PRODUCTION OF EURYCOMALACTONE AND SCOPOLETIN IN DIFFERENT PARTS OF 2 YEARS OLD EURYCOMA LONGIFOLIA (TONGKAT ALI) SEEDLINGS AND TISSUE CULTURE PLANTLETS

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ABSTRACT

Eurycoma longifolia (Tongkat Ali) is one of the most widely used herbs in Malaysia and other Asian country and its demand is increasing rapidly. As raw material supplies from natural resources begin to decreasing, it is necessary to cultivate them widely to meet the demands of the herbal and pharmaceutical industries. For this purpose, seedlings are the main requirement because the harvest of E. longifolia involves the whole tree. The production of seedlings through the tissue culture method is one of the commonly used methods to contribute in increasing the supply of planting material. Plantations for forest species and herbal plants are needed to address the problem of insufficient supply of raw materials to meet the demands of the timber, herbal and pharmaceutical industries. Quality of E. longifolia raw material for the herbal and pharmaceutical product muchly depends on the production of chemical compounds in the planting material. In this study, eurycomalactone and scopoletin is our main focus, since this compounds are also valuable compound of E. longifolia and have substantial effect on human health. Stem and leaves also evaluated because normally for E. longifolia products the roots were harvested. If stem and leaves have comparable amount of bioactive compound so we can save E. longifolia trees from destructive method. In this study, different parts which is stem, roots and leaves of 2 years old E. longifolia seedlings and tissue culture plantlets grown in the nursery were evaluated for the production of eurycomalactone and scopoletin using High Performance Liquid Chromatography (HPLC). The results of chemical analysis showed that eurycomalactone and scopoletin were found in root and stem samples except leaf samples from tissue culture plantlets and seedlings.

Keywords: *E. longifolia, tissue culture plantlets, seedlings, chemical analysis*

INTRODUCTION

Eurycoma is a small genus in the family Simaroubaceae. It is native to Myanmar, Thailand, Indochina and Southeast Asia. The Simaroubaceae family contains many bitter plants. It is locally known as Tongkat Ali, Penawar Pahit or Setunjang bumi. The *E. longifolia* Jack tree does not branch naturally but in certain cases it can produce branching when it is wrecked. Its height can reach 8-10 meters, and the trunk diameter can reach 15 centimeters (Kulip & Wong, 1996). The specialty of this species is the umbrella-shaped concentration of leaves at the upper end of the stem. Therefore, it was also called Payung Ali in some places (Burkill, 1966).

In the last decade, many papers have been published on the chemical composition of *E. longifolia*; for example, *E. longifolia* contains 10-hydroxycanthin-6-one, 9-methoxycanthin-6-one, eurycomanone, eurycomalactone, and eurycomanol that had been reported to have *in vitro* anti-cancer effects (Yunos et al., 2021). These compounds are used as reference markers to standardize *E. longifolia* products (Chan, 2004). The active ingredients are concentrated mainly in the tap roots. Biological studies have shown that *E. longifolia* root has antimalarial (Chan et al., 1986), antiulcer (Tada et al., 1991) and cytotoxic (Kardono et al., 1991) The root of the high-value plant *Eurycoma longifolia* Jack (EL) also has aphrodisiac effects. It is helpful for treating erectile dysfunction (ED), a disease that is accompanied by metabolic syndrome and inflammation. Additionally, it is employed in conventional medicine to treat indigestion, dropsy, fever after childbirth, jaundice, cachexia, and low back discomfort (Rehman et al., 2016). Meanwhile one of the naturally prevalent coumarins that is frequently found in many edible plants and also found in *E. longifolia* is scopoletin, also known as 6-methoxy-7 hydroxycoumarin, and it has a significant impact on human health (Antika et al., 2022). The detection of scopoletin in *E. longifolia* plant samples are reported by Chaingam et al. (2021).

The production of plantlets through the tissue culture method is one of the commonly used methods to contribute in increasing the supply of planting material besides using seeds. Plantations of medicinal plants are needed to address the problem of insufficient supply of raw materials to meet the demands of herbal and pharmaceutical industries. Quality of *E. longifolia* raw material in the herbal and pharmaceutical products mainly depends on the production of chemical compounds in the planting material. In our previous study, roots of *E. longifolia* plantlets contain eurycomanone, 9-methoxycanthine-6-one and canthin-6-one. Eurycomalactone and scopoletin are the main subjects of this study since they are both also useful substances of *E. longifolia* with significant effects on human health. Because the roots are typically taken for *E. longifolia* products, the stem and leaves were also assessed. We can protect *E. longifolia* from destructive methods if the stem and leaves have an equivalent amount of bioactive chemical.

Because of the reason stated above, the objective of this research is to evaluate the production of eurycomalactone and scopoletin in different parts (stem, leaves and roots) of 2 years old seedlings and tissue culture plantlets of *E. longifolia* grown in the nursery. We hope that these two compounds can be found in stem and leaves so we can save *E. longifolia* trees from destructive harvesting.

MATERIAL AND METHODS

Samples collection

E. longifolia mature seeds were collected from Setiu, Terengganu (Figure 1).

Figure 1. E. longifolia mature seeds in Setiu, Terengganu

Ex vitro germination

Seeds were sowing in polybag containing sand (Figure 2) for *ex vitro* germination in the nursery.

Figure 2. Ex vitro germination of E. longifolia seeds in the nursery

Production of *E. longifolia* tissue culture plantlets

Seeds were surface sterilized using Ethanol and Clorox® to produce aseptic culture using method established by Nor Hasnida et al. (2012). *In vitro* germinated plantlets are used as explants for shoot multiplication. MS basal medium containing 0.5 mg/L BAP is used for shoot multiplication. For *in vitro* rooting, ½ MS basal medium containing 1.0 mg/L IBA are used for the production of complete plantlets before acclimatization in the nursery. Establishment of tissue culture plantlets are shown in Figure 3.

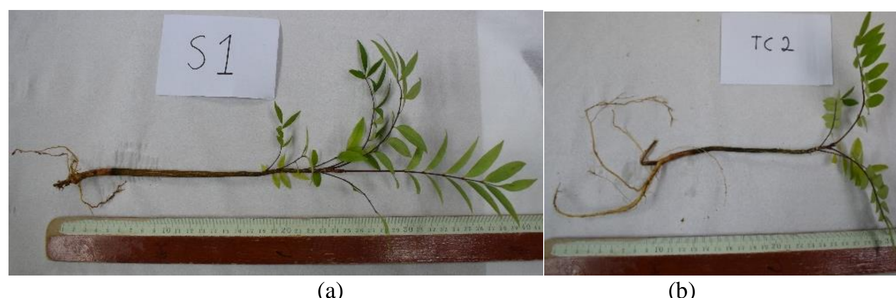
Figure 3. Establishment of E. longifolia tissue culture plantlets using tissue culture techniques, a) Shoot multiplication of E. longifolia in MS basal medium supplemented with 0.5 mg/L BAP, b) In vitro rooting of E. longifolia shoots in ½ MS basal medium plus 1.0 mg/L IBA

(a) (b)

Plant samples

Two years olds seedlings and tissue culture plantlets of *E. longifolia* grown in the nursery (Figure 4) were used as plant samples for chemical analysis.

Figure 4. a) 2 years old seedling of E. longifolia, b) 2 years old tissue culture plantlets of E. longifolia, c) Roots, stem and leaves of E. longifolia samples for chemical analysis



(a) (b)

(c)

Sample preparation

E. longifolia plant samples, seedlings (S) and tissue culture plantlets (T) are divided to three parts (leaves, stem and roots). Fresh weight and dry weight of all the samples were recorded. All samples tested are dried in the oven at a temperature of 40˚C.

A. Test sample preparation

All samples are weighed and dissolved in methanol to produce 100 mg/mL test solution. Each samples were extracted in triplicate (n = 3)**.**

B. Preparation of reference standard solution

Reference standard solution of eurycomalactone and scopoletin with a concentration of 1000 µg/mL are prepared in methanol solution.

C. High Performance Liquid Chromatography (HPLC)

All samples were analysed using High Performance Liquid Chromatography (HPLC) system (WATERS 600 quaternary gradient pump, WATERS 2707 auto sampler and WATERS 2996 PDA) and Luna C18 2535-0088 column (5 µm, 250 mm x 4.6 mm) with two solvent systems: A (0.1% formic acid in water) and B (acetonitrile) as in Table 1. Flowrate were fixed at 1 mL/min with sample volume of 10 µL. Retention time data and clear UV spectrum peak were analysed and recorded.

Table 1 Solvent system for HPLC analysis

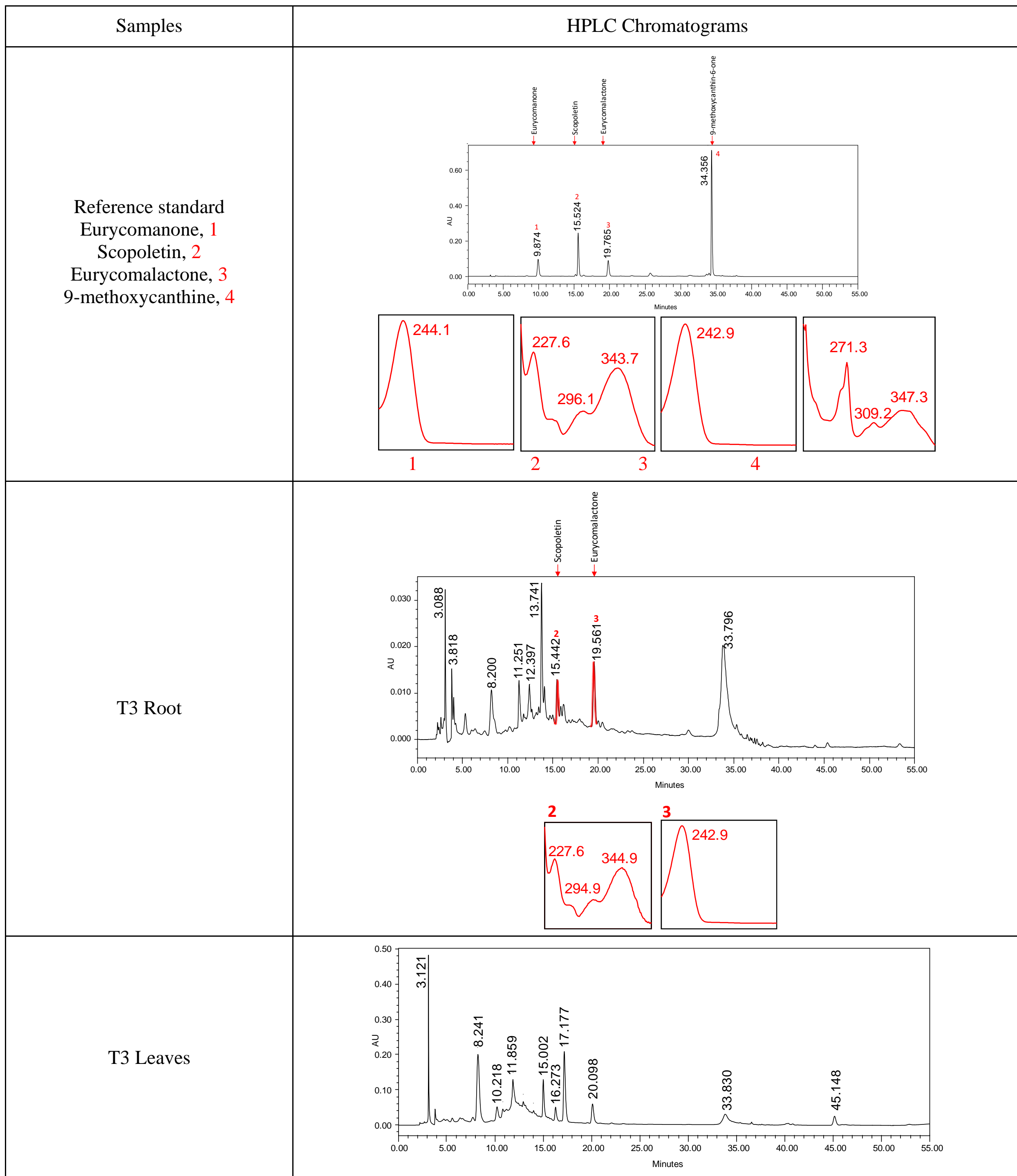
Time (min)	% A (0.1% formic acid in water)	% B (Acetonitrile)
0	90	10
5	75	25
25	70	30
28	0	100
30	0	100
32	5	95
55	5	95

RESULTS AND DISCUSSION

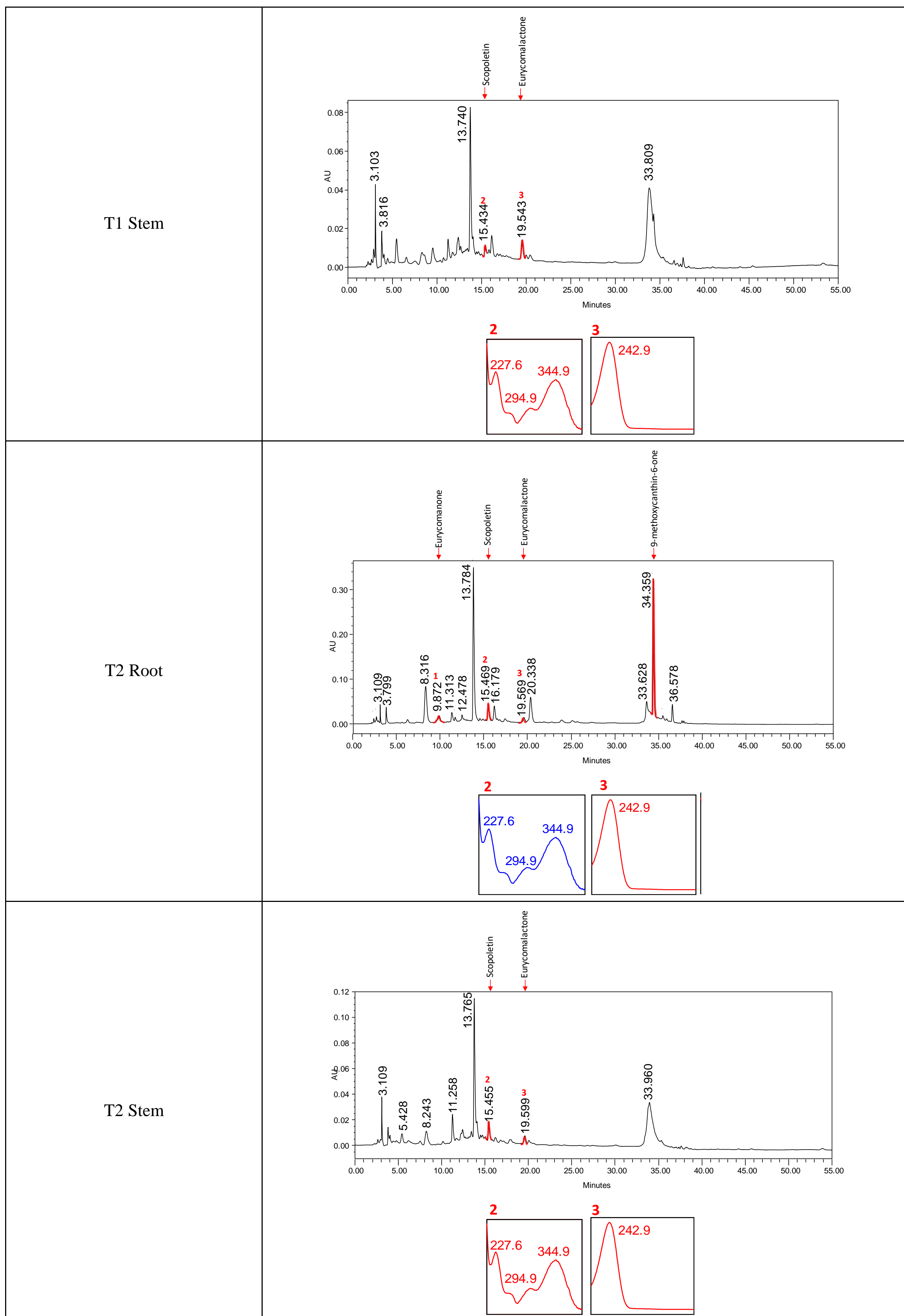
Chemical analysis on extracts of *E. longifolia* seedlings and tissue culture plantlets using HPLC

In this study, extracts of different parts of 2 years olds seedling and tissue culture plantlets of *E. longifolia* which is stem, leaves and roots are analysed using HPLC. HPLC chromatogram profile of eurycomalactone and scopoletin as reference standard and all parts of *E. longifolia* seedlings and tissue culture plantlets are shown in Figure 5.

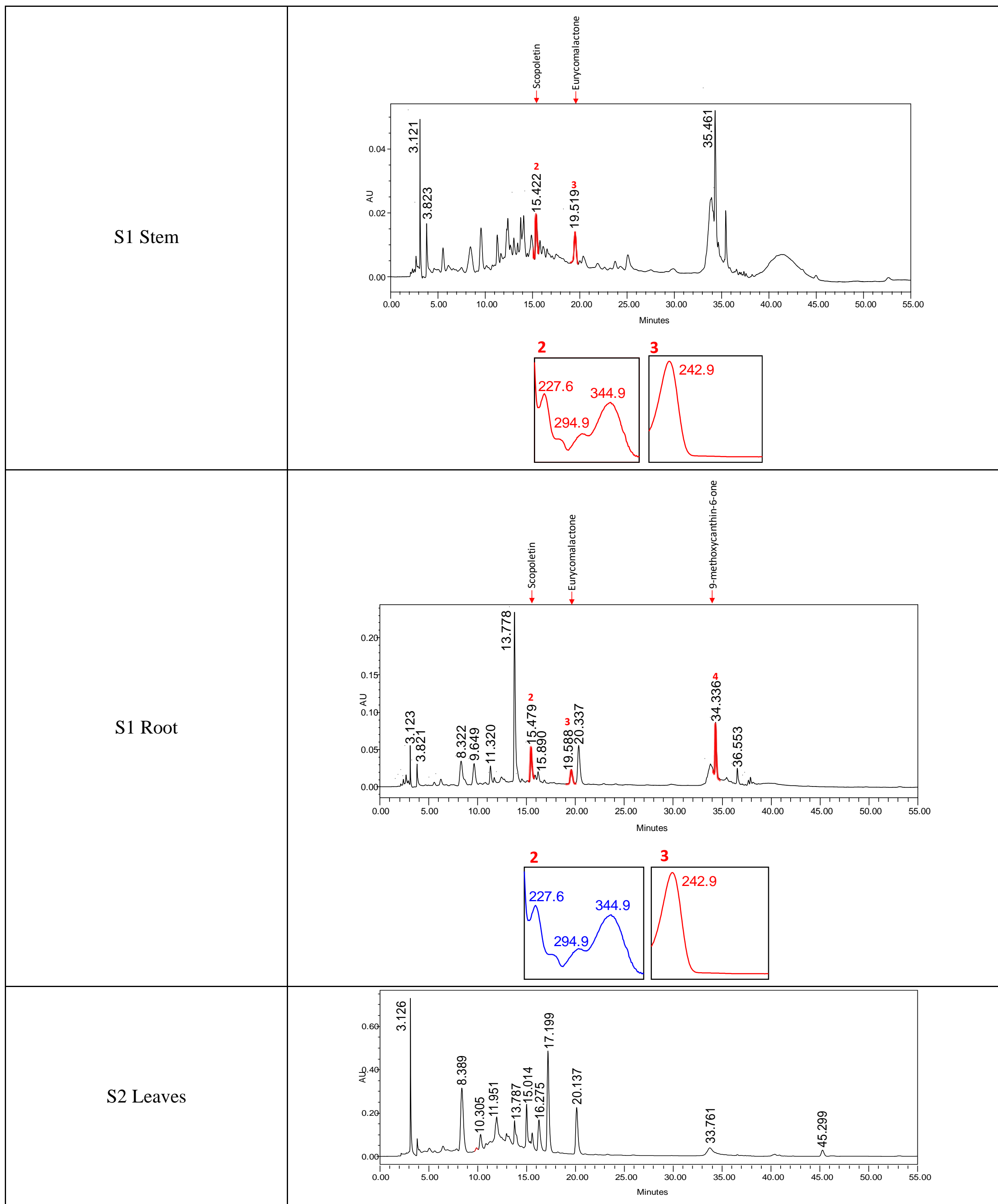
Figure 5. HPLC chromatogram of E. longifolia reference standard and extracts of all parts of seedlings and tissue culture plantlets at 254 nm.

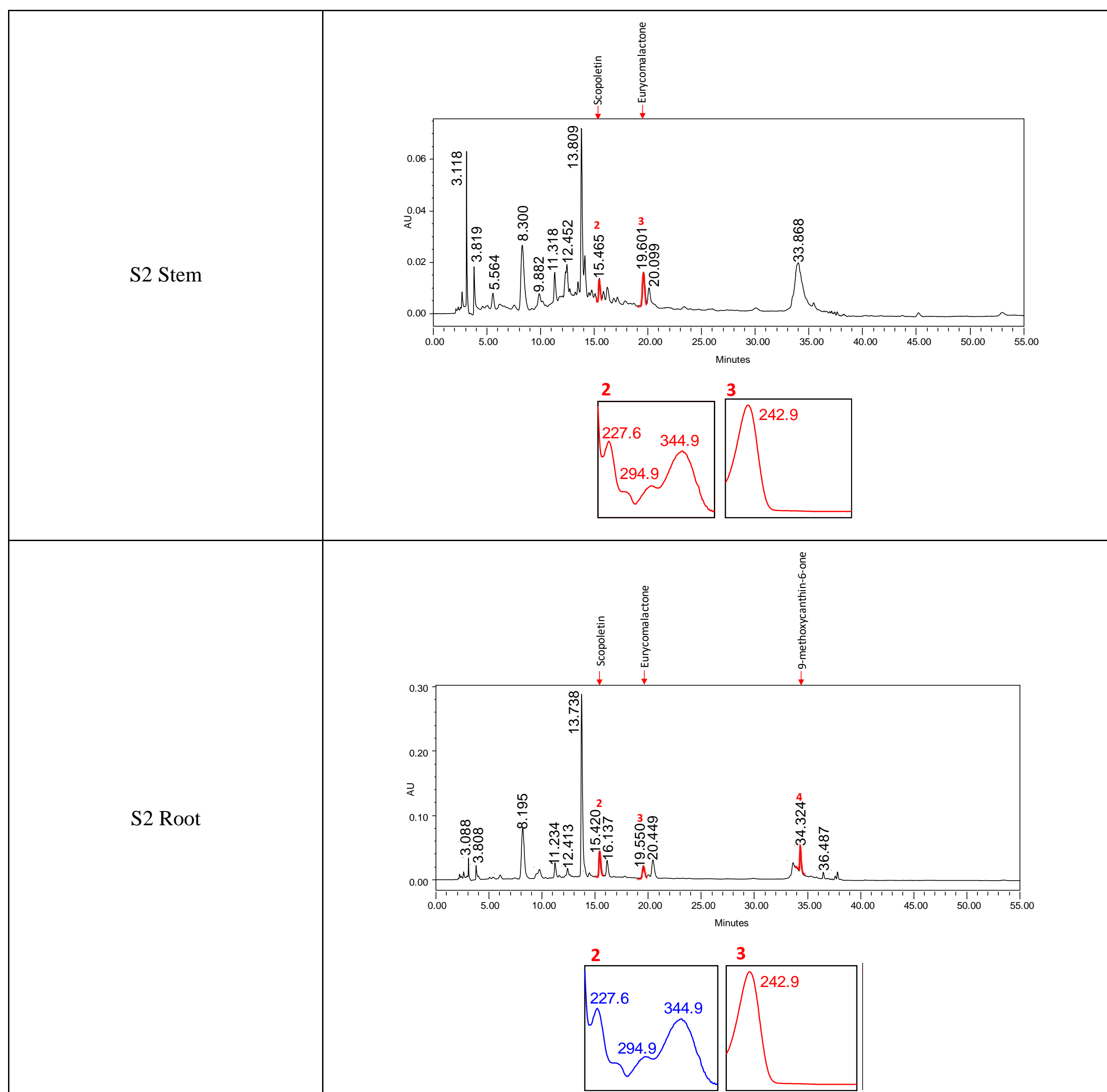


T3 Stem	
T1 Leaves	
T1 Root	


T1 Stem
Scopoletin
Eurycomalactone
AU
3.103
3.816
13.740
15.434
19.543
33.809
Minutes
227.6
344.9
294.9
242.9
T2 Root
Eurycomanone
Scopoletin
Eurycomalactone
9-methoxycanthin-6-one
3.109
3.799
8.316
9.872
11.313
12.478
13.784
15.469
16.179
19.569
20.338
33.628
34.359
36.578
T2 Stem
3.109
5.428
8.243
11.258
13.765
15.455
19.599
33.960

S3 Leaves	
S3 Stem	
S3 Root	


S1 Stem
Scopoletin
Eurycomalactone
AU
Minutes
3.121
3.823
15.422
19.519
35.461
2
3
227.6
344.9
294.9
242.9
S1 Root
Scopoletin
Eurycomalactone
9-methoxycanthin-6-one
AU
Minutes
3.123
3.821
8.322
9.649
11.320
13.778
15.479
15.890
19.588
20.337
34.336
36.553
2
3
4
227.6
344.9
294.9
242.9
S2 Leaves
AU
Minutes
3.126
8.389
10.305
11.951
13.787
15.014
16.275
17.199
20.137
33.761
45.299



HPLC chromatogram profiles of retention time values and UV spectra were used to determine the presence of reference compounds: Eurycomanone, eurycomalactone, 9-methoxycanthine-6-one and scopoletin. Quantitative analysis is carried out on all samples that show the presence of eurycomalactone and scopoletin compounds based on chromatogram profile to determine the percentage of these compounds in the test samples. The concentration and the percentage content of the compounds in the test samples is summarized in Tables 2 and 3.

Eurycomalactone is a powerful NF-B inhibitor with anti-inflammatory effects (Tran et al., 2014). Meanwhile, Chan et al. (1986) reported that eurycomalactone possess antiplasmodial activity against a multi-drug resistant Thailand strain (K-1) of *Plasmodium falciparum*. From the chromatogram profile shown in Figure 5, it was observed that eurycomalactone were detected in root and stem samples from both *E. longifolia* tissue culture plantlets and seedling and its concentration are higher in roots compared to stem samples (Table 2). The highest concentration of eurycomalactone is observed in roots of seedlings (S3=63.67 ± 2.00) followed by roots of tissue culture plantlets (T1=59.33 ± 1.07). The percentage of eurycomalactone in both samples is 0.06%. It was also noted that eurycomalactone concentration in leaf samples were either none available or the concentration is too low and cannot be detected by HPLC.

Accumulation of eurycomalactone in samples of *E. longifolia* tissue culture plantlets are comparable with seedlings. Chaingam et al. (2022) also reported that eurycomalactone is predominant in *E. longifolia* callus culture. The metabolite type and concentration in *E. longifolia* plant extracts, very much be influenced by the processing temperature as well as geographical factors (Rehman et al., 2016).

Table 2 Quantification of eurycomalactone compound in tested samples

Samples	Mean concentration of eurycomalactone ± RSD (ppm)	Mean percentage of eurycomalactone in samples (w/w)
T1 Stem	24.97 ± 1.42	0.02
T1 Root	59.33 ± 1.07	0.06
T2 Stem	13.06 ± 5.05	0.01
T2 Root	25.35 ± 6.15	0.03
T3 Stem	14.89 ± 4.18	0.01
T3 Root	36.62 ± 0.57	0.04
S1 Stem	21.41 ± 1.97	0.02
S1 Root	48.41 ± 1.57	0.05
S2 Stem	32.47 ± 0.31	0.03
S2 Root	50.34 ± 0.40	0.05
S3 Stem	23.59 ± 1.75	0.02
S3 Root	63.67 ± 2.00	0.06

Note: RSD – Relative Standard Deviation

The other compound that is focusing in this study is scopoletin. Scopoletin is the secondary product known as coumarin. It is classified as 1,2-benzopyrones. Plants and fungus both contain this substance. Scopoletin, also known as 6-methoxy-7-hydroxycoumarin, is a naturally produced coumarin that is frequently present in many edible plants and is crucial for maintaining good health in humans (Antika et al., 2022).

In nature, the presence of scopoletin is frequently associated with the plant's defence system against parasite and microbial infection (Tanaka et al., 1983). Numerous therapeutic plants have yielded this compound, which has been isolated (Tal & Robeson, 1985). In numerous species and plant families, scopoletin has been found in a variety of plant parts at varying degrees of concentration. The distribution of scopoletin in different botanical groups, including the Asteraceae, Convolvulaceae, Rubiaceae, Solanaceae, and Moraceae (Antika et al., 2022).

In this study, the production of scopoletin in *E. longifolia* tissue culture plantlets and seedling are evaluated. Results in Table 3 showed that scopoletin were found in all samples except leaves samples from both *E. longifolia* tissue culture plantlets and seedlings. Scopoletin in the leaf samples not detected maybe because of the amount is too low. It was also noted that the concentration of scopoletin are higher in roots samples compared to stems samples from both *E. longifolia* seedlings and tissue culture plantlets. The highest concentration of scopoletin are found in roots of seedlings (S1 = 44.48 ± 2.85) followed by roots of tissue culture plantlets (T1 = 41.34 ± 1.70). But the highest percentage of this compound is found in S3 stem sample (0.16%). Chaingam et al. (2021) also detected scopoletin in *E. longifolia* roots using HPLC-UV method. Scopoletin can be used for the prevention and treatment of various diseases, including inflammation, infectious diseases and metabolic related-diseases, as well as neurogenerative disease (Antika et al., 2022; Chaingam et al., 2021).

Table 3 *Accumulation of scopoletin in* E. longifolia *samples*

Samples	Mean concentration scopoletin ± RSD (ppm)	Mean percentage scopoletin in samples (w/w)
T1 Stem	5.40 ± 3.67	0.01
T1 Root	41.34 ± 1.70	0.04
T2 Stem	13.56 ± 1.07	0.01
T2 Root	38.66 ± 1.21	0.04
T3 Stem	5.91 ± 3.41	0.01
T3 Root	9.90 ± 3.43	0.01
S1 Stem	36.62 ± 0.57	0.04
S1 Root	44.48 ± 2.85	0.04
S2 Stem	9.30 ± 3.06	0.01
S2 Root	40.07 ± 1.02	0.04
S3 Stem	20.04 ± 2.41	0.16
S3 Root	40.30 ± 0.93	0.04

Note: RSD – Relative Standard Deviation

This is the first report on the production of eurycomalactone and scopoletin compounds in *E. longifolia* tissue culture plantlets. *E. longifolia* tissue culture plantlets can be used as an alternative planting material beside seedlings for the establishment of plantation. For the supply of both compounds, either the stem or the root of *E. longifolia* tissue culture plantlets and seedlings can be used.

CONCLUSION

In conclusion, eurycomalactone and scopoletin are only detected in root and stem samples and not detected in leaves samples of *E. longifolia* tissue culture plantlets and seedlings. Leaves of *E. longifolia* seedlings and plantlets are not recommended to use for the extraction of both compounds. Tissue culture plantlets produced can be used as an alternative planting material for the *E. longifolia* plantation since it can also yield *E. longifolia* active ingredient.

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