Intestinal injury can be effectively prevented by *Dunaliella salina* in gamma irradiated rats

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A B S T R A C T

Dunaliella salina (D. salina) is one of the most common microalgae that is used as human food. It is isolated from the salty lakes in El-Fayoum and Lake of Bardawil-Sinai in Egypt and can withstand very high concentrations of salt: The potentiality of *D. salina*, a unicellular biflagellate green alga to protect against intestinal injury induced after radiation exposure was studied. *D. salina* was given orally in doses of 100 and 200 mg/kg to male Wistar rats for 5 days before exposure to 6 Gray (Gy) gamma radiation and continued for a further two days. Rats were sacrificed 24 h later and intestinal segments were dissected out. One segment was examined histologically and another was used to prepare homogenates to assess relevant biochemical parameters reflecting intestinal injury. Radiation exposure led to a rise in the histological damage score, an increase in tissue tumor necrosis factor (TNF-α), interleukin (IL-1β) and thiobarbituric acid reactive substances (TBARS) but a reduction in tissue reduced glutathione (GSH) and in serum citrulline. Pretreatment with either dose of *D. salina* effectively reduced the severity of intestinal mucositis induced by gamma radiation.

1. Introduction

The use of radiotherapy in medicine to treat certain types of tumors is often limited because of potential damage to certain body tissues with a rapidly proliferating nature such as the intestinal mucosa (Hamama et al., 2012). Since the injurious effect of radiation involves oxidative stress mechanisms and the implication of inflammatory processes, the search for natural products that are safe and may be able to protect the intestinal mucosa from radiation injury concentrates on those having anti-inflammatory and anti-oxidant properties.

Microalgae have been used as food for hundreds of years in ancient times in many countries of Asia, Africa and South America. In recent times, these microorganisms have been extensively studied in an effort to discover novel compounds which could eventually be developed to produce therapeutic agents (Spolaore et al., 2006). Among such micro-algae is the alga *Dunaliella salina (D. salina)*, a unicellular marine phytoplankton related to the phylum Chlorophyta (Phadwal and Singh, 2003). It is isolated from the salty lakes in El-Fayoum and Lake of Bardawil-Sinai in Egypt and is rich in carotenoids which accounts for the red coloration of these salty lakes. The β-carotene content of *D. salina* was shown to confer protection against acetic acid-induced small bowel inflammation in rats (Lavy et al., 2003), while the ethanolic extract inhibited inflammatory cytokines in different animal models (Cha et al., 2010; El-Baz et al., 2016). Human studies suggest that β-carotene can provide better quality of life for asthmatic females (Moreira et al., 2004), normalize the enhanced LDL oxidation in patients with diabetes (Levy et al., 2000) and lowered LDL lipid peroxides in male hyperlipidemic smokers (Chao et al., 2002). The antioxidant and immune-modulatory effect of carotenoids has led to investigating their potential application for the prevention of human cancer (Chidambara Murthy et al., 2005). In fact, *D. salina* was reported to protect against radiation damage in children exposed to the Chernobyl disaster on account of its high content of β carotene, conferring anti-oxidant and anti-inflammatory properties (Ben-Amotz et al., 1998). The present study was accordingly designed to study the potential protective effect of *D. salina* against intestinal injury induced by ionizing radiation in rats.

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2. Materials and methods

2.1. Chemicals

Blue-Green medium (BG11) was purchased from Unipath Ltd. (Basingstoke, UK), β-carotene was obtained from Sigma-Aldrich (St. Louis, MO, USA), Enzyme-linked immunosorbent assay (ELISA) rat-specific kits for the determination of citrulline from Cusabio Biotec, (Wuhan, Hubei, China) and that for TNF- α and IL-1β from ID Labs Biotechnology (London, Ontario, Canada). All other chemicals and solvents were of highest analytical grade.

2.1.1. Dunaliella salina

D. salina (Strain NIES-2257) was isolated in our laboratories from samples collected from effluent ponds of the Egyptian Company for Salts and Minerals (EMISAL, El-Fayoum Governorate, Egypt).

The organism was grown in conical flasks containing BG11 nutrient media (Stanier et al., 1971). The media was enriched with 10% NaCl, and the pH was adjusted to pH 7.1 with 1M NaOH or HCl.

D. salina biomass was harvested before the end of the log phase by centrifugation at 6000 rpm for 15 min. Samples were washed twice with water, dried in an oven at 40 °C, ground into a homogenous powder and stored in a refrigerator for further chemical and biological investigation.

2.1.2. Chemical analysis of D. salina dry biomass

Since the main biological activity of *D. salina* resides in its content of carotenoids, lipids and chlorophyll, it was necessary to carry out the chemical analysis of the *D. salina* dry biomass in order to determine the concentration of these constituents (Liu and shen, 2005; Varsano et al., 2006; Lamers et al., 2010; Xu et al., 2018). The carotenoid content was extracted from the algal biomass using ethanol/hexane 2:1 v/v (Shaish et al., 1992) and measured spectrophotometrically at 450 nm using β-carotene as a standard.

Total chlorophyll content was extracted with hot methanol containing magnesium carbonate solution (1%) to prevent chlorophyll degradation and determined spectrophotometrically (Fitzgerald et al., 1971).

Extraction of total lipids was carried out as previously reported (Axelsson and Gentili, 2014) and analysis of the fatty acid methyl esters was conducted according using a Focus gas chromatograph (Thermo Fisher Scientific, Bleiswijk, The Netherlands) (Breuer et al., 2013).

2.2. Animals

Male Wistar rats, each weighing 140–180 g, were purchased from the National Research Centre, Giza, Egypt, and left to acclimatize for 7 days before subjecting them to experimentation at the animal house of the National Centre for Radiation Research and Technology (NCRRT) at an ambient temperature of 25 ± 2 °C, relative humidity of 60–70% and a 12-h light/12-h dark cycle. They were fed on a standard laboratory chow and water ad libitum. The study was approved by the Ethical Committee of the Faculty of Pharmacy, Cairo University, Egypt (Permit PT:184). in accordance with the guidelines set by the European Economic Community (EEC) (revised Directive 86/609/EEC).

2.3. Irradiation of animals

Animals were exposed individually to whole body gamma irradiation at a dose level of 6 Gy at the NCRRT using the Gamma Cell-40 biological irradiator furnished with a Cesium[137] source (Atomic Energy of Canada Ltd; Sheridan Science and Technology Park, Mississauga, Ontario, Canada). The radiation dose rate was 0.46 Gy/min. The radiation dose level of 6 Gy was selected and found to be appropriate to induce intestinal mucositis after pilot experiments carried out in our lab with radiation exposures of 4, 6 and 8 Gy, showing graded extents of injury (Khayyal et al., 2014).

2.4. Experimental design

The rats were blindly allocated to four groups of 8 rats each (n = 8). Group 1 served as control non-irradiated animals, in Group 2 the rats were irradiated at 6 Gy but left untreated, while in Group 3 and 4 the rats were given *D. salina* extract orally in a dose of 100 and 200 mg/kg respectively, daily for 5 days before exposure to radiation and treatment continued for 2 days after irradiation. The doses of *D. salina* were chosen on the basis of preliminary experiments showing graded anti-inflammatory effect with doses ranging from 50 to 200 mg/kg. Furthermore, earlier studies reported that *D. salina* was used in doses ranging from 123 – 615 mg/kg for 8 days in mice to protect them against ultraviolet B-induced corneal oxidative damage (Tsai et al., 2012). A dose of 150 mg/kg was used in diabetic rats (Ruperez et al., 2009), while doses up to 710 mg/kg were used in mice to protect them against carbon tetrachloride hepatotoxicity (Hsu et al., 2008)

On the third day after irradiation, the rats were euthanized by deep ether anesthesia followed by decapitation. Blood was collected to prepare serum samples which were stored at −20 °C until required. One segment from the proximal part of the jejunum was dissected out and kept in 10% formalin for semi-quantitative histological examination. The remaining jejunal tissue was flushed of its content with chilled isotonic saline and stored at −80 °C until needed to prepare intestinal homogenates in appropriate media according to the parameters to be measured.

2.4.1. Histological examination

The proximal jejunal segment fixed in 10% formalin was embedded in paraffin wax, cut serially into 4 μm thick sections, and stained with hematoxylin and eosin (H & E) for light microscopic examination. The sections were examined using an Aristoplan microscope (Leica, Bensheim, Germany) and photographed at x100 magnification.

The histological changes were examined semi-quantitatively as described by Howarth et al. (1996). A damage score of 0–3 (0 = no damage, 1 = mild, 2 = moderate, 3 = severe) was given to each of the following ten criteria in each examined jejunal section: shortening and atrophy of villi, fusion of villi, denudation of villi, activation of glandular epithelium, activation of nuclei of enterocytes, inflammatory cell infiltration, edema in lamina propria, hemorrhage in lamina propria, apoptosis and exudate in the lumen. Accordingly, a maximum overall damage score (ODS) of 30 could be computed for each group.

2.4.2. Assay methods

2.4.2.1. Measurement of intestinal lipid peroxidation.
Lipid peroxides were determined in 10% tissue homogenates prepared in ice-cold 1.15% potassium chloride (Uchiyama and Mihara, 1978). The method depends on the reaction between thiobarbituric acid reactive substance (TBARS) and thiobarbituric acid at an acidic pH to give a pink-colored product that can be measured colorimetrically at a wave length of 535 nm using a Unicam 8625 UV/V spectrophotometer (Cambridge, UK). The TBARS were expressed as nmol/g tissue.

2.4.2.2. Measurement of intestinal reduced glutathione (GSH) content.
The level of GSH was measured according to Beutler et al. (1963). The jejunum tissue was homogenized in ice-cold 10% trichloroacetic acid. The yellow colored complex (5-thio nitro benzoic acid) formed by the reaction of GSH and Ellman's reagent (0.01 M 5,5′-dithiobis-2-benzoic acid solution) was measured spectrophotometrically within 5 min of addition of Ellman's reagent showing maximum absorbance at 412 nm using a Unicam 8625 UV/V spectrophotometer (Cambridge, UK). Results were expressed in mg/g tissue.

2.4.2.3. Measurement of intestinal TNF- α and IL-1β.
Sections of the jejunum tissue were homogenized in phosphate buffer saline (pH 7.4) and the supernatant was separated and used for the estimation of TNF- α
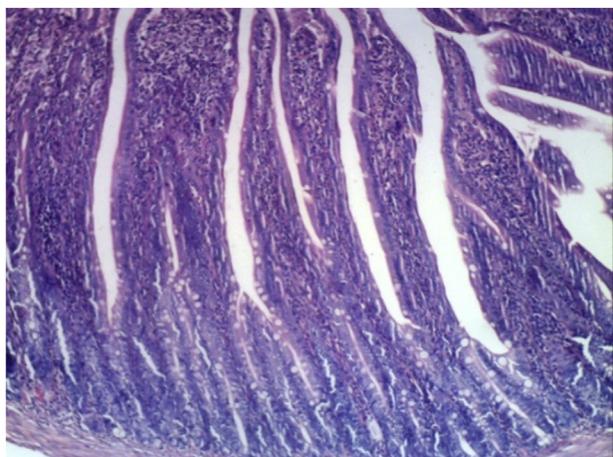
Fig. 1. Histological micrograph of the jejunum of normal rats showing normal structure of mucosa, submucosa, musculosa and serosa. Note the long intestinal villi (H&E x 100).

and IL-1β using a rat specific ELISA kit. The optical density of each sample was measured using an ELISA plate reader (Dynatech R MR 5000, Guernsey, Channel Islands, UK) set at 450 nm. Values were expressed as pg/g tissue.

2.4.2.4. Measurement of serum citrulline. Citrulline in the serum samples was measured using a rat specific ELISA kit and measuring the optical density using the ELISA plate reader mentioned above at a wave length of 450 nm. Values were expressed as (pmol/ml).

2.5. Statistical analysis

All data were expressed as mean values ± standard error mean (SEM). Comparison between the means of the different groups was performed using one-way analysis of variance (ANOVA) together with the Tukey–Kramer multiple comparison test. The assessment of the histological findings was carried out semi-quantitatively and the data then analyzed by Kruskal–Wallis analysis of variance (ANOVA) with a Dunn's post hoc test. In all cases, statistical significance was set at probability values less than or equal to 0.05.

3. Results

3.1. Chemical analysis of D. salina biomass

The total lipid content of the algal mass under the current culture conditions was found to be 7.89 ± 0.40 g%. The total fatty acid content was determined to be 7.32 ± 0.04 mg/g dry weight, of which C16:0 (4.58

± 0.02 mg/g) and C18:0 (0.38 ± 0.06 mg/g) acids were identified as the saturated fatty acids, while C16:1 (0.52 ± 0.00 mg/g), C18:1 (0.63 ± 0.03 mg/g), C18:2 (0.54 ± 0.01 mg/g) and C18:3 (0.66 ± 0.02 mg/g) acids were the unsaturated fatty acids. Total carotenoids were determined to be 3.3 mg/g of dry powder (calculated as β-carotene), while the chlorophyll content (calculated as chlorophyll a) was found to be10 mg/g.

3.2. Histological findings

Normal control rats showed a normal architecture of villi, crypts and enterocytes (Fig. 1). Exposure of rats to acute irradiation led to activation of the glandular epithelium, dense inflammatory infiltration in the lamina propria, and shortening and fusion of villi (Fig. 2). Pre-treatment with *D. salina* at both dose levels protected against these changes nearly to the same extent, showing nearly normal mucosa and villi with few inflammatory infiltrations in lamina propria and slight activation in glandular epithelium (Fig. 3).

The histological damage induced by irradiation was clearly represented by an increase in the ODS score. The protective effect of *D. salina* was further confirmed by the fact that the ODS score was kept within the normal range by both doses used (Fig. 4).

3.3. Biochemical findings

Jejunal homogenates of rats exposed to radiation showed a rise in the level of TBARS by 35% associated with a reduction in the level GSH by nearly 40%. *D. salina* at both dose levels guarded equally against these changes (Fig. 5, Table 1), reflecting its good anti-oxidant activity.

Exposure to radiation further led to a dramatic increase in the inflammation biomarkers, TNF-α and IL-1β, an effect which was also prevented by both doses of *D. salina* (Fig. 6, Table 1).

Exposure to radiation led to a 45% decrease in the serum level of citrulline, an effect which was largely prevented by *D. salina* at both dose levels nearly to the same extent (Fig. 7, Table 1). The changes in the level of citrulline correlated well with the ODS score and the extent of villus atrophy.

From the above results (summarized in Table 1), it can be concluded that a maximal effect was achieved by the dose of 100 mg/kg of *D. salina*. Increasing the dose of the alga led to no further increase in both histological and biochemical parameters measured.

4. Discussion

Gastrointestinal mucosal injury is one of the common side effects of radiotherapy which compromises continuation of therapy. One of the important underlying causes of mucosal injury after exposure to radiation is the excessive generation of reactive oxygen species (ROS) far above the capacity of the inherent cellular anti-oxidant enzyme systems
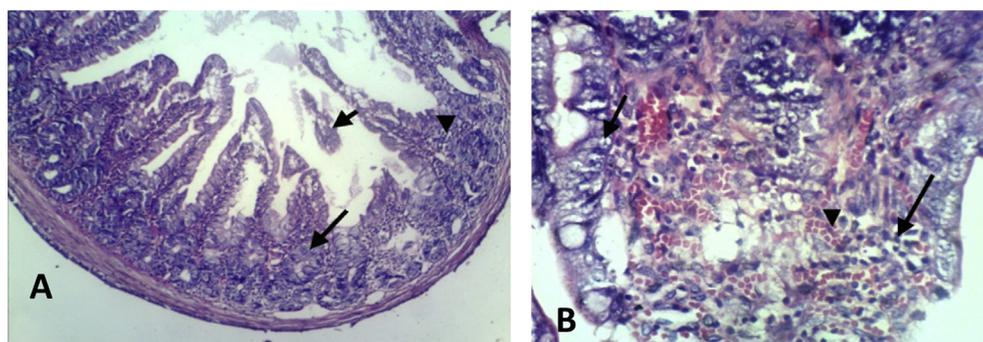
Fig. 2. Histological micrograph of the jejunum of irradiated rats. (A) showing shortening and focal denudation of intestinal villi (small arrow), activation of glandular epithelium (large arrow) and focal inflammatory cell infiltration in lamina propria (arrow head); (B) showing activation of glandular epithelium (small arrow), inflammatory cell infiltration in lamina propria (large arrow) and hemorrhage in lamina propria (arrow head) (H & E x 100).
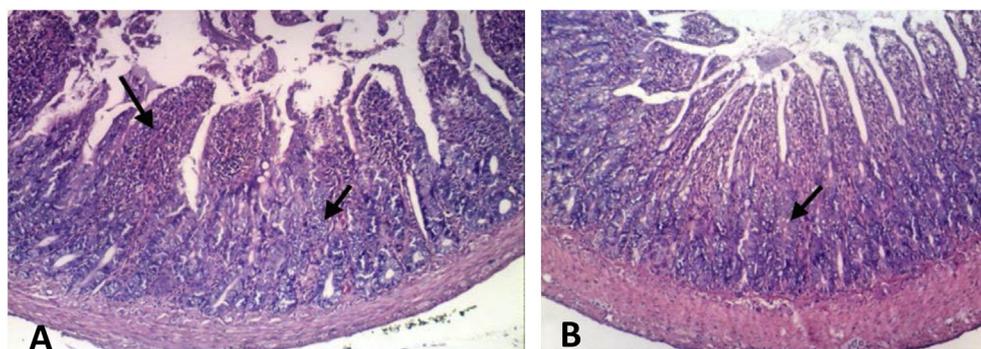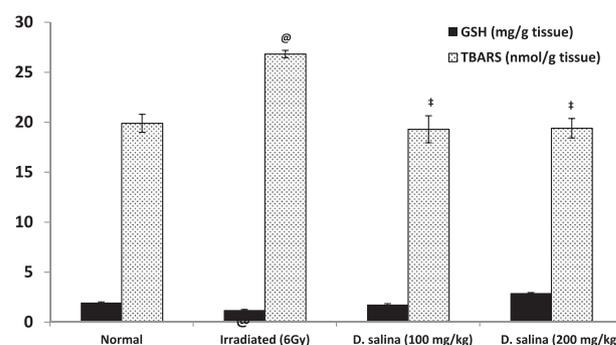
Fig. 3. Histological micrograph of the jejunum of rats treated orally with *D. salina* (A) 100 mg/kg, showing slight activation of glandular epithelium (small arrow) with few inflammatory cells infiltration in lamina propria (large arrow); (B) 200 mg/kg, showing slight activation of glandular epithelium (H & E X 100).
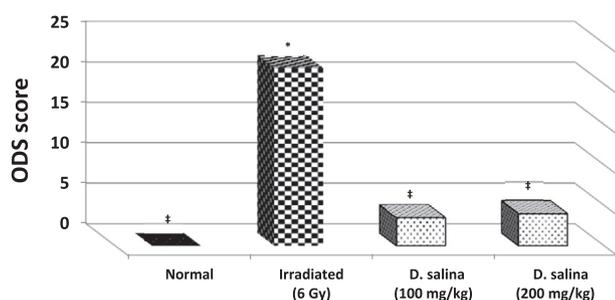
Fig. 4. Semi-quantitative histological assessment of radiation induced intestinal damage in rats after exposure to 6 Gy as influenced by *D. salina* administration. Values are the sum of median scores for ten histological criteria (see text). * indicates $p \leq 0.05$ compared to normal; ‡ indicates $p \leq 0.05$ compared to irradiated rats.

Fig. 5. Effect of *D. salina* on the level of GSH (mg/g tissue) and TBARS (nmol/g tissue) in the jejunal tissue of irradiated rats. All values are expressed as means $\pm$ standard error mean (SEM). @ $p \leq 0.05$ compared to normal, ‡$p \leq 0.05$ compared to irradiated animals.

to compensate for it (Spitz et al., 2004). Oxidative stress was evidenced in this study by the marked increase in lipid peroxidation as shown by the increased level of jejunal TBARS associated with a significant depletion of GSH signifying a reduction of effectiveness of the antioxidant enzyme defense mechanisms to counteract that increase. Algae, in general, possess components that can counteract the harmful effects of ROS, including chlorophyll (Cha et al., 2010) and carotenoids (Bidigare et al., 1993). Accordingly, treatment with *D. salina* was effective in preventing the changes in the levels of TBARS and GSH through its potent antioxidant activity on account of its content of carotenoids (Chidambara Murthy et al., 2005; Tran et al., 2014) and chlorophyll. It has been reported earlier that the protective effects of carotenoids differs from that of chlorophylls. Thus, whereas the former owe their protective effect to their oxidant, antioxidant, redox sensitive cell signaling, induction of gene expression, and provitamin A properties (Elliott, 2005), the latter owe it more to their ability to modulate activation of endogenous xenobiotic detoxification systems and caspases/polymerase pathway as well as by their antioxidant and mutagen trapping properties (Ferruzzi and Blakeslee, 2007). Previous studies suggested that carotenoids and chlorophylls might exert a synergistic and/or additive effect on the antioxidant activity of pepper (Alvarez-Parrilla et al., 2011; Ornelas-Paz

et al., 2013). Thus, the antioxidant activity of *D. salina* might be explained by the synergistic or additive activity of these contents.

Apart from the direct role of ROS in inducing intestinal mucositis after exposure to radiation, they trigger a cascade of inflammatory pathways due to the activation of the NF-κB pathway (Linard et al., 2004; Ong et al., 2010). The latter promotes the expression of multiple pro-inflammatory molecules, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, and pro-IL-1β, and ensures feedback amplification of the NF-κB-dependent signaling pathway (Lee et al., 2014). Therefore, the observed release of TNFα and IL-1β might be due to the activation of NF-κB pathway. Treatment with *D. salina* guarded against the increase in the level of pro-inflammatory cytokines. The anti-inflammatory effect of the algae was reported by earlier studies (Yang et al., 2014; Abdel-Daim et al., 2015). Moreover, previous studies showed that *D. salina* may exert its anti-inflammatory effect through inhibition of iNOS, COX-2, and NF-κB expression, thereby inhibiting the production of the pro-inflammatory cytokines, TNF-α and IL-1β (Lin et al., 2017). Chlorophyll, one of the major content in *D. salina* has been shown to exert anti-inflammatory activity by inhibiting TNF-α gene expression (Subramoniam et al., 2012). Therefore, the anti-inflammatory

Table 1
Effect of *D. salina* on the level of several biochemical parameters in irradiated rats. All values are expressed as means $\pm$ standard error mean. @$p \leq 0.05$ compared to normal, ‡$p \leq 0.05$ compared to irradiated animals.

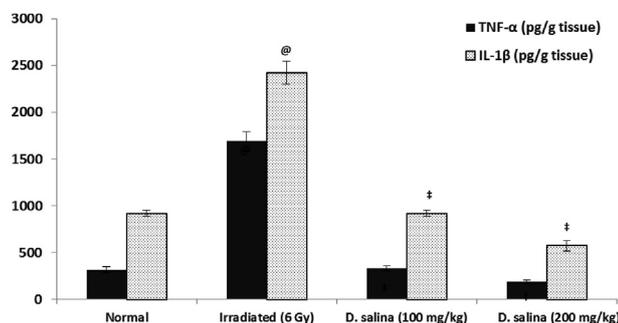| Groups	GSH (mg/g tissue)	TBARS (nmol/gtissue)	IL-1β (pg/g tissue)	TNF-α (pg/g tissue)	Citrulline (pmol/ml)
Normal	1.95 ± 0.06	19.89 ± 0.99	918.90 ± 31.29	316.70 ± 30.73	11.13 ± 0.53
Irradiated	1.19 ± 0.07@	26.82 ± 0.37@	2421.00 ± 125.00@	1692.00 ± 102.20@	6.34 ± 0.22@
D. salina 100 mg/kg	1.76 ± 0.09‡	19.29 ± 1.35‡	919.20 ± 32.68‡	329.20 ± 26.15‡	17.01 ± 0.76‡
D. salina 200 mg/kg	2.99 ± 0.06@‡	19.40 ± 0.97‡	574.40 ± 24.01‡	189.20 ± 16.55‡	16.13 ± 0.77‡

Fig. 6. Effect of *D. salina* on the level of TNF-α and IL-β in the jejunal tissue of irradiated rats. All values are expressed as means ± standard error mean. $^@p \leq 0.05$ compared to normal, $\ddagger p \leq 0.05$ compared to irradiated rats.
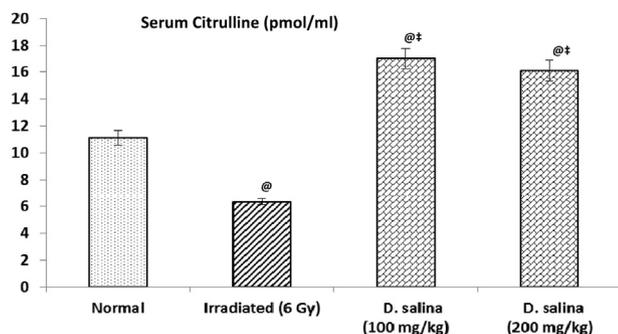

Fig. 7. Effect of *D. salina* on the level of citrulline in the serum of irradiated rats. All values are expressed as means ± standard error mean. $^@p \leq 0.05$ compared to normal, $\ddagger p \leq 0.05$ compared to irradiated animals.

effect of *D. salina* might be attributed to its content of chlorophyll. Moreover, it was suggested that carotenoids, the second important active ingredients in *D. salina*, can modulate inflammatory processes via inactivation of NF-κβ pathway and thereby might have a role in inhibiting the release of TNF-α and IL-1β (Yang et al., 2013).

The release of ROS and inflammatory cytokines has both been shown to contribute to a large extent towards the histological changes induced in the intestinal mucosa of irradiated rats (Hepgul et al., 2010; Cameron et al., 2012). The excessive release of TBARS injures cellular biomolecules such as nucleic acids, proteins, carbohydrates and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation (Sener et al., 2006). Exposure to radiation led to activation of the glandular epithelium, dense inflammatory infiltration in the lamina propria, and shortening and fusion of villi. Our findings are in agreement with earlier studies that showed severe loss of villi and inflammatory cell invasion in the lamina propria after radiation exposure (Sener et al., 2006; El-Ghazaly et al., 2015; Khayyal et al., 2015). The histological damage was reflected by an increase in the ODS score. The observed histological changes could thus be accounted for partly by the increased ROS production induced by radiation and partly due to the increased production of pro-inflammatory mediators.

Therefore, the anti-oxidant and anti-inflammatory properties of *D. salina* are both probably responsible for protecting the intestinal mucosa against the development of these changes. The protection against the radiation-induced histological changes was reflected by the ODS which was retained normal in animals pre-treated with *D. salina*.

Small bowel irradiation results in epithelial cell loss and consequently impairs function and metabolism. Many functional disorders have been reported after exposure to ionizing radiation, including changes in transepithelial transport processes (Lebrun et al., 2002) affecting the absorption of various nutrient substances such as carbohydrates, amino acids, proteins, vitamins, and bile acid (Travis and Menzies, 1992). Some

of these functional changes have been correlated with the epithelial cell mass available for absorption, suggesting a cellular basis in at least part of radiation-induced functional disorders. Citrulline, a metabolic end product of small bowel enterocytes, can be used for quantifying radiation-induced epithelial cell loss (Lutgens et al., 2003). Exposure to radiation led to a decrease in the plasma level of citrulline together with loss of integrity of intestinal mucosal surface and atrophy of the villi and thereby; reduction of the absorptive surface. Similar previous findings showed that the reduction in citrulline level was associated with an epithelial damage after single-dose whole-body irradiation (Khayyal et al., 2015; Lutgens et al., 2003). Pre-treatment with *D. salina* was able to maintain the plasma citrulline level within the normal range possibly by preserving the integrity of the intestinal mucosa.

In conclusion, the present study provides evidence for the beneficial use of *D. salina* in preventing radiation induced intestinal mucositis. A maximal effect was shown with a dose of 100 mg/kg. The results further show that the anti-oxidant and anti-inflammatory activities of *D. salina* as well as its ability to stabilize the intestinal mucosal membrane are the main mechanisms underlying its usefulness against radiation exposure.

Declarations

Author contribution statement

Mohamed T. Khayyal: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Farouk K. El-Baz: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Meselhy R. Meselhy: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gamila H. Ali: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Rania M. El-Hazek: Conceived and designed the experiments; Performed the experiments.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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