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Title page

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Short title: Radio-modifying effect of *Lagenaria*

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Radiation-modifying effect of *Lagenaria siceraria* extract *in-vitro* and *in-vivo*

Abstract:

The cucurbits (prebiotics) were investigated as novel agents for radio-modification against gastro-intestinal injury. The cell-cycle fractions and DNA damage were monitored in HCT-15 cells. A cucurbit extract was added to culture medium 2 h before irradiation (6 Gy) and was substituted by fresh medium 4 h post-irradiation. The whole extract of the fruits of *Lagenaria siceraria (Ls), Luffa cylindrica (Lc)* or *Cucurbita pepo* (Cp) extract enhanced G_2 fractions (42 %, 34 % and 37 % respectively) as compared to control (20 %) and irradiated control (31 %). With cucurbits, the comet tail length remained shorter (*Ls* - 28 µm; *Lc* - 34.2 µm; *Cp* -36.75 µm) than irradiated control (41.75 µm).

For *in vivo* studies, *Ls* extract (2mg/kg body wt.) was administered orally to mice 2 h before and 4 and 24 h after whole body irradiation (10 Gy). *Ls* treatment restored the GSH contents to 48.8 µmol/gm as compared to control (27.6 µmol/gm) and irradiated control (19.6 µmol/gm). Irradiation reduced the villi height from 379 to 350 µm and width from 54 to 27 µm. *Ls* administration countered the radiation effects (length - 366 and width - 30 µm respectively) and improved the villi morphology and tight junction integrity.

This study reveals the therapeutic potential of cucurbits against radiation induced gastro-intestinal injury.

Keywords: Radiation-protection, Cucurbits, *Lagenaria Siceraria,* Gut villi, Tight junctions

Introduction:

Whole body irradiation received during planned or unplanned situations leads to different kinds of syndromes depending on the radiation dose delivered. Radiation dose (3-7 Gy) leads to hemopoietic (HP) syndrome (Suman et al. 2012) and death may occur within 30-60 days. Radiation doses, more than 6 Gy (6-20 Gy) can lead to the damage to gastrointestinal (GI) system and may lead death within few weeks (Donnelly et al. 2010). For HP syndrome, therapeutics like blood transfusion, stem cell transplantation offer reasonable success (Nagayama et al. 2002; Weisdorf et al. 2006). For restoration and repair of HP system, a large number of chemicals like amifostine and gamma-tocotrienol have been synthesized and investigated (Trajkovic et al. 2007; Ghosh et al. 2009). The toxicity and limited therapeutic gain achieved by these agents against HP syndrome yet remains unacceptable clinically. Radiation damage to GI system is however still managed in palliative manner only and no effective treatment of GI syndrome is available till date (Takemura et al. 2014). The development of new agents of synthetic or natural origin has remained elusive in this context and the attention of researchers has been hardly noticeable. Under the present study, we have attempted to investigate the natural dietary cucurbits, against GI syndrome.

Etiology of GI syndrome depends mainly on the damage highly proliferative stem cells, the *crypts of Lieberkuhn*, located at villar bases (Brown 2008). The damaged crypt cells lag in replenishment of the damaged epithelial cells of villi causing shortening of villi height. The denudating villi may be responsible for pathological manifestations like poor

absorption of nutrients (Kau et al. 2011), loss of fluids and electrolytes, perturbation in barrier function (Turner 2009) and microbial infections (Williams et al. 2015; Marchesi et al. 2016).

The synthetic radio-modifying agents target the specific molecular pathways yet are often antagonistic to many co-existing metabolic pathways. Therefore, scientists and clinicians have devised the combinational philosophy which envisages the combining of several agents to achieve better therapeutic gain. The toxicity of even combination modalities has still remained high and thus warranted the use of natural agents in this connection. In fact each plant extract contains hundreds of bioactive molecules which act synergistically in multiple directions. Some of the components of the plant extract may yield direct therapeutic effects while others may concurrently accelerate and reinforce the recovery process and still other may overcome adverse toxic reactions.

Some cucurbits like *Lagenaria siceraria (Ls), Luffa cylindrica (Lc) and Cucurbita pepo* (Cp) have been evaluated for their radio-protective activities. These cucurbits have bioactive molecules as alkaloids, flavonoids, steroids, saponins and glycosides (Irshad et al. 2010). In the previous studies done at our laboratory, these cucurbits displayed significant antioxidant, antimicrobial and anti-inflammatory activities (Sharma et al. 2012; Rawat et al. 2014). The radiation damage is since mainly mediated by generation of free radicals and may lead to the development of several inflammatory and infectious diseases (Arora et al. 2005). Therefore, biological activities (antioxidant, antimicrobial and anti-inflammatory) of these cucurbits were considered important for the radioprotection and it warranted investigations on the radio-modifying efficacy in a holistic manner.

Material and Methods:

Chemicals:

Dulbecco's modified eagle medium (DMEM), Minimum essential medium (MEM), Fetal bovine serum (FBS), Trypsin- EDTA solution, 2,5-diphenyltetrazoliumbromide (MTT), were procured from M/s Himedia (India). NaCl, EDTA, Triton X-100 and Tris were procured from M/s Merck, Mumbai (India). Hematoxylin and Eosin were procured from M/S Fisher Scientific, Mumbai. Propidium iodide and Osmium tetroxide were procured from M/s Sigma Aldrich (USA).

Preparation of Extracts:

Fresh fruits of the cucurbits namely bottle gourd (Ls), sponge gourd (*Lc*) and pumpkin (*Cp*) procured from the local market were thoroughly washed with sterile distilled water several times and 100 g of each plant material was homogenized separately in 100 ml solvent (absolute alcohol and triple distilled water; 50:50, v/v). After 24 h, the homogenate was filtered through a fine strainer having a spread of muslin cloth and thereafter through membrane filter of 0.22 µ. It was further concentrated using rota-vapor. Filtered whole extract of cucurbit fruits was stored at 4°C in air-tight bottles.

Cell Culture:

The human carcinoma cells (HCT-15) procured from National Centre for Cell Sciences, Pune, were cultured in DMEM containing 10% FBS and penicillin/streptomycin (100 µg/mL) at 37^0C in an incubator (CO_2 conc.- 5%). One million cells were inoculated in 5

ml medium contained in a petri-dish having 20 ml capacity. On reaching about 70% confluency, cells were washed with PBS and were trypsinized and sub-cultured.

Animals:

Swiss albino Strain 'A' male mice (6-8 weeks old) weighing about 22 ± 3 g were maintained under controlled laboratory environment (~25 ± 2°C, photoperiod-12 h). Mice were given standard animal feed (Lipton, India) and tap water *ad libitum*. Animals for these experiments were used according to the guidelines of animal ethics committee of Amity University, Noida and INMAS, New Delhi.

Irradiation:

The Teletherapy cobalt machine (Bhabhataron II) at 'Institute of Nuclear Medicine and Allied Sciences' was obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai (India). The dose rate in the irradiation chamber was ~1.9 to 1.86 Gy/minute during the course of these investigations.

For *in vitro* studies, HCT-15 cells were cultured in petri-dishes each containing 5 ml culture medium. Each dish was exposed to gamma irradiation (6 Gy) individually. For *in vivo* studies, whole body irradiation (10 Gy) was delivered to each mouse individually.

Experimental design:

***In vitro*:**

The cells were divided into 3 experimental groups each containing 4 petri dishes:

a) Control group: cells given no treatment

b) Radiation alone group: cells received a dose of 6 Gy

c) Cucurbits + radiation group: 500 µL of cucurbit extract (Ls, Lc or Cp) was added to the cell cultures 2 h before irradiation and fresh medium without the extract was provided at 4 h post-irradiation period.

24 h after the radiation exposure, cells were processed for different experimental parameters.

In vivo:

Three groups of swiss albino strain 'A' male mice, each containing 6 animals, were randomly selected for these experiments. The animals were grouped as under:

i) Control: mice receiving no treatment

ii) Irradiated group: Each mouse received 10 Gy whole body gamma irradiation only

iii) Ls + radiated group: Each mouse received Ls extract at the rate of 2 mg/kg body wt. 2 h before, and 4 and 24 h after irradiation (10 Gy).

On 4th post-irradiation day, the jejunum part of each mouse was taken out and processed for histological study.

For dose mortality response curves, four sets of animals each having 12 mice were selected to see the effect of Ls extract on the survival of mice exposed to gamma irradiation (10 Gy). The animals were grouped as under:

a) Control group

b) Radiation group (10 Gy)

c) Ls treated group (Ls extract administered orally at 0 h, 6 h & 26 h)

d) Ls + radiation group (Ls extract administered 2 h before irradiation and 4 and 24 h after irradiation)

All the animals of each experimental group were kept under controlled environment and were observed for mortality up to 30 days.

Cell cycle analysis:

Cells were processed for cell cycle analysis by following the method described by Pozarowski and Darzynkiewicz (2004). After 24 h of radiation exposure, cells were trypsinized and centrifuged at 2000 g for 10 min. Cell pellet was washed three times and re-suspended in 0.5 ml PBS. Fixation was completed by adding 1.2 mL of 70% cold ethanol for 2 h. The fixed cells were washed with PBS and centrifuged at 2000 ×g for 10 min. After suspending cells in 0.3 mL PBS, DNAase free RNAse (50 mg/mL) was added and incubated for 1 h. After adding 2 µL of propidium iodide (10 mg/mL in PBS), cells were incubated at 4°C for 30 min. DNA contents were analyzed for cell cycle using flow cytometer (Becton and Dickinson) with an excitation wavelength of 488 nm and emission at 670 nm.

Comet assay:

For this, the method described by Singh et al. (1988) was adopted with slight modifications. 24 after the radiation exposure, 100 µL of singled cells suspension was added to 500 µL of 0.8% agarose (Low melting point: 30-35º C) in phosphate-buffered saline (PBS) which is put on a glass slide pre-coated with 1 % agarose having normal-melting point (50- 60º C). Each slide was covered with a cover slip and kept on ice for 5 min. The slides were immersed in ice-cold alkaline lysing solution [2.5 M NaCl, 100 mM

Tris, 100 mM ethylene diamine tetra acetic acid (EDTA), 1 % sodium lauroyl sarcosine sodium salt, 1% Triton X-100 and final pH was adjusted to 10 using 1 N NaOH solution] for at least 1 h at 4° C. The slides were then washed 4-5 times with ice cold Milli Q water and were kept into ice cold buffer (0.2 N NaOH and 200 mM EDTA) for 30 min in the dark at 4°C to unwind DNA. Now slides were incubated for 20 min in ice-cold electrophoresis solution (200 mM NaOH, 500 mM EDTA, pH-13.1), followed by electrophoresis at 25 V (1.25 V/cm) for 25 min. After electrophoresis, the slides were washed and dehydrated with 70 % ice cold ethanol for 5 min and were air dried thereafter. The slides were stained with propidium iodide (50 µg/mL PBS) and kept in dark for 10 min. 50 cells were scored from each slide at a magnification of 400X using a Olympus fluorescence microscope employing excitation at λ 488 nm and emission barrier at λ 515 nm. Quantification of DNA damage was measured microscopically and compared with control slides.

GSH contents:

The glutathione level in the jejunum was determined following the method described by Verma et al. (2011). Briefly, jejunum homogenate was added to 20 % trichloro acetic acid and was centrifuged to collect the supernatant. The supernatant was mixed with 0.3 M Na_2HPO_4 and 5-5, dithiobis-2-nitrobenzoic acid (DTNB) reagent, and allowed to stand for 10 min at the room temperature. The absorbance was taken against blank at 412 nm using a UV-VIS Systronics spectrophotometer.

Histological study:

About 2 cm long pieces of jejunum were taken out using surgical procedure, from a mouse immediately after cervical dislocation and fixed in 10% neutral formalin (pH 7.0–7.6) for 24 hours. The tissue was processed for dehydration and paraffin block making following standard procedure. Microtomy was done to get 5 µ sections which were processed for haematoxylin and eosin (H&E) staining following the method described by Khojasteh et al. (2009) Morphology of the villi was studied under a light microscope. 10 villi per section were assessed and mean value was calculated. Villus morphology, height, width and area were measured at the magnification of 40 × 10 X and compared with the control.

Transmission electron microscopy (TEM)

For Tem, Few pieces of jejunem from each experimental mouse were collected immediately after cervical dislocation and were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. The tissue was stained in 1% uranyl acetate and embedded in Epon following the method described by Soderholm et al. (2002). The staining of sections was done by lead citrate for study under TEM at 80 kV. To evaluate changes in tight junction integrity, the junctional regions of four randomly selected villi were examined in each group.

Statistical Analysis

All the data are presented as mean ± SE and student's t test were applied for determining the statistical significance between different groups.

Results:

Cell cycle analysis:

Effect of different cucurbits on the modulation of radiation induced changes in the different phases of cell cycle has been depicted in Table 1. The control group had 36 ± 0.6 % cells in G_1 phase and gamma irradiation (6 Gy) decreased G_0/G_1 phase cell population to about 32 ± 1.0 %. The extracts of Ls and Cp also decreased the number of cells in G_1 phase further to about 28 % whereas Lc extract did not decrease the G_1 fraction. In the S phase, cells were not influenced significantly by the Ls and Lc treatment but Cp distinctly increased the S phase population to about 24 ± 1.5 as compared to control (19 ± 1.0). In the control group, G_2 fraction was about 20 ± 1.2 % which increased to about 31 ± 2.0 % in radiation-alone group. In Ls, Lc and Cp treated groups, cell population in G_2 phase increased significantly ($P < 0.05$) to about 42 ± 2.0, 34 ± 1.5 and 37 ± 1.0 % respectively.

Evaluation of DNA damage using comet assay:

The tail length of the comet generated during gel electrophoresis of HCT-15 cells, was 4.25 ± 0.47 µm in control and 41.75 ± 1.1 µm in radiation alone group (6 Gy). The cucurbit extracts administered individually before irradiation (-2 h) rendered the tail length significantly shorter than the radiation alone group and measured as 28 ± 1.2 - Ls, 34.2 ± 0.57 - Lc and 36.75 ± 1.18 µm – Cp (Table 2).

Diameter of the comet head in the control group was about 19.3 ± 0.96 µm and decreased to 10.9 ± 1.8 µm in the radiation alone group (Table 2, Fig. 1). Pre-irradiation treatment of HCT-15 cells with cucurbit extract caused reduced decrease in head diameter (16.4 ±

0.68- Ls, 15.75 ± 1.57- Lc and 13.5 ± 0.86 µm- Cp. The ratio of head diameter to tail length was 0.27 in control and 3.5 in radiation alone group. In Ls, Lc and Cp treated groups this ratio remained as 1.38, 1.67 and 2.7 respectively.

GSH contents:

The changes in the amount of total GSH in various experimental groups have been presented in Fig 2. In the control group GSH contents were 27.6 ± 1.3 µmol/gm which decreased to 19.6 ± 0.14 µmol/gm in the irradiated group (10 Gy). Ls treatment (2mg/kg body wt.) increased the GSH contents to 48.8 ± 1.4 µmol/gm which were significantly higher than the control and irradiated group.

Radio-modifying effect of *Lagenaria siceraria* in mice

Mice in the control group (receiving no treatment) and in the Ls extract treated group (2mg/kg body wt.) rendered 100 % survival (30 days and beyond). Irradiation (10 Gy) rendered 33 % survival up to 30th post-irradiation day (Fig. 3). Administration of Ls extract (at -2 h, +4 and +24 h of irradiation). Ls treatment improved the survival of irradiated mice to about 75 % (30th post-irradiation day).

Histological study of gut villi:

The average length and width of the villi in the jejunum region in the control group was 379 ± 0.36 and 54 ± 1.4 µm respectively. Each villus at an average had an area of ~ 64468 ± 2.3 µm^2 (Table 3). The average height and width (VH/VW) ratio of the villi in the control group was 7.01 ± 1.9. On forth post-irradiation day, the average length and width of the villi was observed as 350 ± 0.22 and 27 ± 0.06 µm and the area reduced to

about 29768 ± 0.22 μm^2 in the radiation alone group. This led to the decrease in the ratio to about 12.9 ± 3.1 and the villi looked like thinner flaps

(Fig. 4b) and revealed the increased denudation of the villi epithelium.

Administration of Ls extract (2 h before and at 4 and 24 h post-irradiation periods) restored the height and width of the villi to about 366 ± 0.15 and 30 ± 0.05 µm respectively on forth post-irradiation day (Table 3). The area of the villi was also increased to 34587 ± 0.19 μm^2 as compared to the radiation-alone group (29768 ± 0.22 μm^2). Ls treatment thus improved the height and width ratio (12.2 ± 2.5) than the irradiated group. Ls treatment improved the cellular architecture of the villi to some extent but the damage still remained appreciably marked as compared to the control group (Fig. 4c).

Ultra-structure studies of jejunum sections of mouse intestine:

The tight junctions and desmosomes appeared to have an intact structure in the control group (Fig. 5 a). The radiation-alone (10 Gy) group revealed (on 4^{th} post-irradiation day) that tight junctions between the adjacent cells membrane were disrupted and were not intact (Fig. 5 b). There was partial loss of desmosomes in the intercellular junctions and the electron dense material was scantier in the cells. The density of microvilli on the luminar side of the epithelium reduced.

Ls extract treatment (2 h pre- and 4 and 24 h post-irradiation period) manifested some improvements in the structure of tight junctions as seen on 4^{th} post- irradiation day and the desmosomes were observed to be normal in structure due to Ls treatment. In this Ls + radiation treated group, microvilli were denser and healthier as compared to the radiation-alone group (Fig. 5 c).

Discussion:

Radiation induced GI syndrome involves serious disturbances in luminal environment, cells and tissues of GI tract and the enteric microbiota. In our previous studies, dietary cucurbit fruits (Ls, Lc and Cp) have been shown to act as prebiotics which foster the interaction among enteric microbes (probiotics), diet, cells, tissues and secretions of the GI tract manifesting antimicrobial, antioxidant and anti-inflammatory activity on one hand (Sharma et al. 2012; Rawat et al. 2014) and augment the cellular repair and recovery and enhance cell proliferation to replenish apoptotic and necrotic cells. Oxidative and inflammatory manifestations display parallel situations with respect to the etiology of radiation damage and many acute and chronic ailments of metabolic and oncogenic nature. It was therefore considered rational to investigate the role of cucurbits in the recovery of radiation damage. The radio-protective attributes of cucurbits have not been documented as yet. Therefore, present study was undertaken to explore radio-modifying potential of some dietary cucurbits i*n-vitro* and *in-vivo*.

Cell cycle: The DNA repair and replenishment of damaged and dead cells require augmentation of cell proliferation and G_2 fraction**.** Radiation induced G_2 blockage of cells has been widely reported in the literature and this blockage is to ensure repair and recovery of damage to the cells before allowing them to progress to M-phase (Vucic et al. 2006). Addition of the cucurbit extracts to the irradiated cells has enhanced the accumulation of cells at G_2 check- point as compared to radiation-alone treated group (Table 1). This accumulation is considered necessary to repair the DNA damage maximally which was induced by radiation exposure.

The cucurbit extracts contain hundreds of molecules which remain active in various directions. In our previous studies, cucurbitacin, a characteristic molecule of the family cucurbitaceae, revealed the anti-proliferative effect against HCT-15 cells. Cucurbitacin has also been studied by others and has been reported to enhance accumulation of cells in G_2 phase (Duangmano et al. 2012). The mechanism involving G_2 blockage may be the consequence of reduced formation of spindle microtubules (Dutta and Gupta 2014). Several cell cycle regulators like p53, p21, CDC1, CDC2 have also been reported to affect the cell cycle (Kim et al. 2004). Therefore, it would be interesting to investigate further the role of cucurbits on the expression of various cell cycle regulators.

Comet assay relies on the DNA fragmentation generated by a genotoxic agent. The smaller fragments migrate faster than bigger jump of DNA during gel-electrophoresis and create a typical comet-shaped pattern. In fact, there are several parameters exploited for the evaluation of DNA damage and comet length is considered a reliable parameter in this connection. The irradiated cells revealed a longer tail as compared to un-irradiated control indicating large amount of fragmentation of DNA. Each of the three cucurbits investigated here has been found to reduce the tail length significantly. The Ls extract was more effective in reducing the comet tail length than Lc and Cp. The antioxidant action of these cucurbits could be responsible for such a decrease in tail length because a significant magnitude of damage induced by ionizing radiation results from the action of free radicals (ROS) such as hydroxyl radicals, hydrogen peroxide, superoxide anions etc. The findings of comet assay, where Ls was more effective in reducing the DNA damage followed by Lc and Cp corroborate results of antioxidant activity (Ls > Lc > Cp) of these cucurbits reported earlier (Sharma et al. 2012).

Villi (jejunum): The epithelial cells of villi follow a definite pattern of migration and maturation and are constantly replaced by the new cells produced by the stem cells, *the crypts of Lieberkuhn*. The cells from the crypts migrate towards the tip of villi reaching the top within about 72 h in mice and are thereafter denudated in the gut lumen. The radiation induced effects on gastrointestinal system have been mostly studied by many workers in jejunum (Bing et al. 2014; Zhao et al. 2014) because of very active *crypts of Lieberkuhn* and larger villi in jejunum than in the colon. For reason of comparison we have also studied this region.

The colon which harbors about 10^{14} microbes metabolizes the fiber like inulin (polysaccharides) into short chain fatty acids and also produces many nutrients like vitamin K, B12, riboflavin etc. These molecules may substantially influence the radio-recovery and therefore, studies in the colon tissue deserve further investigations.

Radiation exposure has been reported to induce apoptosis in crypt cells (Matsuu-Matsuyama et al. 2006) and also affects the proliferation of crypt cells adversely. The slow proliferation of crypts cells decreased the replenishment of villi epithelial cells leading to shortening of the height and width of the villi and subsequently the absorptive area of the villus. These changes decreased absorption of the nutrients and essential molecules and this may adversely influence the activity of proliferating and differentiated cells.

Treatment with Ls extract (3 times: 2h pre- and 4 and 24 h post-irradiation) increased the height, width and area of the villi in comparison to radiation alone group (Table 3 and also rendered some improvement in the morphology of the villi (Fig 4c). The exact mode

of action of radio-protection of Ls on gut villi is not known. Our studies on *in-vitro* system (Sharma et al. 2012; Rawat et al. 2012) have demonstrated the antioxidant activity of Ls extract. The endogenous antioxidants like glutathione (GSH), superoxide dismutase may also help in the mitigation of radiation induced free radicals. Ls extract has been shown to up-regulate the production of GSH (Fig. 2). However, these mechanisms alone may not be sufficient to explain the extent of radio-protection. Indeed several other mechanisms may also be concomitantly acting to achieve radio-protection. The Ls extract contains a number of bioactive molecules like flavonoids, alkaloids, saponins etc, which may contribute as important biomolecules for the repair of radiation induced damage. Flavonoids have been demonstrated to act as anti- carcinogenic agents (Seelinger et al. 2008) and thus help in the repair of radiation induced damage and thus provide radio-protection. Radiation has been reported to activate several inflammatory pathways like NF-kB and COX-II (Chung et al. 2010). Ls has already been reported for its strong anti-inflammatory activity (Rawat et al. 2012). However, Ls acting as a radio-protector may have many more aspects like gut-neuronal network which need to be investigated further.

Tight junction: The epithelial cells of the villi are held together by complex structures the 'tight junctions'. The membranes of the two juxta-positioned cells join together and form a dynamic barrier for the microbes. The integrity of tight junction is dependent on the composition and organization of tight junction proteins (Shen et al. 2008) which are composed of trans-membrane proteins (occludin, claudin and junctional adhesion molecule) and cytoplasmic proteins (ZO-1, ZO-2, ZO-3 and cingulin). TJs open and close all the times in response to a variety of stimuli like dietary state, hormonal and neuronal signals, inflammatory mediators and mast cell products, in a regulated manner. However,

dissociation of the protein complex and/or down-regulation of proteins may disrupt the tight junctions (Peerapen and Thongboonkerd 2013) and may lead to the widening of the paracellular passage. The pathogens at this stage may breach the gut epithelium to enter the lamina propria and the blood circulation (Guttman and Finlay 2009). This may lead to immunogenic reactions, onset of infections and several disorders subsequently. Therefore, dietary agents were investigated for managing diarrhea, leaky junctions and the microbial invasion.

Whole body gamma irradiation of 10 Gy disrupted the integrity of tight junctions of adjacent cells (Fig. 5 b). The higher incidence of microbial pathogenicity after whole body lethal irradiation (hemopoietic and gastro-intestinal syndrome) and after exposure of abdomino-pelvic region during radio-therapy (Macnaughton 2000), may also be explained accordingly.

Present study displayed that radiation induced disruptions in tight junctions were recovered by Ls treatment (Fig. 5c). It indicates that Ls extract has some molecules which are very important for regulation of tight junction proteins. In our previous studies, large amount of flavonoids have been found in the Ls. Flavonoids present in the Ls extract (Gangwal et al. 2010) have been reported to enhance the barrier function through regulation of the TJ protein claudin-4 (Amasheh et al. 2008). Therefore, previous studies done in our laboratory has been giving a direction towards the molecular mechanism of Ls extract with respect to epithelial barrier function. Further investigations are necessary to understand the mechanism of recovery by Ls treatment.

Conclusion:

Application of dietary cucurbits for protection against lethal doses of radiation causing GI syndrome has been experimentally demonstrated both *in vitro* and *in vivo*. Many aspects of the mechanism of radio-protection have yet to be understood in more details before cucurbits could be exploited for the development of radio-protective agents.

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Disclosure:

The authors of this research article ***declare that there is no conflict of interests regarding the publication of this manuscript***.

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Tables

Table 1: Effect of cucurbit extracts on the radiation induced changes in cell cycle fractions (%).

Experimental groups	Cell cycle fractions		
	G_1	S	G_2
Control	36 ± 0.6 %	19 ± 1.0 %	20 ± 1.2 %
Radiation (6 Gy)	32 ± 1.0*	22 ± 1.5 %^{NS}	31 ± 2.0 %*
***Lagenaria* + 6 Gy**	28 ± 0.6 %*	18 ± 1.5%^{NS}	42 ± 2.0 %*
***Luffa* + 6 Gy**	36 ± 2.6%^{NS}	20 ± 2.0%^{NS}	34 ± 1.5 %*
***Cucurbita* + 6 Gy**	28 ± 1.0 %*	24 ± 1.5%*	37 ± 1.0 %*

Data represented mean ± SE of three sets of experiments and each experimental group was compared with control. Student's t test was conducted for Significance (*$P < 0.05$, NS- non-significant)

Table 2: Modulation of radiation induced DNA damage by cucurbit extracts in HCT-15 cells.

Groups ↓	Tail length (µm)	Head (µm)	Tail: head
Control	4.25 ± 0.47	19.3 ± 0.96	0.27
Radiation (6 Gy)	41.75 ± 1.1	10.9 ± 1.8	3.5
***Lagenaria* + 6 Gy**	28 ± 1.2*	16.4 ± 0.68*	1.38
***Luffa* + 6 Gy**	34.2 ± 0.57*	15.75 ± 1.57*	1.67
***Cucurbita* + 6 Gy**	36.75 µm ± 1.18*	13.5 µm ± 0.86^{NS}	2.7

Experimental protocol was followed as described in the legend of Fig 1. Comet images provided the data for Table 2. Data represented mean ± SE of three sets of experiments. Comparison was made between radiation and cucurbit (Ls, Lc or Cp) + irradiated groups Student's t test was conducted for significance (*$P < 0.05$, NS- non-significant).

Table 3: Changes in height and width of villi, their ratio and area of different experimental groups.

Group ↓	Villi (µm)		Height/Width	Area
	Height	Width	(μm^2)	
Control	379 ± 0.36	54 ± 1.4	7.01 ± 1.9	64468 ± 2.3
Radiation	350 ± 0.22[a]	27 ± 0.06[a]	12.9 ± 3.1	29768 ± 0.22
Ls + Radiation	366 ± 0.15[b]	30 ± 0.05[b]	12.2 ± 2.5	34587 ± 0.19

(Ls-*Lagenaria siceraria*)

Mice were administered *Lagenaria* extract (at the rate of 2 mg/kg body weight) orally at -2 h, +4 and +24 h of whole body gamma irradiation (10 Gy). On 4th day of radiation exposure, mice were sacrificed to collect the samples which were processed for histological study. Significance level determined by student's t test: [a]$P < 0.05$ control *vs* radiation alone, [b]$P < 0.05$ radiation alone *vs* Ls + irradiated group.

Figure legends

Fig. 1: Images of the comets, stained with propidium iodide, observed through florescent microscopy.

Cucurbit extracts (500 µL) were added to HCT-15 cells (1 million) in culture 2 h prior to irradiation. 4 h after irradiation, the culture medium having cucurbit extract was replaced by fresh medium without cucurbit extract. After 24 h of irradiation, cells were processed for comet assay.

(a) Control (b) Radiation- 6 Gy (c) Ls + radiation (d) Lc + radiation (e) Cp + radiation.

Fig. 2: Effect of *Lagenaria* extract on the radiation induced changes in GSH level in the mice intestine.

Mice were administered *Lagenaria* extract (at the rate of 2 mg/kg body weight) orally at various periods (-2 h, +4 and +24 h of irradiation-10 Gy). On 4^{th} day of irradiation mice were sacrificed to operate out jejunum sections and GSH contents were measured and have been expressed as µmol/gm intestinal tissue. Data in each group represented mean ± SE of three sets of experiments. Significance level determined by student's t test: $^{a}P < 0.05$ control *vs* radiation alone, $^{b}P < 0.05$ radiation alone *vs* Ls + irradiated group.

Fig 3: Effect of *Lagenaria* extract on the survival of gamma irradiated mice.

(Ls- *Lagenaria siceraria*)

Mice were observed for survival till 30 post-irradiation days. Data represented mean ± SE of three independent experiments carried out with 12 animals/group.

Different groups: (a) Control group - without any treatment), (b) Ls treated group - Ls extract administered at 0 h, 6 h & 26 h, (c) Radiation group (10 Gy), (d) Ls + radiation group- Ls extract administered at -2 h and +4 and + 24 h of irradiation

Fig. 4: Images of Hematoxylene & Eosin stained sections of jejunum of mice procured from different groups.

(a) Control showing healthy villi (b) Irradiation group showing disrupted villi (c) Ls + irradiated group showing some improvement in the architecture of villi

Experimental protocol was followed as described in Table 3. Data of Table 3 was computed on the basis of observations received through these images.

Fig. 5: Electron micrograph of the jejunum sections of mice of different groups.

Experimental protocol was followed as described in Table 3. To study the tight junctions, jejunum sections were processed for 'Transmission electron microscopy' on 4th day of irradiation.

(a) Control showing normal structure of tight junction

(b) Radiation treated group showing disrupted tight junction and desmosome

(c) Ls treated group showing some improvement in the structure of tight junction and desmosome

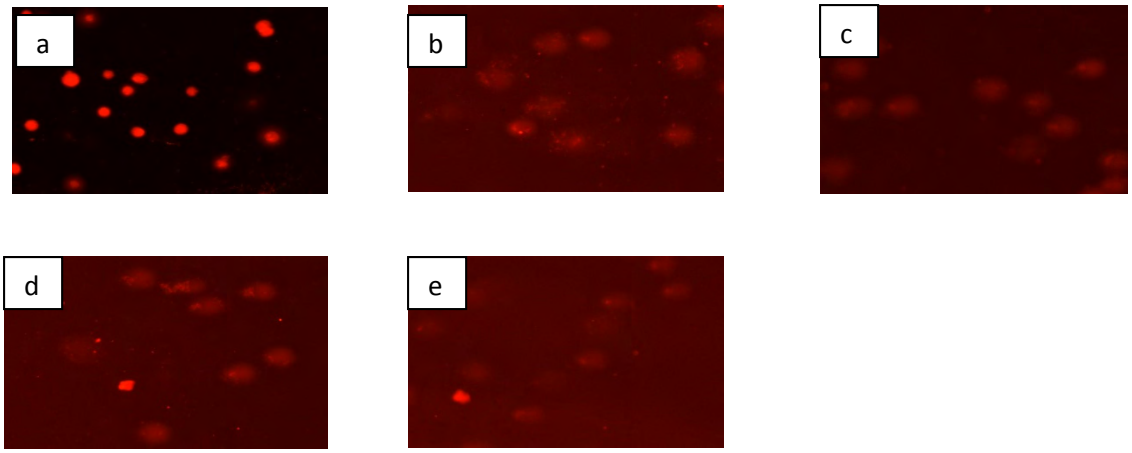

Fig. 1

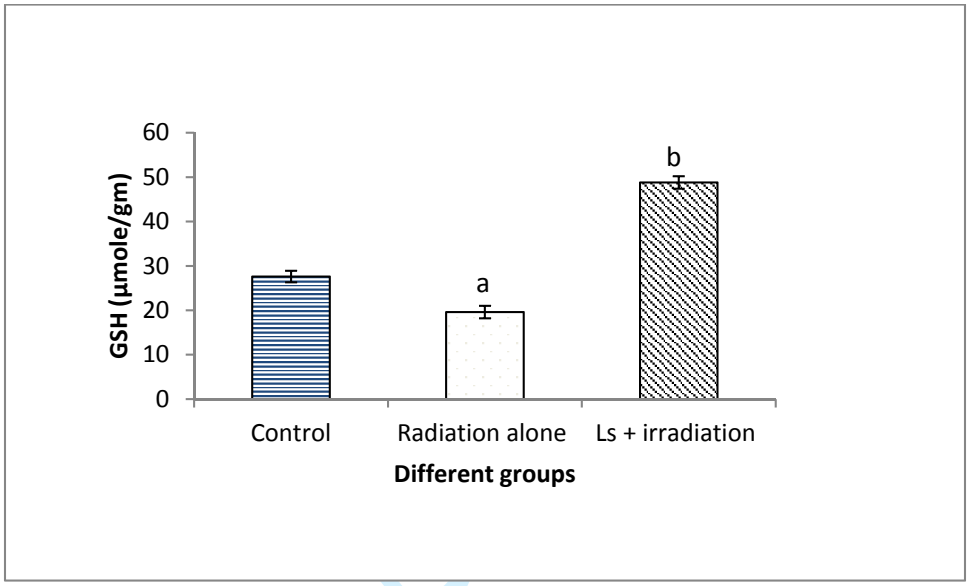
60
50
40
30
20
10
0
GSH (µmole/gm)
a
b
Control
Radiation alone
Ls + irradiation
Different groups

Fig. 2

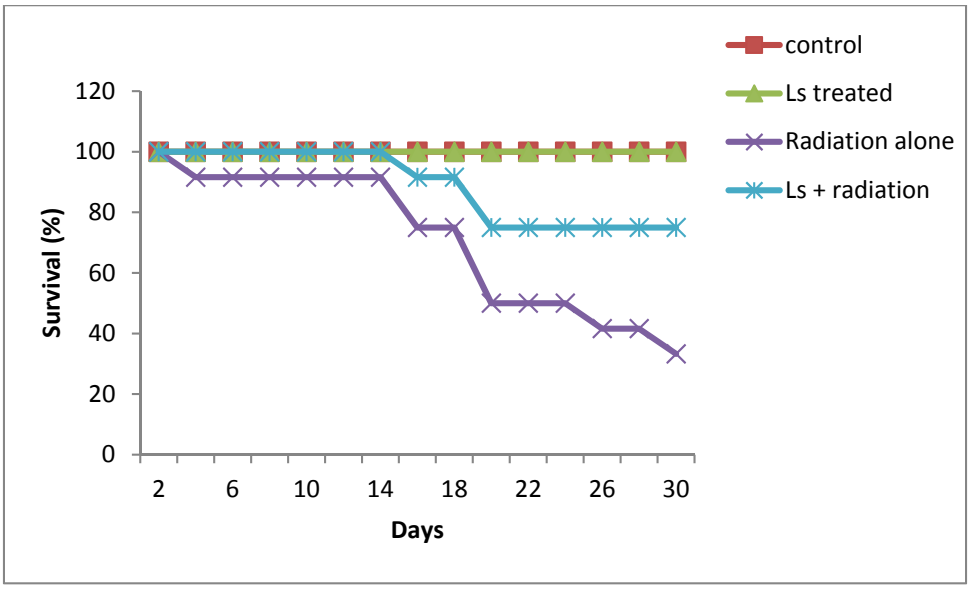
control
Ls treated
Radiation alone
Ls + radiation
120
100
80
60
40
20
0
Survival (%)
2
6
10
14
18
22
26
30
Days

Fig 3

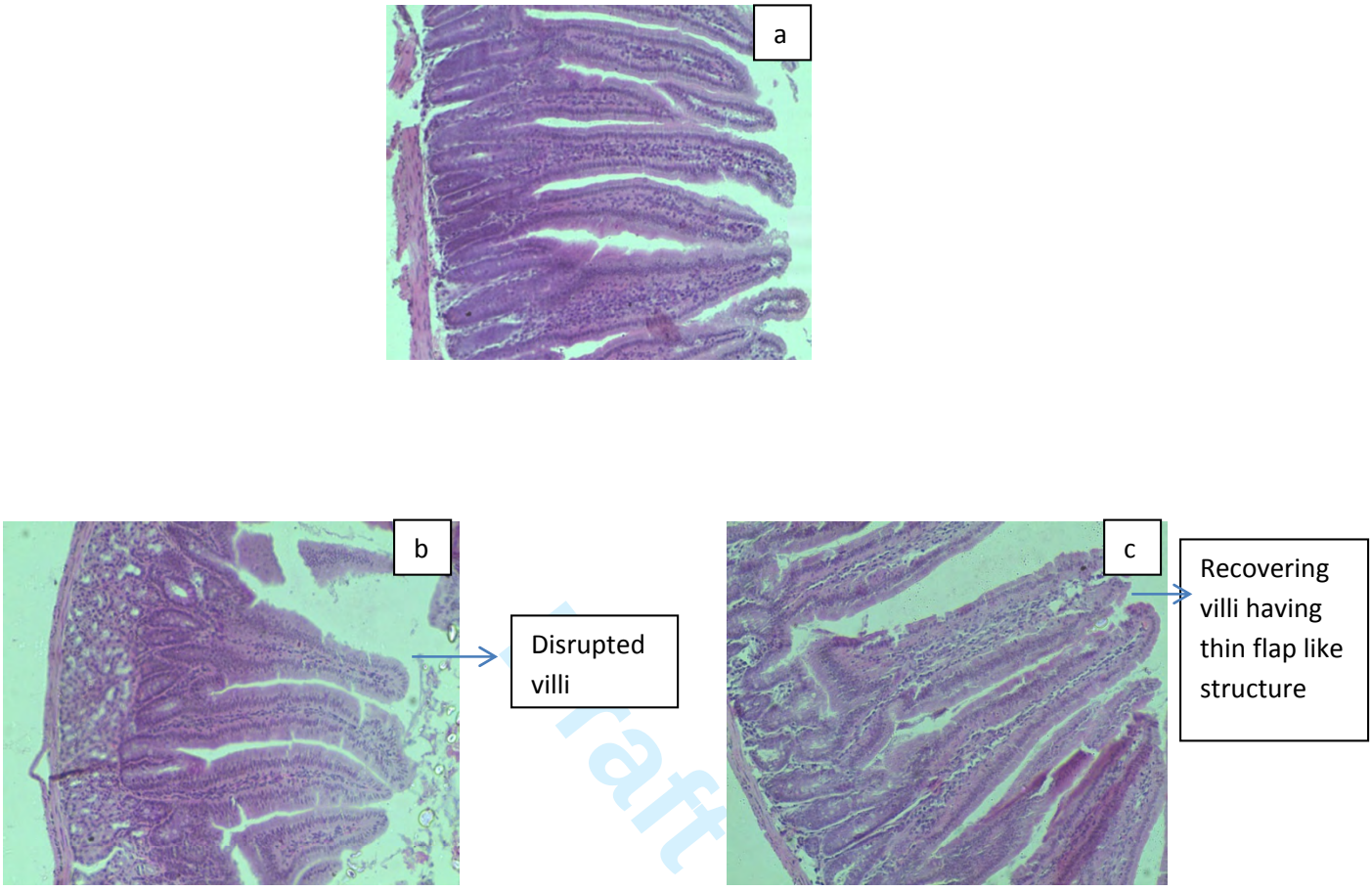
a
b
Disrupted villi
c
Recovering villi having thin flap like structure

Fig. 4

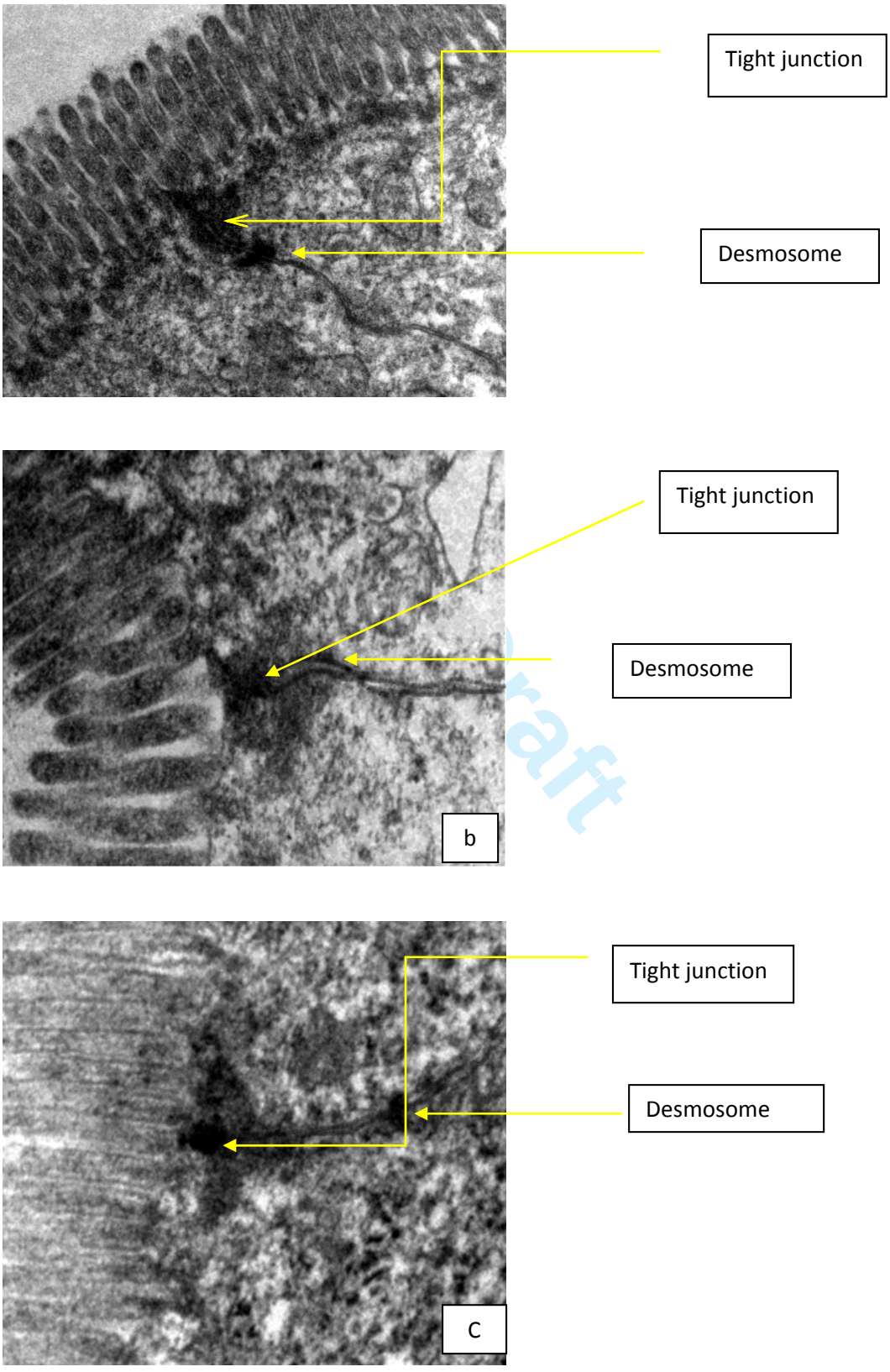
Tight junction
Desmosome
Tight junction
Desmosome
b
Tight junction
Desmosome
C

Fig. 5