Effect of UV Elicitation on Callus Growth, Alkaloid and Terpenoid Contents in *Eurycoma longifolia* Jack

Riksa Parikrama, and Rizkita Rachmi Esyanti

***Abstract*—** Elicitation is a technique to increase secondary metabolites content from plant callus culture. This study was carried out to evaluate *Eurycoma longifolia* callus culture growth and to analyse its secondary metabolites production after elicitation. *E. longifolia* callus culture was grown in Murashige-Skoog (MS) medium added with 2,4-dichlorophenoxyacetic acid (2,4-D) and MS medium with naphthalene acetic acid (NAA). Culture was exposed under UV light 18 hours per day for 28 days as an elicitation method. The results showed that growth of elicitated culture was not significantly lower and the structure was drier and more compact. GCMS analysis showed that *E. longifolia* callus culture in MS medium with NAA and elicited by UV light produced canthin-6-one alkaloid 3.5 times more and pyrrolidine 1.5 times more compared to unelicited culture. This canthin alkaloid was not detected on callus cultured in MS with 2,4-D. Other secondary metabolites produced from elicited callus culture were squalene and 5-(hydroxymethyl)-2-furancarboxaldehyde. Protein profile analysis was performed to confirm which protein involved in those secondary metabolites biosynthesis and affected by elicitation. The study also showed there were proteins with molecular weight ± 37.5 kDa, ± 40 kDa, ± 50 kDa, ± 51 kDa, as putative FPP synthase enzyme, strictosidine synthase, GPP synthase, and ornithine decarboxylase respectively. It can be concluded that UV elicitation is a useful technique to enhance secondary metabolites production from *E. longifolia* callus culture, by generating protein involved in alkaloids and terpenoids synthesis pathway.

***Keywords*—** abiotic elicitation, callus culture, *Eurycoma longifolia*, secondary metabolites, UV.

I. Introduction

EURYCOMA longifolia Jack, known as pasak bumi or tongkat ali, is a widely distributed plant in Indochina region, Southeast Asia, including Malaysia, Thailand, and Indonesia [1]. The extract of E. longifolia can be used as aphrodisiac agent [2], antipyretic [3], antimalaria [4], and anticancer [5]. Medicinal uses of *E. longifolia* come from its various secondary metabolites contents, such as canthin-6-one alkaloid, quassinoid and squalene terpenoid which previous studies showed medicinal uses of these secondary metabolites [2] [5] [6].

Riksa Parikrama, and Rizkita Rachmi Esyanti are with School of Life Sciences and Technology, Institut Teknologi Bandung, Jalan Ganesha no. 10 Bandung, Indonesia (e-mail: riksarama@students.itb.ac.id; rizkita@sith.itb.ac.id)

In vitro culture is a well-known method to grow cell biomass and produce secondary metabolites. However, this method sometimes did not meet the secondary metabolites demand due to low plant's productivity and inefficient production method. In order to tackle this issue, new method are required. One of the methods is elicitation which in previous researches were being used to increase secondary metabolites production from plant cell cultures [10]. Elicitation with NaH_2PO_4 and chitosan on cell suspension culture of *E. longifolia* increased 9-methoxycanthin-6-one alkaloid production [1], while elicitation with chitin on *Morinda citrifolia* cell culture increased anthraquinone production [11]. Furthermore, ultraviolet elicitation on *Catharanthus roseus* culture increased ajmalicine production [12].

Ultraviolet (UV) light were used in this research as elicitor on *E. longifolia* callus culture. UV light can be acts as abiotic stress which triggering plant to perform defense mechanism of this particular stress. Plants responded UV stress to overcome it by producing secondary metabolites [13]. Previous study described that UV light radiation induced gene transcription process of enzymes which involved in secondary metabolites biosynthesis [14]. This study was carried out to evaluate the effect of UV light on *E. longifolia* callus growth and to evaluate secondary metabolites content in *E. longifolia* callus culture.

II. Materials And Methods

A. Callus Preparation

This study used *Eurycoma longifolia* callus culture which has been adapted into in vitro condition, grown and maintained at Plant Physiology Laboratory, School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia. Callus were maintained in solid Zenk medium added with 0.5 ppm IAA and 0.5 ppm kinetin. Then callus were subcultured three times into MS (Murashige-Skoog) medium added with 2.25 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) plus 2 ppm kinetin, later symbolised as Group A; and MS medium added with 0.5 ppm naphthalene acetic acid (NAA) plus 0.5 ppm kinetin, later symbolised as Group B.

B. Elicitation Treatment

Callus of Group A medium (2,4-D plus kinetin) and Group B (NAA plus kinetin) were exposed under ultraviolet light. UV light produced by Philips UV light (200-280 nm wavelength). UV light exposure was given 18 hours per day, from day 1 throughout day 28. Control group were prepared as comparison, which were callus cultures without being elicited with UV light.

C. Biomass Growth

E. longifolia callus dry weight was calculated every 4 days, until last calculation in day 28th. Callus dry weight data were presented in growth curve and statistically analysed by SPSS program, using one-way ANOVA (analysis of variance) and Tukey's test, with 95% of confidence level to find out the significances of different MS medium group (Group A, B, and control).

D. Histochemical Analysis

Cells structure and secondary metabolites content of Group A and B callus, both UV elicited or control, were analysed by histochemical method. This analysis was performed using Dragendorff reagent (alkaloid test) and Neutral Red reagent (terpenoid test). Cells histochemical slides were observed with inverted microscope using 20x to 40x objective lens.

E. Secondary Metabolites Content Analysis

UV elicited and unelicited Group A and B callus were harvested every 4 days (in 28 days of study) to analyse its secondary metabolites content. Thus, it was obtained 7 samples from each group (Group A elicited, Group A unelicited, Group B elicited, and Group B unelicited). Secondary metabolites extraction was performed by dissolving freeze-dried callus with 95% ethanol, then incubated overnight. The extract then filtered by Whatman no. 1 filter paper to obtain fine secondary metabolites extract. Extract then being analysed by gas chromatography–mass spectrometry (GC-MS) method.

F. Protein Analysis

All groups of callus were harvested every 4 days (out of 28 days of study) to analyse its protein. Protein extract was obtained from *E. longifolia* callus which processed with tricloroaceticacid, β-merchaptoetanol, and aceton precipitation. Extract was centrifuged to isolate protein from its solvent. Protein extract dissolved in buffer solution to obtain total protein extract. Samples protein value was determined by Bradford method [15] with 595 nm wavelength.

Protein analysis was performed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method. 0.5 mg (w/v) *E. longifolia* protein samples injected into separating gel, then electrophoresis was performed with 100 W in 2 hours. Thermo Scientific Multicolor Broad Range Protein Ladder no. 26634 was used as protein marker to compare protein bands. Electrophoresis gel was stained by coomasie briliant blue-R, then washed by methanol-acetic acid solution (3:1). Analysis was performed by comparing protein bands from *E. longifolia* callus with protein marker bands.

III. Results And Discussion

The results showed that biomass growth of ultraviolet elicited *Eurycoma longifolia* callus which treated in 2,4-D and kinetin medium (Group A) and also in NAA and kinetin medium (Group B) slightly lower than control group after 28 days of exposure (Fig. 1). However, statistical test revealed that no significant difference between elicited and unelicited callus. This result was slightly different if compared with previous studies which said ultraviolet light stress could decrease plant growth [16] [17].

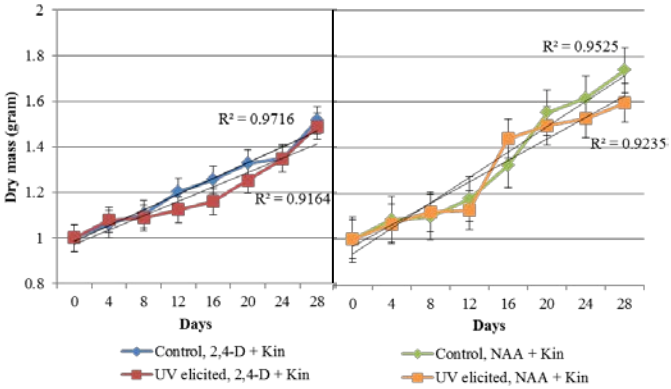

Fig. 1 Growth of *E. longifolia* callus in 28 days

Macroscopic observation of callus structure showed difference between Group A and Group B callus. Group A callus (Fig. 2a) was more friable than Group B callus (Fig. 2c). According to [18], friable callus was formed due to 2,4-D phytohormone and compact callus was formed due to NAA. After UV elicitation, Group A and B callus (Fig. 2b & 2d) became more friable and dry than its control groups. Moreover, the colour was darker after UV elicitation.

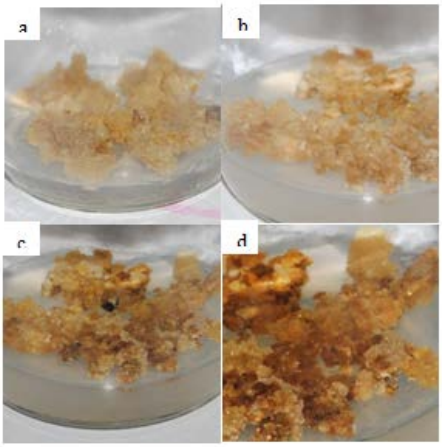

Fig. 2 Callus structure, (a) Group A control, (b) Group A UV elicited, (c) Group B control, (d) Group B UV elicited

Histochemical analysis showed *E. longifolia* callus cells morphology was difference between Group A and Group B, which Group A tend to be longer shape while Group B more rounded (Fig. 3). Dragendorff and Neutral Red reagents test showed positive results, which revealed that both callus group produced alkaloids and terpenoids. This analysis also showed that positive colour intensity of both reagent was denser on UV elicited callus than unelicited callus (more brown and more red) (Fig. 3). This result could be associated with previous result (Fig. 2) which showed darker callus colour after UV elicitation.

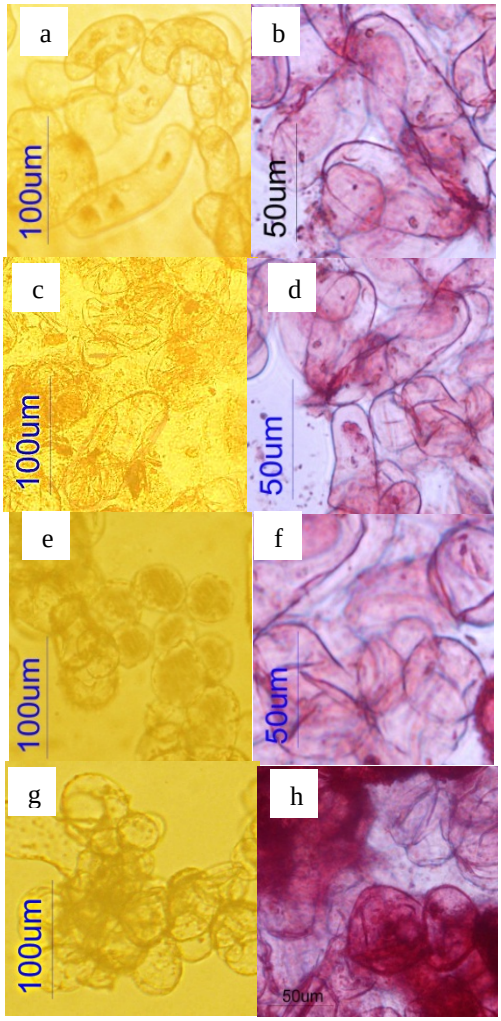

Fig. 3 Histochemical analysis results, (a) (b) Group A control, (c) (d) Group A UV elicited, (e) (f) Group B control, (g) (h) Group B UV elicited, brown is alkaloid, red is terpenoid

Eurycoma longifolia extract analysis which performed by GCMS method revealed almost 100 different compounds. There were secondary metabolites which belong to alkaloid and terpenoid group and contain medical activity. Some of them were canthin-6-one [2], squalene, 5-(hydroxymethyl)-2-furancarboxaldehyde, and isocoumarin [19].

Pyrrolidine was the basic structure of a few type of alkaloids [20]. This compound was produced at most from Group A callus and UV elicited on 24th day of exposure (Table I). Pyrrolidine was alkaloid produced from ornithine amino acid. Protein analysis showed that there was 50 kDa molecular weight protein band when pyrrolidine was founded (Fig. 4b.1). This protein was predicted as ornithin decarboxylase (MW 51 kDa) which involved in decarboxylation of ornithin amino acid to become pyrrolidine [21].

TABLE I
SECONDARY METABOLITES PRODUCTION FROM CALLUS CULTURE

Sec. metabolites	Group / day	Amount (control)	Amount (UV elicited)
Pyrrolidine	A / 24th	45.6%	70.02%
Canthin-6-one	B / 8th	2.65%	9.49%
5-(hydroxymethyl)-2-furancarboxaldehyde	B / 12th	-	26.39%
Isocoumarin	B / 24th	5.36%	-
Squalene	B / 8th	-	6.41%

Canthin-6-one alkaloid was produced at most on 8th day of study from Group B UV elicited callus, which showed 9.49% to 2.65% compared to control group (Table I). Protein analysis of this particular group of callus showed that on 8th day of exposure, there was 40 kDa protein band (Fig. 4d.1). This protein predicted as strictosydine synthase enzyme (MW 43 kDa) which involved in alkaloid biosythesis pathway, including canthin-6-one [22].

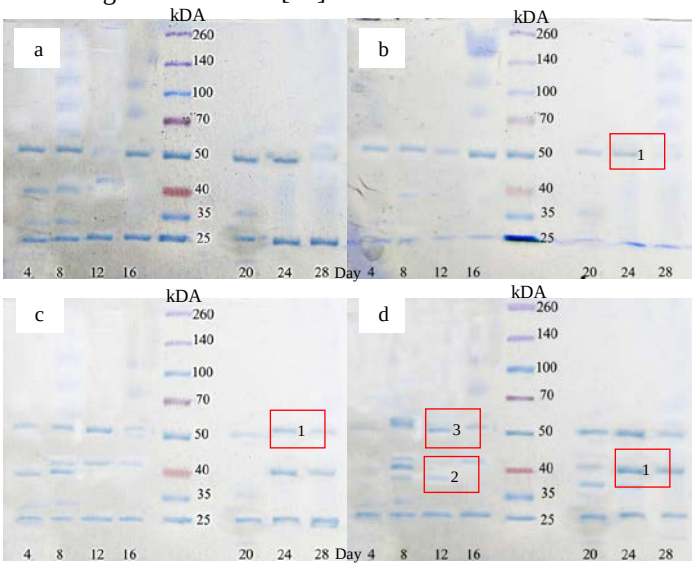

Fig. 4 SDS PAGE analysis results, (a) Group A control, (b) Group A UV elicited, (c) Group B control, (d) Group B UV elicited

5-(hydroxymethyl)-2-furancarboxaldehyde belongs to terpenoid group. Rahmalia *et al*. [19] showed this secondary metabolite could be naturally found in *E. longifolia*. This terpenoid was produced at most from Group B UV elicited callus on day 12th of exposure (Table I). During this day, protein analysis showed that there were 35 kDa and 50 kDa protein bands (Fig. 4d.2 & 4d.3). It was predicted that these bands were FPP synthase enzyme (MW 37.5 kDa) and GPP synthase enzyme (MW 53 kDa) which involved in terpenoids

biosynthesis pathway [23] [24].

This study showed that isocoumarin which belongs to terpenoid groups was not increased after UV elicitation. Isocoumarin content showed most amounts in unelicited Group B callus on day 24th (Table I). During this day, it was produced 50 kDa protein band (Fig. 4c.1) predicted as GPP synthase enzyme which involved in terpenoids biosynthesis pathway [24].

Squalene belongs to terpenoid group, particularly triterpene, which could be found in *E. longifolia* [25] [26]. This study showed that squalene only produced from Group B UV elicited callus on day 8th of exposure (Table I). Protein analysis showed there was 35 kDa protein band produced (Fig. 4d.2). This protein was predicted FPP synthase enzyme (MW 37.5 kDa) which involved in triterpenes biosynthesis [23] [27].

IV. Conclusion

According to this study, it was known that ultraviolet light radiation did not decrease biomass growth of *Eurycoma longifolia* callus culture, but the structure was slightly changed. Alkaloids production from *E. longifolia* callus culture, particularly pyrrolidine and canthin-6-one, and also terpenoids, particularly 5-(hydroxymethyl)-2-furancarboxaldehyde and squalene, could be increased by 18 hours of ultraviolet exposure daily. However, this elicitation method could not be used to increase isocoumarin production.

References

[1] Keng, C. L., Wei, A. Z., Bhatt, A. "Elicitation effect on cell biomass and production of alkaloids in cell suspension culture of the tropical tree *Eurycoma longifolia*". *Research Journal of the Costa Rican Distance Education University*, vol 2 (2), pp. 239-244, 2010.

[2] Kardono, L. B. S., Angehofer, C. K., Tsauri, S., Padmawinata, K., Pezzuto, J.M. Kinghorn, D. "Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia*". *Journal of Natural Products*, vol 54, pp. 1360–1367, 1991.
http://dx.doi.org/10.1021/np50077a020

[3] Nooteboom, L. *Simaroubaceae. In: Steenis JV (ed), Flora Malesiana. Sixth Edition*. Wolters-Woordhoff Publ., pp 197-207, 1972.

[4] Chan, K. L., O'Neill, M. J., Phillipson, J. D., Warhurst, D. C. "Plants as sources of antimalarial drugs. Part 3: *Eurycoma longifolia* Jack". *Planta Med*, vol 52, pp. 105-107, 1986.
http://dx.doi.org/10.1055/s-2007-969091

[5] Kanchanapoom, T., Chumsri, P., Sonchai, S., Kasai, R., Yamasaki, K. "Canthin-6-one alkaloids from callus cultures of *Eurycoma longifolia*". *Natural Medicines*, vol 56 (2), pp. 55-58, 2002.

[6] Miyake, K., Li, F., Tezuka, Y., Awale, S., Kadota, S. "Cytotoxic activity of quassinoids from *Eurycoma longifolia*". *Natural Product Communications*, vol 5 (7), pp. 1009-1012, 2010.

[7] Morris, P. *Secondary Metabolism in Plant Cell Culture*. Cambridge: Cambridge University Press., pp 2-3, 1986.

[8] Ganapathi, B. & Kargi, F. "Recent advances in Indole alkaloid production by *Catharanthus roseus* (Periwinkle)". *Journal of Experimental Botany*, vol 41, pp. 259-267, 1990.
http://dx.doi.org/10.1093/jxb/41.3.259

[9] Mahmood, M., Normi, R., Subramaniam, S. "Distribution of 9-methoxycanthin-6-one from the intact plant parts and callus cultures of *Eurycoma longifolia* (Tongkat Ali)". *Australian Journal of Crop Science*, vol 5 (12), pp. 1565-1569, 2011.

[10] Namdeo, A. G. Plant Cell Elicitation for Production of Secondary Metabolits: A Review. Pharmacognosy Reviews, 1 (1), pp. 69-79, 2007. (Journal)

[11] Dornenburg, H., Knorr, D. "Elicitation of anthraquinonesin *Morinda citrifolia* cell cultures". *Food Biotechnology*, vol 8, pp. 57-65, 1994.
http://dx.doi.org/10.1080/08905439409549868

[12] Prihatini, R. "Pengaruh pencahayaan dan elisitasi sinar ultra violet (UV) terhadap kandungan ajmalisin pada kultur kalus *Catharanthus roseus* (L.) G. Don.". *Tesis Magister, Program Studi Biologi*, Bandung: Institut Teknologi Bandung, 1998.

[13] Ouwerkerk, P. B. F., Hallard, D., Verpoorte, R., Memelink, J.. "Identification of UV-B light-responsive regions in the promoter of the tryptophan decarboxylase gene from *Catharanthus roseus*". *Plant Molecular Biology*, vol 41, pp. 491–503, 1999.
http://dx.doi.org/10.1023/A:1006321100550

[14] Logemann, E., Tavernaro, A., Schulz, W., Somssich, I. E., Hahlbrock, K. "UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley". *Proceedings of the National Academy of Sciences*, vol 97 (4), pp. 1903–1907, 2000.
http://dx.doi.org/10.1073/pnas.97.4.1903

[15] Bradford, M. M. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". *Analytical Biochemistry*, vol 72, pp. 248-254, 1976.
http://dx.doi.org/10.1016/0003-2697(76)90527-3

[16] Alexieva, V, Sergiev, I, Mapelli, S., Karanov, E. "The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat". *Plant, Cell & Environment*, vol 24 (12), pp 1337–1344, 2001.
http://dx.doi.org/10.1046/j.1365-3040.2001.00778.x

[17] Tian, X. R., Lei, Y. B. "Physiological responses of wheat seedlings to drought and UV-B radiation". *Russian Journal of Plant Physiology*, vol 54 (5), pp 676-682, 2007.
http://dx.doi.org/10.1134/S1021443707050160

[18] Zhao, J., Zhu, W. H., Hu, Q., Guo, Y. Q. "Compact callus cluster suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis". *In Vitro Cellular & Developmental Biology – Plant*, vol 37 (1), pp 68-72, 2001.
http://dx.doi.org/10.1007/s11627-001-0013-2

[19] Rahmalia, A., Esyanti, R. R., Iriawati.. "A Qualitative and Quantitative Evaluation of Terpenoid and Alkaloid in Root and Stem of Pasak Bumi *Eurycoma longifolia* Jack". *Jurnal Matematika dan Sains*, vol 16 (1), pp. 49-52, 2010.

[20] Kaur, K., Jain, M., Kaur, T., Jain, R. "Antimalarials from nature". *Bioorg Med Chem.*, vol 17 (9), pp. 3229-3256, 2009.
http://dx.doi.org/10.1016/j.bmc.2009.02.050

[21] Smismans, A., Eizirik, D. L., Pipeleers, D. G. "Interleukin-1beta induces ornithine decarboxylase activity in insulin-producing cells". *Cytokine*, vol 12 (1), pp. 49-54, 2000.
http://dx.doi.org/10.1006/cyto.1999.0524

[22] De Waal, A., Meijer, A. H., Verpoorte, R. "Strictosidine synthase from *Catharanthus roseus*: purification and characterization of multiple forms". *Biochem. J.*, vol 306, pp. 571-580, 1995.

[23] Sagami, H., Morita, Y., Korenaga, T., Ogura, K. "Purification of geranylgeranyl diphosphate synthase from bovine brain". *Acta Biochem Pol*., vol 41 (3), pp. 293-302, 1994.

[24] Tholl, D., Croteau, R., Gershenzon, J. "Partial Purification and Characterization of the Short-Chain Prenyltransferases, Geranyl Diphosphate Synthase and Farnesyl Diphosphate Synthase, from *Abies grandis* (Grand Fir)". *Archives of Biochemistry and Biophysics*, vol 386 (2), pp. 233–242, 2001.
http://dx.doi.org/10.1006/abbi.2000.2212

[25] Itokawa, H., Kishi, E., Morita, H., Takeya, K., Iitaka, Y. "Eurylene, a new squalene-type triterpene from *Eurycoma longifolia*". *Tetrahedron Letters*, vol 32 (15), pp. 1803–1804, 1991.
http://dx.doi.org/10.1016/S0040-4039(00)74334-6

[26] Morita, H., Kishi, E., Takeya, K., Itokawa, H. "Squalene derivatives from *Eurycoma longifolia*". *Phytochemistry*, vol 34 (3), pp. 765–771, 1993.
http://dx.doi.org/10.1016/0031-9422(93)85356-V

[27] Taiz, L., Zeiger, E. *Plant Physiology*. Sunderland: Sinauer Associates, pp 288, 2010.