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Phytochemical Composition, Antioxidant Activity, and Metabolite Profiling of Sequential Extracts from *Vitex trifolia* Leaves with Antibacterial Potential: An Integrated *In vitro* and *In silico* Study

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ABSTRACT

Liligundi *(Vitex trifolia)* are herbal plants that are widely used in ethnomedicine. It contains natural compounds that can fight against oxidation and bacteria. This study aimed to evaluate the phytochemical composition, antioxidant activity, and metabolite profile of sequential extracts from *V. trifolia* leaves and their potential as antibacterial agents using both *in vitro* and *in silico* approaches. Sequential maceration was conducted using n-hexane (HE), ethyl acetate (EAER), and methanol (MER) as solvents. Each extract was evaluated for phenolics, flavonoids, tannins, and antioxidant activity. Antibacterial effects were tested, liquid chromatography-mass spectrometry (LC-MS) profiling was conducted, and *in silico* docking predicted interactions with bacterial targets. Phytochemical analysis showed that the MER extract had the highest levels of phenolics (5732.06±98.56 mg GAE/100 g), flavonoids (2765.32±36.56 mg QE/100 g), and tannins (5057.65±54.72 mg TAE/100 g). It also demonstrated the strongest antioxidant activity with an IC_{50} of 51.61±0.16 ppm, compared to HE (447.70±13.85 ppm) and EAER (188.80±2.33 ppm). Antibacterial activity at 50% concentration against *Staphylococcus aureus* and *Escherichia coli* was moderate for all extracts, with MER showing the largest inhibition zone. Metabolite profiling revealed similarities and differences in the types of compounds found in each solvent used for the extraction. The same compounds appeared in each solvent, namely chrysosplenol C, casticin, and 2-hydroxy-7-isopropyl-1,1,4a-trimethyl-3,4,10,10a-tetrahydro-2h-phenanthren-9-one. *In silico* analysis revealed that MER extract metabolites, vitexin, orientin, and luteolin 7-glucuronide, bind strongly to 3HUN and 1G2A receptors, suggesting antibacterial potential. These findings highlight the therapeutic potential of *V. trifolia* as a source of bioactive compounds and warrant further investigation.

Keywords: *Vitex trifolia*, liligundi, sequential extraction, antibacterial, antioxidant, metabolite profiling

Introduction

Medicinal herbs have long served as a rich source of bioactive compounds with diverse pharmacological properties, including antioxidant and antibacterial activities.[1,2] One potential plant in this regard is Liligundi (*V. trifolia*), which is known to contain flavonoids, alkaloids, tannins, and phenolic compounds that have high biological activities.[3,4] These compounds have an important role in counteracting free radicals that cause oxidative stress and inhibit the growth of pathogenic bacteria that are harmful to human health.[5,6,7] The extraction method used is a key factor in obtaining extracts with a high content of bioactive compounds.[8] Sequential maceration is an effective extraction technique to obtain active compounds based on differences in polarity.[9] This method is carried out in stages using non-polar, semi-polar, and polar solvents so as to extract compounds more selectively and efficiently.[10] With this approach, the bioactive components in *V. trifolia* leaves can be isolated more optimally, increasing their antioxidant and antibacterial potentials. Besides the extraction method, quantitative analysis of bioactive compounds is also an important aspect in determining the effectiveness of the extract. A quantitative analysis of the tannins, flavonoids, and polyphenols in the extract is necessary to determine the contribution of each compound to its biological activity.[11] This test provides useful insights into the chemical composition of the extract and its relationship with its antioxidant and antibacterial properties. Liquid chromatography–mass spectrometry (LC-MS) is employed to identify and characterize the compounds present in *V. trifolia* leaf extracts derived from solvents exhibiting higher bioactivity. The data obtained from LC-MS analysis enabled the determination of the molecular structure of the active compounds, which can then be validated through a molecular docking approach. Molecular docking is utilized to predict the interactions between active compounds and bacterial protein targets or enzymes involved in oxidative stress, offering insights into the extract's mechanisms of antibacterial activity and free radical scavenging.[12,13] This study aimed to investigate the phytochemical composition, antioxidant activity, and metabolite profiling of sequential extracts from *V. trifolia* leaves and to evaluate their antibacterial potential through integrated *in vitro* assays and *in silico* molecular docking analyses. This research represents a novel and

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comprehensive investigation into the phytochemical profile, antioxidant, and antibacterial activities of *V. trifolia* leaf extracts obtained via sequential maceration, combining LC-MS metabolite analysis and *in silico* molecular docking to identify polarity-based bioactive compounds. It is hoped that the results of this study could contribute to the development of herbal medicines based on natural extracts that are more effective and safer to use in the health sector.

Materials and Methods

Plant material

V. trifolia leaf simplisia powder was obtained and identified at the Herbal Materia Medica Laboratory Batu, Malang, East Java, Indonesia (7°52'3.93"S and 112°31'9.41"E) with plant specimen number 000.9.3/7964/102.20/2024.

Extraction

The extraction was performed using a sequential maceration process, beginning with non-polar solvents (n-hexane), followed by semi-polar (ethyl acetate), and finally polar solvents (methanol) (Merck, Germany). A total of 500 grams of powdered material was soaked in 4 L of n-hexane and macerated for 72 hours (3 × 24 hours), after which the mixture was filtered. The residue was then macerated with ethyl acetate solvent and soaked for the same time. The mixture was filtered, and the residue was macerated again with methanol. Filtrates from each maceration process with n-hexane (HE), ethyl acetate (EAER), and methanol (MER) were then concentrated with a rotary evaporator (BUCHI R-300, Swiss).

Determination of total phenol content

The total phenol content of the extracts was analyzed using the Folin-Ciocalteu technique with some modifications.[14] A total of 10 mg of extract was dissolved in 10 mL of methanol (99.9%) (Merck, Germany), homogenized, and centrifuged (NUVE Z10.NF 1200, Turkey) at 3000 rpm for 15 minutes to obtain the supernatant and filtered. Then, 0.4 mL of the filtrate was mixed with 0.4 mL of Folin–Ciocalteu reagent (Merck, Germany) and vortexed (WINA 701, Indonesia). After 5 minutes, 4.2 mL of 5% sodium carbonate solution (Merck, Germany) was added, and the mixture was incubated for 30 minutes. The absorbance of the solution was measured using an ultraviolet-visible (UV/Vis) spectrophotometer (BIOCHROM Libra S60, UK) at 760 nm. A standard curve was prepared using gallic acid solutions ranging from 0 to 140 mg/L. Total phenolic content was calculated as milligrams of gallic acid equivalent per 100 grams of extract (mg GAE/100 g)),[15] using the line equation (y = ax + b) on the gallic acid standard curve.

Determination of total flavonoid content

Analysis of total flavonoid content in each extract was carried out using a colorimetric method with aluminium chloride reagent ($AlCl_3$) (Merck, Germany).[16] A total of 10 mg of sample was dissolved with 10 mL of ethanol (99.9%). Then, 0.5 mL of supernatant was diluted with distilled water and sodium nitrite (5% b/v) (Merck, Germany) and incubated for 5 minutes. The mixture was then added to 0.3 mL of aluminium chloride (10%) (Merck, Germany) and 2 mL of sodium hydroxide (1%) (Merck, Germany) and incubated at room temperature for 30 minutes. The absorbances of the solutions were measured at a wavelength of 415 nm by UV/Vis spectrophotometer (BIOCHROM Libra S60, UK). A standard curve was prepared by dissolving quercetin (Sigma-Aldrich, Germany) in 99.9% ethanol to obtain concentrations ranging from 0 to 140 mg/L. Total flavonoid content was calculated as milligrams of quercetin equivalent per gram of extract (mg QE/100 g), using the equation (y = ax + b) from the quercetin standard curve.

Determination of total tannin content

The Folin-Denis method was used to determine the extract's total tannin content with some modifications.[17] A total of 10 mg of the extract was diluted in 5 mL of phosphate-citrate buffer (Merck, Germany) based on the specified treatment conditions. A 0.25 mL aliquot of the diluted sample was pipetted, then 0.25 mL of Folin-Denis reagent (Merck, Germany) was added. The mixture was vortexed, followed by the addition of 2 mL of 5% Na_2CO_3. The solution was vortexed and then incubated for 30 minutes. The absorbance was measured using a spectrophotometer at a wavelength of 725 nm. The readings were compared with a standard curve using tannic acid (Merck, Germany). Total tannins in the sample were converted into tannic acid equivalents in mg TAE/100 g extract.

Analysis of antioxidant activity

The antioxidant capacity of extracts was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method.[14] Various concentrations (0, 20, 40, 60, 80, 100 ppm) of each extract (1 mL) were mixed in a test tube with 1 mL of DPPH solution (0.1 mM in methanol) (Merck, Germany) and incubated for 30 min. A mixture of 99% methanol (1 mL) and DPPH reagent (1 mL) was used as a control, and methanol (99% v/v) was used as a blank. The absorbances of the samples, controls, and blanks were measured using a UV/Vis spectrophotometer at 517 nm, with each measurement repeated three times. The antioxidant capacity was estimated based on the linear regression equation (y = ax + b).

Antibacterial activity assay

Antibacterial activity testing was performed using the Kirby-Bauer disc diffusion method with some modifications.[18] Each extract was dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) at concentrations of 5%, 25%, and 50%, and 30 µL of each solution was applied onto sterile blank discs (Oxoid, UK). The positive control consisted of chloramphenicol at 30 µg/mL, while the negative control used DMSO, with 30 µL applied to blank discs. Each disc was placed on a Petri dish containing bacterial inoculum and incubated for 24 hours at 37°C, with each treatment repeated three times. Antibacterial activity was characterized by the formation of a transparent zone on the media (zone of inhibition) measured in mm. The strength of antibacterial activity was categorized based on the diameter of the inhibition zone as follows: weak (< 5 mm), moderate (6-10 mm), strong (11-20 mm), and very strong (≥ 21 mm).[19]

Metabolite profiling using ultra-performance liquid chromatography-mass spectrometry

Ten milligrams of the extracts were accurately weighed and dissolved in methanol to a final volume of 10 mL in a volumetric flask. A 5 µL aliquot of the extract solution was then drawn with a microsyringe and injected into the sample holder and ultra-performance liquid chromatography (UPLC) column. Replication was done four times. The liquid sample was converted into droplets as it passed through a needle charged with a positive electrospray ionization (ESI) charge (+). The ions that were generated by the detector were then separated by the Q-ToF analyzer. The eluents used were a mixture of water (formic acid [99.9:0.1]) and acetonitrile (formic acid [99.9:0.1]) with a gradient elution system with an eluent flow rate of 0.2 ml/min. Chromatograms displayed polar compounds first, followed by compounds of lower polarity. The separation results were then read by the QToF-MS detector to produce chromatogram peaks. The chromatogram peaks were then interpreted using the MassLynx V4.1 application. LC System: ACQUITY UPLC H-Class system (Waters, USA); LC Column: ACQUITY UPLC BEH C18 (1.8 µm 2.1 x 50 mm (Waters, USA); Mass Spectrometer: Xevo G2-S QToF (Waters, USA).

Target prediction and docking analysis

In silico analysis was performed on the extract with the most effective antibacterial activity, MER. The compounds identified in the MER from LC-MS analysis for docking were selected based on their biological activity, as estimated using the PASS Online tool from the Way2Drug platform [Way2Drug.com©2011-2022, version 2.0, Moscow, Russia] (www.way2drug.com/passonline/, accessed on January 8, 2024).[20] The PASS program classified biological activity into 'active' (Pa) or 'inactive' (Pi), with estimated probabilities ranging from zero to one. The interpretation of the probability of Pa and Pi is as shown below: (i) if Pa > Pi, the compound is likely to be considered active; (ii) if Pa > 0.7, the compound is likely to show biological activity and has a high probability of being an analogue of a known pharmaceutical drug; (iii) if 0.5 < Pa < 0.7, the compound may have an effect similar to the experimental one but with a lower probability and not similar to a known drug; (iv) if Pa < 0.5, the compound does not correspond to experimental activity but, if confirmed by experimental data, could be a new subject to investigate.[21] Compounds that proceeded to the docking process were those with Pa > 0.5.

The three-dimensional (3D) structures of the compounds were obtained from the PubChem database. The 3HUN target protein structure is the target protein for *S. aureus*,[22] and 1G2A for *E. coli*.[23] Both structures were obtained from the Protein Data Bank (https://www.rcsb.org/) and prepared using UCSF Chimera (v.1.16) software (The Regents of the University of California) to remove water molecules.[24] The grid box coordinates used for docking (PyRx 0.9, The Scripps Research Institute) the target protein 3HUN were set in Vina's search space with center coordinates X = -13.941, Y = 0.167, Z = -19.771 and dimensions (in angstroms) X = 65.927, Y = 59.032, Z = 74.668. Meanwhile, 1G2A was set with center coordinates X = 45.502, Y = -0.757, Z = 15.506 and dimensions (in angstroms) X = 52.678, Y = 55.364, Z = 45.788. The receptor-ligand interactions were visualized and analyzed using Discovery Studio Visualizer (v21.1.0.20298) software (Dassault Systèmes Biovia Corp.).[25]

Statistical analysis

The bacterial inhibition zone data were analyzed for growth inhibition differences using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($p < 0.05$), with Statistical Package for Social Sciences (SPSS) Software (version 23, IBM Corp.).

Results and Discussion

Flavonoid, polyphenol, and tannin contents with IC_{50} values

The analysis showed that sequential extraction with hexane, ethyl acetate, and methanol solvents affected the content of flavonoids, polyphenols, tannins, and IC_{50} values (Table 1). The trend showed that the more polar the solvent used, the higher the bioactive compounds dissolved, and the stronger the antioxidant activity. In the first extraction step, hexane solvent produced the lowest flavonoid, total phenolic, and tannin contents compared to the subsequent steps. This is due to the non-polar nature of hexane, which is more effective in extracting lipophilic compounds.[26] Meanwhile, flavonoids, phenolics, and tannins, which are generally more polar in nature, are not optimally soluble in this solvent.[14]

Table 1: Phytochemical contents and IC_{50} values of *Vitex trifolia* leaf with sequential extraction.

Extract	Flavonoid (mgQE/100 g)	TPC (mg GAE/100 g)	Tannin (mg TAE/100g)	IC_{50} (ppm)
HE	59.33 ± 5.90	230.59 ± 14.68	24.99 ± 4.26	447.70 ± 13.85
EAER	113.33 ± 7.01	567.76 ± 22.25	176.85 ± 10.90	188.80 ± 2.33
MER	2765.32 ± 36.56	5732.06 ± 98.56	5057.65 ± 54.72	51.61 ± 0.16

HE: n-hexane extract; EAER: ethyl acetate extract; MER: methanol extract; TPC: Total phenolic content.

Table 2: Antibacterial activity of *Vitex trifolia* leaf extract by sequential extraction.

Sample	Concentration (%)	Inhibition zone diameter (mm)	
		S. aureus	***E. coli***
HE	5	6.41 ± 0.26[a]	6.08 ± 0.01[a]
	2	6.51 ± 0.29[a]	6.30 ± 0.09[a]
	50	6.60 ± 0.16[a]	6.69 ± 0.49[a]
EAER	5	7.55 ± 0.14[b]	6.93 ± 0.55[a]
	25	7.28 ± 0.17[b]	7.76 ± 0.35[b]
	50	7.69 ± 0.29[b]	7.46 ± 0.08[b]
MER	5	7.93 ± 0.13[b]	6.77 ± 0.43[a]
	25	8.21 ± 0.24[b,c]	8.83 ± 0.17[d]
	50	9.44 ± 0.47[d]	9.39 ± 0.83[d]
Chloramphenicol	-	23.43 ± 0.41[e]	34.30 ± 0.39[e]

HE: n-hexane extract; EAER: ethyl acetate extract; MER: methanol extract; Different letters (a, b, c, d, and e) represent significant differences based on Tukey's test at a p-value < 0.05; Data are presented as mean ± standard deviation (SD); n = 3.

A lower IC_{50} value (50% inhibitory concentration) indicates that the sample has a stronger and greater ability to act as an antioxidant against free radicals. Based on the IC_{50} value of the extract, the antioxidant potential can be categorized as follows: extracts with $IC_{50} < 50$ µg/mL are considered very strong antioxidants; extracts with IC_{50} values between 50 and 100 µg/mL are classified as strong antioxidants; extracts with IC_{50} values between 101-150 µg/mL indicate moderate antioxidant activity; and extracts with $IC_{50} > 150$ µg/mL are classified as weak antioxidants.[27]As a result, the antioxidant activity of the hexane extract is also low, as evidenced by the high IC_{50} value, indicating that this extract has a weak free radical scavenging ability. In the second extraction step, the use of ethyl acetate as a semipolar solvent increased the extraction of phenolic compounds and flavonoids. This is evident from the increase in flavonoid, total phenolic, and tannin contents.[28] Therefore, the antioxidant activity of the ethyl acetate extract was higher than that of the hexane extract, with an IC_{50} value of 188.80±2.33 ppm. This finding indicates that the antioxidant active compounds in *V. trifolia* leaves are mostly semi-polar in nature and are more easily extracted with ethyl acetate than with hexane. The last stage of extraction using methanol, which is the most polar solvent in this step, produced a higher level of flavonoids, total phenolics, and tannins compared to the previous two steps. This observation shows that the main bioactive compounds in *V. trifolia* leaves are highly polar and have high solubility in methanol. In this case, the methanol extract had the lowest IC_{50} value (51.61 ± 0.16 ppm), which indicated that its antioxidant activity was the strongest compared to the hexane and ethyl acetate extracts.

Antibacterial activity

The results of sequential extraction with hexane, ethyl acetate, and methanol solvents showed that the more polar the solvent used, the higher the antibacterial activity of *V. trifolia* leaf extract against *S. aureus* and *E. coli* (Table 2). Tukey's test results showed that there were significant differences ($p \leq 0.05$) between the inhibition of HE, EAER, and MER against *S. aureus*. This significant difference ($p \leq 0.05$) in inhibition was also shown in *E. coli*, especially at concentrations of 25%and 50%, while the 5% concentration was not significantly different ($p \geq 0.05$). At higher concentrations, the content of secondary metabolites, such as flavonoids, tannins, and phenolics, which exhibit antibacterial activity, also increases, leading to a significant enhancement in bacterial growth inhibition.[29] Meanwhile, at a concentration of 5%, the content of active compounds may still be too low to provide a real inhibitory effect, so the difference in inhibition is not significant compared to the control or between extracts.[30,31]

Table 3: LC-MS-based compound identification in the hexane fraction from sequential maceration of *Vitex trifolia* leaves

RT (min)	Area (10^6)	Fit Conf (%)	MS (m/z) $[M+H]^+$	CMW	Molecular formula	Annotated compound
8.73	0.15	96.75	361.09	360.09	$C_{18}H_{16}O_8$	Chrysosplenol C
9.32	0.4	69.42	181.12	180.12	$C_{11}H_{16}O_2$	2-ethyl-3,5-dihydroxy-4-methylbenzaldehyde
9.8	2.99	97.2	375.10	374.10	$C_{19}H_{18}O_8$	Casticin
10.81	0.05	51.87	317.21	316.21	$C_{20}H_{28}O_3$	(1r,4ar,5s,8ar)-5-[2-(furan-3-yl)ethyl]-1,4a-dimethyl-6-methylidene-hexahydro-2h-naphthalene-1-carboxylic acid
11.52	0.24	79.08	301.21	300.21	$C_{20}H_{28}O_2$	2-hydroxy-7-isopropyl-1,1,4a-trimethyl-3,4,10,10a-tetrahydro-2h-phenanthren-9-one
12.18	0.06	92.37	349.23	348.23	$C_{21}H_{32}O_4$	2,5,5,8a-tetramethyl-5'-(2-oxoethylidene)-hexahydro-2h-spiro[naphthalene-1,2'-oxolan]-6-yl acetate
12.75	0.05	62.04	287.23	286.23	$C_{20}H_{30}O$	Ferruginol
13.14	-	-	361.23	360.23	$C_{22}H_{32}O_4$	(2s,3r,4r,4as)-4-[2-(furan-3-yl)ethyl]-4-hydroxy-3,4a,8,8-tetramethyl-3,5,6,7-tetrahydro-2h-naphthalen-2-yl acetate

LC-MS: liquid chromatography-mass spectrometry; RT: Retention time; Fit conf: Fit confidence; MS: Mass spectra; CMW: Calibration mass window.

Table 4: LC-MS-based identification of compounds in the ethyl acetate extract from sequential maceration of *Vitex trifolia* leaves

RT(min)	Area (%)	Fit Conf (%)	MS (m/z) $[M+H]^+$	CMW	Molecular formula	Annotated compound
6.22	1.53	97.12	197.11	196.11	$C_{11}H_{16}O_3$	Loliolide
6.99	0.17	91.93	347.07	346.07	$C_{17}H_{14}O_8$	Syringetin
8.79	6.68	94.10	361.09	360.09	$C_{18}H_{16}O_8$	Chrysosplenol C
9.49	0.55	99.82	331.08	330.08	$C_{17}H_{14}O_7$	3,3'-di-o-methylquercetin
9.63	0.19	90.40	345.09	344.09	$C_{18}H_{16}O_7$	Eupatorin
9.82	41.39	99.74	375.10	374.10	$C_{19}H_{18}O_8$	Casticin
11.5	1.71	99.48	301.21	300.21	$C_{20}H_{28}O_2$	2-hydroxy-7-isopropyl-1,1,4a-trimethyl-

						3,4,10,10a-tetrahydro-2h-phenanthren-9-one
12.53	0.31	90.74	411.36	410.36	$C_{29}H_{46}O$	4,22-Stigmastadiene-3-one
16.11	1.22	99.25	413.37	412.37	$C_{29}H_{48}O$	Stigmasterol

LC-MS: liquid chromatography-mass spectrometry; RT: Retention time; Fit conf: Fit confidence; MS: Mass spectra; CMW: Calibration mass windows.

Table 5: LC-MS-based compound identification in the methanol extract from sequential maceration of *Vitex trifolia* leaves.

RT (min)	**Area (10^6)**	**Fit Conf (%)**	**MS (m/z) $[M+H]^+$**	**CMW**	**Molecular formula**	**Annotated compound**
1.83	0.41	92.45	185.0814	184.0814	$C_9H_{12}O_4$	(4ar,5r)-5-hydroxy-7-(hydroxymethyl)-3h,4h,4ah,5h,6h-cyclopenta[c]pyran-1-one
3.61	0.22	97.09	394.1709	393.1709	$C_{16}H_{27}NO_{10}$	6'-Apiosyllotaustralin
4.55	0.55	99.80	449.1086	448.1086	$C_{21}H_{20}O_{11}$	Orientin
4.88	0.55	97.96	433.1133	432.1133	$C_{21}H_{20}O_{10}$	Vitexin
5.3	0.07	97.16	499.1233	499.1240	$C_{25}H_{22}O_{11}$	3,4-Dicaffeoyl-1,5-quinolactone
5.8	0.21	98.91	463.0880	462.0880	$C_{21}H_{18}O_{12}$	Luteolin 7-glucuronide
8.16	0.05	90.11	301.2189	300.2189	$C_{20}H_{28}O_2$	2-hydroxy-7-isopropyl-1,1,4a-trimethyl-3,4,10,10a-tetrahydro-2h-phenanthren-9-one
8.61	0.51	99.91	361.0931	360.0931	$C_{18}H_{16}O_8$	Chrysosplenol c
9.67	3.6	96.31	375.1062	374.1062	$C_{19}H_{18}O_8$	Casticin
11.48	0.19	99.84	301.2173	300.2173	$C_{20}H_{28}O_2$	(2s,4as,10ar)-2-hydroxy-7-isopropyl-1,1,4a-trimethyl-3,4,10,10a-tetrahydro-2h-phenanthren-9-one
13.32	0.15	97.58	269.2275	268.2275	$C_{20}H_{29}$	Anhydroretinol

LC-MS: liquid chromatography-mass spectrometry; RT: Retention time; Fit conf: Fit confidence; MS: Mass spectra; CMW: Calibration mass window.

The n-hexane extract exhibited the weakest antibacterial activity, with minimal zones of inhibition against *S. aureus* and *E. coli*. Antibacterial activity increased in EAER, which demonstrated a larger zone of inhibition. This suggests that antibacterial compounds more soluble in semi-polar solvents exhibited greater effectiveness compared to those soluble in hexane.[32] Methanol extract demonstrated the most potent antibacterial activity against *S. aureus* and *E. coli*. This finding indicated that the main antibacterial compounds in *V. trifolia* leaves are polar and more effectively extracted using methanol. Overall, multistage extraction allowed the separation of antibacterial compounds based on their polarity, where non-polar compounds in hexane had weaker activity, while semi-polar compounds in ethyl acetate were more active. The most potent antibacterial compounds were found in the methanol extract; thus, this solvent is the best to obtain optimal antibacterial activity from *V. trifolia* leaves. Methanol is widely reported as a solvent for extracts that provide strong antibacterial activity.[33]

Metabolite profiling of Vitex trifolia leaf extracts

The results of LC-MS analysis on *V. trifolia* leaf extracts obtained through sequential extraction showed that the types of compounds detected were different at each solubilization step, representing the effectiveness of the solvent in extracting compounds with certain polarities (Tables 3-5). Liquid chromatography–mass spectrometry profiling of secondary metabolites revealed both differences and similarities in the compounds present in each solvent from the sequential extraction process. The compounds that still emerged in each solvent are chrysosplenol C, casticin, and 2-hydroxy-7-isopropyl-1,1,4a-trimethyl-3,4,10,10a-tetrahydro-2h-phenanthren-9-one. This finding indicates that these three compounds cannot be separated by sequential extraction or remain bound to each solvent. The n-hexane extract contains non-polar compounds, which tend to be lipophilic. At the extraction step with ethyl acetate, more diverse compounds were detected, including semi-polar flavonoids, such as eupatorine.[34] In addition, phytosterol compounds, such as stigmasterol were also observed, suggesting that ethyl acetate is more effective in extracting semi-polar compounds compared to hexane. Meanwhile, MER contains more polar compounds, such as orientin, vitexin, and luteolin 7-glucuronide, as well as several phenolic acid derivatives and flavonoid glycosides.[35] The presence of these compounds suggests that the main bioactive components in *V. trifolia* leaves are more soluble in polar solvents, such as methanol. This solubility likely accounts for the higher antioxidant and antibacterial activities observed in the methanol extract compared to the other extracts. Therefore, sequential extraction made it possible to isolate compounds with different characteristics at each step, which can be used for various pharmaceutical and therapeutic applications.

Target prediction and docking analysis

According to the Probability of Activity (Pa) prediction data using the Way2Drug server on the compounds contained in MER, several important points can be made related to the potential of these compounds as antibacterial and antioxidant agents (Figure 1). Compounds with Pa values above 0.5 indicate significant potential,[36] as observed from the tendency to cluster at the top of the graph with higher activity. However, not all compounds with antioxidant Pa above 0.5 were effective as antibacterials. Some compounds showed a high antioxidant Pa but a low antibacterial Pa. This indicates that effectiveness as an antioxidant does not always correlate with antibacterial activity.[37]

Table 6: Docking of ligand molecules with 3HUN receptor

Compound/ native ligand	**Binding energy (kcal/mol)**	**RMSD (refine unit)**	**H-bonding distance (Å)**	**Interacting residues**
Vitexin	-8.0	1.32	Ser262(4.07), Ser75(3.58), Ser75(2.75), Ser116(2.97)	Van der Waals: Glu297, Ser139, Gly261, Lys78, Ala74, Ser263, Leu115, Glu297 Carbon-Hydrogen Bond: Glu114 Pi-Pi Stacked: Phe241
Orientin	-8.3	1.903	Arg186(4.76), Ser75(4.72), Tyr291(5.01)	Van der Waals: Asp264, Glu183, Ala74, Ala182, Gly181, Ser263, Leu115, Asn141, Ser262, Aer139, Tyr268, Asn117, Phe241, Lys292 Unfavorable Acceptor-Acceptor: Ser116 Pi-Donor Hydrogen Bond: Tyr291
Luteolin 7-glucuronide	-8.6	1.835	Arg300(6.06), Arg186(4.51)	Van der Waals: Tyr268, Glu297, Ala182, Ser263, Thr265, Glu114, Ser116, Ser139, Ser262, Gly261, Ser75, Lys259 Unfavorable Donor-Donor: Asn117, Tyr291, Asp264, Thr260 Pi-Pi Stacked & Amide-Pi Stacked: Phe241, Leu115
Ampicilloic acid (native ligand)	-7.7	1.732	Ser262(2.92)	Van der Waals: Lys78, Ser75, Tyr291, Glu114, Ser116, Ser139, Asn117, Leu115, Gly261, Thr260, Glu297, Asn 141 Pi-Pi Stacked: Phe241
Chloramphenicol (drug)	-5.8	1.557	Glu114(2.97), Arg186(2.66), Glu183(2.12), Ser75(2.92), Ser262 (3.07)	Van der Waals: Phe241, Ser116, Asn141, Ala74, Asn72, Gly181, Ser263, Tyr291, Gly261, Thr260, Ser139 Carbon-Hydrogen Bond: Ser262 Pi-Alkyl: Leu115, Ala182

RMSD: root mean square deviation

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Table 7: Docking of ligand molecules with 1G2A receptor

Compound/ native ligand	**Binding energy (kcal/mol)**	**RMSD (refine unit)**	**H-bonding distance (Å)**	**Interacting residues**
Vitexin	-8.0	1.484	Gly45(3.83), Cys129(4.01, Glu42(6.16),	Van der Waals: Glu87, Leu125, Ile128, Glu88, Glu133, Leu91, Cys90, Gly43, Arg97, Gly89 Pi-Alkyl: Ile86 Pi-sigma: Ile44 Pi-cation:His132
Orientin	-7.8	1.431	Arg97(4.91, 6.19) Cys129(4.03), Glu42(6.66)	Van der Waals: Glu87, Ile86, Glu88, Leu125, Ile128, His132, Glu133, Cys90, Gly45, Leu91, Gly43, Gly89 Pi-sigma: Ile44 Pi-Alkyl: Ile44, Cys129
Luteolin 7-glucuronide	-9.5	1.969	Arg97(2.54, 2.07), Glu95(2.42, 2.62), Gly45(2.07), His132(2.59, Pro94(3.35)	Van der Waals: Cys129, Glu133, Gly43, Gly89, Cys90, Leu161, Leu46, Gln50, Glu41, His7 Pi-sigma: Leu91 Pi-Alkyl: Ile44, Leu91 Pi-Pi stacked: His132
Actinonin (native ligand)	-6.3	1.494	Ile44(3.35, Gly45(2.72), Glu133(5.76, His132(5.42, Leu9(4.62)	Van der Waals: Cys129, Glu88, Arg97, Glu41, Glu95, Gly43, Glu87, Leu46, Gln50 Carbon-Hydrogen Bond: Cys90, Gly89 Unfavourable Donor-Donor & Acceptor-Acceptor: His136, Glu42 Pi-Alkyl: Ile128, Leu125, Ile86
Chloramphenicol (drug)	-5.6	1.325	Gly89(2.13, 2.60), Gln50(1.87), His136(2.41) His132(2.21, Ile44(3.18)	Van der Waals: Cys129, Glu88, Ile128, Ile86, Leu125, Glu87, Gly43, Glu42, Cys90, Leu46, Gly45, Glu133, Arg97 Pi-Alkyl:Leu91

RMSD: root mean square deviation

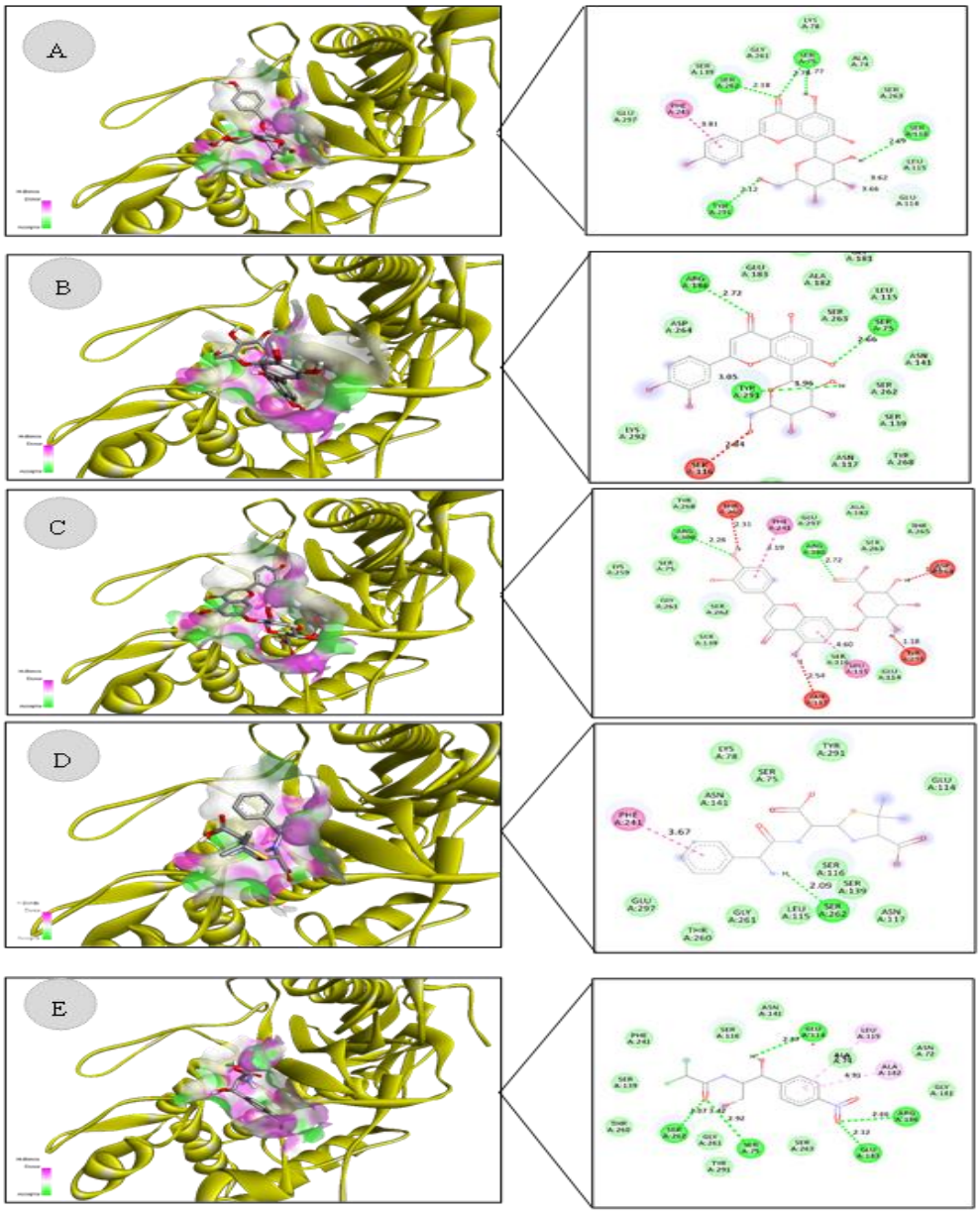

Figure 2: Three-dimensional and two-dimensional visualization of ligand interaction with 3HUN receptor.A: Vitexin; B: Orientin; C: Luteolin 7-glucuronide; D: Ampicilloic acid; E: Chloramphenicol

These results provide a preliminary overview of the compounds' potential, but further studies are needed to confirm their effectiveness. Future research should include both *in vitro* and *in vivo* studies to evaluate the activity of these compounds under controlled laboratory conditions and within complex biological systems. *In silico* approaches, such as molecular docking, can be employed to further understand the potential of these compounds as antibacterials. Therefore, molecular docking was performed on compounds that have Pa > 5, such as orientin, vitexin, and luteolin 7-glucuronide, which were then compared with the drug chloramphenicol and the native ligand actinonin at the 3HUN and 1G2A receptors (Tables 6-7 and Figures 2-3).

Table 6 and Figure 2 of the molecular docking results show that the compounds from *V. trifolia* demonstrated a stronger affinity to the target protein compared to the native ligand ampicilloic acid and the standard drug chloramphenicol with the 3HUN receptor. This is shown by the more negative binding energy values, indicating a more stable complex. Luteolin 7-glucuronide exhibited the lowest binding energy (-8.6 kcal/mol), followed by orientin (-8.3 kcal/mol) and vitexin (-8.0 kcal/mol). For comparison, ampicilloic acid exhibited a binding energy of −7.7 kcal/mol, whereas chloramphenicol demonstrated the weakest affinity at −5.8 kcal/mol. Molecular interaction analysis also showed that *Vitex* compounds formed more interactions with the target residues compared to ampicilloic acid and chloramphenicol. Vitexin interacts through hydrogen bonds with Ser262, Ser75, and Ser116 residues and forms Van der Waals interactions with various other residues. Orientin and luteolin 7-glucuronide also exhibited similar interactions, with the addition of Pi-Pi stacking interactions that may increase the stability of the complex. Orientin is also shown to have antibacterial activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa in vitro*.[38] Thus, these results indicated that compounds from *V. trifolia*, particularly luteolin 7-glucuronide, demonstrated greater potential as antibacterial candidates. Luteolin exerts antibacterial activity against *S. aureus* by inhibiting nucleic acid and protein synthesis, disrupting the bacterial cell membrane, altering cell morphology, and preventing biofilm formation.[39,40]

Table 7 and Figure 3 present the molecular docking results between compounds from *V. trifolia* and the 1G2A receptor of *E. coli*.

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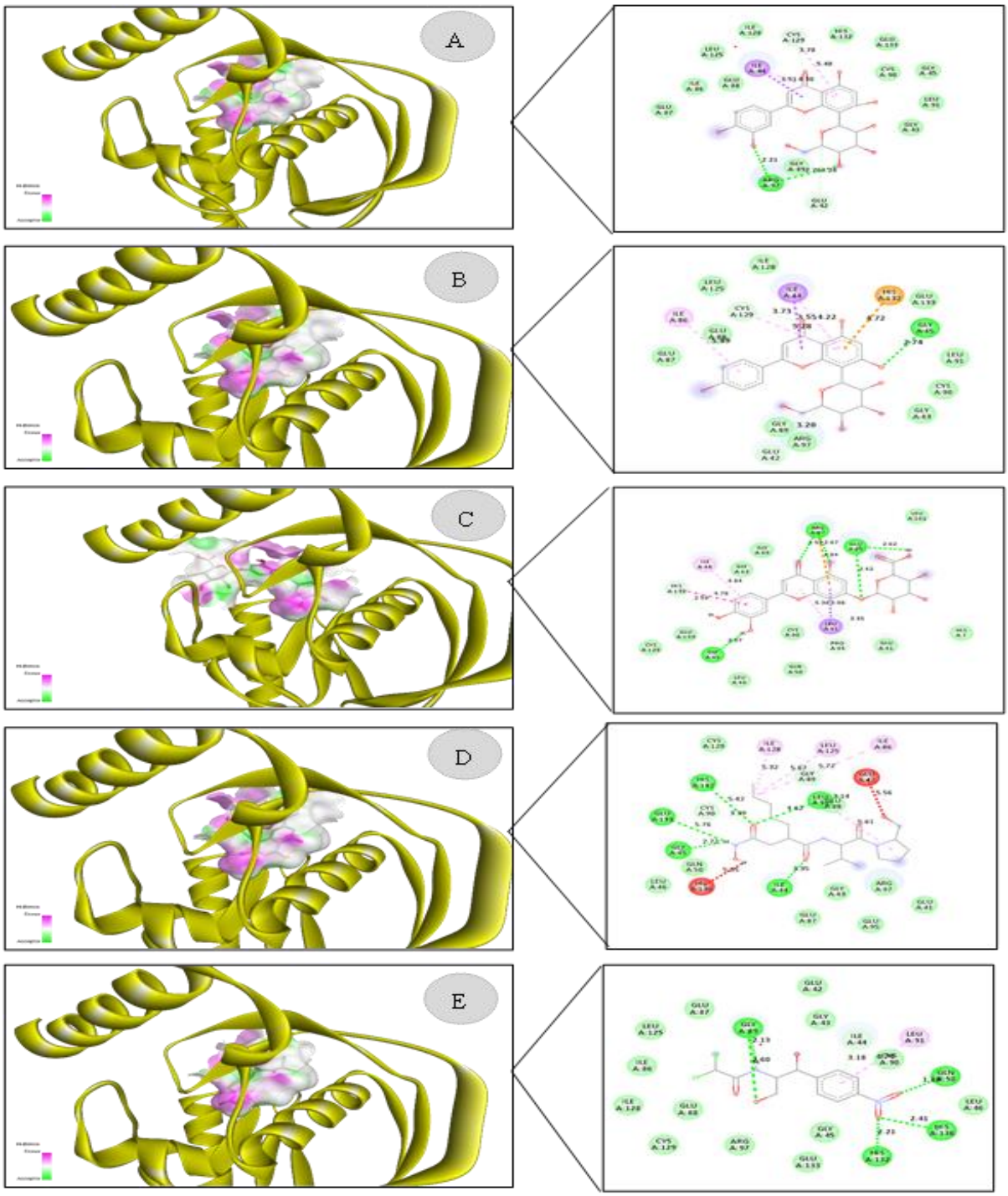

Figure 3: Three-dimensional and two-dimensional visualisation of ligand interaction with 1G2A receptor.A: Vitexin; B: Orientin; C: Luteolin 7-glucuronide; D: Actinonin; E: Chloramphenicol

The results revealed variations in binding energy, hydrogen bonding, and other non-covalent interactions compared to those of the native ligand (actinonin) and the standard drug (chloramphenicol). Luteolin 7-glucuronide showed the lowest binding energy (-9.5 kcal/mol), indicating higher affinity to the receptor compared to actinonin (-6.3 kcal/mol) and chloramphenicol (-5.6 kcal/mol). The compound forms several strong hydrogen bonds with key residues such as Arg97 (2.07-2.54 Å), Glu95 (2.42-2.62 Å), Gly45 (2.07 Å), and His132 (2.59 Å), which show important contributions to the stability of the complex. Additionally, the π–π stacking interaction with His132 may enhance the binding affinity of this compound to the receptor. Vitexin and orientin have binding energies of -8.0 kcal/mol and -7.8 kcal/mol, respectively, which compare favourably with actinonin and chloramphenicol. Both exhibited hydrogen interactions with important residues such as Gly45, Cys129, and Glu42, and interacted via Van der Waals forces with several other residues. However, compared to luteolin 7-glucuronide, the number of hydrogen bonds and their strength were slightly weaker. Docking results revealed that luteolin 7-glucuronide, actinonin, and chloramphenicol shared common hydrogen-bond interactions with the 1G2A receptor, specifically involving residues Gly45 and His132. Luteolin 7-glucuronide binds to Gly45 at a distance of 2.07 Å, shorter than actinonin, which has a distance of 2.72 Å, indicating a stronger interaction. Meanwhile, chloramphenicol did not interact with Gly45.

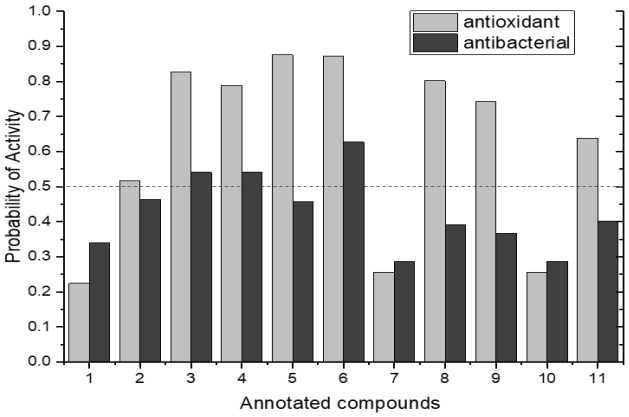

Figure 4: PASS prediction scores for compound 1–11 (MER) as potential antibacterial agent.Pa value greater than 0.5 indicates a strong likelihood of exhibiting *in vitro* activity; MER: methanol.

At residue His132, luteolin 7-glucuronide has a bond distance of 2.59 Å, shorter than actinonin (5.42 Å) but longer than chloramphenicol (2.21 Å), indicating variation in the strength of hydrogen interactions. This difference in hydrogen bond length contributes to the stability of the ligand-receptor complex.[41] The shorter hydrogen bonds, such as in luteolin 7-glucuronide with Gly45 and chloramphenicol with His132, are generally more stable and may increase the affinity of the compound to the receptor. Despite the similarity in interaction residues, each compound exhibits variations in binding strength, which may influence its efficacy as an antibacterial agent.

The docking results showed that the compounds in the *V. trifolia* extract exhibited high affinity to the target protein, but their antibacterial activity in extract form is still lower than that of the standard drug chloramphenicol (Table 2). A key factor is the lower concentration of active compounds in the extract, as it consists of a mixture of various compounds at varying concentrations, whereas synthetic drugs contain pure compounds at optimal doses. Although the compounds in the extract demonstrated antibacterial potential, isolation and optimization of the active compounds are necessary to enhance their effectiveness and develop more potent antibacterial candidates.

Conclusion

Sequential extraction yielded varying results for flavonoids, total phenols, tannins, as well as antioxidant (IC_{50}) and antibacterial activities of *V. trifolia* leaves. The final extraction step using MER demonstrated superior antioxidant and antibacterial activity compared to HE and EAER extracts. Secondary metabolite profiling revealed both unique and common compounds in each extract obtained through the sequential maceration method. Molecular docking suggests that compounds present in the MER extract, such as vitexin, orientin, and luteolin 7-glucuronide, may serve as potential antibacterial agents. Therefore, further research is required to isolate these compounds from *V. trifolia* leaves.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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