

PRELIMINARY PHYTOCHEMICAL SCREENING OF POLYSACCHARIDES CONTENT FOR SELECTION OF HIGH QUALITY PLANTING MATERIALS: *Eurycoma longifolia*

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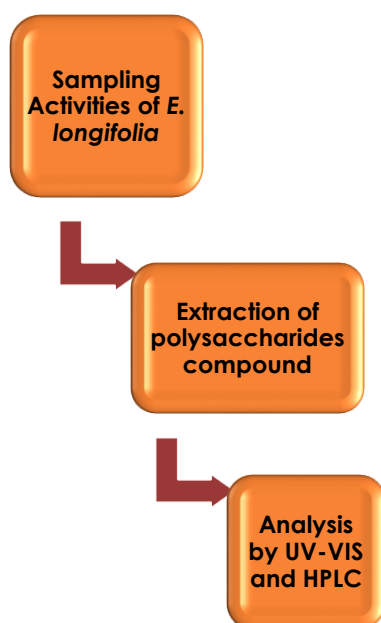
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Graphical abstract



Abstract

Provenance trial of *Eurycoma longifolia* is currently being carried out in order to provide necessary information for breeding strategies, which is crucially needed for the production of high quality planting materials. Assessment of polysaccharides content in the root part of the plant is taken among the important characteristics in the selection of the best provenance. Polysaccharides compound is chosen as bioactive marker due to the non-toxicity nature of the compound and potential pharmacological properties possessed. Polysaccharides compound of the root part is extracted by using water extract procedure from nine selected provenances (Johor, Melaka, Pahang A, Pahang B, Pahang D, Perak, Pulau Pinang, Selangor and Terengganu) of *E. longifolia* collected throughout forest reserves of Peninsular Malaysia. The analysis of the actual weight of the compound is conducted using phenol-sulfuric acid method by UV-VIS and High Performance Liquid Chromatography (HPLC) is used for the detection of structural components of the polysaccharides compound. The assessment showed that polysaccharides content in the root part of Terengganu provenance significantly has the highest with 1.06 % actual weight of polysaccharides (out of 30 g extracted dry roots). Meanwhile, there are four provenances showed undetectable range of polysaccharide content which were Melaka, Pahang A, Pulau Pinang and Selangor. In a nutshell, based on the preliminary assessment of polysaccharide content alone, Terengganu provenance has the potential to be declared among the best provenances that has high quality. The provenance trial also has taken into considerations several important parameters such as the growth performances variation and the tolerance of the plants to pests and diseases besides the phytochemical screening. Provenance trial of *E. longifolia* is still ongoing, data for various parameters are still being collected, and thus it is still too early to derive a conclusion of which is the best provenance.

Keywords: Provenance trial, breeding strategies, bioactive marker, UV-VIS, HPLC

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1.0 INTRODUCTION

Other than molecular genetic markers or DNA profiling, chemical marker has also been used widely for the authentication and identification of genuine herbal usage in the preparation of herbal products. The term "marker compounds" can be defined as standard reference compounds used for the comparison and quality control purposes [1]. In plant improvement program, the use of chemical marker is not only for the identification of correct botanical plant but also for the assessment of the best quality planting materials to provide for huge demand by industries.

According to Rasheed *et al.* (2012), standardization of chemical marker compound is important for the evaluation of quality control of raw materials, detection of the existence of adulterant in herbal based products, and analysis of herbal toxicity level, safety and efficacy of the herbal-based drugs. Consequently, the assessment of the standardized marker compound can be achieved by the estimation of active principal amount present in herbal extracts, the consistency demonstrated by batch to batch extracts or isolation and confirmation of its identity and determination of its quality, purity and detection of nature adulterant by various parameters [1]. Thus, in the use of the plant materials for pharmaceutical purposes, there is a need to establish the fingerprinting profile of a chemical marker compound that shows the amount or percentage of the active principles along with the closely related bioactive principles especially for quality control.

Currently, extensive study on the plant improvement program of *E. longifolia* is being carried out by FRIM. The provenance trial plots of the species have been established at four locations in Peninsular Malaysia which are FRIM's Research Station (SPF) (i) Mata Ayer, Perlis, (ii) SPF Maran, Pahang, (iii) SPF Jelli, Kelantan and (iv) University Technology Malaysia (UTM), Skudai, Johor. Provenance means "origin" and refers to a population of trees that come from a particular location. Provenance trial facilitates selection from populations evolved to specific geographic and climatic conditions within the natural range of the species. In other words, findings in the provenance trial would provide the best match between the environmental conditions and the plant species with various origins [2]. Several important parameters are taken into considerations in the selection of the best provenance including growth performances, tolerance to pests and diseases, and the quality of the phytochemical content.

Therefore, in the assessment of the phytochemical content, eurycomanone and polysaccharides compounds are chosen as the chemical marker which will indicate the best quality provenance. Eurycomanone compound is the only distinct chemical compound found in *E. longifolia* species and not even found in *Entomophthora apiculata* species which is also from the Simaroubaceae family. Thus, for the identification of genuine products of *E. longifolia*,

eurycomanone compound is the most ideal chemical marker. Nevertheless, eurycomanone compound has an issue with toxicity content [3-4] but on the neutral side, this compound has poor bio-availability when taken orally [5]. As for the added value, polysaccharides compound is chosen as another bioactive marker due to the non-toxicity nature and the pharmacological property potential of the compound [6]. Thus, this study adopted polysaccharide as another indicator of bioactive marker in the assessment of high quality provenances for *E. longifolia*.

2.0 EXPERIMENTAL

2.1 Plant Materials and Samples Preparation

Plant materials of *E. longifolia* were harvested from natural forest reserves of nine different provenances namely Johor, Melaka, Pahang A, Pahang B, Pahang D, Perak, Pulau Pinang, Selangor and Terengganu. Only plant samples of similar sizes were collected (average height was 27.3 cm and average diameter was 2.25 mm). The harvested plants were washed and dried in an oven for sample preparation.

2.2 Water Extraction by Double Boiling Procedure

About 30 g dried root of *E. longifolia* was placed in boiling flask with 3 L distilled water. The sample was boiled using heating mantle for two hours (time measurement started once the samples started to boil). After two hours, the sample was filtered using clean cloth and the extract was kept in a container. The sample was boiled with 3 L distilled water again for two hours. Then, the extract was concentrated using rotary evaporator. The concentrated extract was added with ethanol at 1:3 ratio and left overnight in a cold room (4°C).

After the extract was centrifuged with refrigerator centrifuge at 4°C with the speed of 6000 rpm for 10 minutes, the supernatant was discarded and the pellet was diluted with 100 mL distilled water. Followed by freeze-drying process for a day.

2.3 UV-VIS Polysaccharides Content Analysis: Phenol Sulfuric Acid method

A hundred (100) mg freeze-dried samples were placed in 100 mL Schott bottle and 3 mL of 72% sulfuric acid (H₂SO₄) was added into each sample bottle (there were nine samples bottles for total of nine provenances). The samples were left for one hour at 30°C in the fume cupboard. Then, 17 mL of distilled water was added into each sample and autoclaved at 120°C for 120 minutes. The samples were then neutralized with 0.5 M NaOH before evaporated using rotary evaporator to remove excess water. The concentrated samples were dissolved with 80% methanol and filtered to remove salt (Na₂SO₄). The filtrate was evaporated to dryness before being

subjected to UV-VIS detection. The samples were prepared by Phenol-Sulfuric acid method [7] and light absorption at 493 nm was recorded.

2.4 Polysaccharides Structural Components Screening by High Performance Liquid Chromatography (HPLC)

2.4.1 Preparation of the Samples for HPLC

A hundred (100) mg freeze-dried samples were placed in a 100 mL schott bottle and diluted with 1 mL of distilled water. Then, 200 μ L of the diluted samples were added into a 7 mL samples bottles and 800 μ L of 0.3 M NaOH were added. The solution was shaken well and mixed with 1000 μ L polymethylpentene (PMP) solution. The samples were incubated in a water bath at 70°C for 60 minutes. After the samples were cooled, 1000 μ L of 0.3 M HCl were added and mixed well. About 3 mL of chloroform solution were added and being allowed to form a partition layer (partition layer with chloroform was repeated three times) and the upper layer was taken and transferred into the bullet tubes. Then, the bullet tubes were centrifuged by mini micro centrifuge for 5 minutes at room temperature. The solution was then filtered by syringe filter with 0.45 micron (nylon/PTFE) into the HPLC vials and subjected to HPLC screening.

2.4.2 Screening of Polysaccharides Structural Components by HPLC

Monosaccharides compounds of *E. longifolia* samples (galactose, glucose, arabinose, xylose, fructose, mannose and rhamnose) were screened and compared from commercialized synthetic compound [Galactose (Sigma), glucose (Sigma), arabinose (Biochem), xylose (Fluka), fructose (Sigma), mannose (Sigma) and rhamnose (Sigma)].

2.5 Data Analysis

Quantity calculations were made according to the linear calibration curves of standard ($y = mx + c$). The actual weight of polysaccharides content of each provenances were calculated based on the peak area produced by UV-VIS profiles.

3.0 RESULTS AND DISCUSSION

The findings by UV-VIS detection showed that polysaccharides content in the roots of Terengganu was the highest with 1.06 % actual weight of polysaccharides while the lowest was Perak with 0.34 % (Table 1). T-test showed that the actual weight polysaccharides among provenances were significantly different with the p-value = 0.011. There

are four provenances with undetectable range of polysaccharides content which were Melaka, Pahang A, Pulau Pinang and Selangor (Table 1), but the chromatogram profiles revealed the present of monosaccharides compounds (galactose, glucose, arabinose, xylose, fructose, mannose and rhamnose) from all the provenances investigated. However, not all samples of the provenances showed all the profiles of each monosaccharides compounds.

Chromatogram profiles from all provenances showed that glucose was at the highest peak indicating the high level of the compound present. However, interestingly profile (Figure 1) from Selangor provenance showed fructose was at the highest peak followed by glucose. On the other hand, there were five provenances (Johor, Pahang B, Pahang D, Perak and Melaka) showed non-detection of arabinose compound from the profiles observed.

In this study, young wildings of *E. longifolia* with the estimated age of one to two years collected from natural forest reserves were used as samples. Thus, the exact age of the plant samples are unknown, but the sampling materials were standardized according to the size of the plants in order to minimize the variation. Polysaccharides compound is a primary metabolite compound, it is generally not affected by abiotic factors. Nevertheless, the age of plant might play an important role in the formation of primary metabolites compounds.

Of now, growth performance data collected (data not shown) revealed that Terengganu provenance has the best survival rate out of 10 provenances investigated throughout four trial plots in Peninsular Malaysia. On the other hand, eurycomanone content present in Terengganu provenance is also the highest [8]. While Pahang B provenance, which is the second highest for polysaccharides content is the best provenance according to the growth performance increment. The quality of phytochemical content in plants is an added characteristic to their quality assessment of the selected plants apart from growth performances.

The main considerations of using polysaccharides content as another marker for the selection of high quality planting materials are the non-toxicity nature of polysaccharides compound and the pharmacological property potential of the compound [6]. In recent years, studies on traditional medicines have uncovered that polysaccharides are the major components contributing to the pharmacological activities such as anti-fatigue [9], antioxidant activities [10] and hepatoprotective effect [11]. Besides, polysaccharides compound including proteins, tannins, alkaloids, terpenoids, flavonoids and saponin are the important components of herbal products that are recognized by modern pharmacological researches [6].

Table 1 Percentage and actual weight of polysaccharide content in roots of *E. longifolia* from different provenances

Provenance	Dry Polysaccharide Fraction (g)	Polysaccharides Content (%)	Actual Weight Polysaccharides (%)	SD	RSD
Johor	0.318	41.5	0.44	0.007	2.231
Pahang B	0.633	35.8	0.76	0.008	2.808
Pahang D	0.302	40.8	0.41	0.012	4.133
Perak	0.265	39.1	0.34	0.001	0.410
Terengganu	0.939	34.0	1.06	0.005	1.938
Melaka	0.574	ND	ND	NA	NA
Pahang A	0.820	ND	ND	NA	NA
Pulau Pinang	0.130	ND	ND	NA	NA
Selangor	0.103	ND	ND	NA	NA

SD = Standard Deviation, RSD = Relative Standard Deviation, ND = non detected, NA = Not Applicable

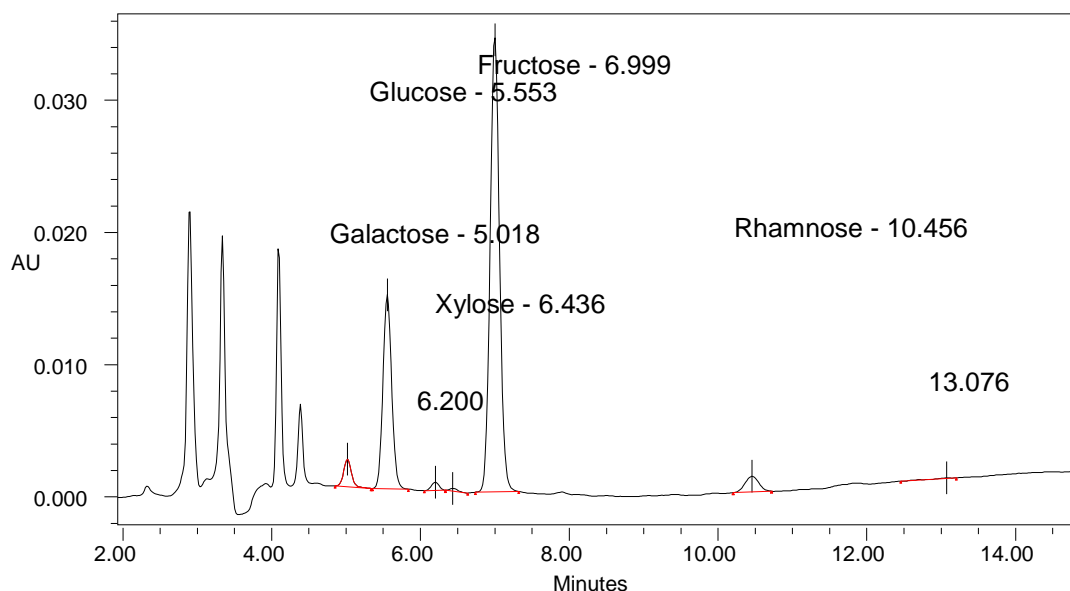


Figure 1 HPLC profiles of monosaccharides compounds of *E. longifolia* (galactose, glucose, arabinose, xylose, fructose, mannose and rhamnose) extracted from Selangor provenance

4.0 CONCLUSION

In root of *E. longifolia*, it is safe to conclude that the highest value of polysaccharides content in the plant would be the best indicator for selection of high quality provenance. This study indicates that Terengganu provenance has significantly the highest content of polysaccharides, followed by Pahang B, Johor, Pahang D and Perak. In addition, growth performances and the occurrences of pests and diseases data at the four trial plots are still being collected and monitored. Ultimately, the provenance with the best growth performance and the best quality of phytochemical content will be declared as the high quality provenance and selected planting material.

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