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CHARACTERIZATION OF PRIMARY AND SECONDARY METABOLITES OF LEAF AND STEM EXTRACTS FROM *EURYCOMA LONGIFOLIA* JACK

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ABSTRACT

This study evaluates the primary and secondary metabolite profiles of *Eurycoma longifolia* Jack (EL) stems and leaves to determine whether it can be utilized for therapeutic purposes as the roots. A total of six types of extracts were tested. The extracts showed high content of glycosaponins, polysaccharides, proteins and phenolics. The presence of flavonoids and phospholipids was also detected. High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC) analysis showed the presence of bioactive marker of EL root, eurycomanone and 14,15ß-dihydroxyklaineanone in stem and leaf extracts. Primary and secondary metabolites identified were reported to associate with the enhancement of ergogenic and aphrodisiac activities in animal and human subjects. The result shows that stem and leaves of *E. longifolia* has the potential for therapeutic purposes.

Keywords: *Eurycoma longifolia*; eurycomanone; 14,15ß-dihydroxyklaineanone; aphrodisiac.

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1. INTRODUCTION

Eurycoma longifolia Jack (locally known as Tongkat Ali, Genus: Eurycoma; Family, Simaroubaceae) is one of the most popular tropical herbal plants. Indigenous to Southeast Asian countries, it is found in several parts of Malaysia, Indonesia, and Vietnam. This plant also found in certain patches in regions of Cambodia, Myanmar and Thailand. The tree starts bearing fruits after 2–3 years, yet complete maturation takes up to 25 years. Its long, twisted roots are harvested. The roots used in folk medicine for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency [1]. In Malaysia, water extract of *E. longifolia* famously consumed to improve strength, male virility and sexual prowess [2]. Extensive studies on root have been published. It contains quassinoid alkaloid, tannins, high molecular weight polysaccharides, glycoprotein, mucoproteins and mucopolysaccharides. Three novel C19 and C18 quassinoids, eurycolactones A-C isolated from roots of *E. longifolia* [3-4]. The glycoprotein components exert anti-cancer, pro-fertility, aphrodiasic and anti-aging properties based on animal studies [9]. *E. longifolia* root extract elevated the testosterone levels, reduced the bone resorption marker and upregulated OPG gene expression of the orchidectomised rats [5]. It exhibited strong cytoxicity toward human breast cancer (MCF-7) cell lines and displayed potent antimalarial activity against the resistant *Plasmodium falciparum* [6].

The aboveground part of E. longifolia, stem and leaves was far less studied. During the collection of raw material, the collectors have always ignored these parts. Stem and leaves account about 50-70% of total plant biomass that most of the time gone waste. In order to evaluate the potential of stem and leaves for therapeutic uses, characterization of primary and secondary metabolite profiles stem and leaf extracts was carried out. Identification and determination of bioactive compounds were also performed.

2. RESULTS AND DISCUSSION

2.1. Total Metabolites in *E. Longifolia*

In order to investigate the metabolite profile of *E. longifolia* stem and leaf extracts, six type of extracts were prepared; leaf-water extract-maceration (TALW-M), leaf-water extract-reflux (TALW-R), leaf-ethanol extract (TALEW), stem-water extract-maceration (TASW-M),

stem-water extract-reflux (TASW-R), and stem-ethanol water (TASEW). Detail on extract can be seen in experimental section. Total metabolite content presented in Fig. 1. In general, stem extracts contain higher amount of polysaccharides compared to leaf extracts. Phenolics compounds are account for most of antioxidant activities in plants [7]. Stem extracts contain more phenolic in general but TALW-R possess the highest total phenolic content at 41.82 mg/g. TALEW shows highest content of flavonoid for 31.37 mg/g. The second highest was recorded in TASW-M for 9.31 mg/g while in stem it was found in the lowest content which is only 2.24 mg/g. Flavonoid is belongs to group polyphenolic compound and they widely exist in plant kingdom and displayed positive correlation between increased consumption of flavonoid and reduced risk of cardiovascular and cancer diseases [8].

In determination of total phospholipids, ethanol water extracts for both leaf (TALEW) and stem (TASEW) show higher content of phospholipids at 12.71 mg/g and 6.84 mg/g, respectively. For total protein, maceration was found to be the most effective method of water extraction for both stem and leaves. The protein content is in order of TASW-M (68.11 mg/g)>TALW-M (38.24mg/g)>TALEW (23.17mg/g)>TALW-R (22.59mg/g)>TASW-R (10.14 mg/g)>TASEW (1.01mg/g). Previous study done by [16] found that 0.3868 mg/mL and 0.9573 mg/mL of highly water soluble protein were extracted from root of *E.longifolia* from Perak and Pahang. This may be because the extraction method is likely to be a promising technique to extract a relatively low abundance of plant proteins.

Saponin exhibit a wide range of pharmacological activities which includes espectorant, anti-inflammatory, vasoprotective, hypocholesterolemic, immunomodulatory, hypoglycaemic, molluscicidal, antifungal, antiparasitic, anticonvulsant and other central nervous activities [9] Total glycosaponin was found highest in leaves as compared to stem. The order of saponin content is TALEW (258.60mg/g) > TASW-M (188.00mg/g) >TASEW (139.70 mg/g) > TALW-M (121.50 mg/g) > TALEW (78.70 mg/g) > TASEW (64.73 mg/g). *E. longifolia* root was found to have total saponin in at 1.0769 g in multiple-steps of extraction [10]. To the best of our knowledge, there is no reported study for quantification of metabolites in leaf and stem of *E. longifolia*. So, the data obtain from this study can be used as references for metabolites content in *E. longifolia*.

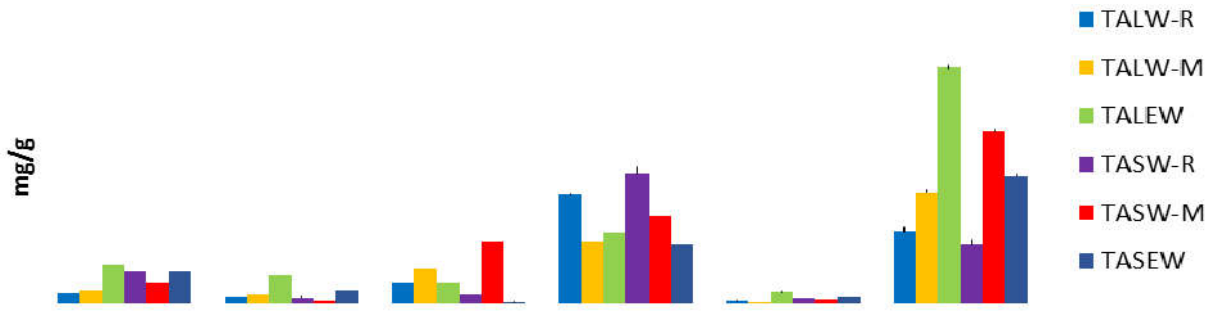

Fig.1. Total metabolites in *E. longifolia* extracts

2.2. High Performance Thin Layer Chromatography (HPTLC) Analysis

HPTLC is an analysis tools for plants material assessment, which allows a wide numbers of compound analysis both efficiently and cost effective [11]. Fig. 2 and Table 1 show that bioactive compounds of Eurycomanone (EU), 13α(21)-Epoxyeurycomanone (EE) and 14,15ß-dihydroxyklaineanone (DK) can be observed as dark bands under 254nm visualization while SP was observed as fluorescent under 254nm and 366nm. The reference markers position was observed at Rf value 0.12 for EE, 0.14 for EU, 0.50 for DK, 0.68 for SP (Fig. 3). EE and EU can be detected as dark band in leaf extracts, while in stem it was not clearly seen. DK can be seen to present in all stem and leaf extracts. SP was observed as fluorescent band in all extracts under 366nm while in 254nm the samples were not fluorescent. The samples' chromatograms indicated the presence of the spots with similar colour and the exact R_f value as the standard. Based on the chromatogram obtained, it can be concluded that two out of five markers was clearly can be detected in HPTLC which are DK and SP. HPLC was then conducted to further confirm these markers in *E. longifolia* stem and leaf extracts.

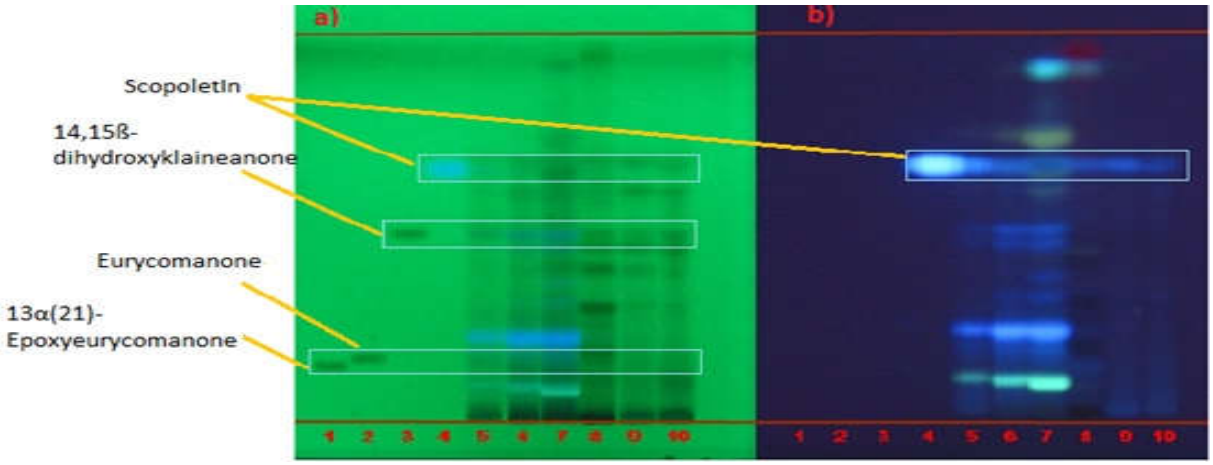

Fig.2. HPTLC profiling of *E.longifolia* extracts for stem and leaves visualized under a) 254nm b) 366nm

Table 1. Sample for HPTLC analysis

Number	Marker/Sample
1	Eurycomanone (EU)
2	13α(21)-Epoxyeurycomanone (EE)
3	14,15ß-dihydroxyklaineanone (DK)
4	Scopoletin (SP)
5	stem water extract-reflux
6	stem water extract-maceration
7	stem 50% ethanol water
8	leaf 50% ethanol water
9	leaf water extract-maceration
10	leaf water extract-reflux

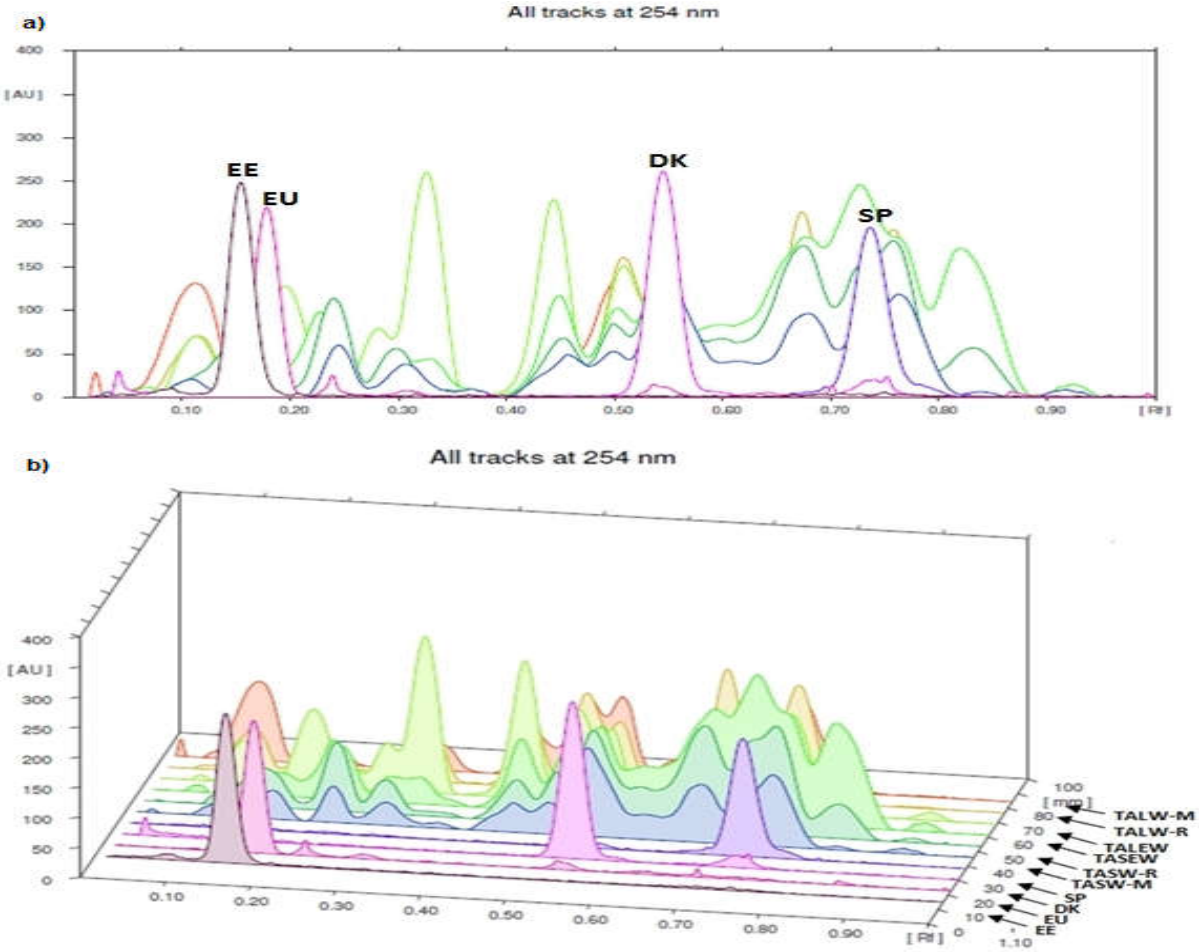

Fig.3. HPTLC densitogram profile of *E. longifolia* extracts in stem and leaves a) 3D HPTLC densitogram b) 2D HPTLC densitogram

2.3. High Performance Liquid Chromatography (HPLC)

2.3.1. Validation of HPLC Method

HPLC method was developed and validated using four markers which are (EU), (EE), (SP) and (DK) in order to detect the present of these markers in stem and leaves samples. The markers were selected as reference because they have been reported as the major quassinoids in *E. longifolia* and displays a wide range of physiological properties *in vitro* or *in vivo* experiments. All of the markers were established by using gradient mobile phase consisting of 0.1% formic acid and acetonitrile with UV detection at 254nm. The marker compounds were separated within 25 minutes. The standards were settled and eluted at ±7.4, ±8.4, ±14.4 and ±15.7 min with respect to EE, EU, DK and SP (Fig. 4). A new HPLC method was developed and validated for the quantification of markers in *E. longifolia* stem and leaf extracts.

2.3.1.1. Selectivity

The selectivity of the method was determined by comparing the retention time of Eurycomanone (EU), 13α(21)-Epoxyeurycomanone (EE),14,15ß-dihydroxyklaineanone (DK) and Scopoletin (SP) obtained in extracts with those of the reference compounds. Retention time of were at 7.48 ±0.003, 8.40± 0.006, 14.43 ± 0.009 and 15.7± 0.007 respectively. The standards in *E. Longifolia* extracts were eluted at 7.47 ± 0.010, 8.40 ± 0.006, 14.42 ± 0.005 and 15.71 ± 0.014 min respectively. Spiking the extract with standards increased the peak area of the compounds without any shift in the retention time and appearance of the extra peaks; this further confirms the identity of the compounds and methods selectivity of the system.

2.3.1.2. Linearity

Linearity was achieved at 0.976-500 µg/ml and presented in terms of regression coefficient (R^2) for EU, EE, DK and SP were 0.999±0.0001, which indicate good linearity of the proposed method.

2.3.1.3. Precision

Precision is presented in terms of %RSD of retention time and peak area (n = 5) of standards. The retention time for EU, EE, DK and SP were at 7.48 ± 0.003, 8.40 ± 0.006, 14.43 ± 0.009 and 15.7 ± 0.007 min with %RSD of was less than 0.12, 0.13, 0.13 and 0.08 % respectively.

The percentage RSD of the peak area was calculated in the concentration range 7.8125-125 µg/ml and the average was less than1.20, 1.18, 0.98 and 0.97 % (Table 2-5).

Table 2. Precision analysis of 13α(21)-Epoxyeurycomanone (EE) in HPLC method. The %RSD was calculated for intraday and interday. Retention time (RT) and peak area (PA) are presented as mean ± SD (n = 5)

Precision	**Concentration**	**PA (mAU*S)**		**RT (min)**	
	µg/ml	AV±SD	%RSD	AV±SD	%RSD
Intraday	125	1429.3 ± 13.846	0.97	7.49 ± 0.004	0.05
	62.5	736.14 ± 5.723	0.78	7.49 ± 0.004	0.05
	31.25	354.42 ± 4.044	1.14	7.49 ± 0.003	0.05
	15.625	166.75 ± 0.919	0.55	7.49 ± 0.004	0.05
	7.8125	83.876 ± 0.210	0.25	7.49 ± 0.003	0.04
Interday	125	1459.29 ± 17.486	1.19	7.47 ± 0.008	0.11
	62.5	709.93 ± 6.462	0.91	7.47 ± 0.008	0.11
	31.25	346.19 ± 1.409	0.40	7.47 ± 0.008	0.11
	15.625	173.68 ± 1.600	0.92	7.47 ± 0.008	0.11
	7.8125	86.58 ± 0.887	1.02	7.47 ± 0.007	0.09

Table 3. Precision analysis of Eurycomanone (EU) in HPLC method. The %RSD was calculated for intraday and interday. Retention time (RT) and peak area (PA) are presented as mean ± SD (n = 5)

Precision	**Concentration**	**PA (mAU*S)**		**RT (min)**	
	µg/ml	AV±SD	%RSD	AV±SD	%RSD
Intraday	125	1026.3±11.419	1.11	8.41±0.003	0.04
	62.5	519.83±1.622	0.31	8.41±0.003	0.04
	31.25	256.26±2.682	1.05	8.41±0.004	0.05
	15.625	128.59±0.766	0.60	8.41±0.004	0.05
	7.8125	65.06±0.340	0.52	8.41±0.004	0.04
Interday	125	1053.93±11.408	1.08	8.39±0.009	0.11
	62.5	525.69±3.026	0.58	8.39±0.009	0.12

	31.25	260.88±1.874	0.72	8.39±0.008	0.10
	15.625	132.65±1.297	0.98	8.39±0.008	0.08
	7.8125	66.62±0.782	1.17	8.39±0.008	0.11

Table 4. Precision analysis of 14,15ß-dihydroxyklaineanone (DK) in HPLC method. The %RSD was calculated for intraday and interday. Retention time (RT) and peak area (PA) are presented as mean ± SD (n = 5)

Precision	**Concentration**	**PA (mAU*S)**		**RT (min)**	
	µg/ml	AV±SD	%RSD	AV±SD	%RSD
Intraday	125	1628.57±11.569	0.71	14.43±0.008	0.05
	62.5	824.35±2.512	0.30	14.44±0.003	0.02
	31.25	413.15±1.004	0.24	14.44±0.005	0.04
	15.625	208.42±1.147	0.55	14.44±0.003	0.02
	7.8125	132.00±0.481	0.36	14.44±0.003	0.02
Interday	125	1672.68±16.200	0.97	14.42±0.017	0.12
	62.5	848.86±5.353	0.63	14.42±0.015	0.10
	31.25	422.22±3.779	0.89	14.42±0.013	0.09
	15.625	214.41±2.091	0.98	14.42±0.011	0.08
	7.8125	108.07±0.926	0.86	14.42±0.010	0.07

Table 5. Precision analysis of Scopoletin (SP) in the HPLC method. The %RSD was calculated for intraday and interday. Retention time (RT) and peak area (PA) are presented as mean ± SD (n = 5)

Precision	**Concentration**	**PA (mAU*S)**		**RT (min)**	
	µg/ml	AV±SD	%RSD	AV±SD	%RSD
Intraday	125	1323.96±8.852	0.67	15.72±0.006	0.04
	62.5	667.28±0.936	0.14	15.72±0.004	0.03
	31.25	334.58±0.590	0.18	15.72±0.007	0.04
	15.625	169.54±0.782	0.46	15.72±0.006	0.04
	7.8125	85.56±0.259	0.30	15.72±0.005	0.03
Interday	125	1348.81±12.172	0.90	15.70±0.007	0.07

62.5	687.99±6.902	1.00	15.70±0.007	0.07
31.25	339.75±2.808	0.83	15.70±0.008	0.05
15.625	173.69±1.667	0.96	15.70±0.010	0.04
7.8125	86.92±0.579	0.67	15.70±0.010	0.04

2.3.1.4. Accuracy

Accuracy was determined as a percentage recovery of standards at three concentrations. The mean percentage recovery at 100, 50 and 25 µg/ml for EE, EU, DK and SP were 101-102 ± 0.50 %, 95-103 ± 0.94 %, 98-102 ± 0.94 % and 98-103 ± 1.21 % respectively (Table 6). The results are presented as average percentage recovery ± SD.

Table 6. Accuracy of *E. longifolia* at 25, 50 and 100 µg/mL. Results are showed as average percentage recovery of the reference compounds ± SD (n = 3)

Compound	% Recovery		
	25 µg/mL	**50 µg/mL**	**100 µg/mL**
EE	101.70 ± 0.61	101.08 ± 0.83	101.49 ± 0.07
EU	95.82 ± 0.69	103.41 ± 0.56	103.24 ± 0.63
DK	100.56 ± 1.18	102.04 ± 0.92	97.73 ± 0.72
SP	101.57 ± 1.45	97.58 ± 1.73	102.52 0.46

2.3.1.5. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated through the slope and standard deviation method by using the linear regression equations of the reference compounds. The calibration data is summarized in Table 7.

Table 7. Summary of Eurycomanone (EU), 13α(21)-Epoxyeurycomanone (EE), 14,15ß-dihydroxyklaineanone (DK) and Scopoletin (SP) data. The regression equation is (y = ax + c), where (a) is the slope and (c) is the y intercept. The data are presented as average ± SD (n = 5)

Compound	A	c	LOD (µg/mL)	LOQ (µg/mL)	R^2
EE	11.634±0.129	9.000±3.338	0.945±0.011	2.863±0.032	0.9999
EU	8.323±0.0902	1.983±0.893	0.353±0.004	1.069±0.011	0.9999
DK	13.165±0.111	6.536±2.054	0.515±0.004	1.561±0.013	0.9999

SP	10.680±0.105	4.797±1.979	0.611±0.006	2.136±0.156	0.9999

2.4. Quantification of Markers Compound in *E. Longifolia* Extracts

The result shows that in leaf extracts, EE was only present in 50% ethanol extract (TALEW) and was not detected in maceration (TALW-M) and reflux (TALW-R) extracts whereas other markers were detected (Fig. 5). DK was present in highest content (1.00 %) in 50% ethanol (TALEW) extract, followed by maceration (TALW-M) at 0.98% and reflux (TALW-R) shows the lowest content of all markers.

In stem extracts, it was found that EE was only present in 50% ethanol (TASEW) extract as in leaves extract. However, EU shows highest in 50% ethanol extract (TASEW) for 1.00% followed by reflux (TASW-R) 0.83%. While in maceration (TASW-M), EU shows the lowest content for 0.61%. In addition, DK shows highest in 50% ethanol leaf (TALEW) extract for 1.00% as compared to maceration and reflux. SP was shows to only present in maceration (TASW-M) for 0.44% in stem extracts while it was present in all leaf extracts with the highest detected in maceration extracts (TALW-M). According to [12], Eurycomanone and its derivatives present in highest amount among the detected quassinoids. Whereas, the concentration of alkaloids was increased when the roots of *E. longifolia* were extracted at 100°C. The chromatograms showed a good separation of four markers compound in the system.

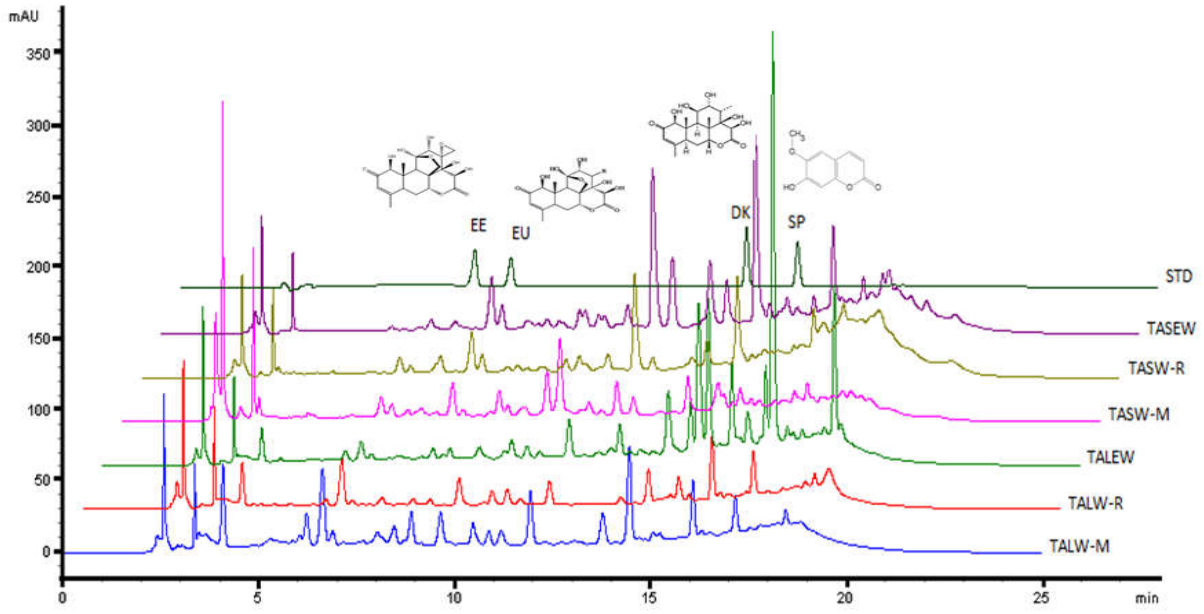

Fig.4. HPLC chromatogram in stem and leaf extracts of *E. longifolia* for four markers compound

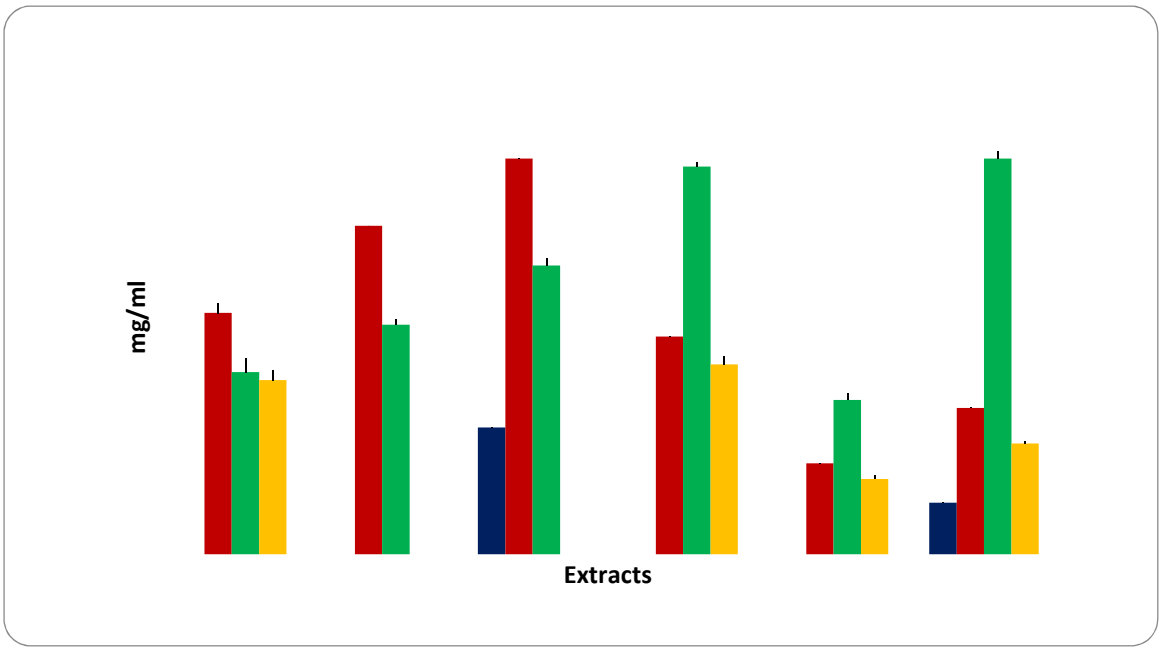

Fig.5. Concentration of markers in *E. longifolia* extracts

3. EXPERIMENTAL

3.1 Preparation of Plant Material

The leaves and stem of *E. longifolia* was supplied by Herbagus Trading Sdn. Bhd, Kepala Batas, Penang, Malaysia. The dried stem and leaves were washed thoroughly with water to keep clean. It was then grinded into a fine powder using an electric grinder (Restch, Germany). The powder was kept in air tight container until further use.

3.2. Preparation of Extracts from Different Parts of *E. Longifolia*

Two different parts (leaves and stem) were obtained from the plant material. Only water and ethanol were used as the solvents for extraction. Extracts were prepared by using maceration and reflux methods of each part with 100% water while sohxlet method was prepared using 50% ethanol water with 1:10 ratio.

3.2.1. Maceration

Two hundred gram of powdered *E. longifolia* materials was macerated using water with 1:10 ratio at 60˚C for 24 hours. The extracts were filtered and collected until 72 hours, and then concentrated using rotary evaporator. Extracts were kept in 4^{o}C until further use.

3.2.2. Reflux

Two hundred gram of powdered material of *E. longifolia* was weighed and placed in round

bottom flask. Two liter of water was added and the flask was reflux for 8 hours, left to cool and filtered. It was repeated until 24 hours. The extracts were filtered and concentrated using rotary evaporator. Extracts were kept in 4^{o}C until further use.

3.2.3. Sohxlet

Two hundred gram of powdered material of *E. longifolia* was weighed and placed in sohxlet apparatus which was wrapped with filter paper. Two liter of 50% ethanol water was added in the round bottom flask and the solvents were left to cycles until clear. The extracts were filtered and concentrated using rotary evaporator. Extracts were kept in 4^{o}C until further use.

3.3. Total Protein

Method was done based on method proposed by [13]. Ten milligram of extract was weighed and added with 1mL of distilled water. The solution was sonicated and centrifuged for 5 min at 8000rpm. A series of bovine serum albumin (BSA) concentrated was prepared (0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2mg/ml). Twenty microliter of solutions and standards were added with 1 ml Bradford reagent. All the samples were incubated at room temperature for 5 min and absorbance was measured at 595 nm against blank.

3.4. Total Glycosaponin

One gram of extract was mixed with 50ml methanol (Merck, USA) in 250ml round bottom flask. The mixture was refluxed for 30 minutes and filtered. The residue was refluxed again in 50ml methanol twice and the filtrate of the 3 cycles was concentrated using rotary evaporator (Buchi, Germany) to approximately 10mL. Subsequently, 50mL acetone (Merck, USA) was added to the extract and the saponins were precipitated at the bottom of weighed beaker. The precipitate was collected and dried in oven (Memmert, Germany) at 100˚C to constant weight. Calculation of total glyscosaponins was done using the following formula:

Percentage of glycosaponins = (Wp/Ws) x 100

where Wp: weight of precipitate (g) and Ws: weight of sample (g).

3.5. Total Polysaccharides

The method of total polysaccharides was reported by [14]. Ten milligram of extracts was dissolved in 100 mL water. One milliliter of solution was added with 5% phenol reagent (Sigma-Aldrich, USA) and 5 mL of H_2SO_4 concentrated. The mixture was incubated for 10

min at room temperature. Glucose was used as a standard in range of 10-100µL and absorbance was measured at 488 nm against blank. Total polysaccharides were calculated as average percentage ± SD, (n = 3). The yield of polysaccharides in the samples was finally calculated based on the standard calibration curve.

3.6. Total Phenolics

Colometric assay was used to estimate the total phenolics contents as described by [15]. Thousand ppm extracts were prepared in methanol (Merck, USA) and 100 µL of the extract solution was added to 750 µL of Folin-Ciocalteau phenol reagent (diluted at 1:10 in distilled water) (Sigma-Aldrich, USA). The sample were incubated for 5 minutes in the dark condition, 750 µL of sodium carbonate (Sigma-Aldrich, USA) solution of 60 mg/mL was added and further incubated for 90 min at 30˚C in the dark condition. The absorbance of the samples was taken at 725 nm. All samples were prepared and analyzed in triplicate. Gallic acid (Sigma-Aldrich, USA) prepared in the range 3.91-125 µg/mL was used as a positive control and was used to construct the standard calibration curve. The total phenolics content in the sample extracts were calculated based on the linear regression equation and the results are shown as average percentage of total phenolics in the extracts (n = 3).

3.7. Total Phospolipid

Total phospholipids were determined according to method proposed by [16]. 27g of ferric chloride (Sigma-Aldrich, USA) were dissolved in 1L of water at the test reagent. Approximately 2 mg of extract were weighed and dissolved in 2 mL chloroform. 1mL of thiocyanate reagent was added, vortex for 1 minute and centrifuged at 2700 rpm for 5 min. The lower layer was then removed and absorbance was measured at 488nm. Phosphatidylcholine (Sisma-Aldrich, USA) was prepared in the range of 10-100 µg/mL and same procedure was applied to construct calibration curve. Total phospholipids were calculated by means of 3 replicates and total phospholipids were expressed as average percentage ± SD.

3.8. Total Flavonoids

Total flavonoids determination was conducted using aluminium chloride method as described by [17]. Five milligrams of *E.longifolia* extracts were dissolved in 1 mL of methanol. 500µL

of extracts were then mixed with 1.5mL methanol. Hundred microliters of 10% Aluminium chloride and 100 µL of 1M potassium acetate were added and mixed with the extracts. The samples were incubated at room temperature for 30 minutes. The absorbance was measured at 415 nm against blank that considered of all reagent and samples except AlCl3. The calibration curve was plotted by preparing quacertin solution at ranges of 3.125-100 µg/mL. Total flavonoids were calculated as average percentage ± SD, (n = 3).

3.9. High Performance Thin Layer Chromatography (HPTLC) Analysis

Qualitative HPTLC was carried out on HPTLC plates (10x10cm) silica gel 60 F254 (Merck, USA). Five microliters of extracts (50mg/mL) and marker compound (0.5 µg/mL) were applied to HPTLC glass plate as 5mm bands with 80 mm space from lower edge. Eurycomanone (EU), 13α(21)-Epoxyeurycomanone (EE), Scopoletin (SP) and 14,15ß-dihydroxyklaineanone (DK) were used as reference markers. The plates were then developed in a glass chamber, which was saturated with the solvent mixture for 10 minutes before developing to a distance of 80mm. The solvent mixture used was toluence: ethyl acetate: formic acid: methanol (3:3:0.5:0.8).

3.9.1. Development and Validation of High Performance Liquid Chromatography (HPLC) Method in *E. Longifolia* Extracts

3.9.1.1. HPLC Conditions for Quantification of Markers

A gradient HPLC system was developed to improved separation of references compound from the solvent front and peaks from extracts. High Performance Liquid Chromatography 1100 series, Agilent, USA equipped with deggaser, quaternary pump, auto sampler, column oven and ultraviolet (UV) detector was used. Separation was done using Zorbax eclipse plus RP-C18, 250x4.6 mm, 5.0 µm particle sizes, (Agilent, USA). The mobile phase consisted of A (acetonitrile (Merck, USA)) and B (0.1% formic acid (Merck, USA)) in water, the elution program was gradient for 25 minutes (Table 1), the flow rate was maintained at 1 mL/min and injection volume was 10 µL. The column temperature was set at 30°C and UV detector was operated at wavelength 254 nm.

Table 8. HPLC gradient mobile phase operating parameters

Time (min)	Flow Rate (ml/min)	%A	%B
0	1.0	90	10
10	1.0	80	20
15	1.0	70	30
20	1.0	90	10
25	1.0	90	10

3.9.1.2. Preparation of Stock Solutions

Each standards 1mg (Eurycomanone (EU), 13α(21)-Epoxyeurycomanone (EE),14,15ß-dihydroxyklaineanone (DK) and Scopoletin (SP)) was dissolved in 1mL HPLC grade methanol (Merck, USA) to produce a standard concentration of 1000 ppm. From standard stock solution, serial dilution of 500-0.24 ppm were prepared for calibration curves. Dried extract of *E. longifolia* (5 mg) was weighed accurately and dissolved in methanol (1 ml) filtered through 0.45 mm syringe filter.

3.9.1.3. Validation of the HPLC Methods

The method was validated based on ICH guidelines. The following validation characteristics were evaluated; selectivity, linearity, precision, accuracy and the limits of detection and quantification (LOD and LOQ).

3.9.1.4. Linearity

Linearity was determined by injecting 10 µL of the standards mixture in a concentration range 0.2-500 µg/ml. The calibration curves were obtained by plotting the peak area versus concentration for each individual compound. Linearity was determined as the regression coefficient (R^2) of calibration graphs.

3.9.1.5. Selectivity

The selectivity method was determined by comparing retention time of markers obtained in the sample with that of reference compound, and by spiking the extract with reference compound.

3.9.1.6. Precision

Precision was determined as relative standard deviation (%RSD) of peak area and retention

time. The standard compound was analyzed at 5 concentrations points in range 7.8125-125 µg/ml. The intraday and interday precisions were calculated (n = 5).

3.9.1.7. Accuracy

The accuracy in this study was determined as a percentage recovery of markers at 100, 50 and 25 µg/ml, added to the *E. longifolia* extract at 5000 µg/ml. Peak areas of the compound in the extracts (B), the individual reference compounds (C) and their combinations (A) were recorded. Percent recovery was calculated as in equation below [19] and the results are presented as mean ± SD (n = 3).

% Recovery = $\{(A - B)/C\}100$

3.9.1.8. Limit of Quantification (LOQ) and Limit of Detection (LOD)

The LOD and LOQ were calculated using slope and standard deviation method as described in ICH guidelines [18].

LOD = $(3.3 \times \delta)/S$

LOQ = $(10 \times \delta)/S$

where $\delta$is the standard deviation of the linear regression equation and S is the slope of the linear regression equations.

3.9.2. Quantification of Markers in *E. Longifolia*

E. longifolia extracts at 10 µl were injected and the peak area corresponding to markers were recorded. Concentration of markers compound in the samples were calculated by applying the linear regression equation of the standard calibration curve, and the content (%wt/wt, n = 3) was calculated as below [18]:

Content (%wt/wt) = (Cf/Cs)100

where Cf is the found concentration and Cs is concentration of the sample.

This experimental section can be divided into subsections, the contents of which vary according to the subject matter of the article. It must contain all the information about the experimental procedure and materials used to carry out experiments.

4. CONCLUSION

Extracts of *E. longifolia* stem and leaf have potential metabolites comparable to that of root.

This finding suggests that stem and leaf can be developed for therapeutic uses. Further research will facilitate a wider use of *E. longifolia* stem and leaf. Data collected in this work can be the basis on the standardization of *E. longifolia* stem and leaf toward development of potential applications.

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