INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY, BIOLOGY AND CHEMISTRY

Research Article

Standardization parameters, Internal Transcribed Spacer nucleotide sequence and their anti-malarial activity of *Eurycoma longifolia* Jack

S. Katib, N. Ruangrungsi, W Chaijaroenkul and K. Rungsihirunrat*.

College of Public Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand.

ABSTRACT

Determination of pharmacognostic parameters plays a significant role for standardization of medicinal plants. The objective of recent study aims to examine the standardization parameters of *Eurycoma longifolia* Jack in Thailand, the pharmacognostic specifications of foreign matter, acid insoluble ash, total ash, ethanol-soluble content and water content of *Eurycoma longifolia* Jack were evaluated according to World Health Organization guideline. The ITS region was amplified by PCR and sequenced. Dried root of *Eurycoma longifolia* Jack was exhaustively extracted with ethanol and the obtained clued extract were analyzed with thin layer chromatography. The *in vitro* activity of the extract against *P. falciparum* strain 3D7 and K1 were also evaluated using SYBR green I-based assay. The results indicated that the ethanolic extract of dried root showed a great antimalarial activity against both *P. falciparum* 3D7 and K1 strain with the IC_{50} of 2.16 µg/ml and 1.79 µg/ml respectively. The PCR amplification of ITS region generated the PCR product approximately 750 base pairs (bp) in size. The alignment of ITS sequences showed 98-99% homology, 12 single nucleotide polymorphisms and 23 Indels were detected. The Combination of standardization parameters, biomolecular method and biological activity are useful for correct identification and quality control of *Eurycoma longifolia* Jack.

Keywords: *Eurycoma longifolia* Jack, standardization parameters, nucleotide sequence, internal transcribed spacer (ITS) region, antimalarial

1. INTRODUCTION

The increasing demand in herbal medicine has stimulated the improvement in analysis and quality control of their herbal materials and products. To ensure reproducible quality of any herbal plants, requirements and methods for research and evaluation are essential. *Eurycoma longifolia* Jack is a popular medicinal plant in the family Simaroubaceae consists of 200 species. It is indigenous to Southeast Asia including Thailand. All parts of *E. longifolia* Jack has been traditionally used for its antimalarial, antipyretic, antidiabetic, Aphrodisiac, and antimicrobial activities [1-5]. Roots extracts have also been reported for enhancing testosterone levels. In modern dietary supplements, *E.longifolia* can be use to improve sexual function, restore hormonal balance, and improve physical and mental energy due to their chemical composition that have different effects in the body [6-7]. A wide range of chemical components have been isolated and characterized form *Eurycoma longifolia* [8-10]. Although these plants have been widely used in traditional medicine, there is still limited scientific report undertaken in their standardization for safety reason. Moreover, many closely related species have similar morphology and growth conditions as well as many other variables such as the storage condition, environmental condition, and the harvest processing may affect the analysis and quality control of the herbal materials and products. Therefore, additional of molecular method have been developed for medicinal plants authentication. Sequence variation of the Internal transcribe spacer (ITS) region in the

nuclear DNA has been applied widely for identification in species level and can be further applied for investigation the adulteration of herbal drugs [11].

Standardization of dried root of *E. longifolia* Jack was carried out in the present study. Combination of several characteristics such as standardization, biological activity and DNA based analysis have been introduced in this study for correct identification and quality control of this Thai herbal plant. This study aims to examine the standardization parameters, ITS region sequence variation and their antimalarial activity of *Eurycoma longifolia* Jack in Thailand. The information from this study will provide data which is useful in the correct identification and authentication and may help in preventing its adulteration.

2. MATERIAL AND METHODS

2.1 Plants collection

Whole plants of E. longifolia Jack were collected from several localities in Thailand. Plants were authenticated by botanical expert and comparing with the herbarium specimens at Forest Herbarium Thailand (BKF). All samples are deposited at College of Public Health Sciences, Chulalongkorn University.

2.2 Standardization parameters

Dried root of *E. longifolia* Jack were collected from 15 Thai traditional drug stores in 11 provinces in Thailand and evaluated according to World Health Organization (WHO) quality control method for medicinal plant materials [12]. The pharmacognostic specifications of foreign matter, acid insoluble ash, total ash, ethanol-soluble content and water content were determined and presented as mean ± SD. Thin layer chromatography (TLC) of *E. longifolia* Jack roots ethanolic extract was also evaluated. The TLC chromatogram was captured by a digital scanner (Hewlett Packard Deskjet F2280).

2.3 Antimalarial sensitivity assay

Dried root of plant material was grounded to powder and exhaustively extracted with ethanol using Soxhlet apparatus. The extract was filtered through filter paper then concentrated and stored at -20^{o}C. The *in vitro* activity of the extracts were evaluated against *P. falciparum* strain 3D7 (chloroquine sensitive) and K1 (chloroquine resistant) using SYBR green I-based assay [13]. The extract was dissolved in 50% ethanol to give the concentration of 1 mg/ml and then diluted with complete media to obtain the concentration range 0-100 µg/ml. Dihydroartemisnin (DHA) was used as positive control. Fifty microlitre parasite suspension and 50 µl of different concentration of the extracts were mixed together in 96 well plate and incubated at 37ºC under a gas mixture of 50% N_2, 5% O_2 and 5% CO_2 for 2 days. One hundred microlitre of fluorescent haemolysis reagent (0.01% of fluorescent dye SYBR Green I in lysis buffer) was added to each well and incubated the plates in the dark for 1 hours. Fluorescence intensity was determined at the excitation and emission wave lengths of 485 and 530 nm, respectively. Mean values were calculated from the triplicate results. The percent growth inhibition was calculated and the IC_{50} was determined from a log dose-response analysis using the CalcuSyn^{TM} computer program.

2.4 DNA extraction and amplification of ITS region

Genomic DNA was individually extracted from the fresh young leaves of *E. longifolia* Jack using a modified CTAB technique [14]. The ITS region was amplified using a pair of universal primer ITS4 and ITS5 [15]. Amplifications were carried out in 20 µl reaction mixture containing of 1x PCR buffer [100mM Tris-HCl (pH 8.8), 500mM KCl, 0.8% (v/v) Nonidet P40], 2.5mM $MgCl_2$, 0.1mM dNTPs, 0.1µM of each primer, 0.5 Unit of *Taq* polymerase (Fermentas) and 1 µl of DNA template. The PCR amplification reaction conditions were as following: an initial denaturation for 5 min at 95^{o} C, 30 cycles of denaturation at 95^{o} C for 30 second, annealing at 55^{o} C for 30 second and extension at 72^{o} C for 30 second, and then follow by a final 5 min extension at 72^{o} C. The PCR products were evaluated by 1.5% agarose gel electrophoresis, stain with ethidium bromide and visualized under UV transilluminator. The amplified PCR products were then purified by PCR purification kit (QIAGEN) prior sequencing of both sense and antisense stand. ClustalW program was used to multiple aligned the sequence of the ITS region.

3. RESULTS

3.1 Standardization parameters

The pharmacognostic study of *E. longifolia* Jack was determined from 15 Thai traditional drug stores from 11 provinces of Thailand (**Table 1**) and TLC fingerprinting of ethanolic extract was showed in **Figure 1**.

3.2 Antimalarial activity

The susceptibility of *P. falciparum* strain K1 and 3D7 to the ethanolic extract of *E. longifolia* Jack root was investigated *in vitro* based on SYBR Green I assay. The IC_{50} value (drug concentration that inhibits the parasite growth by 50%) was shown in **Table 2.** Dihydroartemisnin was used as positive control.

Table1
The pharmacognostic constant values determination in *E. longifolia* Jack

Specification	Mean ± SD	Min - Max
Foreign matter	0.168 ± 0.179	0 - 0.705
Acid-insoluble ash	0.696 ± 0.064	0.503 - 0.889
Total ash	1.973 ± 0.039	1.857 - 2.088
Ethanol-soluble extractive	1.733 ± 0.178	1.199 - 2.267
Water-soluble extractive	4.888 ± 0.376	3.760 - 6.017
Loss on drying	7.515 ± 0.252	6.760 - 8.271
Volatile oil content	0	0
Water content	13.352 ± 0.839	10.836 - 15.869

Table 2
IC_{50} of *Eurycoma longifolia* Jack extracts against *P. falciparum* strain 3D7 and K1

	IC_{50} (µg/ml)	
	3D7	K1
E. longifolia Jack root ethanolic extracts	2.16	1.79
Dihydroartemisnin	1.97	1.46

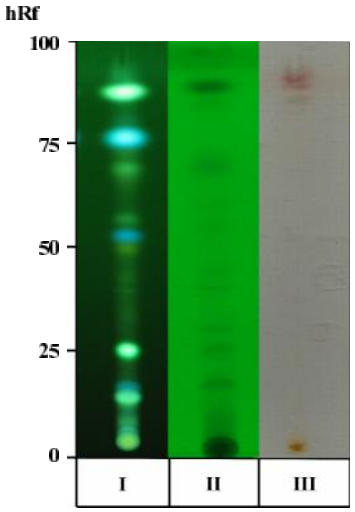

Figure 1
TLC fingerprinting of ethanolic extract from dried root of *E. longifolia* Jack using solvent system; chloroform: methanol = 9:1
Panel I: Detection under UV light (366 nm), Panel II: Detection under UV light (254 nm), and Panel III: Detection with Anisaldehyde.

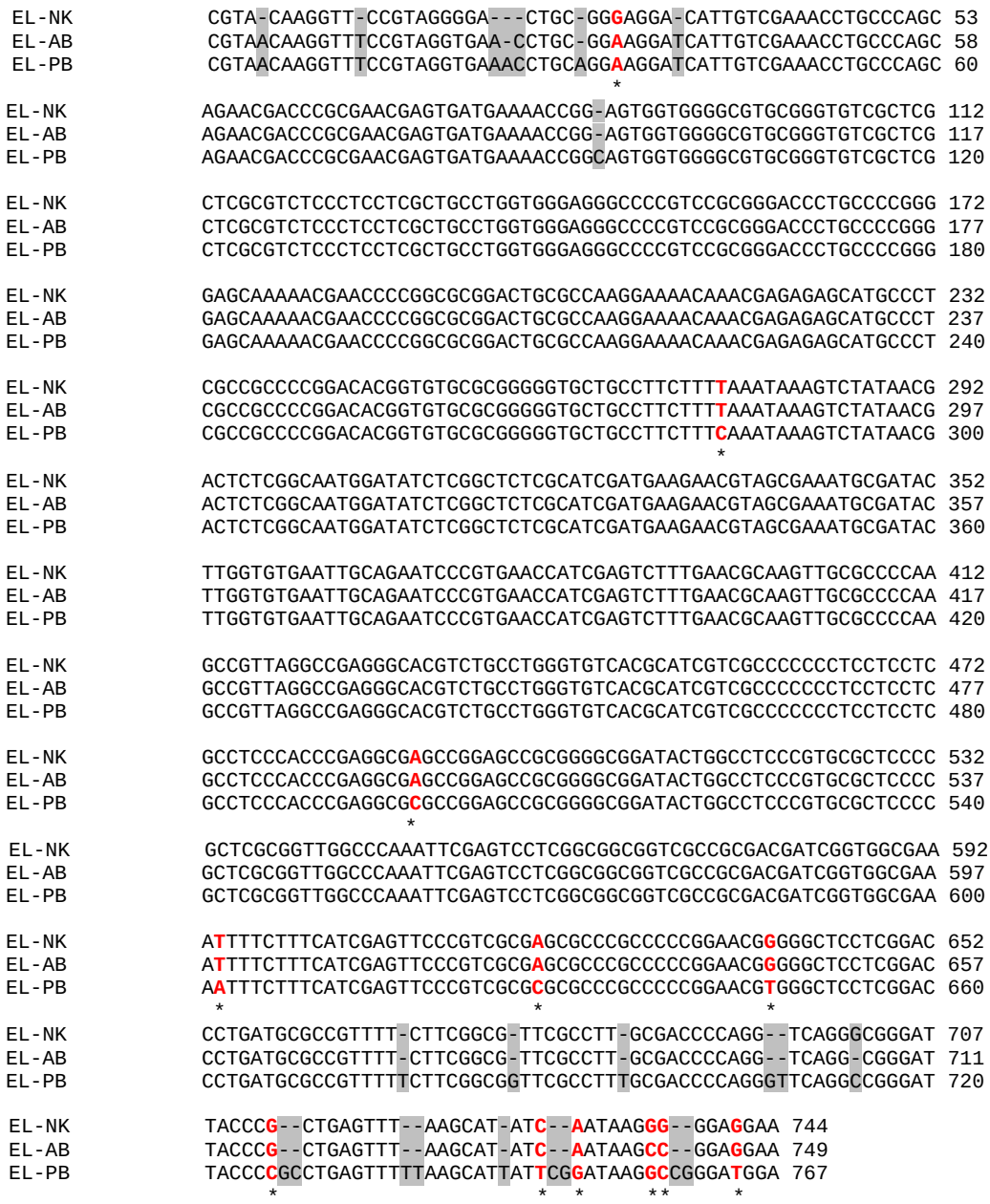

Figure 2
The ITS nucleotide sequence of *E. longifolia* Jack.
(Gray box indicated indels,* indicated SNP)

3.3 Variation of ITS region

An approximately 750 base pair in size of PCR product was obtained from the ITS region amplification from 3 location (EL-NK, EL-AB, and EL-PB). Analysis of sequence alignment of *E. longifolia* Jack showed 98-99% sequence homology (**Figure 2**). There are 12 single nucleotide polymorphisms (SNPs) and 23 Indel**s.**

4. DISCUSSION AND CONCLUSION

Medicinal plants have been long history used for treatment of various diseases. Correct identification and quality control of the starting material is essential for herbal preparation. However, the major problem of employing medicinal plant for traditional usages is the lack of adequate supporting scientific information on their safety, quality and efficacy. Standardization

is a system to ensure the safety and quality of medicinal product. Determination of pharmacognostic parameters such as determination of ash residues, extractive values, and water content plays a significant role for standardization of medicinal plants. Based on the method recommended by World Health Organization, physicochemical parameters of 15 *E. longifolia* Jack were evaluated as present in Table 1. The water content value was 13.352 ± 0.839, the less value of water content could prevent the medicinal plant from microorganism growth through storage [16]. The ash value used to find out quality and purity of medicinal plant. The total ash and acid insoluble ash value were 1.973 ± 0.039 and 0.696 ± 0.064 respectively. The solubility value of *E. longifolia* Jack in water extraction is higher (4.888 ± 0.376) when compared with ethanolic extraction (1.733 ± 0.178). The extractive values can be estimate the solubility of the chemical constituents in the particular solvent. According to the results, ethanolic extracted of *E. longifolia* Jack showed active antimalarial activity both 3D7 and K1 with the IC_{50} of 2.16 µg/ml and 1.79 µg/ml respectively, suggesting that this medicinal plant has a potential to be an antimalarial agent when compare to dihydroartemisnin, a standard antimalarial drug. Previous study indicated that extraction of *E. longifolia* showed antimalarial activity against multidrug resistant strain of *Plasmodium falciparum* under *in vitro* conditions[17-18]. *E. longifolia* root extracts inhibited six *P. falciparum* isolates at 1.25-5.00 ug/ml [19]. However, studies on antimalarial activities of *E. longifolia in vivo* are still limited. Therefore, additional of DNA technologies have been applied for medicinal plant authentication and combinations of various methods have been employed for herbal drug technology development. Identifying of specific regions in the DNA can be used for different level of authentication [20] as the genetic composition is unique for each species and is not affected by physical and environmental factors. Further study should be performing for investigation sequence variation in this plant for more information and developing the genetic marker for species identification of their substitution and adulterants materials.

In conclusion, combination of various methods such as standardization, biological activity, and DNA based method is an effective tool for determination the identity of this herbal plants; *E. longifolia* Jack.

5. ACKNOWLEDGEMENTS

This work was scholarly supported by the scholarships for the Ph.D. programme, Mae Fah Luang University. The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and The Herbal Remedies and Alternative Medicine of Special Task Force for Activating Research (STAR) under 100 Years of Chulalongkorn University Fund. The authors would like to thanks College of Public Health Sciences, for facilities support.

6. REFERENCES

1. Bhat R, Karim AA, Tongkat Ali (Eurycoma longifolia Jack): A review on its ethnobotany and pharmacological importance. Fitoterapia, 2010; 81(7): 669-679.
2. Rashid M, Sachin K, Bahar A, Medicinal uses of Eurycoma longifolia: a review. The Pharma Research, 2009; 2(1): 70-78.
3. Varghese CP, Ambrose C, Jin SC, Lim YJ, Keisaban T, Antioxidant and anti-inflammatory activity of Eurycoma longifolia Jack, A traditional medicinal plant in Malaysia. International Journal of Pharmaceutical Sciences and Nanotechnology, 2013; 5(4): 1875-1878.
4. Kuo PC, Damu AG, Lee KH, Wu TS, Cytotoxic and antimalarial constituents from the roots of Eurycoma longifolia. Bioorg Med Chem, 2004; 12(3): 537-44.
5. Kumar1MR, Kavitha K, Dhanaraj SA, Traditional uses and potential health benefits of Eurycoma longifolia available in Malaysian region – a review. International Journal of Pharmaceutical Research and Development, 2014; 6(4): 130-137.
6. Malviya M, Jain S, Guota VB, Vyas S, Recent studies on aphrodisiac herbs for the management of male sexual dysfunction-a review. Acta Pol Pharm, 2011; 68(1): 3-8.
7. Zanoli P, Zavatti M, Montanari C, Baraldi M, Influence of Eurycoma longifolia on the coplatory activity of sexually sluggish and important male rats. J Ethnopharmacol, 2009; 126(2): 308-313.
8. Bedir E, Abou-Gazar H, Ngwendson JN, Khan IA, Eurycomaoside: a new quassinoid-type glycoside from the roots of Eurycoma longifolia. Chem Pharm Bull (Tokyo), 2003; 51(11): 1301–1303.
9. Kuo PC, Shi LS, Damu AG, Su CR, Huang CH, Ke CH, Wu JB, Lin AJ, Bastow KF, Lee KH, Wu TS, Cytotoxic and antimalarial β carboline alkaloids from the roots of Eurycoma longifolia. J Nat Prod, 2003; 66(10):1324–1327.

10. Ang HH, Hitotsuyanagi Y, Fukaya H, Takeya K, Quassinoids from Eurycoma longifolia. Phytochem, 2002; 59(8): 833–837.
11. Roonyamarai W, Rungsihirunrat K, Vipunngeun N, Ruangrungsi N, Microscopic and molecular analyses of selected Morinda species in Thailand**.** Asian J Trad Med, 2011; 6(3):118-126.
12. World Health Organization (WHO), Quality control method for medicinal plant materials. Geneva: World Health Organization. 1998.
13. Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, Davidson E, Roepe PD, Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. Antimicrob Agents Chemother, 2004; 48(5): 1807–1810.
14. Doyle JJ, Doyle JL, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 1987; 19: 11-15.
15. White TJ, Bruns T, Lee S, Taylor J, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications. 1990. pp. 315-322.
16. Pandey MK, Singh GN, Sharma RK, Lata S, Standardization of Yakrit Plihantak Churna: An Ayurvedic Poly herbal formulation. IJPSR, 2012; 3(1): 171-176.
17. Chan KL, O'neill MJ, Phillipson JD, Warhurst DC, Plants as sources of antimalarial drugs, Part 31- Eurycoma longifolia. Planta Medica, 1986, 52(2): 105-107.
18. Hout S, Chea A, Bunb SS, Elias R, Gasquet M, Timon-David P, Balansard G, Azas N, Screening of selected indigenous plants of Cambodia for antiplasmodial activity. J Ethnopharmacol, 2006; 107(1): 12–18.
19. Ang HH, Chan KL, Mak JW, Effect of 7-day daily replacement of culture medium containing Eurycoma longifolia Jack constituents on the Malaysian Plasmodium falciparum isolates. J Ethnopharmacol, 1995; 49(3): 171–175.
20. Xue HG, Zhou SD, He XJ, Yu Y. Molecular authentication of the traditional Chinese medicinal plant Euphorbia pekinensis. Planta Med, 2007; 73(1): 91-93.