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Morphological and Biochemical Responses of *Eurycoma longifolia* Callus to Gamma Irradiation

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ABSTRACT

Eurycoma longifolia is commonly used as medicinal plant in South East Asia, particularly for its aphrodisiac property. In this study, the mutation of *Eurycoma longifolia* callus cultures was induced by gamma irradiation at 20, 40, 60, 80 and 100 Gray (Gy). After three weeks of irradiation, the morphological and biochemical changes of the callus were examined. The radiation sensitivity test determined that 60 Gy was the optimum dose to cause a 50% of decline in the fresh weight (FW) of *E. longifolia* callus. Even though there was no significant difference on the colour and texture of irradiated and non-irradiated calli, the FW of the calli decreased gradually with the increase of gamma doses. The total soluble protein content recorded 17, 57 and 30% higher than the non-irradiated calli in 20, 40 and 60 Gy, respectively. A further increase of gamma dose to 80 and 100 Gy caused a decrease of 64% in total soluble protein content. Contrarily, gamma irradiation generally brought about a decrease in total phenolic and total flavonoids content. The lowest total phenolic content (0.05 ± 0.01 mg gallic acid equivalents/g FW) was recorded in calli exposed to 40 Gy while the lowest total flavonoid content (0.13 ± 0.01 mg catechin equivalents equivalents/g FW) was obtained in calli irradiated at 60 Gy.

Keywords: callus culture, *Eurycoma longifolia*, gamma irradiation, *in vitro* mutagenesis, total flavonoids content, total phenolic content
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **MS**, Murashige and Skoog

INTRODUCTION

Eurycoma longifolia, from the Simaroubaceae family, is one of the commonly used medicinal plants in South East Asia such as Myanmar, Thailand, Laos, Cambodia, Indonesia and Malaysia. Traditionally, it was used to treat fever, diarrhea, malaria, sexual insufficiency and dysentery (Kuo *et al.* 2004; Bhat and Karim 2011). Besides, it has been proven to have aphrodisiac activity, cytotoxic, anti-malarial, anti-tumor promoting and anti-parasitic properties (Hussein *et al.* 2005a; Chua *et al*. 2011). Nowadays, the increment in harvesting wild-grown *E. longifolia* for medicinal usage has led to rapid thinning of natural populations (Osman *et al*. 2003). In addition, the low yield of the active compound of *E. longifolia* plant worsens the problem of insufficient plant supply (Mohtar *et al*. 2007).

In vitro mutagenesis can be defined as the induction of mutation in cell cultures by the use of chemical or physical mutagens. Gamma irradiation is a type of physical induced mutagen which was commonly used for *in vitro* mutagenesis. Introduction of ionizing radiation on cell culture can induce wide range of molecular damage included cell inactivation, chromosomal rearrangement, mutation (Jagetia and Venkatesha 2006) and DNA damage (Nishiguchi *et al*. 2012). The gamma ray used in this process produces free radical when it interacts with the molecule in cell. These free radicals will damage the components of plant cells, and have been reported to affect the anatomy, biochemistry, and physiology of the plant (Wi *et al.* 2007). *In vitro* mutagenesis can result in a range of genetically stable variation, which was useful in crop improvement by using gamma irradiation (Jain 2000). In fact, it has contributed to genetic improvement in several plants such as *Ananas comosus* (El-Sayed *et al.* 2007) and *Hyoscyamus niger* (Sharma *et al.* 1989). The *in vitro* mutagenesis can change either one or few specific traits of cultivar, and is able to contribute to the fruit improvement without upsetting the requirement of the fruit industry or the consumers (Predieri 2001). Even though Khan *et al*. (2005) and El-Beltagi *et al*. (2011) reported that mutagenesis was able to increase the production of secondary metabolites in plants, there are still not many reports studying the effects of gamma irradiation on medicinal plants, especially *E. longifolia*. To date, the approaches applied to improve *E. longifolia* are still at a very preliminary stage, mainly focused on plant cell and tissue culture techniques such as callus cultures (Maziah and Rosli 2009) and somatic embryos (Hussein *et al*. 2005b). Recently, a preliminary study on protoplast isolation targeted for crop improvement by somatic hybridization was reported (Ling *et al*. 2010b). In view of that, this study was conducted in order to determine the morphological and physiological responses of *E. longifolia* callus after exposed to gamma irradiation at different doses.

MATERIALS AND METHODS

Callus cultures

In this study, the callus cultures were induced and maintained as described by Hussein *et al*. (2005). Briefly, approximately 0.2 g of two-week old leaf-derived callus of *E. longifolia* was cultured in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose (Sigma Aldrich, St. Louis, USA), 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 0.8% (w/v) agar (Copens, Kuala Lumpur, Malaysia).

Gamma irradiation

The gamma irradiation was performed at Malaysia Nuclear Agency. Calli were exposed to gamma ray treatment by using

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Cobalt-60 source at the dose rate of 4.042 kGy/hr. Different gamma dose (20, 40, 60, 80, and 100 Gy) were used to irradiate the callus. After gamma irradiation, the callus was transferred into the fresh MS medium containing 1 mg/L 2.4-D and maintained in the culture room at 25 ± 2°C, light intensity at 15 µmol m^{-2} s^{-1} and a 16-h photoperiod.

Radiation sensitivity test

After three weeks of gamma irradiation, radiation sensitivity test was conducted on the callus of *E. longifolia* based on the fresh weight of the callus. It was used to determine the gamma dose which could allow 50% decrement in fresh weight (FW). Apart from that, the effect of gamma irradiation towards the morphological responses (colour, survival percentage and texture of callus) on callus was also recorded.

Biochemical responses

1. Sample extraction for total soluble protein

The irradiated and non-irradiated calli were ground with mortar and pestle, and extracted with protein extraction buffer at the ratio of 3 mL of protein extraction buffer to 1 g of the sample. The extract was then centrifuged at 10,000 rpm, 4°C for 30 min. The supernatant was collected and used to determine the total soluble protein content and specific activity of peroxidase.

2. Determination of total soluble protein content

The total soluble protein of the irradiated and non-irradiated callus was determined using the Bradford method (Bradford 1976). A total of 20 µL of the sample extract, 80 µL of the protein extraction buffer and 5mL of the protein reagent were mixed. As for blank, 20 µL of the sample extract was substituted with distilled water. The absorbance was then measured at 595 nm using UV-spectrophotometer (Spectronic Genesys 20, Madison, USA). A standard calibration curve was constructed using bovine serum albumin (BSA) (Sigma Aldrich, USA) as standard at 0, 20, 40, 60, 80 and 100 µg/mL. The absorbance reading of the samples was then compared to the standard calibration curve and expressed in milligram per gram FW (mg/g FW) of plant material.

3. Sample extraction for total phenolic and total flavonoids content

The irradiated and non-irradiated calli were ground with 80% (v/v) methanol at the ratio of 1 g of sample to 10 mL of methanol. The extracts were then ultra-sonicated for 20 min. After that, 2 mL of the extract was centrifuged for 15 min at 10,000 rpm. After centrifugation, aliquot was used for the determination of total phenolic content and total flavonoids content.

4. Determination of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu assay. In this method, 1 mL of the supernatant and 1 mL of Folin-Ciocalteu's reagent (R&M Chemicals, Essex, UK) was added into 9 mL of deionised water and the solution was shaken. After 5 min, 10 mL of 7% (w/v) sodium carbonate (Sigma, USA) was added into the solution. The solution was further diluted with 25 mL of deionised water. After incubated for 1 hr, the absorbance reading was measured at 750 nm. The blank was prepared by replacing the supernatant in the solution above with deionised water. The calibration curve was constructed using gallic acid (Sigma-Aldrich) as standard at the concentrations of 0.000, 0.002, 0.004, 0.006, 0.008 and 0.010 mg/mL. The absorbance reading of the sample was compared to the standard calibration curve and the total phenolic content was expressed in milligram gallic acid equivalent per gram FW of plant material (mg GAE/g FW).

5. Determination of total flavonoids content

The aluminium chloride colorimetric assay was used to determine the total flavonoids content. In this assay, 1 mL of the supernatant, 4 mL of deionised water and 0.3 mL of 5% (w/v) sodium nitrate (Fluka, Munich, Germany) were mixed. After 5 min, 0.3 mL of 10% (w/v) aluminium trichloride (Sigma Aldrich) was added into the solution. At the 6^{th} minute, 2 mL of 1 M sodium hydroxide (R&M Chemicals) was added and the solution was diluted to 10 mL with deionised water. The absorbance reading was recorded at 510 nm. The blank was prepared by replacing the supernatant in the solution above with deionised water. A standard curve was constructed to measure the amount of total flavonoids content by using catechin (Sigma-Aldrich) as standard at 0.000, 0.002, 0.004, 0.006, 0.008 and 0.010 mg/mL. The absorbance reading of the samples were compared to the standard calibration curve and it was further expressed in milligram catechin equivalent per gram FW of plant material (mg CE/g FW).

Statistical analysis

In this study, triplicates were used for each treatment and the experiment was repeated twice. One-way ANOVA and Tukey's Honestly Significant Difference (HSD) test at $P < 0.05$ were used to identify the differences in the mean of the parameter of morpho-logical and physiological changes for callus sample via SPSS software (version 11.5) (SPSS Inc. USA).

RESULTS AND DISCUSSION

Radiation sensitivity test

In the present study, the yellowish callus of *E. longifolia* remained the same colour after gamma irradiation (**Fig. 1**). These findings were in accordance to the studies on *Orthosiphon stamineus* callus (Ling *et al.* 2010a). In contrast, green tea, in which it presented significant changes and improvement in colour at 20 kGy (Kim *et al*. 2006). The radiation sensitivity test of *E. longifolia* callus was conducted by comparing the increment of FW between gamma irradiated callus and non-irradiated callus after three weeks of gamma irradiation treatment. From **Fig. 2**, the callus FW decreased gradually with the increment of gamma irradiation dose. This might be attributed by reduced amount of endogenous growth regulators, especially the cytokines, as a result of break down and lack of synthesis, due to irradiation (Omar *et al*. 1993). Meanwhile, Wi *et al.* (2007) stated that the growth inhibition that induced by the high dose irradiation could have been attributed to the cell cycle arrest at G2/M phase during somatic cell division and various damages in the entire genome. Besides, it was determined

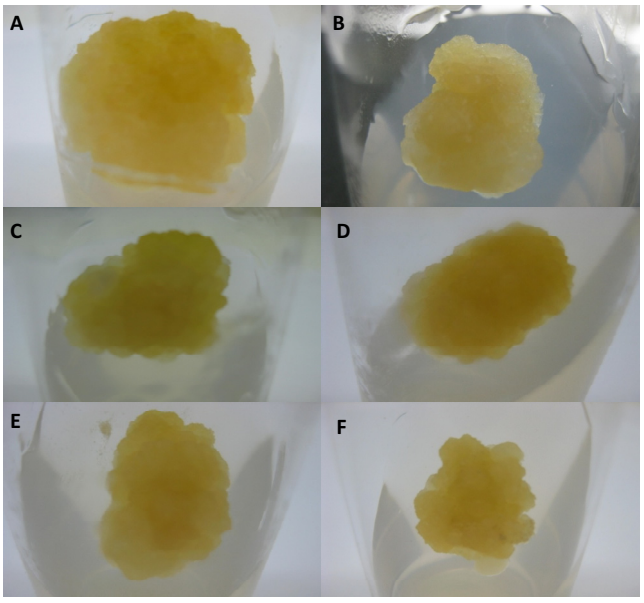

Fig. 1 The effects of gamma irradiation on the morphological changes of *E. longifolia* callus after three weeks of irradiation. (**A**) 0 Gy, (**B**) 20 Gy, (**C**) 40 Gy, (**D**) 60 Gy, (**E**) 80 Gy, (**F**) 100 Gy.

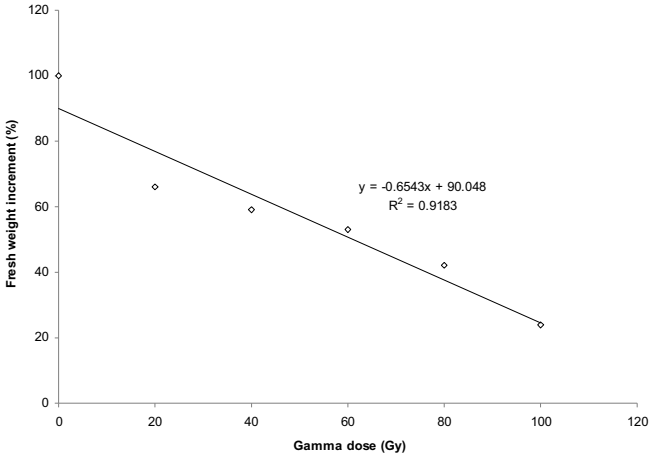

Fig. 2 Radiation sensitivity test of *E. longifolia* callus based on fresh weight increment after three weeks of gamma irradiation.

that at 60 Gy, a 50% of decrease in the FW of the *E. longifolia* callus was observed. These results were in accordance with the radiation sensitivity test done on *O. stamineus* that recorded the LD_{50} of 72.5 Gy (Ling *et al.* 2008a). The effect of gamma irradiation could be highly species dependent as studies on *Citrus sinesis* reported the LD_{50} of 27 Gy (Ling *et al.* 2008b).

Determination of total soluble protein content

The total soluble protein content in *E. longifolia* callus varied depending on the irradiation doses applied. Generally, most of the gamma irradiated calli showed the increment in total soluble protein content as reported by El-Beltagi *et al*. (2011) on *Rosmarinus officinalis* L.

Results showed that the callus irradiated at 40 Gy gave the highest value of total soluble protein, a value about 57% of increment as compared to that detected in the non-irradiated callus (**Fig. 3**). Contrarily, in *O. stamineus* callus, protein content was generally lower as compared to non-irradiatied control after treatment with 10 to 50 Gy (Ling *et al*. 2010a). Zbikowska *et al*. (2006) stated that the gamma irradiation may be able to rupture the covalent bonds in target protein molecules. The damages included loss of nitrogenous base, hydrogen bond breakage between chains, single-strand breakage, double-strand breakage in base cross-linking within the double helix, cross-linking to other DNA molecules and cross-linking to protein molecules (Maity *et al*. 2008). The cross-linking of the chain influences the tertiary structure of protein and their physiochemical properties. Furthermore, the proteolytic enzyme would break down and recycle the abnormal, misfolded protein (Hameed *et al*. 2008). Therefore, the total protein present in *E. longifolia* callus might be degraded after the gamma irradiation by the disruption of covalent bond.

Apart from that, the solubility of the protein could be another factor that contributed to low total soluble protein in callus that irradiated at 80 and 100 Gy. The unfolding and denaturation of protein after gamma irradiation could lead to increase surface hydrophobicity of the protein by exposing nonpolar groups (Shawrang *et al*. 2008). The hydrophobic interaction leads to aggregation, followed by coagulation and precipitation. This may increase its degradability (Shawrang *et al*. 2008). Thus, the water binding activity (solubility) may be decreased after gamma irradiation and it may reduce the total soluble protein content.

Determination of total phenolic content

Generally, the gamma irradiation treatment decreased the total phenolic content in the callus of *E. longifolia*. Among all the irradiated calli, the callus irradiated at 20 Gy possessed the highest total phenolic content. It showed 0.07 ± 0.01 mg GAE/g FW or 28.7% decrement as compared to the non-irradiated callus (**Fig. 4**). Meanwhile, the callus that irradiated with 80 and 100 Gy also produced similar amount of total phenolic content, in which both displayed 0.07 ± 0.01 mg GAE/g FW. Furthermore, a significant difference was observed between the non-irradiated callus and the callus that irradiated at 40 and 60 Gy through the Tukey's HSD ($P > 0.05$). The lowest total phenolic content was recorded on the callus that irradiated at 40 Gy. It showed 50% of decrement as compared to the non-irradiated callus.

Some of the studies showed similar observation, in which the total phenolic content was decreased after gamma irradiation. In a study by Toledoa *et al*. (2007), a significant decline in total phenolic content was recorded in soya bean (*Glycine max*) seeds at 2 and 4 kGy. Furthermore, Khattak *et al*. (2008) reported a drop in the amount of total phenolic compounds in dehydrated rosemary after irradiation at 10 and 30 kGy. The decrease of total phenolic content in *E. longifolia* callus after gamma irradiation might be due to unreleased phenolic compounds from glycosidic components (Lee *et al*. 2009). Low phenolic content after irradiation could also indicate that the gamma doses applied were unable to degrade the larger phenolic compounds into smaller ones (Harrison and Were 2007).

During irradiation, the water would most possibly be degraded and form reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radicals (Wi *et al*. 2007). The hydroxyl radical would be able to oxidize the phenolic compound that composed of aromatic ring and bearing one or more hydroxyl substituent (Muchuweti *et al*. 2007). The intermediated radical is further oxidized in the presence of oxygen and carbonyl groups are then formed (Schindler *et al*. 2005). Hydroxycyclohexadienyl radicals would be formed as a result of further reaction of the hydrogen radical and the benzene ring system. Some

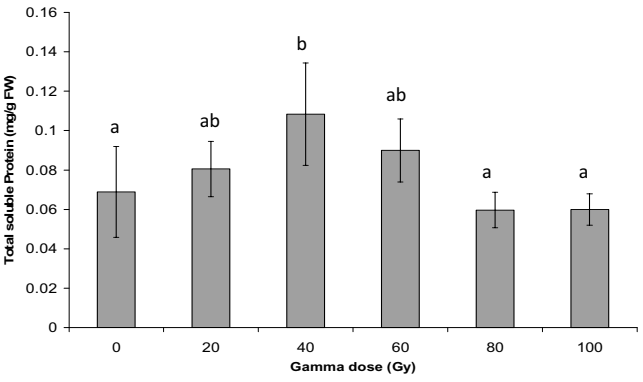

Fig. 3 The effects of gamma irradiation on the total soluble protein of *E. longifolia* callus after three weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($P < 0.05$). Error bars indicate the mean ± standard deviation (n = 3).

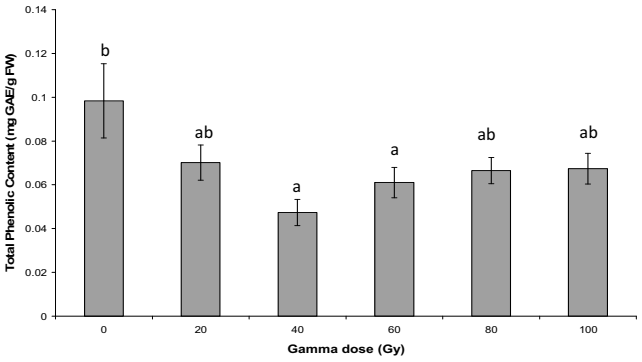

Fig. 4 The effects of gamma irradiation on the total phenolic content of *E. longifolia* callus after three weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($P < 0.05$). Error bars indicate the mean ± standard deviation (n = 3).

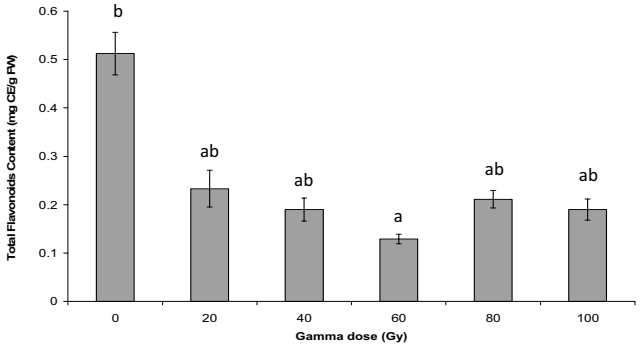

Fig. 5 The effects of gamma irradiation on the total flavonoids content of *E. longifolia* callus after three weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($P < 0.05$). Error bars indicate the mean ± standard deviation (n = 3).

part of the aromatic ring is hydroxylated by the addition of oxygen to form OH-adducts. With these, the structure of the phenolic compound could be disrupted and degraded easily under gamma irradiation (Schindler *et al*. 2005).

Determination of total flavonoids content

The gamma irradiation decreased the total flavonoids content in the entire irradiated callus, particularly the callus that irradiated at 60 Gy, which displayed the most decrement (64%) (**Fig. 5**). A significant difference was observed between the callus that irradiated at 60 Gy and non-irradiated callus through Tukey's HSD ($P > 0.05$). Meanwhile, non-irradiated callus displayed the highest total flavonoids content, which was 0.51 ± 0.04 mg CE/g FW. Apart from that, the gamma irradiation also reduced the total flavonoids content in callus that irradiated with 20, 40, 80 and 100 Gy.

Most of the study regarding the effects of gamma irradiation on total flavonoids content indicated a contradict results as compared to the present study. Hussain *et al*. (2008) reported that the content of one of the flavonoid compounds, anthocyanin was significant higher than the non-irradiated peaches after irradiation treatment at 1.1 kGy. Similarly, total flavonoid content was significantly increased in *Centell asiatica* after exposure to acute gamma irradiation (Moghaddam *et al*. 2011). Nevertheless, gamma irradiation treatment (0.8 kGy) on diced onions increased the total quercetin content, another type of flavonoid compound (Patil *et al*. 1999). Even though there was no detailed study that could explain the decreasing pattern of total flavonoids content in plant sample subjected to gamma irradiation, it could be postulated that the different plant type and tissue as well as environment condition might affect the response of plant towards the gamma irradiation.

CONCLUSIONS

It has been widely reported by various research that gamma irradiation induces the antioxidant activity such as superoxide dismutases and catalases. Thus, diphenylpicrylhydrazyl (DPPH) test is strongly recommended to investigate the antioxidant activity in irradiated callus. Moreover, the gamma irradiation treatment could cause some changes in biochemical content especially the proteins, which will influence the metabolism of the plant indirectly. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. Therefore, it is suggested that protein profiling studies using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) should be carried out in order to determine the biochemical marker for mutagenesis. Furthermore, mutation in gene may produce a certain type of trait in plant. Therefore, it is vital to study the mutated genes which may play an important role in the recombinant DNA technology. In order to study the gene expression, agarose gel electrophoresis is strongly recommended to identify the gene pattern in the irradiated callus of *E. longifolia*.

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REFERENCES

Bhat R, Karim AA (2010) Tongkat ali (*Eurycoma longifolia* Jack): A review on its ethnobotany and pharmacological importance. *Fitoterapia* **81**, 669-679

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254

Chua LS, Mohd Amin NA, Neo JCH, Lee TH, Lee CT, Sarmidi MR, Abdul Aziz R (2011) LC–MS/MS-based metabolites of *Eurycoma longifolia* (Tongkat Ali) in Malaysia (Perak and Pahang). *Journal of Chromatography B* **879**, 2909-3919

El-Beltagi SH, Ahmed O, El-Desouky W (2011) Effect of low doses γ-irradiation on oxidative stress and secondary metabolites production of rosemary (*Rosmarinus officinalis* L.) callus culture. *Radiation Physics and Chemistry* **80**, 968-976

El-Sayed OEE, Rizkalla AA, Sabri SRS (2007) *In vitro* mutagenesis for genetic improvement of salinity tolerance in wheat. *Research Journal of Agriculture and Biological Sciences* **4**, 337-383

Hameed A, Shah TM, Atta BM, Haq MA, Sayed H (2008) Gamma irradiation effects on seed germination and growth, protein content, peroxidase and protease activity, lipid peroxidation, in desi and kabuli chickpea. *Pakistan Journal of Botany* **40**, 1033-1041

Harrison K, Were LM (2007) Effect of gamma irradiation on total phenolic content yield and antioxidant capacity of almond skin extracts. *Food Chemistry* **102**, 932-937

Hussain PR, Meena RS, Dar MA, Wani AM (2008) Studies on enhancing the keeping quality of peach (*Prunus persica* Bausch) cv. Elberta by gamma-irradiation. *Radiation Physics and Chemistry* **77**, 473-481

Hussein S, Ibrahim R, Ling APK, Fadzillah NM, Daud SK (2005a) Multiple shoots formation of an important tropical medicinal plant. *Eurycoma longifolia* Jack. *Plant Biotechnology* **22**, 349-351

Hussein S, Ibrahim R, Ling APK, Fadzillah NM, Daud SK (2005b) Micropropagation of *Eurycoma longifolia* Jack via formation of somatic embryogenesis. *Asian Journal of Plant Sciences* **4**, 472-485

Jagetia GC, Venkatesha VA (2006) Mangiferin protects human peripheral blood lymphocytes against γ-radiation–induced DNA strand breaks: A fluorescence analysis of DNA unwinding assay. *Nutritional Research* **26**, 303-311

Jain M (2000) Tissue culture-derived variation in crop improvement. *Euphytica* **118**, 153-166

Khan MTH, Ather A, Gambari R (2005) The role of metabolic engineering for the production of secondary metabolites of plants. *Minerva Biotecnologica* **17**, 127-131

Khattak KF, Simpson TJ, Ihasnullah (2008) Effect of gamma irradiation on the microbial load, nutrient composition and free radical scavenging activity of *Nelumbo nucifera* rhizome. *Radiation Physics and Chemistry* **77**, 473-481

Kim JK, Jo C, Hwang HJ, Park HJ, Kim YJ, Byun MW (2006) Colour improvement by irradiation of *Curcuma aromatica* extract for industrial application. *Radiation Physics and Chemistry* **75**, 449-452

Kuo PC, Damu AG, Lee KH, Wu TS (2004) Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. *Bioorganic and Medicinal Chemistry* **12**, 537-544

Lee JW, Kim JK, Srinivasan P, Choi J, Kim JH, Han SB, Kim D, Byun MW (2009) Effect of gamma irradiation on microbial analysis, antioxidant activity, sugar content and colorof ready-to-use tamarind juice during storage. *LWT - Food Science and Technology* **42**, 101-105

Ling APK, Ong JXC, Hussein S, Harun AR (2010a) Morphological and physiological responses of *Orthosipohom stamineus* callus to gamma irradiation at different doses. *World Journal of Agricultural Sciences* **6**, 58-66

Ling APK, Phua GAT, Tee CS, Hussein S (2010b) Optimization of protoplast isolation protocols from callus of *Eurycoma longifolia*. *Journal of Medicinal Plants Research* **4,** 1778-1785

Ling APK, Chia JY, Hussein S, Harun AR (2008b) Physiological responses of *Citrus sinensis* to gamma irradiation. *World Applied Sciences Journal* **5**, 12-19

Ling APK, Lai AG, Hussein S, Harun AR (2008a) Physiological responses of *Orthosiphon stamineus* plantlets to gamma irradiation. *American-Eurasian Journal of Sustainable Agriculture* **2**, 135-149

Maziah M, Noormi R (2009) The production of 9-methoxycanthin-6-one from callus cultures of (*Eurycoma longifolia* Jack) Tongkat Ali. *Methods in Molecular Biology* **547**, 359-369

Moghaddam SS, Jaafar H, Ibrahim R, Rahmat A, Abdul Aziz M, Philip E (2011) Effects of acute gamma irradiation on physiological traits and flavonoid accumulation of *Centella asiatica*. *Molecules* **16**, 4994-5007

Mohtar MA, Kumaresan S, Sarmidi MR, Aziz RA (2007) Modelling and

optimization of *Eurycoma longifolia* extraction utilizing a recirculating flow extractor. *Journal of Applied Science* **7**, 2168-2173

Muchuweti M, Kativu B, Mupure CH, Chidewe C, Ndhlala AR, Benhura MAN (2007) Phenolic composition and antioxidant properties of some spices. *American Journal of Food Technology* **2**, 414-420

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-479

Nishiguchi M, Nanjo T, Yoshida K (2012) The effects of gamma irradiation on growth and expression of genes encoding DNA repair-related proteins in Lombardy poplar (*Populus nigra* var. italica). *Journal of Environmental Radioactivity* **109**, 19-28

Omar MS, Yousif DP, Al-Jibouri AJ, Al-Rawi MS, Hameed MK (1993) Effect of gamma rays and sodium chloride on growth and cellular constituents of sunflower (*Helianthus annus* L.) callus culture. *Journal of the Islamic Academy of Sciences* **6**, 69-72

Osman A, Jordan B, Lessard PA, Muhammad N, Haron MR, Riffin NM, Sinskey AJ, Rha CK, Housman DE (2003) Genetic diversity of *Eurycoma longifolia* inferred from single nucleotide polymorphisms. *Plant Physiology* **131**, 1294-1301

Patil BS, Pike LM, Howard LR (1999) Effect of gamma irradiation on quercetin on onion (*Allium cepa* L.) cultivars. *Subtropical Plant Science* **51**, 16-22

Predieri S (2001) Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture* **64**, 185-200

Schindler M, Solar S, Sontag G (2005) Phenolic compounds in tomatoes-natural variations and effect of gamma-irradiation. *European Food Research Technology* **221**, 439-445

Sharma JR, Lal RK, Misra HO, Gupta MM, Ram RS (1989) Potential of gamma radiation enhancing the biosynthesis of tropane alkaloids in black henbane (*Hyoscyamus niger* L.). *Euphytica* **40**, 253-258

Shawrang P, Nikkhah A, Zare-Shahneh A, Sadeghi AA, Raisali G, Moradi-Shahrebabak M (2008) Effects of gamma irradiation on chemical composition and ruminal protein degradation of canola meal. *Radiation Physics and Chemistry* **77**, 918-922

Toledo TCF, Canniatti-Brazaca SG, Arthur V, Piedade SMS (2007) Effects of gamma radiation on total phenolics, trypsin and tannin inhibitors in soybean grains. *Radiation Physics and Chemistry* **76**, 1653-1656

Wi SG, Chung BY, Kim JS, Kim JH, Baek MH, Lee JW, Kim YS (2007) Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron* **38**, 553-564

Zbikowska HM, Nowak P, Wachowicz B (2006) Protein modification caused by a high dose of gamma irradiation in cryo-sterilized plasma: Protective effects of ascorbate. *Free Radical Biology Medicine* **40**, 536-542