STUDY ON PHYTOCHEMICAL CONSTITUENTS AND SOME BIOLOGICAL ACTIVITIES OF THE ROOT OF *EURYCOMA LONGIFOLIA* JACK

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Abstract

Eurycoma longifolia Jack is a plant species that belongs to the Simaroubaceae family, and it is also known locally as Bittu Bark in Myanmar, Piak and Tung Saw in Thailand, and Tongkat Ali in Malaysia. The objective of this study was to evaluate the phytochemical constituents and some biological activities of root of *E. longifolia*. The phytochemical constituents were investigated by the test tube and TLC profiling methods. Some biological activities such as antimicrobial, antioxidant, and cytotoxicity activities were determined by using the Agar Well Diffusion method, DPPH assay and Brine shrimp lethality bioassay**.** The antioxidant activity of ethanol extract (IC_{50} = 204.4 µg/mL) was shown to be more potent than watery extract (IC_{50} = 765.1 µg/mL) of *E. longifolia* root. The ethyl acetate extract of the sample was found to have more potent antimicrobial activity against all eight tested microorganisms in the inhibition zone diameter range (24 -32 mm) than those of ethanol, methanol, and watery extracts. Moreover, the cytotoxic effect of ethanol extract (LC_{50} = 233.35 µg/mL), which showed substantial toxicity was slightly more toxic than the positive control of standard potassium dichromate (LC_{50} = 244.46 µg/mL).

Keywords: *Eurycoma longifolia* Jack, antioxidant activity, antimicrobial activity, cytotoxicity

Introduction

Traditional medicine has employed medicinal plants to treat a variety of ailments. However, using these herbs improperly can have negative side effects. Therefore, it is necessary to conduct scientific research to investigate the pharmacological characteristics of diverse plants. One of the most well-liked medicinal herbs in Southeast Asia, *E. longifolia*, also known as Tongkat ali in Malaysia and locally, Bittu Bark in Myanmar, was the subject of this study. It is a member of the family Simaroubaceae. It is a tall, slender tree that resembles a shrub and grows in sandy soil. It has pinnately shaped compound leaves that are green in colour. The leaves have smooth edges and are opposite or subopposite, lanceolate to ovate-lanceolate. The blooms are small, unisexual, reddish, and tightly packed. When ripe, the ovoid fruits turn a dark reddish brown colour (Keng, 1978). More practical formulations, including additives blended with tea and coffee, have recently been available. Its main constituents include terpenoids, alkaloids, and quassinoids. Pharmacological testing on *E. longifolia* has demonstrated that it has anti-malarial, anticancer, anti-inflammatory, and other properties (Shaheed *et al*., 2016).

The imbalance between reactive oxygen and nitrogen RON species synthesis and antioxidant defence leads to oxidative stress. Oxidative stress is linked to ageing and a number of illnesses, including cancer and chronic obstructive pulmonary disease (Hassan, 2015). The main goal of this study is to identify some biological processes, such as antioxidants related to lowering oxidative stress and antimicrobial activity to treat infectious disorders and food poisoning caused on by consuming foods infected by pathogenic bacteria.

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Figure 1. Photographs of plant and roots of ***Eurycoma longifolia*** **Jack**

Materials and Methods

Collection of Sample

The sample was collected from Dawei Township, Tanintharyi Region, Myanmar in October, 2021. Then, the sample was authenticated at the Department of Botany, University of Yangon. The collected sample was washed with water and air dried as shown in Figure 1. The dried roots were then ground into a powder using a grinding machine. The powdered sample was stored in an air-tight container to prevent moisture changes and other contaminations.

Chemicals

Methanol, ethanol, ethyl acetate, 2,2-diphenyl-1-picryhydrazyl (DPPH), potassium dichromate, sodium hydroxide, caffeine, distilled water, Trypticase soy broth from Difco U.S.A, Muller Hinton agar (Hi-Media) and triple sugar iron from Becton, U.S.A,

Instruments

Quartz cuvette (4 mL), UV-visible spectrophotometer (UV-7504), a shaker, an autoclave (Tomy Seiko Co., Ltd, Tokyo, Japan), petri dish, spirit burner, polyethylene plastic bag, a refrigerator and an incubator

Preparation of Extracts

About 50 g of dried powdered samples of roots of ***Eurycoma longifolia*** was extracted by using appropriate solvents (EA, EtOH and MeOH) and sonicating the samples three times in 1000 mL of each solvent for 1 h. The filtrate was concentrated in a rotatory evaporator to obtain the extracts.

Preparation of Watery Extract

A watery extract of the dried powdered sample was obtained by boiling 20 g of sample with 300 mL of distilled water for about 30 min on a hot plate, and it was filtered. Then, the filtrate was concentrated by evaporation on a hot plate.

Preliminary Phytochemical Tests

Preliminary phytochemical tests were performed by using a reported method. A preliminary phytochemical investigation was used to classify phytoconstituents embedded in the samples, through a reported method (M-Tin Wa, 1972; Trease and Evans, 1980; Shriner *et al*., 1980).

Phytochemical Investigation by TLC Profiling

A sample was prepared by diluting the crude extract of methanol and then applying usually 1-10 μL volumes to the origins of a TLC plate, 2 cm above its bottom, using capillary tubes. After

the application of the sample on the plate, the plates were kept in a TLC glass chamber (solvent saturated). And then, the mobile phase was allowed to move through the adsorbent phase up to the plate. TLC was performed for the presence of alkaloids, coumarins, phenols, steroids, and terpenoids.

In Vitro Antioxidant Activity by DPPH Free Radical Scavenging Assay

Preparation of DPPH solution

DPPH (4.732 mg) was completely dissolved in ethanol (100 mL). The prepared solution was placed in the brown reagent bottle and was stored in the refrigerator for no more than 24 h.

Determination of antioxidant activity

Using a DPPH radical scavenging test, the antioxidant activity of ethanol and watery extracts was evaluated (Marinova and Batchvaro, 2011). First, a stock solution was made by mixing 20 mg of each extract with 20 mL of ethanol. After that, this stock solution was serially diluted at five different concentrations (1000, 500, 250, 125, and 62.5 µg/mL). An oxidant was a solution of 4 mg of DPPH in 100 mL of ethanol. Ascorbic acid, which possesses strong antioxidant properties, was used as a positive control. After that, 1.5 mL of the tested sample solution and 1.5 mL of the DPPH solution were combined and incubated for 30 min. Then, the absorbance was measured spectrophotometrically at 517 nm in triplicate. The following equation was used to determine the amount of inhibition of the DPPH free radical:

$$\% \text{ RSA} = \frac{A_{DPPH} - (A_{\text{sample}} - A_{\text{blank}})}{A_{DPPH}} \times 100$$

Where, % RSA = % radical scavenging activity
A_{DPPH} = absorbance of DPPH in EtOH solution
A_{sample} = absorbance of sample and DPPH solution
A_{blank} = absorbance of sample and EtOH solution

Screening of Antimicrobial Activity of the Sample by Agar Well Diffusion Method

By using the agar well diffusion method, the antimicrobial activity of four crude extracts from the roots of *E. longifolia* Jack was assessed against eight strains of microorganisms, including *Agrobacterium tumefaciens*, *Bacillus pumilus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Micrococcus luteus*, C*andida albicans*, and *Escherichia coli* at Chemistry Department Pathein University's.

Determination of Antimicrobial Activity by Agar Well Diffusion Method

The Agar Well Diffusion method was used for antimicrobial evaluation. Firstly, the tested microorganisms were inoculated in Muller Hinton Broth at 37 °C for overnight. On the next day, the overnight broth culture was diluted with normal saline. Muller Hinton agar plates were prepared and sterilized by autoclaving at 121°C for 15 min. The broth inoculums were evenly spread out with sterile cotton swabs on the agar plates to obtain the uniform inoculum. After the plates were inoculated, 8 mm diameter wells were made on the agar medium by using a sterile cork. The wells were then filled with the respective extract solutions with the concentration (100 mg per 1 mL). The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested. Plates were incubated for 24 h to allow diffusion through the agar medium to form zones of inhibition (Perez, *et al*, 1990).

Determination of Cytotoxicity Activity

Sample solutions and solutions of standard potassium dichromate and caffeine were made up at various concentrations between 50 and 800 µg/mL. Separate clean vials were filled with sample solutions corresponding to 50, 100, 200, 400, and 800 µg/mL. Each vial was filled with a mixture of 9 mL of artificial seawater and 1 mL of a sample at various concentrations. Ten live brine shrimp nauplii were put into each vial by using a Pasteur pipette. For control solutions, a similar procedure was used. All vials were incubated for 24 h at room temperature with light. In control and test vials, the number of shrimp that survived was counted, and median lethal concentrations (LC_{50}) were computed using the Microsoft Excel programme (Meyer *et al*., 1982).

Results and Discussion

Phytochemical Constituents of *E. longifolia* Jack Root

The phytochemical screening of *E. longifolia* was preliminarily done by the test tube methods. Alkaloids, α-amino acids, carbohydrates, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, flavonoids, and terpenoids were found in the root of *E. longifolia*, whereas tannins, and cyanogenic glycosides were absent. From TLC profiling of methanol extract, the classes of phytochemical constituents, such as alkaloids, terpenoids, steroids, phenolic compounds, and coumarins, are found to be present.

***In vitro* Antioxidant Activity of Crude Extracts from Root of *E. longifolia* Jack by DPPH Free Radical Scavenging Activity**

Antioxidant activity of ethanol and watery extracts from root of *E. longifolia* was evaluated by DPPH free radical scavenging activity. This activity was used to determine antioxidant activity based on the scavenging activity from the donation of hydrogen or radicals by using stable DPPH in the absorbance at 517 nm. In this study, the IC_{50} values of ethanol and watery extracts were 204.4 μgmL^{-1} and 765.1 μgmL^{-1} while ascorbic acid showed 4.4 μgmL^{-1}. Among the two extracts, the ethanol extract showed higher antioxidant activity than the water extract. The results, showed antioxidant activity in order to reduce oxidative stress in the body based on these relationships. These results are shown in Tables 1and 2 and Figures 2 and 3.

Table 1. Average % Radical Scavenging Activity and IC_{50} Values of Crud Extracts from the Root of *E. longifolia* Jack

Extracts	% RSA ± SD at Different Concentration (µg/mL)					IC_{50} (µg/mL)
	62.5	125	250	500	1000	
(Ethanol extract)	33.23± 3.39	39.82± 1.82	55.83± 1.12	70.50± 0.38	91.42± 1.47	204.4
(Watery extract)	31.58± 0.15	33.38± 0.26	37.87± 0.62	44.90± 1.26	54.49± 2.96	765.1

Table 2. % RSA (Radical Scavenging Activity) of Standard Ascorbic Acid

Sample	% RSA ± SD at Different Concentration (µg/mL)					IC_{50} (µg/mL)
	1.25	2.5	5	10	20	
Ascorbic acid	23.67± 1.26	27.39± 0.15	55.4± 0.82	87.43± 1.38	96.16 ± 0.00	4.4

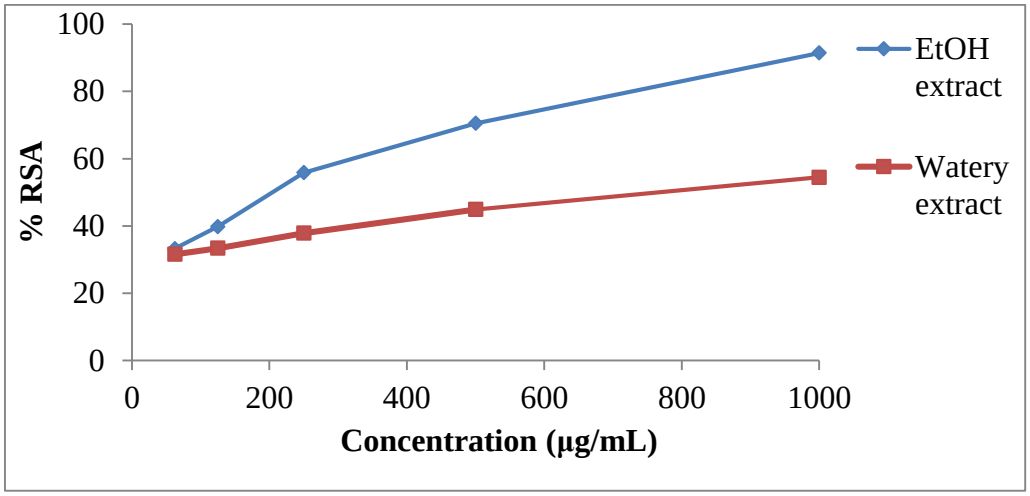

Figure 2. DPPH free radical scavenging activities of crude extracts of *E. longifolia* Jack at different concentrations

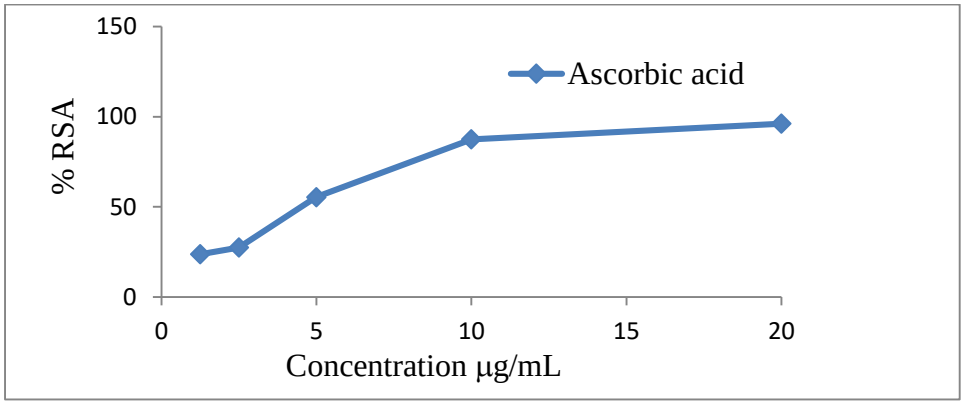

Figure 3. DPPH free radical scavenging activities of standard ascorbic acid

Antimicrobial Activity of Crude Extracts of *E. longifolia* Jack Root

The diameter of the inhibition zone including the well diameter (8 mm) was measured to determine the antimicrobial activity. In this study, three Gram negative bacteria (*A. tumefacies, P. fluorescens, and E. coli*), four Gram positive bacteria (*B. subtilis, B. pumilus, M. luteus, and S. aureus*), and the fungus *C. albicans* were evaluated against four extracts (EtOAc, EtOH, MeOH, and aqueous) of *E. longifolia* Jack. The activity of the examined microorganisms increased with increasing inhibition zone diameter. The results showed that all of the extracts had potent antimicrobial activity, with inhibition zone diameter varying from 18 to 32 mm on all tested microorganisms. On five of the tested microorganisms (*A. tumefacies*, *B. pumilus*, *B. subtilis*, *C. albicans*, *S. aureus*), the activity of the EtOAc extract was more potent than the positive controls (chloramphenicol and nystatin), comparable to two of the tested microorganisms (*E. coli*, *M.luteus*) and less potent than one of the tested microorganisms (*P. fluorescens*). On the seven tested bacteria, the ethanol and methanol extracts of *E. longifolia* Jack root showed the maximum activity, while

the watery extract displayed the highest activity on four selected microorganisms. These results are shown in Figures 4 and 5, and Table 3.

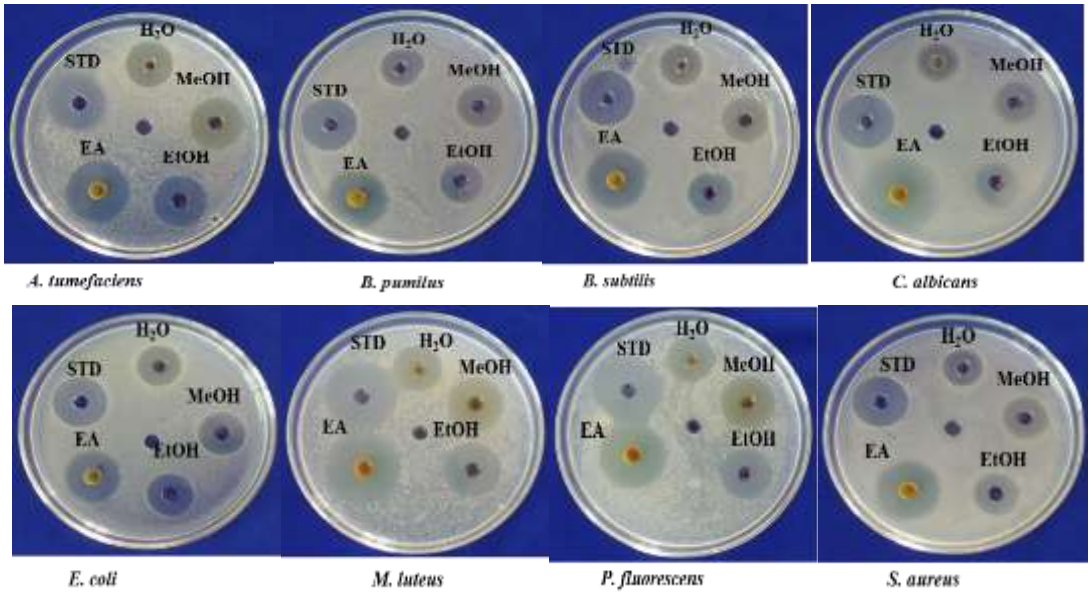

Figure 4. Screening of antimicrobial activities of the root of *E. longifolia* Jack

Table 3. Inhibition Zone Diameter of the Root of *E.longifolia* Jack Against Eight Different Microorganisms

Extracts	Inhibition Zone Diameter (mm) against eight microorganisms							
	A. tumefacies	***B. pumilus***	***B. subtilis***	***C. albicans***	***E. coli***	***M. luteus***	***P. fluorescens***	***S. aureus***
Watery	21(+++)	18(++)	19(++)	19(++)	21(+++)	21(+++)	20(+++)	19(++)
MeOH	23(+++)	21(+++)	21(+++)	19(++)	21(+++)	24(+++)	24(+++)	20(+++)
EtOH	24(+++)	20(+++)	21(+++)	19(++)	21(+++)	25(+++)	22(+++)	20(+++)
EtOAc	27(+++)	26(+++)	26(+++)	32(+++)	24(+++)	30(+++)	29(+++)	26(+++)
*STD	25(+++)	25(+++)	25(+++)	-	24(+++)	30(+++)	31(+++)	24(+++)
**STD				24(+++)				

Diameter of agar well = 8 mm
(-) no activity
(+) 9 mm ~ 14 mm, Low activity
(++) 15 mm ~ 19 mm, medium activity
(+++) 20 mm above, high activity

***STD = chloramphenicol for bacteria**
****STD = nystatin for fungus**

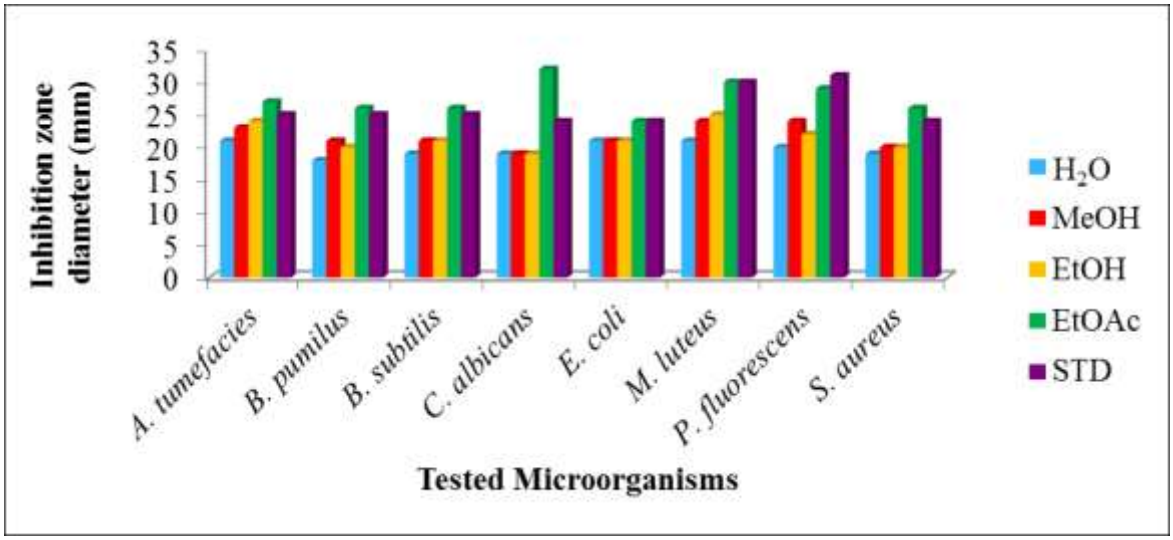

Figure 5. Inhibition zone diameters for crude extracts of root of *E. longifolia* Jack

Investigation of Cytotoxicity Activity of the Root of *E. longifolia* Jack by Brine Shrimp Lethality Bioassay

By using a brine shrimp lethality test, the cytotoxicity of ethanol and watery extract of the root of *E. longifolia* Jack was evaluated. The ethanol extract of the root of *E. longifolia* Jack was found to be an LC_{50} value of 233.35 µg/mL, while the watery extract was a value of 254.42 µg/mL. In comparison to the two extracts, the ethanol extract showed greater toxicity. The LC_{50} value of the ethanol extract was 233.35 µg/mL, which was significantly more toxic than the positive control of standard potassium dichromate (244.46 g µg/mL), which also showed substantial toxicity. These results are shown in Table 4 and Figure 6.

Table 4. Results of Cytotoxicity on Ethanol and Watery Extracts of Roots of *E.longifolia* Jack (Myanmar)

Sample	Death % of Brine Shrimp in Various Concentrations (µg/mL)					
	50	**100**	**200**	**400**	**800**	**LC_{50}**
EtOH	6.67 ±0.57	23.33 ±0.57	43.33 ±0.57	83.33 ±0.57	100 ±0.00	233.35
Watery	13.33 ±1.15	23.33 ±0.57	33.33 ±0.57	70.00 ±1.00	96.67 ±0.57	254.42
*$K_2Cr_2O_7$	23.33 ±0.57	33.33 ±0.57	43.33 ±0.57	73.33 ±1.15	96.67 ±0.57	244.46
**Caffeine	0.00	0.00	6.67±0.82	16.67±0.94	30±0.47	>1000

*positive control = $K_2Cr_2O_7$ ** negative control = Caffeine

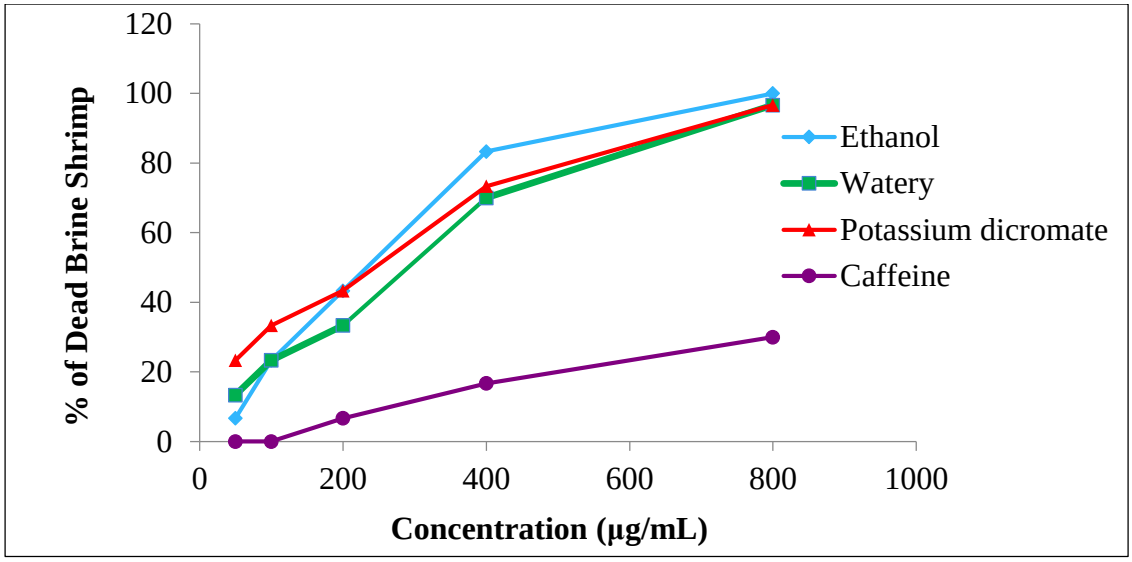

Figure 6. Cytotoxic activity of *E. longifolia* Jack root

Conclusion

From the overall assessment of the chemical investigation into the root of *E. longifolia*, the following inferences could be concluded. The preliminary phytochemical tests revealed the presence of alkaloids, α-amino acid, carbohydrate, glycoside, organic acid, phenolic compounds, saponin, starch, steroid, flavonoids and terpenoids, but the absence of cyanogenic glycoside, and tannins in the root of ***E. longifolia*** Jack. From the DPPH radical scavenging assay, the ethanol extracts (91.42 ± 1.47) µg/mL showed higher antioxidant activity than watery extract (54.49 ± 2.96) µg/mL on the concentration of 1000 µg/mL. And then**,** all of the extracts showed potent antimicrobial activity, with inhibition zone diameters ranging from 18 to 32 mm on the all tested microorganisms. Among these extracts, EtOAc extract exhibited higher potency than positive control (chloramphenicol, nystatin) on five tested microorganisms, similar to two tested microorganisms and weaker than one tested microorganism. These results suggest that ethyl acetate fraction would be potential to be used for isolation of bioactive constituents. Moreover, the present study on cytotoxic activity also indicated that the LC_{50} values of ethanol (233.35 µg/mL) and watery extract (254.42 µg/mL) were significantly cytotoxic in comparison with positive control standard potassium dichromate solution (244.46 µg/mL). Thus, the extracts of this plant will be reliable for the treatment of numerous antioxidant - related diseases and many other diseases because of its cytotoxicity activity. According to these findings, the extracts of *E. longifolia* Jack root (Myanmar) will be effective in the treatments of a variety of disorders including bacterial infections, inflammation and cancers**.**

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