In Vitro Antibacterial Activity of Eurycoma Longifolia Jack (Tongkat Ali) Root Extract

Faisal GG[a], Zakaria SM[b], Najmuldeen GF[c]

[a]*Department of Basic Medical Science, Kulliyyah of Dentistry, International Islamic University Malaysia,*
[b]*Department of Biomedical Science, Kulliyyah of Allied Health Science, International Islamic University Malaysia,*
[c]*Faculty of Chemical and Natural Resources Engineering, University Malaysia Pahang, Malaysia.*

ABSTRACT

Introduction: Currently, researchers are aiming to explore herbal plants to replace synthetic drugs because herbal plants contain high active compounds and fewer side effects. Our study was done to determine the antibacterial activity of *Eurycoma longifolia Jack (E. longifolia)* root using ethanol based extract. **Methods:** Five types of pathogenic bacterial strains were used; Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative (*Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*). Disc diffusion assay and Minimum Inhibitory Concentration (MIC) tests were used to determine the inhibition zone and turbidity of suspension which reflects the antibacterial activity of the extract. **Results:** The ethanolic extract of *E. longifolia Jack* root extract showed positive results against Gram-positive bacteria (*S. aureus* and *B. cereus*) and Gram-negative (*S. typhi*). *B.cereus* and *S.typhi* showed inhibition zone values of 11.76mm and 14.33mm at the extract concentration of 150mg/ml that were higher than the positive control values (9.00, 12.67mm) respectively. However, *E. coli* and P. *aeruginosa* did not show any inhibition by the ethanol-based extract. **Conclusion:** From the results we can conclude that *E.Longifolia* root extract possesses antibacterial activity that can be further explored to produce new medicinal products.

KEYWORDS: *Eurycoma longifolia Jack*, antibacterial, root extract

INTRODUCTION

Exploring herbal plant is becoming an ultimate aim to cure health problems.[1] Herbal and traditional medicines have already been used since thousands of years ago and showed improvement in health. Currently researchers are exploring the medicinal benefits of herbal plants because they contain high amounts of biologically active compounds and produce less side effects[2] and also to overcome the emergence of multidrug bacterial resistance.[3] E*urycoma longifolia Jack (E. longifolia)* also known as Tongkat Ali, Bidara Pahit, Bba binh, and Hau phat (Vietnamese) can be found in Malaysia, Cambodia, Sumatra and Borneo. It belongs to family Simaroubacae and has a very bitter taste. *E. longifolia Jack* root is known to have many medical properties such as aphrodisiac,[4,5] anticancer[6] and antimalarial.[7]

Corresponding author:

Asst. Prof. Dr. Ghasak Ghazi Faisal
Kulliyyah of Dentistry
Basic Medical Sciences Unit
Jalan Sultan Ahmad Shah
Indera Mahkota
Kuantan
Pahang, Malaysia
Telephone: +60105442293
Email: drghassak@yahoo.com

There is an increasing demand especially from pharmaceutical company to search for new medicine to treat diseases, while reducing side effects associated with synthetic drugs. Hence, plant-based medicine is a good choice as it contains a lot of secondary metabolites with the greatest effects on biological activities.[8] According to Nadia *et al.* (2012),[9] isolation of secondary metabolites from the root of *E. longifolia Jack* showed the presence of eurycomanone, eurycomanol, eurycomalactone, cathine-6-one alkaloid, phenolic components, tannins, quassionoids, and tripertenes. In this study, the main goal was to explore the antibacterial activity of *E. longifolia Jack* root using an ethanol based extraction method.

MATERIALS AND METHODS

Plant materials: The *E. longifolia Jack* root used in this study was purchased from a certified supplier. The sample was dried in the oven at 40°C until constant weight was achieved. After that, the dried plant materials were grinded into powder using a dry grinder and 35.7404 g was obtained. The active compounds of the sample were extracted with absolute ethanol around its boiling point at 60 to 65 °C using Soxhlet extraction method. It was done for approximately 24 hours. Then, the extracts were dried under vacuum and reduced pressure using rotary evaporator at 60°C and at a pressure of 175 mbar. This was done to evaporate the solvents and to obtain the crude extracts. The weight of final extract was 11.8 g and the percentage yield of ethanol extract of *E.*

longifolia Jack was 33%. The residue obtained were placed in universal bottle and stored in freezer at -4°C until further test was conducted. Finally, the crude extracts of root *E. longifolia Jack* materials were used in the antibacterial assay. The working stocks for the crude extract of root of *E. longifolia Jack* were prepared at 50 mg/ml, 100 mg/ml and 150 mg/ml by dissolving 100 mg, 200 mg and 300 mg of the crude extracts into 2 ml of 25% ethanol respectively. Erythromycin and Ciprofloxacin containing discs were used as positive controls for treatment of Gram-positive and Gram-negative bacteria respectively.

Bacterial strains: Reference strains of human pathogens were used, including Gram-positive bacteria, *Bacillus cureus* (ATCC 25923) and *Staphylococcus aureus* (ATCC 14778), while Gram-negative bacterial strains were *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Salmonella thypi* (from Institute of Medical Research Health Ministry, IMR). For the anti-bacterial assay, the bacteria were grown on the Muller Hinton (MH) broth and agar and put in the incubator at 37 °C and observed the turbidity.

Adjustment of Microorganism Number and Inoculums Preparation: Direct Colony suspension method was used to prepare inoculation suspension.[10,11] Barium Sulfate (0.5 McFarland) of standard suspension was prepared by adding 0.5 part of 0.048M $BaCl_2$ to 99.5 parts of 0.18M H_2SO_4 and agitated vigorously until a homogenous suspension was obtained. The turbidity of the suspension was verified by measuring the optical density at 600 nm (OD_{600}) by the spectrophotometer. Proper dilutions were done to get an absorbance value of 0.008 - 0.10 which corresponded to 0.5 McFarland standards. Under aseptic condition, five colonies isolated by ignition-sterilized inoculation loop from 24 hour cultivated agar plates of each microbe were suspended separately in 20 ml pre-warmed (37oC) broth medium (Nutrient broth (NB) for bacterial strains and kept in screw-cap bottles and incubated at 37°C. During the incubation period, aliquots of 1 ml were taken from the culture at hourly intervals and optical density (OD_{600} for bacterial suspension) were measured using spectrophotometer. Finally, the resultant broth suspensions contained 10^7 CFU/ml for bacterial spp. was used for all experiments performed. Suspensions were always agitated thoroughly before OD measurement and inoculation.

Agar Disc Diffusion Assay: The Kirby-Bauer Disc Diffusion method was chosen to determine the anticipated antimicrobial activity of *E. Longifolia Jack* root.[11] Five pathogenic bacterial strains were swabbed into the MH agar to grow the bacteria for a night in the incubator at 37 °C. The filter paper discs loaded with crude extract of *E. longifolia Jack* root and reference antibiotic-containing disc (5 µg/disc ciprofloxacin and 5 µg/disc erythromycin) were laid down on the inoculated agar plates using sterile forceps with gentle pressing to ensure a good adherence to the agar surface. The discs were distributed to be at least 15mm from the edge of the plate and no closer than 24mm from center to center. Finally, the plates were inverted upside downward and incubated at 37°C for 24 hour. After the incubation period, the zone of inhibition (mm) around each disc was measured using ruler and compared with reference antibiotics used.

Broth Microdilution test (MIC): Microdilution method[12] was used in this study to identify the inhibition growth of test organisms through the minimal concentration of root extract. Serial double-fold dilutions were carried out in sterile 96-well plate. First, all wells to use were filled with 180 µl of Muller-Hinton broth containing microorganisms. Then, 20 µl of sample extract were transferred to the first well and mixed. Three fold serial-dilution was performed by transferring 100 µl of the mixture in the first well into the next consecutive wells, until the end of the row. The last well, 100 µl of the mixture were discharged, so the total volume of each well was 100 µl. The microplate was incubated at 37 °C for 18 – 24 hours. The MIC value was determined by comparing the turbidity of the mixture in test wells with blank wells. To decrease the occurrence of bias, the test was assessed by two examiners who were blinded to the type of mixture. The test of all sample and control were performed in triplicates.

RESULTS

Table I. Antibacterial activity profile of ethanol extract of *Eurycoma longifolia Jack root* using disc diffusion method

	Zone of inhibition (mm) (Mean ± SD)					
	Extract concentration			Positive Control		Negative Control
Bacteria	50 mg/ml	100 mg/ml	150 mg/ml	Erythromycin	Ciprofloxacinc	25% Ethanol
S. aureus	5.67 ±2 .05	8.78 ± 1.13	10.67 ± 0.82	11.78 ± 0.79	- -	
B. cereus	5.89 ± 1.80	9.44 ± 1.42	11.67 ± 1.50	9.00 ± 1.05	- -	
S. typhi	8.44 ± 0.50	9.33 ± 0.82	14.33 ± 0.47	-	12.67 ± 3.13	-
E. coli	- -	- -			8.39 ± 0.42	-
P.aeruginosa	- -	- -			12.28 ± 0.47	-

According to the results in Table I, we have found that the root extract possess antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella typhi* which was demonstrated by a zone of inhibition of growth which increased with the increase in the concentration of the extract. However no antibacterial activity was detected against *Escherichia coli* and *Pseudomonas aeruginosa*.

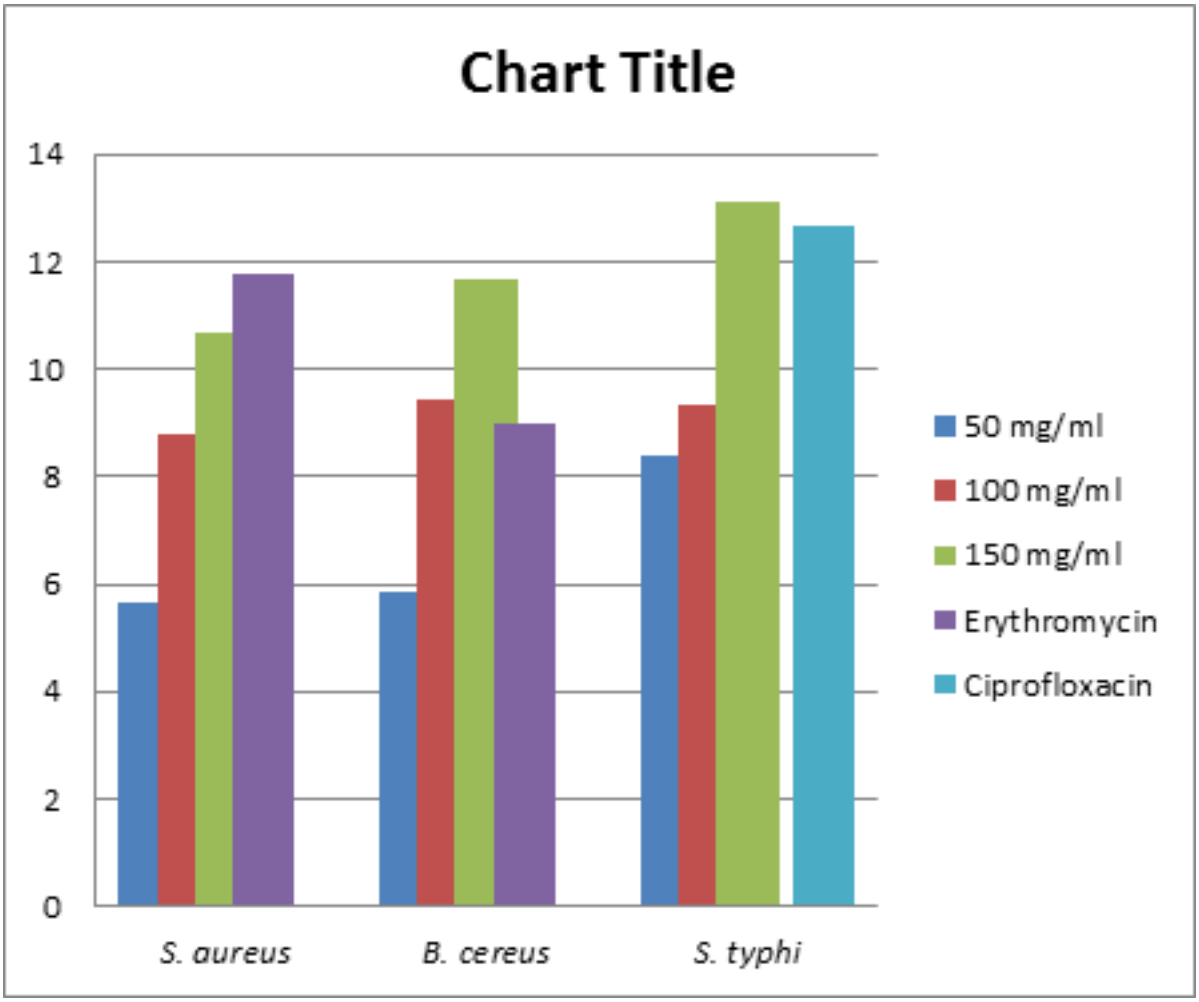

Figure 1: Diameter of inhibition zone (mm) against human pathogenic bacteria using different concentrations of E.L root extract compared to positive control (Erythromycin and ciprofloxacin).
Figure 1 shows that the highest inhibition zone is achieved with the highest concentration of extract 150mg/ml. The inhibition zones for *B.cereus* and *S.typhi* at extract concentration of 150mg/ml are higher than that of the positive control.

Table II. Minimum inhibitory concentration (MIC) of E.L root extract on pathogenic bacteria

Concentration of plant extract (mg/ml)	Staphylococcus aureus	Bacillus cereus	Salmonella typhimirium
200	+	+	+
100	+	+	+
50	+	+	+
25	+	+	+
12.5	-	+	-
6.25	-	-	-
3.125	-	-	-
1.560	-	-	-

+ = clear
- = turbid

DISCUSSION

Our study showed positive results as there were inhibition zones of bacterial growth. Ethanol extracts of *E. longifolia Jack* root displayed antibacterial activity against *pathogenic Gram+ve and some Gram -ve bacteria*. Our results are new in documenting anti-bacterial activity of the root extract using alcohol and heat mediated extraction. Previous research on the antibacterial properties of *E.longifolia* is limited to a few studies that have different results some of which are contradictory to others. The study by Farouk *et al*.[13] showed that the leaves and stems of Eurycoma longifolia possess antibacterial activity while the roots did not show any activity. Another study by Farouk *et al*.[14] also indicated that only the leaves part of *E. longifolia Jack* shows the antibacterial activity. Only one study which was done by Danial et al.[15] reported that there was inhibition of the growth of pathogenic bacteria by *E.longifolia Jack* root extract and that study used a different extraction method. On the contrary, Tzar *et al*.[16] stated that there was no antibacterial activity of *E. longifolia* root extract. This concludes that many factors contribute for plants to show its therapeutic properties, for example the mode of processing of samples to obtain the crude extract and the right handling before, after and during the experiment was conducted.

In our study we used ethanol extraction method. In general, alcoholic extracts displayed higher antibacterial and antifungal activity than aqueous extract.[17] Our method of extraction and solvent used were different from the previous mentioned studies, which might be the reason behind the different results that we obtained. We chose Soxhlet method as our method of extraction because it produces higher crude extract yield when compared to other methods.[18] Besides, it also reduces the usage of solvent and produces high purity product. Moreover, the hot continuous extraction can release more secondary metabolites, especially flavonoids, eurycomanone, eurycomanol, eurycomalactone, quanissoids, and triterpenes.[19]

Staphylococcus aureus is a gram positive bacteria which causes a range of diseases from minor skin infections, such as folliculitis, scalded skin syndrome, and life threatening disease like pneumonia, meningitis, food poisoning and sepsis.[20] The inhibition zones value for *S. aureus* recorded for ethanol root extract for three concentrations were 5.67 ± 2.05 mm (50 mg/ml), 8.78 ± 1.13 mm (100 mg/ml) and 10.78±0.79 mm (150 mg/ml) Table I. However, all inhibition zone values recorded was lower than positive control values (erythromycin) (Figure 1).

Bacillus cereus is a pathogenic bacteria that can cause diarrhoea, gangrene and ocular infection.[21] *B. cereus* inhibition zone recorded for ethanol root extract for three concentrations were 5.89 ± 1.80 mm (50 mg/ml), 9.44 ± 1.42 mm (100 mg/ml), and 11.67 ± 1.50 mm (150 mg/ml) (Table I). The inhibition zone value at 150 mg/ml was higher than positive control values (erythromycin) which means that the extract has a strong antibacterial effect against this bacteria (Figure 1).

The incidence of antibacterial resistant bacteria causing food borne infection, like *S. typhi* has remarkably increased and lead to death.[22] In addition, the contamination of fresh fruits and vegetables has increased the incidence of food-borne outbreaks.[23] *S. typhi* was the only gram-negative bacteria that showed inhibition of growth by the ethanol root extract. Besides, compared to other bacteria's inhibition zones, *S. typhi* showed the highest inhibition zone values which were 8.44 ± 0.50 mm (50 mg/ml), 9.33 ± 0.82 mm (100 mg/ml) and 14.33 ± 0.47 mm (150 mg/ml) (Table 1). The inhibition zone value at 150 mg/ml was higher than the positive control values (ciprofloxacin). At 150 mg/ml extract concentration, we found that *B. cereus* and *S. typhi* had higher inhibition zones compared to the positive control values (Figure 1).

Regarding other gram negative bacteria, no inhibition zone was recorded for *E. coli* and *P. aeruginosa* (Table I). This may be due to the fact that gram-negative bacteria has an inner layer and outer layer containing thick lipid bilayers that lead to its selective permeability[24] and make it more resistant.

In order to measure the strength of the exact inhibitory concentration, minimal inhibitory concentration testing (MIC) was performed according to positive results in the disc diffusion assay. The minimal inhibitory concentration was identified by the clearness of the turbidity of the well. The identification was done by comparing the test well to the control well by observing with naked eyes in sufficient light source. The procedure was conducted by two examiners who were blinded for the type of mixture being examined to reduce bias. From the result, the clear well was chosen until the lowest concentration and was recorded.

According to the MIC of *E. longifolia Jack* root against gram-positive and gram-negative pathogenic bacteria (Table II), it inhibited the visible growth of *S. aureus, B. cereus* and *S. typhi* from descending concentrations of 200-12.5 mg/ml where the mixture of bacterial culture and the extract produced clear solutions. The lowest MIC value of the extract was detected against gram-positive bacteria which was *Bacillus cereus* at a value of 12.5 mg/ml. All solutions became turbid or cloudy at the concentration of 12.5 - 6.25 mg/ml. Thus, the lowest MIC of the extract that inhibited the growth of all three pathogenic bacterial strains was 25 mg/ml.

CONCLUSION

Our study showed that ethanol-based Eurycoma longifolia Jack root extract has antibacterial properties on the following pathogenic strains:

S.aureus, B. cereus, and *S. typhi*. Thus, the results from this study have revealed the potential antimicrobial activity of E.longifolia. Further studies on isolating bioactive compounds using bioassay guided approach are recommended to ensure the active compounds presence from *E. longifolia Jack*. There are many aspects that influence the antibacterial activities such as part of plant, methods of extraction and types of solvents used to get the crude extract.

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