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Ameliorative efficacy of bioencapsulated *Chironomous* larvae with Shilajit on Zebrafish (*Danio rerio*) exposed to Ionizing radiation

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Highlights

- Radioprotective effects of Shilajit were evaluated using Zebrafish.
- Zebrafish exposed with X-Ray at a single acute dose of 1 Gy.
- Morphological, behavioral and clinical pathology were assessed.
- Antioxidant levels and DNA damage were assessed.
- Shilajit have significant radioprotective and antioxidant enhancing capability.

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ABSTRACT

Using Zebrafish (*Danio rerio*) as a model organism, we evaluated the radioprotective and antioxidant effects of the Indian traditional medicine Shilajit exposed to X-Ray. The Zebrafish were divided into three experimental groups and control group, each group containing ten fish. The three experimental fish groups, group I, group II and group III were fed with 3, 5 and 7 ppm shilajit encapsulated *Chironomous* larvae and group IV served as a control fed with non- encapsulated larvae. After 60 days of feeding trial, fish were irradiated with X-Ray at a single acute dose of 1 Gy. 72 h of post-irradiation, each experimental fish were observed for its morphological, behavioral, clinical symptoms, antioxidant levels and DNA damage were evaluated. Among the experimental groups 5 ppm shilajit encapsulated *Chironomous* larvae fed fish group shows the most significant radioprotective effects compared with control and other experimental fish groups. The present study indicates that shilajit have significant radioprotective and antioxidant enhancing capability. The humus substance of shilajit may be the factor responsible to react with radiation-derived or radiation related reactive species on zebrafish.

1. Introduction

Nowadays, many synthetic compounds like Metformin (Xu et al., 2015), PEGylated ceria nanoparticles (Li et al., 2015) and natural products like Resveratrol (Zhang et al., 2013) and Cinnamic acid (Cinkilica et al., 2014) are having ameliorative properties that are immediately administrated before irradiation to reduce injuries caused by ionizing radiation. Presently "Amifostine" is the only radioprotector that has been clinically approved by the US Food and Drug Administration (US FDA) for reducing the side effects (xerostomia) in patients undergoing radiotherapy. Besides, cysteine and cysteamine are tested for their anticancer properties by several researchers that offer good protection, but are relatively toxic (Koukourakis, 2002). Thus it is imperative to find less toxic, more effective radioprotectors to overcome the plethora of adverse effects in patients undergoing radiotherapy.

An exemplary radioprotector should be non-toxic and also should protect the non-target cells. Numerous natural dietary ingredients were found to safeguard cells from damage induced upon ionizing radiation (Arora et al., 2008). It is well known that antioxidant vitamins such as ascorbic acid and vitamin E protect cellular DNA and membranes from radiation-induced damage (Noroozi et al., 1998; Konopacka and Rzeszowska-Wolny, 2001; Kumar et al., 2002; Jagetia, 2007). Recently,

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several flavonoids, polyphenols and phenolic acids have become more popular as diet compounds due to their beneficial impact on human health. During radiotherapy, ionizing radiation absorbed by living cells produce chemical and biological changes via Reactive Oxygen Species (ROS) production that may damage nucleic acids, proteins and lipids. Thus side effects induced during radiotherapy or accidental exposure to ionizing radiation should be fairly minimized using a suitable radio-protectant.

Shilajit is a natural mineral originated in India is widely used in Indian traditional medicine (Agarwal et al., 2007). Shilajit plays a vital role as a rejuvenator and potential immuno-stimulant and capable of enhancing the antioxidant properties (Musthafa et al., 2016) and eliminates free radicals due to the presence dibenzo- -pyrones, carotenoids, indigoids, metallo-humus substance like fulvic acids and fusims (Ghosal, 1990; Kwon et al., 2004; Ghosal, 2006). Moreover, it contains certain organic compounds and vitamins like B1 and B12 (Frolova and Kiseleva, 1996; Al-Himaidi and Mohammed, 2003). Shilajit is able to regulate the activity of body functional components and fluids (Agarwal et al., 2007; Heinrich, 2007). It is also used as an immuno stimulant and anabolic food additives (Schepetkin, 2003).

Beneficial effects of Shilajit have been studied by many researchers. It has used in the treatment of peptic ulcer (Goel et al., 1990; Ghosal et al., 1998) as a memory enhancer, neuro-protective, anti-inflammatory, and anti-oxidant effects (Goel et al., 1990; Acharya et al., 1998; Agarwal et al., 2007). It is found to elevate the levels of antioxidant enzymes in corpora striatum after exposure to toxicants (Bhattacharya, 1995) and also significantly decreased carrageenan-induced edema in rats (Ghosal, 1990). These effects may be related to the anti-inflammatory and neuroprotective effects of Shilajit (Goel et al., 1990). Few studies have reported to increase of proinflammatory cytokines such as TNF-α, IL-0β, and IL-6 (Johansson, 2000). Shilajit induced ameliorative efficacy upon ionizing radiation exposure has not been studied.

Zebrafish (*Danio reri*) has emerged as a popular vertebrate model organism in the field of radiobiology, biomedical and toxicology research due to substantial degree of gene homology. An attractive and potential live food *Chironomous* larvae was subjected to bioencapsulation with Shilajit and fed to Zebrafish during the entire experimental period. Because, the ornamental fishes in particular Zebrafish prefer livefeed source than the formulated feed and even the movement of an organism attracts the fishes to chase and feed. Also, fishes can digest easily the Shilajit available in the encapsulated form instead of the freestate. Therefore, the present study is the first attempt to evaluate the of bioencapsulated *Chironomous* larvae with Shilajit on Zebrafish (*Danio rerio*) against single acute dose of X-Ray.

2. Materials and methods

2.1. Collection and care of experimental fish

The freshwater Zebrafish (*Danio rerio*) adults were procured from a commercial fish farm and transported to the laboratory in oxygenated bags and released into 10 L aquarium tank filled with dechlorinated tapwater.

2.2. Bioencapsulation of Chironomous larvae with Shilajit

Shilajit (mineral pitch) is white in colour dried matrix purchased from Annai Aravindh Herbals, Chennai, India. Shilajit was dispersed in purified water and used without any further purification. Three different concentrations of shilajit solutions [3 ppm (group I), 5 ppm (group II), 7 ppm (group III) and 0 ppm (control group IV)] were prepared in 1 l glass beaker. In each beaker 50 numbers of *Chironomous* larvae were introduced and allowed for 24 h for proper enrichment. The same procedure was repeated for 60 days.

2.3. Experimental design and feeding schedule

After 24 h of Shilajit encapsulated *Chironomous* larvae were introduced into the experimental aquarium tanks (Ten Zebrafish for each experimental group) and control fish (non irradiated) group fed with non-enriched *Chironomous* larvae for two times a day (7 h and 16 h). The unfed dead individuals were removed from the experimental tanks. The experimental duration was restricted to 60 days. At the end of the experiment, fish were subjected to irradiation with single acute dose of X- Ray.

2.4. X-ray irradiation

After 60th day of feeding experiment, fishes were irradiated with a single dose of 1.0 Gy X-ray from a Model Vision 100 X–Ray system (100 mA Mobile X-Ray Machine; Serial No.: V14152479), with fish placed inside a 1000 ml sterilized glass beaker with a homogeneous field of 200 ml (1.5 cm depth), at m away from the beam source. This is a non-filtered X-ray source and this dose was delivered by 100 keV over a 5-min period (*i.e.,* a dose rate of 0.2 Gy/min). The X-Ray irradiation facility utilized at The Excellent Clinical Research Laboratory, Chennai, recognized by Atomic Energy Regulatory Board (AERB, Govt. of India; AERB Authorization No.: AERB/18/02).

2.5. Behavioral study and monitoring of morphological & clinical symptoms

72 h of post irradiation, the fish were monitored their changes in the behaviour (hyperactivity, loss of balance, rate of swimming, rate of food intake and convulsions) and external morphology & clinical symptoms (emaciation, hampered breathing, skin lesions, exophthalmia, haemorrhages, fading of body colour, withdrawal of fins and mortality) were observed following the standard protocol of (Mishra and Mohanty, 2008).

2.6. Assessment of antioxidant parameters

72 h of post irradiation, the blood samples (5 fish) were drawn from the caudal vein of Zebrafish with a heparinized syringe. tration of 5000 IU heparin sodium salt in 1 ml. An aqueous solution of heparin sodium salt at 0.01 ml per 1 ml blood was used to stabilize the samples. Blood plasma obtained from cooled centrifuged blood samples (4 °C, 837 × *g*) was stored at –80 °C until use. Biochemical indices including SOD, GPx, and GR were determined using a VETTEST 8008 analyzer (IDEXX Laboratories Inc., Maine, USA). To test the accuracy of the method applied, Ransod Control SD 126 and Control SC 692 (Standard) were analyzed for SOD, GPx and GR respectively. The concentration of Total superoxide dismutase (SOD) was observed by the method of Marklund and Marklund (1974). involving the autoxidation of pyrogallol and was assessed spectrophotometrically at 420 nm. Glutathione peroxidase (GPx) concentration was determined based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with glutathione reductase (GR). The extinction coefficient of 6.22 mM cm^{-1} was used to determine the specific activity (Lawrence and Burk, 1976). The concentration of GR was found spectrophotometrically, measuring NADPH oxidation at 340 nm. Activity was expressed as international milliunits (mU) per milligram of protein.

2.7. Assessment of DNA damage

72 h of post irradiation, the peripheral blood samples (5 fish) were collected just above the lateral line system with a disposable syringe previously washed with 0.1. M EDTA to prevent clotting. The single-cell gel electrophoresis (comet) assay was performed to detect DNA damage as described by Singh et al. (1988) with slight modification. Experiments were conducted using molecular grade and DNAse-free reagents (Sigma Aldrich, USA) were used throughout the experiment. On the

sterilized slides, two solutions, 0.5% normal-melting agarose (NMA) and 0.5% low-melting agarose (LMA), were prepared in Ca^{2+}-, Mg^{2+}-free PBS. NMA (0.1 ml) was used for the first layer, and a suspension of 1000 cells in 75 µl LMA + 10 µl PBS was used for the second layer. Finally, a third layer of 85 µl LMA was added. Slides were immersed in freshly prepared ice-cold lysis solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM Na2 EDTA,10 mM Tris–HCl pH 10, 1% Triton X-100% and 10% DMSO) to lyse the cells and to denature the DNA. Slides were incubated for 1 h at 4 °C in the dark, and then placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (1 mMNa2 EDTA, 300 mM NaOH, pH 13) to cover the slides, and the slides were maintained in high-pH buffer for 20 min to denature the DNA and expose the alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (300 mA). The above treatments were performed in an ice bath. Following electrophoresis, the slides were washed gently in neutralization buffer (0.4 M Tris–HCl, pH 7.5) to remove the alkali and detergents. The slides were drained well and dehydrated by dipping into absolute ethanol for 5 min and air-dried for storage. 100 µl of Vista Green DNA Staining Solution was loaded onto the slides that were subsequently covered with a cover slip and analyzed with an Olympus BX50 fluorescence microscope at 20× magnifications for comet visualization. All of the steps described above were conducted in the dark to prevent induction of additional DNA damage. A total of 100 cells were scored for each sample, and the captured images were analyzed using comet score image analysis software. Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (% tail DNA) were measured. Olive tail moment (tail length× tail DNA content) was calculated using the following formula:

$$M_{\text{Tail Olive}} = (CG_{\text{Tail}} - CG_{\text{Head}}) \times \% \ DNA_{\text{Tail}}$$

Where $M_{\text{Tail Olive}}$ is the Olive tail movement, CG_{Tail} the center of the gravity of the tail, CG_{Head} the center of gravity of the head, and % DNA in the tail compared to the head were determined.

2.8. Statistical Analysis

The data of each parameter were expressed as the mean ± standard error of mean (SEM) and the impact of experimental bioencapsulated diets were tested using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison test using SPSS (version 16 for windows). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Behavioral changes

The results of the present study pertaining to behavioral changes of irradiated fish are presented in Table 1. After irradiation, the fish were lethargic and timid at the same time motions were uncoordinated and dwelled at the bottom of the aquarium in 3 ppm shilajit bioencapsulated *Chironomous* larvae fed fish group compared to control and other experimental groups. Similarly, the food intake was also decreased in the same experimental fish group. From this study, it is inferred that Shilajit has maximum radioprotectivity in behavioral changes in concentration of 5 ppm and moderate protective effects was found in concentration of 7 ppm.

3.2. Morphological and clinical symptoms

Studies on the morphological and clinical symptoms shows that, there were 8 major parameters such as emaciation, withdrawal of fins, skin lesions, fading of body colour, haemorrhages, exophthalmia, hampered breathing and mortality were observed (Table 1). 48–72 h of post irradiation bilateral exophthalmia and haemorrhages were observed approximately on various parts of the body. The most severe haemorrhages were observed in the region of gills and pectoral fins. The fading out of the body colour and withdrawal of fins can be observed from the 3rd day of post irradiation. The decrease of food intake led to marked emaciation. 3 ppm shilajit bioencapsulated *Chironomous* larvae fed fish group was found to have severe to moderate symptoms in all the parameters of morphological and clinical symptoms whereas, 7 ppm group fish were found to have mild hampered breathing, haemorrhages and fading of colour. The best result was found in the 5 ppm group which did not exhibit any of the afore mentioned parameters even mildly.

Table 1
Clinical symptoms, Morphological and Behavioral changes in Shilajit bioencapsulated *Chironomous* larvae fed Zebrafish (*Danio rerio*) after exposed to 1 Gy X-Ray radiation.

Parameters	Control	3 g kg^{-1}	5 g kg^{-1}	7 g kg^{-1}
Emaciation	-	+	-	-
Hampered Breathing	-	++	-	+
Exophthalmia	-	+	-	-
Haemorrhages	-	+	-	-
Fading of Body Colour	-	++	-	+
Alopecia of Fins & Scales	-	+	-	-
Hyperactivity	-	++	+	+
Loss of Balance	-	+++	-	-
Rate of Swimming	-	++	-	-
Rate of food intake	-	++	+	+
Convulsions	-	+++	+	-
Mortality	-	+	-	-

-: None (Zero percent), +: mild (< 10%), ++: moderate (10–50%), +++: severe (< 50%).
Data represented mean of three groups (Ten fish/group).

3.3. Anti-oxidant parameters

The results of the antioxidant (SOD, GPx, and GR) levels in the blood samples of 1 Gy X-Ray irradiated Zebrafish, control and Shilajit-treated groups were shown in Figs. 1, 2 and 3. The SOD (6.6 ± 1.9 mU/mg protein), GR (55.3 ± 3.7 mU/mg protein) and GPx (52.9 ± 2.9 mU/mg protein) levels were significantly enhanced ($p < 0.05$) in the blood samples of X-Ray irradiated Zebrafish fed with 5 ppm shilajit bioencapsulated *Chironomous* larvae, when compared to the control and other experimental groups. The moderate increase of antioxidants were seen in 7 ppm and mild increase noticed in fish group fed with 3 ppm shilajit bioencapsulated *Chironomous* larvae compared with control groups.

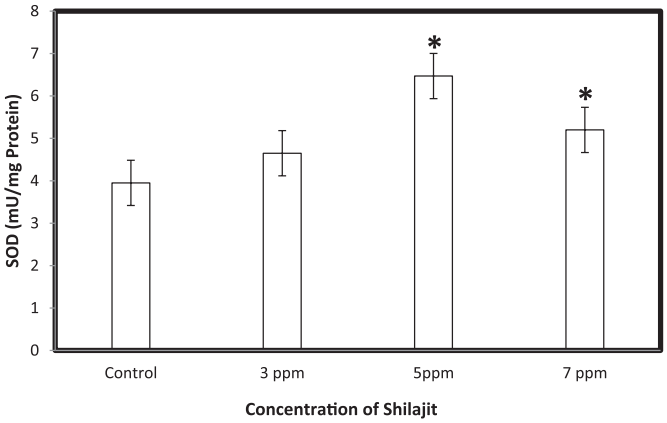

Fig. 1. Total superoxide dismutase (SOD) activity of Zebrafish (mean ± SEM, n = 6) fed Shilajit bioencapsulated *Chironomous* larvae with different concentration (3, 5, and 7 ppm). Significant different ($p < 0.05$) from the control are indicated by asterisks.

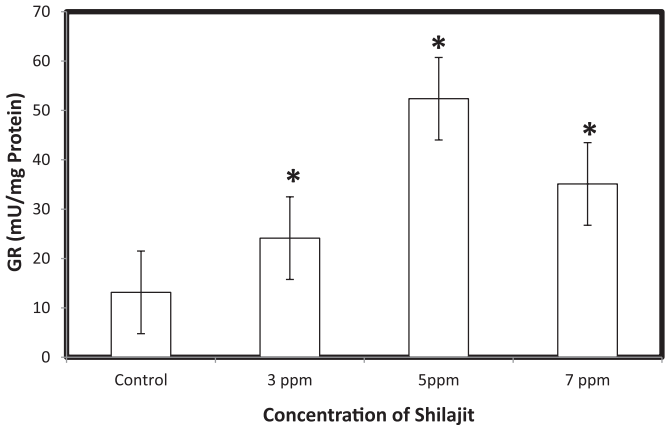

Fig. 2. Glutathione Reductase (GR) activity of Zebrafish (mean ± SEM, n = 6) fed Shilajit bioencapsulated *Chironomous* larvae with different concentration (3, 5, and 7 ppm). Significant different ($p < 0.05$) from the control are indicated by asterisks.

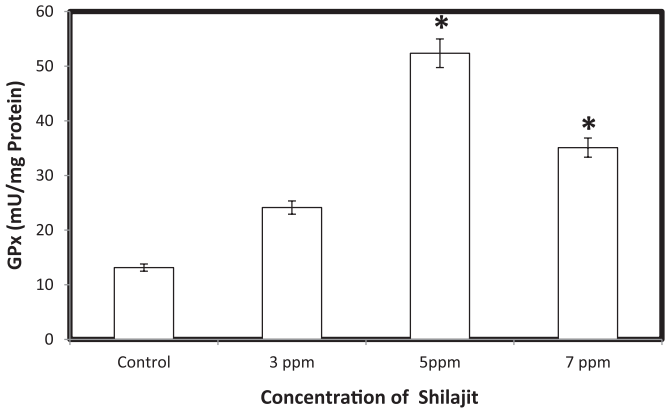

Fig. 3. Glutathione peroxidase (GPx) activity of Zebrafish (mean ± SEM, n = 6) fed Shilajit bioencapsulated *Chironomous* larvae with different concentration (3, 5, and 7 ppm). Significant different ($p < 0.05$) from the control are indicated by asterisks.

3.4. DNA damage assessment

Ionizing radiation effects on DNA damage was validated by comet assay method. DNA damage were analyzed with comet score 1.5 software to obtain parameters such as comet length, tail length and percentage DNA in the tail. A significant ($p < 0.05$) higher percentage (96%) of tail DNA was found in X-Ray irradiated fish group fed with 3 ppm Shilajit encapsulated *Chironomous* larvae due to decreased antioxidant levels simultaneously, severe DNA damage was reflected. In 7 ppm group fish were found to have moderate tail DNA (46%) whereas in the 5 ppm group fish exhibit mild (38%) tail DNA (Fig. 4).

4. Discussion

After radiotherapy, cancer patients undergo innumerable side effects particularly damages in the integument, emaciation condition and alopecia (loss of hair). Genes responsible for the development of scales and fins in fish are similar to that seen in skin, hair and tooth development of mammals (Kondo et al., 2001; Sharpe, 2001; Xu et al., 2014). Radioprotective effects of Shilajit were reported here for the first time in the Zebrafish, an animal model. Hence, we evaluated the ameliorative efficacy of bioencapsulated *Chironomous* larvae with Shilajit on Zebrafish (*Danio rerio*) exposed to X-Ray. For 60 day feeding trail, three different concentrations (3 ppm, 5 ppm and 7 ppm) of Shilajit bioencapsulated *Chironomous* larvae were fed to Zebrafish along with control group. Among the experimental groups, 5 ppm Shilajit bioencapsulated *Chironomous* larvae fed fish group shows the maximum radioprotective effects followed 7 ppm whereas moderate and severe morphological,

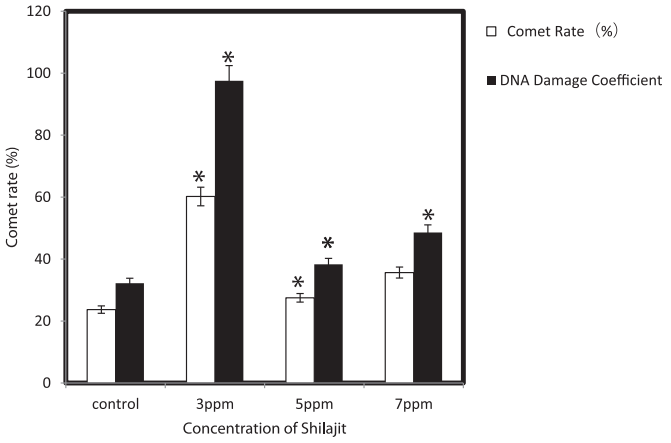

Fig. 4. Comet rate and DNA damage coefficient of Zebrafish (mean ± SEM, n = 6) fed Shilajit bioencapsulated *Chironomous* larvae with different concentration (3, 5, and 7 ppm). Significant different ($p < 0.05$) from the control are indicated by asterisks.

behavioral and clinical signs were observed in 3 ppm compared to the control group. No, previous studies reported on Zebrafish therefore, the novel compound Shilajit could be used to cancer patients prior to radiotherapy to enhance the immune system, antioxidants and mucus membrane eventually minimize the side effects instead of using synthetic drugs.

The SOD activity was significantly enhanced in X-Ray irradiated fish fed bioencapsulated *Chironomous* larvae with 5 ppm Shilajit during the experimental period. The superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then reduced to water. This detoxification pathway is the result of multiple enzymes with SOD catalyzing the first step and then CAT, GPx and various peroxidases while removes hydrogen peroxide (Singh et al., 1988). GPx is identified as a very potent antioxidant that protects the body from damage due to oxidation by free radicals (Sies, 1997). Antioxidants provide cells with a comprehensive defense from ROS-induced damage. The antioxidant enzymes such as SOD, CAT and GPx are the first line defense mechanism against oxidative stress which converts superoxide radicals into hydrogen peroxide and then into water and molecular oxygen; this facilitates to maintain the lowest possible levels of ROS in the cell, and is recognized as an essential component of an organism's self-maintenance (Rotruck et al., 1973).

DNA strand break is a major effect of ionizing radiation. According to Harrison (2013), the DNA molecule is the primary target of ionizing radiation within the cell and biological effects of radiation originate mostly DNA damage. Ionizing radiation can induce DNA damage by changing the molecular chemical structure either directly or indirectly via radiation-generated reactive radicals. The comet assay technique is considered as a rapid, sensitive and relatively simple method for detecting DNA damage at the level of individual cells. X-ray, as a physical mutagen, can induce single-strand breaks, alkali-labile sites and double-strand breaks that can be detected by the comet assay. In addition, the cellular capacity to repair DNA damage can be quantified by the comet assay. Hence, this assay may also be used to observe the repair kinetics of DNA damage induced by X-rays. To our knowledge, no studies exist regarding the radio-protective effects of Shilajit in Zebrafish lymphocytes. The increase in comet tail-size after irradiation observed in this study is thought to be due to DNA strand-breaks. Shilajit encapsulated larvae fed fish groups prior to X-Ray irradiation decreased the genetic damage index and the percentage of damaged cells, indicates that Shilajit prevented the formation of DNA damage. In this study, Shilajit was encapsulated with different concentrations (3, 5 and 7 ppm) in the livefeed *Chironomous* larvae which was fed to 1.0 Gy X-Ray irradiated Zebrafish and the results inferred that 5 ppm Shilajit encapsulated *Chironomous* larvae fed Zebrafish shows better protection against irradiation followed by 7 ppm experimental group compared to control

group.

According to traditional Indian knowledge, Shilajit acts as a tonic, laxative, expectorant, diuretic, anti-bilious, immuno-modulator, lithotriptic, and anti-hypertensive medicine when given orally; when applied externally it acts as an antiseptic, analgesic, deobstruent and germicide. Intra peritoneal administration of Shilajit at doses of 20 and 50 mg/kg per day for 21 days induced a dose dependent increase of antioxidants such as SOD, CAT, and GPx activities in the frontal cortex and striatum of rats when compared with the control because of the presence of dibenzo-α-pyrones and fulvic acid (Schepetkin, 2003). Recent studies report that Shilajit possesses immunomodulatory capabilities such as increasing white blood cell activity eliciting different degrees of murine peritoneal macrophages and activating splenocytes of animals at early and later stages of tumour growth (Agarwal et al., 2007).

Recently, Zhang et al. (2013) reported that Resveratrol, a polyphenolic natural compound and potent antioxidant which ameliorates Ionizing radiation induced long-term Hematopoietic Stem Cell injury in the bone marrow of mice by effectively inhibiting radiation induced NOX4 expression and ROS production. Also, the role of Resveratrol in modulating sensitivity of insulin, reducing the level of insulin-like growth factor-1, increasing AMP-activated protein kinase and PGC-1a activity has been observed which indirectly targets on HSC self-renewal by regulating HSC senescence (Baur, 2006; Pirola and Frojdo, 2008). On the other hand, a synthetic drug metformin (250 mg/kg/day) ameliorates IR-induced HSCs injury through decrease the ROS production in podocytes and aortic endothelial cells of mice irradiated with 4.0 Gy of ^{137}Cs at the dose rate of 0.78 Gy/min (Xu et al., 2015). According to Li et al. (2015), the polyethylene glycol coated ceria nanoparticles inevitably ameliorates the gamma radiation (^{60}Co) irradiated (20 Gy/min at the dose rate of 41.4 Gy/h) induced effects on human liver cells. These above mentioned natural and synthetic compounds active ameliorative mechanism mainly due to the up-regulation of SOD1, SOD2, CAT and GPx1 mRNA in HSCs and higher enzymatic activity of SOD, CAT, GPx resulting in decreased oxidative damage of DNA induced by ROS. Our recent study (Musthafa et al., 2016) also confirmed that 4 g kg^{-1} shilajit enriched diet enhances the immunity and antioxidants in the freshwater prawn *Macrobrachium rosenbergii*. In agreement with these previous reports we observed that the humic substance like fulvic acid, humins and humic acids of Shilajit are the active constituents which are the factor responsible for the induction of antioxidant enzymes on X-Ray irradiated Zebrafish.

Our findings suggest that pre-treatment with 5 ppm Shilajit encapsulated *Chironomous* larvae inhibit X-Ray induced oxidative stress and DNA damage on Zebrafish by modulating the expression of antioxidant enzymes. Shilajit can be used as an efficacious medical radiation countermeasure, because it is a natural, safe traditional drug that has been used extensively in the clinic. Thus, we believe that Shilajit can function as a radioprotectant to non-target cells and significantly enhance the therapeutic efficacy of radiation therapy for cancer and thus may exhibit as a promising radioprotectant without any side effects.

Conflict of interest statement

None of the authors has any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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