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Article

The effect of woody endocarpium of walnut alcoholic extract on acetic acid-induced ulcerative colitis in rats

Zakieh Keshavarzi[1,2], Aleme Ashekar[2], Mehran Vatanchian[3], Alireza Abbaspour[4], Bahram Bibak[1, 2], Morteza Behnamfar[2], Saeid Barzegar[5], Farzaneh Shakeri[1,2] *

[1]Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran, [2]Department of Physiology and Pharmacology, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, [3]Department of Anatomical, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, [4]Department of Biochemistry, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran, [5]Department of Pathology and Laboratory Sciences, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran

Various pharmacological effects including anti-inflammatory and anti-oxidant properties were shown for woody endocarpium of walnut alcoholic extract (WEW). In the study, the effect of the WEW extract in acetic acid induced ulcerative colitis in rats was evaluated. Thiol, glutathione peroxidase (GPX), malondialdehyde (MDA), superoxide dismutase (SOD) and gastric acid levels and pathological changes in the colon were investigated in the control group (C), ulcerative colitis group (UC), UC groups treated with WEW extract (10, 20, and 50 mg/kg) and sulfasalazine. Levels of gastric acid, MDA and pathological scores in colon were increased but SOD, GPX and thiol levels were decreased in UC animals compared to those of the control group ($p<0.001$). Treatment with the highest concentration of extract significantly improved level of thiol and pathological scores compared to the UC group ($p<0.05$ to $p<0.001$). Treatment with the two higher concentrations of extract also significantly decreased acid level compared to the UC group ($p<0.01$ to $p<0.001$). There was significant improvement in MDA due to treatment with the all concentrations of the extract ($p<0.001$). Sulfasalazine treatment also significantly improved most parameters compared to the UC group but did not changed pathological scores ($p<0.05$ to $p<0.001$). These results indicated a possible preventive therapeutic effect for the WEW extract on UC.

Keywords: WEW. Oxidative stress. Gastric acid. Inflammation. Ulcerative colitis.

INTRODUCTION

The walnut plant belongs to the genus Juglans from Juglandaceae family of the order Juglandales. Walnut is a fruit that has been known and cultivated since ancient times. Its plantation is spread over all regions of Turkey (Gedikli, 2006). The seeds, green husks, and leaves of the walnut are a rich source of phenolic compounds such as flavonoids, phenolic acids, and naphthoquinones (Pereira *et al.*, 2008; Zhao *et al.*, 2014). Walnut is a valuable source of nutrients with cholesterol-free contents, substitutes for animal proteins, high concentrations of unsaturated fatty acids, linoleic acid and linolenic acid, and polyunsaturated fatty acids that are essential for a healthy life (Aydın, Gökçe, Yılmaz, 2015). In Iranian folk medicine, walnut is a well-documented remedy for treatment of several diseases such as infections, inflammations, and diabetes (Nasiry, Khalatbary, Ahmadvand, 2017). Several therapeutic effects such as anti-oxidative (Almeida *et al.*, 2008), anti-inflammatory and antinociceptive (Erdemoglu, Küpeli, Yeşilada, 2003), anti-carcinogenic (Carvalho *et al.*, 2010), anti-microbial (Rather *et al.*, 2012), and anti-fungal (Noumi *et al.*, 2010) were reported for this plant. It

***Correspondence:** F. Shakeri. [1]Natural Products and Medicinal Plants Research Center. North Khorasan University of Medical Sciences, Bojnurd, Iran. ORCID: 0000-0003-4813-6030. [2]Department of Physiology and Pharmacology School of Medicine. North Khorasan University of Medical Sciences, Bojnurd, Iran. Phone: +98 58 31513051. E-mail: f_1366_sh@yahoo.com.

was reported that walnut contained high concentrations of a-tocopherol, which have strong anti-oxidant effect preventing the process of lipid oxidation (Amaral *et al*., 2005; Köksal *et al*., 2006). In addition, walnut consumption has also been shown to decrease the plasma concentration of C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), (Salas-Salvadó *et al*., 2008).

Chemical compositions and nutritional of WEW include phenolic compounds (gallic acid, phthalic acid, catechin, vanillin, ethyl gallate, dihydroquercetin, kaempferol, taxifolin-3-*O*-α-L-arabinofuranoside, quercetin-3-rhamnoside, quercetin-3-*O*-(4$^{//}$-*O*-acetyl)-α-L-rhamnopyranoside, blumenol B, propyl gallate and vanillic acid), fatty acids (octanoic acid, decylic acid, lauric acid, myristic acid, pentadecanoic acid, palmitic acid and margaric acid), amino acids (lysine, phenylalanine, threonine, isoleucine, leucine, valine and aspartic acid), monosaccharides (mannose, rhamnose, ribose, glucuronic acid, trehalose and galacturonic acid) and mineral element (k, Na, Ca, Mg, Fe, Cu, Zn, Mn and Se), (Table I), (Hu *et al*., 2019).

Ulcerative colitis is a chronic disease of the large intestine, which is characterized by acute and chronic inflammation of the mucosa, ulceration of the colon, bloody diarrhea, rectal bleeding, abdominal pain, cramping and weight loss (Shih, Targan, 2008). Etiology and pathogenesis of ulcerative colitis is unidentified and depends on multiple immune, genetic, and environmental factors, reactive oxygen species and gastrointestinal infections (Loftus, 2004). Oxidative stress is an important factor in the pathogenesis of ulcerative colitis disease (Pavlick *et al*., 2002). It was reported that enhanced oxidative stress in colonic mucosal and decreased antioxidant defense caused tissue damage and inflammation of the colon in the patients with ulcerative colitis (Mehrabani *et al*., 2011). Most of the current therapies for ulcerative colitis include non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, immunomodulators, and selective cyclooxygenase-2 (COX-2) inhibitors (Strober, Ludviksson, Fuss, 1998). Although many types of treatments have good outcomes, additional therapeutic approaches are needed because they produce adverse effects which have reduced their clinical applications.

Therefore, the present study aimed to evaluate the effect of the woody endocarpium of walnut (WEW) alcoholic extract on the levels of gastric acid, malondialdehyde (MDA), glutathione peroxidase (GPX), superoxide dismutase (SOD) and thiol and colon pathological scores on acetic acid-induced ulcerative colitis in rats.

TABLE I - Chemical compositions and nutritional attributes of WEW

Phenolic	Fatty acids	Amino acids	Monosaccharides	Minerals
Gallic acid	Octanoic acid	Lysine	Mannose	k
Phthalic acid	Decylic acid	Phenylalanine	Rhamnose	Na
Catechin	Lauric acid	Threonine	Ribose	Ca
Vanillin	Myristic acid	Isoleucine	Glucuronic acid	Mg
Ethyl gallate	Pentadecanoic acid	Leucine	Trehalose	Fe
Dihydroquercetin	Palmitic acid	Valine	Galacturonic acid	Cu
Kaempferol	Margaric acid	Aspartic acid	Xylose	Zn

(continues on the next page...)

TABLE I - Chemical compositions and nutritional attributes of WEW

Phenolic	Fatty acids	Amino acids	Monosaccharides	Minerals
Taxifolin-3-O-α-L-arabinofuranoside	Stearic acid	Serine	Galactose	Mn
Quercetin-3-rhamnoside	Arachidic acid	Glutamate	Arabinose	Se
Quercetin-3-O-(4//-O-acetyl)-α-L-rhamnopyranoside	Heneicosanoic acid	Glycine		
Blumenol B	Behenic acid	Alanine		
Propyl gallate	Tricosanoic acid	Cystine		
Vanillic acid	Lignoceric acid	Tyrosine		

MATERIAL AND METHODS

Plant collection and extraction

Walnut was purchased from Bojnurd city, North Khorasan province, Iran, in July 2017 and identified by botanists in the herbarium of Ferdowsi University of Mashhad (Herbarium No. 44540-FUMH).The WEW were grounded to powder (100 g), mixed with 96% ethanol at a ratio of 1:10 (powder to ethanol) and left for 3 days at 37°C with occasional shaking and stirring. The mixture was then filtered and the resulting liquid was concentrated under reduced pressure at 45°C in an Eyela (Heidolph, Germany) rotary evaporator.

WEW extract characterization

Phenolic compounds of the ethanolic extract of WEW were identified by liquid chromatography-mass spectrometry (LC-MS) using a Waters Alliance 2695 HPLC-Micromass Quattro micro API Mass Spectrometer. Five polyphenols were identified in this extract, they were Gallic acid, Protochatechuic acid, Epicatechin, Catechin and Vanillic acid (Figure 1).

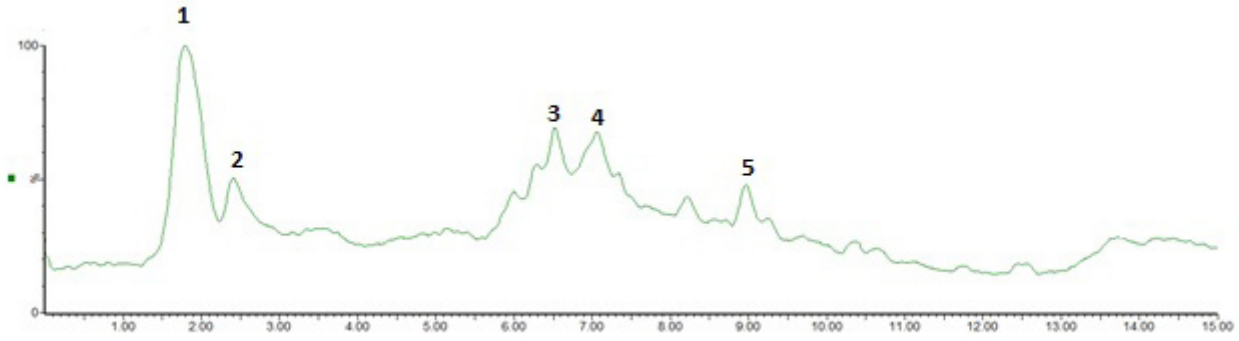

FIGURE 1 - The chromatographic separation of bioactive compounds from ethanolic extract of WEW.

Animals

Experiments were performed using adult male Wistar rats (200-250g) prepared from Animal house, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran. The animals were kept in cages receiving clean filtered air (Maximiser, Thoren Caging System Inc., Hazleton, PA, U.S.A.) under standard condition at 22±2 ^{0}C and regular 12 hr/12 hr light/dark cycles. They also had free access to food and water ad libitum during experimental period.

Induction of ulcerative colitis in rat

Animals were anesthetized by intraperitoneal injection of thiopental (50mg/kg), and colitis was induced by intra-colonic administration of 4% acetic acid (2 ml) through a lubricated catheter under thiopental anaesthesia (Keshavarzi *et al*., 2018) and treatment was started post-colitis induction. The instillation site was about 8 cm from the anal verge into the rectum. Rats were maintained in trendelenburg position for 30 seconds to prevent the leakage of the acid. Control group rats received the saline intraperitoneally. Rats were acclimatized to laboratory conditions for 8 days before the start of experimental procedures and maintained in a well-ventilated cage under standard protocols. Study protocol was approved by ethical committee of North Khorasan University of Medical Sciences (Ethics allowance No. 950015) and experiments were performed in compliance with the regulations of the Institute of Laboratory Animals Resources Commission on Life Sciences. On the 8th day, animals were sacrificed and colon samples were collected and stored at -80 ^{0}C until analysis.

Experimental groups

Animals were randomly divided into the six groups (n=7 in each group) including: (1) control group (group C) without induction of ulcerative colitis; (2) ulcerative colitis group (group UC) which was induced by acetic acid; (3) UC group treated with sulfasalazine 200 mg/kg (group S); and (4-6) UC groups treated with WEW extract 10, 20, and 50 mg/kg (groups WEW). The WEW extract and sulfasalazine were injected intraperitoneally for the 8-day after induction period of the ulcerative colitis.

Measurement of malondialdehyde (MDA) level

Malondialdehyde (MDA) level, as an index of lipid peroxidation, was measured. MDA reacts with thiobarbituric acid (TBA) as a thiobarbituric acid reactive substance (TBARS) to produce a red complex with the maximum absorbance at 535 nm. For MDA measurement, 2 mL of TBA/trichloroacetic acid (TCA)/HCl was added to 1 mL of tissue homogenate and the mixture was heated in a water bath for 40 min. Then, the mixture was centrifuged at 1000 g for 10 min. Finally, the absorbance was measured at 535 nm (Eftekhar *et al*., 2019).

Measurement of thiol level

Total thiol concentration was measured using reagent DTNB which reacts with the thiol to produce a yellow coloured complex with a peak absorbance at 412 nm. One ml Trisethylene diamine tetraacetic acid (EDTA) buffer (pH 8.6) was added to 50 µl serum in 1 ml cuvettes and sample absorbance was read at 412 nm against Tris-EDTA buffer alone (A1). Twenty microlitre DTNB reagents (10 mmol in methanol) were then added to the mixture and keep it in laboratory temperature for 15 min and the sample absorbance was read again (A2). The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (mmol) was calculated using the following equation (Shakeri, Boskabady, 2017):

Total thiol concentration (mM) = (A2–A1–B)×1.07/0.05×13.6.

Measurement of SOD and GPX levels

Levels of SOD and GPX in colon tissue were measured, using the Randox assay kits, and the content of GPX and SOD were given as U/g protein. Estimation of protein content follows the method of Lowry *et al.* (1951).

Measurement of gastric acid level

Gastric acid concentration was measured as previously described by washout method (Rafsanjani *et al*., 2007). The animals were completely deprived of food for 24 hours before the test. Then they were laparotomized, and by creating a hole in the duodenum, the cannula was inserted into the duodenum and then pushed into the stomach. To prepare the specimens, first, 1 ml of the physiological saline solution was injected into the stomach, and at the end of 15 minutes, was drawn. The amount of acid was measured immediately after sample collection using a manual titrator at laboratory temperature.

Colon macroscopic damage evaluations

After sacrificing the animals using ether overdose, a segment of the colon, 8 cm in length and 3 cm proximal to the anus was excised, opened longitudinally and washed in saline buffer. The criteria for macroscopic evaluation relied on a previously validated scoring system (0-4). The scores were: 0=no ulcer; 1=mucosal erythema only; 2=mild mucosal edema, slight bleeding or slight erosion; 3=moderate edema, bleeding ulcers or erosions; and 4=severe ulceration (Millar *et al*., 1996).

Assessment of colon histological damage

For histological examination, colon tissues were separately fixed in 10% formalin, dehydrated, paraffin embedded, processed, sectioned as 4 μm-thick sections, and stained with haematoxylin and eosin (HE), (Shahrokhi *et al*., 2018).

Statistical Analysis

The data of oxidant and antioxidant biomarkers, level of gastric acid and colon macroscopic damage were quoted as mean ± SEM. Statistical comparisons among and within groups were performed using one-way analysis of variance (ANOVA) with Kruskal- Wallis test. The results were considered statistically significant if the p value was less than 0.05. InStat (GraphPad Software, Inc, La Jolla, USA) was used for data analysis.

RESULTS

The effect of extract on oxidant and antioxidant biomarkers

The levels of MDA were significantly increased while SOD, thiol and GPX levels decreased in ulcerative colitis animals compared to the control group ($p<0.05$ to $p<0.001$; Figures 2 and 3). MDA levels in ulcerative colitis animals treated with all concentrations of WEW extract, and thiol level in the group treated with the highest concentration were significantly improved compared to those of untreated ulcerative colitis group ($p<0.001$ for both cases; Figures 2b and 3a).

Sulfasalazine treatment also significantly reduced MDA but increased SOD, thiol and GPX levels compared to the ulcerative colitis group ($p<0.05$ to $p<0.001$; Figures 2 and 3). GPX and thiol levels in the groups treated with the two lower concentrations of WEW extract were significantly different with those of the control group ($p<0.05$ to $p<0.001$; Figures 2b and 3b). The effect of the lowest concentration of WEW extract on thiol and its two lower concentrations on GPX level were significantly lower than those of sulfasalazine ($p<0.05$ to $p<0.01$; Figures 2b and 3b). However, the effects of the highest concentration of WEW extract on thiol level were significantly higher than that of sulfasalazine ($p<0.05$; Figure 2b).

The effect of the high concentration of WEW extract (50 mg/kg) on thiol and GPX levels were significantly higher than the low concentration (10 mg/kg), ($p<0.01$ to $p<0.001$; Figures 2b and 3b). In addition, there was a significant difference between the effects of high (50 mg/kg) and medium (20 mg/kg) concentrations of WEW extract on thiol level ($p<0.001$; Figure 2b).

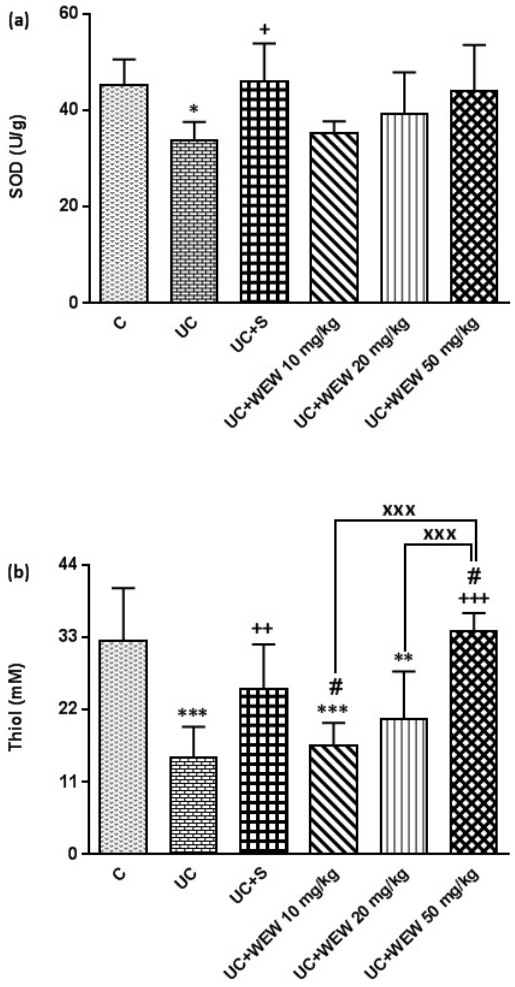

FIGURE 2 - SOD (a) and Thiol (b) levels in the colon tissue of control (C), ulcerative colitis group (UC), ulcerative colitis rats treated with sulfasalazine (UC+S) and woody endocarpium of wulnut extract (UC+WEW), (n=7 in each group). Data are presented as mean ± SEM values. * p<0.05, ** p<0.01 and *** p<0.001 shows significant differences compared to group C. + p<0.05, ++ p<0.01 and +++ p<0.001 show significant differences compared to group UC. # p<0.05 shows significant differences compared to group UC+S. xxx p<0.001 show significant differences between the three concentrations WEW. Statistical analyses were performed using ANOVA with Tukey-Kramer's post-test.

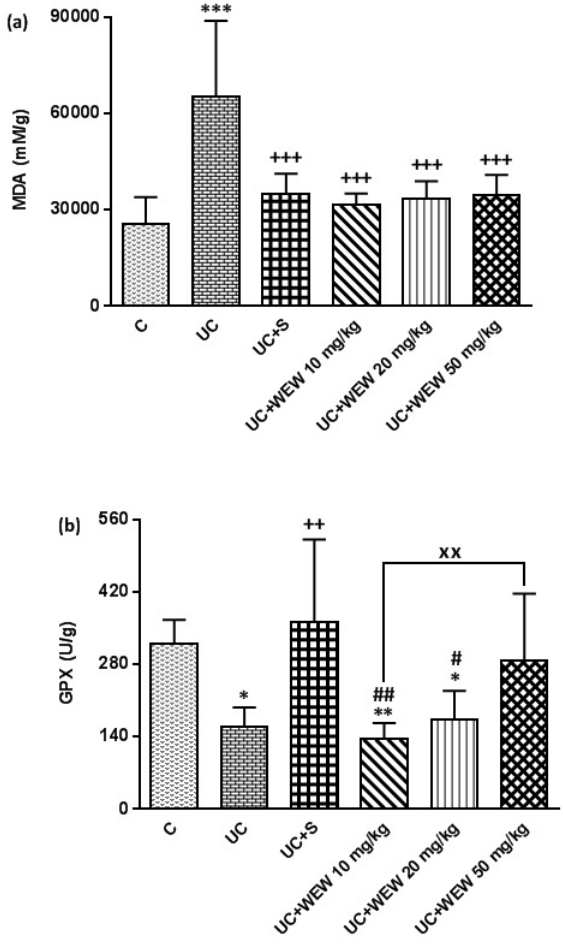

FIGURE 3 - MDA (a) and GPX (b) levels in the colon tissue of control (C), ulcerative colitis group (UC), ulcerative colitis rats treated with sulfasalazine (UC+S) and woody endocarpium of wulnut extract (UC+WEW), (n=7 in each group). Data are presented as mean ± SEM values. * p<0.05, ** p<0.01 and *** p<0.001 shows significant differences compared to group C. ++ p<0.01 and +++ p<0.001 show significant differences compared to group UC. # p<0.05 and ## p<0.01 shows significant differences compared to group UC+S. xx p<0.01 show significant differences between the three concentrations WEW. Statistical analyses were performed using ANOVA with Tukey-Kramer's post-test.

The effect of extract on gastric acid level

The level of gastric acid was significantly increased in the ulcerative colitis animals compared to those of the control group (p<0.05; Figure 4a). Treatment with the two higher concentrations of WEW extract and sulfasalazine were significantly improved level of gastric acid compared to those of untreated ulcerative colitis group (p<0.01 to p<0.001; Figure 4a). No significant

difference was seen between treated groups and control group on gastric acid level.

The effect of the lowest concentration of WEW extract on gastric acid level was significantly lower than those of sulfasalazine ($p<0.05$; Figure 3a). The effect of the high (50 mg/kg) concentration of WEW extract on gastric acid level was significantly higher than the low (10 mg/kg) concentration ($p<0.001$; Figure 4a).

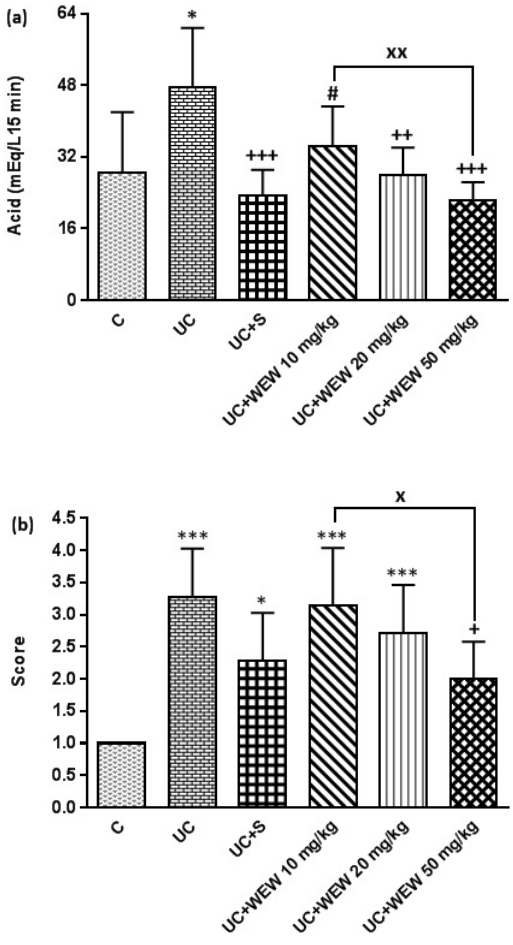

FIGURE 4 - Acid level (a) and pathological (b) scores in the colon tissue of control (C), ulcerative colitis group (UC), ulcerative colitis rats treated with sulfasalazine (UC+S) and woody endocarpium of wulnut extract (UC+WEW), (n=7 in each group). Data are presented as mean ± SEM values. * $p<0.05$ and *** $p<0.001$ shows significant differences compared to group C. + $p<0.05$, ++ $p<0.01$ and +++ $p<0.001$ show significant differences compared to group UC. # $p<0.05$ shows significant differences compared to group UC+S. x $p<0.05$ and xx $p<0.01$ show significant differences between the three concentrations WEW. Statistical analyses were performed using ANOVA with Tukey-Kramer's post-test.

The effect of extract on pathological scores and histopathological

The scores of pathological changes in ulcerative colitis group were significantly increased compared to the control group ($p<0.001$; Figure 4b). Pathological scores in ulcerative colitis group treated with the highest concentration of WEW extract were significantly reduced compared to the untreated ulcerative colitis group ($p<0.05$; Figure 4b).

Sulfasalazine treatment was reduced pathological scores compared to the ulcerative colitis group. However, this change was not statistically significant. Pathological scores in groups treated with the two lower concentrations of WEW extract and sulfasalazine were significantly different with those of the control group ($p<0.05$ to $p<0.001$ Figure 4b). There was no significant difference in pathological scores among sulfasalazine-treated group and groups treated with the three concentrations of WEW extract.

The effect of the high (50 mg/kg) concentration of WEW extract on pathological scores was significantly higher than the low (10 mg/kg) concentration ($p<0.05$; Figure 4b).

Figure 5 shows a specimen of colon photograph of each studied group. Colonic tissue histopathological evaluation from the ulcerative colitis group showed severe submucosal edema and crypt loss, while the control group showed a preserved mucosal architecture. The morphological characteristics of the ulcerative colitis group treated with the highest concentration of the WEW extract (50mg/kg) revealed significant amelioration of colonic tissue injury.

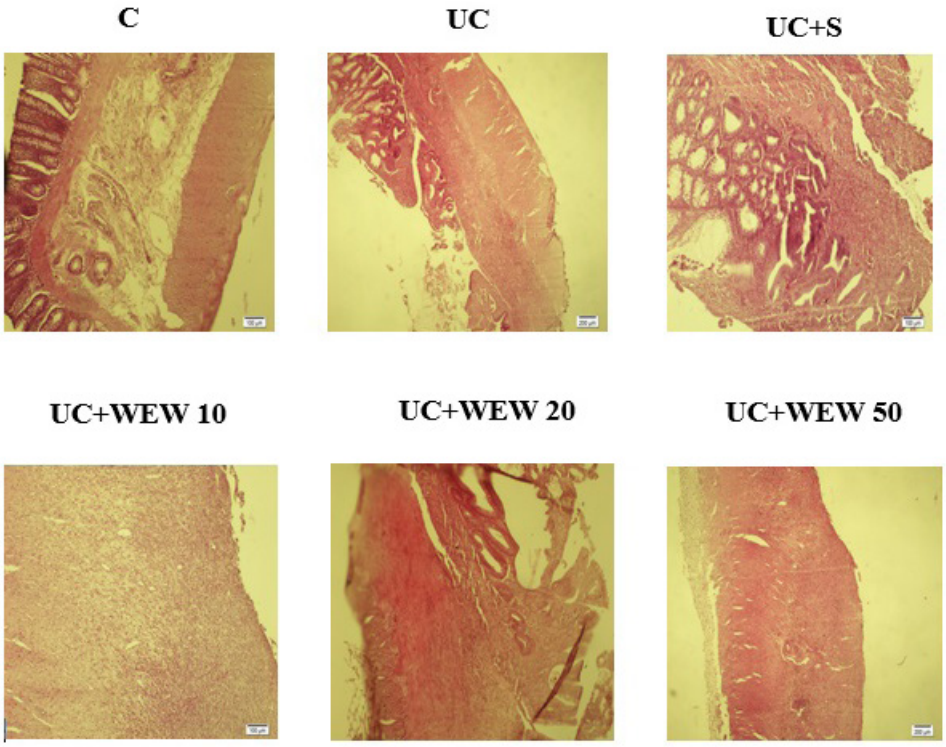

FIGURE 5 - Photographs of colon specimens under a light microscope (X40), in control (C), ulcerative colitis group (UC), ulcerative colitis rats treated with sulfasalazine (UC+S) and woody endocarpium of wulnut extract (UC+WEW).

DISCUSSION

This study evaluated the healing effects of the WEW ethanolic extract against acetic acid-induced ulcerative colitis by measuring tissue histopathology, gastric acid and MDA, SOD, GPX and thiol level in rats. This study found that treatment with the WEW ethanolic extract could lead to enhancement in colonic antioxidant capacity and a decrease in inflammation and acute colonic injury induced by acetic acid, which is dose-dependent. The results were confirmed by histopathological examinations. Concentration-dependent effects of the WEW ethanolic extract could be another reason indicating the preventive effect of plant in colon inflammation in the ulcerative colitis rats. Sulfasalazine is mainly used for treatment of inflammatory bowel disease (IBD), including UC and Crohn's disease (Karaca *et al.*, 2010). Sulphasalazine is composed of sulphapyridine, which has antibacterial activity and 5-aminosalicylate (5-ASA), which has anti-inflammatory potency (Klotz, 1985). We used sulfasalazine as a reference drug, and found that the 50 mg/kg dosage of the extract in most parameters was more effective than sulfasalazine.

The model of acetic acid induced colitis shares many of the histologic features of the ulcerative colitis in human beings including mucosal edema and submucosal ulceration (Sharon, Stenson, 1985). The destruction of colon structure and mucosa barrier is due the chemical stimulation, enhanced vessel permeability, increased inflammatory mediators, and reactive oxygen species (ROS), (Carty *et al.*, 2000).

There is strong evidence that oxidative stress plays a vital role in the IBD initiation and continuance (Kruidenier, Verspaget, 2002). Increased production of oxidants in ulcerative colitis subjects has been reported by human studies (Babbs, 1992). Imbalance between oxidant and antioxidant parameters in ulcerative colitis induced oxidative damage (Sahebari *et al.,* 2015), which is a characteristic feature of colitis (Droge, 2002).

In the current experiment, MDA level as indicator of oxidative stress were significantly increased but SOD, GPX and thiol levels decreased in the ulcerative colitis

animals compared to the control group. Similarly, previous evidences also indicated an imbalance in oxidant/antioxidant enzymes balance towards oxidative conditions in ulcerative colitis animals (Balmus *et al*., 2016; Lih-Brody *et al*., 1996). GPX is involved in different mechanisms including the synthesis and repair of DNA, recycling of vitamins C and E, prevention of free radicals-induced damage, improvement of the antioxidant activity of vitamin C, and facilitation of the transport of amino acids and plays a principle role in detoxification (Chavan *et al*., 2005). SOD, levels are reduced in the inflamed intestinal tissues as free radicals affect the intestinal epithelium (Kandhare *et al*., 2013). Oxidative damage of the colitis imbalances the catalytic activity resulting in the inflammation and oxidative damage to the colonic mucosa (Circu, Aw, 2012). Findings of this study showed that treatment of ulcerative colitis animals with the extract of WEW resulted in a significant reduction in MDA but increased SOD, GPX and thiol levels. Consistently, previous studies on the effect of the hydro-ethanolic extract of walnut on MDA and SOD levels in CCl4-induced liver damage in the rat had comparable results with those of the present study (Aydın, Gökçe, Yılmaz, 2015; Eidi *et al*., 2013). A recent study has demonstrated that dietary administration of walnut (6% and 9% w/w) significantly increased SOD, catalase and GPX in transgenic mouse model of alzheimer disease, which confirms the findings of our study (Pandareesh, Chauhan, Chauhan, 2018). Increased level of thiol has been observed in rat that feeded with the walnut extract (10% w/w), (Olabiyi, Oboh, Adefegha, 2017) which supports the results of the present study.

Extensive studies have shown that many extra-intestinal tissues including skin, intestines, kidneys, and stomach are also affected by ulcerative colitis (Greenstein, Janowitz, Sachar, 1976). Also, inflammation of the duodenum, gastric and esophageal ulcers is also seen in children suffering from the ulcerative colitis, which indicates a link between these gastric and colon disorders (Ruuska *et al*., 1994). Therefore, due to the fact that ulcerative colitis is a complex disorder that can affect many tissues, including the stomach. In the present study, the effect of walnut extract on the gastric secretory response and histopathological changes in ulcerative colitis model induced by acetic acid was designed in male rats.

Our results also showed increased pathological scores and gastric acid level in the ulcerative colitis animals. The same results were observed in ulcerative colitis model induced by acetic acid in rats (Keshavarzi *et al*., 2018), which confirms the induction of ulcerative colitis in animals. Treatment with the various concentrations of WEW extract resulted in a significant protection against the most ulcerative colitis-related colon pathological damages and gastric acid level. Similar to our study, this condition was reversed in another study using ethanolic extract of the walnut (10 and 20 mg/kg, intraperitonealy) in acetic acid-induced experimental colitis in the rat (Keshavarzi *et al*., 2019). Moreover, the administration of walnuts (0, 3.5, 7 and 14% g/kg, orally) reduced overall colitis scores in a rat model of dextran sulfate sodium-induced acute colitis in the mice (Nakanishi *et al*., 2019). These differences may be due to some limitations of the present study, such as the number of animals and short term period of the treatment protocol.

All the above-described studies, in line with the current experiment, support the preventive therapeutic effect of the WEW extract on the ulcerative colitis.

The results of the present study showed that WEW extract improves the oxidative stress and colon inflammation in the ulcerative colitis rats. These results suggest a therapeutic effect for the WEW extract against the IBD through both antioxidant activities and preventive effects on the colon inflammation. However, further studies are needed to evaluate the effects of the plant and its constituents on animal models of the ulcerative colitis as well as human studies.

In conclusion, the results of the present study indicated a preventive effect for WEW extract on oxidative markers and colon pathological damages in ulcerative colitis rats, which were comparable but more specific, to the effect of sulfasalazine at used concentrations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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