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Sex hormones and testicular cell proliferation in *sargassum virgatum* - administered rats exposed to gamma rays

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Infertility may be attributed to a series of environmental factors such as heat, severe exposure to pesticides, radiation, radioactivity, and other hazardous substances. We scrutinized the protective and antioxidative effects of both aqueous and ethanolic extracts of the marine brown macroalga *Sargassum virgatum* extracts (*S. virgatum*) in the irradiated rats. The animals were subjected to 3.5 Gy as a single dose of gamma radiation (γ), *S. virgatum* extracts 100 and 400 mg/kg^{-1} were daily administrated at the 24 h post- gamma radiation treatment and was then injected daily for 14 days. The blood and testicles samples were taken 15 and 60 days post the radiation. According to a proposed therapeutic dose, the irradiated rats were administrated with *S. virgatum* and then the effect of these treatments on the parameters correlated to oxidative damage of the rat testes were assessed. Blood biological analysis manifested that *S. virgatum* extracts are able to ameliorate the reduction in the serum testosterone caused by gamma rays. Furthermore, *S. virgatum* could significantly decrease FSH and LH. In contrariwise, treatment of the irradiated rats with *S. virgatum* increased the total and free testosterone in serum. Moreover, microscopic analysis reinforced that *S. virgatum* extracts able to prevent histological damage in the testes of irradiated rats. Moreover, treatment of gamma rays promoted to an augmentation in the expression of Proliferating Cell Nuclear Antigen Gene (PCNA). Interestingly, the *S. virgatum* treatment also exhibited a significant improvement in the testicular function in irradiated rats. Additionally, our results substantiated the prospective effects of *S. virgatum* extracts in attenuating gamma rays-induced oxidative damage, in particular in testicular tissue. It could be postulated that *S. virgatum* as a multifunctional dietary supplement could exert a modulatory role in radiation-induced testis and biochemical and histological changes through its antioxidant properties.

Keywords: γ- rays; brown algae; antioxidant; PCNA; testis and DNA fragmentation

INTRODUCTION

The studies have demonstrated that 30-50% of human infertility is due to infertility in males (Agarwal et al., 2015; Yao et al., 2016). Although hormonal causes of male infertility are well known, the inability to diagnose and treat certain forms of infertility is due to the loss of sufficient information about intracellular mechanisms that produce important factors needed to regulate spermatogenesis.

Exposure of animals to ionizing radiation leads to a series of successive physiological and biochemical changes known as acute radiation syndrome, which is dependent on the exposure dose and may lead to death. In addition to previous damages, there is damage to the blood

system, which is a major factor in the mortality rate after exposure to acute radiation (Garau et al., 2011).

The testis is known to be one of the most sensitive radiation devices in the body. With the appearance of new radiotherapy methods, there is a significant improvement in the survival rate of cancer patients represented in experimental animals. Therefore, it is recommended to protect the reproductive capacities and genetic characteristics of the germ cells of these mammals from radiation damage (Osovets et al., 2011). It is worth noting that apoptosis in the testis plays an important role in reducing the number of germ cells (Alves and Oliveira, 2017). Furthermore, external disturbances such as chemotherapy or radiation cause stress, which in turn leads to increased testicular cell death, which increases the chance of total germ cell loss. Therefore, the exposure to doses as low as 0.1 Gy is known to cause damage to the spermatogonia cells.

At present, the search for more efficient radiotherapy has been intensified due to the increased use of ionizing radiation in radiation therapy to treat malignant tumors. Radiotherapy alone can cause long-term azoospermia (Meistrich, 2013 and Ahmad and Agarwal, 2017). Many studies have shown that local or total exposure to gamma rays causes testicular damage to both humans and animals (Akleyev, 2016). Radiotherapy, one of the most important methods of cancer treatment, relies on the generation and use of reactive oxygen species (ROS) to eradicate tumors (Chen et al., 2017), and in the process, non-target tissues are also damaged. ROS produces toxic free molecules in conjunction with biological molecules within the organism and leads to lipid peroxidation as well as severe DNA damage (Dayem et al., 2017). Moreover, these free radicals can also change the balance of internal protection systems, such as glutathione and enzymatic antioxidant defense systems (Patlevič et al., 2016). The endogenous antioxidants may be insufficient to reduce free radicals induced by radiation. Antioxidant supplements may have the potential to reduce an individual's susceptibility to oxidative damage from such agents. Seaweeds are known to be a good supply of several health-promoting bioactive compounds capable of acting on a wide spectrum of diseases and/or disorders beyond their role of basic nutrition, and therefore are being marketed as functional foods or nutraceuticals (Mishra et al., 2015; Wells et al., 2017; Boi et al., 2017). Even though the utility of *Sargassum* species in Occidental countries have barely been estimated (used mainly as fertilizer, for animal feed, and for alginate extraction) (McHugh,2003), in Asia, the experimental use of *Sargassum* species is used to treat a variety of diseases embracing skin, thyroid disease, atherosclerosis, bronchitis (Liu et al., 2012). Despite many other common uses, there is a lack of scientific evidence on their pharmacological properties, with scarce data existing on its effective results as well as the mechanisms of action. *Sargassum virgatum* is known to produce molecules with potent antioxidant properties (Pinteus et al., 2017); however, it is important to understand if it has potential to protect human cells on oxidative stress conditions. *Sargassum* is a rich source of bioactive compounds with a wide range of health benefits. However, a systemic phytochemical study of these *Sargassum* species could help to determine the biological activity of each species for medicinal uses (Yip et al., 2018). This seaweed, in particular, represents one of the common algal flora growing in the Egyptian Red Sea coasts (Djakouré et al., 2017). Seaweeds or large algae contain a large variety of metabolic compounds and biologically active compounds, such as polysaccharides, polyunsaturated fatty acids, and other phenolic compounds (Perez et al., 2016).

Therefore, the present study was designed to estimate the possible protective effects of *S. virgatum* against oxidative stress induced by gamma irradiation in male Wister albino rats by measuring certain biochemical parameters such as testosterone free and total, LH and FSH hormones as well as, proliferation marker (PCNA) in testicular tissues. Whether *S. virgatum extracts* has any significant role in radiation-induced testes dysfunction *in vivo* by studying its effects on the histopathological study of testes before and after the treatments.

MATERIALS AND METHODS

Animals

Experiments were performed during November- January 90- day-period using Wistar male rats were obtained from the Nuclear Research Center (Cairo, Egypt). The animals were initially of 3-month age maintained under standard conditions (in plastic rat cages provided with food and water *ad libitum*; and They were staying at room temperature with a normal light / dark cycle. The guidelines of the Institutional

Animal Ethics Committee for experimental research in small animals have been strictly followed (Kasatkina and Kaplansky, 2000). In particular, when blood samples were taken, the rats were anesthetized by injection of 60 mg/kg of sodium phenobarbital via the intraperitoneal membrane.

Algal sampling, identification and extraction

Specimens of the brown seaweed *Sargassum virgatum* were collected on October 26th 2017 from the littoral zone of rocky shorelines in Hurghada city (27° 15′ 58.45″ N, 33° 48′ 57.09″ E), the Red Sea coast of Egypt. The collected seaweeds were washed successively in the field and then by tap water and distilled water, then were air-dried under shade for 2 weeks. The dried algal material was ground to 2 mm or smaller particle size.

The samples were pulverized to a fine powder using an electric blender and then stored well in sterile clean plastic bags in the refrigerator until further studies. Specimens of *S. virgatum* were morphologically identified following the relevant literature adopted by Silva et al. (1996). Based on preliminary studies using different polar and non-polar solvents (i.e., hexane, ethyl acetate, ethanol and water), the polar solvent "ethanol" was found to be the most effective one for the extraction process of antioxidant polyphenols, and the ethanolic extract of *S. virgatum* (1: 10 w/v) was prepared according to the method of O'Sullivan et al., (2014). Ground seaweed was suspended in methanol (EtOH) (99%) and the extraction process was carried out by incubation in an orbital shaker (Unimax 2010, Heidolph Instruments, Schwabach, Germany) at 175 rpm. The crude algal methanolic extract was filtered after 6 h incubation through Whatman No.1 filter paper (Whatman International, Ltd., Maidstone, England), and then the seaweed material was re-suspended in ethanol (1: 10 w/v) and filtered after 24 h to ensure exhaustive extraction occurred. The solvent extracts were combined and methanol was removed by a rotary evaporator at (Büchi R-200, Marshall Scientific, Minnesota, USA) at 40°C. The *S. virgatum-EtOH and aqueous extracts were* stored at -20 ºC in liquid nitrogen until further use.

Gamma-radiation

The entire body of rats were exposed to gamma rays a single dose of (3.5 Gy) gamma- ray with a dose rate of 0.7 rad/Sec. For this purpose, the source of cesium source (^{137}CS) source, Gamma Cell-40 biological irradiator, at the National Centre for Radiation Research and Technology (NCRRT), Cairo, Egypt. The animals were exposed to a single dose of (3.5 Gy) γ -ray (with a dose rate of 0.7 rad/Sec.

Study design

The experiment was based on dividing the animals into two main experimental groups (n=90). The first set was kept as an untreated control group and this consists of fifteen rats. The second group consisted of seventy- five rats were exposed to a single dose of γ- radiation (3.5 gray) whole body to induce testicles injury (Ogilvy-Stuart & Shalet, 1993). The irradiated animals were subdivided into five subgroups (each containing 15 rats). The first subgroup (GII A) was sustained without treatments until the end of the experiment (14 days) (Irradiated group). The second subgroup (GII B) received the first intraperitoneally (IP) treated with the *S. virgatum* aqueous extract 100 mg/kg BW at the next day post- gamma radiation treatment. The third subgroup (GII C) received the *S. virgatum* aqueous extract 400 mg/kg^{-1} at the next day post-gamma radiation treatment.

The fourth subgroup (GII D) received the *S. virgatum* ethanol extract 100 mg/kg^{-1} at the next day post- gamma radiation treatment, daily for 14 days. The fifth subgroup (GII E) received the *S. virgatum* ethanol extract at dose 400 mg/kg^{-1} at the next day post- gamma radiation treatment, daily for 14 days.

In order to confirm radiation induced testicular failure and to evaluate the outcome of four extractions, blood samples from each animal were collected from orbital venous plexus. The serum was separated by centrifugation at 3000 g for 15 min, divided into aliquots and stored at -20º C for biochemical assays.

Acute toxicity studies of *S. virgatum extract*s

Acute toxicity (LD50) of *S. virgatum* was determined using the method of Lorke, (1983). Thirty rats were used for this test in two groups. In the first one, the rats consisted of concentrations of 10, 100 and 1000 mg/kg of the *S. virgatum* intraperitoneally. The second group included high concentrations of (1600, 2900 and 5000 mg/kg). It should be noted that each concentration of the previous concentrations included the number of five rats. The rats were observed for 48 hours for any mortality.

$LD_{50} = M_0 + M_1 / 2$

Where M_0 = The highest dose of test substance that does not give any mortality, M 1 = The lowest dose of test substance that give mortality.

Blood samples Collection and Processing

Serum was separated by centrifugation at 3000 g for 15 minutes and kept frozen at −80°C until assessment of Testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

Estimation of Follicle stimulating hormone (FSH)

FSH was determined by radioimmunoassay (RIA) procedures using commercial kits purchased from (DIAsource Immunoassays', Louvain-la-Neuve, Belgium) on Gamma Counter RIA Star Model.

The assay is based on that the antibodies are labeled with radioisotopes which are used to bind antigens present in the samples. When a positive sample is added to the tubes, radioactively labeled (labeled with I^{125} radioisotopes) antibodies bind to the free epitopes of antigens and form an antigen-antibody complex. On the contrary, unbound labeled antibodies are removed by a second reaction with a solid phase antigen. The amount of radioactive remaining in the solution is direct function of the antigen concentration.

Estimation of LH hormone

LH was determined by immunoradiometric assay (IRMA) procedures using commercial kits purchased from (Institute of Isotopes Co. Ltd., Budapest, Hungary) on Gamma Counter RIA star model.

The ^{125}I labelled signal-antibody binds to an epitope of the LH molecule spatially different from that recognized by the biotin-capture antibody. The couple antibodies react simultaneously with the antigen present in standards or samples, which leads to the formation of a capture antibody - antigen - signal antibody complex, also referred to as a "sandwich". During an hour incubation period with shaking immuno-complex is immobilized to the reactive surface of streptavidin coated test tubes. Reaction mixture is then discarded, test tubes washed exhaustively, and the radioactivity is measured in a gamma counter. The concentration of antigen is directly proportional to the radioactivity measured in test tubes. By constructing a calibration curve plotting binding values against a series of calibrators containing known amount of LH, the unknown concentration of LH in samples can determined.

Estimation of Testosterone hormone

Testosterone was determined by radioimmunoassay assay (RIA) procedures using commercial kits purchased from (DIA Source Immunoassays, Louvain-la-Neuve, Belgium) on Gamma Counter RIA Star Model.

The assay is based on a fixed amount of ^{125}I labeled steroid competes with the steroid to be measured present in the sample or in the calibrator for a fixed amount of antibody sites being immobilized to the wall of a polystyrene tube. Neither extraction nor chromatography is required because of the high specificity of the coated antibodies. After 3 hours' incubation at 37°C, an aspiration step terminates the competition reaction. The tubes are then washed with 3 ml of wash solution and aspirated again. A calibration curve is plotted and the testosterone concentrations of the samples are determined by dose interpolation from the calibration curve.

Estimation of Testosterone free hormone

Testosterone free was determined by radioimmunoassay assay (RIA) procedures using commercial kits purchased from (DIA source Immunoassays, Louvain-la-Neuve, Belgium) on Gamma Counter RIA Star Model.

The Free Testosterone (FT) CT RIA obeys the law of mass action according to the following equation:

$$\text{Free} \begin{cases} FT \\ {}^{125}I\text{-}FT \end{cases} + Ab \rightleftharpoons \text{Bound} \begin{cases} Ab\text{-}FT \\ Ab\text{-}{}^{125}I\text{-}FT \end{cases}$$

Since the concentrations of ^{125}I - FT and coated antibodies are constant; the advancing state of the equation depends on the concentration of FT (Manni et al., 1985). The amount of ^{125}I - FT bound to the coated tube is inversely proportional to the concentration of FT in the sample. Following the incubation, the tube is aspirated to remove excess unbound labeled T. Sample concentration is read from a calibration curve.

Histological Examination

Autopsy samples were taken from the testes of rats in different groups and taking care to handle specimens gently to minimize trauma to the delicate seminiferous tubules. Immersion of whole testis in Bouin's fixative (150 mL of saturated picric acid (Sigma), 50 mL of 37% formaldehyde, and 10 mL of glacial acetic acid) over 10% neutral-buffered formalin for 24 h and further pathologic examination. The sections were

processed for light microscopic study using the wax-embedding method. Briefly, the tissues were washed in phosphate buffer and dehydrated through a graded series (70%, 80%, 90% and 95%) of ethanol (BDH Chemical Limited, Poole, England) for one and half hours each. It was then passed through two changes of absolute ethanol (99.7%) for an hour each. The tissues were cleared in xylene (BDH Chemical Limited, Poole, England) and impregnated with molten wax overnight. Paraffin wax tissue blocks were prepared for sectioning at 4 µm thickness by sledge microtome (Sorvall, U.S.A). The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin &eosin stain for routine examination through the light electric microscope (Banchroft et al., 1996).

Immunohistochemically staining

Anti-proliferating cell nuclear antigen (PCNA)

The testis tissues were fixed in Bouin's, embedded in paraffin and sectioned at 4 µm thickness. Immunocytochemical reactions were performed according to the technique described by Ramos-Vara, (2005). The procedure involved the following steps: the paraffinized sections were mounted on glass slides and deparaffinized. The antigen sites were unmasked by washing the slides with boiled water followed by treatment with 0.03% H_2O_2 in absolute methanol for 10 min to quench endogenous peroxidase activity. Section samples were incubated overnight at 4 °C with (1:50) polyclonal rabbit anti-polyclonal rabbit anti-proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To remove the unbound primary antibodies, section samples were washed with phosphate buffer saline (PBS). Afterwards, samples were incubated with goat-derived secondary anti-rabbit antibody conjugated to horseradish peroxidase at 37 °C for 30 min. Antigen-antibody interactions were finally detected by incubating samples with the chromogen 3,3′-diaminobenzidine tetrachloride (DAB-H_2O_2) at room temperature for 10 min as substrate. Testis sections were visualized using 400× magnification lens (Nikon Eclipse E200-LED, Tokyo, Japan).

Fertility assessment (Breeding)

All groups were tested daily for estrous cycles using vaginal lavage techniques. Vaginal lavage was performed in the morning by flushing the vagina with 20 µl of distilled water and subsequently aspirated, then smeared onto a glass slide. The flushed vaginal fluid was fixed with 70% ethanol and examined microscopically using methylene blue stain. Male rats from all groups (n = 5 in each group) were mated with age related, and sexually experienced females at about 60-day post-natal for up to 3 sexual cycles (3 estrus). One male was housed with a group of 2 or 3 females. Vaginal swabs were used to ensure the presence of sperms to assess mating. The pregnant females at 14–15 d of gestation were isolated. Number of pregnant females and newborn pups were counted and kept with their mother until two post-natal to check breastfeeding and eventual lethality.

Statistical Analysis

Data are presented as the mean ± SE and were analyzed by Two-way analysis of variance (ANOVA) followed by Duncan as a post-hoc test. All analysis was performed using Statistical Analysis System (SAS), Version 9.2 (Freund et al., 1986). In all statistical tests, a value of $P<0.05$ was considered significant (Freund et al., 1986).

RESULTS

From the results of the measurement of acute toxicity of *S. virgatum* extracts, whether aqueous or ethanolic extract, it is evident that no deaths were produced throughout the experiment when treated with these extracts. In addition, the LD50 for each extracts about >3000 mg/kg.

Table 1 discloses the effect of radiation dose used on rats, where irradiated rats which exposed to this dose showed a significant a significant increase of FSH and LH above 100% either 15- or 60-days post irradiation. Oppositely, the same of the irradiated dose caused a significant reduction in the total and free testosterone compared to control rats at 15- and 60-days' post-irradiation. This reduction was over than 70% either 15- or 60- days post irradiation. Interestingly, two weeks of *S. virgatum* extracts treatment had a significant ($p < 0.05$) effect in terms of preventing this reduction in the previous hormone compared to the irradiated rats (Table 1). On the other hand, *S. virgatum* extracts significantly decreased both the FSH and LH levels compared with that in the irradiated control rats at 15- and 60-days' post-irradiation. Additionally, interactions between different treatments and periods referring to FSH, total and free testosterone levels were significant ($\rho<0.05$).

Table 1: Effect of S. *virgatum* on the sex hormones of irradiated rats

Treatment	Period	Control	Irradiated	Water extract		Ethanol extract		F1	F2	F3
				100	400	100	400	Treatment (T)	Periods (P)	T X P
FSH (mIU/ml)	15 d	$3.97^{i} \pm 0.01$	$14.82^{a} \pm 0.13$	$12.20^{c} \pm 0.11$	$10.98^{d} \pm 0.07$	$8.22^{g} \pm 0.09$	$9.82^{f} \pm 0.09$	2834.21	340.69	25.43
% change			273.30% ↑	17.68% ↓	25.91% ↓	44.53% ↓	33.74% ↓	P= 0.001	0.001	0.001
	60 d	$4.02^{i} \pm 0.02$	$14.33^{b} \pm 0.08$	$10.54^{e} \pm 0.18$	$9.97^{f} \pm 0.10$	$6.64^{h} \pm 0.06$	$8.38^{g} \pm 0.05$			
% change			256.47% ↑	26.45% ↓	30.43% ↓	53.66% ↓	41.52% ↓			
LH (mIU/ml)	15 d	$4.48^{f} \pm 0.14$	$14.19^{a} \pm 0.55$	$10.22^{b} \pm 0.20$	$9.28^{c} \pm 0.17$	$7.54^{d} \pm 0.25$	$8.34^{e} \pm 0.11$	243.46	18.04	1.63
% change			216.74% ↑	27.98% ↓	34.60% ↓	46.86% ↓	41.23% ↓	P= 0.001	0.001	0.16
	60 d	$4.57^{f} \pm 0.05$	$13.47^{a} \pm 0.57$	$9.43^{b} \pm 0.28$	$8.19^{c} \pm 0.15$	$6.76^{d} \pm 0.13$	$7.32^{e} \pm 0.14$			
% change			194.75 ↑	30% ↓	39.20% ↑	49.81% ↓	45.66% ↓			
Total Testosterone (ng/dl)	15 d	$719.60^{a} \pm 1.23$	$168.55^{i} \pm 8.46$	$255.85^{h} \pm 4.18$	$316.80^{g} \pm 6.36$	$433.22^{e} \pm 3.42$	$387.34^{f} \pm 6.05$	2378.41	932.0	95.59
% change			76.58% ↓	51.79% ↑	51.79% ↑	87.96% ↑	157.03% ↑	P =0.001	0.001	0.001
	60 d	$720.05^{a} \pm 0.80$	$168.25^{i} \pm 8.00$	$394.38^{f} \pm 6.19$	$479.85^{d} \pm 4.92$	$570.81^{b} \pm 4.09$	$513.48^{c} \pm 4.39$			
% change			76.63% ↓	134.68% ↑	185.20% ↑	239.26% ↑	205.19% ↑			
Free Testosterone (pg/ml)	15 d	$37.68^{a} \pm 0.22$	$5.58^{h} \pm 0.16$	$8.47^{g} \pm 0.20$	$11.85^{f} \pm 0.31$	$17.59^{d} \pm 0.26$	$14.81^{e} \pm 0.24$	4449.23	514.24	51.48
% change			85.19% ↓	51.79% ↑	112.37% ↑	215.23% ↑	165.41% ↑	P =0.001	0.001	0.001
	60 d	$37.46^{a} \pm 0.14$	$6.08^{h} \pm 0.12$	$11.85^{f} \pm 0.31$	$14.88^{e} \pm 0.36$	$23.06^{b} \pm 0.36$	$19.72^{c} \pm 0.11$			
% change			83.77% ↓	94.90% ↑	144.74% ↑	279.28% ↑	224.34% ↑			

Data expressed as mean ± SE. The groups are statistically significant (P<0.05) as compared with control, using Two-way ANOVA followed by Duncan's test as a post-hoc test. Different superscript letters in the same row indicate significant differences at p< 0.05.

Table 2: Assessment of bucks' fertility

Groups	No. of dams	Fertility capacity %	Mean No. of Pups
Control	10	100%	8^{a}
Irradiated (IR)	10	0	0^{d}
IR/ water extract 100 mg/kg	3	20	3^{c}
IR/ water extract 400 mg/kg	10	100	4^{b}
IR/ ethanol extract 100 mg/kg	10	100	8^{a}
IR/ ethanol extract 400 mg/kg	10	100	6^{ab}

The fertility capacity was expressed as a percentage of pregnant females, n= 10, fertility was expressed as the number of pups per mated females. Fertility values represent the median; they were compared by Kruskal-Wallis's test followed by Dunn' multiple comparisons as a post-hoc test, and differences were considered significant when P< 0.05. a or b: Significantly different from control or radiation group, respectively at P< 0.05

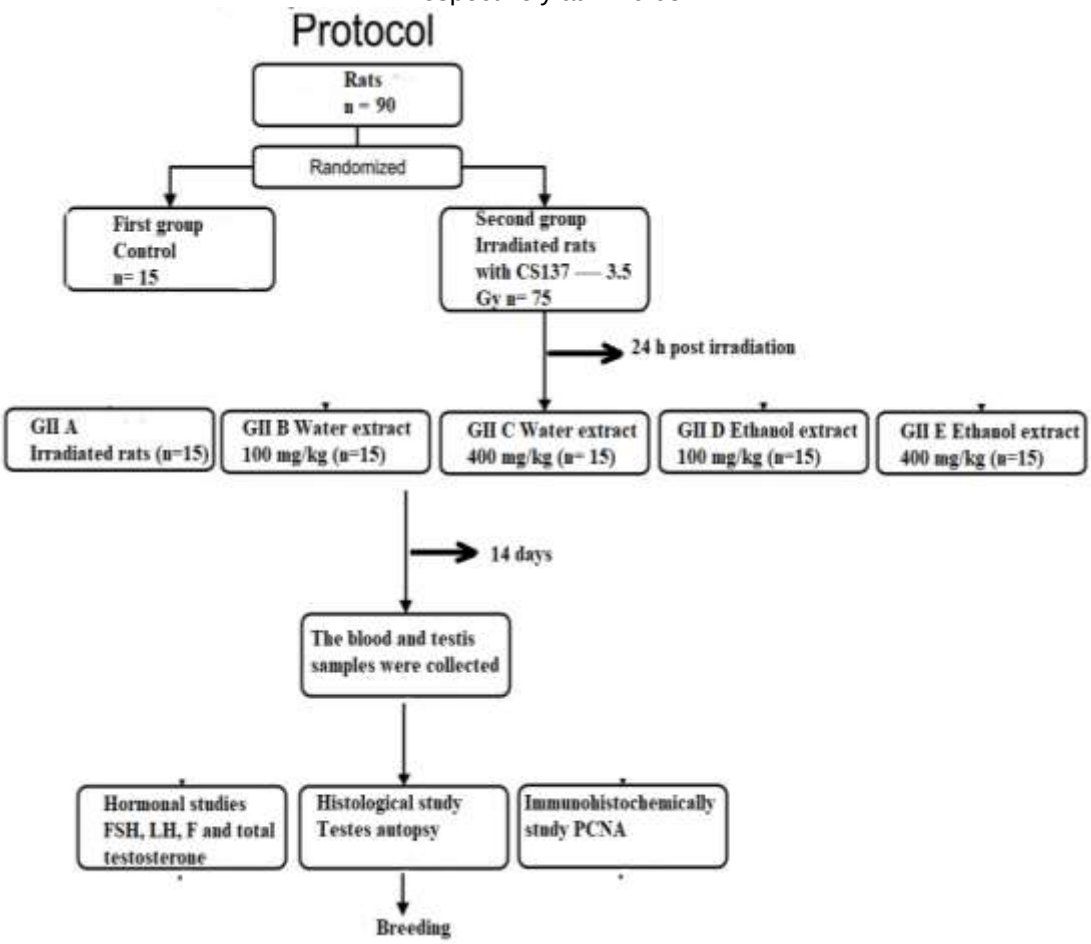

Figure1: Schematic diagram of the experimental design.

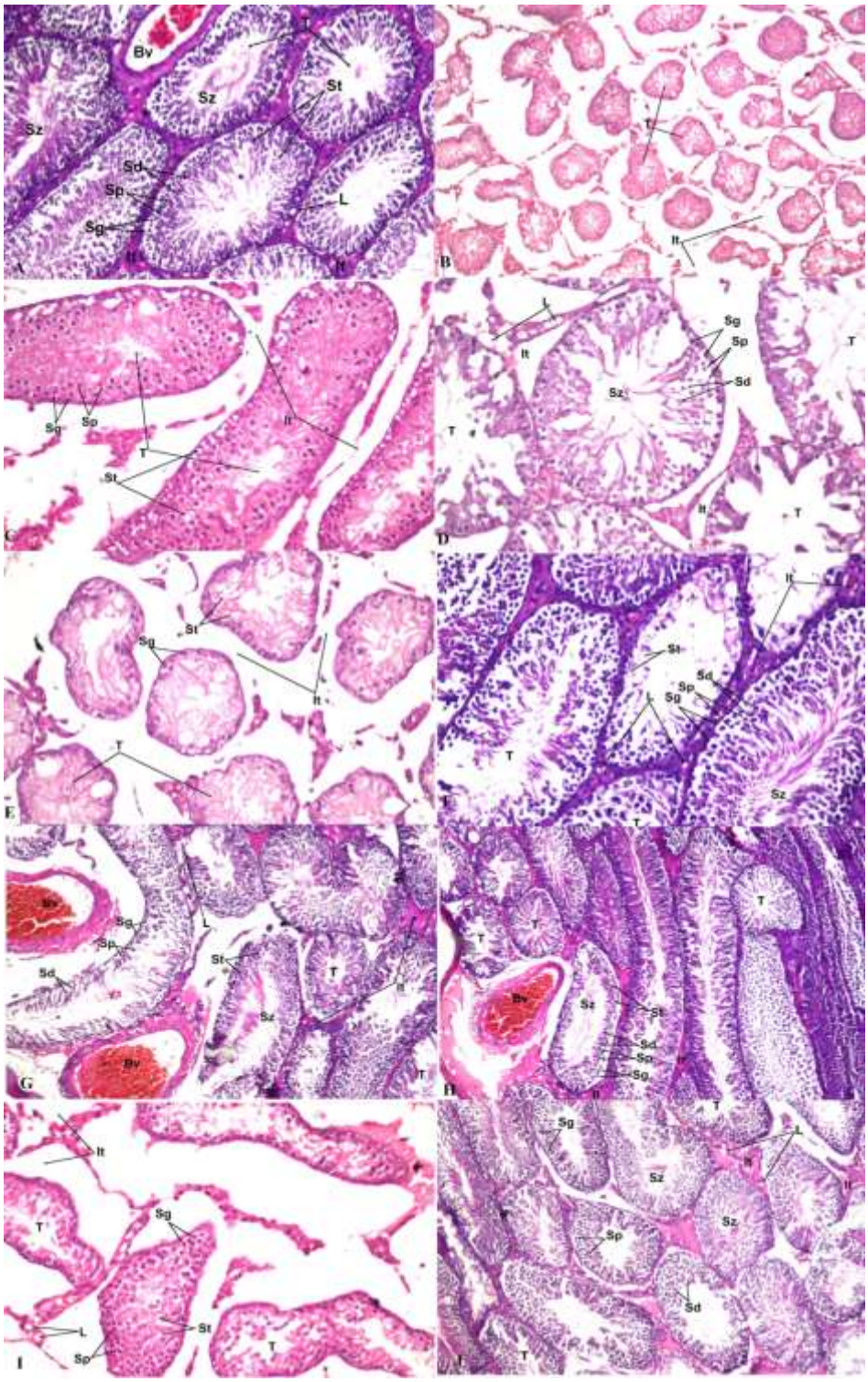

Figure 2: Impact of water and ethanol S. virgatum extract at dose 100 and 400 mg/kg-1 on the histology of the testis in irradiated rats. (A) Control rats; showing typical testicular architecture; (B) Irradiated rats showing severe testicular damage. (C & D) *S. virgatum* water extract -treated rats at dose 100 mg/ kg-1 post 15 days; showing typical spermatogenic cells in the seminiferous tubules; (E & F) *S. virgatum* water extract -treated rats at dose 400 mg/ kg-1 post 60 days ; (G & H)

irradiated- ethanol *S. virgatum* extract treated group at dose 100 mg/kg-1 post 15 days and (I & J) irradiated- ethanol *S. virgatum* extract treated group at dose 400 mg/kg-1 post 60 days, ameliorated the defects of the spermatogenic cells in the seminiferous tubules caused by irradiation. Bv: Blood vessels; T: Seminiferous tubules; It: Interstitial tissue; L: Leydig cells; St: Sertoli cells; Sg: Spermatogonia; Sp: Primary Spermatocytes; Sd: Spermatids; Sz: Spermatozoa. H & E X 40.

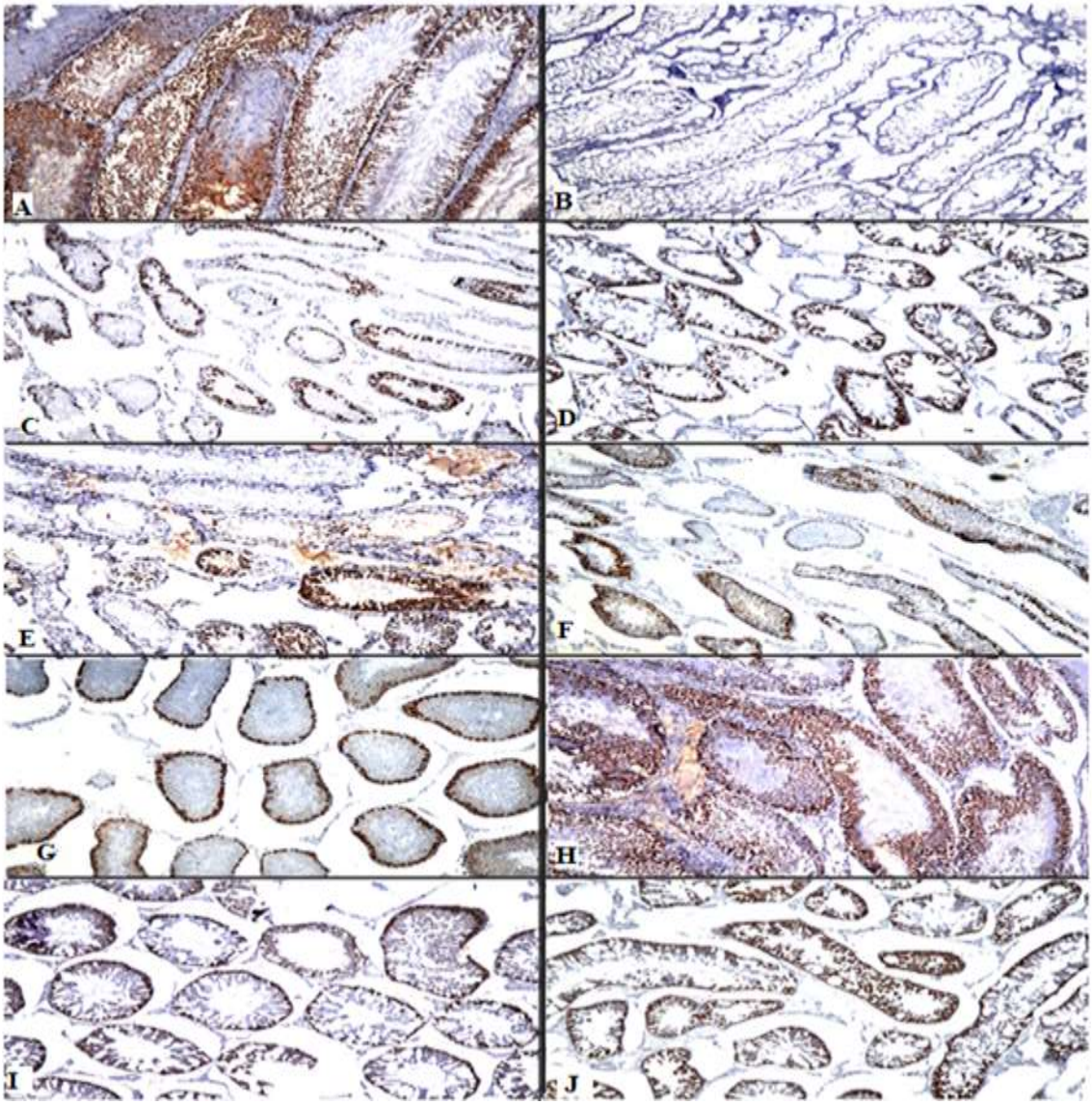

Figure 3: Evaluation of Immunohistochemical Staining PCNA-positive cells was strongly detected in the spermatogonia and early-stage spermatocytes of the control rats (A). The number of PCNA-positive germinal cells was lower in irradiated group than control group (B). The PCNA index was significantly decreased in the irradiated group, compared to that of controls (C &D): *S. virgatum* extract -treated rats at dose 100 mg/ kg^{-1} post 15 days; showing the number of PCNA-positive germinal cells in regenerated tubules was markedly increased; as well as, post 60 days respectively. *S. virgatum* water extract -treated rats at dose 400 mg/ kg^{-1} post 15 & 60 days; (E & F); irradiated- ethanol *S. virgatum* extract treated group at dose 100 & 400 mg/kg^{-1} and (G- J), ameliorated the defects post 15 & 60 days after radiation + extracts group respectively. Figure no 3 is not sighted in text

On the other hand, there is no significant in the interactions between different treatments and periods referring to LH levels in all the treatments and the control group.

The improvement with ethanol extract was better than water extract in the previous hormones either 15- or 60- days post treatment (Table 1).Figure 1 shows a diagram of the experiment and the rat groups, divided according to the in vivo experiment.

There was no histopathological alteration observed and the normal histological structure of the tubules with multiple sperm in the tubular lumen is shown in Figure 2 A. Degenerative change and a considerable number of thickened were detected in some seminiferous tubules and the tubular lumens were empty of spermatozoa after 15- or 60-days post irradiation (Figure 2 B). In animals treated with *S. virgatum* extracts, regained normal seminiferous tubules lined by several layers of spermatogenic cell series after 14 of the treatment (Figure 2C- J).

Figure (3) illustrates, for all groups, the cells that were immunoreactivity positive for the Proliferating Cell Nuclear Antigen Gene (PCNA), once again showing that both the seminiferous tubules and Although spermatogonia and primary spermatocytes mainly contained PCNA immunoreactivity cells, secondary spermatocytes or spermatids appeared to have lower responses of PCNA. It is clear from Figure 3C, D that the number of PCNA-positive germinal cells was markedly reduced in irradiated rats compared to control rats, and that PCNA staining was potentially circumscribed to spermatogonia and primary spermatocytes with no reaction was noticed with PCNA immunostaining either in the secondary spermatocytes or spermatids. Finally, *S. virgatum* extracts treated irradiated rats exhibited plenty of PCNA immunostained cells in the tissues of the testis, indicating high expression of PCNA (Figure 3, comparing E- H to C & D). The irradiated rats that were treated with *S. virgatum* at 400 mg/kg^{-1} either water or ethanol extracts however, showed fewer positive nuclei for PCNA in the spermatogenic epithelium cells (Figure 3 C & D, comparing E to T).

The signs of recovery were significantly showed after the treatment of *S. virgatum* than those of irradiated animals. *S. virgatum* treatment prevent these oxidative stresses in testis cells at 15- and 60-days' post-irradiation.

Breeding and assessment of fertility

Estrous cycle was carried out one week prior to the beginning of the mating period. All rats showed normal cyclist (4–5-day estrous cycle); however, most irradiated rats were arrested at the estrous and proestrus-estrous phase. In the irradiated group, mating with age related females resulted in significant decline in male fertility than control ones. In contrast, treatment of irradiated females with *S. virgatum* preserved their fertility and increased the ability of males to fertilize their females to become pregnant to 100%. Additionally, no differences were found in gestation length or offspring morphologies among treatment groups.

DISCUSSION

This study was conducted to peruse the therapeutic effect of *S. virgatum* on gamma-radiation-induced damage to testes and epididymal spermatozoa. We have identified the extent of changes in the structure of rat testes and epidermal sperm parameters subsequent of γ-Radiation.

The present study demonstrated that γ-Radiation -induced infertility caused a concatenation of testicular dysfunctions, and such functional deficits like the dilapidation of seminiferous tubules and the dwindling of seminiferous tubules and the loss of spermatogenic cells can be ameliorated by treatment with *S. virgatum*.

As a useful cellular proliferation marker, PCNA expression has been used to characterize spermatogonia and early- juncture primary spermatocytes in all stages of the seminiferous tubules of the testis tissues (Kang et al., 1997). In our study, PCNA was exceedingly expressed in spermatogonia and early- phase spermatocytes of the control group rats, and its expression decreased in the diabetic rats. *S. virgatum* treatment significantly increased PCNA expression in the testis tissues of irradiated rats, as evidenced by immunohistochemically staining.

Oxidative stress is regarded as an important mediator of apoptosis, (Zhao et al., 2013), and *S. virgatum* – acting as an antioxidant has excellent reactive oxygen species (ROS)-scavenging ability.

Irradiation can cause temporary azoospermia in mouse and this effect is reversible after eight weeks. In the present study, γ- Radiation treatment caused a marked reduction in serum testosterone level, and sex hormone-binding globulin by means of androgenic receptors occupation (Laurent et al., 2016). Sertoli cells (SCs) are the main somatic cells of testis which play a major role in cytoarchitectural organization

of the seminiferous tubules and, more importantly, govern the differentiation of germ cells (GCs). In the present study, total and free testosterone decreased in rats at different time intervals of exposure to γ- rays as compared to control rats. Marzban et al., (2017) reported that irradiation of 2 Gy γ-rays to the testis of the rats considerably influenced the quantitative of spermatogonia, primary gametocyte, round spermatid, spermatozoa, seminiferous tube and lumen diameters, thickness of the epithelial tissue, Leydig's cell nuclear diameter and volume, epithelial tissue height, and apoptotic cells ($p<0.05$). Oxidative stress in the testes can disrupt the estrogen capacity of the Leydig cells. The capacity of germinal cells (germ) is different from normal spermatozoa, leading to impaired spermatogenesis and infertility through the oxidation of proteins, lipids, and germ cells' DNA (Aitken & Roman, 2008).

Sobhani et al., (2015) reported that none of the doses of the *Sargassum* extract might increase the percentage of normal spermatozoan morphology. Therefore, our report is consistent with the results of other researchers including Patra et al., (2008), Ye et al., (2008) who stated that the *Sargassum* extract inhibits the free radicals.

Simultaneous suppression of both testosterone and FSH with GnRH antagonists (GnRH-ant) reverses the radiation-induced block in spermatogonial differentiation in rats (Shetty et al., 2006). In the present study, Gamma ray's exposure correlated with increased levels of FSH and LH. Because androgen and FSH receptors are generally believed to be absent from germ cells including spermatogonia, LH & FSH must produce their effects by acting on somatic cells. The actual physiological and molecular mechanism of the LH and FSH inhibition of spermatogonial differentiation after irradiation is under investigation. FSH can exert its direct inhibitory action only on Sertoli cells because they are the only ones with the receptor, and hence a reasonable approach is to examine FSH- and T-regulated Sertoli cell genes (Shetty et al., 2006). Maintaining LH serum levels is very important for initiating and supporting spermatogenesis, hence degeneration of Sertoli cells and germinal cells may be due to high concentrations of circulating LH (Sarkar et al., 2000 and Izumi et al., 2005).

These results clearly demonstrate the anti-peroxidative role of the *S. virgatum*. In fact, phlorotannins are the major phenolic compounds of brown algae with an antioxidant role (Koivikko et al., 2008). It was reported that the aqueous and ethanol extracts of *Sargassum* significantly inhibits the increase of FSH and LH. Considering the attainable antioxidant effects of the extract of *Sargassum* and the fact that no studies were conducted on the effects of this extract on human semen parameters, this study investigated the effects of concentrations of 250 and 500 mg/mL of *Sargassum* extract on the amount of reactive oxygen species (ROS) and human sperm parameters after cryopreservation (Sobhani et al., 2015). The free radical of DPPH is stable and widely used to evaluate the free radical scavenging ability of natural compounds. The DPPH radical-scavenging activity is conceivably due to a hydrogen-donating ability (Dawidowicz et al., 2012). We analyzed the chemical compositions of *both S. virgatum* either water or ethanolic extracts exhibited good antioxidant activity and ABTS radicals' inhibition was recorded (977.40 and 1031.37 µg TE/ ml respectively) (data unpublished). Furthermore, the ethanolic extract showed higher antioxidant activity in the DPPH than water extract (181.90 µg TE/ ml) (data unpublished). Additionally, both extracts have total phenols (159.17 and 371.94 mg GAE/ml respectively). Additionally, the most abundant compounds in *S. virgatum* were Gallic acid, Cateachin, Chlorogenic, and Cinnamic in both extracts.

Free radical-scavenging is one amongst the far-famed mechanisms by that antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical scavenging activity of specific compounds (Amarowicz et al., 2004). These results indicate that improvement through the use of ethanolic extract was somewhat better than the water extract.

CONCLUSION

In conclusion, the fertile status of γ- rays treated male rats was recovered by treatment with *S. virgatum* at two different doses and two different extract methods (water and ethanol extracts) in this study. Animals treated with *S. virgatum* showed modulated the testosterone, FSH and LH, especially at 60 days.

These results show that *S. virgatum* could be both a rich and functional source for the treatment of infertility. Furthermore**,** our experimental data have shown that *S. virgatum* treatment could preserve the morphology of testes; restore the expression of PCNA, and inhibit death of testicular cells in irradiated rats. The competence of *S.*

virgatum to release oxidative stress illustrates its potential as a promising therapeutic agent for the treatment of oxidative stress-mediated testicular dysfunction in irradiated rats.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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