Pharmacogn J. 2021; 13(3): 787-791
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Research Article

The Effect of Eurycoma Longifolia Jack (Tongkat Ali) Root Extract on salivary *S. Mutans, Lactobacillus* and *Candida Albicans* Isolated from High-Risk Caries Adult Patients

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ABSTRACT

Introduction: The roots of *E. longifolia jack* (E.L.) or Tongkat Ali have been used in traditional medicine as well as supplements and food additives. Many chemical compounds have been detected in extracts of its roots which are believed to be responsible for its medicinal properties. In this study, our objectives were to study the effects of EL root extracts on the growth of *Streptococcus Mutans, Lactobacillus* and *Candida Albicans* isolated from saliva of adult patients with high caries risk. **Materials and Methods**: The ethanolic extract of the root of this plant was tested against saliva isolated *Streptococcus Mutans, Lactobacillus* and *Candida Albicans* via disc diffusion assay at a concentration of 200mg/mL. The minimum inhibitory concentration was carried out by the standard broth microdilution method. Cell viability of test microorganisms against different concentration of the extract and inhibition zones were calculated. **Results**: Disk diffusion assay showed positive zones of inhibition for all test microorganisms with S. mutans, Lactobacillus and C. albicans exhibiting zones of inhibition of 8.3 ± 0.7mm, 12.4 ± 2.4mm and 21.4 ± 2.7mm respectively. For minimum inhibitory concentration, the test microorganisms were tested at concentration of 250mg/mL, 125mg/mL, 62.5mg/mL, 31.3mg/mL and 0mg/mL. The minimum inhibitory concentration showed that MIC of S. mutans was at 62.5mg/mL, Lactobacillus at 125mg/mL and C. albicans at 31.3mg/mL. Lastly, the cell viability results supported the MIC determined prior. **Conclusion**: Ethanol-based *E. longifolia* Jack root extract has an antimicrobial effect on the following microorganisms isolated from the saliva of high-risk caries adult patients: *S. mutans, Lactobacillus* and *C. albicans.*

Key words: *Eurycoma longifolia* Jack, Inhibition, Salivary isolate, Antimicrobial effect.

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History

- Submission Date: 03-02-2021;
- Review completed: 19-03-2021;
- Accepted Date: 05-04-2021.

DOI : 10.5530/pj.2021.13.100

Article Available online

http://www.phcogj.com/v13/i3

INTRODUCTION

Natural product extracts have been known and used for thousands of years for their health benefits[1]. In order to combat multi drug bacterial resistance researchers continuously are experimenting

herbal plants because they also contain high amounts of bioactive compounds and produce less side effects.[2]

Eurycoma longifolia Jack is an herbal medicinal plant of South-East Asian origin, popularly recognized as 'Tongkat Ali.' The plant appear to be a medium-sized and slender. It can reach 10 metres in height and is often unbranched. The root of this plant has been used as a form of traditional medicine in South East Asian region. This plant have been used as supplements, as well as food and drink additives.

Among the many effective medicinal values of the root of this plants are sexual enhancement property for males, as well as antipyretic, antimalarial, antibacterial, and antitumor properties.[3-6] In animal studies, the glycoprotein components has been shown to exert anti-cancer, anti-aging, aphrodisiac and pro-fertility properties. Research on E.L has shown testosterone enhancing effect, anti-stress and anti-aging effect with normalizing growth hormone and anti-oxidant activity.[7]

Worldwide dentition data collected by WHO reported that dental caries is unfortunately a prevailing health problem faced even in the more industrialized parts of the world. Dental caries is defined as a multifactorial transmissible, infectious, disease characterized by complex interaction between dental plaque (biofilm) with fermentable dietary carbohydrates on tooth surface over time.[8] Streptococcus *mutans* are Gram-positive bacteria that reside in the human mouth and, more specifically, in the multispecies biofilms on the surfaces of teeth. They have been identified as a pathogen in the development of dental caries. They are highly acidogenic, producing short-chain acids which soften hard tissues of teeth.[9]

Apart from *Streptococcus Mutans*, another microorganism is commonly implicated in the development of dental caries. They are called *Lactobacilli*. *Lactobacilli* are important part of the oral flora, they are fundamental microbes in the formation of dental caries.[10] These bacteria can cause

Cite this article: bin Ramzi MI, bin Kosnin MH, Faisal GG, Arzmi MH, Kusumawardani A, Sabere ASM, *et al.* The Effect of Eurycoma Longifolia Jack (Tongkat Ali) Root Extract on salivary *S. Mutans, Lactobacillus* and *Candida Albicans* Isolated from High-Risk Caries Adult Patients. Pharmacog J. 2021;13(3): 787-91.

a decrease in environmental pH until values are less than 4.5 which is less than the critical pH of 5.5.[11]

Caries risk assessment is a form of assessment done in order to determine the probability of a person to have dental caries. The concept of this assessment is grounded on the well-known fact that dental caries is a disease. Dental caries is considered a lifestyle-based disease therefore factors such as habits, socioeconomic background, attitude, etc., strongly influence the disease process and progress.[12] According to the American Dental Association (ADA) caries risk assessment, for a patient to be categorized as high caries risk patient, the patient must either clinically present with 3 or more carious lesions or restorations in the last 36 months, have teeth missing due to carious lesions in the past 36 months or have severe dry mouth (xerostomia).

Lastly, another microorganism has been commonly implicated in posing health risks are the *Candida sp.* fungi. *Candida* is an important member of the oral microflora and is present in a high percentage of the general population. It is also recognized as an opportunistic microorganism that has the ability to cause oral diseases such as oral candidiasis. Candida *albicans* is the main causative agent of oropharyngeal candidiasis.[13]

For this research, the objective is to investigate the ability of the *E. longifolia* Jack ethanolic extract to reduce the growth of *S.mutans, lactobacillus and C.albicans* isolated from saliva of high risk caries patients.

MATERIALS AND METHOD

Ethical approval

Prior to commencement of sample collection from patients at IIUM Dental Polyclinic, ethical approval was obtained from the IIUM Research Ethics Committee (IREC) on 19 March 2018. (Ref: IIUM/504/14/11/2/IREC 2018-056).

Sample collection

Three samples were collected from adult patient with high caries risk (CRA form modified from American Dental Association, 2011; Scottish Intercollegiate Guideline Network, 2000). The selected patients were briefed with the experiment and consent was obtained prior to sample collection. Following that, 3 mL of unstimulated saliva was collected in a sterile container prior identification of microorganisms.

Identification of microorganisms

To identify *S. mutans* and *Lactobacillus* sp., 100 µL of the unstimulated saliva was smeared onto both strips of caries risk test (CRT) kit and incubated at 37 °C for 24 to 72 hours. Meanwhile, to identify *C. albicans*, the 100 µL of unstimulated saliva was inoculated on CHROMagar followed by incubation at 37 °C for 24 to 72 h in aerobic condition. Finally, the morphology including the surface appearance, margin, forms, elevation and colour of the colony were recorded.

Growth of microorganisms

Using a sterile wire loop, isolated bacteria (*S. mutans* and *Lactobacillus* sp.) and *C. albicans* were inoculated on Brain Heart Infusion (BHI) agar and Yeast Extract Peptone Dextrose (YEPD) agar, respectively using single colony dilution streaking method. Finally, the plates were incubated at 37 °C for 24 h in aerobic environment.

Preparation of stock cultures

Stock cultures preparation of Streptococcus mutans and Lactobacillus

Using a sterile cotton swab, 3 colonies of *S. mutans* or *Lactobacillus* sp. from BHI agar were inoculated in a sterile tube containing 10 mL of BHI broth. The suspension was the mixed thoroughly using vortex mixer and incubated at 37 °C for 4 to 5 hours. Following incubation, the turbidity of the growth suspension was measured and standardised using spectrophotometer to an optical absorbance 0.1 at a wavelength of 620 nm (OD_{620nm} 0.1) that were equivalent to 10^8 cells mL^{-1}, and 10^7 cells mL^{-1} of *S. mutans* and *Lactobacillus* sp., respectively. Finally, the standardised stock cultures were aliquoted into 1 ml sterile vials and stored at -20 °C for long time storage.

Preparation of Candida albicans stock culture

Using a sterile cotton swab, three colonies of *C. albicans* from YPD agar were inoculated in a sterile tube containing 10 ml of YEPD broth. The suspension was the mixed thoroughly using vortex mixer and incubated at 37 °C for 3 to 4 h at 37 °C. Following incubation, *C. albicans* suspension was standardised to give OD_{620nm} 0.1 that was equivalent to 10^6 cells mL^{-1}. The standardised stock cultures were finally pipetted into 1 ml sterile vials and stored at -20 °C for long time storage.

Preparation of the Eurycoma Longifolia Jack plant extracts

Eurycoma longifolia Jack roots were purchased from a certified supplier. The roots were grinded and extracted with ethanol using Soxhlet method, then the excess ethanol was removed by drying in a rotary evaporator.

Antimicrobial activity of Eurycoma longifolia Jack

The susceptibility of *S. mutans*, *Lactobacillus* sp. and *C. albicans* towards E. *Longifolia Jack* plant extract were determined using disc diffusion test and broth dilution methods.

Disc diffusion assay

Disc diffusion test was carried out. Initally, *S. mutans*, *Lactobacillus* spp. or *C. albicans* colonies were suspended into 5 mL of 0.85% (v/v) of sterile saline. The optical density of the cell suspension was then standardized to an OD_{550nm} of 0.1 to give 10^8 cells mL^{-1}, 10^7 cells mL^{-1} and 10^6 cells mL^{-1} of *S. mutans*, *Lactobacillus* sp. and *C. albicans*, respectively. Following that, 100 µL of the suspension was pipetted out and evenly swabbed on Mueller-Hinton (MH) agar (BD, USA). Chlorhexidine (120 µg), ampicillin (120 µg), nystatin (10 µg) and *E. longifolia* extract (20 mg) impregnated paper discs were aseptically placed onto the agar plate and incubated overnight at 37 °C. Finally, the diameter of growth inhibition zone was measured. A disc containing sterile distilled water was also included which serve as the negative control.

Minimal inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) assay was carried out by the standard broth microdilution method in YPD medium according to the Clinical and Laboratory Standards Institute (CLSI) reference method M27-A3. A sterile 96-well plate was labelled W1–W7 horizontally and numbered vertically. Using sterile pipette, 100 µL of YEPD broth was added to W2 through W7 while 100 µL of *E. longifolia* extract (20 mg mL^{-1}) was added into W1 and W2. The plate was slowly agitated to mix the content. Using a sterile pipette, 100 µL of W2 was transferred to W3. Following a thorough mixing, 100 µL of W3 was transferred to W4 and the procedure was continued through until W6. After mixing, 100 µL from W6 was discarded. Lastly, 100 µL of *S. mutans*, *Lactobacillus* sp. or *C. albicans* suspension that was standardized to an OD_{550nm} of 0.1 was aseptically added to W2 through W7. W7 that received no antimicrobial agent and W1 that did not contain microorganism served as the respective negative and positive control for the experiment. The plate was incubated for 24 h at 37 °C. The MIC endpoint was determined as the lowest concentration that caused a significant diminution (≥50% inhibition) of growth relative to the untreated groups.

Viability assay

Initially, the biofilm was scraped off to remove adhered cells in the 96-well plate as prepared in MIC. Later, 100 µL of the yeast and bacterial inocula were diluted in YPD broth and BHI broth, respectively. Then, each suspension was serially diluted and inoculated on a similar media and incubated at 37 °C for 24 h. Finally, the total number of colony forming unit (CFU) was determine and recorded.

Statistical analyses

Statistical analyses were conducted on SPSS version 25 to compare the susceptibility of of *C. albicans*, *S. mutans* and *Lactobacillus* sp. towards *E. longifolia Jack* using paired ANOVA post-hoc Tukey HSD. The data were considered significant when P<0.05.

RESULTS

Inhibition zone of *C. albicans, S. mutans* and *Lactobacillus sp.*

In this current study, results showed that both *C. albicans* ATCC and *C. albicans* caries isolate were susceptible to *E. longifolia Jack* (200 mg) with no significant difference was observed (P>0.05) between the two strains (Figure 1, Table 1). In addition, *S. mutans* and *Lactobacillus* sp. isolated from caries patient were also susceptible towards *E. longifolia Jack*, with significantly more inhibition observed in *Lactobacillus* sp. than *S. mutans* (P<0.05). The study also exhibited no inhibition of *C. albicans* in both strains towards ampicillin (120 µg). Similarly, no inhibition of *S. mutans* and *Lactobacillus* sp. was observed when treated with nystatin (10 µg). We also observed that *C.albicans* of both strains were significantly more susceptible towards *E. longifolia Jack* than *S. mutans* that was isolated from caries patient. Interestingly, the inhibition of caries isolated *C. albicans* towards *E. longifolia Jack* was significantly higher than chlorhexidine (120 µg; P<0.05), however, no significant different was observed on *C. albicans* ATCC strain (P>0.05).

Minimum inhibitory concentration of *S. mutans, Lactobacillus sp* and *C. albicans* towards *E. longifolia.*

The present study showed that the minimum inhibitory concentration (MIC) of caries isolate *S. mutans,* caries isolate *Lactobacillus* sp., *C. albicans* ATCC and caries isolate *C. albicans*, towards *E. longifolia* Jack extract were 62.5mg/mL, 125 mg/mL, 125 mg/mL and 31.3 mg/mL, respectively. Caries isolate *C. albicans* exhibited more susceptible towards *E. longifolia* extract compared to *C. albicans* ATCC (Table 2). Similarly, caries isolate S. *mutans* exhibited more susceptibility to the extract more than caries isolate *Lactobacillus* sp.

Cell viability of *C. albicans, S. mutans* and *Lactobacillus sp.* towards *E. longifolia*

The present study showed that caries isolated *C. albicans* formed more colony forming unit (CFU) compared to *C. albicans* ATCC significantly after 24 h incubation at 37 °C when grown in YPD broth (P<0.05; Table 3). Furthermore, *Lactobacillus* sp. formed significantly more biofilms than *S. mutans* isolated from dental caries.

A significant decrease of viable cells was observed for *C. albicans* ATCC when treated with 62.5 mg/mL of *E. longifolia* extract compared to 31.5 mg/mL (P<0.05). However, no significant difference was observed between the two concentrations of *E.longfolia* in caries isolated *Lactobacillus* sp. (P>0.05; Table 3).

Table 1: Inhibition zone of *C. albicans* ATCC, caries isolates *C. albicans*, caries isolates *S. mutans* and caries isolates *Lactobacillus* sp. towards *E. Longifolia Jack* water-based extract, ampicillin, nystatin and chlorhexidine after 24 h incubation at 37 °C. The study was repeated in three biological replicates and three technical replicates (N=9). Standard deviation (SD) is given in the parenthesis.

Samples	Inhibition zone (mm) (Mean ± SD)			
	E. Longifolia Jack ethanol-based extract (200 mg/disc)	Ampicillin (120 µg/disc)	Nystatin (10 µg/disc)	Chlorhexidine (120 µg/disc)
C. albicans ATCC	15.0 (3.9)	0	20.1 (4.2)	12.6 (1.5)
Caries isolates *C. albicans*	21.4 (2.7)	0	24.4 (1.3)	15.8 (2.0)
Caries isolates *S. mutans*	8.3 (0.7)	18.1 (0.8)	0	20.3 (4.7)
Caries isolates *Lactobacillus* sp.	12.4 (2.4)	31.0 (1.3)	0	26.7 (7.3)

Table 2: Minimum inhibitory concentration (MIC) of *C. albicans* ATCC, caries isolates *C. albicans*, caries isolates *S. mutans* and caries isolates *Lactobacillus* sp. towards *E. Longifolia Jack* water-based extract after 24 h incubation at 37 °C. Symbols (+) showed positive inhibition and (–) showed negative inhibition. The study was repeated in three biological replicates and three technical replicates (N=9).

Concentration of E. longifolia Jack extract (mg/mL)	Minimum Inhibitory Concentration (MIC)			
	C. albicans ATCC	Caries isolate C. albicans	Caries isolate S. mutans	Caries isolate Lactobacillus sp.
250	+	+	+	+
125	+	+	+	+
62.5	-	+	+	-
31.3	-	+	-	-
0	-	-	-	-

Table 3: Cell viability of *C. albicans* ATCC, caries isolates *C. albicans*, caries isolates *S. mutans* and caries isolates *Lactobacillus* sp. towards *E. Longifolia Jack* water-based extract based on cfu/mL after incubation for 24hr at 37 °C. The study was repeated in three biological replicates and three technical replicates (N=9). Standard deviation (SD) is given in the parenthesis.

Concentration of E. longifolia Jack extract (mg/mL)	Colony forming unit (CFU/mL)			
	C. albicans ATCC	Caries isolate C. albicans	Caries isolate S. mutans	Caries isolate Lactobacillus sp.
250	0	0	0	0
125	0	0	0	0
62.5	1.6×10^6 (1×10^5)	0	0	7.3×10^7 (9×10^6)
31.3	6.9×10^6 (2.2×10^6)	0	2.0×10^8 (9×10^7)	7.5×10^7 (1×10^6)
0	12.4×10^7 (3×10^5)	11.0×10^7 (2×10^5)	3.5×10^8 (5×10^6)	7.1×10^7 (4×10^6)

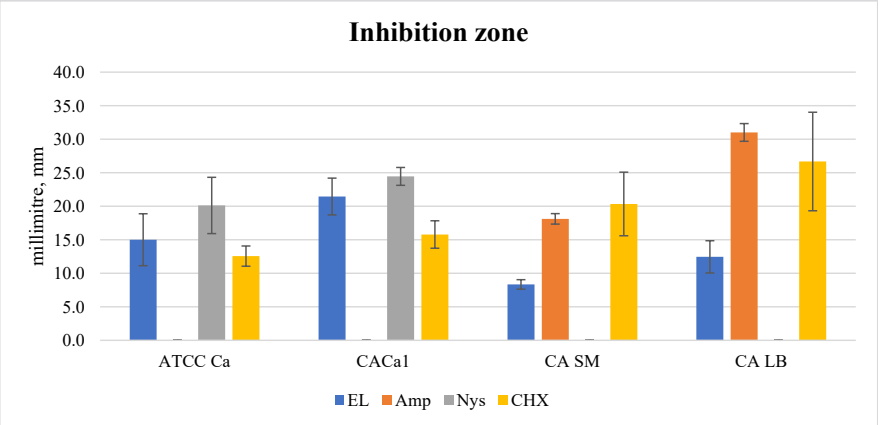

Figure 1: Inhibition zone of *C. albicans* ATCC, caries isolates *C. albicans*, caries isolates *S. mutans* and caries isolates *Lactobacillus* sp. towards *E. Longifolia Jack* water-based extract, ampicillin, nystatin and chlorhexidine after 24 h incubation at 37 °C. The study was repeated in three biological replicates and three technical replicates (N=9).

DISCUSSION

In a previous study, the results of the antibacterial activity on different parts of *E. longifolia* in relative to positive control antibiotics against Gram-positive bacteria (*S. aureus, M. leteus, E. faecalis, B. subtilis*) and Gram-negative bacteria (*P. vulgaris, E. coli, S. thyphi*) showed antibacterial activity on the leaves and stem of E. longifolia and not the root.[14]

However, much recently, *E. longifolia* Jack had been discovered that the root of this plant species has exhibited antibacterial and antifungal effects. *E. longifolia* Jack's crude root extract was an effective antibacterial agent with large inhibition zones against five pathogenic strains of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis and Shigella felxneri*.[15]In another study, ethanolic root extract of *E. longifolia* Jack was tested against two fungi species namely *C. albicans* and *Aspergillus fumigatus*. It was found that *E. longifolia* Jack extract exhibited positive zones of inhibition for both fungi strains.[16]

In this study, ethanol is used as solvent in extracting the active compounds of *E. longifolia* plant. A phytochemical screening of *E. longifolia* root extract has shown the presence of alkaloids, phenolic compounds, terpenoids, flavonoids, cardiac glycosides and proteins.[17] Flavonoids well known for their excellent antioxidant properties as well as anti-inflammatory effects.[18] In addition to this, the detection of terpenoids have a great implication to the antimicrobial activity of *E. longifolia* because terpenoids have been referred to as antibiotics, insecticidal, anthelmintic and antiseptic in pharmaceutical industry.[19] Alkaloids on the other hand believed to wide range of therapeutic effect including analgesic, bactericidal, antimalarial and antispasmodic activity.[20]

The results from this study contradicts the study done by Farouk et al 2007 as well as Tzar et al in 2010. This difference in findings may be as a result of the difference in method of extraction of the root of *E. longifolia* Jack plant.

Lactic acid bacteria (LAB) like any other bacteria can exchange genes to enhance their survival in antibiotic-containing habitats.[21] In order for horizontal gene transfer to occur via mobile genetic elements, there must be close contact between LAB and other bacteria in the intestine, mucosa or in food. Antibiotic resistance can therefore explain the possibility of why *Lactobacillus* in our experiment appear to have a high minimum inhibitory concentration and is less sensitive to the effects of the *E. longifolia* Jack extract.

As for safety of this plant to be used as a composition in oral health promotion, many studies and reviews have been done to assess the plant's toxicity profile and safe dosage. So far, safety studies that have been carried out showed that if Tongkat Ali (E. longifolia) concentrations are used at therapeutic level, there appear to not have any detrimental effects on human spermatozoa in vitro.[22] In animal studies, no negative effect on the offspring could be found (malformations, body weight or number of the offspring)[23] In addition to this, a study conducted by Li *et al*. shows the calculated acceptable daily intake (ADI) for *E. longifolia*

extract, was up to 1.2g/adult/day. This investigation is useful for the development of the product as well as regulating safety dosage.

Generally, it is evident from available literatures that *E. longifolia* Jack possess adequate therapeutic potential and could be further exploited commercially. Studies have proven that the plant, *E. longifolia*, is safe when used at therapeutic levels. Based on the results of this study, inhibitory activity of cariogenic microorganisms has been confirmed. This suggests that it has a great potential to be used in oral health promotion because it is safe, nontoxic and environment friendly as in does not contaminate the environment.

CONCLUSION

As a conclusion, the results in this study proved that ethanol-based *E. longifolia* Jack root extract has a good potential to reduce caries causing microorganisms and *Candida albicans* that may cause other oral infections.

Therefore, the *E. longifolia* extract could be futher explored as an alternative to be used as part of constituent in daily oral health care products such as dentifrices and mouthwash.

ACKNOWLEDGEMENT

We would like to thank the University Malaysia Pahang and International Islamic University Malaysia for funding this work through the sustainable research collaboration grant number: SRCG20-025-0025.

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Cite this article: bin Ramzi MI, bin Kosnin MH, Faisal GG, Arzmi MH, Kusumawardani A, Sabere ASM, *et al*. The Effect of Eurycoma Longifolia Jack (Tongkat Ali) Root Extract on salivary *S. Mutans, Lactobacillus* and *Candida Albicans* Isolated from High-Risk Caries Adult Patients. Pharmacog J. 2021;13(3): 787-91.