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Full Length Research Paper

Micropropagation and production of eurycomanone, 9-methoxycanthin-6-one and canthin-6-one in roots of *Eurycoma longifolia* plantlets

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Eurycoma longifolia **or Tongkat Ali is a well known medicinal plant in Malaysia. The plants are used as main portion in herbal preparation, and have been extensively harvested. Generally, most of the raw materials required by the industries are heavily collected from the natural forests, thereby resulting in the uncontrolled exploitation of the plant in the wild. Hence, there is a need to ensure adequate supply of quality *E. longifolia* for the use of the related industries and for conservation purposes. Micropropagation has proved to be an alternative for the multiplication of medicinal and aromatic plants and it can allow the future commercial use of *E. longifolia* in the herbal industries. In this study, *E. longifolia* plantlets were successfully mass-produced using axillary shoot multiplication techniques, and the production of eurycomanone, 9-methoxycanthin-6-one and canthin-6-one compounds of *E. longifolia* were detected in roots of tissue culture plantlets.**

Key words: *E. longifolia*, Tongkat Ali, micropropagation, eurycomanone, 9-methoxycanthin-6-one, canthin-6-one.

INTRODUCTION

Eurycoma belongs to the Simaroubaceae family, which contains many bitter plants. Naturally, it can be found in Asia region. There are two *Eurycoma* species found in Malaysia: *E. longifolia* Jack. and *E. apiculata* Benn. (Burkill, 1966), although more scientific research are focusing on *E. longifolia* Jack. It is locally known as Tongkat Ali, penawar pahit or setunjang bumi (Perry, 1980). The height can reach up to 8 to 10 m, while the diameter of the stem could be about 15 cm (Burkill, 1966). *E. longifolia* roots were commonly used in Malaysia and Indonesia for malaria or fever, wounds, ulcers and pain (Burkill, 1966). *E. longifolia* is also known as herb for energy or male aphrodisiac (Gimlette and Thomson, 1977). Many scientific researches show that this plant has anti-malarial activity, anti-ulcer, cytotoxic activity and antipyretic activities. Biological activities are caused by chemical compounds such as quassinoid, squalene and triterpene (Chan et al., 1986). *E. longifolia* is among the most widely used herbs in Malaysia and its demand is increasing rapidly. It is therefore necessary to plant this tree to meet the demands of the industry. This plant usually reaches maturity at five years and bears only a little fruit. For this purpose, seedlings are the main requirements for planting.

Several bioactive compounds have been identified from *E. longifolia*, such as eurycomanone, 9-methoxycanthin-

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Abbreviations: MS, Murashige and Skoog; **WPM**, woody plant medium; **BAP**, N6-benzyladeninepurine; **IBA**, indole-3-butyric acid.

6-one, 14, 15-β-dihydroxyklaineanone and 13,21-epoxyeurycomanone. These compounds are usually used as standard markers for the standardization of *E. longifolia* products (Chan, 2004). Since the active compounds are extracted mainly from the roots, excessive usage and destructive method have resulted in decrease populations of *E. longifolia* in the forest. Therefore, urgent measures are required to conserve and replenish plants growing in the wild. Rapid propagation method is necessary to meet the increasing demand for planting materials of *E. longifolia* for commercial production of its by-products. In addition, planting materials are also required for conservation activities.

This study was aimed at the successful propagation of *E. longifolia in vitro* and the comparison of the production of *E. longifolia* compounds in roots of tissue culture plantlets and wild plants.

MATERIALS AND METHODS

In vitro germination

The source of *E. longifolia* explants used in this study was obtained from FRIM research station in Maran, Pahang, Malaysia. *E. longifolia* fruits were surface sterilized using 70% (v/v) ethanol and a drop of Tween 20 for 3 min, and then washed with 50% (v/v) Chlorox® plus a drop of Tween 20 for 15 min. Then, the fruits were washed with sterile distilled water five times. The seed coat was peeled off and seeds were cultured in germination media, Murashige and Skoog (MS) basal (Murashige and Skoog, 1962) and woody plant (WPM) basal media (Lloyd and McCown, 1980) supplemented with cytokinins 6-benzylaminopurine (BAP) at a concentration between 0.0 to 2.5 mg/L, 30 g/L sucrose and 0.3% (w/v) gelrite at the pH value of 5.8. The cultures were incubated at 25°C in the light condition for germination *in vitro*.

Shoots multiplication

Due to the limited number of shoots, nodal segment was used as explants for shoot multiplication study. Nodal segments (1.5 cm) were cultured in MS media containing BAP and kinetin (KIN) at a concentration of 0.0 to 2.0 mg/L, 30 g/L sucrose and 0.3% (w/v) gelrite at the pH value of 5.8. All cultures were incubated at 25°C in the light. The production of new shoots was observed after eight weeks in culture. Data were analyzed using statistical software MINITAB Release 15.

Roots induction and acclimatization

For *in vitro* rooting, individual shoots produce (4 to 5 cm) were rooted in half MS medium containing indole-butyric-acid (IBA) at a concentration of 0.0 to 10.0 mg/L. All cultures were incubated under light at 25°C. The percentage of roots produced and the number of roots per shoot were recorded after eight weeks in culture. Data were analyzed using statistical software MINITAB Release 15.

Rooted shoots were removed from the bottle and washed with distilled water to remove the agar. Plantlet was subsequently immersed in 1% (w/v) fungicides (MENDATE 50 WP) for 5 min and rinsed with distilled water for three times. Plantlets were planted in sandy soil for three weeks under the plastic cover and the percentage of survival was recorded. Finally, plantlet was then transferred into pots containing sand and soil at 1:3 ratios.

Chemical analysis

Roots of one year old *E. longifolia* tissue culture plantlets and roots from matured plants were used as plant samples for chemical analysis. They were then harvested from these samples and dried overnight in an oven. The dried roots were extracted with methanol and concentrated using rotary evaporator. Next, the methanol extracts was each dissolved in high-performance liquid chromatography (HPLC) grade methanol and the solution was filtered through a membrane filter (pore size 0.45 µM) prior to HPLC analysis using a waters HPLC system (Waters Delta 600 with 600 Controller) with photodiode array detector (Waters 996). A Phenomenex-Luna (5 µM) column was used (4.6 mm i.d. × 250 mm) and for elution of the constituents, a gradient of two solvents denoted as A and B was employed; A was 0.1% aqueous formic acid, whereas B was acetonitrile. Initial conditions were 35% A and 65% B, with a linear gradient reaching 45% A at time (t) = 1 min. This was followed by an isocratic elution until t = 10 min, after which the programme returned to the initial solvent composition at t = 15 min and continued for another 10 min. The column temperature was maintained at 40°C. The flow rate used was 1.0 ml/min and the injection volume was 10 µL. The retention time and UV spectrum of major peaks were analysed.

RESULTS AND DISCUSSION

In vitro germination

New shoots first appeared after two weeks in culture for both MS and WPM basal media regardless of the different BAP concentration used (Table 1). Individual and multiple shoots first appeared after three to five weeks of culturing. Bud formation of new shoots was often seen forming near the elongated axillary bud. It was observed that in WPM medium, the average number of shoots produced per explants was higher than that in MS media. WPM medium supplemented with 2.0 mg/L BAP induced a higher number of shoots (2.75 ± 0.5 shoots per explants) compared with other media. On average, the maximum number of three shoots appeared in the cultures that produced multiple shoots. Naturally, *E. longifolia* seeds germinate and produce only one shoot. However, when germinated *in vitro*, multiple shoots were formed and this may be due to the presence of cytokinins in the cultivation media. In general, cytokinins can overcome apical dominance, release of lateral shoot buds from dormancy and increase the production of shoots (Loc et al., 2005).

Shoots induction and multiplication

George (1993) stated that one or more cytokinins were usually added to the media at the beginning of the study, in order to promote the development of axillary buds and

Table 1. Effect of BAP in MS and WPM basal on *in vitro* germination of *E. longifolia.*

BAP (mg/L)	Mean. no. of shoots per explants ± SD	
	MS	WPM
0.0	1.00 ± 0.00[a]	1.00 ± 0.00[c]
0.05	1.25 ± 0.50[a]	2.25 ± 0.50[ab]
0.5	1.00 ± 0.00[a]	2.00 ± 0.00[ab]
1.0	1.25 ± 0.50[a]	1.75 ± 0.96[bc]
2.0	1.50 ± 0.58[a]	2.75 ± 0.50[ab]
2.5	1.25 ± 0.50[a]	1.00 ± 0.00[c]

Mean number with the same letter did not show significant difference ($p<0.05$) (Duncan's multiple range test).

Table 2. Effect of different concentration of BAP and Kinetin in MS basal medium on shoot multiplication of *E. longifolia.*

Media	Mean number of shoot ± SD
MS basal	1.04 ± 0.02[a]
MS + 0.5 mg/L Kinetin	1.09 ± 0.03[a]
MS + 1.0 mg/L Kinetin	1.11 ± 0.04[a]
MS + 2.0 mg/L Kinetin	1.16 ± 0.06[a]
MS + 0.5 mg/L BAP	1.82 ± 0.14[b]
MS + 0.5 mg/L BAP + 0.5 mg/L Kinetin	1.53 ± 0.09[b]
MS + 0.5 mg/L BAP + 1.0 mg/L Kinetin	1.59 ± 0.09[b]
MS + 1.0 mg/L BAP	1.75 ± 0.10[b]
MS + 1.0 mg/L BAP + 0.5 mg/L Kinetin	1.80 ± 0.16[b]
MS + 1.0 mg/L BAP + 1.0 mg/L Kinetin	1.76 ± 0.11[b]
MS + 2.0 mg/L BAP	1.64 ± 0.22[b]

Mean number with the same letter did not show significant difference ($p<0.05$) using Tukey's test. 75 explants were cultured in each concentration.

to reduce the dominancy of apex in shoot culture. Regulatory effects of a combination of two plants cytokinins were also included in this study which was the combination of BAP and kinetin (Table 2). MS basal were used for shoot multiplication study because in the earlier experiment that we conducted, the *E. longifolia* shoots were multiplied better in MS basal than in WPM basal (data not shown). Table 2 shows that the mean number of shoots per explants produced from cultures on media containing KIN was lower than those on media containing BAP. In this experiment, MS basal supplemented with 0.5 mg/L BAP gave the highest mean number of shoots compared to mean number of shoots in other concentration. These observations showed that the number of shoots produced during shoot multiplication following repeated sub-cultures at five week intervals on these media over a period of one year was maintained without any evidence of decline. According to Haliza (2000), in *Acacia auriculiformis*, low concentration of BAP (0.1 and 0.5 mg/L BAP) was also optimal for shoots multiplication. High concentration of cytokinin in the medium will result in the production of many stunted shoots, and the shoots produced will fail to elongate and become hyperhydric (George, 1993).

This study also shows that not all cytokinin have a positive influence on shoots production during micro-propagation of *E. longifolia*. This was supported by the significantly more number of shoots produced with the higher BAP concentration used as compared to KIN. Similar observation on the positive effects of BAP on inducing shoot production had been reported in *Cunila galioides* (Fracaro and Echeverrigay, 2001). Reddy et al. (1998) also reported that KIN did not significantly improve shoot length and number of shoots produced. Moreover, KIN has not been widely used in tissue culture of woody species (Chishimba et al., 2000) as BAP is more stable in tissue culture media compared with kinetin (George, 1993). This result is, however, in contrast with Hussein et al. (2005). Their findings show that KIN has significant effects on the *E. longifolia* shoots produced compared to

Table 3. Effect of different concentration of IBA in ½ MS medium on rooting of *E. longifolia* shoots.

IBA (mg/L)	Mean numbner of rooted shoots ± SD	Mean number of roots per shoot ± SD
0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
0.5	0.05 ± 0.03^{a}	0.13 ± 0.07^{ab}
1.0	0.11 ± 0.04^{a}	0.24 ± 0.09^{ab}
2.5	0.28 ± 0.05^{b}	0.91 ± 0.21^{b}
5.0	0.43 ± 0.06^{bc}	2.04 ± 0.37^{c}
7.5	0.33 ± 0.06^{b}	1.03 ± 0.23^{b}
10.0	0.56 ± 0.06^{c}	2.24 ± 0.31^{c}

Mean number with the same letter did not show significant difference ($p<0.05$) using Tukey's test. 75 explants were cultured in each concentration.

Table 4. Survival rates of plantlets from different concentration of IBA observed after 3 weeks of transfer to nursery.

IBA concentration (mg/L)	Survival rates (%)
0.0	54.1
0.5	57.4
1.0	75.0
2.5	74.6
5.0	59.6
7.5	30.4
10.0	39.4

BAP and zeatin. In addition, the numbers of *E. longifolia* shoots produced in this study were lower than those obtained by Hussein et al. (2005). This may be due to the different type of the explants used; nodal segment was used in this study, while the other research used the shoot tip. Type of explants also plays important role in multiple shoot induction.

In vitro rooting of *E. longifolia*

In this study, the individual shoots of *E. longifolia* were cultured in ½ MS medium containing indole-3-butyric acid (IBA)S ranging from 0.0 to 10.0 mg/L. The result for *E. longifolia in vitro* rooting is shown in Table 3. The highest mean number of shoots rooted was recorded in media containing 10.0 mg/L IBA with mean 0.56 ± 0.06 that was significant compared to the other media. However, it was not significant with media containing 5.0 mg/L IBA. As for the number of roots per shoot, it was found that the media containing 10.0 mg/L IBA produced the highest number of roots per shoot (2.24 ± 0.31), which was significant compared to the other media.

Rooting *in vitro* actually depends on the genotype of a plant. Sometimes rooting can occur naturally during the micropropagation process, so that the use of rooting media can be avoided. In some species, basal media can also promote the formation of roots (George and Sherrington, 1984). ½ MS media was used in the *in vitro* rooting process of *E. longifolia* shoots since it was found that lower mineral salts concentration help to increase the percentage of rooting and root length or number, in other species. However, the reaction can vary between cultivars of the same species. It was suggested that the ratio of carbon/nitrogen and nitrogen compounds in the auxins' metabolism affects the rooting process. More also, limited nutrients affect the production and development of the whole root system of plants *in vivo* (Karhu, 1997).

Hussein et al. (2005) reported that only the MS media containing 0.4 and 0.5 mg/L IBA showed the formation of roots from the *E. longifolia* shoots. A total of 90% root formation was shown in the MS media containing 0.5 mg/L IBA and 40% in the 0.4 mg/L IBA. Different observations were noted in this study since different media were used.

Acclimatization of plantlets in the nursery

Rooting and plantlet acclimatization of *E. longifolia* was the most difficult stage of this protocol since *E. longifolia* plantlets do not always react well when rooting *in vitro* and during acclimatization. Table 4 shows the percentage of plantlets survival observed after three weeks of transfer into the sandy media. It was found that plantlets planted in ½ MS medium containing 1.0 mg/L IBA showed the highest percentage of survival (75.0%) and the lowest percentage of survival in 7.5 mg/L IBA, with 30.4% percentage. This shows that although the number of roots per shoot produced in ½ MS medium containing 10 mg/L IBA was the highest in culture, this does not guarantee the survival rate when the plantlets are transferred to the nursery. George (1993) mentioned that *in*

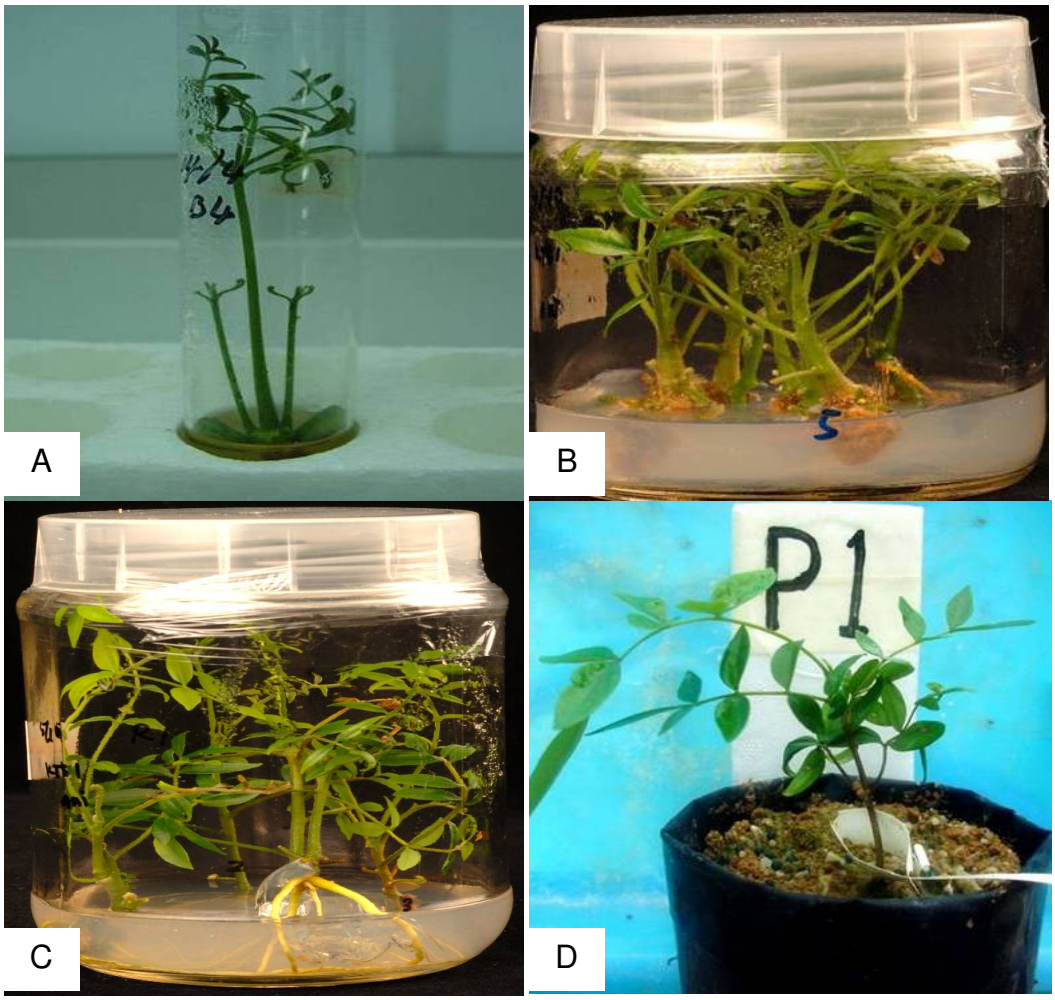

Figure 1. The sequence of the *E. longifolia* micropropagation from axillary shoots. (A) Shoots induction of *E. longifolia* in woody plant medium (WPM) containing 2.0 mg/L BAP. (B) Shoots multiplication of *E. longifolia* in MS media containing 0.5 mg/L BAP. (C) Root induction of *E. longifolia* in ½ MS media containing 10.0 mg/L IBA. (D) Acclimatization of *E. longifolia* plantlet in nursery.

vitro roots' main weakness is the difficulty to induce fully functional root system when the plants are transferred to the ground. *In vitro* roots usually have less hairy roots and vascular tissue, while secondary cam-bium tissue would not develop until they were released from the culture. In addition, *in vitro* roots may be injured when transferring the plants from the culture bottle to the nursery, thereby increasing the possibility for infectious diseases caused by fungus or bacteria. In addition, when the plantlets were transferred to the ground many roots that were formed during *in vitro* die without the formation of new roots. Beside that, *in vitro* leaves have fewer layers of wax on the inside and outside leaves and decrease the survival percentage of plantlets during acclimatization (George, 1993).

Figure 1 shows the sequence of the *E. longifolia* micro-propagation from axillary shoots. This technique used axillary buds from nodal cultures derived from *in vitro* germinated plantlets. Most flowering plants and woody plants were commercially propagated using axillary shoot. Pierik (1987) stated that axillary shoot technique is generally easier than other techniques. In addition, the multiplication rate is much faster, genetically stable and growth of plant resulting from this technique is better than other micropropagation techniques, with minimum variation.

Production of eurycomanone, 9-methoxycanthine-6-one and canthin-6-one in roots of *E. longifolia* plantlets and matured plant

Methanol extract of *E. longifolia* dried roots from tissue culture plantlets and matured root of *E. longifolia* were

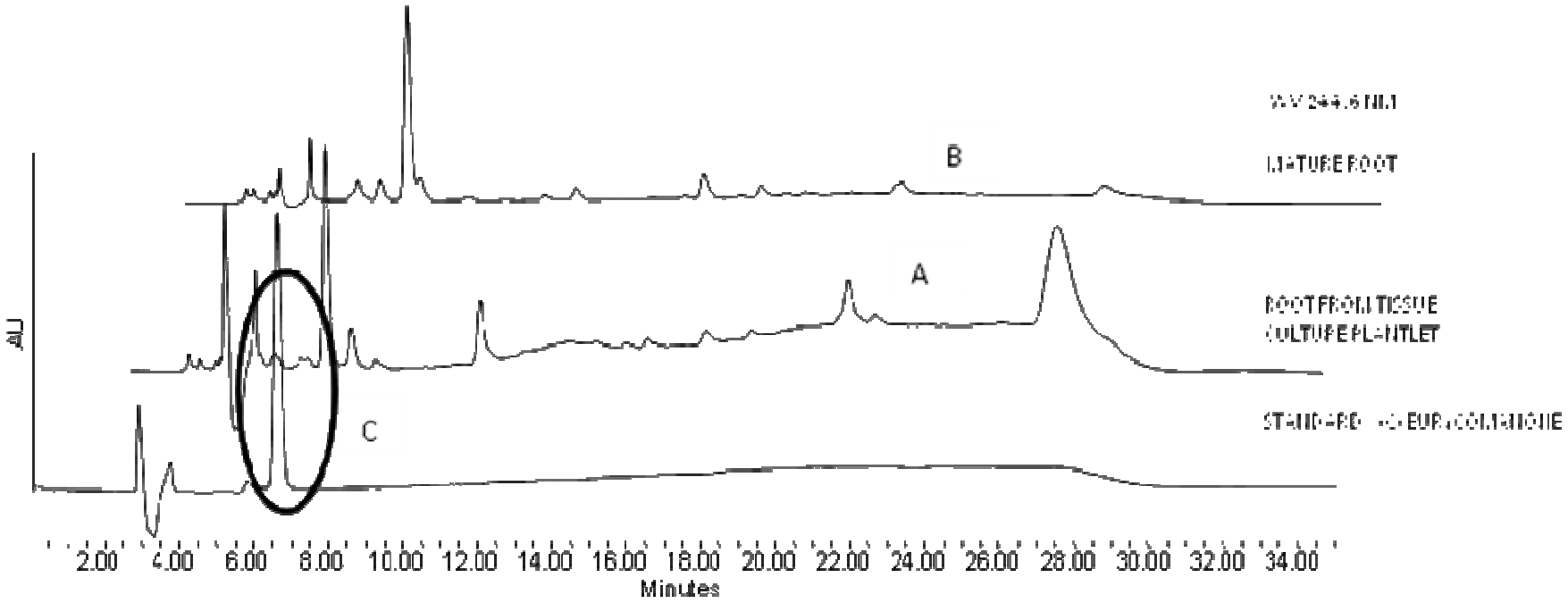

Figure 2. HPLC profile of methanol extract of two *E. longifolia* samples at 244.6 nm wavelength using eurycomanone compound as standard. A) Dried roots extract from tissue culture plantlets. B) Dried roots extract from mature *E. longifolia*. C) Eurycomanone peak at RT 6.577.

subjected to HPLC analysis. Observations were on the differences of the HPLC profile and the content of *E. longifolia* compounds in the samples. Three compounds were quantitatively determined; eurycomanone, canthin-6-one and 9-methoxycanthin-6-one. The others were not identified owing to lack of authentic references. In this study, eurycomanone peak was observed in RT 6.577, canthin-6-one in RT 25.263 and 9-methoxycanthin-6-one in RT 26.697 based on the standard HPLC profile. HPLC profiles of these two samples were similar (Figures 2 and 3) and the compounds that existed in the matured root were also present in the roots of *E. longifolia* tissue culture plantlets. Through the HPLC profile, it can be observed that in addition to the three main compounds, there are other compounds present that have yet to be identified and are still in research progress.

Furthermore, the concentration of the three main compounds was determined based on the standard HPLC profile (not shown). As shown in the Figure 4, the contents of the three compounds were relatively higher in *E. longifolia* plantlets than in the mature roots. Among the three compounds, the content of eurycomanone was found to be higher in dried roots of tissue culture plantlets (120.76 ppm/mg) compared with matured root of *E. longifolia* (101.26 ppm/mg). The yield of secondary metabolite in tissue culture plantlets was relatively higher than that *in vivo* as was reported for other secondary metabolites. Karam et al. (2003) reported that yield of rosmarinic acid in *in vitro* sage was higher than that in leaves or roots of greenhouse grown plants. Meanwhile, Mostafa et al. (2010) reported that *in vivo* leaves of *Arbutus andrachne* L. contained higher arbutin content (0.3 to 0.81% w/w) than *in vitro* leaves (0.09% w/w). Chemical constituents in the tissue

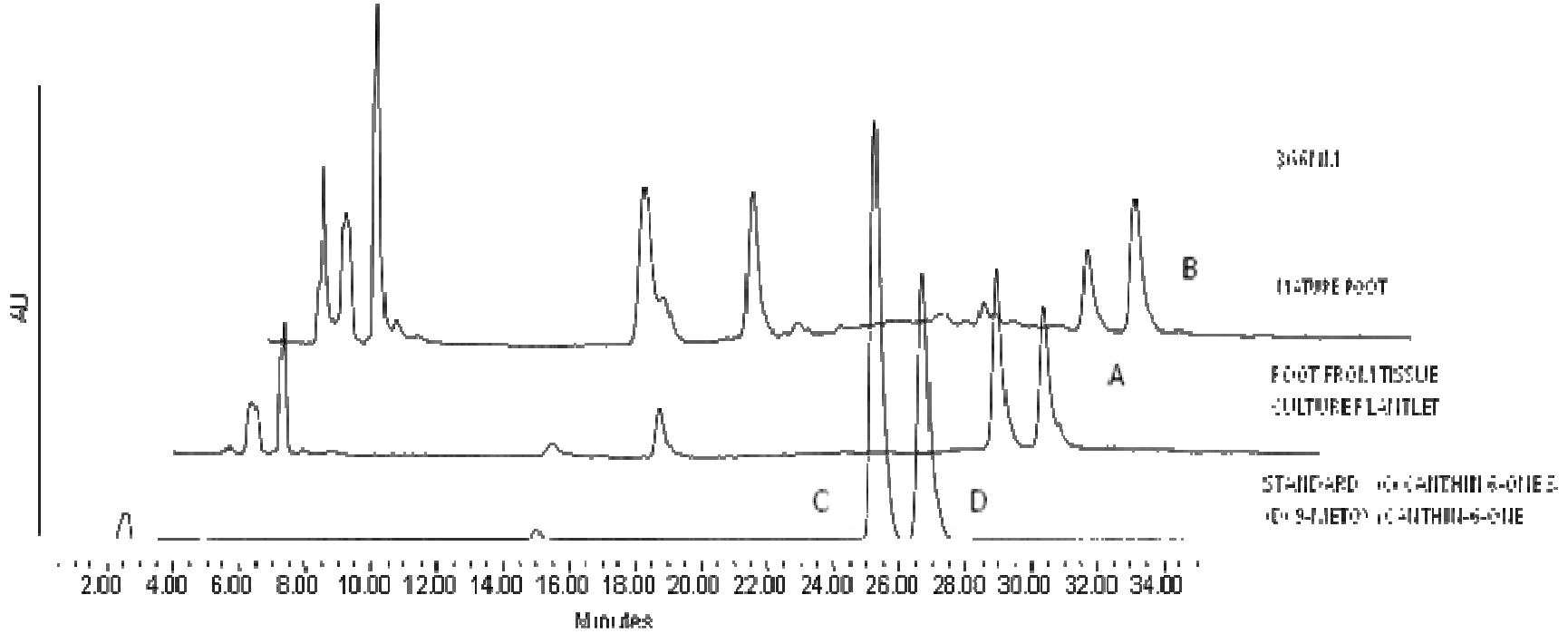

Figure 3. HPLC profile of methanol extract of two *E. longifolia* samples at 366 nm wavelength using 9-methoxycanthin-6-one and canthin-6-one as standard. (A) Dried roots extract from tissue culture plantlets. (B) Dried roots extract from matured *E. longifolia*. (C) Canthin-6-one peak at RT 25.263. (D) 9-Methoxycanthin-6-one peak at RT 26.697.

factors. Effect of chemical constituents on tissue culture plantlet's growth and secondary metabolite production is one of the major focuses in tissue culture study. For example, Demeyer and Dejaegere (1989) reported that nitrogen treatment showed positive effects on the increase of hyoscyamine in root culture of *Datura stramonium*.

This study provides techniques for rapid multiplication of *E. longifolia* starting from the sterilization techniques to the plantlets acclimatization in nursery. Results of this study show that *E. longifolia* plantlets also contain *E. longifolia* compounds and axillary shoot multiplication techniques produced true to type plantlets.

Conclusion

In this paper, we reported an effective mass propagation system for *E. longifolia* that is easy to establish and maintain. This *in vitro* strategy has the potential to hasten the development of a genetically stable system for the enhanced production of secondary metabolite in culture as well as facilitating cultivation, thereby alleviating pressure on wild population of *E. longifolia* plants.

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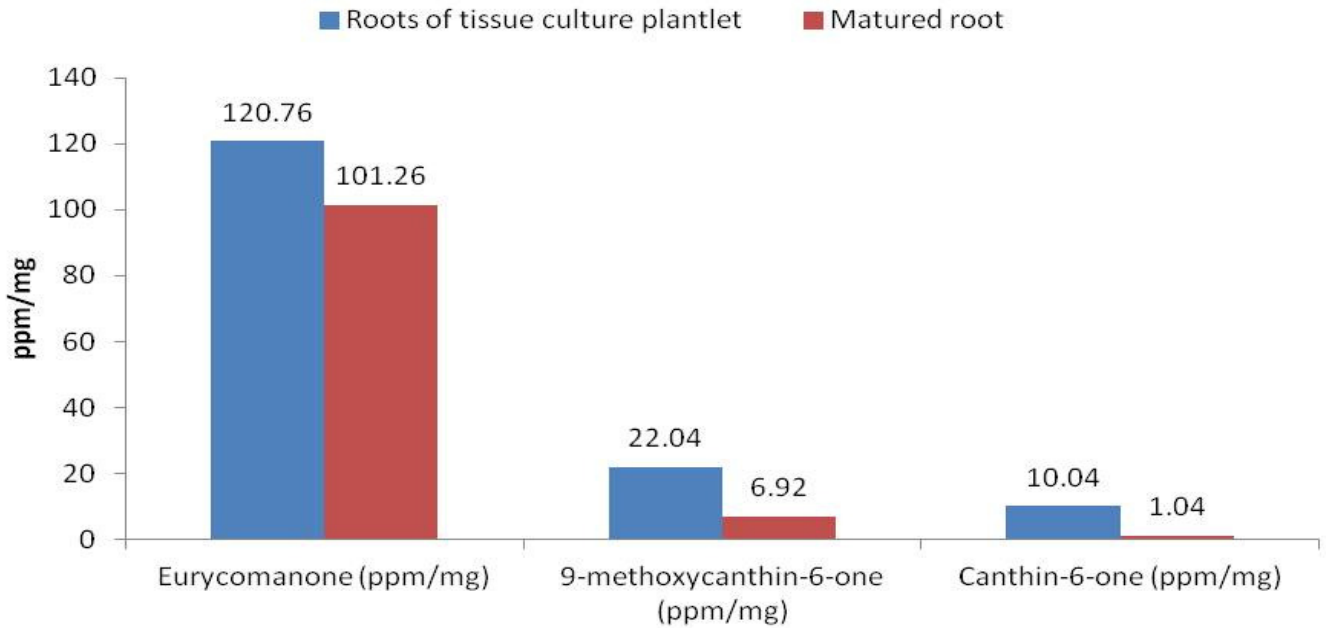

Figure 4. Concentration of eurycomanone, 9-methoxycanthin-6-one and canthin-6-one in dried roots of *E. longifolia* tissue culture plantlets and matured root.

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