incidence is increasing throughout the world. It is triggered by an irregular immune response connected with environmental, genetic, and intestinal microbiota imbalance [(1)]. The imbalance of cytokines modulated by activated immune cells should be the primary factor that causes spread superficial inflammatory damage in UC

[2]. Activation of these infiltrating immune cells result in the release of various pro-inflammatory mediators that play a pivotal role in tissues destruction and propagation of the inflammatory responses [3]. Although wide spectrum of medical treatment of UC such as corticosteroids, immunosuppressants, and biological drugs, undesirable effects and incomplete efficacy of currently used therapies is a continual challenge, so, there is a need to new and safer therapies for UC [4].

Artemisinins are a family of anti-malarial agents originally derived from *Artemisia annua L*. [5]. Artemisinin are blood schizonticides active against *Plasmodium falciparum*, including multidrug resistant strains. In addition to its clinical anti-malarial effect, artemisinin has been evaluated in animal models of autoimmune diseases, allergic disorders, cancers and septic inflammation [6]. Nevertheless, inadequate data are accessible with reference to the therapeutic outcome of artemisinin in induced colitis. Hence, we aimed to assess the protective effect of artemisinin versus experimental colitis.

Methods

Materials

Animals: Forty adult albino male rats (200-220 g) were supplied from the Animal House of College of Science/Thi-Qar University. Animal were housed five per cage for 7 days before any experiments started to acclimatize to the animal room conditions of controlled temperature 28-30 ^{o}C, with a 12 hr light /12 hr dark cycle and had access to laboratory chow pellet and were allowed to drink tap water ad libitum. All ethical themes of the studies on animals were considered carefully and the experimental protocol was approved by the Institutional Review Board (IRB) in the college of medicine of Al-Nahrain University.

Chemicals and drugs: Acetic acid (AA) and diethyl ether (BDH Chemical Ltd., England), dimethyl Sulphoxide (DMSO), sulfasalazine and artemisinin (Sigma–Aldrich), immunohistochemistry (IHC) kits (Abcam/UK), were purchased.

Experimental design

This study was performed on 40 adult albino – Wister male rats. Animals were divided into four group (n=10/group). Group I kept as normal control and received no treatment. Group II, III and IV were subjected to the induction of colitis by rectal administration of 4% AA (v/v). One hour after the induction of colitis group II was given 1 ml of 1% DMSO orally; group III and IV were treated orally with artemisinin 100 mg/kg and sulfasalazine 100 mg/kg respectively for 7 days.

Induction of UC

Rats previously subjected to starvation for at least 24 hr before the induction of colitis but they were allowed free access to tap water; during starvation, rats were kept in cages provided with a wide wire-mesh floor to avoid coprophagy [7]. On the day of the experimental colitis induction water was interrupted two hours before the procedure according to the method described by Manna et al. [8] with slight modification. Briefly, under light ether anesthesia rats were administered 5 ml/kg of 4% AA solution by transrectally using a flexible silicone plastic tube with an external diameter of 2 mm was inserted into the colon to 8 cm. Then, rats were holed in head down position for 2 min to prevent AA solution leakage. Control animals submitted the same procedure using equal volume of normal saline instead of AA solution.

Preparation of drugs

The sulfasalazine and artemisinin freshly prepared before administration on the day of the experiment. Investigated drug (artemisinin) suspend in 1% DMSO and the standard sulfasalazine was suspended in distilled water. The dose of artemisinin 100 mg/kg was selected according to other studies reporting cytokines suppression effect of artemisinin [9]. Sulfasalazine was used as standard therapy in a dose of 100 mg/kg [10].

Assessment of colitis

After the ending of experiment, animals were sacrificed by high dose of diethyl ether inhalation and rapidly dissection of the abdomen, thereafter the colon was extracted. The colon specimens were opened longitudinally, cleansed gently using normal saline, and observed normally for macroscopic assessment. At last, the colon samples were fixed in 10% formalin for histopathological and immunohistochemistry examination.

Clinical evaluation

Colon edema

The colon sample of each animal was incised along its mesenteric border and gently washed; the colon edema was determined by measuring the colon weight by a sensitive balance after plotting the tissue on a filter paper to discard excess water. It was used as indicator of tissue edema and the intensity of colitis [11].

Disease Activity Index (DAI)

The DAI defined by Mao et al. [12] was used to estimate the disease clinically, which include bodyweight loss {(0) no reduction or weight gain; (1) 1-5% reduction; (2) 6-10% reduction; (3) 11-20% reduction; (4) more than 20% reduction}. The grades of stool consistency {(0) Normal; (2) loose; (4) diarrhea}, and rectum bleeding {(0) normal; (2) mild; (4) severe bleeding}. The DAI was calculated as the sum of total scores.

Macroscopic colonic score

The colonic samples were examined visually. The macroscopic score based on the clinical features of the colon according to scoring system ranging from 0-6 as follows: 0 = absence of inflammation; 1 = redness or swelling; 2 = swelling and redness; 3 = one or two ulcers; 4 = one large ulcer or more than two ulcers; 5 = initial necrosis; 6, severe necrosis [13].

Histopathological examination

The colonic samples were fixed in 10% formalin at room temperature. Dehydration, paraffin embodiment, and deparaffinization were done on the specimen, prepare 4 µm thick section from each colonic sample and stained with hematoxylin and eosin (H&E). Slides were examined and scored for histopathological evaluation in a blinded fashion by experienced histopathologist and results evaluated according to scoring system ranging from 0-3 (0: normal,1: focal, 2: zonal, 3: diffuse), which estimated the extension of: epithelial damage, and/or glandular crypts dilation, loss of goblet cells, inflammatory cells infiltration, crypt abscesses, edema, mucosal hemorrhage and dysplasia [14].

Immunohistochemistry

IHC techniques exhibit the benefit of directly demonstrating cells in the affected tissue [15]. The IHC reactions were produced by the presence of specific antibodies, concomitantly the estimation of the production of a number of biochemical markers in intestinal tissue specimens that were paraffin-embedded so as to measure the colonic cytokines, adhesion molecule, and oxidative stress markers. Quantification of IHC was carried out in accordance to the following semi quantitative scores [16]: 0: no staining, 1: ≤ 25%; 2: 26-50%; 3: 51-75%; and 4: 76-100%; that based on the percentage of positively stained cells.

Statistical analysis

All the data were presented as mean ± standard deviation. Unpaired t test was used for comparison of means of two groups, while ANOVA (analysis of variance) with post hoc Tukey test were used for comparison of means of parameters among groups. Statistical package for social sciences (SPSS) version 23 were used to analyze the results. P value less than 0.05 were considered significant [17].

Results

Effect of artemisinin on macroscopic scores

The colonic mucosa of colitis untreated rats showed edematous inflammation, extensive ulceration and necrosis versus normal colonic mucosa of healthy group, the rats that administered artemisinin or sulfasalazine orally treatment produce significant reduction to the

DAI and colonic weight. In addition, both drugs significantly ($p<0.01$) decrease the macroscopical score as shown in table 1.

Effect of artemisinin on histopathological scores

The present study exhibits the histological changes in untreated colitis, primarily showed mucosal ulceration and necrotic tissue, heavy mononuclear inflammatory infiltrate, complete depletion of goblet cells as displayed in figure (1&2). Furthermore, both sulfasalazine and artemisinin treated groups evolve significant ($p<0.01$) attenuate in the histopathological score as evidenced by mucosal regeneration and glandular formation; moderate inflammation and moderate depleted goblet cells as displayed in figure (3). However, there were no significant difference between both sulfasalazine and artemisinin in their histopathological scores (Table 1).

Table 1. Macroscopic and histopathological parameters in study and healthy control groups

Macroscopic and Histopathological Parameters	Groups (n=10/group)			
	Healthy control	Colitis + DMSO	Colitic rats treated with Sulfasalazine	Colitic rats treated with Artemisinin
Colonic weight (g)	1.27±0.15 A	3.13+0.2 B	1.61±0.07 C	1.6±0.08 C
Disease activity index	0.0+0.0 A	9.67+1.51 B	2.0+2.11 C	2.0+1.0 C
Macroscopic score	0.0±0.0 A	3.5+0.84 B	1.7±1.34 C	1.8±0.92 C
Histopathology	0.0±0.0 A	2.83+0.41 B	1.5±0.71 C	1.8±0.63 C

Comparison expressed by letters; different letters denote significant difference. The expression of values as mean ±Standard deviation (SD)

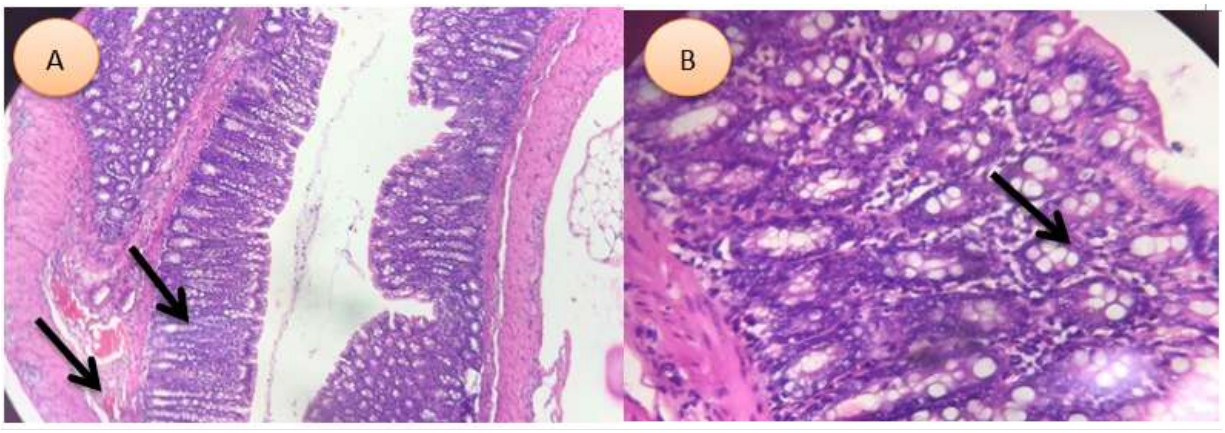

Figure 1. Histological section through colonic wall showing normal mucosal and submucosal pattern with no evidence of inflammation and preservation of colonic gland with goblet cells (arrow); A: 20X; B: 40 X; H and E stain

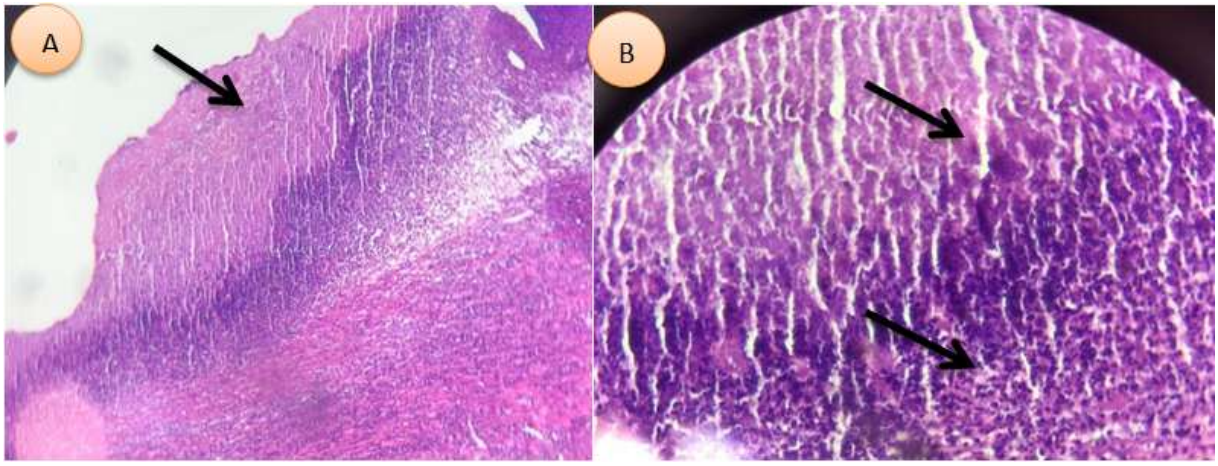

Figure 2. Histological section through colonic wall showing mucosal ulceration and necrotic tissue; heavy mononuclear inflammatory infiltrate in experimentally induced colitis in rat(arrow); A: 20X; B: 40 X; H and E stain

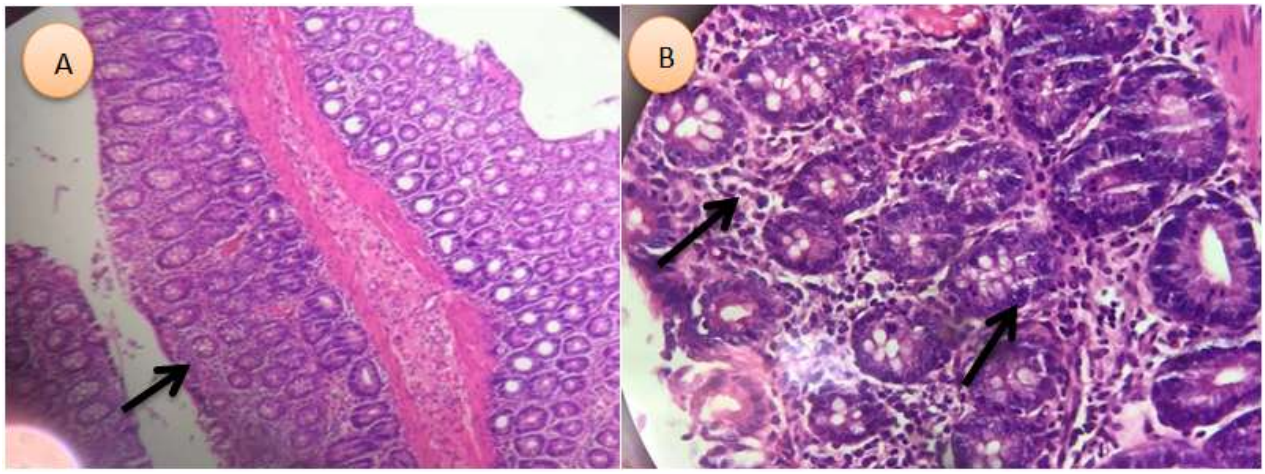

Figure 3. Histological section through colonic wall showing the effects of treatment after 7 days in which, there is evidence of mucosal regeneration and glandular formation; mild inflammation and slightly depleted goblet cells (arrow); A: 20X; B: 40 X; H and E stain

Effect of artemisinin on the cytokines; TNF-α and IL-4

As shown in Table 2, colonic levels of TNF-α and IL-4 showed significant elevation after AA introduction compared to those of control group; these values were significantly ($p<0.01$) diminished in rats treated with artemisinin and sulfasalazine. While there was no significant difference between both sulfasalazine and artemisinin in their IHC expressions scores of cytokines TNF-α and IL-4.

Effect of artemisinin on the myeloperoxidase

After artemisinin and sulfasalazine treatment, the high colonic myeloperoxidase (MPO) level in the induced group was found to be significantly ($p<0.01$) decreased. However, there was no significant difference between sulfasalazine and artemisinin in their IHC expressions scores of MPO (Table 2).

Effect of artemisinin on the E-selectin (CD62)

The elevated colonic CD62 in the colitis group was found to be significantly ($p<0.01$) decreased after artemisinin and sulfasalazine treatment (Figure 4). However, artemisinin exhibited a better reduction results in the scoring of the CD62 parameter compared with the sulfasalazine group displayed in table 2.

Table 2. Immunohistochemical score for cytokines, oxidative stress and adhesive molecule in study and healthy control groups

Cytokines, oxidative stress and adhesive molecule Parameters	Groups (n=10/group)			
	Healthy control	Colitis + DMSO	Colitic rats treated with Sulfasalazine	Colitic rats treated with Artemisinin
Tumor necrosis factor-α	1.0±0.0 A	3.33+0.52 B	1.0±0.0 A	1.0±0.0 A
Interleukine-4	1.0±0.0 A	3.67+0.52 B	1.5±0.53 C	1.8±0.42 C
Myeloperoxidase	1.0±0.0 A	3.5+0.84 B	1.6±0.52 C	1.8±0.63 C
E-selectin	1.0±0.0 A	3.67+0.52 B	1.7±0.48 C	1.4±0.52 C

Comparison expressed by letters; different letters denote significant difference. The expression of values as mean ±Standard deviation (SD)

Discussion

The present study showed that artemisinin significantly reduced DAI and colonic weight in experimentally induced colitis in rats, this finding is comparable to finding of Yang et al. [18] and Chen et al. [19], who approved that Artesunate (derivative of artemisinin) reduced DAI in in an experimentally induced colitis in mice. Furthermore, in this work, artemisinin reduced macroscopic score and histopathological changes of colon in experimentally induced colitis and this finding in accordance with the finding of Yang et al. [18]. Additionally, Chen et al. [19] suggested that artesunate significantly decreased histopathological scores in an experimentally induced colitis. The beneficial improvement in histopathological score in artemisinin treated group may be attributed to the effective role of artemisinin on the pro-inflammatory and oxidative markers, which have been evaluated in the current study.

The anti-inflammatory effects of artemisinin have been widely supported, involving suppression of toll-like receptors (TLRs), nuclear factor-kB (NF-kB), signal transducer and activator of transcription (STAT), which are key factors mediate immune-inflammatory response [20]. Increased TNF-α tissue level is characteristic feature of colitis and many other chronic inflammatory diseases [21]. After an initial damage to mucosal epithelial barrier, TNF-α is secreted by T cells, macrophages, and intestinal mucosal cells causing release of chemokines and cytokines [22]. IL-4 is a key immunoregulatory T helper type 2 cytokine which direct immune reactions, its dysregulation may participate to many inflammatory diseases, including ulcerative colitis [23]. The present study found that administration of artemisinin caused a significant reduction in IHC expression of TNF-α and IL-4 in colonic mucosa of experimentally induced colitis in rats, this finding comparable with the findings of Yuan et al. [24]. Artemisinin family drugs were found to block TNF-α production from Lipopolysaccharide-stimulated peritoneal macrophage by suppress nuclear

translocation of NF-Kb [25], one proposed protective mechanism of artemisinin is by reduced the infiltration of macrophages, neutrophils and CD4+ T cells, and suppressed the expression of immunocyte related chemokines (IL-1β, IL-6 and TNFα) in rosacea-like dermatitis mouse model [24].

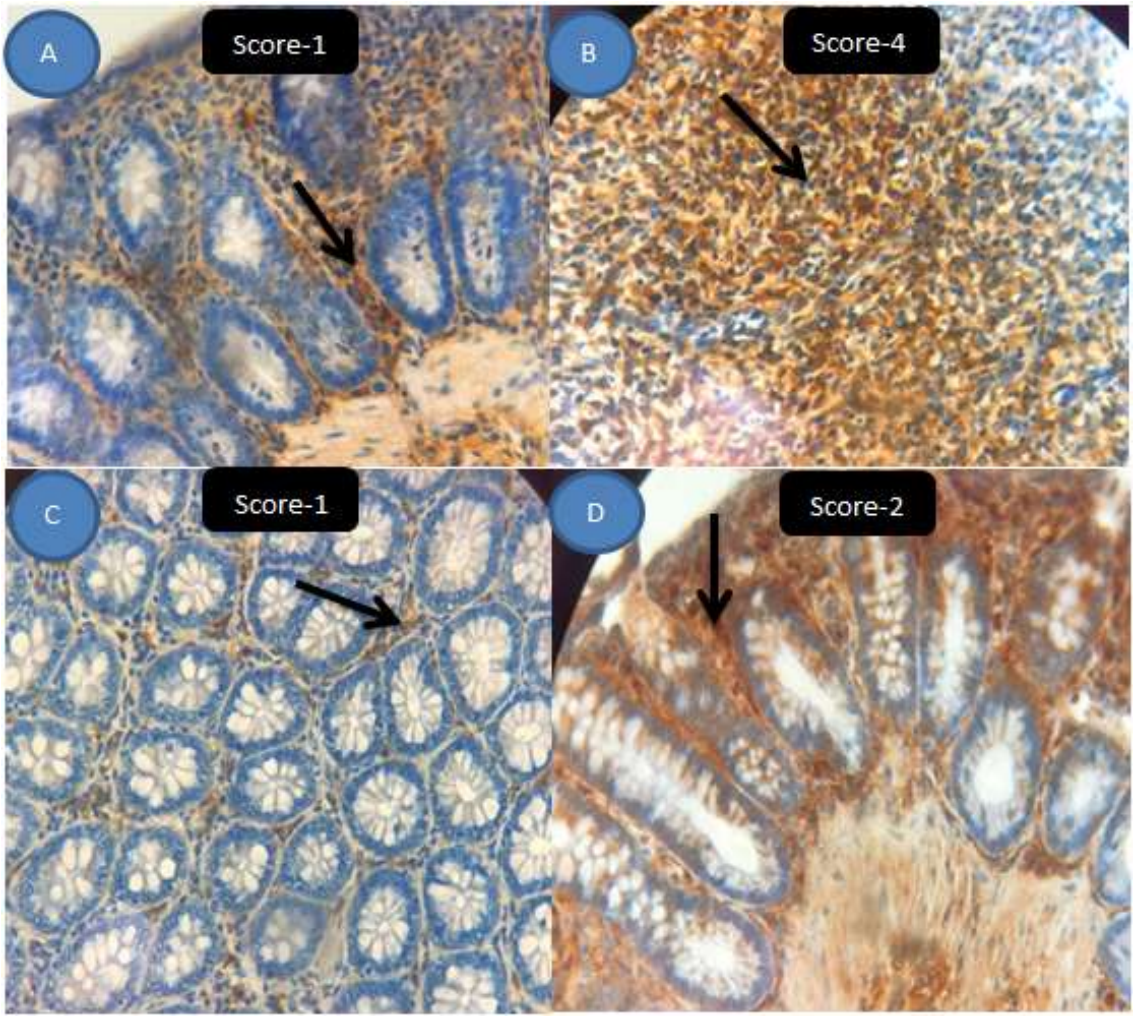

Figure 4. Immunohistochemical expression of: (A) Tumor necrosis factor-α (TNF-α) reveals membranous and secretory pattern (brown color in the stromal cells(arrow)); (B) Interleukine-4 reveals secretory pattern (brown color in the stromal cells (arrow)); (C) Myeloperoxidase (MPO) reveals cytoplasmic pattern (brown color in the stromal cells(arrow)); (D) CD 62 reveals membranous pattern (brown color in the stromal cells(arrow))

MPO is an enzyme essentially create in neutrophils and has been used as an effective indicator of granulocyte cells infiltration to the inflammatory site [26], however, MPO is active in inflamed mucosa in UC patients and participate to the progress of malignancies [27]. The present study has also been shown that administration of artemisinin caused significant reduction in IHC expression of oxidative marker (MPO) in colonic mucosa and this finding agrees with previous studies that has been confirmed that artemisinin protect retinal neuronal cells against oxidative stress [28] and pretreatment with artemisinin reduced the subsequent glutamate-induced elevate of

mitochondrial reactive oxygen species (ROS) and total intracellular ROS levels [29].

Selectins participate in the primary phases of leukocytes rolling to the epithelium of blood vessel, and endothelial selectin plays a major role in the emigration of leukocytes to the vascular wall and their adhesion to the endothelial cells [30]. The current study demonstrated that artemisinin elicited significant reduction in IHC expression of adhesion molecules (E selectin) in rat colonic mucosa in comparison with untreated colitis group and this finding is corresponding with Wang et al. (2016) who showed artemisinin can suppress the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in TNF-α-stimulated human umbilical vein endothelial cells, which likely mediated through the suppression of the NF-κB and mitogen-activated protein kinase (MAPK) pathways in vitro [31].

In conclusion, the results demonstrated that artemisinin has a therapeutic effect through lowered of the inflammatory mediator's TNF-α, IL4 and MPO, downregulation of E-selectin which is comparable to that of sulfasalazine in colitis induced by AA in the rat model. These beneficial effects of artemisinin may be useful in patients suffering from UC.

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Author contribution

Dr. Abdullah: coordinated study recruitment, implementation and progress of this study and helped with data interpretation and manuscript drafting. Dr. Abd and Dr. Qasim supervised the study and participated in its design and interpretation.

Conflict of interest

The authors have no conflicts of interest to declare.

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