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Research Paper

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Cytotoxic Activity of Cell Suspension Cultured and Plant Derived Crude Extracts of Tongkat Ali (*Eurycoma longifolia* Jack) against Human Cancer Cell Lines

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***Abstract*—** There are more than 200 different known cancers that afflict humans. Many researchers currently prioritize their research towards development of anticancer treatments with plant derived products. Our study aims to investigate the anticancer potential of Tongkat Ali (*Eurycoma longifolia*) extracts derived from the cell suspension cultures and the roots, stems and leaves of the mature plants (7 years old) against three human cancer cell lines: A549 (lung cancer), HCT116 (colon cancer) and SKBR3 (breast cancer). Results obtained showed that the chloroform, ethyl acetate and n-butanol extracts derived from the cell suspension cultures exhibit cytotoxicity activities with wide variation of IC_{50} values ranging from 40.9 to 932.1 µg/ml. While the three different solvent extracts derived from the root, stem and leaves of the mature plant exhibit better cytotoxicity activity with IC_{50} between 32.9 and 109.9 µg/ml. The cytotoxicity activity of the water extracts for the cell cultures could not be determined due to cell viability of the cancer cell lines were above 50%. While the water extracts obtained from the root, stem and leaves showed cytotoxicity activity with IC_{50} values of 33.7 – 214.3 µg/ml. Extracts from the dried cell suspension cultures indicate having similar compounds with the root extracts separated using thin layer chromatography. Hence the cell suspension cultures of *E. longifolia* can be used as one of the tools for the production of bioactive compounds and as an alternative means for prevention of its eventual extinction. It can also be established as the potential materials source besides the different plant parts for the preparation of anticancer plant-based products.

Keywords— Cell suspension culture, Tongkat Ali, Eurycoma longifolia, anticancer, cytotoxicity activity

I. INTRODUCTION

Tongkat Ali *(Eurycoma longifolia* Jack), a member of the Simaroubaceae family, is a tropical rainforest medicinal plant found mainly in Malaysia, Indonesia, Vietnam, Thailand, Laos and Cambodia. It is an evergreen tree which can grow up to 10 metres tall with flowering and fruiting only once a year [1]. In South-East Asian regions, the roots are used as folk medicine for the treatment of sexual dysfunction, malaria, cancer, diabetes, anxiety, constipation, fever and also as a health tonic [2]. *E. longifolia* root extracts have been reported to possess aphrodisiac, analgesic, antipyretic, antiulcer, antimalarial, antifungal and anticancer properties [3, 4, 5]. The plant extracts had also been reported to possess high cytotoxicity activity against P388 leukaemia cells, human lung cancer (A549) cell line, human breast cancer (MCF-7) cell line and human liver cancer [6, 7]. The root extract was reported to have protective effect in rats against hepatotoxicity [8]. It was also recommended as an alternative treatment for male osteoporosis [9]. Various alkaloids such as quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, tirucallane-type triterpenes, squalene derivatives and biphenylneolignans were successfully isolated from the roots and leaves of *E. longifolia.* These bioactive metabolites were reported to be the effective therapeutic agents for various diseases [6, 10, 11]. They were also found to be responsible for the cytotoxicity and mutagenicity effects [12,13], anti-hypertriglyceridemia, anti-inflammatory and analgesic activities in animal models [5], antitumor activity against fibrosarcoma cells [14] and tyrosinase inhibition activity [15].

There are more than 200 *E. longifolia* products registered with the National Pharmaceutical Control Bureau of Malaysia. These products are available as health food either in the form of raw crude root powder or as capsules mixed with other herbs. Approximately 21,000 kg of *E. longifolia* are being harvested from the rainforest in Malaysia to meet the market demand of more than 54,000 kg per year [16]. Its genetic diversity is decreasing and facing the possibility of extinction due to widespread

indiscriminate harvesting to meet the high market demand for the preparation of various herbal products. To overcome the eventual extinction of important medicinal plant species, various *in vitro* culture techniques have been used by various researchers. *In vitro* propagation technique was used as a means for conservation of *Maerua Arenaria*, an important medicinal climbing shrub in India (17) while clonal propagated *Withana somnifera* L. Dural seedlings were used for the production of therapeutically valuable compounds [18]. Micropropagation of *E. longifolia* via embryogenesis technique was proposed for production of planting materials [19]. While root cultures, callus and cell suspension cultures were established for the production of bioactive metabolites from this species [20, 21, 22, 23, 24, 25].

With the increasing number of cancer cases worldwide, and the decreasing population of this valuable *E. longifolia* plant, an alternative cell suspension culture method is proposed as the supply of material source for the preparation of anti-cancer products. Hence, the present study aims to investigate the cytotoxicity activity of the crude extracts derived from the cell suspension cultures and compared with the roots, stem and leaves of seven year old mature plant of *E. longifolia* against three human cancer cell lines: human alveolar adenocarcinoma (lung cancer) cell line (A549), colon cancer cell line (HCT116) and breast cancer cell line (SKBR3). It is also to determine whether the cell suspension culture technology can be used as an alternative tool for the production of the bioactive compounds with anti-cancer properties and alternatively as a means for the prevention of eventual extinction of *E. longifolia.*

In the current study, Section I comprises of brief introduction on the demand, traditional and pharmaceutical uses of Tongkat Ali (*E. longifolia*) and the establishment of its cell suspension cultures as an alternative means for the production of crude extract with anticancer properties. Section II describes the methodology used for the preparation of the crude extracts derived from the cultured cells and that of the mature plant of *E. longifolia* and the cytotoxicity tests for the prepared crude extracts. Section III explains the results obtained and discussion. While Section IV concludes the research findings.

II MATERIALS AND METHODS

Plant Materials

The roots, leaves and stem of *E. longifolia* were obtained from a mature (7 years old) plant grown at School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The collected samples were air dried in an air-conditioned room with temperature maintained at 25 ± 2°C until constant weight was attained. Three different cell suspension cultures were prepared from three different callus cell lines (Eu5, Eu6 and Eu12) derived from the leaf explants. Each cell culture was established by inoculating 0.5g of the friable callus in 25 ml of MSBs liquid medium, a modified MS medium formulated by Siregar et al. [24]. The pH of the culture medium was adjusted to 5.7-5.8 prior to autoclave at 121^{o} C and 1.05kg/cm^3 for 13 minutes with Elite Sterilizer (EAC400°C). The cultures were maintained at 23 ± 2°C with continuous illumination with light intensity of 32.5μE $m^{-2}s^{-1}$. The cultures were placed on an orbital shaker and agitated at 120rpm for 13 days. The cells were then harvested and air dried in air-conditioned room at 25 ± 2°C until constant weight was obtained. The plant materials intended for further studies were then grinded to power form.

Preparation of crude extracts

Successive extraction methods were performed on the six samples separately to obtain chloroform, ethyl acetate, n-butanol and water extracts. The six samples under studied were the dried root, leaf and stem of seven years old plant and the dried cells of Eu5, Eu6 and Eu12 cell lines. An amount of 35 g for each dried sample was weighed and extracted three times with methanol under reflux at 60°C for 30 minutes. The extracted solutions were filtered and concentrated *in vacuo*. This was then partitioned between chloroform and water (2:1) to yield the chloroform extract and the aqueous layer. The latter was then partitioned with ethyl acetate and water (2:1) followed by n-butanol and water (2:1) yielding the ethyl acetate, n-butanol and water layers. All the layers obtained were evaporated to dryness *in vacuo*.

Culture of Human Cancer Cell lines

Cell lines of human alveolar adenocarcinoma (A549), colon cancer (HCT116) and breast cancer (SKBR3) were obtained from the American Type Culture Collection (ATCC). They were cultured in 25 cm^3 TPP flask containing DMEM medium (Sigma) for A549 cell line, McCoy's 5A modified medium (Sigma) for HCT116 cell line and RPMI-1640 medium (Sigma) for SKBR3 cell line all supplemented with 10% foetal bovine serum under incubation conditions of 5% CO_2-95% humidified air at 37°C.

Cytotoxicity assay

The cultured human cells were plated at a concentration of 5 x 10^3 cells/well in 96-well plates and incubated for 24 hours. The cells were then treated with the crude extracts of the four solvent layers prepared at five different concentrations (0.0625 – 1.0 mg/ml) and left to incubate for 72 hours. The growth inhibitory effects of the crude extracts from the four solvent layers were evaluated with a WST-1 colorimetric assay. WST-1 dye was added at a concentration of 10% and incubated for 1 hour. Absorbance was determined at 450 nm with a reference wavelength of 630 nm using a micro-plate reader. The experiment was repeated three times for each solvent sample and the 50% growth inhibitory concentration (IC_{50}) was calculated according to the inhibitory effect model curve (Microsoft Excel).

Thin Layer Chromatography

Crude extracts of chloroform, n-butanol and ethyl acetate layers were separated using thin layer chromatography

glass plates coated with Silica gel 60 F254 to observe the difference in compounds between extracts originating from cell suspension cultures and the different plant parts. For chloroform layer, a solvent system of $CHCl_3$:MeOH (19:1) was used for separation while for ethyl acetate layer a solvent mixture of $CHCl_3$:MeOH (3:1) was used. Separation of n-butanol layer was done in a solvent system of $CHCl_3$:MeOH:H_2O (7:3:0.5). The spots were observed under irradiation of UV lamp (366 nm). The R_f value for each compound was determined as:

$$R_f = \frac{\textit{Distance from baseline travelled by solute}}{\textit{Distance from baseline travelled by solvent front.}}$$

Statistical Analysis

Comparison of the extract yield and the IC_{50} values between the six studied materials of *E. longifolia* were analysed using One-way Analysis of Variance (ANOVA) followed by mean comparison using the Tukey HSD test at $p \leq 0.05$. Statistical analysis was performed using SPSS ver 20.0 software for windows.

III RESULTS AND DISCUSSION

Extraction yields of plant materials

Successive extraction method conducted on the dried cells derived from the cell suspension cultures and the different plant parts of *E. longifolia* had successfully yielded four different layers of extracts with varying amount. Extracts derived from the dried cells of three different lines were of higher yield as compared to those obtained from the dried plant materials except for the ethyl acetate and n-butanol layers of leaf extract which were more than double the amount as compared to that derived from the cell suspension cultures. It could also be seen that the water and chloroform extracts from the dried cells of each cell line produced significantly higher yield than that obtained from the dried plant materials. The yields were also found to be different among the three cell lines except those of chloroform extracts which was not significantly different among the three cell lines. The yield of the leaf extracts was also 3.6 to 10 times higher than those of root and stem extracts. In general, the variation of extract yield for each solvent layer obtained from the cultured cells was small while the extract yield from the three different plant parts had a high variation (Table 1). The higher yield of crude extracts from the dried cultured cells than the plant materials could be due to the simple structures of cultured cells that are made up mainly of cell wall, plastids, cytoplasm, nucleus and vacuoles. While the leaf, root and stem of *E. longifolia* mature plant are made up of specialized plant cell types and tissues which include parenchyma cells, collenchyma cells, sclerenchyma cells, and the conducting cells, xylem and phloem [26, 27]. The cell wall and contents of the cultured cells are more easily broken down when extracted with the various organic solvents. The presence of specialized differentiated tissues of the leaf, root and stem making it harder to extract the contents from the fibrous woody plant parts.

Cytotoxicity activity of the crude extracts

The different solvent extracts derived from the different plant parts of *E. longifolia* mature plant generally showed higher and consistent cytotoxic effect against the human alveolar adenocarcinoma cells (A549) with low IC_{50} values ranging between 32.9 and 61.9 µg/ml as compared to the suspension cultured cells with great variation of IC_{50} values ranging from 52.5 to 932.1 µg/ml. Only n-butanol crude extract of suspension cultured cell line Eu5 and the ethyl acetate crude extract of cell line Eu6 showed good cytotoxicity activity with IC_{50} values of 55.4 and 52.5 µg/ml respectively (Table 2).

Cytotoxicity activity of the different solvent crude extracts of *E. longifolia* against the human colon cancer cell lines HCT116 showed similar trend in which the extracts obtained from the root, stem and leaf of the mature plant possessed stronger cytotoxicity activities as that of the suspension cultured cells except the ethyl acetate extract of cultured cell line Eu6 showed high cytotoxicity activity with IC_{50} of 52.5 µg/ml (Table 3).

The different solvent crude extracts derived from the different plant parts and the cultured cells of *E. longifolia* also showed good cytotoxicity activity against the human breast cancer cell line SKBR3 except the ethyl acetate extract of Eu12 cultured cells, the water extracts of the three different cultured cells (Eu5, Eu6 and Eu12) and the chloroform and water extract of stem samples. Those crude abstracts that indicate strong cytotoxicity activity possessed IC_{50} values between 34 and 78.5 µg/ml (Table 4).

The cytotoxic activity of water crude extracts derived from the dried cell of suspension cultures could not be detected because cell viability of all the three cancer cell lines used in this study was above 50%. This could either mean that the cytotoxic compounds in these water crude extracts were absent or in trace amounts. These water crude extracts were sticky in nature and this characteristic indicate the presence of high sugar content which could also be the cause of no cytotoxic activity as sugar supports the growth of cancer cells by stimulating the division of cancer cells [28]. The presence of high sugar content could have masked the cytotoxic activity of the bioactive compounds present in the water crude extracts.

Table 1. Crude extract yields obtained from the extractions of the dried samples using four different solvents

Sample	Chloroform extract (mg/g)	Ethyl acetate extract (mg/g)	n-Butanol extract (mg/g)	Water extra (mg/g)
Cell line Eu5	40.4 a	9.8 b	6.7 c	242.4 b
Cell line Eu6	46.7 a	8.1 c	8.5 b	201.0 c
Cell line Eu12	45.2 a	6.2 d	6.6 c	263.6 a
Root	7.7 c	6.0 d	4.3 d	40.1 e
Stem	9.2 c	2.3 e	2.5 e	15.7 f
Leaf	29.9 b	21.6 a	17.1 a	66.2 d

Mean values for each column followed by different alphabet were significantly different at $p \leqslant 0.05$

Table 2. Cytotoxicity effect of different *E. longifolia* crude extracts against human lveolar adenocarcinoma cell line A549

Sample	IC_{50} (µg/mL)			
	Chloroform extract	Ethyl acetate extract	n-Butanol extract	Water extract
Cell line Eu5	124.4 b	297.6 a	55.4 b	Cannot
Cell line Eu6	98.2 c	52.5 c	913.7 a	be
Cell line Eu12	194.2 a	104.8 b	932.1 a	detected
Root	42.3 d	33.7 d	33.3 c	41.5 b
Stem	51.7 d	39.6 d	50.6 b	61.9 a
Leaf	51.3 d	33.7 d	32.9 c	42.8 b

Mean values for each column followed by different alphabet were significantly different at $p \leqslant 0.05$

Table 3. Cytotoxicity effect of different crude extracts of *E. longifolia* against human colon cancer cell line HCT116

Sample	IC_{50} (µg/mL)			
	Chloroform extract	Ethyl acetate extract	n-Butanol extract	Water extract
Cell line Eu5	144.1 a	120.1 b	114.1 b	Cannot
Cell line Eu6	131.3 b	52.5 c	101.7 c	be
Cell line Eu12	85.7 c	177.8 a	170.3 a	detected
Root	37.5 e	33.7 d	35.8 d	41.5 b
Stem	79.7 c	36.2 d	36.3 d	91.3 a
Leaf	47.1 d	35.9 d	40.0 d	38.0 b

Mean values for each column followed by different alphabet were significantly different at $p \leqslant 0.05$

Table 4. Cytotoxicity activity of different solvent crude extracts of *E. longifolia* against human breast cancer cell line SKBR3

Sample	IC_{50} (µg/mL)			
	Chloroform extract	Ethyl acetate extract	n-Butanol extract	Water extract
Cell line Eu5	54.5 b	56.2 b	42.4 b	Cannot
Cell line Eu6	55.9 b	40.9 c	46.9 b	be
Cell line Eu12	59.7 b	142.4 a	63.3 a	detected
Root	35.2 c	33.8 d	42.8 b	78.5 b
Stem	109.9 a	39.5 c	41.7 b	214.3 a
Leaf	51.2 b	34.0 d	33.4 c	33.7 c

Mean values for each column followed by different alphabet were significantly different at $p \leqslant 0.05$

The roots of *E. longifolia* are normally used for the preparation of traditional medicine and commercial herbal-pharmaceutical products because it was believed that the roots contained the most potent bioactive compounds [29, 30]. Two big herbal plant extract manufacturing companies in Malaysia, Bionutrica and Naturalin, only prepare *E. longifolia* root extracts for sale. Many researchers involved in studying *E. longifolia* mainly using the root extracts as their research materials. The root extracts were reported to have significant cytotoxic effects on ovarian cancer (Caov-3), prostate cancer (DU-145), epidermoid carcinoma (KB), rhabdosarcoma (RD) and breast cancer (MCF-7) cell lines with selectivity on bovine normal kidney (MDBK) cell line [31]. The root extract was also used to study its effect on Corpus Cavernosum of rat [3]. The leaves were only used for the preparation of concoction for washing body itches while the root and its barks were used for the preparation of traditional herbal medicine for the treatment of various ailments [16]. Although all plant parts of *E. longifolia* were used for traditional therapeutic purposes but only the roots were believed to contain the most valuable components with medicinal properties [32]. However, our study showed that the extracts derived from the leaves and the stem of *E. longifolia* mature plants also exhibited strong cytotoxicity activities comparable with the root extract. While certain cell lines of *E. longifolia* showed high cytotoxicity activities with low IC_{50} values comparable with the mature plant parts. Hence, extracts from the selected cell culture of *E. longifolia* showed great potential to be developed as an anticancer agent.

Thin layer chromatography

From the thin layer chromatography plates for all three different solvents (Figure 1), it could be seen that the R_f values established (Table 5) for the crude extracts derived from the dried cell suspension cultures were similar as that of the roots of *E. longifolia* mature plant. This indicated that some of the compounds present in both crude extracts were similar. For example, the chloroform crude extracts derived from the cell suspension cultures and the roots had five similar R_f values while the ethyl acetate crude extracts had six similar R_f values and n-butanol layer had three. Results also showed that there were many different R_f values between the crude extracts of the cell suspension cultures as compared to the leaf and stem extracts of the plant. This hence indicated that they produced different bioactive compounds. For all the three different solvent crude extracts of the three different suspension cell lines (Eu5, Eu6 and Eu12), the number and position of spots were similar with similar R_f values (Table 5). Hence, this showed that crude extracts derived from the dried cell suspension cultures contained similar compounds but in different quantities and different IC_{50} values for the cytotoxic activity against different cancer cell lines (Table 1 - 4). This again strengthened the theory that the different genetic makeup between the different cell lines did affect the rate of expression and production of cytotoxic secondary metabolites. This is a promising result as it means that the production of secondary metabolites in cell suspension cultures is possible for *E. longifolia* as there have been many instances where secondary metabolites can only be produced in organized tissue such as that reported for vincristine, morphine and scopolamine [33]. The results of the TLC separation also showed that in the crude extracts of dried plant materials, root and stem contained quite similar compounds but extracts derived from the leaf was very different. However, the IC_{50} values of leaf extracts were still comparable to the root extracts and this could mean that although the compounds were different, the cytotoxic activity was still present but due to different cytotoxic compounds.

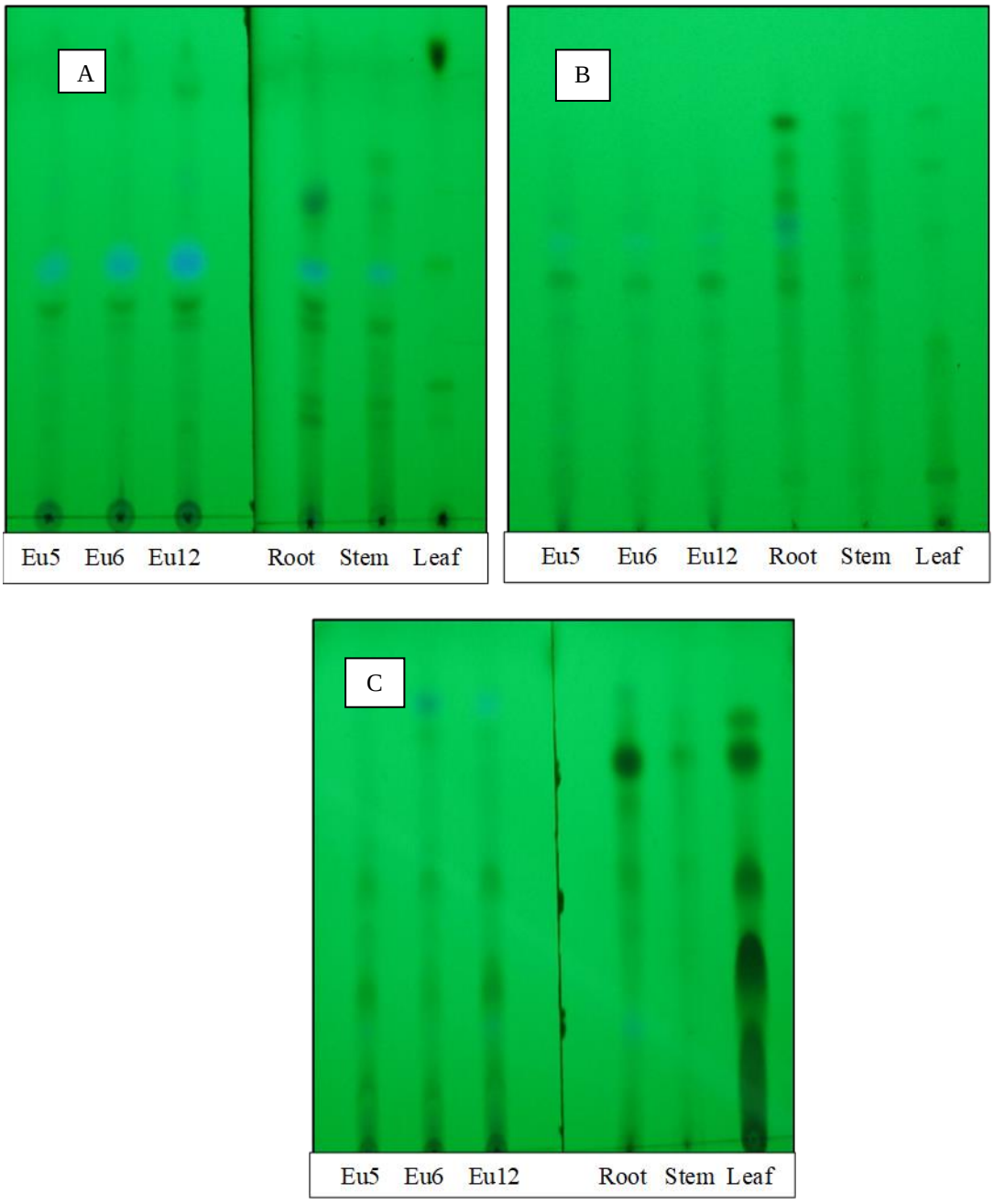

Figure 1: Images of the chromatography plates after thin layer chromatography separation of the different layers from crude extracts of cell suspension culture and plant parts
A: Chloroform layer
B: n-butanol layer
C: Ethyl acetate layer

Table 5 R_f values from the thin layer chromatography separation of the different solvent crude extract of cell suspension culture and different plant parts of *E. longifolia* Jack

Sample	R_f Values		
	Chloroform layer	Ethyl acetate layer	n-Butanol layer
Cell line Eu5	0.19	0.12	0.39
	0.23	0.20	0.46
	0.36	0.26	0.49
	0.40	0.36	
	0.48	0.45	
	0.80	0.69	
	0.85	0.75	
Cell line Eu6	0.19	0.12	0.39
	0.23	0.20	0.46
	0.36	0.26	0.49
	0.40	0.36	
	0.48	0.45	
	0.80	0.69	
	0.85	0.75	
Cell line Eu12	0.19	0.12	0.39
	0.23	0.20	0.46
	0.36	0.26	0.49
	0.40	0.36	
	0.48	0.45	
	0.80	0.69	
	0.85	0.75	
Root	0.19	0.20	0.39
	0.23	0.36	0.46
	0.36	0.45	0.49
	0.40	0.57	0.57
	0.48	0.63	0.59
	0.60	0.75	0.66
Stem	0.19	0.45	0.39
	0.23	0.63	0.66
	0.36		
	0.48		
	0.60		
	0.67		
Leaf	0.19	0.12	0.08
	0.25	0.26	0.28
	0.50	0.45	0.46
	0.87	0.63	0.58
		0.69	0.66

Hence, with appropriate *in vitro* culture conditions, it is possible to induce the cultured cells of a plant to produce any substance that is characteristic of the parent plant. This was proven in our present study whereby we employed the leaf-derived callus to initiate the cell suspension cultures of *E. longifolia* and the different R_f values between the suspension cultured cells and the leaf crude extracts of the parent plant did indicate the bioactive compounds produced were different but highly similar to that of the root crude extract (Table 5). The roots of *E. longifolia* are mainly used for the preparation of herbal remedy products and health supplements as a single ingredient or as a mixture with other herbs as compared to its leaves and stem [30]. Several classes of bioactive compounds have been identified and isolated from the roots of *E. longifolia* and they are mainly the quassinoids, eurycomanone, canthin-6-one alkaloids , β-carboline alkaloids, squalene derivatives and biphenylneolignans [6, 10, 11, 20, 34, 35, 36, 37]. Some of these constituents were shown to possess cytotoxic, antimicrobial, antimalarial, anti-inflammatory, enhancing endurance exercise performance, prevention and treatment of male osteoporosis and aphrodisiac property and analgesic property [5, 6, 9, 12, 29, 30, 31, 38, 39].

IV CONCLUSION

The present investigation revealed that extracts prepared from the dried root, stem and leaves of mature *E. longifolia* plant exhibit higher and consistent cytotoxic activity than the extracts derived from the dried cell suspension cultures with the root extracts having the most cytotoxic activity. However, secondary metabolites produced from the cell suspension cultures are similar with the root extracts with potent IC_{50} values. It is hence indicated that the cell suspension culture of *E. longifolia* Jack can be developed as a potential and sustainable material source for the preparation of anticancer products. In the cell suspension culture technology, no destruction of *E. longifolia* plants in their natural habitat is involved. It can hence be concluded that the cell culture technology can be used as an alternative tool for the prevention of eventual extinction of *E. longifolia* plants. Embarking in large scale culture of the *E. longifolia* cells would be our future scope of study for better production of the useful secondary metabolites. The success of cell culture technology application would eventually reduce the gap of market demand of *E. longifolia*, it also prevent the eventual extinction of this plant in the tropical forests ecosystem.

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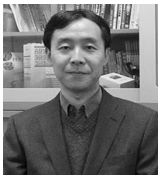

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