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Research Article
Estimation of Antioxidant, Anticancer and Cytotoxic Properties of *Eurycoma longifolia* Jack

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Abstract

Background and Objective: *Eurycoma longifolia* roots hold traditional medicinal value, but scientific evaluation of their bioactivity and safety is lacking. This study investigated the antioxidant and anticancer potential of *E. longifolia* extracts and assessed cytotoxicity. **Materials and Methods:** Methanol and ethyl acetate extraction were used to obtain *E. longifolia* root extracts. Antioxidant activity was measured by DPPH assay and antiproliferative and cytotoxic effects were assessed against various cell lines. A one-way ANOVA was conducted to compare the IC_{50} values among the different groups. **Results:** The highest DPPH radical scavenging activity was detected in methanol extract (IC_{50} = 65.50±6.74 µg/mL) and ethyl acetate extract (IC_{50} = 463.52±59.81 µg/mL). The methanol extract displayed potent cytotoxicity against all tested cell lines, with IC_{50} values ranging from 4.71-6.70 µg/mL. The ethyl acetate extract exhibited moderate cytotoxicity towards the non-cancerous LLC-MK2 cell line (IC_{50} = 25.00±5.64 µg/mL), but retained high cytotoxicity against the cancer cell lines (MDA-MB-231 and HeLa), with IC_{50} values of 6.09±1.32 and 6.70±1.87 µg/mL, respectively. **Conclusion:** Methanol extract displayed strong antioxidant and antiproliferative activity, but also cytotoxicity in both cancerous and non-cancerous cells. Further research using *in vivo* models is needed to assess safety and identify specific bioactive compounds for responsible future use.

Key words: Antioxidant activity, anticancer activity, cytotoxic activity, *Eurycoma longifolia* Jack, methanol extraction

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Eurycoma longifolia, a plant in the Simaroubaceae family, has a long history of use in traditional medicine in Southeast Asia (Thailand, Myanmar, Laos, Cambodia, Vietnam, Malaysia and Indonesia)[1,2]. Locally, it is known as "Pralaipueak" in Thailand. *Eurycoma longifolia* root extracts have demonstrated various bioactivities, including aphrodisiac, antimalarial, antimicrobial, antidiabetic, anti-inflammatory, antinociceptive and antipyretic properties[2,3]. Notably, the root is the most commonly used part for medicinal purposes[4-6]. Phytochemically, *E. longifolia* root extracts are rich in secondary metabolites, such as phenolic compounds, terpenoids and flavonoids[2,7]. In contrast, the stem extract contains a broader range of compounds, including cardiac glycosides, proteins and alkaloids, alongside the metabolites found in the root[2,7]. Among these, flavonoids are particularly noteworthy due to their antioxidant, anti-inflammatory, anticancer and antimicrobial activities[2,7]. The antioxidant capacity of plants is often attributed to the presence of phenolic compounds and flavonoids[2,7].

Antioxidants are crucial in neutralizing free radicals, which are generated by various sources such as endogenous metabolism, environmental pollutants and sunlight exposure[8]. Free radicals can damage cellular components, impacting DNA, blood vessels and protein synthesis (enzymes, prostaglandins)[8]. Previous studies have reported the antioxidant properties of *E. longifolia* root in its crude extract in different solvents (aqueous and ethanol)[9-11]. However, little work has been done comparing different solvent extraction methods for their impact on antioxidant and anticancer properties. Therefore, this study investigated the use of ethyl acetate (non-polar solvent) and methanol (polar solvent) to extract *E. longifolia* root and analyze their effects on antioxidant and anticancer properties using the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and MTT assay, respectively.

MATERIALS AND METHODS

Study area: The study was conducted at Department of Microbiology and Chemistry, Silpakorn University, Nakhon Pathom, Thailand, between June, 2023 to July, 2024.

Sample preparation: Ten *Eurycoma longifolia* roots were collected from a site located at Wat Prangkasi, Tha Kha-nun, Thong Pha Phum, Kanchanaburi, Thailand (14.65230°N, 98.67051°E). To remove adhering soil and impurities, the roots were subjected to a washing process using water flow. Subsequently, the roots were dried in an oven (Memmert, Schwabach, Germany) at a controlled temperature of 60°C. Finally, the *E. longifolia* roots were chopped into smaller pieces. A precisely weighed quantity of 200 g of dried *E. longifolia* root pieces was subjected to extraction using a simple maceration technique. Each extraction employed 1000 mL of either methanol or ethyl acetate as the solvent, conducted in a conical flask under constant shaking at 150 rpm for 24 hrs. The extraction process was replicated three times under identical conditions. Subsequently, the solution was filtered using a cotton filter. The collected filtrate was concentrated using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure to yield a dry residue. The resulting crude extracts of *E. longifolia* roots were then employed for further studies. The percentage yield of the methanol extract was determined to be 4.30% (w/w), while the ethyl acetate extract yielded 7.37% (w/w). The yields of crude extracts were stored in -20°C freezer for further use.

2,2-diphenyl-1-picrylhydrazyl assay: The radical scavenging activity of the *E. longifolia* root crude extracts was evaluated using the DPPH assay, with slight modifications based on the method described by Blois[12]. Four varying concentrations of the crude extract were prepared: 18.75, 37.5, 75 and 150 µg/mL. A 0.1 mM DPPH solution was prepared by dissolving 1.9 mg of DPPH in methanol and adjusting the final volume to 100 mL with methanol. To protect the solution from light exposure, it was stored in an aluminum-wrapped bottle. One milliliter of the prepared 0.1 mM DPPH solution was added to each test tube containing 3 mL of a specific concentration of the *E. longifolia* extract solution. The resulting mixtures were incubated in the dark at room temperature for 30 min. Following the incubation period, the absorbance of each reaction mixture was measured at 517 nm using a UV 1650 PC-Shimadzu B UV-visible spectrophotometer (Shimadzu, Osaka, Japan). All measurements were performed in triplicate. Ascorbic acid was used as positive control. The radical scavenging activity of the extracts was then calculated using the following formula[12]:

$$\text{DPPH scavenged (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The IC_{50} values were determined and reported.

Determination of the cytotoxicity assay: To assess *in vitro* cytotoxicity, the crude extract was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay[13]. Breast cancer MDA-MB-231 and cervical cancer

HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were incubated with media containing 1-512 µg/mL of crude extracts for 24 hrs. Cells were washed once before adding 50 µL FBS-free medium containing 5 mg/mL MTT. After 4 hrs of inoculation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 µL DMSO. The optical density was measured at 540 nm. The percentage induction of cytotoxicity against cancer cell lines was determined[13]. The experiments were performed in triplicate and data are expressed as the Mean±Standard Deviation. To evaluate the compound's selectivity towards cancer cells, a non-cancerous LLC-MK2 cell line was included in the assay. Selectivity indices (SI) were calculated as the ratio of the IC_{50} (concentration inhibiting 50% of cell growth) values obtained in the LLC-MK2 and cancer cells. Higher SI values indicate greater selectivity of the compound for cancer cells, with minimal cytotoxicity towards healthy cells. The doxorubicin hydrochloride (Thermo Fisher Scientific, Massachusetts, USA) served as positive controls for cytotoxicity.

Statistical analysis: All experiments were performed in triplicate. Data were expressed as Mean±Standard Deviations (SD). Statistical analyses were performed using SPSS for Windows version 11.01 (SPSS Inc., Chicago, Illinois, USA). Treatment effects were analyzed using one-way ANOVA, followed by Duncan's multiple range tests. A $p<0.5$ was used to indicate significance.

RESULTS

The antioxidant activity of different solvent extraction of *E. longifolia* roots was determined. The highest DPPH radical scavenging activity was detected in methanol extract (IC_{50} = 65.50±6.74 µg/mL) and ethyl acetate extract (IC_{50} = 463.52±59.81 µg/mL) (Table 1). The results showed that the scavenging ability increased towards the methanol extract with increasing polarity of the solvent. However, ascorbic acid exhibits a significant ($p<0.5$) antioxidant property with IC_{50} value found to be 4.09±0.52 µg/mL.

This study investigated the antioxidant activity of two solvent extracts obtained from the roots of *Eurycoma longifolia*. The DPPH radical scavenging assay was employed to assess antioxidant capacity. The methanol extract exhibited the strongest free radical scavenging activity, with an IC_{50} value of 65.50±6.74 µg/mL (Table 1). This value was significantly lower ($p<0.05$) compared to the ethyl acetate extract (IC_{50} = 463.52±59.81 µg/mL), indicating a greater antioxidant capacity in the more polar methanol extract. For reference, ascorbic acid, a well-known antioxidant, displayed a significantly lower IC_{50} value (4.09±0.52 µg/mL), highlighting its superior free radical scavenging ability.

The cytotoxicity of crude extracts derived from *E. longifolia* roots was evaluated using three cell lines: A non-cancerous rhesus monkey kidney cell line (LLC-MK2) and two cancer cell lines (MDA-MB-231, breast cancer and HeLa, cervical cancer). The methanol extract displayed potent cytotoxicity against all tested cell lines, with IC_{50} values ranging from 4.71-6.70 µg/mL (Table 2). In contrast, the ethyl acetate extract exhibited moderate cytotoxicity towards the non-cancerous LLC-MK2 cell line (IC_{50} = 25.00±5.64 µg/mL), but retained high cytotoxicity against the cancer cell lines (MDA-MB-231 and HeLa), with IC_{50} values of 6.09±1.32 and 6.70±1.87 µg/mL, respectively (Table 2). Notably, the selectivity indices (SI) of these crude extracts for the cancer cells were significantly lower compared to doxorubicin hydrochloride.

Table 1: Antioxidant activities of different solvent extractions of *E. longifolia* roots

Solvent extraction	IC_{50} (µg/mL)*
Methanol	65.50±6.74[a]
Ethyl acetate	463.52±59.81[b]
Ascorbic acid	4.09±0.52[c]

*Values are expressed as Mean±SD of three parallel measurements and values within a column followed by different letters are significantly different ($p<0.5$)

Table 2: IC_{50} values and selectivity indices (SI) of different solvent extractions of *E. longifolia* roots against non-cancerous and cancer cell lines

	IC_{50} (mg/mL)			SI	
Test substances	LLC-MK2*	MDA-MB-231	HeLa	MDA-MB-231	HeLa
Methanol extraction	6.70±1.05[a]	4.71±0.53[a]	6.07±0.85[a]	1.42	1.10
Ethyl acetate extraction	25.00±5.64[b]	6.09±1.32[b]	6.70±1.87[a]	4.10	3.70
Doxorubicin hydrochloride	101.81±16.72[c]	6.25±1.14[b]	4.23±1.43[b]	16.29	24.07

*LLC-MK2: Human breast cancer cell line, MDA-MB-231: Human breast cancer, HeLa: Human cervical carcinoma cell line, values are expressed as Mean±SD of three parallel measurements and values within a column followed by different letters are significantly different ($p<0.5$)

DISCUSSION

The incorporation of medicinal herbs as food additives is gaining traction due to their perceived health benefits. This surge in popularity coincides with the increased availability of plant-based products in the market. This trend is likely driven by the presence of bioactive compounds in herbs, such as phenolic acids and flavonoids, which have demonstrated therapeutic potential in various disease models. *Eurycoma longifolia* root decoctions have been used traditionally to manage various ailments, including diarrhea, fever, gastric ulcers, glandular swelling, osteogenic pain, persistent dry cough, dropsy and chronic hypertension. Additionally, the roots are employed as an appetite stimulant and nutritional supplement[2,14,15]. Extensive research has explored the bioactivity of *E. longifolia* using a variety of solvents. Methanol and ethyl acetate were used for extraction due to their known effectiveness in extracting a wide variety of phytochemicals. The resulting extracts were then compared for their antioxidant and cytotoxic properties.

Among the tested solvents, methanol extraction of *E. longifolia* roots exhibited the lowest DPPH radical scavenging activity. However, the IC_{50} values of both methanol and ethyl acetate extracts were statistically lower compared to ascorbic acid (4.09 µg/mL), indicating a significant free radical scavenging capacity. The DPPH radical is a stable free radical known for its solubility in methanol and characteristic absorption at a wavelength of 515-520 nm. This property allows for the measurement of antioxidant activity by monitoring the color change of the DPPH solution upon reduction by antioxidants[16]. According to the classification system established by Blois[12], samples exhibiting an IC_{50} value for DPPH free radical scavenging activity lower than 50 µg/mL are considered very strong antioxidants, 50-100 µg/mL was a strong antioxidant, 101-150 µg/mL was a medium antioxidant, while a weak antioxidant with IC_{50}>50 µg/mL. Based on this categorization, the IC_{50} values of the methanol extracts of *E. longifolia* roots indicate that they possess strong antioxidant properties. However, it is important to note that this method has limitations and may not fully reflect the complete antioxidant capacity of the extract compared to a well-established reference like ascorbic acid. These findings suggested that higher polarity solvents might extract components from *E. longifolia* roots with stronger antioxidant capacity as measured by the DPPH assay. Further investigations using complementary antioxidant assays and exploring a wider range of solvent polarities are warranted to obtain a more comprehensive understanding of the antioxidant profile of *E. longifolia* root extracts. In agreement with studies investigating the DPPH radical scavenging activity of *E. longifolia* root extracts reveal a potential influence of solvent polarity. Mohamad and Ismail[10] reported a 38.90% scavenging activity at 1 mg/mL using an aqueous extract, while Varghese *et al.*[9] observed a higher activity (61.13%) at a lower concentration (250 µg/mL) with a 70% hydroalcoholic extract.

Eurycoma longifolia has been shown to exhibit cytotoxic and antiproliferative effects against various human cancer cell lines, including those derived from lung, breast and cervical cancers[2]. *In vitro* studies have demonstrated the cytotoxic potential of *E. longifolia* extracts against various cancer cell lines. Thu *et al.*[17] reported that Eurycomanone, a prominent bioactive compound in *E. longifolia* roots, exhibited potent dose-dependent anticancer effects on lung and breast cancer cells (A-549 and MCF-7), but demonstrated moderate activity against gastric (MGC-803) and intestinal cancer cells (HT-29). Present study investigated the cytotoxic properties of methanol and ethyl acetate extracts from *E. longifolia* roots against cancerous and non-cancerous cell lines. Interestingly, the results revealed cytotoxicity in both cancerous and noncancerous cell lines. This observation contrasted with the findings of Nurhanan *et al.*[18], who reported selective cytotoxicity of various *E. longifolia* root extracts (methanol, n-butanol and chloroform) against a panel of cancer cell lines (KB, DU-145, RD, MCF-7 and CaOV-3) with no significant effect on the MDBK kidney (normal) cell line[18]. These contrasting results warrant further investigation to elucidate the underlying reasons for the observed discrepancies. Factors such as variations in extraction methods, cell line specificities and the presence of cytotoxic compounds unique to our extracts could contribute to the observed differences. Future studies employing standardized protocols and a wider range of cell lines would be valuable to obtain a more comprehensive understanding of the cytotoxic profile of *E. longifolia* root extracts.

Despite its historical use in traditional medicine, *E. longifolia* extracts have demonstrated promising biological activities, including antioxidant and anticancer properties. However, alongside these potential benefits, a thorough evaluation of its safety profile is crucial for responsible use. Our *in vitro* study using a methanol extract of *E. longifolia* roots suggests a concentration-dependent effect, with potential cytotoxicity observed below the IC_{50} value (6.70 mg/mL). While this finding provides preliminary data, it is important to acknowledge the limitations of *in vitro* studies. These models often do not fully replicate the complex interactions that occur within a living organism. Therefore, further *in vivo* investigations are warranted to

comprehensively assess the safety profile of *E. longifolia* extracts. These studies should explore potential dose-dependent toxicities and identify safe consumption ranges. Additionally, pharmacokinetic studies would be valuable to understand the absorption, distribution, metabolism and excretion of *E. longifolia* extracts within the body. A comprehensive understanding of the therapeutic potential and safety profile of *E. longifolia* extracts is essential for its responsible use in traditional and potentially modern medicine.

CONCLUSION

Eurycoma longifolia holds significant importance in traditional medicine, with its roots used for various purposes. Current study revealed promising results, demonstrating strong antioxidant and anticancer activities associated with the methanol extract of *E. longifolia* roots. However, these findings are balanced by the observation of cytotoxic activity, even in non-cancerous cell lines. The presence of cytotoxic activity underscores the importance of investigating the safety profiles of *E. longifolia* extracts. While traditional use may suggest a level of safety and a comprehensive scientific evaluation is necessary.

SIGNIFICANCE STATEMENT

Eurycoma longifolia Jack, a traditional herbal medicine, has been shown to possess antioxidant and anticancer properties. Current study demonstrates the efficacy of crude root extracts in inhibiting oxidative stress and cancer cell growth. While the methanol extract exhibited significant cytotoxic activity against cancer cells, it also demonstrated toxicity in non-cancerous cell lines. These findings highlight the importance of further research to elucidate the safety profile of *E. longifolia* extracts, ensuring their potential use in safe and effective therapeutic applications.

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