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Full Length Research Paper

Gelam honey acting as a radioprotectant agent in gamma-irradiated human diploid fibroblasts

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In this study, the effect of Gelam honey against radiation-induced DNA damage and cell survival rate of human diploid fibroblasts (HDFs) was evaluated. The degree of damaged DNA was determined by Comet assay while Clonogenic assay was used to evaluate cell survival rate. Irradiated Gelam honey at the concentration of 6 mg/ml was used to treat HDFs pre-, during- and post-exposure to 1 Gy of gamma-rays to evaluate its radioprotectant properties. Comet assay showed that exposure to gamma-rays caused a significant increase in total DNA damage in a dose dependent manner ($p<0.05$). Pre-treatment with Gelam honey at 6 mg/ml decreased the degree of damaged DNA significantly ($p<0.05$) which was not observed when Gelam honey treatment was given during- and post-irradiation. Clonogenic assay showed the percentage of survival fraction of HDFs decreased significantly with increasing dose of gamma-rays exposure ($p<0.05$). Cell survival rate however was significantly increased when HDFs were treated with Gelam honey pre- and during-irradiation. These findings indicated that pre-treatment with Gelam honey protected against radiation-induced DNA damage and enhanced cell survival rate. In conclusion, Gelam honey may acts as a radioprotectant agent in gamma-irradiated human diploid fibroblasts.

Key words: Gelam honey, radioprotectant, human diploid fibroblasts (HDFs), DNA damage, cells survival.

INTRODUCTION

Ionizing radiation such as gamma-rays can trigger the formation of free radicals which induces biological damage even at a very low dose (Prasad, 2005). It can also cause damage to DNA via its direct or indirect effects. The direct effects involved the interaction between ionizing reaction and biological molecules, which results in breaking of single stranded or double stranded DNA and DNA cross linking. Meanwhile, the indirect effect is due to ionization of water molecules in the cells during ionizing process. This ionization process will lead to production of short-lived free radicals, which will interact further with other biological molecules in the cell including DNA (Borek, 2004; Yusof, 2001). Therefore, to protect the cells from damage and to maintain cell functions, antioxidants are required to scavenge these free radicals.

The application of radioprotective compounds has been studied since the earliest days of the nuclear era, due to the possibility of radiation accident that may occur during handling of the radioactive source. Depending on the dose of exposure, the effect of radiation ranges from nausea and vomiting to immune system failure which leads to death (Singh et al., 2005). A report by International Atomic Energy Agency (IAEA) and World Health Organization (WHO) (2000) indicated that there were 405

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radiation accidents worldwide between 1944 and 1999. Approximately, 3000 persons were injured, with 120 fatalities. Although radiation accidents were rare, during the last few years the number of accidents has increased. Thus the application of radioprotectant agent might help to prevent radiation injury due to acute or long-term effects of radiation. Moreover, it has also been considered of using radioprotectant agent during radiotherapy as radiotherapy treatment is toxic to both cancer cells as well as healthy cells. According to Grdina et al. (2002), the ideal radioprotectant agent is one that can protect normal cells while maintaining tumor sensitivity during radiotherapy treatment.

One of the natural antioxidants that have been used since ancient times in traditional medicine apart from its nutritional values is honey (Martos et al., 2008). Honey is a natural sweetener with complex mixture of sugars such as glucose and fructose. It also contains minerals, vitamins, enzymes, flavonoids and phenolic compounds, making it a good source of antioxidant. The antioxidant bioactive components included both enzymatic and non-enzymatic antioxidants such as glucose oxidase, catalase, ascorbic acid, flavonoids and phenolic acids (Baltrušaitylê et al., 2007; Bertoncelj et al., 2007). Blasa et al. (2007) revealed that the binding of flavonoids in honey to red blood cell membrane can protect the erythrocytes against oxidative damage. While, the water fraction of honey acts as an outside protector to erythrocyte membranes. Honey was also shown to remove reactive oxygen spesies (ROS) in cultured endothelial cells subjected to oxidative stress (Beretta et al., 2007). The radioprotective effect of honey was shown by Biswal et al. (2003) who reported that patients who received honey supplement before undergoing radiotherapy to the head and neck region showed significantly lower induced-mucositis as compared to non honey-supplemented patients.

Gelam honey was derived from nectar of Gelam tree (*Melaleuca* spp.) in deep forest of Malaysia. It contains several antioxidant active compounds such as gallic, ferulic, caffeic, benzoic and cinnamic acids (Aljadi and Kamaruddin, 2004) and was shown to have wound healing property (Rozaini et al., 2004; Aljady et al., 2000). Gelam honey has been shown to stimulate fibroblast cells as well as to activate epithelialization in animal model (Kassim et al., 2010). Nevertheless, no study has been conducted to determine the property of Gelam honey as a radioprotectant agent on human fibroblast cells.

Therefore, the aim of this study was to determine the ability of Gelam honey as a radioprotectant agent in irradiated HDFs by determining the degree of damaged DNA and cell survival fraction.

MATERIALS AND METHODS

Reagents

1,1-Diphenyl-2-picryl-hydrazil (DPPH), iron (II) sulphate, glacial acetic acid, sodium acetate trihydrate, 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) and ferric trichloride hexahydrate were purchased from Sigma (St. Louis, USA).

Sterilization of Gelam honey

Malaysian Gelam honey (*Melaleuca* spp.) was purchased from Department of Agriculture, Batu Pahat, Johor, Malaysia. The Gelam honey was packed in plastic bottles and sent to SINAGAMA, Malaysian Nuclear Agency for sterilization process using Cobalt-60 source (Model JS10000). The irradiation process was carried out at the dose of 25 kGy. The irradiated and non-irradiated honeys were then kept in the dark at room temperature.

1,1-diphenyl-2-picryl-hydrazil (DPPH) free radical-scavenging assay

The free radical-scavenging activity of Gelam honey was determined by 1,1-diphenyl-2-picryl-hydrazil (DPPH) as described by Meda et al. (2005). Irradiated (25 kGy) and non-irradiated gelam honey were used at concentration ranges between 0 to 100 mg/ml (w/v). Approximately, 0.09 mg/ml of DPPH was prepared in methanol, whereas various concentrations of honey were prepared using distilled water and were added into falcon tube containing 1.5 ml of DPPH reagent. The mixture was shaken vigorously and allowed to stand in dark at room temperature for 10 min. The absorbance was measured at 517 nm wavelength using a UV/VIS spectrophotometer (Shimadzu, Japan). The percentage of DPPH scavenging-activity was calculated using the following equation:

$$\text{Free radical-scavenging activity } (\%) = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100$$

whereas; $Abs_{control}$ is the measurement taken from sample without honey. Abs_{sample} is the measurement taken from sample which contains honey.

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant power in Gelam honey was measured based on the reduction of ferric tripyridyltriazine (Fe-TPTZ) complex to ferrous form, which has an intense blue color. The method used in this study as described by Blasa et al. (2006) with slight modifications. The $FeSO_4.7H_2O$ standard was prepared at various concentrations (0 to 1000 µM). Gelam honey at concentration ranges between 0 to 100 mg/ml (w/v) was prepared using distilled water. Standard or honey sample of 40 µl was added into 1.2 ml FRAP reagent. The mixture was shaken vigorously and left in the dark at room temperature for 10 min. The absorbance of each solution was measured at 593 nm wavelength using UV/VIS spectrophotometer (Shimadzu, Japan). The standard curve for $FeSO_4.7H_2O$ was plotted as mean FRAP value (µM) against concentration of $FeSO_4.7H_2O$ (mg/ml). Mean FRAP value for each sample was determined from the standard curve and was expressed in µM unit.

Cell culture and treatment protocol

Primary HDFs were derived from foreskins of three 9 to 12 year-old boys after circumcision. Written informed consents were obtained from parents of all subjects. The samples were aseptically collected and washed several times with 75% alcohol and phosphate buffered saline (PBS) containing 1% antibiotic–antimycotic solution (PAA, Austria). After removing the epidermis, the pure dermis was

cut into small pieces and transferred into a falcon tube containing 0.03% collagenase type I solution (Worthington Biochemical Corporation, USA). Pure dermis was digested in the incubator shaker at 37°C for 6 to 12 h. Then, cells were rinsed with PBS before being cultured in Dulbecco Modified Eagle Medium (DMEM) (Flowlab™, Australia) supplemented with 10% fetal bovine serum (PAA, Austria), 10,000 µg/ml penicillin/streptomycin (Gibco, USA), 250 µg/ml amphotericin B (PAA, Austria), 100 mg/ml gentamycin (PAA, Austria) and incubated in 5% CO_2 atmosphere at 37°C. The HDFs used in this study were within passage 4 to 6. This research has been approved by the Universiti Kebangsaan Malaysia Ethical Committee (Approval Project Code: FF-287-2009).

Treatment with Gelam honey

Several groups of HDFs within passage 4 to 6 were treated with irradiated Gelam honey (w/v). The concentration of Gelam honey selected was based from the cytotoxicity study. There were 6 different groups of HDFs viz; non-irradiated and non-treated group (untreated control), irradiated HDFs (positive control) and HDFs treated with Gelam honey (negative control). The other 3 groups represent HDFs treated with Gelam honey before exposure to gamma-radiation, HDFs treated with Gelam honey before and during exposure to gamma-radiation and HDFs treated with Gelam honey after exposure to gamma-radiation. The Gelam honey treatment was carried out for 24 h in CO_2 incubator. The level of DNA damage and cell survival fraction were determined by Comet and Clonogenic assays, respectively.

Cytotoxicity study

The cytotoxicity study of HDFs treated with Gelam honey at concentrations ranging from 0 to 10 mg/ml after 24 h incubation was determined by MTT assay. Cells were plated at 2×10^4 in 96-well plate and incubated overnight. Then the medium was replaced with new medium containing various concentrations of Gelam honey and incubated for 24 h at 37°C, in 5% CO_2 HDFs at passage 6 were seeded into 96-well micro titer plates and incubated at 37°C, in 5% CO_2 incubator. After 24 h of growth, cells were treated with irradiated Gelam honey at various concentrations (0 to 10 mg/ml; w/v). The cultures were then incubated for a further 24 h. Cells were assayed for viability using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT).

Screening of HDFs radio sensitivity

HDFs were exposed to gamma-rays at 0 to 10 Gy using Cobalt-60 source ELDORADO 8 (Atomic Energy of Canada Limited) at Secondary Standard Dosimetry Laboratory (SSDL), Malaysian Nuclear Agency. The dose rate was 0.28 Gy/min and the distance between source and tissue culture flask was 80 cm. The level of DNA damage and cell survival percentage were determined by Comet and Clonogenic assays, respectively. The lowest dose that showed significant increased in damaged DNA was selected for the subsequent experiments.

Comet assay

The level of DNA damage and mean tail moment of HDFs were analyzed by alkaline Comet assay as described by Fairburn et al. (1995). All steps were carried out in ice and under subdued lighting. The non-treated and Gelam honey-treated HDFs at passage 6 were harvested and placed in eppendorf tubes which were kept in ice during and after irradiation process to prevent DNA lesions repair (Giorgio et al., 2000). After irradiation, cell suspension was mixed gently with 0.6% low melting agarose at 37°C and pipetted onto 0.6% normal agarose layer. The electrophoresis process was conducted for 20 min at 25 V with current at 300 mA. As for HDFs treated with Gelam honey after exposure to gamma-irradiation, HDFs were exposed within the culture flask and were incubated for 24 h before subjected to Comet assay. Another group of irradiated HDFs was also prepared as a reference to this group.

DNA damage for each slide was analysed by fluorescence microscopy (Carl Zeiss, Germany) with visual inspection of tail length of nuclei. The cell nuclei were classified into five categories: (0); undamaged (nuclei without Comet tail), (1); low damaged (nuclei with Comet tails up to two fold longer than nucleus diameter), (2); damaged (nuclei with Comet tail two to three fold longer than nucleus diameter), (3); highly damaged (nuclei with Comet tails three fold longer than nucleus diameter), and (4); severely damaged (cell nuclei was almost not visible with long and dispersed Comet tails). At least 300 cells per slide were counted and two slides were prepared for each treatment. A total damage score was determined by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade according to methods described by Heaton et al. (2002). Total DNA damage score was calculated as follows:

$$\text{Total DNA damage} = [(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]$$

Where;

n_0 = cells with score 0, n_1 = cells with score 1, n_2 = cells with score 2, n_3 = cells with score 3, n_4 = cells with score 4.

The mean tail moment which represents the tail length and intensity of DNA was determined using TriTek CometScore™ Software. It is calculated by multiplying the distance between center tail mass and center head mass with percentage of DNA in the tail (Bowden et al., 2003).

Clonogenic assay

The ability of a single cell to grow into a colony was determined as described by Franken et al. (2006) with slight modification. The Gelam honey-treated and non-treated HDFs at passage 6 were harvested and 50 cells were seeded into six-well plates. After 14 h incubation, the cells were exposed to gamma-rays and incubated for 2 weeks to allow the formation of macroscopic colonies. The colonies were stained with a mixture of 6.0% glutaraldehyde (Sigma, USA) and 0.5% crystal violet (Acros, USA) (1:1). Plating efficiency (PE) and surviving fraction (SF) were determined using the following equation:

$$PE = \frac{\text{Number of colonies formed in control}}{\text{Number of cells seeded}}$$

$$SF = \frac{\text{Number of colonies formed after treatment}}{\text{Number of cells seeded X PE/100}}$$

Statistical analysis

Data are expressed as mean $\pm$ S.D in each group. Statistical analysis using SPSS statistical software version 16 and Student's *t*-test were applied to determine the significant differences among the groups. A value of $p<0.05$ was considered significant.

RESULTS

Antioxidant properties of Gelam honey

The free radical-scavenging activity and total antioxidant power in Gelam honey increased significantly with increasing concentrations of the honey ($p<0.05$). Gelam honey showed significant difference in free radical-scavenging activity in a dose dependent manner ($p<0.05$) as indicated by the DPPH assay. However, no significant difference was observed between irradiated and non-irradiated Gelam honey (Figure 1).

Similar results were observed for FRAP assay which showed significant difference in total antioxidant power for Gelam honey in a dose dependent manner ($p<0.05$). Comparison between irradiated and non-irradiated honey showed a significant difference in total antioxidant power at 50, 90 and 100 mg/ml honey ($p<0.05$) (Figure 2).

Cytotoxicity study

The percentage of viable cells decreased significantly with increasing concentrations of Gelam honey treatment as compared to non-treated HDFs ($p<0.05$) (Figure 3). Although there was no significant difference among the concentrations of Gelam honey used in this study, concentration at 6 mg/ml was selected for subsequent experiments as it represents the highest percentage of viable cells.

Screening of HDFs radio sensitivity

The degree of damaged DNA and the survival rate of individual cell in response to different doses of gamma-rays exposure are summarized in Table 1. HDFs exposed to gamma-rays showed a significant increase in total DNA damage in a dose dependent manner ($p<0.05$). Similarly, the mean tail moment was significantly increased with increasing dose of gamma-rays exposure ($p<0.05$).

Clonogenic assay showed the percentage of survival fraction of HDFs decreased significantly with increasing dose of gamma-rays exposure ($p<0.05$). The decrease in the survival percentage of HDFs was visualized as reduction of colonies formed in the plate. A significant difference on the percentage of survival fraction was also observed in HDFs exposed to gamma-rays at 2 Gy as compared to 1 Gy exposure ($p<0.05$). No colony was observed for HDFs exposed to 9 and 10 Gy of gamma-rays.

Effects of Gelam honey on radiation-induced DNA damage

HDFs were treated with 6 mg/ml of Gelam honey for 24 h pre-, during- and post-irradiation with 1 Gy of gamma-rays. Results showed that the level of damaged DNA increased significantly in irradiated HDFs as compared to untreated control ($p<0.05$) (Figure 4). Gelam honey-treated HDFs however showed a significant reduction in the level of damaged DNA compared to untreated control and irradiated HDFs ($p<0.05$). Similar reduction in the level of damaged DNA was observed in HDFs pre-treated with Gelam honey ($p<0.05$) compared to irradiated HDFs but higher as compared to untreated control ($p<0.05$). However, HDFs treated with Gelam honey during- and post-irradiation showed increased level of damaged DNA as compared to untreated control ($p<0.05$).

The effects of Gelam honey on mean tail moment is shown in Table 2. The mean tail moment increased significantly in irradiated HDFs ($p<0.05$). Gelam honey-treated HDFs however showed a significant reduction in mean tail moment as compared to untreated control and irradiated HDFs ($p<0.05$). Similar reduction in mean tail moment was observed in HDFs pre-treated with Gelam honey ($p<0.05$) compared to irradiated HDFs but higher as compared to untreated control ($p<0.05$). HDFs treated with Gelam honey during- and post-irradiation showed increased mean tail moment as compared to untreated control ($p<0.05$).

Effects of Gelam honey on cell survival rate

The survival rate of HDFs when treated with Gelam honey before-, during- and post-exposure to 1 Gy of gamma-rays is shown in Figure 5. A significant reduction in cell survival rate was observed in HDFs exposed to 1 Gy of gamma-rays as compared to control ($p<0.05$). Cell survival rate however, increased significantly in Gelam honey-treated HDFs as compared to untreated control and irradiated HDFs ($p<0.05$). Similar increase in cell survival rate was observed in HDFs pre-treated with Gelam honey ($p<0.05$) compared to irradiated HDFs. HDFs treated with Gelam honey during irradiation also showed increased cell survival as compared to irradiated HDFs ($p<0.05$). Similar protective effect however was not observed when treatment of Gelam honey was given post-irradiation.

DISCUSSION

Honey was reported to have anti microbial properties (Ainul et al., 2005; National Honey Board, 2005). It may also contain yeasts and spores-forming bacteria due to poor handling during harvesting and transportation (Snowdon and Cliver, 1996). In this study, we compared the free radical-scavenging activity and total antioxidant power between sterilized and non-sterilized Gelam honey. The radiation dose used to sterilize Gelam honey in this study was 25 kGy of gamma-rays, the dose accepted for sterility as stated by Medical devices directorate (Lambert, 2004) which did not destroy the anti

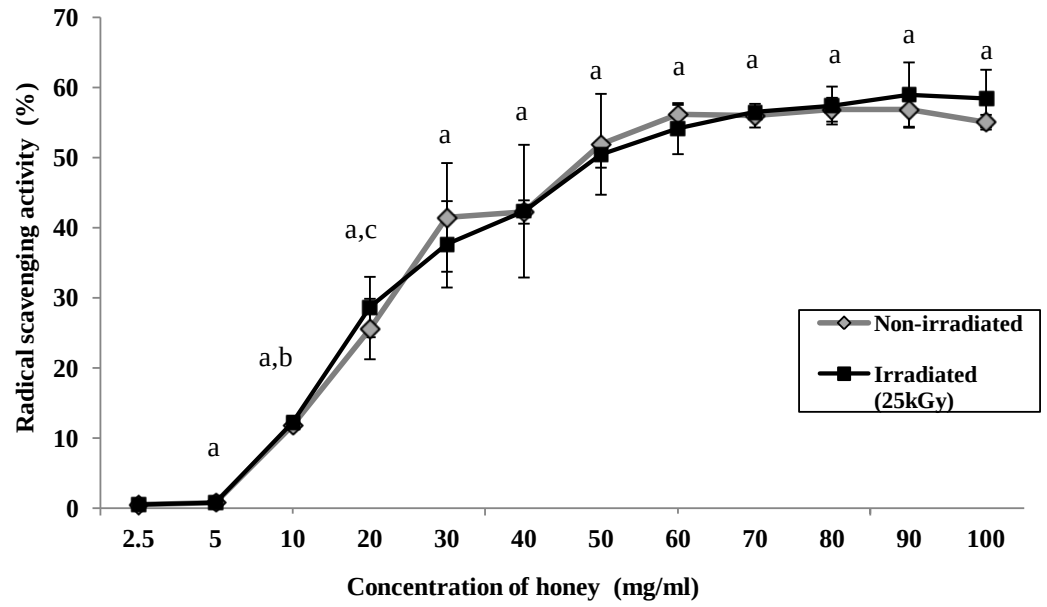

Figure 1. Comparison of free radical scavenging activity between various concentrations of irradiated (25 kGy) and non-irradiated Gelam honey determined by DPPH assay. Results are expressed as mean ± S.D (n = 9). [a]Denotes p<0.05 compared to 2.5 mg/ml honey, [b]p<0.05 compared to 10 mg/ml honey, [c]p<0.05 compared to 20 mg/ml honey.

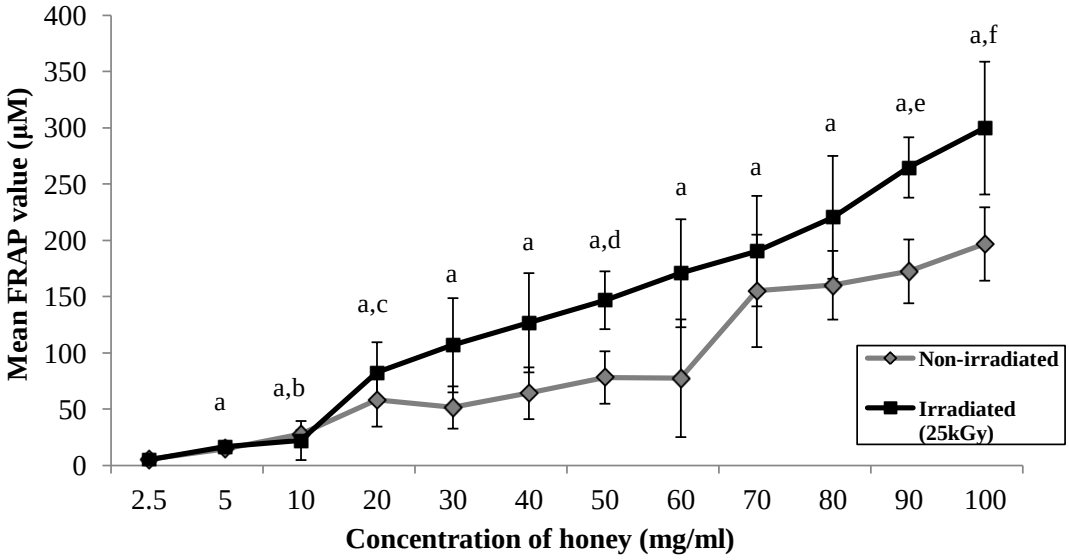

Figure 2. Comparison of total antioxidant power between various concentrations of irradiated (25 kGy) and non-irradiated Gelam honey determined by FRAP assay. Results are expressed as mean ± S.D (n = 9). [a]Denotes p<0.05 compared to 2.5 mg/ml honey, [b]p<0.05 compared to 10 mg/ml honey, [c]p<0.05 compared to 20 mg/ml honey, [d]p<0.05 compared to 50 mg/ml non-irradiated honey, [e]p<0.05 compared to 90 mg/ml non-irradiated honey, [f]p<0.05 compared to 100 mg/ml non-irradiated honey.

bacterial activity in honey (Molan and Allen, 1996). Our results showed no significant difference in free radical-scavenging activity between irradiated and non-irradiated Gelam honey while higher total antioxidant power was observed at 50, 90 and 100 mg/ml of irradiated Gelam honey as compared to non-irradiated honey. Previous studies have shown that Gelam honey contains antioxidant (Aljadi and Yusof, 2004) and irradiation of honey causes enhanced antioxidant activities and flavonoid (Hussein et al., 2011).

The cytotoxicity study of Gelam honey was carried out to determine the minimum concentration that might be

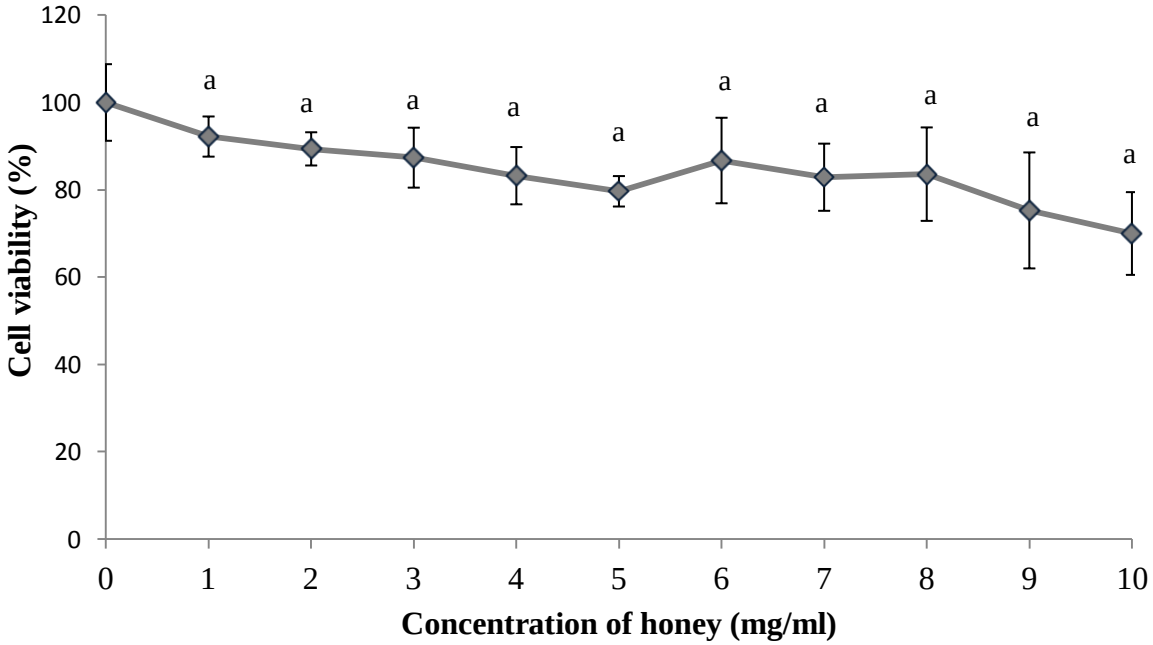

Figure 3. Cells viability of Gelam honey-treated HDFs determined by MTT assay. Cells viability was significantly decreased with Gelam honey treatment compared to untreated control. Results are expressed as mean ± S.D (n = 9). [a]Denotes p<0.05 compared to untreated control.

Table 1. DNA damage in HDFs after gamma-rays exposure was measured in arbitrary unit and mean tail moment. The survival rate of HDFs was measured as percentage of survival fraction.

Radiation dose (Gy)	Comet assay (arbitrary unit)	Comet assay (mean tail moment)	Clonogenic assay (Percentage of survival fraction)
0	116.17±41.2	2.32±0.7	100.0±0.0
1	327.67±36.3[a]	3.01±1.2	69.95±17.3[k]
2	395.83±84.4[a]	5.26±1.3[e,f]	37.22±11.1[k,l]
3	550.83 ±80.8[a]	5.97±1.0[e]	35.38±12.1[k]
4	624.50±74.6[a]	7.34±1.3[e]	27.57±11.2[k]
5	846.33±65.7[a,b]	11.75±4.1[e,g]	19.92±14.4[k]
6	985.83±30.3[a,c]	17.24±2.8[e,h]	6.65±6.6[k]
7	1024.67±15.4[a]	20.21±2.1[e]	5.80±1.0[k]
8	1032.00±10.2[a]	22.00 ±1.0[e]	8.02±8.0[k]
9	1137.33±25.9[a,d]	28.68±3.1[e,i]	0[k]
10	1155.33±30.4[a]	39.23±9.9[e,j]	0[k]

Results are expressed as mean ± SD (n = 6). [a]Denotes p<0.05 compared to 0 Gy, [b]p<0.05 compared to 4 Gy, [c]p<0.05 compared to 5 Gy, [d]p<0.05 compared to 8 Gy, [e]p <0.05 compared to 0 Gy, [f]p <0.05 compared to 1 Gy , [g]p <0.05 compared to 4 Gy, [h]p <0.05 compared to 5 Gy, [i]p <0.05 compared to 8 Gy, [j]p <0.05 compared to 9 Gy, [k]p <0.05 compared to 0 Gy and [l]p<0.05 compared to 1 Gy.

cytotoxic to HDFs to ensure that honey itself does not affect cell growth when used in the study. Our results showed that cell viability decreased as the concentrations of honey increased. Although Gelam honey treatment at concentration of 1 mg/ml maintained cells viability at 92.2%, but its scavenging activity and total antioxidant power were less than 0.46% and 5 µM, respectively. On the other hand, the free radical-scavenging activity and total antioxidant power of Gelam honey at concentration of 10 mg/ml were 12.2% and 22.0 µM, respectively but MTT assay showed cells viability was low (70.0%) when HDFs were treated with the same concentration of Gelam honey. Therefore, for the subsequent experiments, we used 6 mg/ml of Gelam honey as it maintained cells viability at 86.7% with scavenging activity of 0.7% and total antioxidant power of 16.3 µM.

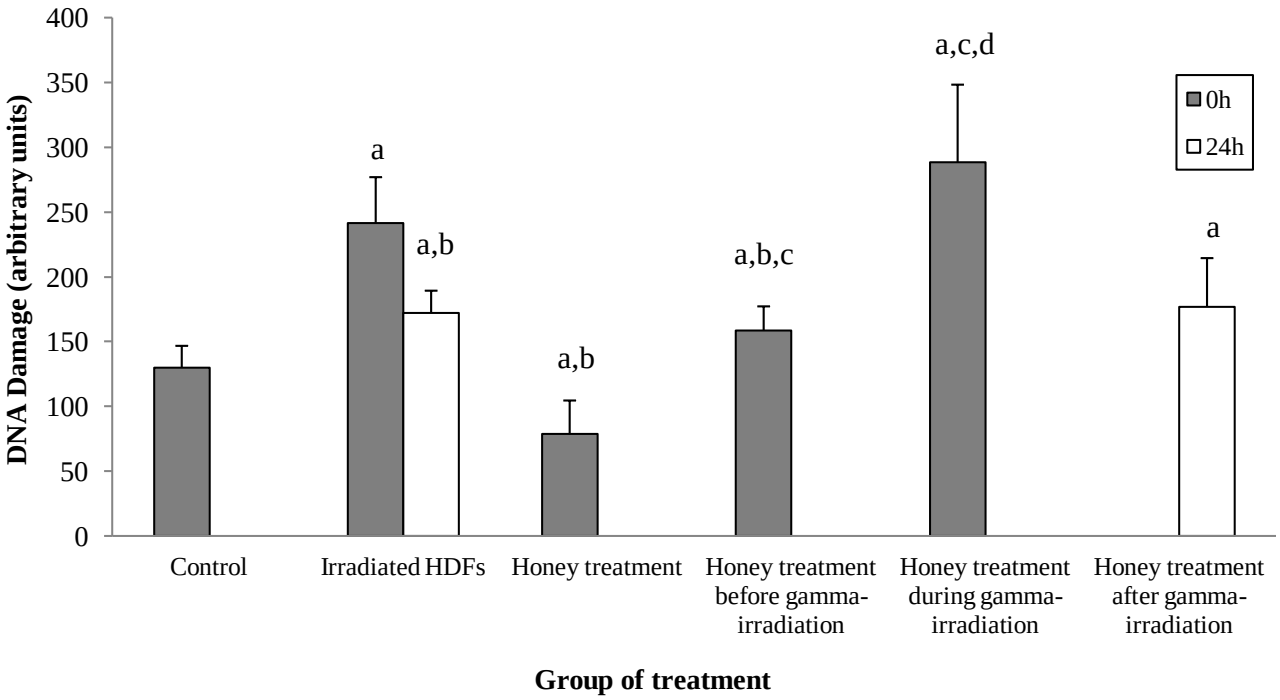

Figure 4. Effects of Gelam honey against irradiation-induced DNA damage. HDFs were treated with 6 mg/ml of Gelam honey for 24 h pre-, during- and post-irradiation with 1 Gy of gamma rays. The level of damaged DNA increased significantly in irradiated HDFs as compared to control. Gelam honey-treated HDFs showed a significant reduction in the level of damaged DNA compared to control and irradiated HDFs. Similar reduction in the level of damaged DNA was observed in HDFs pre-treated with Gelam honey compared to irradiated HDFs but higher as compared to control. HDFs treated with Gelam honey during- and post-irradiation showed increased level of damaged DNA as compared to control. Results are expressed as mean ± S.D (n = 9). [a]Denotes $p<0.05$ compared to untreated control, [b]$p<0.05$ compared to irradiated HDFs, [c]$p<0.05$ compared to honey treatment, [d]$p<0.05$ compared to HDFs pre-treated with honey.

Table 2. Effects of Gelam honey treatment on the degree of damaged DNA.

Group of sample	Comet assay (arbitrary unit)	Comet assay (mean tail moment)
Control	129.67±17.0	0.11±0.14
Irradiated HDFs	241.67±35.3[a]	0.24±0.06[a]
HDFs treated with Gelam honey	78.50±26.0[a,b]	0.07±0.01[a,b]
HDFs treated with Gelam honey before radiation	158.67±18.5[a,b,c]	0.14±0.02[a,b,c]
HDFs treated with Gelam honey continuously during radiation	288.67±59.8[a,c,d]	0.23±0.05[a,c,d]
24 h post-radiationIrradiated HDFs	176.83±37.6[a,b]	0.18±0.02[a,b]
HDFs treated with Gelam honey after radiation	172.00±17.3[a]	0.14±0.03[a]

Results are expressed as mean± S.D (n = 6). [a]Denotes $p<0.05$ compared to control, [b]$p<0.05$ compared to irradiated HDFs, [c]$p<0.05$ compared to Gelam honey-treated HDFs, [d]$p<0.05$ compared to HDFs pre-treated with Gelam honey.

Screening for HDFs radiosensitivity was carried out to determine the optimum dose for gamma-irradiation. Therefore prior to treating the HDFs with Gelam honey, the level of damaged DNA and the percentage of cells survival were determined post exposure to increasing doses of gamma-rays. Comet and Clonogenic assays were performed to determine total DNA damage and cells survival as both assays could detect the direct and indirect effects of gamma-radiation. The alkaline comet assay chosen in this study is a predictive method as it is rapid and sensitive in measuring DNA damage in each individual cell. This assay was able to detect DNA single-strand break, double strand breaks and alkaline labile sites in the DNA (Olive and Banath, 2006). Several researchers have used this method to determine the relationship between radiosensitivity and DNA damage

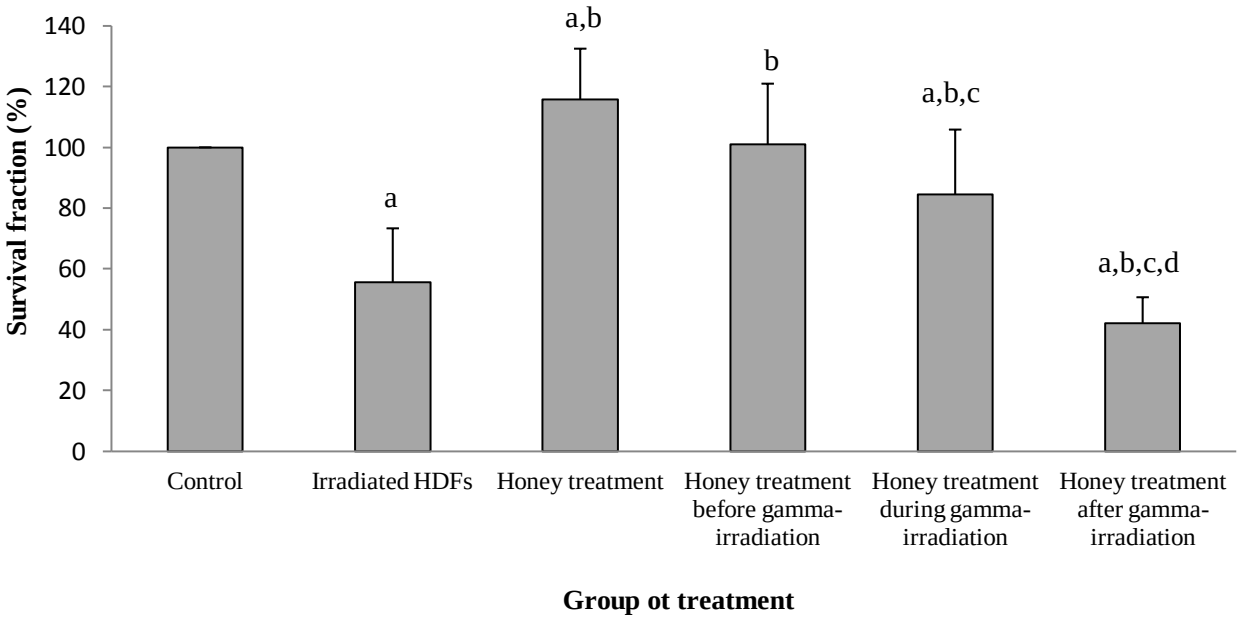

Figure 5. Effects of gamma-irradiation and Gelam honey treatment on survival fraction of HDFs. A significant reduction in cell survival rate was observed in HDFs exposed to 1 Gy of gamma-rays as compared to control. Cell survival rate increased significantly in Gelam honey-treated HDFs as compared to control and irradiated HDFs. Similar increased in cell survival rate was observed in HDFs pre-treated with Gelam honey compared to irradiated HDFs. HDFs treated with Gelam honey during irradiation also showed increased cell survival as compared to irradiated HDFs. Results are expressed as mean ± S.D (n = 9). [a]Denotes $p<0.05$ compared to untreated control, [b]$p<0.05$ compared to irradiated HDFs, [c]$p<0.05$ compared to honey treatment, [d]$p<0.05$ compared to HDFs pre-treated with honey.

(Dunne et al., 2003; McKeown et al., 2003). Our results showed there were increased in damaged DNA and mean tail moment in a dose dependent manner with gamma-rays exposure. The severity of the damaged DNA was increased as the radiation dose was increased. Clonogenic assay has been used in decades to detect cell reproductive death after exposure to ionizing radiation or treatment with cytotoxic agents (Franken et al., 2006). This *in vitro* cell survival assay was based on the ability of a single cell to grow into a colony after cells were incubated for 2 weeks. The colonies of HDFs formed after 14 days of incubation decreased in a dose dependent manner with gamma-rays irradiation. Similar results were reported when primary culture of normal human fibroblasts from dermis (NHF-d) was exposed to gamma-radiation using ^{137}Cs source. Laurent et al. (2005) showed that the mean tail moment and cell survival percentage of NHF-d decreased when gamma-radiation dose was increased.

Based on the results from Comet and Clonogenic assays, we decided to use 1 Gy of gamma-radiation dose for the subsequent experiments. Therefore, in the subsequent experiments, HDFs were treated with 6 mg/ml of Gelam honey pre-, during- and post-exposure to 1 Gy of gamma-rays. Our results showed that pre-treatment with Gelam honey produced a significant protective effect against irradiation-induced DNA damage in HDFs.

Ionizing radiation generates reactive oxygen species such as hydrogen peroxide that can induce a wide range of molecular lesions in cells. Previous study showed antioxidant nutrients and phytochemicals may protect against ionizing radiation (Weiss and Landauer, 2003). Honey may be able to protect against oxidative stress-induced DNA damage as it contains catalase and non-enzymatic compounds such as flavonoids (Blasa et al., 2007). The reduction of hydrogen peroxide to water and oxygen by catalase may protect against DNA damage (Bansal et al., 2005). Similar protective effect however was not observed when HDFs were treated with Gelam honey during gamma-irradiation process or post-exposure to gamma-rays.

The percentage of cells survival was increased in HDFs pre-treated with Gelam honey and HDFs treated with Gelam honey during gamma-irradiation while post-treatment did not produce similar protective effect. This finding indicated that Gelam honey protects against the direct effect of gamma-radiation.

The best time to take honey supplement for protection against radiation-induced DNA damage has never been studied before. According to Lanphier (2011), the atomic energy commission recommended to take two tablespoons a day of sodium alginate supplements to protect against radiation. However, the dosage should be

increased to two full tablespoons of sodium alginate four times daily to ensure that there is a continual supply in the gastrointestinal tract during or after exposure to radiation. This report indicated that the requirement for antioxidant supplement varies at different time of radiation treatment. In practice, radiation workers and patients that undergo radiotherapy treatment are encouraged to take antioxidant pill within 30 to 60 min before exposure to radiation (Prasad, 2005). Scientific data to support the needs to take antioxidant supplement before or during exposure to radiation is lacking. Therefore, further research is needed to study the molecular mechanism of antioxidant supplement in preventing radiation damage in cells.

Conclusion

Gamma-radiation induced DNA damage and decreased cells survival rate of HDFs. Pre-treatment with Gelam honey protects against radiation-induced DNA damage and increased cells survival rate. Therefore, Gelam honey may be able to act as a radioprotectant agent against ionizing radiation in HDFs.

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