Songklanakarin J. Sci. Technol.
37 (1), 49-53, Jan. - Feb. 2015

SJST
http://www.sjst.psu.ac.th

Original Article

Additions of precursors and elicitors improve geranylgeraniol production in *Croton stellatopilosus* callus cultures

Juraithip Wungsintaweekul[1,3]*, Piyawan Sangchuay[1], Siriwan Phongprueksapattana[2], Wijittra Kaewnoi[3], and Damrong Kongduang[4]

[1] *Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences,*

[2] *Scientific Equipment Center (SEC),*

[3] *Excellent Research Laboratory, Phytomedicine and Pharmaceutical Biotechnology Research Center (PPBRC), Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.*

[4] *Department of Science and Mathematics, Faculty of Agro Industrial Technology, Rajamangala University of Technology Isan, Kalasin Campus, Mueang, Kalasin, 46000 Thailand.*

Received: 3 July 2014; Accepted: 4 December 2014

Abstract

Strategies for enhancing GGOH production in *Croton stellatopilosus* callus culture included additions of precursors (sodium acetate-NA, sodium pyruvate-NP, mevalonic acid lactone-MVA) and elicitors (methyl jasmonate-MJ, acetylsalicylic acid-ASA, yeast extract-YE). Treated cells were evaluated for their GGOH contents by GC-FID and compared with the non-treated cells as controls. Additions of NA (25 mg/L), NP (50 mg/L) and MVA (100 mg/L) resulted in an enhancement of GGOH productivity to 0.61 mg/g DW, 0.52 mg/g DW and 0.70 mg/g DW, respectively, compared to the control culture (0.29 mg/g DW). Callus cultures elicited with MJ at 30 mg/L for 24 h stimulated GGOH production to 0.35 mg/g DW compared to the control culture (0.07 mg/g DW). Cells also responded to ASA (20 mg/L, 2 days) and YE (0.25 g/L, 4 days) and produced GGOH contents of 0.46 mg/g DW and 1.37 mg/g DW, respectively. This study has shown that isoprenoid precursors and conventional elicitors enhanced GGOH production in the *C. stellatopilosus* callus culture.

Keywords: *Croton stellatopilosus*, callus culture, elicitation, geranylgeraniol, precursor addition

1. Introduction

Croton stellatopilosus Ohba (plaunoi) belongs to the Euphorbiaceae family and is widely distributed in the South-east Asian region (Esser and Chayamarit, 2001). Some promising pharmacological activities of plaunotol have emerged to make this plant of interest to medical research. Plaunotol has been used as a gastro-protective agent since it inhibited the growth of *Helicobacter pylori* and accelerated an ulcer healing effect (Ogiso *et al.*, 1985; Koga *et al.*, 1996). Plaunotol is a hydroxylated product of geranylgeraniol (GGOH), which is produced from geranylgeranyl diphosphate (GGPP)-a general precursor for the biosynthesis of diterpenes (Tansakul and De-Eknamkul, 1998; Sitthithaworn *et al.*, 2001).

Two decades ago, attempts to produce plaunotol and related substances via a biotechnological approach were made, but unfortunately, only GGOH has been reported to

* Corresponding author.
Email address: juraithip.w@psu.ac.th

accumulate in plant cell cultures. Several studies have been reported using leaf explants, growing with Murashige and Skoog (MS) medium that did produce plaunotol. They also suggested the type of culture has effect on plaunotol production (Morimoto and Murai, 1989). A callus culture was shown to be more suitable for plaunotol production than a cell suspension culture (Kongduang *et al.*, 2014). However, the production yield still did not meet requirements and the cell line was unstable. Previously, we have reported that a *C. stellatopilosus* suspension culture, grown in MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/L benzyladenine (BA) produced GGOH (1.14 mg/g fresh weight), phytosterols and fatty acids (Wungsintaweekul *et al.*, 2007).

Based on the hypothesis that the plant cell culture may lack precursors in the metabolic pool and that therefore, the cells were unable to produce plaunotol, increasing the GGOH production may flux into the reaction product of diterpene biosynthesis. In the present study, we aimed to enhance the production of GGOH using precursor addition and elicitation. In this study, we chose to add sodium acetate, sodium pyruvate and mevalonic acid lactone. For elicitation, common elicitors such as methyl jasmonate, acetylsalicylic acid and yeast extract were added.

2. Materials and Methods

2.1 Callus culture

Young plaunoi leaves were soaked in 70% (v/v) ethanol for 3 min, followed by sterilization in 0.5% (w/v) sodium hypochlorite for 5 min and thoroughly washed with sterile distilled water. The sterile leaves were excised and placed on Murashige and Skoog (MS) (Murashige and Skoog, 1962) agar medium containing 2 mg/L 2,4-D, 1 mg/L kinetin and 3% (w/v) sucrose solidified with 0.8% (w/v) plant agar. The cultures were maintained under a 16-h photoperiod (2000 lux) at 25±2°C. After 4 weeks, the calli formed were maintained by subculture every 3 weeks on MS agar medium containing 2 mg/L BA and 2 mg/L NAA, 1% (w/v) sucrose and 0.2% (w/v) gellan gum. The homogenous callus culture was used as the plant material for the various treatments.

2.2 Growth curve

The growth of the homogenous callus culture was evaluated by constructing a growth curve. At least 4 independent callus cultures were prepared and collected every 10 days. Cells were harvested and immediately frozen in liquid N_2 and dried by lyophilization. The dried cell mass was weighed in gram and its growth index calculated as shown in the equation. The growth curve was constructed by plotting the growth index against the age of the culture.

$$\text{Growth index} = \frac{(\text{weight at measured date} - \text{initial weight})}{\text{initial weight}}$$

2.3 Precursor addition

Sodium acetate (NA), sodium pyruvate (NP) and mevalonic acid lactone (MVA), each at final concentrations of 10, 25, 50, 100 mg/L, were fed to the cells at the 10th day of culture. Callus cultures were incubated on shelves under a 16 h/day light cycle for 2 weeks. Callus cells were harvested and thoroughly washed with water then immediately frozen in liquid N_2 and dried by lyophilization. Samples were investigated in triplicate.

2.4 Elicitor treatment

Methyl jasmonate (MJ) was prepared in 0.2% ethanol to obtain a stock solution. Acetylsalicylic acid (ASA) and yeast extract (YE) were prepared in water. Solutions were filter sterilized with a membrane (0.22 μm). Callus cells were elicited with MJ and ASA at final concentrations of 10, 20, 30 mg/L, and with YE at 0.1, 0.25 and 0.5 g/L. Elicitations were added on day 15th for 1, 2 and 4 days. Callus cells were harvested and thoroughly washed with water then immediately frozen in liquid N_2 and dried by lyophilization. Samples were investigated in triplicate.

2.5 Quantification of GGOH content

The GGOH content was determined using the GC-FID method as described in Kongduang *et al.* (2012). Briefly, lyophilized powder (50 mg) was extracted with 10 mL of absolute ethanol and 1% (w/v) NaOH, sonicated for 5 min followed by reflux for 1 h, and then centrifuged. The supernatant was collected and its volume was adjusted to 14 mL to obtain *ca*. 70% (v/v) ethanol. To obtain a lipophilic fraction, the supernatant was partitioned with *n*-hexane (10 mL; 3 times), pooled and evaporated to dryness. Prior to GC analysis, the residue was dissolved with *n*-hexane (0.2 mL) and centrifuged. The GC-FID conditions for GGOH determination were: column DB1 dimethylsiloxane (GC-Hewlett Packard, CA, USA), 30 m x 0.32 mm i.d., a 0.25 μm film thickness, a flow of helium at 1.0 mL/min, a split mode (10:1) injection, a temperature program from 220°C (hold 3 min) to 280°C (rate 10°C/min) and held for 6 min, an injector temperature of 280°C, and a detector temperature of 300°C, the sample size was 1.0 μL. The GGOH was eluted at 6.7 min. The area under peak was integrated and converted to a concentration by using GGOH calibration curve. The GGOH content was expressed as mg per g dry weight (DW).

2.6 Statistical analysis

All data were presented as a mean value ± standard deviation (SD). Statistical analysis was performed using the SPSS 15.0 (Tukey HSD analysis).

3. Results and Discussion

The green callus culture was induced from *C. stellatopilosus* leaves. Figure 1 shows the steps of establishment of a green callus culture according to Kongduang *et al.* (2014). The callus culture was firstly induced by placing explants on MS medium supplemented with 2 mg/L 2,4-D, 1 mg/L kinetin and 3% (w/v) sucrose (Vongcharoensathit and De-Eknamkul, 1998). To induce green callus culture, the callus was subcultured to MS agar medium containing 2.0 mg/L NAA and 2.0 mg/L BA, 1% (w/v) sucrose and 0.2% (w/v) gellan gum. Only green cells were selected and subcultured to a new medium every three weeks. At this stage, the callus culture was characteristically friable, with a slow growth rate and greenish tissues (Figure 1). The growth curve of the green callus culture was determined and is shown in Figure 2. The growth index reflected the growth characteristic of the callus culture within 30 days. Lag phase was the first five days of culture following the linear phase, and then the growth index was exponentially increased after 10 days of culture. The growth declined and entered a stationary phase after 15 days of culture. Previously, the growth and terpenoid accumulation characteristics were described from this type of culture (Kongduang *et al.*, 2014). Similar pattern of growth characteristic was found in our callus culture. Terpenoid contents including phytol, GGOH, plaunotol and phytosterols were reported but in microgram level per gram dry weight of callus culture. Therefore, the green callus culture was manipulated in order to improve the terpenoid production. We chose the approaches of addition of possible precursors and elicitation for exploitation.

From growth curve, precursors were added to the cells on days 10 and elicitors were added on days 15. To enable the growing cultures to assimilate and utilize the fed precursors, cells were harvested on days 25 of culture. For the elicitation, the callus cultures were exposed to elicitors for 1, 2 and 4 days in order to compare the effects of different amounts of the elicitors and the time response to production of the GGOH.

Because isoprene units, in higher plant, can be supplied from the mevalonate (MVA) pathway and the methylerythritol (MEP) phosphate pathway (Schwarz, 1994), sodium acetate (NA) and mevalonic acid lactone (MAL) were supplied as precursors for MVA pathway, while sodium pyruvate (NP) was supplied as a precursor for the MEP pathway. Nevertheless, NP could be transformed to acetyl CoA by complex enzymes, pyruvate dehydrogenase, prior entering to citric acid cycle (Guan *et al.*, 1996). Based on the hypothesis that all genes and enzymes exist in the callus cultures, then application of precursors could enhance the GGOH and probably plaunotol productions. The results in Figure 3 indicate that the cells could utilize NA, MAL and NP for the GGOH biosynthesis. NA (25 mg/L) promoted a doubling of GGOH production (0.61±0.09 mg/g DW; $P<$ 0.01) compared to the control but a further increase in the amount of NA caused a reduction of GGOH production. For NP treatment, the most GGOH production was 0.52±0.02 mg/g DW at 50 mg/L NP application (Figure 3B). Higher amount of NP also slightly suppressed the GGOH production. Interestingly, application of MVA, an immediate precursor of isoprene unit, enhanced the GGOH production in a concentration-dependent manner. Cells treated with 100 mg/L MVA produced and accumulated GGOH content up to 2.4 times (0.70±0.05 mg/g DW) higher than the control culture (Figure 3C). The results denote that all fed precursors were utilized by the callus culture and transformed to GGOH. Moreover, the callus cells preferred to utilize sodium acetate and mevalonic acid lactone, precursors of the MVA pathway, rather than sodium pyruvate, a precursor of the MEP pathway. By our GC method, the amount of plaunotol was under the detection limit. The absence of plaunotol may be argued by the absence of GGOH 18-hydroxylase, an enzyme that catalyzing GGOH to plaunotol in the cells (Tansakul and De-Eknamkul, 1998). Moreover, plaunotol and GGOH 18-hydroxylase were reported to localize in chloroplasts (Tansakul and De-Eknamkul, 1998; Sitthithaworn *et al.*, 2006).

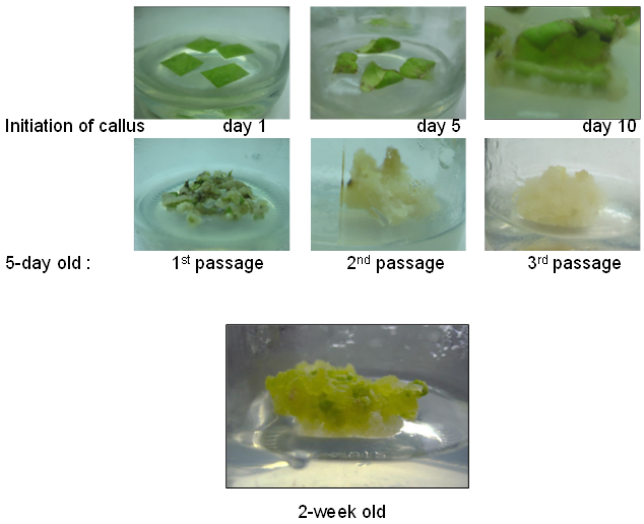

Figure 1. Establishment of a green callus culture from *C. stellatopilosus* leaf explants.

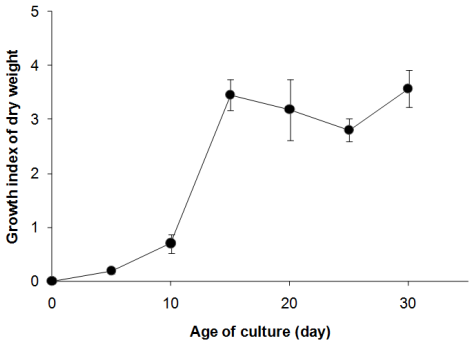

Figure 2. Growth curve of a *C. stellatopilosus* callus culture.

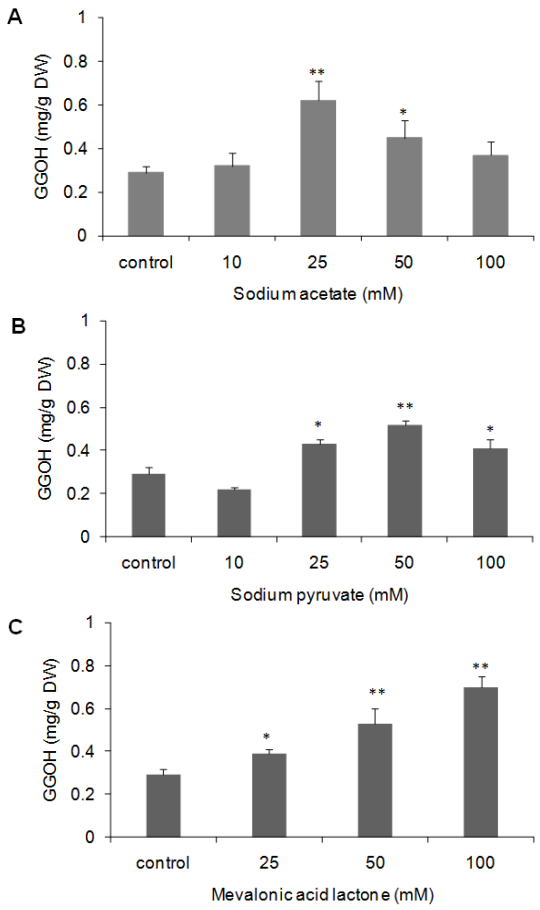

Figure 3. GGOH content of callus culture cells after addition of precursors. A. sodium acetate (NA); B. sodium pyruvate (NP); C. mevalonic acid lactone (MVA). **; * indicate $P<0.01$ and $P<0.05$, respectively.

The transmission electron micrograph (TEM) shown in Kongduang *et al.* (2008) indicated that the callus culture contained immature chloroplast. Suppression of the GGOH production by high amounts of NA and NP could be explained by a feedback inhibition of the rate-limiting step enzyme in isoprenoid biosynthesis (Spurgeon and Porter, 1981). Following the GGOH biosynthesis by a molecular biology approach should be investigated in order to understanding the fluxing mechanism of precursor at the cellular level.

Theoretically, genes involved in GGOH synthesis sometimes exist in the cell culture but may not be expressed and functioned properly. Previously, the relationship of the occurrence of the mRNAs and type of *C. stellatopilosus* cultures has been reported (Kongduang *et al.*, 2014). mRNAs encoding 1-deoxy-D-xylulose 5-phosphate synthase, 2*C*-methyl-D-erythritol 4-phosphate synthase and geranylgeranyl diphosphate synthase are present only in the leaves and green callus culture, but not in the cell suspension culture. In this study, the green callus culture was used as plant material, in which it is presumed the genes involved in terpenoid biosynthesis exist. Elevation of mRNAs of genes may enhance the GGOH production in the cells. Thus, the conventional elicitors such as methyl jasmonate (MJ), acetylsalicylic acid (ASA) and yeast extract (YE) were added to the callus culture. As shown Figure 4, the concentrations and time exposures of each elicitor were varied during treatments. For MJ elicitation, the callus cells responded to produce the GGOH content mostly after elicitation with MJ at 30 mg/L for 24 h, up to 4.9 times higher than the control (0.35±0.09 mg/g DW) ($P<0.01$). Unlike MJ, late response was found in the groups of ASA and YE treatments. ASA at concentration of 20 mg/L treated for 2 days significantly enhanced the GGOH production (0.46±0.16 mg/g DW; $P<0.01$); however, longer treatment caused a repressed effect. A similar effect was seen with YE elicitation. At 0.25 g/L YE and stimulated for 4 days has mostly produced GGOH when compared with controls (1.37± 0.07 mg/g DW; $P<0.05$). Yeast extract elicited the cells to produce secondary metabolites with unknown compositions like microbial cell walls (Siddiqui *et al.*, 2013). It has been shown to increase secondary metabolite production in many cell cultures (for review see Namdeo, 2007). Nevertheless, the role of yeast extract in GGOH production in *Croton stellatopilosus* callus cultures is still unknown.

In conclusion, additions of isoprenoid precursors and elicitors improve the GGOH production in *C. stellatopilosus*

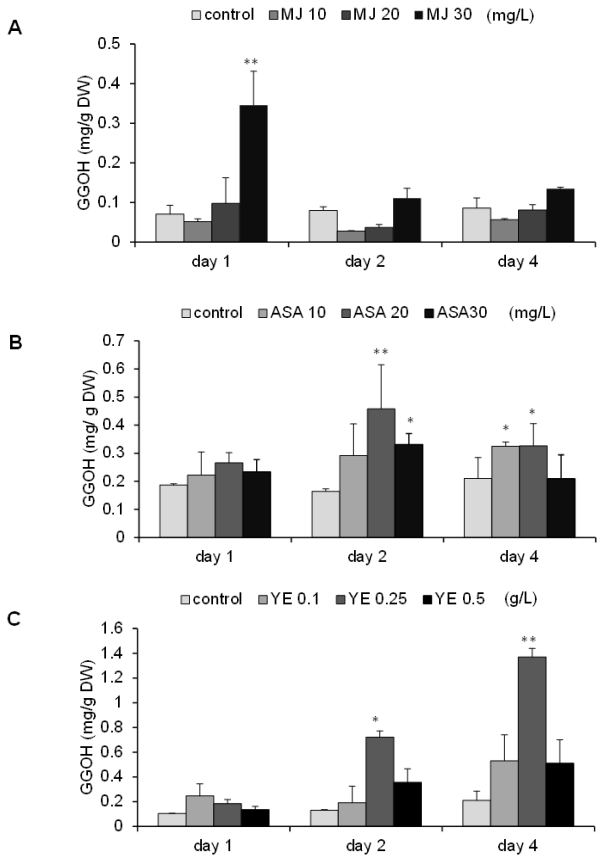

Figure 4. GGOH content of callus culture cells after elicitation. A. methyl jasmonate (MJ); B. acetylsalicylic acid (ASA); C. yeast extract (YE). **; * indicate $P<0.01$ and $P<0.05$, respectively.

green callus cultures. These results prove that callus cultures are able to produce GGOH but lack precursors in the metabolic pool. Elicitors including methyl jasmonate, acetylsalicylic acid and yeast extract increased GGOH production. Generally, application of both precursor and elicitor may result in tremendous increases the amounts of secondary metabolite production (Baldi and Dixit, 2008). But high accumulations of metabolites have effect on the feedback inhibition in plant biosynthesis. Besides, some metabolites such as GGOH at high amount are toxic to the cells and cause cell death (Yoshikawa *et al.*, 2009). Therefore, the benefit of using both precursors and elicitors in case of *C. stellatopilosus* needs to be further investigated.

Acknowledgements

This research was supported by Prince of Songkla University (grant no. PHA550336S). The authors thank Dr. Brian Hodgson for suggestions and assistance with the English.

References

Baldi, A. and Dixit, V.K. 2008. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Bioresource Technology. 99, 4609-4614.

Esser, J. and Chayamarit, K. 2001. Two new species and a new name in Thai *Croton* (Euphorbiaceae). Thai Forest Bulletin (Botany). 29, 51-57.

Guan, Y., Rawsthorne, S., Scofield, G., Shaw, P. and Doonan, J. 1995. Cloning and characterization of a dihydrolipoamide acetyltransferase (E2) subunit of the pyruvate dehydrogenase complex from *Arabidopsis thaliana*. Journal of Biological Chemistry. 270(10), 5412-5417.

Koga, T., Kawada, H., Utsui, Y., Domon, H., Tshii, C and Yasuda, H. 1996. *In vitro* and *in vivo* antibacterial activity of plaunotol, a cytoprotective antiulcer agent, against *Helicobacter pylori*. Journal of Antimicrobial Chemotherapy. 37, 919-926.

Kongduang, D., De-Eknamkul, W., Sitthithaworn, W. and Wungsintaweekul, J. 2014. Terpenoid content and transcription profile analysis in callus and suspension cultures of *Croton stellatopilosus*. Journal of Plant Biochemistry and Biotechnology. 23(1), 61-68.

Kongduang, D., Wungsintaweekul, J. and De-Eknamkul, W. 2008. Biosynthesis of β-sitosterol and stigmasterol proceeds exclusively via the mevalonate pathway in cell suspension cultures of *Croton stellatopilosus*. Tetrahedron Letters. 49, 4067-4072.

Kongduang, D., Wungsintaweekul, J. and De-Eknamkul, W. 2012. Established GC-FID for simultaneous determination of diterpenes and phytosterols in Plaunoi (*Croton stellatopilosus* Ohba). Songklanakarin Journal of Science and Technology. 34(6), 623-628.

Morimoto, H. and Murai, F. 1989. The effect of gelling agents on plaunotol accumulation in callus cultures of *Croton sublyratus* Kurz. Plant Cell Reports. 8, 210-213.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio-assay with tobacco tissue culture. Plant Physiology. 15, 473-497.

Namdeo, A.G. 2007. Plant cell elicitation for production of secondary metabolites: A review. Pharmacognosy Reviews. 1(1), 69-79.

Ogiso, A., Kitazawa, E., Kobayashi, S., Komai, T., Matsunuma, N., Kataumi, S. 1985. Plaunotol (CS-684), a new antiulcer agent. Sankyo Kenkyusho Nenpo. 37, 1-39.

Schwarz, M.K. 1994. Terpene biosynthesis in *Ginkgo biloba*: a surprise story. Doctoral Dissertation. Nr. 10951, Swiss Federal Institute of Technology Zurich, Switzerland.

Siddiqui, Z.H., Mujib, A., Mahmooduzzafar, Aslam. J., Hakeem, K.R. and Parween, T. 2013. In vitro production of secondary metabolites using elicitor in *Catharanthus roseus*: A case study. In Crop Improvement New Approaches and Modern Techniques, Hakeem, K.R., Ahmad, P., and Ozturk, M. Eds. Springer Science, New York, U.S.A., pp 401-420.

Sitthithaworn, W., Kojima, N., Viroonchatapan, E., Suh, D.Y., Iwanami, N., Hayashi, T., Noji, M., Saito, K., Niwa, Y. and Sankawa, U. 2001. Geranylgeranyl diphosphate synthase from *Scoparia dulcis* and *Croton sublyratus* plastid localization and conversion to a farnesyl diphosphate synthase by mutagenesis. Chemical and Pharmaceutical Bulletin. 49, 197-202.

Sitthithaworn, W., Potduang, B. and De-Eknamkul, W. 2006. Localization of plaunotol in the leaf of *Croton stellatopilosus* Ohba. ScienceAsia 32, 17-20.

Spurgeon, S.L. and Porter, J.W. 1981. Biosynthesis of isoprenoid compounds. Porter, J.W., Spurgeon, S.L., Eds.; John Wiley and Sons: New York, U.S.A., pp 1-53.

Tansakul, P., and De-Eknamkul, W. 1998. Geranylgeraniol-18-hydroxylase: The last enzyme on the plaunotol biosynthetic pathway in *Croton sublyratus*. Phytochemistry 47(7), 1241-1246.

Vongcharoensathit, A. and De-Eknamkul, W. 1998. Rapid TLC-densitometric analysis of plaunotol from *Croton sublyratus* leaves. Planta Medica. 64, 279-280.

Wungsintaweekul, J., Sriyapai, C., Kaewkerd, S., Tewtrakul, S., Kongduang, D. and De-Eknamkul, W. 2007. Establishment of *Croton stellatopilosus* suspension culture for geranylgeraniol production and diterpenoid biosynthesis. Zeitschrift für Naturforschung. 62c, 389-396.

Yoshikawa, N., Yamada, J., Tsuno, N.H., Okaji, Y., Kawai, K., Tsuchiya, T., Yoneyama, S., Tanaka, J., Shuno, Y., Nishikawa, T., Nagawa, H., Oshima, N. and Takahashi, K. 2009. Plaunotol and geranylgeraniol induce caspase-mediated apoptosis in colon cancer. Journal of Surgical Research. 153(2), 246-253.