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SAFFRON (*CROCUS SATIVUS*) AMELIORATES TNBS-INDUCED COLITIS IN RATS VIA DOWNREGULATION OF INFLAMMATORY CYTOKINES TNF-α AND IL- 10, CASPASES-3 GENE EXPRESSION AND OXIDATIVE STRESS IN EXPERIMENTAL RATS

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ABSTRACT

Ulcerative colitis (UC) is a chronic replacing disorder of the gastrointestinal tract by inflammation and tissue damage. Saffron, a spice derived from the flower of Crocus sativus, is rich in carotenoids . In the current study, we aimed to investigate the potential alleviating effects of Saffron extract against TNBS-induced colitis in rats. saffron extract (80 ml/kg body weight was administered daily by oral gavages in ulcerative colitic rats. Administration of saffron extract mitigated the colonic levels of TNF-a and IL- 10 cytokines. The attenuation of saffron extract to colon injury was also associated with suppression of oxidative stress via reduction of lipid peroxides and nitric oxide along with boosting the antioxidant defenses through restoration of colon glutathione. In addition, caspases-3 gene expression activity, an apoptotic marker, was inhibited. Furthermore, addition of sucrose on the diet of colitic rats increases the colonic inflammation, without incidence on the antioxidant status of colitic rats, indicating that the increase in oxidative stress is directly related to TNBS rather than sucrose.

Key words: Saffron extract - Colitis- TNBS- Caspase-9 gene expression - Sucrose - Cytokines

INTRODUCTION

Natural products are of particular interest as chemo-preventive agents because of their low toxicity and potent efficacy. Saffron (*Crocus sativus*) is a species belonging to the Iridaceae family cultivated in Europe, Turkey, Central Asia, India, China and Algeria , it has oxytocic and anti-carcinogenic properties . Saffron can be used as a traditional medicine and spice, natural food color and flavor. It enhances the absorption and bioavailability of other drugs [1].The color of saffron comes from water-soluble carotenoids, including crocetin (8,8′-diapocarotenedioic acid) and crocins (mono-, di-, and triglycosyl esters of crocetin; Picrocrocin, a beta-d glucoside of safranal, that is responsible for the saffron bitter taste and is its second most abundant component .Safranal , a terpene aldehyde, a major volatile oil that comprises as much as 60–70% of stigma essential oils, it is responsible for the distinctive aroma of this spice and is produced from dehydration of picrocrocin during the drying process [2]. Furthermore, saffron contains protein, moisture, fat, riboflavin and thiamine vitamins, minerals, crude fiber, and sugars, including reducing sugars, pentosans, pectin, gums, starch and dextrins [3].Ulcerative Colitis (UC) is a disease of large intestine known as Inflammatory Bowel Diseases (IBD). Colitis' means that there is inflammation in the colon and if this becomes severe enough, the lining of the colon is actually breached and ulcers may form [4]. The pathogenesis of IBD involves changing of the colon epithelial barrier which is followed by inflammations and immune responses into intestinal flora in a context of genetic tendency. TNF-a, is a main immuno regulatory cytokine that increase the inflammatory response by enhancing of cytokines making, arachidonic acid metabolites ,ROS and proteases [5] . Oxidative stress is the main cause of pathophysiology of the IBD, on the other hand, using of antioxidant compounds is helpful in

reducing colon injury [6,7]. The aim of current work was evaluating the protective role of aqueous extract of saffron against the cytotoxicity, and oxidative stress in colon tissue in rats fed high sucrose diet with a single administration of TNBS

MATERIALS AND METHODS

Drugs

TNBS (2, 4, 6- trinitrobenzen sulphonic acid) was of analytical pure grade and purchased from El-Gomhuria Co. Cairo, Egypt.

Preparation of plant extract

Saffron was obtained from local market, we used the maceration method to soak 10 g of saffron stigmas in 500 ml ethanol (80%, v/v) for 3 days . Then filtered and concentrated under pressure at 40 °C. The extraction was prepared fresh daily for oral administration by gavages [8].

Colitis Induction

A rubber catheter was used for colitis induction, the method involves insertion of the catheter 8 cm into the anus, then left for 15 seconds before deduction thus preventing expulsion of injected solution. A single intra colonic dose of 10 mg of TNBS (was dissolved in 0.25 ml of 50% ethanol (v:v) was enough for colitis induction. At the end of experimental period, rats were scarified, blood samples were collected and centrifuged, and colons were dissected [9].

Animals

Thirty two adult male albino rats weighing 180-200g were used . Animals were kept on a natural light/dark cycle and given food and tap water *ad libitum*. Rats were grouped in 4 experimental groups as follow:

Group (1): control group, fed basal diet
Group (2): colitic rats, fed basal diet
Group (3): colitic rats fed basal diet, containing 30% sucrose (Sucrose is replaced with the corn starch).
Group (4): colitic rats fed basal diet, containing 30% sucrose and given oral supplementation of Saffron extract (80 mg/kg body wt. by oral gavage) daily[10]

Determination of Colon/ Body Weight Ratio

Remove 8 cm of the colon starting from rectum, longitudinally opened and cleared of feacal remains if present, using 0.9% saline , dried then weighted. The ratio of the 8 cm segment distal colon weight was calculated as an index of colonic tissue edema [11]. A sample of distal colon specimen was homogenized in 3 ml 0.5% hexadecyltrimethyl ammonium bromide in 50mM phosphate buffer (pH 6), enzyme stability was maintained by keeping the sample in ice bath during homogenization, then centrifuged at 4000 r.p.m for 15 minutes at 4°C; the resulting supernatant was used for the determination of biochemical parameters.

Biomarkers of Inflammation

Alkaline phosphatase ALP activity ,Serum nitric oxide NO levels and Myeloperoxidase activity (MPO), were determined by commercial kits.

Estimation of inflammatory cytokines (TNF-a and IL-10).

TNF-a and IL-10 levels in colon homogenate supernatants were measured using ELISA kits (R&D systems incorporation, USA).

Biochemical analysis

Glutathione-s-transferase (GST) , Glutathione reductase (GR) , Thioredoxin Reductase (TrxR) activities and colon tissue Caspase-9 gene was determined by commercial kits.

Histopathological examination

Colon samples were fixed in 10% formal saline prior to wax embedding. Sectioning and staining with haematoxylin and eosin for histological evaluation of colonic damage by light microscopy [12]

Statistical Analysis

The data were statically analyzed by using the statistical software package SPSS for windows (Version 20). The significance of differences between more than two groups was evaluated by one way analysis.

RESULTS

Figures 1,2,3 revealed that, no significant variation in food intake in G1, compared to other groups. A significant variation observed in average body weight of G1 comparing this result with other group's values. TNBS administration lowers the final average body weight, while addition of sucrose in diet G3 shows a significant variation compared to the G1, oral administration of saffron extract showed announced improvement of final body weight as compared to both G2 and G3. The

produced damage inside rat colon by TNBS was confirmed by a marked elevation in the colon/body weight ratio compared to the G1. Adding sugar to rat's diet increase this ratio as compared to colitic rats.It is clear from Figures 4,5,6 that colitic rats posses significant elevation in colonic MPO, ALP , NO compared to G1. Meanwhile, daily administration of Saffron extract (G4) causes significant decrease in MPO level comparing it to groups G2 and G3. A slight reduction was observed in ALP activity in saffron treated group G4 as compared to G2 and G3. Saffron administration supports marked reduction in NO high levels in G2 rats, addition of 30% sucrose increases NO level.To gain an insight into the effect of Saffron extract on animal inflammatory status with TNBS colitis, the levels of inflammatory cytokines (TNF-a and IL-10) were assessed. TNF-a and IL-10 levels showed as a remarkable increase, as compared to the G1 (Figures 7,8). While saffron administration effectively lowered TNF-a and IL-10 contents, as compared G2. However, it seems that sucrose addition to diets increase TNF-a and IL-10 levels as compared to sucrose free colitic group (G3).The obtained data presented in Figures 9,10,11,12 revealed that, administration of TNBS-induced colitis which is manifested by caused significant increase in colonic GST activity. However, TrxR, GR, Caspase-9 in colon tissues were significantly decreased when compared with normal control group. Administration of Saffron extract to colitic rats significantly decreased GST activity. On the other hand, saffron treatment enhanced the activity of TrxR, GR and Caspase-9 in colon tissues when compared with either TNBS-treated group.

Figures (1,2,3): results of food Intake, final body weight and colon/body weight ratio in all treatments

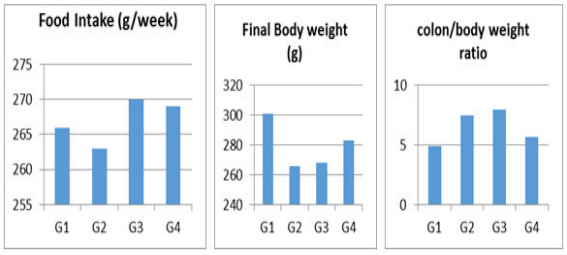


Figure 1 **Figure 2** **Figure 3**

Figures (4,5,6)
Effect of oral administration of Saffron extract on biomarkers of inflammation in rat colon tissues

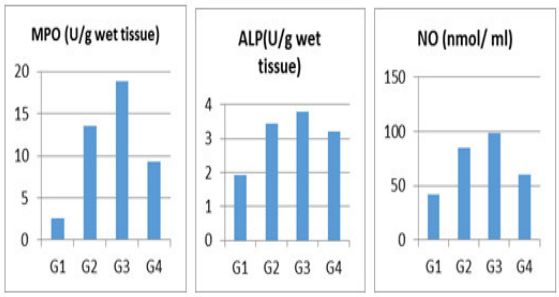


Figure 4 **Figure 5** **Figure 6**

Figures (7,8)
Effect of oral administration of Saffron extract on biomarkers of inflammation in rat colon tissues (TNF-a ,IL-10)

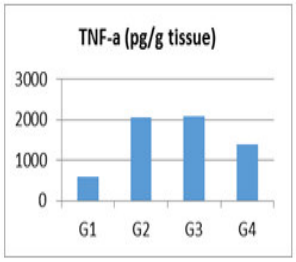


Figure 7

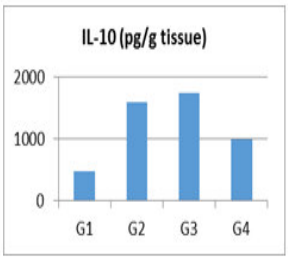


Figure 8

Figures (9,10,11,12)
Effect of oral administration of Saffron extract on biomarkers of oxidative stress and caspase-9** gene expression **in colon tissue of colitic rats and their control

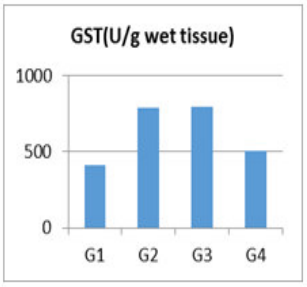


Figure 9

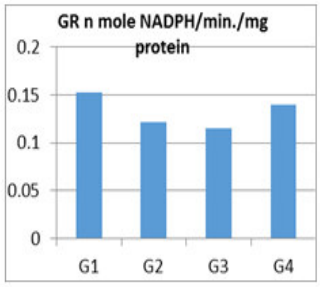


Figure 10

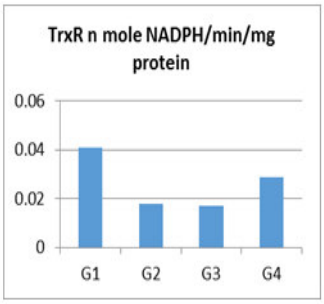


Figure 11

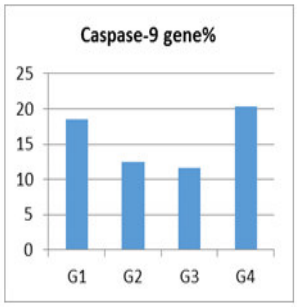


Figure 12

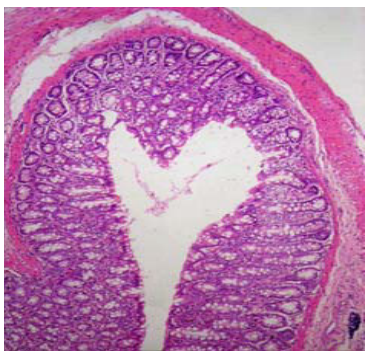


Figure (13)(a)
A photomicrograph of colon section in healthy control group showing no histopathological alteration and the normal histological structure was recorded

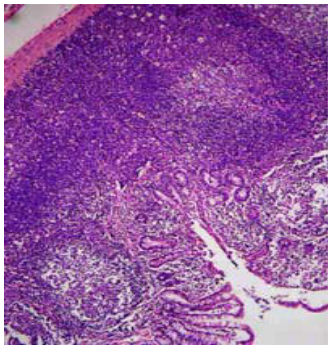


Figure (13) (b)
A photomicrograph of colon section in TNBS induced colitic group, showing serious mucosal injury included diffuse of inflammatory cell infiltration in the mucosa

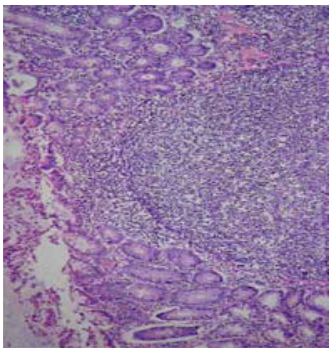
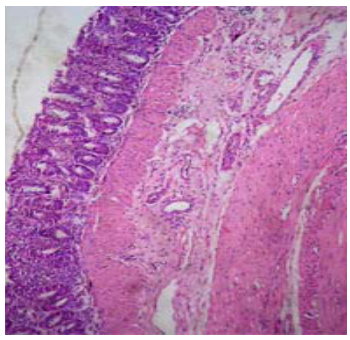


Figure (13) (c)
A photomicrograph of colon section in colitic group supplemented with 30% sucrose showing focal ulceration of the colonic mucosa extending through the mucosa



Figure(13) (d)
A photomicrograph of colon section in TNBS group , diet supplemented with 30 % sucrose and rats treated either saffron extract showing that, Saffron treatment enhanced the tissue damage and produced the surface of mucosa without ulceration.

DISCUSSION

This study confirms the intestinal anti-inflammatory and antioxidant activity of saffron extract, at a dose (80 mg/kg body wt.), in the TNBS model of rat colitis.Results demonstrated a beneficial effect of saffron extract in ulcerative colitis, as evidenced by effective modulation of the severity and extent of colon injury along with histopathological attenuation of the alterations caused by TNBS. Saffron extract supplementation suppressed the oxidative stress induced by TNBS via inhibition of elevated lipid peroxides and NO and restoration of GSH and GR in colon tissues .TNBS reduced the final body weight in all colitic groups , addition of sucrose in diet , causes significant differences in G3 compared to the control group. The produced damage in rat colon by TNBS was confirmed by a increased colon/body weight ratio. The impairment in the epithelial barrier function causes the pathophysiology of colon, increasing the access of luminal agents into

the intestinal tissue. Moreover, this injury weakens the normal absorptive capacity and inappropriate secretion of intestinal fluid and electrolytes, this may cause abdominal cramps, excessive gas and diarrhea [13] .This effects occurs in both animal and human model with intestinal inflammations [14]. Inflammatory response in colitic animals are represented as loss of body weight and diarrhea [15] and accompanied by increase in colon/body weight ratio. Saffron oral administration of concentrate brought about a noteworthy diminishment of colon/body weight proportion showing calming impact of saffron concentrate the large bowl.A significant elevation in colonic MPO, ALP , NO was shown in colitic rats posses comparing results to G1. Meanwhile, daily administration of saffron extract (G4) resulted in a significant decrease in MPO level as compared to groups G2 or G3. A slight reduction observed in ALP activity in G4 when comparing to G2 and G3, while saffron administration caused noticeable reduction in ATP and NO in G2 rats. NO considered as inflammation product that is required for of homeostasis maintenance in different organ systems and is a major agent for destroying pathogenic invaders. Diets contain 30% sucrose show a pronounced increase in inflammation. On the other hand, saffron extract has many active cpompiuns like including the crocin and tricrocin, pykrvkrvsyn, and safranal [3] .These active constituent have antioxidant properties which is important in extenuating lowered insulin production , it acts on insulin resistance preventing diabetes complications. The hypoglycemic effect of saffron extract seems to be exerted by mechanisms such as, reducing insulin resistance , stimulating of glucose uptake by peripheral tissues , and inhibition of intestinal glucose absorption .Colonic inflammation was additionally portrayed by an increase in ALP level, which has been attributed principally to leucocyte. ALP activity was previously reported to be significantly correlated with colitis inflammations[16]. TNBS-prompted colitis looks like most components of human IBD concerning a few histological modifications including mucosal intrusion of polymorphonuclear cells with excessive generation of inflammatory mediators that inflict injury to colon tissues [17]. MPO enzyme is a marker for neutrophil infiltration is the which is stored in azurophilic granules and released upon neutrophil activation and degranulation [18] . Inflammation increased the colonic MPO activity significantly , because saffron extract contains active anti-inflammatory compounds, thus reduces MPO levels in comparison with both non-treated colitic rats and sucrose supplemented rats. The data obtained from a previous study [19] showed that dietary crocin suppresses chemically induced colitis and colitis-related colon carcinogenesis in mice, by inhibiting inflammation, the mRNA expression of certain pro-inflammatory cytokines and inducible inflammatory enzymes.Inflammatory cytokines, TNF-an and IL-10 were surveyed, results portrayed a checked upregulation of the inflammatory status with expansions in the levels of the proinflammatory TNF-a in rats with TNBS-induced colitis, which are predictable with previous literatures [20,21,22].Saffron reduced TNF-a and IL-10 levels, as compared G2. However, it seems that sucrose addition to diets increase TNF-a and IL-10 levels as compared to sucrose free colitic group (G3). Endotoxins are toxic molecules that produced by intestinal microflora, the presence of excess carbohydrates in intestines initiates colonic inflammation, by penetrating the epithelial barrier, and stimulating the mucosal immune reactions . This produces pro-inflammatory cytokines, such as IL-10, IL-6 and TNF-α, and other mediators, causing the inflammatory activation of the mucosal immune system[23] .Activated neutrophils produce reactive oxygen and nitrogen species within intestinal mucosa inducing oxidative stress, which plays a significant role in the pathogenesis of ulcerative colitis. Moreover, TNF-a is released from macrophages in the early inflammatory response playing an important role in TNBS-induced colitis and it is considered the regulator key of the inflammatory cascade in ulcerative colitis . [24].Moreover, the level of inflammation and harm prompted by TNBS was paralleled to low levels of the anti-inflammatory cytokine IL-10 in colonic specimens[24]. Aftereffects of this study pronounced that adding sucrose in animal diets by 30% will build irritation and creates additional oxidative anxiety. Several animal studies have found a promoting effect of dietary sucrose on colon tumor developing [25,26]. In spite of the fact that, sucrose is hydrolyzed to fructose and glucose, after ingestion, sucrose expanded increased colon cell proliferation more than fructose and glucose. Previous studies [27,28] demonstrated that dietary sucrose act as a co-initiators or promoters in the formation of colon tumors.Oxidative status of the colon was calculated by the total GST,GR and TrxR . G2 characterized by a significant elevation in colonic GST accompanied by significant reduction in TrxR and GR. TNBS is a potent DNA damaging agent and carcinogenic that induces intestinal and colonic tumors in rodents [29] . Due to the absence of significant difference between G2

and G3, the increase in oxidative stress is directly related to TNBS rather than sucrose.It is clear that glutathione reductase (GR), is the major reductase catalyze the reduction of oxidized glutathione in glutathione system [31] .While, glutathione-S-tranferases (GST) is multifunctional enzymes, which play a key role in cellular detoxification[32] . In addition, thioredoxin reductase (TrxR), is the enzyme catalyze the NADPH-dependent reduction of thioredoxin and is noticed to play an important role in multiple cellular events related to carcinogenesis[33].Administration of saffron extract to colitic rats significantly decreased GST activity. On the other hand, saffron treatment enhanced the activity of TrxR, GR and caspase-9 in colon tissues when compared with either TNBS-treated group. These obtained results was in agreement with a previously published study [34] which reported that , crocetin (saffron's component) inhibited cellular reactive oxygen species generation. Caspase-9 in colon tissues was significantly decreased in G2 when compared to G1. Similarly, previous work [35] demonstrated that, caspase-9 was shown to be down regulated in colon cancer specimens in comparison with normal mucosa tissues. Also, another work reported that, decreased caspase-3 positive cells were evident in TNBS induced group as compared to control group. Saffron treatment enhanced the value of caspase-9 gene expression in colon tissues when compared with TNBS-induced colitic group. Meanwhile, the highest antioxidant capacity of saffron was shown by crocin**.** Crocin displayed a positive effect on the cognitive function via an antioxidant mechanism and inhibitory activity on amyloid-β aggregation[37] **.**Reactive oxygen species such as hydrogen peroxide, superoxide anion radical, peroxyl radical, hydroxyl radical, are the most important sources of oxidative damage in human body. The antioxidant effect of saffron extracts may occur due to interaction with enzymes or with signal transduction of free radicals in the monocytes. Moreover, safranal has lower antioxidant activity than crocetin and dimethylcrocetin [38]**.**Histopathological analysis showed that colon section in TNBS induced colitic group, showing serious mucosal injury included diffuse of inflammatory cell infiltration in the mucosa. The use of TNBS, altered the colon architectures, whereas daily administration of saffron extract is a protective factor of these organs in mitigating its harmful effect in experimental colitis, on the other hand, addition of dietary sucrose showed focal ulceration of the colonic mucosa extending through the muscularis mucosa. Dietary sucrose is a promoter in colon tumors formation[39].The genotoxic effect of sucrose in rat colon was previously studied [40,41] and indicated that the genotoxicity of sucrose is not related to oxidative DNA damage or altered DNA repair, but increased oxidative damage may still take place in other macromolecules leading to indirect effect on DNA. Histological examination of G4 showed that saffron treatment enhanced the tissue damage and produced the surface of mucosa without ulceration [42] **,** this may due to its major constituent, crocin by reducing the growth of colorectal cancer cells .

CONCLUSION

Oral administration of aqueous extract of saffron, protects the rat colon from experimental colitis induced by TNBS injection, by decreasing in oxidative stress associated with inflammation. Addition of sucrose on the diet of colitic rats increases the colonic inflammation, while sucrose has no incidence on the antioxidant status of colitic rats, indicating that the increase in oxidative stress is directly related to TNBS rather than sucrose. TNBS caused histological alterations rat colon mucosal cells, while saffron extract supplementation protects colonic mucosal cells against damage.

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