

Article

LC-MS/MS-Based Phytochemical Profiling and Biological Activities of *Eurycoma longifolia* Jack Extracts with Cosmeceutical Potential

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Abstract

This study investigates the optimization of extraction conditions, phytochemical contents, biological activities, and cosmeceutical potential of *Eurycoma longifolia* Jack extracts obtained from wild and cultivated plant roots, stems, and leaves. HPLC analysis revealed that eurycomanone was predominantly accumulated in the root extracts. Among the extraction solvents, 70% ethanol exhibited the most effective extraction of bioactive compounds. Leaf extracts showed the highest total flavonoid content (255.71–269.67 mg quercetin/g sample) and total phenolic content (20.34–20.80 mg gallic acid/g sample). Furthermore, 70% ethanol leaf extracts demonstrated strong antioxidant activity with DPPH IC₅₀ values of 6.37 ± 0.74 and 7.95 ± 0.86 µg/mL for wild and cultivated samples, respectively, and potent tyrosinase inhibitory activity with IC₅₀ values of 50.71 ± 3.92 and 55.80 ± 4.75 µg/mL. The extracts also exhibited selective antibacterial activity against Gram-positive bacteria. Moreover, the in silico molecular docking of biflorin against the 2QVD protein exhibited the highest binding affinity (−7.4 kcal/mol). Anti-inflammatory activity was evidenced by nitric oxide inhibition in LPS-stimulated RAW 264.7 macrophages without significant cytotoxicity. Overall, 70% ethanol extracts, particularly from leaves and roots, exhibited promising biological activities and may serve as potential natural ingredients for future cosmeceutical applications. However, further studies are required to confirm their efficacy and safety before practical applications can be considered.

Keywords: *Eurycoma longifolia* extracts; antioxidant; antibacterial; anti-inflammatory; eurycomanone; cytotoxicity



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1. Introduction

Eurycoma longifolia, commonly known as Tongkat Ali in Thailand, is a medicinal plant belonging to the family Simaroubaceae and is widely distributed throughout Southeast Asia, particularly in Thailand, Malaysia, and Indonesia [1]. Traditionally, this plant has been used in folk medicine for the treatment of fever, fatigue, malaria, inflammation, sexual dysfunction, and various infectious diseases [2]. The roots of *E. longifolia* are especially recognized for their rich phytochemical constituents, including quassinoids, alkaloids, flavonoids, phenolic compounds, and triterpenes, which contribute to diverse biological activities [1].

Recent studies have demonstrated that *E. longifolia* possesses several pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and anti-aging activities [3,4]. These biological effects are closely associated with bioactive compounds such as eurycomanone, eurycomanol, and canthin-6-one alkaloids. Among these compounds, eurycomanone is considered one of the major quassinoids and has been widely used as a chemical marker for quality control of *E. longifolia* extracts [4].

Oxidative stress and inflammation are major factors involved in skin aging and various chronic diseases. Natural antioxidants derived from medicinal plants have attracted increasing attention due to their ability to scavenge free radicals and inhibit inflammatory mediators with lower toxicity, compared to synthetic compounds [5]. In addition, natural tyrosinase inhibitors originating from plant extracts have become important candidates for cosmetic and pharmaceutical applications related to skin whitening and hyperpigmentation treatment [3].

Extraction conditions, particularly solvent polarity and ethanol concentration, significantly influence the recovery of phytochemicals and biological activities of herbal extracts. Previous reports have indicated that hydroethanolic solvents are more effective than water or absolute ethanol alone for extracting phenolic and flavonoid compounds from *E. longifolia* [6]. Furthermore, recent extraction technologies have focused on improving extraction efficiency and preserving bioactive compounds for medicinal and cosmetic applications [7].

In Thailand, *Eurycoma longifolia* is considered a valuable medicinal plant with increasing demand for pharmaceutical and cosmeceutical applications. However, this species requires a relatively long cultivation period before harvesting, and excessive collection from natural habitats may contribute to the depletion of wild populations. Community-based cultivation has therefore emerged as a practical approach to support sustainable utilization and conservation of this medicinal resource. Despite its importance, scientific evidence comparing the phytochemical characteristics and biological activity of *E. longifolia* obtained from wild populations and cultivated sources remains limited, particularly for different plant parts. Determining whether cultivated materials possess comparable bioactive potential to wild plants is essential for promoting cultivated sources as sustainable alternatives to reduce harvesting pressure on natural populations.

Although numerous studies have investigated the pharmacological properties of *E. longifolia* roots, limited information is available regarding the comparative phytochemical profiling and bioactivities of different plant parts obtained from wild and community-cultivated sources in Thailand. This study aimed to investigate the optimal extraction conditions for different parts of wild and cultivated *E. longifolia*; evaluate their phytochemical contents; establish the mechanism for anti-inflammatory, antioxidant, antibacterial, and tyrosinase inhibitory activities using molecular docking; determine cytotoxicity profiling; and quantify eurycomanone content using high-performance liquid chromatography (HPLC) analysis. In addition, the study explored the potential application of *E. longifolia* extracts in cosmetic formulations through preliminary in vitro experiment-based screenings and generated scientific evidence to support the sustainable utilization and conserva-

tion of this slow-growing medicinal plant through the promotion of cultivated resources. Therefore, this study addresses a key gap by directly comparing wild versus cultivated *E. longifolia* sources across multiple plant parts and showing that cultivated materials can provide comparable or even superior bioactive profiles in selected assays. A particular novel aspect is the inclusion of leaves as sustainable non-root material, demonstrating strong antioxidant, tyrosinase inhibitory, antibacterial, and anti-inflammatory activities, thereby offering a less destructive alternative to root harvesting.

2. Experimental Procedures

2.1. Plant Materials, Preparation of Extracts, LC-MS/MS Profiling

Wild and cultivated *Eurycoma longifolia* samples were collected from the Tamot District, Phatthalung Province, Thailand, and voucher specimens (No. CTTM-00906) were deposited at the Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The roots, stems, and leaves were separated, washed with distilled water, and dried at room temperature before being ground into fine powder. The extraction procedure was modified from previous studies reported by Abdul Wahab et al. [1,6]. Briefly, dried plant powders were extracted using ethanol at different concentrations (0%, 50%, 70%, and 95%) with a solid-to-solvent ratio of 1:10 (*w/v*). The mixtures were macerated for 5 days at room temperature with intermittent shaking (WS-500, Beijing, China) twice daily to improve extraction efficiency. After extraction, the solutions were filtered and concentrated using a rotary evaporator (Hei-Var-ML, Schwabach, Germany). The crude extract yield was calculated as the percentage yield of extract (% yield extract).

LC-MS/MS-based profiling of *E. longifolia* root and leaf extracts in positive and negative ionization modes was performed using quadrupole time-of-flight liquid chromatography–mass spectrometry (Nexera 40 UHPLC; Quadrupole Time-of-Flight 9050; Shimadzu, Kyoto, Japan) with a Shim-pack Velox C18 column (2.7 μm ; 2.1 $\times$ 100 mm), acquisition software (Lab Solution version 5.128), and processing software (LabSolutions Insight Explore version 4.2). In brief, the extract was dissolved in 95% ethanol at a concentration of 1.8 to 1.9 $\mu\text{g/mL}$ and analyzed using mobile phase (0.1% formic acid in water (A): acetonitrile: methanol (B)) at a flow rate of 0.3 mL/min in a gradient mode (0 to 6 min [10% B]; 6 to 14 min [10 to 15% B]; 14 to 15 min [100% B]; 15 to 21 min [10–100% B]; and 21 to 25 min [10% B]) with an injection volume of 2 μL . The column temperature was maintained at 40 $^{\circ}\text{C}$, nebulizing gas flow (3.0 L/min), interface temp (300 $^{\circ}\text{C}$), *m/z* MS scan range of 100 to 1500, *m/z* MS/MS scan range (50–1500), collision energy of 35, and precursor intensity threshold of 10,000.

2.2. HPLC Analysis of Eurycomanone

Quantitative analysis of eurycomanone was performed using HPLC following the method described by Farouk [8]. The chromatographic system consisted of a C18 column (Merck, Darmstadt, Germany) with acetonitrile: methanol (1:1) as the mobile phase at a flow rate of 1.5 mL/min. Detection was carried out using a diode array detector at 254 nm. The concentration of eurycomanone in each extract was calculated using a standard calibration curve as presented in Figure 1.

2.3. FTIR Analysis

The extracts obtained from different parts of *Eurycoma longifolia* Jack were dissolved in DMSO (10% *w/v*) and analyzed using Fourier-transformed infrared (FTIR) spectroscopy (BRUKER IFS-66 V, Ettlingen, Germany) to identify the functional groups present in the extracts. FTIR spectra were analyzed over the wavelength range of 600 to 3800 cm^{-1} . The characteristic absorption bands of the extracts were evaluated to determine the major chemical functional groups associated with the phytochemical constituents.

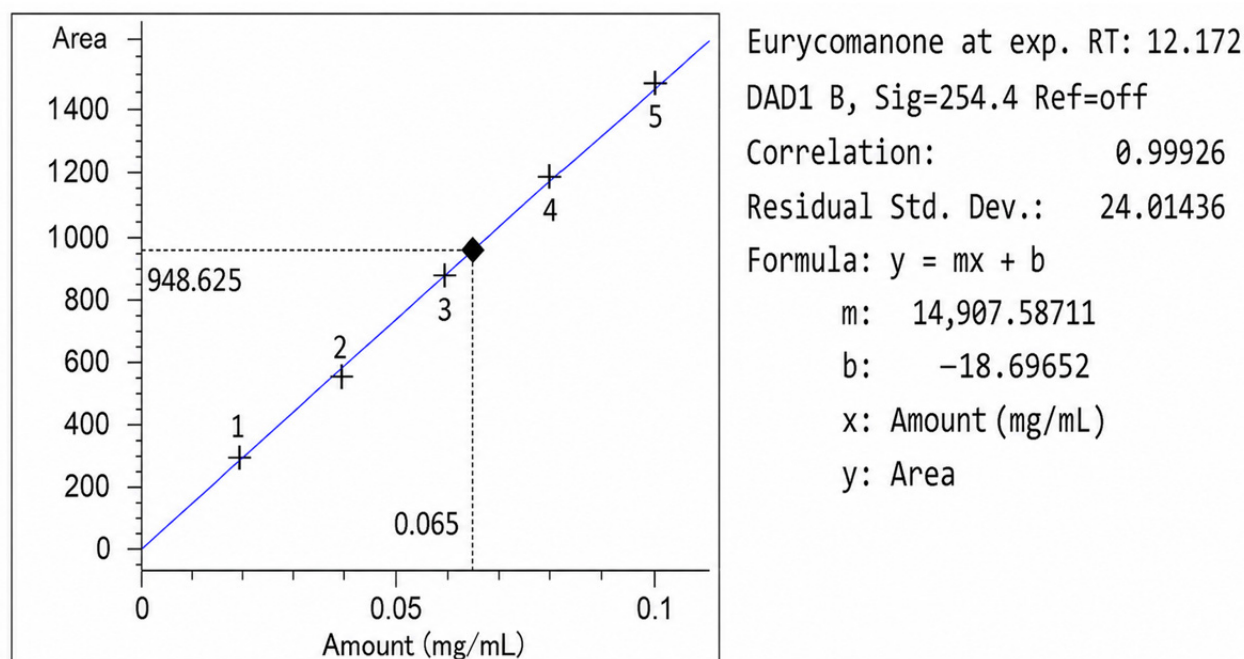


Figure 1. Standard calibration curve from HPLC chromatograms of *Eurycoma longifolia* Jack extracts.

2.4. Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method [9] with slight modifications. Briefly, the extract solution was admixed with Folin–Ciocalteu reagent and sodium carbonate solution. After incubation for 40 min, absorbance was measured at 725 nm using a UV–Vis spectrophotometer (Biohit 830, Biohit, Helsinki, Finland). Gallic acid was used as the standard, and the results were expressed as milligrams of gallic acid equivalent per gram sample (mg GAE/g sample).

2.5. Total Flavonoid Content

Total flavonoid content (TFC) was evaluated using the aluminum chloride colorimetric assay [9]. The extract was mixed with aluminum chloride solution, and absorbance was measured at 415 nm. Quercetin was used as the calibration standard, and the results were expressed as milligrams of quercetin equivalent per gram sample (mg QE/g sample).

2.6. Antioxidant Activity Assays

2.6.1. DPPH Radical Scavenging Assay

The antioxidant activity of the extracts was evaluated using the DPPH radical scavenging assay [10]. Different concentrations of the extracts (4–512 $\mu\text{g/mL}$) were mixed with DPPH solution and incubated in the dark for 30 min. The absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Biohit 830, Biohit, Helsinki, Finland). The inhibitory concentration required to scavenge 50% of DPPH radicals (IC_{50}) was calculated.

2.6.2. ABTS Radical Scavenging Assay

The ABTS radical scavenging activity was determined following the method [10]. The ABTS $\bullet$ + working solution was prepared by mixing ABTS stock solution with potassium persulfate and incubating in the dark. The extract solutions were mixed with ABTS reagent, and absorbance was measured at 734 nm using a UV–Vis spectrophotometer (Biohit 830, Biohit, Helsinki, Finland). L-ascorbic acid was used as the positive control.

2.6.3. Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was determined according to the method [11]. Briefly, mushroom tyrosinase enzyme and L-DOPA substrate were mixed with sample extracts in a microplate. The absorbance was measured at 475 nm using a UV–Vis spectrophotometer (Biohit 830, Biohit, Helsinki, Finland) based microplate reader. The IC₅₀ value was calculated as the concentration required to inhibit 50% of tyrosinase activity. L-ascorbic acid was used as the positive control.

2.6.4. Antibacterial Activity

Antibacterial activity was evaluated using the agar well diffusion method according to the Clinical and Laboratory Standards Institute [12]. The bacterial strains used in this study included *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Sterile wells were prepared on Mueller–Hinton (BD Difco, Sparks, MD, USA) agar plates inoculated with bacterial suspension. Extract solutions (2.5 mg/mL) were added to the wells and incubated at 37 °C for 24 h. The inhibition zone diameter was measured in millimeters. Vancomycin and gentamicin were used as positive controls, while 2.5% DMSO served as the negative control.

2.7. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were determined using the broth microdilution method [12]. Serial dilutions of the extracts were prepared in Mueller–Hinton broth and inoculated with bacterial suspensions. After incubation at 37 °C for 24 h, MIC values were determined as the lowest concentration showing no visible bacterial growth. Aliquots from wells without visible growth were sub-cultured onto agar plates to determine MBC values.

2.8. Biocompatibility Assay

The hemolytic activity of the formulation was evaluated using a modified method [13]. Briefly, 5 mL of fresh blood was mixed with 10 mL of phosphate-buffered saline (PBS), and red blood cells (RBCs) were isolated by centrifugation at 10,000 rpm for 10 min. The RBC pellets were washed five times with PBS and subsequently diluted in 50 mL of PBS to obtain the RBC suspension. For positive and negative controls, RBCs were incubated with 0.5% Triton-X and PBS, respectively. The RBCs were incubated with the formulation at concentrations of 500 and 1000 µg/mL at room temperature for 1 h. After incubation, the mixtures were centrifuged at 10,000 rpm for 3 min. A 100 µL aliquot of the supernatant from each sample was transferred to a 96-well plate, and the absorbance was measured at 570 nm using a microplate reader.

Cytotoxicity of the extracts was evaluated using the MTT assay [13]. HEK293, Ha-CaT, and HeLa cells were cultured and treated with various concentrations of extracts (1.95–62.5 µg/mL). After incubation, the MTT solution was added and further incubated. Formazan crystals were dissolved with DMSO, and absorbance was measured at 570 nm using a UV–Vis spectrophotometer (Biohit 830, Biohit, Helsinki, Finland). Cell viability was calculated relative to untreated control cells.

2.9. Anti-Inflammatory Activity

Anti-inflammatory activity was assessed using RAW 264.7 macrophage cells stimulated with lipopolysaccharide (LPS) following the method [13]. Cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and antibiotics. After treatment with extracts and LPS stimulation, nitric oxide (NO)

production was measured using the Griess reagent assay. The absorbance was measured at 560 nm using a microplate reader (Biohit 830, Biohit, Helsinki, Finland).

2.10. Preparation of Cream Formulation Containing *Eurycoma longifolia* Extract

The cream formulation was prepared using a phase separation method (Table 1). Phase B ingredients were weighed into a 50 mL beaker, while Phase A ingredients were weighed into a 250 mL beaker. The oil phase (Phase B) was heated on an electric hot plate (IKA C-MAG HS 7, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 70–72 °C with continuous stirring until completely dissolved and homogeneous. Subsequently, the oil phase was slowly added to the aqueous phase (Phase A) with continuous mixing using a homogenizer until a uniform emulsion was obtained. After the formulation cooled and became homogeneous, Phase C ingredients, including *Eurycoma longifolia* extract and cucumber extract, were incorporated and mixed thoroughly to obtain the final cream formulation.

Table 1. Composition of cream formulation containing *Eurycoma longifolia* extract.

Ingredients	Amount (% <i>w/w</i>) Phase
Milli-Q water	73.7 A
Butylene glycol	5.0 A
Glycerin	3.0 A
Trehalose	3.0 A
Cyclopentasiloxane	3.0 B
1,2-Hexanediol	2.0 A
Squalane	2.0 B
Phenyl trimethicone	2.0 B
Caprylyl methicone	2.0 B
Ammonium acryloyldimethyltaurate/VP copolymer	1.0 A
Phenoxyethanol (and) caprylyl glycol (and) sorbic acid	1.0 A
<i>Eurycoma longifolia</i> root extract	1.0 C
Tocopheryl acetate	0.5 C
Carbomer Ultrez 21	0.4 A
Polyglyceryl-3 methylglucose distearate	0.2 B
Disodium EDTA	0.1 A
Cucumber extract	0.1 C
Fragrance	q.s. C

2.11. Molecular Docking of Selected Phytochemicals Against 2QVD Protein

Molecular docking studies of chlorogenic acid, magnolioside, and biflorin against the target protein (PDB ID: 2QVD) were performed using PyRx version 0.8. The three-dimensional crystal structure of 2QVD was retrieved from the Protein Data Bank and prepared by removing water molecules and co-crystallized ligands, followed by the addition of polar hydrogen atoms. The chemical structures of chlorogenic acid, magnolioside, and biflorin were obtained from PubChem, energy-minimized, and converted into PDBQT format. A docking grid center (X = 25.8883, Y = 44.5064, Z = 1.1164) and a spacing of 0.3750 angstroms were defined around the active binding site of the protein, and docking simulations were carried out using default Vina parameters. The binding poses with the lowest binding energies were selected as the most favorable conformations. Protein–ligand interactions, including hydrogen bonding, hydrophobic contacts, and other non-covalent interactions, were subsequently analyzed and visualized using Discovery Studio Visualizer to assess the binding affinity and interaction patterns of the compounds with the target protein.

2.12. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 10.5). Data are presented as mean $\pm$ standard deviation (SD) from triplicate experiments ($n = 3$). Differences among groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A p -value < 0.05 was considered statistically significant.

3. Results and Discussion

Eurycoma longifolia Jack, commonly known as Tongkat Ali, is a renowned medicinal plant native to Southeast Asia, traditionally valued for its aphrodisiac and anti-malarial properties. While root extracts have dominated pharmacological research for sexual dysfunction and exercise recovery, emerging evidence suggests that leaves, stems, and roots contain diverse bioactive compounds with significant potential for cosmetic applications. The plant harbors quassinoids, canthin-6-one alkaloids, tirucallane triterpenes, and squalene derivatives, which exhibit antioxidant, antibacterial, and anti-inflammatory activities. These properties are critical for cosmetic formulations targeting skin protection, anti-aging, and antimicrobial control.

Recent studies demonstrate that alcoholic and acetone extracts from leaves and stems possess potent antibacterial activity against Gram-positive and Gram-negative bacteria, whereas root extracts showed limited antibacterial efficacy. This differential activity across plant parts warrants systematic investigation into their formulation potential. With increasing demand for natural, sustainable cosmetic ingredients, exploring non-root parts of *E. longifolia* offers a sustainable approach while conserving the heavily harvested roots. This study evaluates the leaves, stems, and roots of *E. longifolia* for cosmetic suitability, focusing on antioxidant capacity, antibacterial activity, and skin-beneficial phytochemical profiling to inform results and discussion on their dermatological applications.

The polarity of extraction solvents plays an important role in recovering phytochemicals from medicinal plants. Hydroethanolic solvents can dissolve both polar and semi-polar compounds, thereby improving extraction yield and recovery of bioactive constituents [14]. Previous studies also reported that 50–70% ethanol effectively extracted phenolic compounds and flavonoids from medicinal plants due to improved solvent penetration into plant tissues [6]. Therefore, 50% and 70% ethanol were selected for further biological activity studies.

The results of LC-MS/MS-based profiling of *E. longifolia* root and leaf extracts in positive and negative ionization modes are presented in Tables 2 and 3, respectively. The LC-MS/MS profiling of *E. longifolia* root extract in negative ionization mode identified 502 compounds and, in positive ionization mode, 105 compounds, while the leaf extract identified 423 compounds and 108 compounds in positive ionization mode. LC-MS/MS profiling of *E. longifolia* root and leaf extracts suggested a chemically diverse profile suitable for cosmeceutical applications, with identified constituents spanning phenolic acids, flavonoids, fatty acids, lipids, and alkaloid-like compounds. The presence of chlorogenic acid, neochlorogenic acids, glycosides (quercetin), apigenin, and kaempferol-type derivatives is particularly relevant due to the wide range of pharmacological properties of these compounds, including antioxidant, anti-inflammatory, and skin-protective activities, which are desirable in anti-aging and photoprotective formulations. Fatty acids and lipid derivatives such as oleic acid, linoleamide, vaccenic acid isomers, octadecenoic acid derivatives, and octyl palmitate may support emollient, barrier-repair, and skin-conditioning properties, enhancing topical performance.

Table 2. LC-MS/MS-based profiling of *Eurycoma longifolia* root extract in positive and negative modes using a Shimadzu quadrupole time-of-flight liquid chromatography–mass spectrometry (LC-MS/MS) instrument.

Name of Compounds from Root Extract in Negative Mode	Mass	Assign Score (%)
13-Oxo-octadecadienoic acid	294.21	100.0
9-KODE	294.21	100.0
(10E,12E,15E)-9-Hydroxy-10,12,15-octadecatrienoic acid	294.21	100.0
2-Ethoxy-1-phenoxy-2-decanol	294.21	100.0
(6E,9E,11E)-13-Hydroxy-6,9,11-octadecatrienoic acid	294.21	100.0
11-Hydroxy-6,9,12-octadecatrienoic acid	294.21	100.0
9-(Nona-1',3'-dienoxy) non-8-enoic acid	294.21	100.0
13-Hydroxy-octadecatrienoic acid	294.21	100.0
2,7-Octanedione, 4,4-dimethyl-3-[2-(1-hydroxy-1-methylethyl)-3-methyl-3-butenylidene]	294.21	100.0
Trans-vaccenic acid	282.25	0.0
Cis-vaccenic acid	282.25	0.0
Oleic acid	282.25	0.0
Octadec-12-enoic acid	282.25	0.0
Isooleic acid	282.25	0.0
Cys-Arg-Asp	392.14	50
Arg-Cys-Asp	392.14	50
His-Gln-Glu	412.17	40
Gln-Glu-His	412.17	40
His-Ala-Ala-Asp	412.17	40
Lys-His-Gln-Ala	482.26	40
Arg-Val-Asn-Pro	484.27	21.4
Malyngamide R	582.30	50
L-seryl-L-valyl-L-lysyl-L-seryl-L-tyrosine	582.30	16.7
L-valyl-L-asparaginyl-L-valyl-L-histidyl-L-seryl-L-seryl-L-leucine	754.39	68.8
1-[(2E)-2-Buten-1-yl]-8-(3,5-diethyl-1H-pyrazol-1-yl)-3-methyl-7-pentyl-3,7-dihydro-1H-purine-2,6-dione	412.25	100
Ethyl 2-[(5-amino-6-[(2,2,6,6-tetramethylpiperidin-4-yl) amino] pyrimidin-4-yl) amino] benzoate	412.25	100
Isobutyl 4-[(5-amino-6-[(2-(1-piperidinyl) ethyl) amino]-4-pyrimidinyl) amino] benzoate	412.25	100
4-(Isobutylamino)-6-[2-[4-(2-methoxyethyl)-1-piperazinyl] ethoxy]-2-quinazolinecarbonitrile	412.25	100
N',N''-[1,4-Phenylenedi(Z)methylidene]bis [2-(1-piperidinyl) acetohydrazide]	412.25	100
Ledebouriellol	374.13	87.6
5-Hydroxymatairesinol	374.13	87.6
Linoleamide	279.25	86.8
Mochiquinone	278.15	35.6
Tris(chloropropyl)phosphate	326.00	17.2
Crispatene	398.24	12.5
Felixinin	398.24	12.5
Tetraphenylhydrazine	336.16	11.4
Val-Leu-Arg-Lys	514.35	4.1
Leu-Phe-Leu-Phe	538.31	0
Phe-Phe-Ile-Leu	538.31	0
Lys-Thr-Tyr-Lys	538.31	0

Table 3. LC-MS/MS-based profiling of *Eurycoma longifolia* leaf extract in positive and negative modes using a Shimadzu quadrupole time-of-flight liquid chromatography–mass spectrometry (LC-MS/MS) instrument.

Name of Compounds from Leave Extract in Negative Mode	Mass	Assign Score (%)
Magnolioside	354.09	100.0
Biflorin	354.09	100.0
Neochlorogenic acid	354.09	100.0
Chlorogenic acid	354.09	100.0
3-Nitro-4-[4-(4-nitrophenyl)-1-piperazinyl]benzoic acid	372.10	100.0
2-[2-[(2S)-2,3-dihydro-1,4-benzodioxin-2-ylcarbonyl] hydrazino]-2-oxoethyl 5-methyl-2-pyrazinecarboxylate	372.10	100.0
N'-[3-(4-butoxy-3-ethoxyphenyl) acryloyl]-4-oxo-3-propyl-3,4-dihydro-1-phthalazinecarbohydrazide	492.33	100.0
N-[2-Cyclobutyl-7-[(10-methoxy-3,4-dihydro-2H-1,5-benzoxazocin-5(6H)-yl) carbonyl]-1-methyl-1H-benzimidazol-5-yl]-2-methoxyacetamide	492.33	100.0
N-[(5,6-dimethyl-1H-benzimidazol-2-yl) methyl]-2-[1-(2-methoxyethyl)-3-(3-methoxyphenyl)-2,5-dioxo-3-pyrrolidinyl]-N-methylacetamide	492.33	100.0
Stecholide E	492.33	100.0
4-Acetoxy-2,3-bis(3,4,5-trimethoxybenzyl)-1-butanol	492.33	100.0
Met-Val-Arg-Arg	560.32	100.0
Ethyl N-[(4-ethylphenyl) [(2-methylcyclopropyl) (N~2~-[(2-methyl-2-propanyl) oxy] carbonyl] glutaminy] amino] acetyl]-β-alaninate	560.32	100.0
L-isoleucyl-L-alanyl-L-α-glutamyl-L-lysyl-L-threonine	560.32	100.0
Benzyl N-[[1-(N~2~-acetyl-N~6~-[(2-methyl-2-propanyl) oxy] carbonyl)-L-lysyl]-3-piperidinyl] carbonyl]-β-alaninate	560.32	100.0
N-(benzylsulfonyl)-3-cyclohexyl-D-alanyl-N-[(1-carbamimidoyl-4-piperidinyl) methyl]-L-prolinamide	560.32	100.0
2-Butyne-1,4-diylbis(oxy-2,1-ethanediyl) bis(4-{[3-(aminomethyl) benzyl] carbamoyl}-1-piperazinecarboxylate)	722.37	100.0
D-(–)-quinic acid	192.06	83.3
Pyroglutamylhistidine	266.10	85.7
Lignoceric acid	368.36	0
Octyl palmitate	368.36	0
Cetyl octanoate	368.36	0
8-C-glucosylgenistein	432.10	80
β-D-genistin	432.10	80
Sophoricoside	432.10	80
Cosmosiin	432.10	80
Apigetrin	432.10	80
Vitexin	432.10	80
Genistin	432.10	80
Brovincamine	432.10	33.3
Cys-Cys-Cys-His	464.09	87.5
Gossypitrin	464.09	75.0
Icariside	464.09	75.0
Quercetin-3-glucoside	464.09	75.0

Table 3. Cont.

Name of Compounds from Leave Extract in Negative Mode	Mass	Assign Score (%)
Hyperin	464.09	75.0
Myricitrin	464.09	75.0
Demeclocycline	464.09	75.0
Hyperoside	464.09	75.0
Val-Met-Phe-Pro	492.23	50
Ser-Met-Lys-Gln	492.23	50
Pro-Val-Phe-Met	492.23	50
Lys-Asn-Thr-Met	492.23	50
Leupyrrin C	724.39	81.8
Monanchocidin A	859.62	0
(3E)-6-[2-(dimethylamino) ethoxy]-3-imino-1,2,4-benzotriazin-4(3H)-ol-1-oxide	265.11	100.0
6-[(2Z)-2-(3,3-dimethyl-5-oxocyclohexylidene)hydrazino]-1,2,4-triazine-3,5(2H,4H)-dione	265.11	100.0
(1S,2S,3R)-3-(6-amino-9H-purin-9-yl)-1-(hydroxymethyl)-2-methyl-1,2-cyclobutanediol	265.11	100.0
2-Amino-9-[[[(3R,4S)-3,4-dihydroxycyclopentyl] methyl]-3,9-dihydro-6H-purin-6-one	265.11	100.0
(1R,2R,3S,5R)-3-(4-amino-1H-pyrazolo [3,4-d] pyrimidin-1-yl)-5-methoxy-1,2-cyclopentanediol	265.11	100.0
2-Amino-9-[(1R,2R,4S)-2-hydroxy-4-(hydroxymethyl) cyclopentyl]-3,9-dihydro-6H-purin-6-one	265.11	100.0
8-methyl-2'-deoxyadenosine	265.11	100.0
Linoleamide	279.25	96.9
Asp-His-His-Val	506.22	51.9
Trp-lys-cys-val	534.2	21.7
Lys-Met-Ala-Trp	534.2	17.7
Lys-Cys-Val-Trp	534.2	21.7
Lys-Ala-Met-Trp	534.2	17.7
Lys-Thr-Tyr-Lys	538.31	2.9
Phe-Phe-Ile-Leu	538.31	1.5
Leu-Phe-Leu-Phe	538.31	1.5
Trp-Lys-Met-Val	562.29	16.3
Met-Trp-Lys-Val	562.29	16.3
Phe-Leu-Phe-His	562.29	9.1
Phe-Leu-His-Phe	562.29	9.1
Met-Thr-Arg-Arg	562.29	8.7
Lys-Glu-Trp-Met	592.26	22.6
Glu-Lys-Trp-Met	592.26	22.6
Thr-Trp-Asp-Trp	606.24	17.8
Ser-Trp-Trp-Glu	606.24	17.8
Phe-Trp-Glu-Gln	608.25	21.0
Tyr-Arg-Pro-Trp	620.30	14.2
Pro-Trp-Arg-Tyr	620.30	14.2
Glycyl-L-leucyl-L-phenylalanyl-L-tryptophyl-L-aspartic acid	636.29	21.4

Table 3. Cont.

Name of Compounds from Leave Extract in Negative Mode	Mass	Assign Score (%)
Tyr-gly-pro-gly-phe-pro	636.29	21.4
Arg-Trp-Glu-Phe	636.29	16.9
Phe-Arg-Trp-Glu	636.29	16.9
Haplophytine	652.28	57.1
Tyr-Trp-Glu-Arg	652.28	27.6
Ala-Phe-Ser-Ser-Trp-Gly-NH ₂	652.28	27.6
Sileneoside H	700.36	2.0

The LC-MS/MS-based profiling of *E. longifolia* root and leaf extracts suggested the presence of several unique low-molecular-weight metabolites and peptide-like ions, indicating broad metabolic coverage and possible synergistic bioactivity. In positive and negative-ion modes, the dual-platform LC-MS/MS approach improved confidence in compound annotation by capturing metabolites with different ionization behaviors, which is important for untargeted phytochemical profiling. For cosmeceutical applications, such a profile is valuable because it supports the idea that the extract may provide multifunctional benefits, including antioxidant defense, reduction of oxidative stress, and support for skin health.

The extraction yields of wild and cultivated *E. longifolia* obtained using different ethanol concentrations are presented in Table 4. Among all plant parts, leaf extracts exhibited the highest extraction yields, followed by roots and stems, respectively. The crude extracts obtained from all samples showed a dark brown, viscous appearance similar to tar-like resin. For wild plant samples, extraction with 50% ethanol provided the highest yield in roots (2.88%), stems (3.03%), and leaves (14.74%), whereas 70% ethanol showed the second-highest extraction efficiency. Similar results were observed in cultivated samples, in which 50% ethanol produced the highest extraction yields in roots (3.12%), stems (3.09%), and leaves (18.99%). These findings indicate that hydro-ethanolic solvents enhanced extraction efficiency better than absolute ethanol or water alone.

Table 4. Extraction yield of *Eurycoma longifolia* Jack extracts obtained from wild and cultivated plants using different ethanol-water ratios.

Source	Plant Part	Ethanol: Water Ratio (% v/v)	Extract Weight (mg)	Extraction Yield (%)	Extract Appearance
Wild	Root	0:100	400	1.60	Sticky, tar-like
		50:50	720	2.88	
		70:30	610	2.44	
		100:0	330	1.43	
Wild	Stem	0:100	170	0.67	Sticky, tar-like
		50:50	760	3.03	
		70:30	710	2.81	
		100:0	360	1.44	
Wild	Leaf	0:100	1280	10.48	Sticky, tar-like
		50:50	1810	14.74	
		70:30	1480	12.12	
		100:0	820	6.54	
Cultivated	Root	0:100	430	1.72	Sticky, tar-like
		50:50	780	3.12	
		70:30	550	2.20	
		100:0	310	1.35	
Cultivated	Stem	0:100	190	0.76	Sticky, tar-like
		50:50	810	3.09	
		70:30	650	2.51	
		100:0	340	1.33	
Cultivated	Leaf	0:100	1190	9.58	Sticky, tar-like
		50:50	2360	18.99	
		70:30	2080	16.91	
		100:0	810	6.22	

Quantitative analysis using HPLC revealed that roots contained the highest quantity of eurycomanone, followed by stems and leaves. Cultivated root extracts contained 1.53 ± 0.07 mg/mL eurycomanone, whereas wild root extracts contained 1.30 ± 0.00 mg/mL (Figure 2). Eurycomanone is a major quassinoid compound widely recognized as a bioactive marker compound of *E. longifolia*. This compound has been associated with various pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer effects [1,15]. The high concentration of eurycomanone in root extracts supports the traditional use of the roots as the primary medicinal part of the plant.

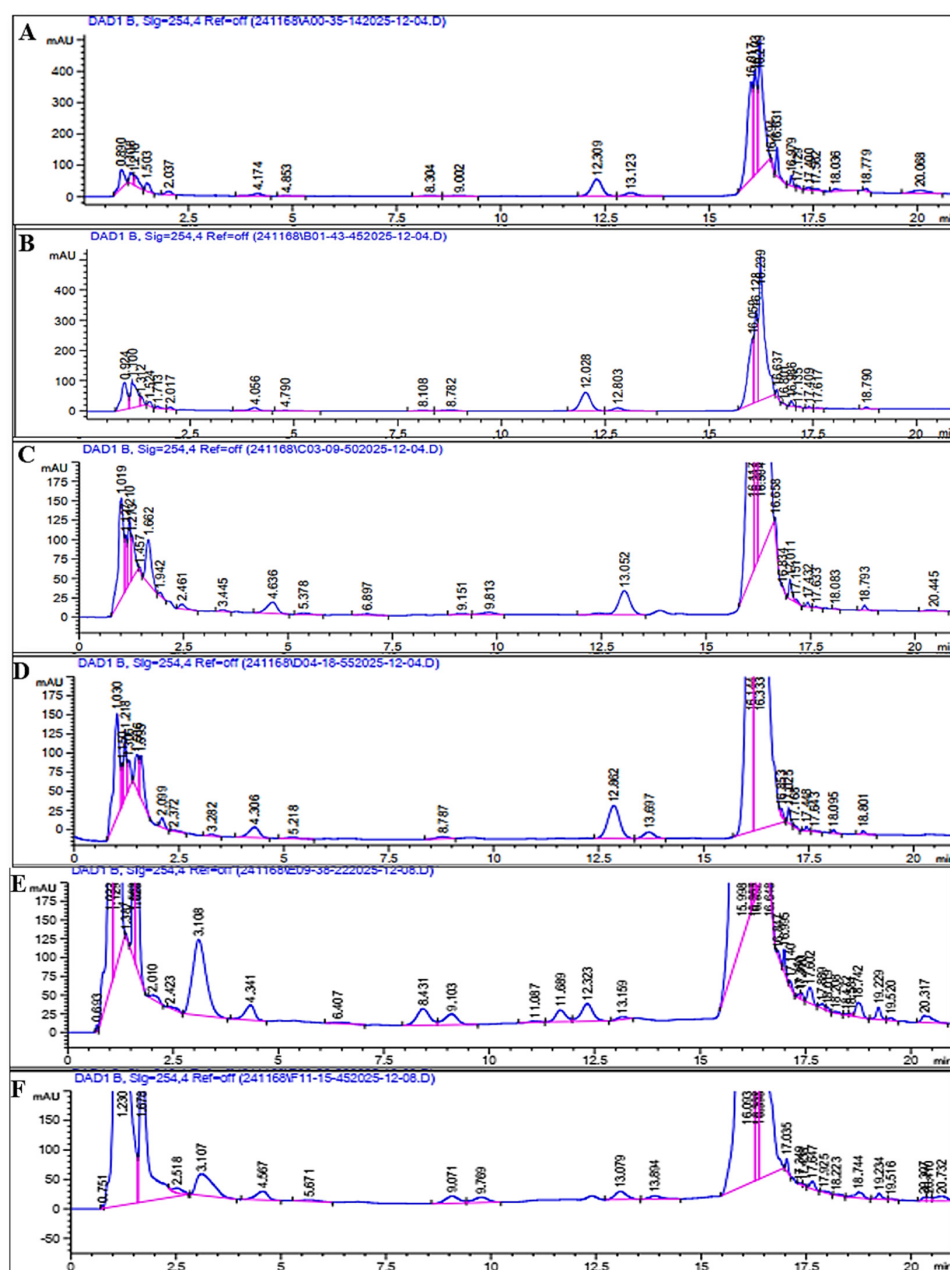


Figure 2. HPLC chromatograms of *Eurycoma longifolia* Jack extracts from wild and cultivated sources extracted with 70% ethanol for the determination of eurycomanone content. Chromatographic analysis was performed using an acetonitrile:methanol (1:1, v/v) mobile phase at a flow rate of 1.5 mL/min and detected at 254 nm using a diode array detector (DAD). (A) wild root extract, (B) cultivated root extract, (C) wild stem extract, (D) cultivated stem extract, (E) wild leaf extract, and (F) cultivated leaf extract.

The FTIR spectra of *Eurycoma longifolia* Jack extracts were acquired at 600–3800 cm^{-1} to identify the peaks corresponding to stretching or bending vibrations arising from the functional groups of bioactive compounds within the extract (Figure 3). The extracted plant parts demonstrated similar spectral peaks at $\sim 3400 \text{ cm}^{-1}$, $\sim 3000 \text{ cm}^{-1}$, $\sim 2900 \text{ cm}^{-1}$, $\sim 1660 \text{ cm}^{-1}$, $\sim 1400 \text{ cm}^{-1}$, $\sim 1300 \text{ cm}^{-1}$, $\sim 1000 \text{ cm}^{-1}$ corresponding to O-H stretching/H-bonded, aromatics C-H stretching, alkenes C-H stretching, C=O stretching, C-C stretching, N-O symmetric stretching, and C-O stretching, respectively. Furthermore, DMSO alone showed sharp peaks around $\sim 2996 \text{ cm}^{-1}$, 1300 cm^{-1} , and 1020 cm^{-1} ascribed to C-H stretching vibrations, bending vibrations of methyl C-H_3 , and stretching of S=O bonds, respectively. Thus, the presence of a sharp peak in each plant part extract confirms the presence of DMSO at 1020 cm^{-1} . The broad absorption bands observed in the spectra are consistent with phenolics, flavonoids, and other oxygenated metabolites that may contribute to the observed biological effects. Moreover, the presence of molecules with different chemical structures was assessed based on differences in spectral peak distribution in the fingerprint region of the infrared spectrum.

The phytochemical contents and antioxidant activities of *E. longifolia* extracts are summarized in Table 5. The results demonstrated that 70% ethanol extraction yielded the highest TPC and TFC in both wild and cultivated samples. Among all plant parts, the leaves showed significantly higher TFC and TPC values than the roots and stems. Wild leaf extracts obtained using 70% ethanol showed the highest TFC ($269.67 \pm 3.04 \text{ mg QE/g sample}$) and TPC ($20.34 \pm 0.26 \text{ mg GAE/g sample}$), while cultivated leaf extracts showed comparable values of $255.71 \pm 3.04 \text{ mg QE/g sample}$ and $20.80 \pm 0.16 \text{ mg GAE/g sample}$, respectively. These findings suggest that leaves are an important source of phenolic and flavonoid compounds.

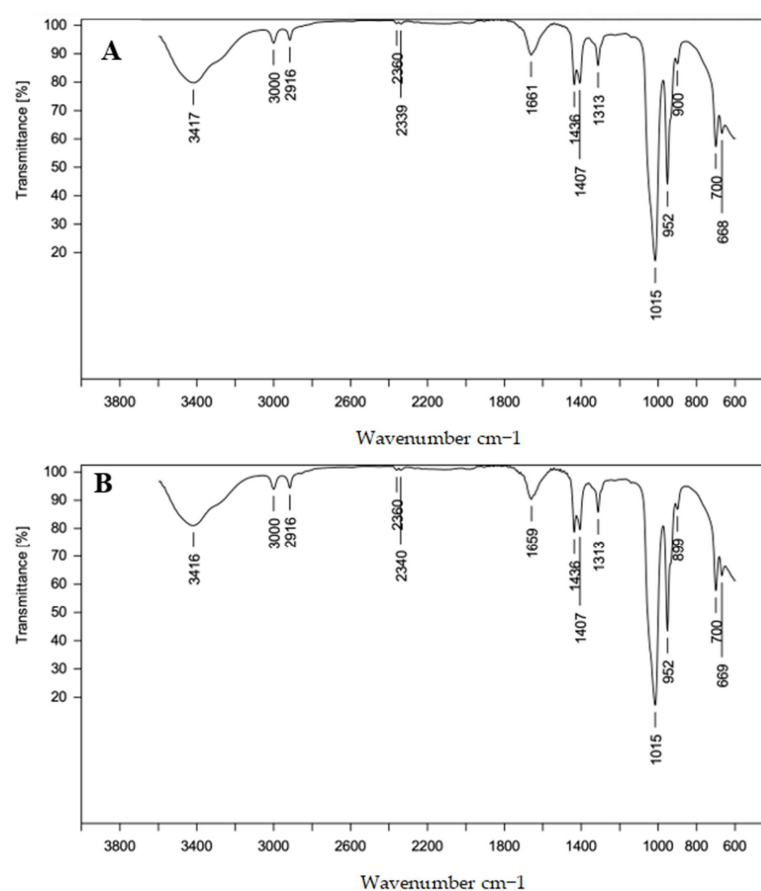


Figure 3. Cont.

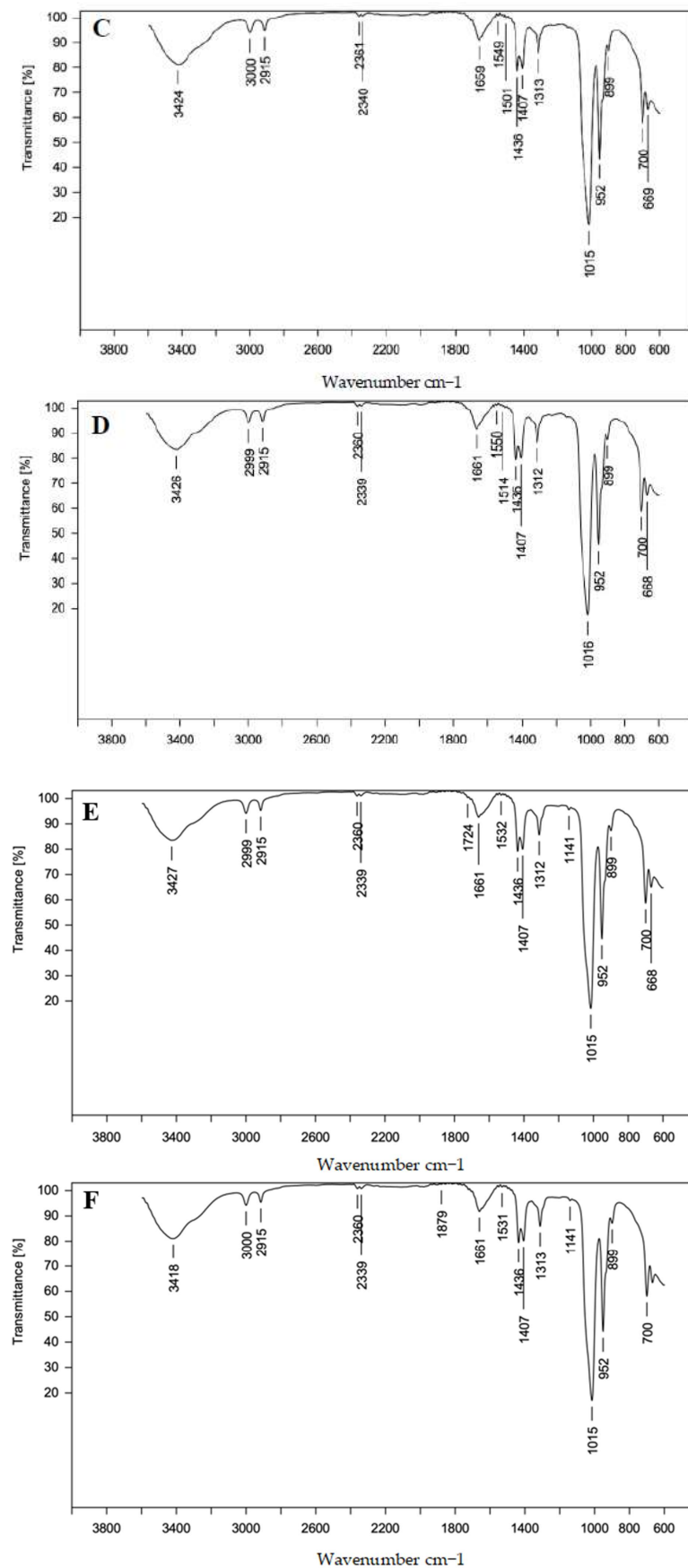


Figure 3. Cont.

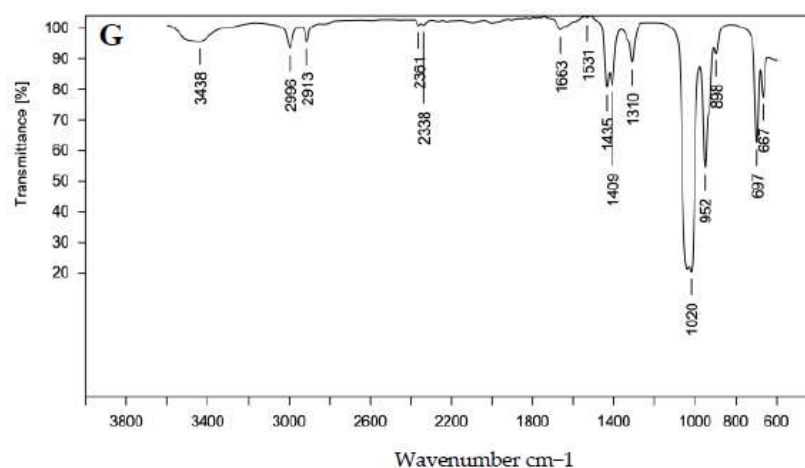


Figure 3. Structural and functional FTIR peaks with stretching and bending of *Eurycoma longifolia* Jack extracts from different parts, such as wild root extract (A), cultivated root extract (B), wild stem extract (C), cultivated stem extract (D), wild leaf extract (E), cultivated leaf extract (F), and DMSO (G).

Table 5. Total phenolic content (TPC), total flavonoid content (TFC), antioxidant activities, and tyrosinase inhibitory activity of *Eurycoma longifolia* Jack extracts from wild and cultivated plants.

Source	Plant Part	Ethanol: Water Ratio (% v/v)	TFC (mg Quercetin/g Sample)	TPC (mg Gallic Acid/g Sample)	DPPH IC ₅₀ (μg/mL)	ABTS IC ₅₀ (μg/mL)	Tyrosinase IC ₅₀ (μg/mL)
Wild	Root	0:100	45.08 ± 2.35	7.34 ± 0.18	125.61 ± 0.80	238.14 ± 1.76	96.57 ± 4.83
		50:50	48.73 ± 2.22	6.98 ± 0.16	140.46 ± 1.06	257.14 ± 1.06	111.65 ± 2.78
		70:30	53.94 ± 1.13	9.58 ± 0.84	123.78 ± 0.83	218.58 ± 1.43	95.84 ± 2.01
		100:0	49.88 ± 2.56	8.00 ± 0.44	156.22 ± 0.83	287.78 ± 1.10	134.66 ± 2.91
Wild	Stem	0:100	68.00 ± 3.48	7.59 ± 0.36	130.16 ± 1.36	117.56 ± 0.95	106.02 ± 3.01
		50:50	59.15 ± 2.84	6.76 ± 0.22	174.93 ± 1.54	138.72 ± 1.52	151.10 ± 4.01
		70:30	69.77 ± 2.66	9.37 ± 0.15	127.22 ± 1.21	114.39 ± 1.27	78.73 ± 4.07
		100:0	62.17 ± 1.60	6.19 ± 0.18	166.45 ± 1.28	116.59 ± 1.31	108.34 ± 3.74
Wild	Leaf	0:100	262.79 ± 4.38	19.09 ± 0.43	17.33 ± 0.70	86.38 ± 0.96	139.99 ± 1.86
		50:50	224.98 ± 3.66	19.91 ± 0.13	15.98 ± 0.83	63.28 ± 1.01	58.51 ± 3.85
		70:30	269.67 ± 3.04	20.34 ± 0.26	6.37 ± 0.74	57.53 ± 1.10	50.71 ± 3.92
		100:0	230.60 ± 3.13	18.79 ± 0.16	16.68 ± 1.04	67.01 ± 0.79	153.22 ± 3.37
Cultivated	Root	0:100	44.56 ± 1.74	6.09 ± 0.75	134.40 ± 0.72	249.04 ± 1.30	98.54 ± 3.63
		50:50	48.10 ± 2.08	8.03 ± 0.52	173.18 ± 0.76	309.07 ± 1.70	84.96 ± 2.93
		70:30	62.06 ± 1.43	9.84 ± 0.59	132.49 ± 0.88	248.84 ± 1.12	81.79 ± 2.69
		100:0	49.56 ± 1.43	8.19 ± 0.29	169.41 ± 1.05	262.28 ± 1.73	118.13 ± 2.31
Cultivated	Stem	0:100	57.90 ± 1.72	8.05 ± 0.29	132.57 ± 1.35	128.94 ± 1.26	96.37 ± 3.65
		50:50	55.92 ± 1.60	6.75 ± 0.16	144.13 ± 1.08	123.12 ± 1.55	196.56 ± 3.55
		70:30	87.38 ± 2.19	10.19 ± 0.18	107.95 ± 1.08	98.01 ± 1.25	87.97 ± 4.30
		100:0	74.88 ± 2.48	6.47 ± 0.28	110.37 ± 1.32	104.69 ± 1.02	125.01 ± 3.13
Cultivated	Leaf	0:100	241.23 ± 2.08	19.15 ± 0.96	22.76 ± 0.79	89.44 ± 0.90	186.48 ± 2.72
		50:50	206.13 ± 3.26	20.14 ± 0.20	8.77 ± 0.70	58.68 ± 0.90	71.38 ± 3.16
		70:30	255.71 ± 3.04	20.80 ± 0.16	7.95 ± 0.86	43.90 ± 0.85	55.80 ± 4.75
		100:0	209.35 ± 2.39	18.83 ± 0.94	14.22 ± 1.11	52.78 ± 0.83	119.68 ± 3.13
Standard compound	Quercetin	—	—	—	2.52 ± 0.95	—	—
Standard compound	L-ascorbic acid	—	—	—	—	7.43 ± 1.04	75.77 ± 1.67

Values are expressed as mean ± standard deviation (n = 3).

The antioxidant activities determined by DPPH and ABTS radical scavenging assays revealed that 70% ethanol extracts exhibited the strongest antioxidant activity, indicated by the lowest IC₅₀ values. Wild leaf extracts showed the most potent DPPH scavenging activity, with an IC₅₀ value of 6.37 ± 0.74 μg/mL, while cultivated leaf extracts exhibited an IC₅₀ value of 7.95 ± 0.86 μg/mL. Similar trends were observed for ABTS radical scavenging activity.

The high antioxidant activities are attributed to elevated phenolic and flavonoid contents in the leaf extracts. Phenolic compounds are well known for their hydrogen-donating and electron-transfer capacities, which contribute to free radical scavenging activity [16]. In addition, the ortho-dihydroxyl structure in the B-ring of flavonoids enhances antioxidant capacity by stabilizing free radicals [17]. These findings are consistent with previous reports demonstrating strong antioxidant activities of *E. longifolia* extracts rich in phenolic constituents [1,4].

Tyrosinase inhibitory activities of the extracts are presented in Table 5. The results demonstrated that 70% ethanol extracts exhibited strong tyrosinase inhibitory effects in all plant parts. Wild leaf extracts showed strong inhibition with an IC_{50} value of $50.71 \pm 3.92 \mu\text{g/mL}$, followed by cultivated leaf extracts ($55.80 \pm 4.75 \mu\text{g/mL}$). Interestingly, these values were lower than those of the positive control, L-ascorbic acid ($75.77 \pm 1.67 \mu\text{g/mL}$), suggesting notable tyrosinase inhibitory activity under the present in vitro assay conditions. Although the 70% ethanolic leaf extract showed the strongest inhibition among the tested samples, its IC_{50} value remained higher than those typically reported for strong cosmetic standards such as kojic acid or arbutin [18]. L-ascorbic acid was used as the positive control in the present study; however, other established cosmetic whitening agents, such as kojic acid and arbutin, were not included and should be considered in future investigations for a more comprehensive comparison. Tyrosinase is a key enzyme involved in melanin biosynthesis, and inhibition of this enzyme is widely employed as a preliminary screening approach for identifying compounds with potential depigmenting properties [19]. The strong inhibitory activity observed in leaf extracts may be associated with high levels of phenolic compounds such as quercetin and kaempferol, which are known to interfere with tyrosinase activity [20]. Moreover, the hydroxyl groups present in phenolic compounds may interact with the active site of tyrosinase through hydrogen bonding, thereby suppressing enzymatic activity [21].

The antibacterial activities of *E. longifolia* extracts are summarized in Figure 4. The results showed that the root and stem extracts exhibited no antibacterial activity against the tested bacterial strains. However, leaf extracts from both wild and cultivated samples demonstrated inhibitory effects against *Staphylococcus epidermidis* with inhibition zone diameters of $7.06 \pm 1.04 \text{ mm}$ and $7.70 \pm 0.30 \text{ mm}$, respectively. No inhibitory activity was observed against *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa* using the agar diffusion assay. Therefore, the antibacterial activity of the tested extracts was considered selective and moderate, with the most evident effect observed against the Gram-positive bacteria, *Staphylococcus epidermidis*. In contrast, activity against Gram-negative bacteria was weak or absent under the conditions tested. Furthermore, MIC and MBC analyses revealed that leaf extracts exhibited inhibitory effects against Gram-positive bacteria, while relatively weaker effects were observed against *E. coli*. Leaf extracts showed MIC/MBC values of 256/256 $\mu\text{g/mL}$ against *S. epidermidis* and 512/512 $\mu\text{g/mL}$ against *S. aureus* (Table 6). The observed inhibitory effects against Gram-positive bacteria may be partly attributed to the presence of phenolic compounds and flavonoids, which have been reported to affect bacterial cell wall integrity and membrane permeability [22]. Gram-negative bacteria generally exhibit greater resistance due to the presence of an outer membrane that limits the penetration of phytochemicals. Thus, the pattern suggests that the extracts contain constituents with limited but meaningful antibacterial potential, which may be more effective against Gram-positive organisms due to their simpler cell wall structure and greater susceptibility to phytochemicals.

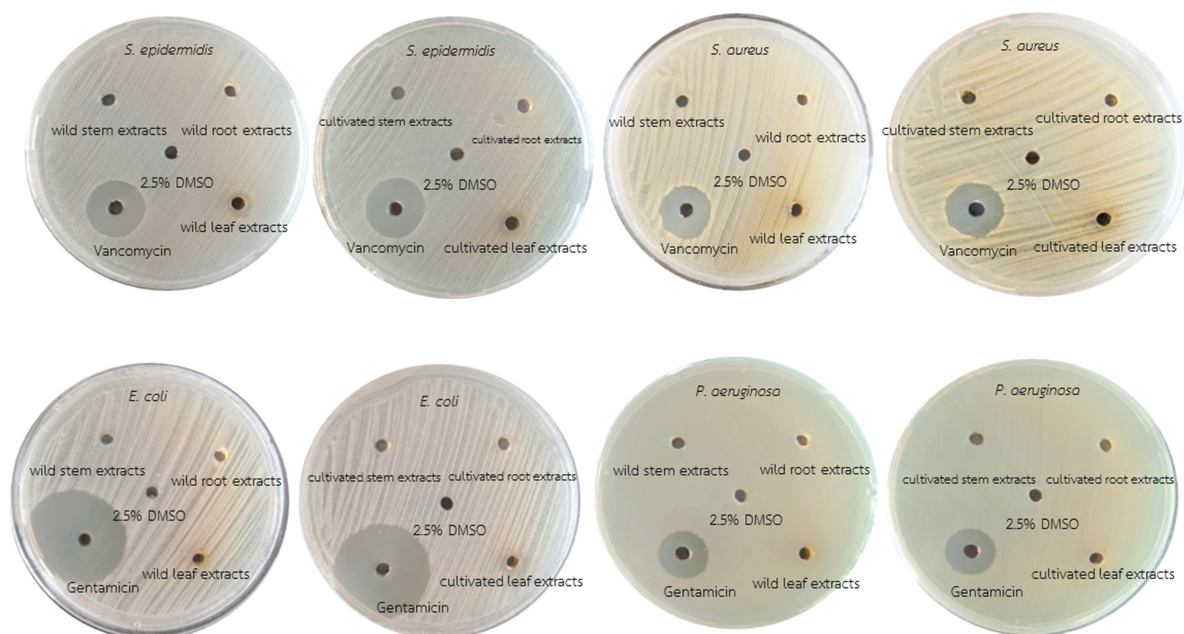


Figure 4. Antibacterial activity of *Eurycoma longifolia* Jack extracts from wild and cultivated sources extracted with 70% ethanol against *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 was determined by the agar well diffusion assay. Vancomycin and gentamicin were used as positive controls, while 2.5% DMSO was used as the negative control. The leaf extracts exhibited inhibitory activity against *S. epidermidis*, whereas no inhibitory effect was observed against the tested Gram-negative bacteria.

Table 6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Eurycoma longifolia* Jack extracts from wild and cultivated sources extracted with 70% ethanol.

Test Microorganisms	Wild Root Extract		Wild Stem Extract		Wild Leaf Extract		Cultivated Root Extract		Cultivated Stem Extract		Cultivated Leaf Extract		Vancomycin		Gentamicin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>S. aureus</i> ATCC 25923	>2048	>2048	>2048	>2048	512	512	>2048	>2048	>2048	>2048	512	512	0.25	0.5	–	–
<i>S. epidermidis</i> ATCC 12228	>2048	>2048	>2048	>2048	256	256	>2048	>2048	>2048	>2048	256	256	0.5	0.5	–	–
<i>E. coli</i> ATCC 25922	>2048	>2048	>2048	>2048	512	1024	>2048	>2048	>2048	>2048	512	1024	–	–	0.25	0.5
<i>P. aeruginosa</i> ATCC 27853	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	>2048	–	–	0.25	0.5

Values are expressed in µg/mL; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration.

The biocompatibility results demonstrated that the tested extract lysed red blood cells in a dose-dependent manner (Figure 5). Less than 1% cell lysis was observed when cells were exposed to 31.25–500 µg/mL for the wild stem, cultivated stem, wild leaf, cultivated leaf, and phosphate buffer groups; however, wild root and cultivated root showed higher lysis (above 1%) when tested between 31.25 and 500 µg/mL. These results suggest that the tested extracts are biocompatible. According to a previous study, <2% hemolysis is classified as non-hemolytic, hemolysis between 2 and 5% is classified as slightly hemolytic, and >5% hemolysis is labeled as hemolytic [13,21]. Cytotoxicity evaluation using the MTT assay demonstrated that the extracts exhibited low toxicity toward HaCaT, HEK293, RAW264.7, and HeLa cells (Figures 6–9). Cell viability remained above 80% at all tested concentrations, indicating that the extracts were relatively safe for normal mammalian cells. The low cytotoxicity observed in this study supports the potential application of *E. longifolia* extracts in pharmaceutical and cosmetic formulations. Similar findings were

previously reported by Taechowisan et al. [4], who demonstrated low cytotoxicity and promising biological activities of *E. longifolia* extracts.

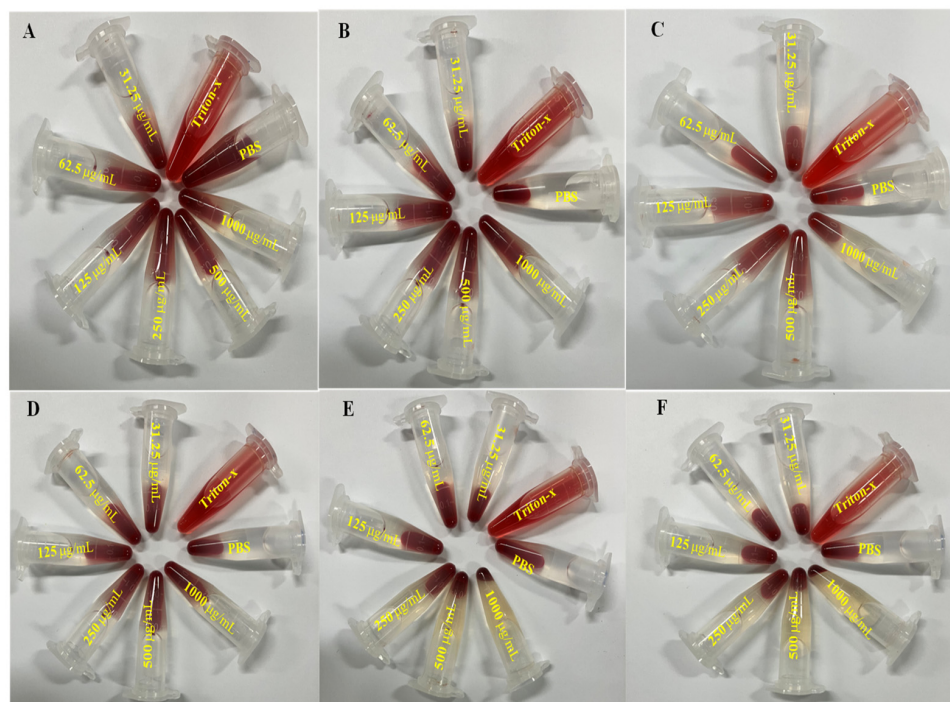


Figure 5. Biocompatibility assessment of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (B), wild stem (C), cultivated stem (D), wild leaf (E), and cultivated leaf (F) against red blood cells. Cells were treated with different concentrations of the extracts (31.25–1000 µg/mL) for 24 h.

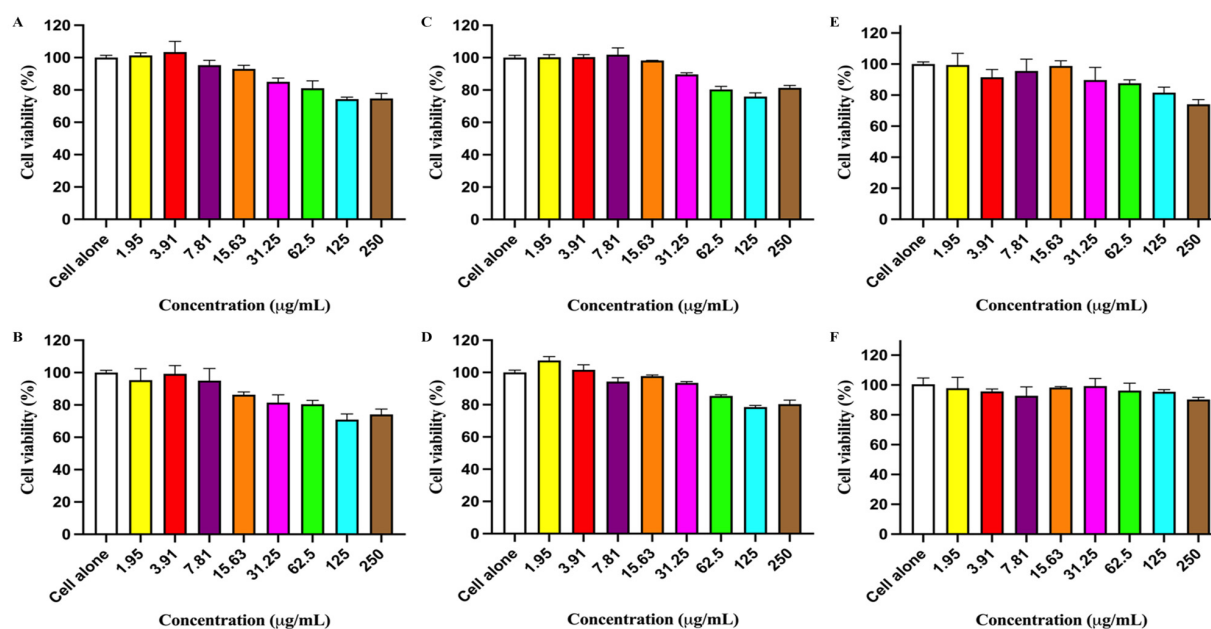


Figure 6. Cytotoxicity assessment of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (B), wild stem (C), cultivated stem (D), wild leaf (E), and cultivated leaf (F) against HaCaT cells using the MTT assay. Cells were treated with different concentrations of the extracts (1.95–250 µg/mL) for 24 h. Cell viability was expressed as the percentage relative to the untreated control group. The extracts showed low cytotoxicity toward normal and cancer cell lines, with cell viability remaining above 80% in most tested concentrations. Values are expressed as mean $\pm$ SD (n = 3).

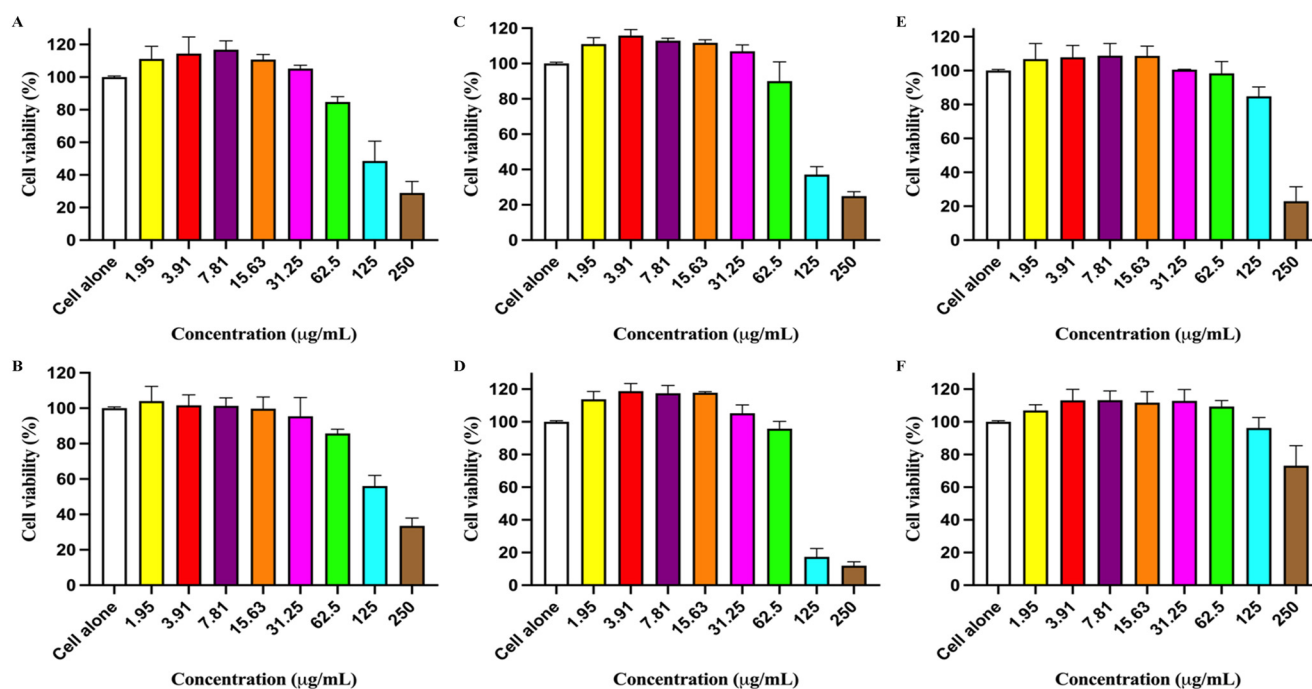


Figure 7. Cytotoxicity assessment of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (B), wild stem (C), cultivated stem (D), wild leaf (E), and cultivated leaf (F) against HEK293 cells using the MTT assay. Cells were treated with different concentrations of the extracts (1.95–250 µg/mL) for 24 h. Cell viability was expressed as the percentage relative to the untreated control group. The extracts showed low cytotoxicity toward normal and cancer cell lines, with cell viability remaining above 80% in most tested concentrations. Values are expressed as mean $\pm$ SD (n = 3).

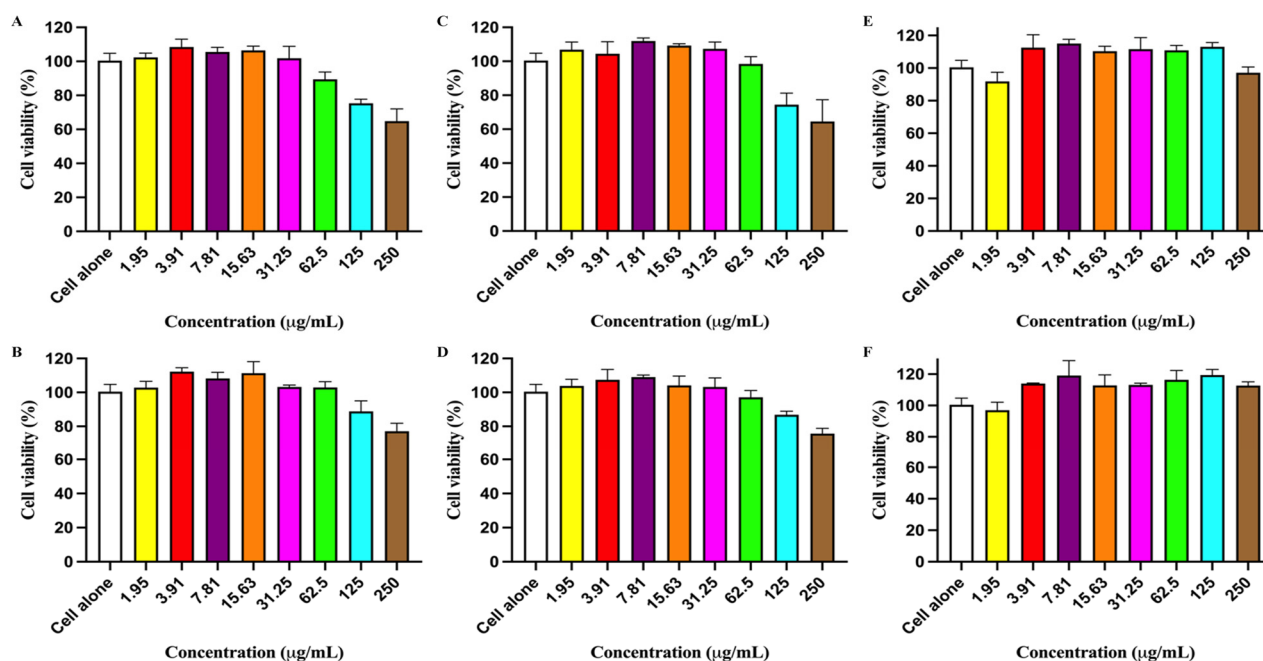


Figure 8. Cytotoxicity assessment of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (B), wild stem (C), cultivated stem (D), wild leaf (E), and cultivated leaf (F) against RAW264.7 cells using the MTT assay. Cells were treated with different concentrations of the extracts (1.95–250 µg/mL) for 24 h. Cell viability was expressed as the percentage relative to the untreated control group. The extracts showed low cytotoxicity toward normal and cancer cell lines, with cell viability remaining above 80% in most tested concentrations. Values are expressed as mean $\pm$ SD (n = 3).

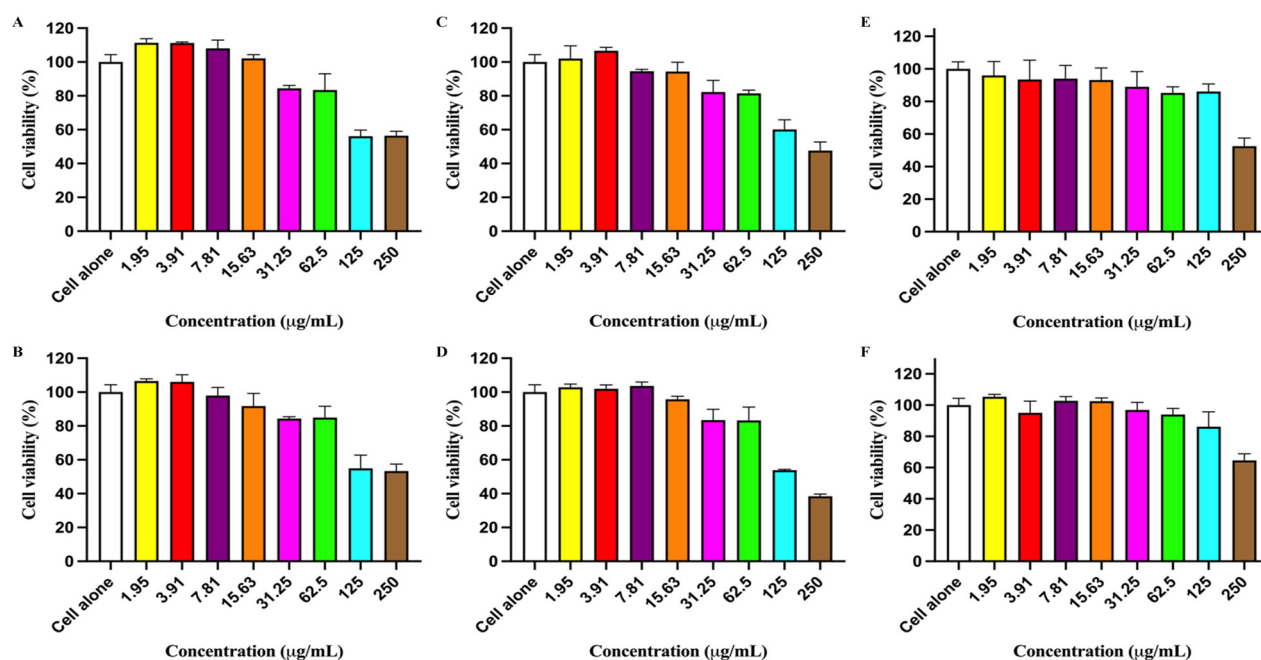


Figure 9. Cytotoxicity assessment of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (B), wild stem (C), cultivated stem (D), wild leaf (E), and cultivated leaf (F) against HeLa cells using the MTT assay. Cells were treated with different concentrations of the extracts (1.95–250 µg/mL) for 24 h. Cell viability was expressed as the percentage relative to the untreated control group. The extracts showed low cytotoxicity toward normal and cancer cell lines, with cell viability remaining above 80% in most tested concentrations. Values are expressed as mean $\pm$ SD ($n = 3$).

The anti-inflammatory effects of the extracts were evaluated using LPS-stimulated RAW 264.7 macrophage cells by measuring nitric oxide (NO) production. At a concentration of 125 µg/mL, the root, stem, and leaf extracts significantly reduced NO production compared with the LPS-treated control group. Wild leaf extracts exhibited a strong inhibitory effect with NO inhibition of 77.82%, followed by cultivated root extracts (71.35%) (Figure 10). These findings indicate that *E. longifolia* leaf extracts possess potent anti-inflammatory properties. Excessive NO production induced by inducible nitric oxide synthase (iNOS) is associated with inflammatory responses and tissue damage. Suppression of NO production suggests inhibition of inflammatory mediators [3]. The anti-inflammatory effects observed in this study may be related to the quassinoids, flavonoids, and phenolic compounds present in the extracts.

The physicochemical properties and color stability of the root extract formulation and base formulation were evaluated before and after six freeze–thaw (FW) cycles. The results are presented in Table 7. Although leaf extracts exhibited stronger biological activity, the root extract was selected for formulation development because the root is currently the only part of *E. longifolia* listed in the CosIng database as a cosmetic ingredient. Moreover, the root extract contained the highest eurycomanone content, a recognized bioactive marker of *E. longifolia*, which further supports its selection as a standardized formulation candidate. The present formulation study, therefore, aimed to assess the feasibility and stability of a cosmetically recognized *E. longifolia* ingredient, while leaf extracts remain promising candidates for future development as sustainable non-root cosmetic actives. The root extract formulation showed a significant decrease in viscosity after FW cycling, from 770.40 ± 28.65 cP to 656.00 ± 30.33 cP ($p < 0.05$). The observed viscosity reduction of approximately 14.85% remained within the acceptable range for cosmetic formulation stability, as viscosity changes below 20% are generally considered physically acceptable for semisolid cosmetic products [23]. The reduction in viscosity suggests that repeated temperature fluc-

tuations affected the internal structure of the formulation, leading to decreased resistance to flow. Similar findings have been reported in cosmetic emulsions subjected to accelerated stability conditions, where freeze–thaw stress can disrupt the emulsion network and reduce formulation consistency. In terms of color parameters, the L^* value significantly decreased from 49.83 ± 0.40 to 47.53 ± 0.34 after FW cycling, indicating that the formulation became darker following storage under stress conditions. However, no significant changes were observed in a^* , b^* , chroma (C^*), or hue angle (h°) values ($p > 0.05$), suggesting that although slight darkening occurred, the overall color tone remained relatively stable. The darkening effect may be associated with oxidation or degradation of phytochemical constituents in the root extract during repeated exposure to low and high temperatures. Previous studies have reported that phenolic compounds and natural pigments in plant extracts are susceptible to oxidative reactions during storage, leading to color changes in topical formulations. In contrast, the base formulation exhibited no statistically significant differences in viscosity or color parameters after six FW cycles ($p > 0.05$). The viscosity remained relatively stable, changing only slightly from 1273.00 ± 10.39 cP to 1220.00 ± 72.08 cP. Likewise, minimal changes were observed in L^* , a^* , b^* , C^* , and h° values (Table 7). These results indicate that the formulation base possessed good physical and color stability under accelerated storage conditions. The absence of significant changes in the base formulation suggests that the instability observed in the root extract formulation was primarily related to the incorporation of the plant extract rather than the emulsion system itself. Freeze–thaw cycling is commonly used as an accelerated stability test to predict the long-term stability of cosmetic and pharmaceutical emulsions. Repeated freezing and thawing can promote phase separation, droplet aggregation, and structural breakdown, which subsequently affect viscosity and appearance. Therefore, maintaining stable viscosity and color properties after FW testing is considered an important indicator of formulation stability.

Table 7. Physicochemical properties and color parameters of root extract cream formulation and base formulations before and after six freeze–thaw cycles.

Sample	Condition	Appearance	Viscosity (cP) at 100 rpm	L^*	a^*	b^*	C^*	h°
Root extract cream formulation	Initial (25 °C)		770.40 ± 28.65^a	49.83 ± 0.40^a	7.56 ± 0.16^a	37.88 ± 0.89^a	38.62 ± 0.85^a	78.70 ± 0.42^a
	After 6 FW cycles		656.00 ± 30.33^b	47.53 ± 0.34^b	7.69 ± 0.43^a	37.08 ± 0.29^a	37.87 ± 0.37^a	78.30 ± 0.55^a
Base formulation	Initial (25 °C)		1273.00 ± 10.39^a	72.09 ± 0.10^a	-2.64 ± 0.01^a	-1.42 ± 0.03^a	3.01 ± 0.01^a	208.24 ± 0.63^a
	After 6 FW cycles		1220.00 ± 72.08^a	71.14 ± 2.61^a	-2.67 ± 0.05^a	-1.38 ± 0.70^a	2.89 ± 0.10^a	202.87 ± 7.94^a

Values are expressed as mean $\pm$ SD ($n = 3$). Different superscript letters within the same column indicate significant differences at $p < 0.05$ according to Tukey's multiple comparison test. FW = freeze–thaw cycles.

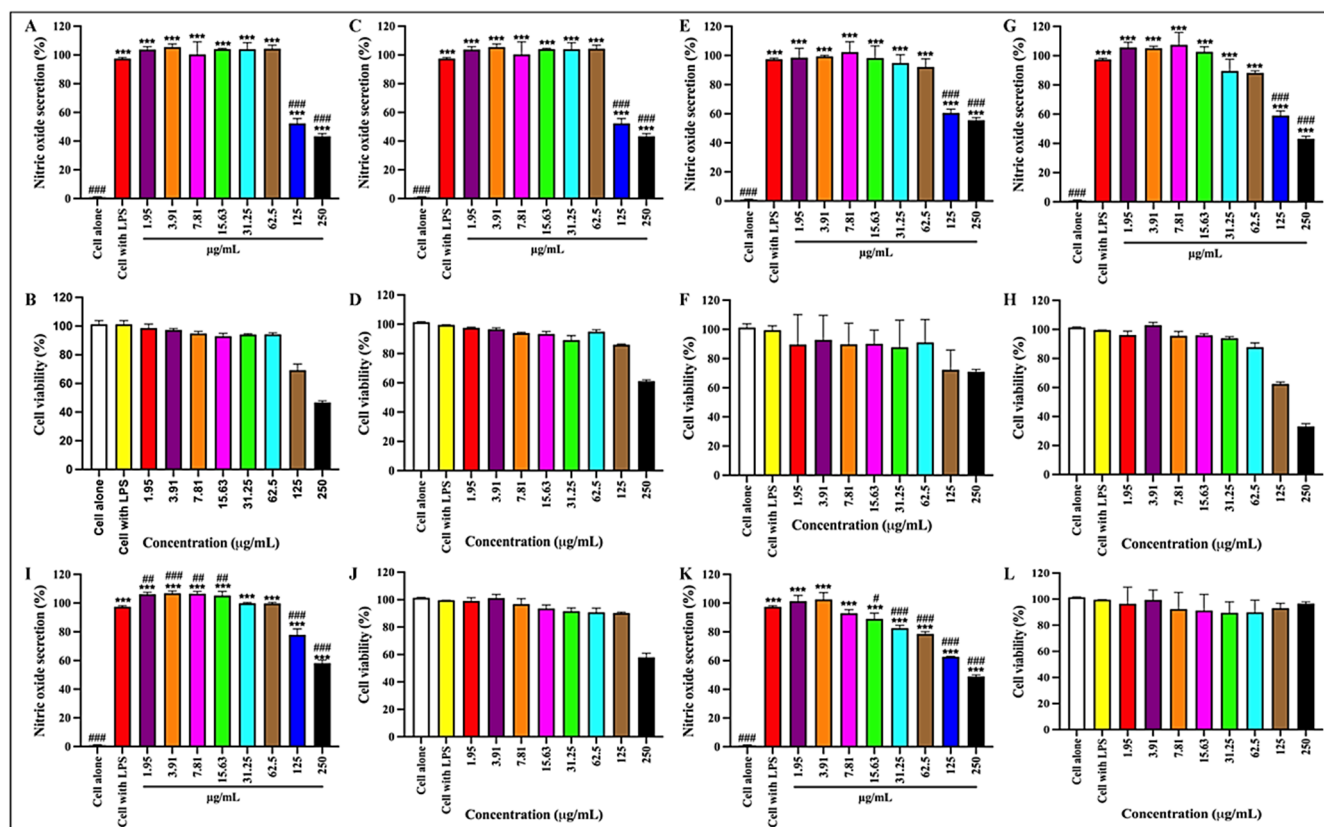


Figure 10. Anti-inflammatory activity of *Eurycoma longifolia* Jack extracts from wild root (A), cultivated root (C), wild stem (E), cultivated stem (G), wild leaf (I), and cultivated leaf (K) sources was determined by inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells at a concentration of 250 µg/mL. Moreover, after testing NO, the same plates were subsequently quantified for cytotoxicity (anti-inflammatory activity of *Eurycoma longifolia* Jack extracts from wild root (B), cultivated root (D), wild stem (F), cultivated stem (H), wild leaf (J), and cultivated leaf (L) sources). The wild leaf extract exhibited the highest NO inhibitory activity, followed by wild root, cultivated root, wild stem, cultivated stem, wild leaf, and cultivated leaf extracts. Values are expressed as percentage inhibition of nitric oxide production and as mean $\pm$ SD (n = 3). Statistical significance was determined by one-way ANOVA and Dunn's multiple comparison test, indicated as # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. the LPS-stimulated group; and *** $p < 0.001$ vs. the control group.

The antibacterial activity of the cream formulation containing *E. longifolia* Jack root extract was evaluated by determining the MIC and MBC against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 under freeze–thaw cycle and 25 °C storage conditions (Table 8). The results demonstrated that the cream formulation exhibited antibacterial activity against both tested bacterial strains. Under both freeze–thaw cycle and 25 °C storage conditions, the formulation showed the same MIC and MBC values. For *S. aureus* ATCC 25923, the MIC and MBC values were 16 and 32 mg/mL, respectively. Meanwhile, for *E. coli* ATCC 25922, the MIC and MBC values were 8 and 32 mg/mL, respectively. The reference antibiotics showed stronger antibacterial activity than the cream formulation. Vancomycin exhibited MIC and MBC values of 0.25 and 0.5 µg/mL, respectively, against *S. aureus*, while gentamicin exhibited MIC and MBC values of 0.25 and 0.5 µg/mL, respectively, against *E. coli*. These findings indicate that the formulation retained its antibacterial effects after stability testing. However, as the observed activity occurred at relatively high concentrations, further studies are required to clarify its practical significance in topical applications.

Table 8. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of a cream formulation containing *Eurycoma longifolia* Jack root extract against tested bacterial strains.

Test Microorganisms	Condition	MIC (mg/mL)	MBC (mg/mL)
<i>S. aureus</i> ATCC 25923	Freeze–thaw cycle	16	32
	25 °C	16	32
<i>E. coli</i> ATCC 25922	Freeze–thaw cycle	8	32
	25 °C	8	32
Vancomycin	25 °C	0.00025	0.0005
Gentamicin	25 °C	0.00025	0.0005

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration.

Molecular docking analysis against the target protein 2QVD demonstrated favorable binding interactions for all three phytoconstituents (Figure 11), with binding affinities ranging from -7.0 to -7.4 kcal/mol. Among the tested compounds, biflorin exhibited the highest binding affinity (-7.4 kcal/mol), followed closely by magnolioside (-7.3 kcal/mol) and chlorogenic acid (-7.0 kcal/mol). Although the reference drug diclofenac showed a slightly stronger binding affinity (-7.8 kcal/mol), the docking scores of the phytochemicals were comparable (Table 9), suggesting their potential to interact effectively with the active site of the target protein. The relatively high affinity of biflorin may be attributed to its quinone-based aromatic scaffold, which can establish hydrophobic and π -mediated interactions within the binding pocket, thereby enhancing complex stability. Magnolioside and chlorogenic acid possess multiple hydroxyl groups capable of forming hydrogen bonds with key amino acid residues, contributing to favorable ligand–protein recognition and stabilization. Mechanistically, the ability of these compounds to occupy the active site and establish strong non-covalent interactions may interfere with substrate binding or catalytic activity of the target protein, ultimately modulating downstream inflammatory pathways. The docking results therefore support the potential of biflorin, magnolioside, and chlorogenic acid as bioactive molecules with possible anti-inflammatory activity, warranting further validation through molecular dynamics simulations and in vitro studies.

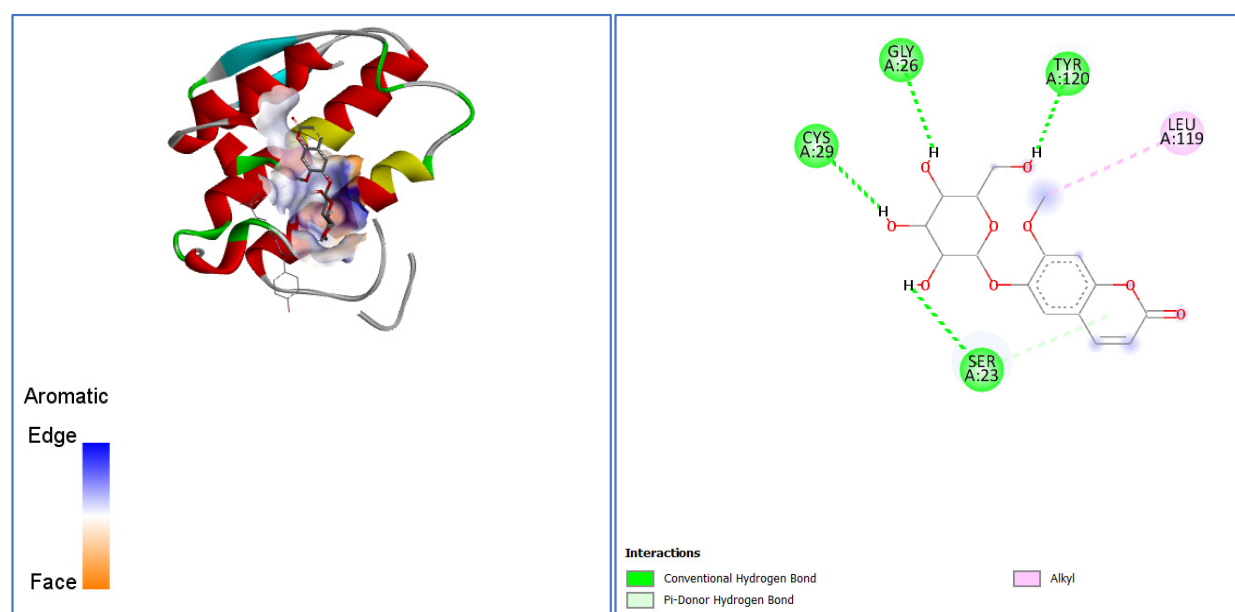
**Figure 11.** 2D and 3D interactions of biflorin against 2QVD.

Table 9. Docking score of selected phytochemicals against the inflammatory protein.

Sr. No.	Ligand Name	Binding Affinity (Kcal/mol)
1	Chlorogenic acid	−7
2	Biflorin	−7.4
3	Magnolioside	−7.3
4	Diclofenac	−7.8

While the molecular docking results provide supportive computational evidence that the selected phytochemicals may interact favorably with the target protein, they do not prove the mechanism of action. In silico docking is a predictive approach that helps to generate hypotheses about possible ligand–protein interactions; however, these findings require further validation by complementary biochemical, enzymatic, and cellular studies before any mechanistic conclusions can be drawn.

4. Conclusions

This study demonstrated that *Eurycoma longifolia* extracts obtained from different plant parts exhibited diverse biological activities depending on extraction conditions and plant sources. Among the tested solvents, 70% ethanol was identified as the most suitable extraction solvent for recovering bioactive compounds, particularly phenolic and flavonoid constituents, from the roots, stems, and leaves of both wild and cultivated plants. Leaf extracts showed the highest total phenolic and flavonoid contents as well as the strongest antioxidant and tyrosinase inhibitory activities. In particular, wild and cultivated leaf extracts exhibited potent DPPH and ABTS radical scavenging activities and strong tyrosinase inhibition, suggesting their potential as preliminary candidates for further investigation in anti-aging and depigmenting research. In addition, leaf extracts demonstrated antibacterial activity against Gram-positive bacteria, especially *Staphylococcus epidermidis*, while root extracts showed promising anti-inflammatory activity by suppressing nitric oxide production in LPS-stimulated RAW 264.7 macrophage cells. Cytotoxicity evaluation confirmed that the extracts exhibited low toxicity toward normal mammalian cells, supporting their preliminary safety for pharmaceutical and cosmeceutical applications. Furthermore, high-performance liquid chromatography analysis revealed that root extracts contained the highest amount of eurycomanone, a major quassinoid compound associated with the medicinal properties of *E. longifolia*. Overall, the findings suggest that *E. longifolia*, particularly leaf and root extracts obtained using 70% ethanol, represents a promising source of bioactive compounds with antioxidant, anti-inflammatory, antibacterial, and tyrosinase inhibitory activities. The molecular docking study suggests that biflorin, magnolioside, and chlorogenic acid exhibit promising binding interactions with the 2QVD target protein, supporting their potential as natural anti-inflammatory agents for further experimental validation. These findings provide a basis for further studies exploring their pharmaceutical and cosmeceutical potential. Future studies should focus on compound isolation, mechanism of action, formulation stability, safety assessment, and in vivo efficacy evaluation before practical applications can be considered.

5. Limitations

The formulation study is basic and preliminary, as further experimental consideration is required to evaluate long-term stability, retention of active compounds, microbial challenge, and skin compatibility before any practical cosmetic application can be proposed.

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