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/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 (Jaegita 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
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 5 mL 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 mortar and pestle, and extracted with water. The calibration curve was constructed using gallic acid (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 was 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).

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 10 mL of the solution. At the 6th 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 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 callus sample via SPSS software (version 11.5) (SPSS Inc. USA).

**Results and discussion**

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 morphological and physiological changes for callus sample via SPSS software (version 11.5) (SPSS Inc. USA).

**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...
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 sinensis 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-irradiated 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 physicochemical 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 an 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 decrease 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 50 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

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

**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 ($n = 3$).
Interaction with gamma irradiation on 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).

**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 result 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 expression, agarose gel electrophoresis is strongly recommended to identify the gene pattern in the irradiated callus of *E. longifolia*.

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**REFERENCES**


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


Mohtar MA, Kumaraesn S, Sarmidi MR, Aziz RA (2007) Modelling and
Effects of gamma irradiation on *E. longifolia* callus. Hussein et al.

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


