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Molecular Insight, Antioxidant, and Cytotoxicity Evaluation of *Vitex Trifolia* Leaves against EGFR L858R/T790M and Wild Type in Lung Carcinoma

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This study investigates the chemical properties of *Vitex trifolia* and its antioxidant and anticancer effects against the non-small cell lung cancer (NSCLC). The chemical constituents of extract were analyzed using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS). Antioxidant activity was evaluated in vitro, while cytotoxicity was determined with a WST-8 assay. Molecular docking simulations were performed with AutoDock Vina. GC-MS identified 144 compounds, and LC-HRMS revealed 26 flavonoids. Antioxidant capacity ranged from 5.39 ± 0.26 to 102.18 ± 0.18 mg ascorbic acid equivalent and 7.99 ± 0.79 to 113.11 ± 0.19 mg Trolox equivalent per g extract. The extract exhibited affinity for NSCLC with the EGFR L858R/T790M mutation, with an IC_{50} of 4.67×10^{-11} µg/mL compared to rociletinib (1.23×10^{-12} µg/mL). Docking studies indicated that artemetin, casticin, and vitexilactone may interact similarly to rociletinib. This study represents the first integrated chemical, biological, and in silico evaluation of *V. trifolia* leaf extract, which successfully identified flavonoids with dual antioxidant and anticancer activity against NSCLC. These findings suggest the potential clinical value of *V. trifolia* bioactive compounds as alternative therapeutic agents for NSCLC, particularly in EGFR mutant cases that are resistant to standard treatments.

1. Introduction

Cancer is a multifaceted disease marked by the unregulated proliferation of cells, often driven by alterations in multiple signaling pathways across various tumor types. Notably, cancer cells possess the ability to metastasize to distant organs even after the complete excision of the primary tumor.[1] Despite significant advancements in cancer research, the prevalence and impact of cancer continue to rise.[2,3] Although treatment modalities—such as chemotherapy, radiotherapy, and surgery—remain effective, they frequently result in adverse effects on normal cells, underscoring the need for alternative, targeted treatment strategies. A promising avenue involves the exploration of natural compounds, many of which have demonstrated potential as effective chemotherapy agents,[4] with elucidated mechanisms of action.[5] However, numerous plant species that have not been systematically investigated[6–8] still present opportunities for drug discovery.

In the case of *Vitex trifolia*, over 180 metabolites have been identified from various plant parts, including terpenoids (monoterpenes, sesquiterpenes, diterpenes, triterpenes, and phytosterols), ecdysteroids, flavonoids, lignans, phenylpropanoids, anthraquinones, and fatty acids, along with xanthones derived from endophytic fungi associated with its fruit.[9–11] However, the chemical profile of *V. trifolia* leaf remains inadequately characterized.

Prior research has reported diverse pharmacological properties for *V. trifolia*, including antibacterial, anti-inflammatory,

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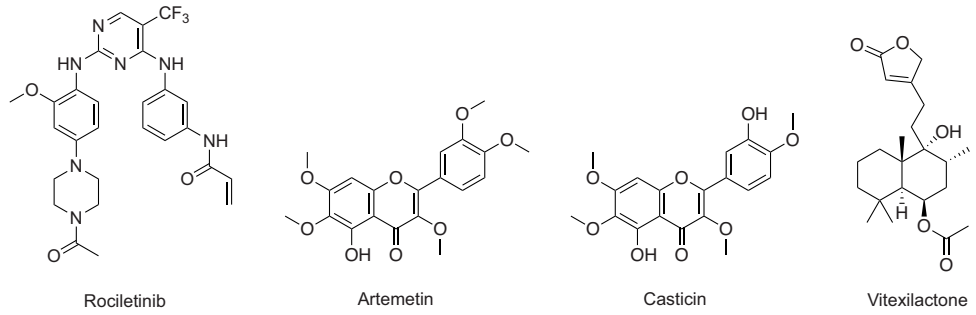

Figure 1. Structure of ligands in the molecular docking simulation.

antioxidant,[12] hepatoprotective, and anticancer activities. For instance, a study demonstrated that at a concentration of 25 µg/mL, *V. trifolia* exhibited significant inhibitory effects on the T47D breast cancer cell line.[13] An in vivo assessment of cytotoxicity utilizing methanol, ethyl acetate, and chloroform extracts employed the brine shrimp bioassay method. Results indicated that the methanolic extract exhibited the highest cytotoxic activity, with a half-lethal concentration (LC_{50}) of 140 mg/mL, followed by ethyl acetate and chloroform at an LC_{50} of 165 and 180 mg/mL, respectively, with potassium dichromate serving as a positive control.[14] Additionally, the methanol extract showed significant cytotoxicity against Hep-G2 cells, with a half inhibitory concentration (IC_{50}) value of 6 µg/mL.[14]

In vitro assay of dichloromethane extracts from *V. trifolia* leaves indicated notable toxicity against various cancer cell lines, including SQC-1 UISO, OVCAR-5, HCT-15 COLADCAR, and KB, with ED_{50} values of 2.2, 2.9, 1, and 1.9 mg/mL, respectively. These findings emphasize the potential of *V. trifolia* leaf extracts as a source of bioactive metabolites with cytotoxic properties.[15] Moreover, *V. trifolia* leaf extract exhibited inhibitory effects on cell proliferation in HCT116 and A549 cell lines.[16] A549 cells are a widely used in vitro model representing alveolar type II pulmonary epithelial cells in lung adenocarcinoma and are frequently employed to assess the general cytotoxic effects of candidate compounds in lung cancer.[17] However, to enhance translational relevance, it is equally important to evaluate cancer cell lines that harbor clinically significant genetic alterations. H1975 cells carry the EGFR L858R and T790M mutations, which are well known to drive resistance to first- and second-generation tyrosine kinase inhibitors. By incorporating both A549 and H1975 models, the evaluation of *V. trifolia* provides a more comprehensive understanding of its therapeutic potential—capturing not only general effects on NSCLC biology but also its activity against EGFR-driven resistant phenotypes of clinical importance.

The present study aims to comprehensively profile the chemical constituents of *V. trifolia* leaves utilizing gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-high resolution mass spectrometry (LC-HRMS). The ethanolic extract of *V. trifolia* leaves provided by ultrasonic-assisted extraction (UAE) will be evaluated for its antioxidant and anticancer potential targeting epidermal growth factor receptor (EGFR) L858R/T790M mutations in non-small cell lung cancer (NSCLC) through in vitro cytotoxicity assays. Molecular docking simulation supporting the molecular interaction of artemetin, casticin, and vitexilactone (Figure 1) found in the extract toward EGFR L858R/T790M and wild type.

2. Results

2.1. Ultrasound-Assisted Extraction

The UAE method is a novel strategy for extracting secondary metabolites because it is rapid and requires less solvent, making it a green extraction method.[18,19] The findings of *V. trifolia* leaf extraction showed a yield of 7% with an hour of extraction time. Although the yield of extract was not particularly high, the active chemical components were recovered effectively owing to ultrasonics' ability to break down leaf cell walls optimally.

2.2. GC-MS Profiling

The chemical profile of the extract was examined using a Shimadzu GC-MS QP-2010, which included an autosampler (AOC-20s) and an autoinjector (AOC-20i) with an SH-Rxi-5MS Sill column (30 m, 0.25 mm, and 0.25 µm). The detailed procedure is provided in the Supporting Information. The GC-MS analysis revealed 144 chemical components, as indicated in Table S1 and Figure S1. The chemical compounds with retention time, molecular formula, molecular weight, and area included in Table 1 are dominant compounds with an area of at least 1%.

The dominant chemical components from the extract were found to be tetrahydroedulan, which has the highest area, almost 12%. The second highest chemical component is stigmast-5-en-3-ol (3.beta., 24s), with an area percentage of 8.40%, followed by caryophyllene, 8.36%.

2.3. LC-HRMS Profiling

The analysis utilized a Vanquish HPLC system equipped with a binary pump and a high-resolution Q-Exactive Orbitrap mass spectrometer (Thermo Scientific, Rockford, IL, USA). The separation was achieved on a Thermo Scientific Acclaim VANQUISH C18 column (150 mm × 2.1 mm ID × 2.2 µm). The mobile phases comprised LC-MS-grade water with 0.1% formic acid (A)

Table 1. The chemical compound of *V. trifolia* leaves extract by GC-MS.

No	Retention time (min)	Compound	Molecular formula	Molecular weight	Peak area (%)
1	8.123	Eucalyptol	$C_{10}H_{18}O$	154.25	3.41
2	13.351	3-cyclohexene-1-methanol,. alpha.,.alpha.,4-trimethyl-, acetate	$C_{12}H_{20}O_2$	196.29	3.33
3	14.375	Caryophyllene	$C_{15}H_{24}$	204.35	8.36
4	20.539	17-Norkaur-15-ene, 13-methyl-, (8.beta.,13.beta.)-	$C_{20}H_{32}$	272.4681	2.35
5	21.807	Naphthalene, decahydro-1,1,4a-trimethyl-6-methylene-5-(3-methyl-2,4-pentadienyl)-, [4a	$C_{20}H_{32}$	272.47	2.18
6	22.221	1-Naphthalenepropanol, alpha.-ethenyldecahydro-2-hydroxy-.alpha.,2,5,5,8a-pentamethyl-	$C_{20}H_{34}O$	290.5	3.32
7	22.583	1-(4-isopropylphenyl)-2-methylpropyl acetate	$C_{11}H_{14}O_2$	178.23	5.08
8	24.749	1-Naphthalenepropanol, alpha.-ethenyldecahydro-2-hydroxy-.alpha.,2,5,5,8a-pentamethyl-	$C_{20}H_{36}O_2$	308.5	1.35
9	24.972	Androsta-4,16-dien-3-one	$C_{19}H_{26}O$	270.4	1.85
10	25.595	Kolavelool	$C_{20}H_{34}O$	290.5	2.30
11	28.087	Thunbergol	$C_{20}H_{34}O$	290.5	1.63
12	28.791	2,6,10,14,18,22-tetracosahexaene, 2,6,10,15,19,23-hexamethyl-	$C_{30}H_{50}$	410.73	1.52
13	29.430	(1*R*,2*R*,4a*S*,8a*S*)-1-(2-(Furan-3-yl)ethyl)-2,5,5,8a-tetramethyldecahydronaphthalen-1-ol	$C_{30}H_{52}O$	428.7	2.07
14	29.753	1,3-Propanedione, 1,3-bis(tricyclo[3.3.1.1(3,7)]dec-1-yl)-	$C_{23}H_{32}O_2$	340.5	4.73
15	29.929	Gamma.-Sitosterol	$C_{29}H_{50}O$	414.7067	6.98
16	30.050	Tetrahydroedulan	C_4H_8O	72.11	11.57
17	30.387	Stigmast-5-en-3-ol, (3.beta.,24*S*)-	$C_{29}H_{50}O$	414.71	8.40
18	34.335	Rotundifuran	$C_{22}H_{34}O_4$	362.5	1.33
19	34.724	(11*E*,13*Z*)-11813-labdadien-8-ol	$C_{20}H_{34}O$	290.48	2.02
20	36.700	1,4-Methanoazulen-9-ol, decahydro-1,5,5,8a-tetramethyl-, [1*R*-(1.alpha.,3a.beta.,4.alpha.	$C_{15}H_{26}O$	222.37	1.85
21	36.766	Lupeol	$C_{30}H_{50}O$	426.7	1.65
22	39.290	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	$C_{33}H_{54}O_2$	482.8	1.40
23	41.151	Betulinaldehyde	$C_{30}H_{48}O_2$	440.7	1.16

and LC-MS-grade methanol with 0.1% formic acid (B). Complete procedures are presented in the Supporting Information. In the chemical component analysis performed using LC-HRMS, a variety of potential compounds were identified, with particular attention given to flavonoid compounds. The ethanol extract of V. trifolia leaves indicated the presence of 26 flavonoid compounds, as shown in Table 2. Figure 2 illustrates 15 flavonoid derivatives generated by modifications to the positions of the hydroxy and methoxy substituents. Figure 3 depicts three types of flavonoids associated with glucose. Meanwhile, the total number of flavonoids linked to pyran amounts to eight, as illustrated in Figure 4.

2.4. Antioxidant Capacity

The extract's antioxidant capacity was assessed using a variety of methodologies, including total oxidant and antioxidant capacity (TOAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, ferric-reducing antioxidant power (FRAP), and cupric-reducing antioxidant capacity (CUPRAC), which is critical for providing a more comprehensive and accurate view of a sample's antioxidant potential.

Different ways react differently to certain antioxidants. For example, certain approaches may be more sensitive to antioxidants that work on free radical reactions, whilst others focus on antioxidants' capacity to protect biological components such as lipids or proteins. A more comprehensive antioxidant activity profile can be generated by employing various techniques.[12]

In the antioxidant activity evaluation, ascorbic acid and trolox were used as reference standards in all antioxidant activity test methods. Both reference standards were made into concentration series to produce a linear regression equation, as shown in Figure 5. The linear regression equation of each standard reference is used to calculate the extract's antioxidant capacity. The antioxidant capacity of the extract is presented in Table 3.

2.5. Cytotoxicity Activity

The IC_{50} values of the extract and rociletinib against NSCLC cell lines (H1975 and H441) were evaluated utilizing the WST-8 assay. The cells corresponding to H1975 are characterized by the EGFR L858R/T790M mutations, representing a variant of NSCLC with dual mutations, whereas the cells associated with H441 are of the EGFR wild type. The cells were cultivated in 96-well plates using a medium enriched with 10% FBS and 100 IU/mL of PS. After a 24-h incubation period at 37 °C within a 5% CO_2 environment, the cells attained a density of 2.5×10^3 cells per well. Subsequently, the cells were exposed to a range of concentrations of the extract or rociletinib for a duration of 48 h. Cell viability was assessed employing the Cell Counting

Table 2. The flavonoid compounds of *V. trifolia* leaves extract by LC-HRMS.

No	Retention time (min)	Compound	Molecular formula	$[M + H]^+$ (*m/z*)	Area
1	9.843	5,7-dihydroxy-3′,4′,5′-trimethoxyflavanone	$C_{18}H_{18}O_7$	347.11124	1,920,006,820
2	6.058	Hesperetin	$C_{16}H_{14}O_6$	303.08572	6,467,929.361
3	7.022	2-(4-Hydroxyphenyl)-5,6,7,8-tetramethoxy-2,3-dihydro-4*H*-chromen-4-one	$C_{19}H_{20}O_7$	361.12711	78,582,227.96
4	9.693	2-(3,4-dimethoxyphenyl)-5,7-dihydroxy-6-methoxy-4*H*-chromen-4-one	$C_{18}H_{16}O_7$	345.09589	136,434,085.7
5	9.371	2-(3,4-dihydroxyphenyl)-5-hydroxy-7-methoxychroman-4-one	$C_{16}H_{14}O_6$	303.08585	35,954,460.99
6	7.599	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3,4-dihydro-2*H*-1-benzopyran-4-one	$C_{15}H_{12}O_6$	289.07037	13,026,611.92
7	11.482	2-(2,4-Dimethoxyphenyl)-7-hydroxy-5-methoxy-2,3-dihydro-4*H*-chromen-4-one	$C_{18}H_{18}O_6$	331.11694	40,242,280.6
8	8.637	3,5-Dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydro-4*H*-chromen-4-one	$C_{17}H_{16}O_7$	333.09616	111,003,741.2
9	10.123	Casticin	$C_{19}H_{18}O_8$	375.10608	13,982,842.9
10	8.024	3-Methoxy-5,7,3″,4″-tetrahydroxy-flavone	$C_{16}H_{12}O_7$	317.06522	21,715,479.65
11	9.036	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6-dimethoxy-4*H*-chromen-4-one	$C_{18}H_{16}O_8$	361.09039	1,642,413,522
12	7.656	Kaempferol	$C_{15}H_{10}O_6$	287.05463	103,430,476.2
13	7.583	Quercetin	$C_{15}H_{10}O_7$	303.04950	12,147,964.25
14	9.306	2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-6-methyl-4*H*-chromen-4-one	$C_{16}H_{12}O_7$	317.06482	16,665,250.41
15	5.935	Cynaroside	$C_{21}H_{22}O_{11}$	449.10706	147,051,058.6
16	10.123	Isoharmnetin	$C_{16}H_{12}O_7$	317.06461	13,982,842.9
17	5.109	(1ξ)-1,5-Anhydro-1-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-8-yl]-D-galactitol	$C_{21}H_{20}O_{11}$	449.10703	29,601,878.63
18	4.955	Hesperetin 7-glucoside	$C_{22}H_{24}O_{11}$	465.13837	18,965,534.75
19	6.718	Scutellarin	$C_{21}H_{18}O_{12}$	463.08636	359,971,507.2
20	5.111	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-8-[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]-6-(3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)-4*H*-chromen-4-one (non-preferred name)	$C_{26}H_{28}O_{15}$	581.14862	11,903,904.13
21	5.699	3-(3,4-dihydroxyphenyl)-7-hydroxy-8-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)-4*H*-chromen-4-one	$C_{21}H_{20}O_{10}$	433.11215	173,469,295.4
22	5.604	6-(4,5-dihydroxy-6-(hydroxymethyl)-3-((2,3,4-trihydroxycyclohexyl)oxy)tetrahydro-2*H*-pyran-2-yl)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one	$C_{26}H_{28}O_{14}$	565.15436	9,077,830.435
23	6.580	1,5-Anhydro-1-[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-6-yl]-6-O-[(2*E*)-3-(3,4-dihydroxyphenyl)-2-propenoyl]hexitol	$C_{30}H_{26}O_{14}$	611.13837	60,311,486.81
24	7.905	5-(5-Hydroxy-3,6,7-trimethoxy-4-oxo-4*H*-chromen-2-yl)-2-methoxyphenyl β-D-glucopyranoside	$C_{25}H_{28}O_{13}$	537.15985	12,264,677.56
25	6.711	5,7-dihydroxy-2-(3-hydroxy-4-{[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}phenyl)-4*H*-chromen-4-one	$C_{21}H_{20}O_{11}$	449.10724	10,352,037.99
26	5.474	Sakuranin	$C_{22}H_{24}O_{10}$	449.14319	860,836,652.7

Kit-8 (Dojindo, Kumamoto, Japan), in accordance with the manufacturer's instructions.[3,20] Based on the data in Figure 6, the extract has a lower cytotoxic activity against H1975 than the positive control, rociletinib ($p < 0.01$). Meanwhile, the extract and rociletinib exhibit comparable cytotoxicity against H441.

2.6. Molecular Docking Studies

This study employed the AutoDock Vina package for molecular docking to examine the interaction between the ligand and receptor complex.[21,22] A grid box parameter was defined to constrain the ligand's position and conformation around the receptor site. All other parameters were left at their default settings in AutoDock Vina.[23] The Broyden–Fletcher–Goldfarb–Shanno (BFGS) algorithm was utilized as the optimization parameter. The parameter settings adhered to protocols similar to as delineated in prior studies,[24,25] which is explained in more detail in the Supporting Information. Molecular docking analysis revealed that all the investigated compounds had identical binding energies to the target receptor. However, rociletinib has the lowest binding energy to the 3IKA receptor compared to other chemical compounds. Meanwhile, of the three chemical compounds of *V. trifolia*, vitexilactone showed the lowest binding energy (−7.7 kcal/mol). Table 4 shows vitexilactone's binding affinity to 3IKA, which demonstrates its selectivity to EGFR L858R/T790M and discrimination against EGFR wild type.

Visualization of the binding model between the ligand and the receptor target shown in Figure 7, reveals that artemetin and rociletinib have similar binding models to the amino acid residues on the 3IKA receptor, including hydrogen and hydrophobic bonds (Tables 5 and 6). Meanwhile, casticin and

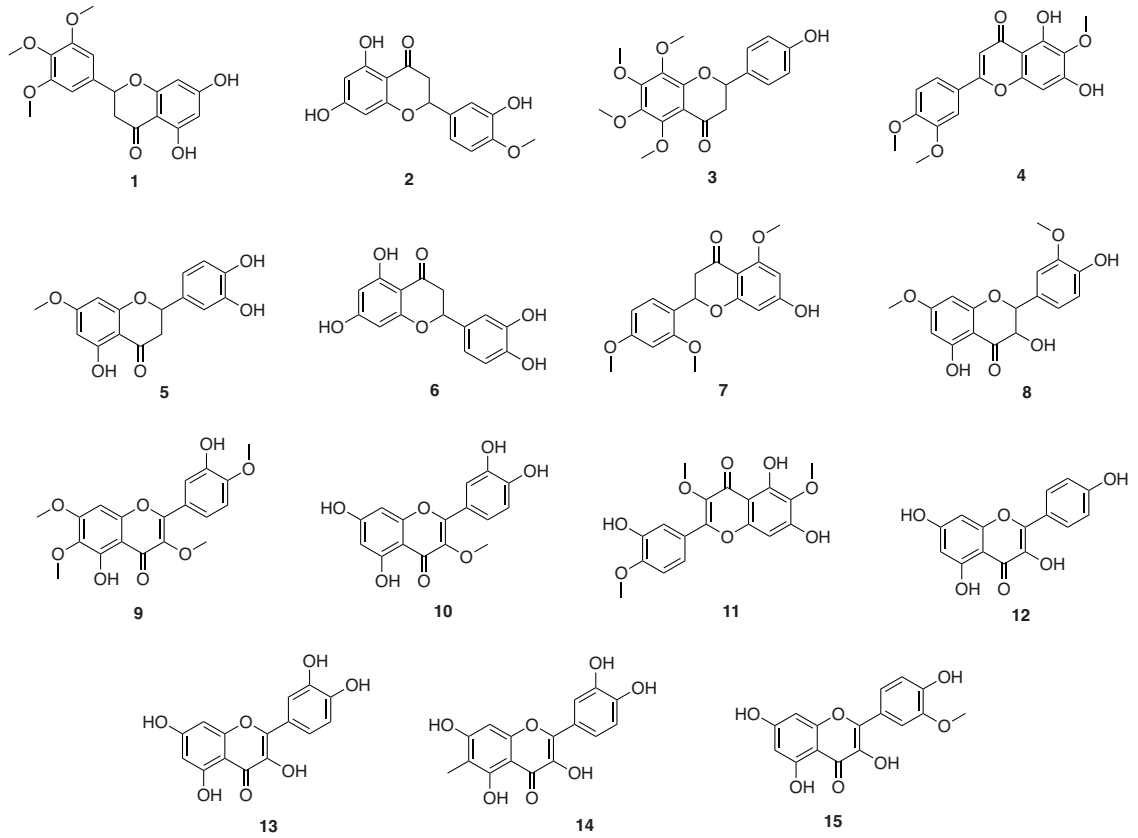

Figure 2. Flavonoids in the extract of *V. trifolia* leaves.

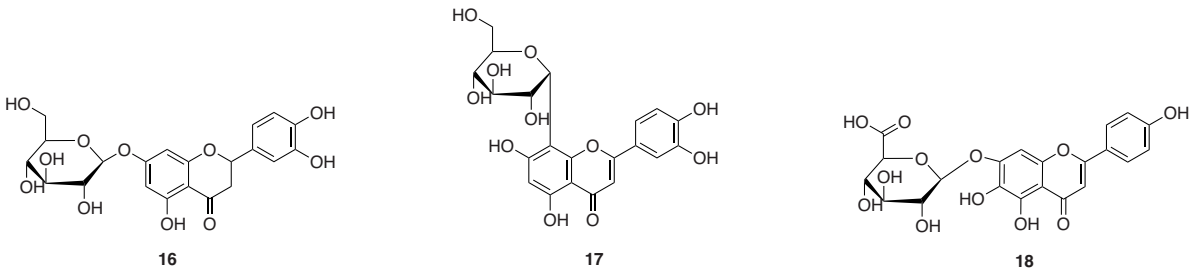

Figure 3. Flavonoids with a glucose substituent in the extract of *V. trifolia* leaves.

vitexilactone reveal no hydrogen bonds with the 3IKA receptor. Although vitexilactone lacks hydrogen bonds with the 3IKA receptor, it is the sole ligand with a salt bridge on the receptor, specifically at residue 745A (LYS), distance 3.9, and the carboxylate group.

Casticin, on the other hand, forms a hydrogen bond with the 6D8E receptor, much like artemetin, as shown in Table 4. In contrast to hydrophobic interactions, all three ligands have distinct interactions with several amino acid residues, displayed in Tables S3 and S4.

3. Discussion

V. trifolia is a shrub or small tree that can reach heights of up to 6 m and is found in various parts of Asia, including China, India, Indonesia, Sri Lanka, Singapore, and Australia.[11] It has a long history of use in traditional medicine, particularly for treating asthma and respiratory ailments.[10,26] Although all parts of the plant, such as the fruit, leaves, roots, flowers, and stems, possess medicinal properties, the fruit is the most researched.[12]

This plant is rich in secondary metabolites, notably terpenoids (especially labdane-type diterpenes) and flavonoids, which are identified as its main bioactive compounds.[27] Pharmacological studies have validated its therapeutic benefits, demonstrating significant antioxidant, anti-inflammatory, hepatoprotective, anticancer, antimicrobial, antiviral, anti-malaria, and antispasmodic activities, as well as some insecticidal effects.[12,28] These results highlight the diverse health benefits of *V. trifolia* and its significance in traditional medicine.

Currently, the extraction method employed to evaluate *V. trifolia* plants is a common extraction method, such as solvent

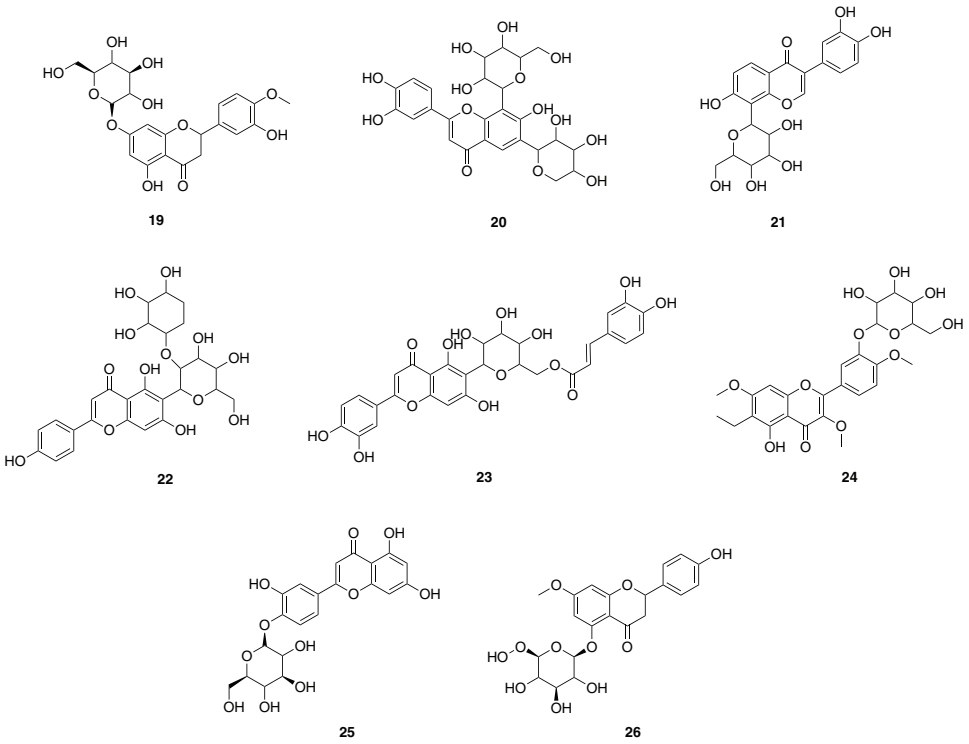

Figure 4. Flavonoids with a pyran substituent in the extract of *V. trifolia* leaves.

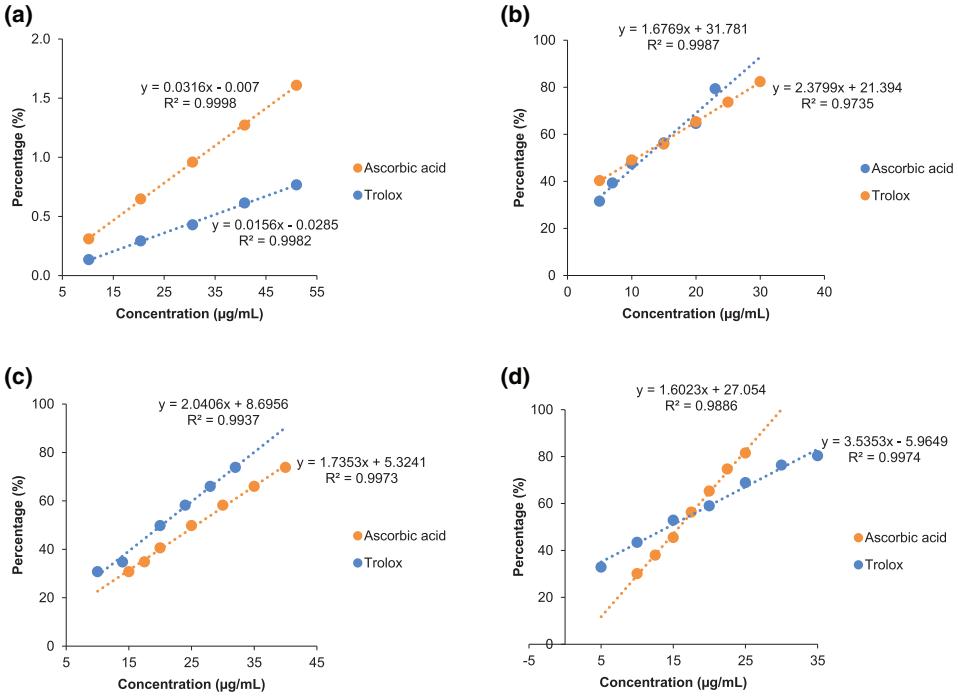

Figure 5. Linear regression curves of concentration versus percent inhibition for standard references across different assays: TOAC a), DPPH radical scavenging b), FRAP c), and CUPRAC d) tests.

extraction. Various existing methods for extracting bioactive chemicals from plants, including solvent extraction,[8] mechanical extrusion, supercritical extraction, and microwave extraction. Compound extraction using these methods has limitations, such as the need for large volumes of solvent in solvent extraction, low yield in mechanical extrusion, costly expenditure in supercritical fluid extraction, and the requirement for an aqueous phase in microwave-assisted extraction.[29] When compared to these procedures, UAE provides advantages such as reduced time and energy requirements, extraction at low temperatures,

Table 3. Antioxidant capacity of *V. trifolia* leaves extract by different methods.

Methods	AEAC (mg/g extract)	Average	TEAC (mg/g extract)	Average
TOAC	4.91	5.35 ± 0.24	8.02	7.99 ± 0.05
	5.41		7.99	
	5.40		7.97	
	5.39		7.91	
	5.65		8.04	
DPPH	103.16	102.13 ± 0.99	113.45	113.83 ± 0.37
	102.72		114.41	
	102.28		113.77	
	102.72		114.09	
	101.39		113.77	
	100.51		113.45	
FRAP	86.24	86.81 ± 0.25	94.83	98.89 ± 3.57
	85.80		94.53	
	85.37		97.48	
	86.67		102.88	
	86.67		96.01	
	87.09		97.77	
CUPRAC	70.44	71.03 ± 1.20	88.59	89.40 ± 0.74
	70.44		89.56	
	72.21		90.05	
	75.72		91.01	
	74.84		89.56	
	72.21		89.08	

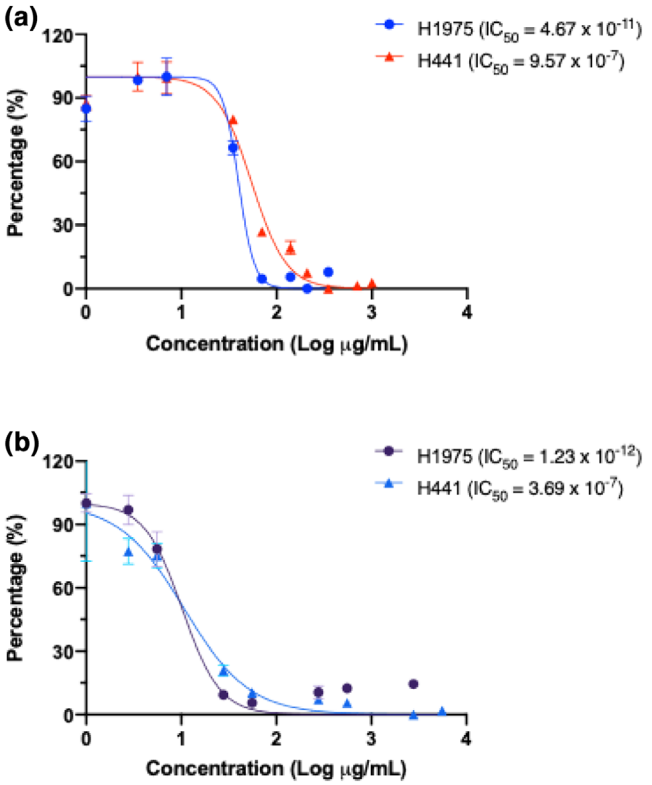

Figure 6. Cytotoxicity activity of *V. trifolia* leaves extract a) and rociletinib b) toward H1975 and H441 cell lines.

Table 4. The binding energy (kcal/mol) of ligands in complex with the receptors.

Compound	3IKA (EGFR L858R/T790M)	6D8E (EGFR Wild type)
Rociletinib	−8.1	–
Artemetin	−6.9	−6.8
Casticin	−7.2	−6.8
Vitexilactone	−7.7	−7.0

and retention of extract quality. UAE extracts bioactive chemicals from plant samples with high-intensity sound waves. Ultrasonic waves disturb plant tissues by physical pressures generated during acoustic cavitation, and they aid in the rapid release of extractable components in solvent by boosting mass transfer.[30]

Tetrahydroedulan is the most abundant chemical component found in *V. trifolia* leaf extract obtained using UAE extraction. This molecule has a fundamental structure in the form of a hydrogenated cyclohexane ring. It is commonly employed as a precursor in the synthesis of chemical compounds in the development of drugs. The substance (3b,24*S*)-Stigmast-5-En-3-Ol is the second most abundant compound in *V. trifolia* leaf extract by GC-MS. It can be found naturally in a variety of beta-sitosterol-rich plants. This chemical has been utilized in a number of cosmetic products as well as to cure skin cancer.[31] Furthermore, this molecule has anti-inflammatory, anticholesterolemia,[32] and antidiabetic.[33]

Caryophyllene, a terpenoid molecule present in numerous essential oils from diverse plants, is the third most important chemical component. Caryophyllene has a unique chemical structure and is classified as a sesquiterpene, containing 15 carbon atoms. Caryophyllene can bind to the CB2 receptor in the human endocannabinoid system. This interaction has anti-inflammatory and pain-relieving properties, making caryophyllene a promising candidate for the treatment of chronic inflammatory disorders, such as arthritis and rheumatoid arthritis. This is one feature that distinguishes caryophyllene from other terpenoids: its effects are similar to cannabinoid substances like cannabidiol, but without the intoxicating properties.[34,35]

Several studies have also revealed that caryophyllene has antioxidant properties, which indicates that it can protect cells from free radical damage.[35] This is consistent with our in vitro antioxidants evaluation in Table 3, as well as prior research demonstrating that an ethanol extract of *V. trifolia* leaves can reduce free radicals using a variety of approaches.[12] Furthermore, caryophyllene has been shown to have anticancer properties in various animal models,[36,37] while further research is needed to confirm this effect in humans.

The LC-HRMS profiling identified numerous chemical compounds, with a particular focus on flavonoids. A total of 26 flavonoid compounds were detected with subclasses, some of which were bound to sugar or pyran groups (Figures 2–4). Flavonoids are phytochemical substances that have a wide range

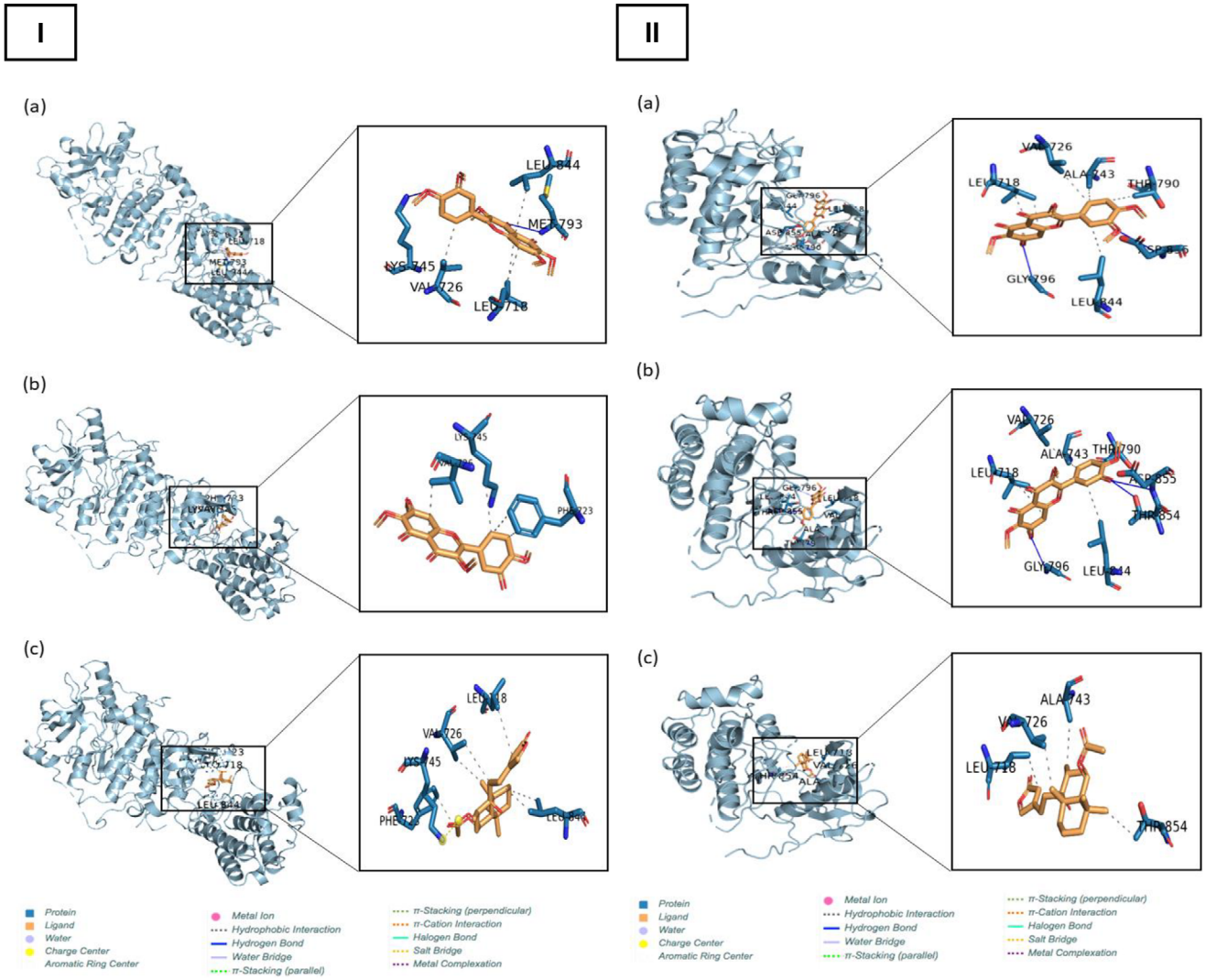

Figure 7. Ligand conformation at the catalytic site of (I) 3IKA and (II) 6D8E: a) Artemitin, b) Casticin, and c) Vitexilactone. The web server PLIP (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index), gathered with PyMOL software (https://pymol.org/2/), visualizes the cartoon and stick models representing the protein and ligand.

Table 5. Hydrogen bonds of ligands in complex with the receptor 3IKA.

Compound	Residue	Amino acid	Distance H–A (Å)	Distance D–A (Å)	Donor angle	Donor atom	Acceptor atom
Rociletinib[22]	745A	LYS	2.2	3.14	153	470 [N3+]	6014 [O2]
	793A	MET	3.21	3.72	114.35	5998 [Npl]	904 [O2]
	797A	CYS	2.83	3.36	112.65	933 [Nam]	6005 [O3]
Artemetin	745A	LYS	3.08	4.08	164.84	386 [N3+]	4943 [O3]
	793A	MET	3.18	4.09	154.07	740 [Nam]	4925 [O3]

of health-promoting pharmacological actions. Their major functions are antioxidant, anti-inflammatory, anticancer, cardioprotective, osteoblast cell proliferation, and neuroprotective.[38,39] The therapeutic potential of flavonoids has sparked interest in the development of natural medications and health supplements for the prevention and treatment of various ailments, particularly heart disease, diabetes, cancer, and neurological disorders.[40]

These sugar and pyran substituents significantly influence the stability and biological activity of the flavonoids. Flavonoids attached to sugar groups, known as flavonoid glycosides, exhibit a range of pharmacological effects that depend not only on the inherent properties of the flavonoids but also on how the sugar is bonded. This sugar moiety impacts the solubility, distribution, and bioavailability of the flavonoids.[41] Flavonoid glycosides are frequently reported to have potent anti-inflammatory proper-

Table 6. Hydrophobic interactions of ligands in complex with the receptor 3IKA.

Compound	Residue	Amino acid	Distance	Ligand atom	Protein atom
Rociletinib[22]	718A	LEU	3.67	6004	230
	718A	LEU	3.57	5996	231
	801A	TYR	3.68	6031	974
	804A	GLU	3.82	6031	1012
	844A	LEU	3.93	6001	1432
Artemetin	718A	LEU	3.76	4932	182
	718A	LEU	3.95	4926	184
	726A	VAL	3.45	4940	232
	844A	LEU	3.89	4926	1169
Casticin	723A	PHE	3.73	4939	214
	723A	PHE	3.52	4940	215
	726A	VAL	3.91	4926	233
	745A	LYS	3.75	4940	384
Vitexilactone	718A	LEU	3.95	4951	184
	723A	PHE	3.75	4943	214
	726A	VAL	3.31	4930	232
	726A	VAL	3.82	4944	233
	844A	LEU	4.00	4929	1169
	844A	LEU	3.72	4945	1168

ties. It can inhibit inflammatory pathways, such as the synthesis of prostaglandins and pro-inflammatory cytokines. The sugar groups alter the solubility and distribution of flavonoids, which helps boost their anti-inflammatory effects within the body.[42,43]

Flavonoid glycosides, such as rutin and quercetin-3-glucoside, possess antioxidant, and anticancer properties, including the ability to inhibit angiogenesis, induce apoptosis, and reduce cancer cell proliferation. The sugar moieties attached to the flavonoid structure can modify its configuration, enhancing its affinity for cell receptors or enzymes involved in regulating cell growth pathways, such as the p53, CDK, and p21 pathways.[44]

Flavonoids that are bound to pyran groups, also known as pyrano-cyclic flavonoids, contain a pyran ring as part of their chemical structure. This pyrano ring forms a heterocyclic chain and significantly influences the physicochemical properties, stability, and biological activity of the flavonoid.[41] The pyran group interacts with various biological components in the body, modulating the pharmacological effects of the flavonoid. Pyrano-cyclic flavonoids are particularly beneficial in protecting the central nervous system. The pyran group within the flavonoid structure helps stabilize the compound, extending its protective effects against oxidative stress, which is commonly associated with neurological disorders like Alzheimer's and Parkinson's diseases. Additionally, pyrano-cyclic flavonoids can enhance cognitive performance and protect neurons from damage.[45]

Among the three antioxidant testing methods, the DPPH method demonstrates that the extract possesses the highest antioxidant potential. A higher antioxidant capacity value signifies greater potential. Multiple studies categorize extracts with an equivalence value exceeding 100 mg/g as having very high antioxidant activity (referensi, apak).

There is currently no evidence to suggest that *V. trifolia* leaves extract has a cytotoxic effect against NSCLC cell lines. In this investigation, two NSCLC cell lines were used: H1975 and H441. In the cytotoxicity test, the extract had a significantly higher IC_{50} than rociletinib ($p < 0.01$). This is because rociletinib is a pure compound that acts as a selective inhibitor of H1975, corresponding to the EGFR double mutation L858R/T790M (Figure 6). In contrast to the cytotoxic activity against H441 that corresponds to the EGFR wild type, the IC_{50} values of the extract and rociletinib were comparable. Thus, a single chemical component of the extract may have a stronger cytotoxic potential than rociletinib.

Several active chemical components of *V. trifolia*, including artemetin, casticin, and vitexilactone, have been identified as potential cytotoxic agents.[46–49] Thus, the three chemical components were used as ligands in a molecular docking study to target the EGFR double mutant L858R/T790M and the EGFR wild type. The docking results are consistent with the in vitro cytotoxicity test, in which rociletinib outperformed other ligands (Table 4). The binding energy of rociletinib is the lowest among the other ligands, at −8.1 kcal/mol, followed by vitexilactone at −7.7 kcal/mol. The lower the binding energy, the more likely the ligand will attach to the target receptor. However, the properties of the bond formed by rociletinib and the EGFR double mutant residue are comparable to those of the artemitin bond. In addition, only artemetin forms hydrogen bonds with the 3IKA receptor. This differs from casticin and vitexilactone, which only have hydrophobic interactions with 3IKA receptor residues (Tables 5 and 6). Although docking provides valuable insights into potential ligand–receptor interactions, it offers only a static view and does not account for conformational flexibility, binding stability, or the dynamic behavior of protein–ligand complexes under physiological conditions. Incorporating molecular dynamics (MD) simulations in future work would allow validation of docking results, provide a more accurate prediction of binding affinity and stability, and strengthen the translational relevance of the identified compounds from *V. trifolia* as potential anticancer agents.

The evaluation of the sample's activity is limited to in vitro assays, which inherently have several limitations. In vitro systems involve only cells or tissues maintained under controlled conditions and therefore do not replicate the complex interactions among organs, the immune system, metabolism, and excretory processes within a living organism. Moreover, in vitro data do not provide information on the absorption, distribution, metabolism, and excretion (ADME) of a compound, all of which are critical for determining its actual efficacy and toxicity in vivo. Consequently, further evaluation using in vivo models is necessary in future studies.

The clinical significance of this study lies in demonstrating that *V. trifolia* leaf extract contains flavonoids with both antioxidant and anticancer activity, specifically against NSCLC harboring EGFR L858R/T790M mutations, which are commonly associated with resistance to current tyrosine kinase inhibitors.

The identification of compounds such as artemetin, casticin, and vitexilactone that interact in a manner comparable to rociletinib suggests a potential role for *V. trifolia* as a source of novel agents capable of targeting drug-resistant lung cancer. In addition, the extract's strong antioxidant activity may provide further clinical benefit by reducing oxidative stress, which contributes to tumor progression and therapy resistance. Together, these findings highlight *V. trifolia* as a promising candidate for development as a supportive or alternative therapeutic strategy for NSCLC patients who have limited options after resistance to standard EGFR-targeted therapies.

4. Conclusion

Several chemical components were effectively identified from *V. trifolia* leaf extract using GC-MS and LC-HRMS. Some of the prominent compounds discovered exhibit pharmacological action, particularly as antioxidant and anticancer agents in several target cell lines. Furthermore, artemetin, casticin, and vitexilactone contained in *V. trifolia* contribute to the extract's potential as an inhibitor of NSCLC cell lines H1975 and H441, which represent the EGFR L858R/T790M mutation and the EGFR wild type, respectively. The cytotoxic potential data were supported by molecular docking simulations that revealed similarities in the hydrogen bond model and hydrophobic interactions with target receptors between artemetin and rociletinib. Thus, the chemical components of *V. trifolia* leaf extract shows great promise for development as an anticancer agent through different methods.

Supporting Information

Experimental materials, methods, and molecular docking results are provided in the Supporting Information.[3,20–25,50–56]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Flavonoid · Lung cancer · Molecular modeling · Mutant · Radical reactions

[1] A. L. Couffignal, M. Lapeyre-Mestre, C. Bonhomme, R. Bugat, J. L. Montastruc, *Therapie* **2000**, *55*, 635–641.
[2] R. L. Siegel, K. D. Miller, N. S. Wagle, A. Jemal, *Ca-Cancer J. Clin.* **2023**, *73*, 17–48.
[3] M. Fawwaz, K. Mishiro, A. Arwansyah, R. Nishii, K. Ogawa, *Bioimpacts* **2024**, *14*, 27774–27774.
[4] D. J. Newman, G. M. Cragg, *J. Nat. Prod.* **2016**, *79*, 629–661.
[5] G. M. Cragg, J. M. Pezzuto, *Med. Principles Prac.* **2015**, *25*, 41–59.
[6] J. H. Elizondo-Luévano, R. Gomez-Flores, M. J. Verde-Star, P. Tamez-Guerra, C. I. Romo-Sáenz, A. Chávez-Montes, N. E. Rodríguez-Garza, R. Quintanilla-Licea, *Plants (Basel)* **2022**, *11*, 2862.
[7] N. A. Shafie, N. A. Suhaili, H. Taha, N. Ahmad, *F1000Res* **2020**, *9*, 187;
[8] M. I. Yusuf, S. Susanty, M. Fawwaz, *Pharmaco. J.* **2018**, *10*, 686-690.
[9] X. Peng, F. Sun, G. Li, C. Wang, Y. Zhang, C. Wu, C. Zhang, Y. Sun, S. Wu, Y. Zhang, H. Zong, R. Guo, H. Lou, *J. Agric. Food Chem.* **2021**, *69*, 11216-11224.
[10] N. Kamal, N. S. Mio Asni, I. N. A. Rozlan, M. A. H. Mohd Azmi, N. W. Mazlan, A. Mediani, S. N. Baharum, J. Latip, S. Assaw, R. A. Edrada-Ebel, *Plants* **2022**, *11*, 1944.
[11] J. Mottaghipisheh, M. Kamali, A. H. Doustimotlagh, M. H. Nowroozzadeh, F. Rasekh, M. H. Hashempur, A. Iraji, *Front. Pharmacol.* **2024**, *15*, 1322083.
[12] M. Fawwaz, B. Purwono, Y. Sidiq, Arwansyah, M. I. A., Fitriana, M. P., A. Fajriani, A. T. Kusuma, Amirullah, A. A. M., A. Sarfandi, M. Baits, *ChemistrySelect* **2024**, *9*, e202403834.
[13] M. Dai, A. Fadhilah, J. Rahmawati, A. M. Forentin, T. Usia, M. Maryati, *Pharmaco. Magazine* **2018**, *14*, 359-363.
[14] S. El-Kousy, M. Mohamed, S. Mohamed, *Life Sci. J.* **2012**, *9*, 670-677.
[15] M. M. Hernández, C. Heraso, M. L. Villarreal, I. Vargas-Arispuro, E. Aranda, *J. Ethnopharmacol.* **1999**, *67*, 37–44.
[16] M. Mathankumar, R. Tamizhselvi, V. Manickam, G. Purohit, *Toxicol Int* **2015**, *22*, 114-118.
[17] D. Li, X. Zhang, Z. Song, S. Zhao, Y. Huang, W. Qian, X. Cai, *Immunol. Cell Biol.* **2024**, *102*, 557–569.
[18] L. Shen, S. Pang, M. Zhong, Y. Sun, A. Qayum, Y. Liu, A. Rashid, B. Xu, Q. Liang, H. Ma, X. Ren, *Ultrason. Sonochem.* **2023**, *101*, 106646.
[19] K. Kumar, S. Srivastav, V. S. Sharanagat, *Ultrason. Sonochem.* **2021**, *70*, 105325.
[20] M. Fawwaz, M. Pratama, A. Hasrawati, H. Widiastuti, Z. Abidin, *Biointerface Res. Appl. Chem.* **2021**, *11*, 11293–11302.
[21] O. Trott, A. J. Olson, *J. Comput. Chem.* **2010**, *31*, 455-461.
[22] M. Fawwaz, K. Mishiro, B. Purwono, R. Nishii, K. Ogawa, *J. Pharm. Pharmacogn. Res.* **2024**, *12*, 231–242.
[23] G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, *J. Comput. Chem.* **2009**, *30*, 2785–2791.
[24] A. Arwansyah, A. R. Arif, G. Syahputra, S. Sukarti, I. Kurniawan, *Mol. Simul.* **2021**, *47*, 988-1001;
[25] T. Sumaryada, Arwansyah, A W. Roslia, L. Ambarsari, A. Kartono, in 1st International Conference on Biomedical Engineering (IBIOMED), **2016**, pp. 1-4.
[26] P. Koilkonda, J. Kottapalli, b. Raju, b. Prasad, R. Arora, *Med. Aroma. Plant Sci.Biotechnol.* **2009**, *3*, 6-14.
[27] C.-X. Yan, Y.-W. Wei, H. Li, K. Xu, R.-X. Zhai, D.-C. Meng, X.-J. Fu, X. Ren, *J. Ethnopharmacol.* **2023**, *308*, 116273.
[28] S. Tandon, A. K. Mittal, A. K. Pant, *Fitoterapia* **2008**, *79*, 283–286.
[29] S. Samaram, H. Mirhosseini, C. P. Tan, H. M. Ghazali, S. Bordbar, A. Serjouie, *Food Chem.* **2015**, *172*, 7–17.
[30] M. Ashokkumar, *Ultrason. Sonochem.* **2015**, *25*, 17–23.
[31] D. O. Moon, M. O. Kim, Y. H. Choi, G. Y. Kim, *Cancer Lett.* **2008**, *264*, 181–191.
[32] H. X. Wang, T. B. Ng, *Life Sci.* **1999**, *65*, 2663–2677.
[33] S. Sujatha, S. Anand, K. N. Sangeetha, K. Shilpa, J. Lakshmi, A. Balakrishnan, B. S. Lakshmi, *Int. J. Diabetes Mellitus* **2010**, *2*, 101–109.
[34] E. Aly, M. A. Khajah, W. Masocha, *Molecules* **2019**, *25*, 106.
[35] L. F. S. Gushiken, F. P. Beserra, M. F. Hussni, M. T. Gonzaga, V. P. Ribeiro, P. F. de Souza, J. C. L. Campos, T. N. C. Massaro, C. A. Hussni, R. K. Takahira, P. D. Marcato, J. K. Bastos, C. H. Pellizzon, *Oxid. Med. Cell Longev.* **2022**, *2022*, 9004014.
[36] A. Di Sotto, R. Mancinelli, M. Gullì, M. Eufemi, C. L. Mammola, G. Mazzanti, S. Di Giacomo, *Cancers* **2020**, *12*, 3034.

[37] K. Fidyt, A. Fiedorowicz, L. Strządała, A. Szumny, *Cancer Med.* **2016**, *5*, 3007–3017.
[38] A. Ullah, S. Munir, S. L. Badshah, N. Khan, L. Ghani, B. G. Poulson, A. H. Emwas, M. Jaremko, *Molecules* **2020**, *25*, 5243.
[39] M. Fawwaz, E. Wahyudin, M. N. Djide, *Int. J. PharmTech Res.* **2014**, *6*, 666–670.
[40] H. Hasnat, S. A. Shompa, M. M. Islam, S. Alam, F. T. Richi, N. U. Emon, S. Ashrafi, N. U. Ahmed, M. N. R. Chowdhury, N. Fatema, M. S. Hossain, A. Ghosh, F. Ahmed, *Heliyon* **2024**, *10*, e27533.
[41] M. C. Dias, D. Pinto, A. M. S. Silva, *Molecules* **2021**, *26*.
[42] B. Soto-Blanco, in *Herbal Biomolecules in Healthcare Applications* (Eds: S. C. Mandal, A. K. Nayak, A. K. Dhara), Academic Press, Cambridge, MA **2022**, pp. 239-282.
[43] M. I. Nassar, S. A. Aboutabl el, D. M. Eskander, M. H. Grace, E. D. El-Khrisy, A. A. Sleem, *Pharmacognosy Res.* **2013**, *5*, 17.
[44] M. Farhan, A. Rizvi, M. Aatif, A. Ahmad, *Metabolites* **2023**, *13*, 481.
[45] A. Calderaro, G. T. Patanè, E. Tellone, D. Barreca, S. Ficarra, F. Misiti, G. Laganà, *Int. J. Mol. Sci.* **2022**, *23*, 14835.
[46] K. Carbone, F. Gervasi, L. Kozhamzharova, N. Altybaeva, E. Sönmez Gürer, J. Sharifi-Rad, C. Hano, D. Calina, *Front. Mol. Biosci.* **2023**, *10*, 1157558.
[47] W. X. Li, C. B. Cui, B. Cai, H. Y. Wang, X. S. Yao, *J. Asian. Nat. Prod. Res.* **2005**, *7*, 615-626.
[48] W. X. Li, C. B. Cui, B. Cai, X. S. Yao, *J. Asian Nat. Prod. Res.* **2005**, *7*, 95-105.
[49] Y. C. Song, X. Zhang, G. Y. Lei, C. X. Dang, *Zhonghua Yi Xue Za Zhi* **2010**, *90*, 703-707.
[50] A. Biswas, S. Dey, A. Xiao, Y. Deng, Z. M. Birhanie, R. Roy, D. Akhter, L. Liu, D. Li, *Chem. Biol. Technol. Agri.* **2023**, *10*, 64.
[51] N. González-Silva, Y. Nolasco-González, G. Aguilar-Hernández, S. G. Sáyago-Ayerdi, Z. Villagrán, J. L. Acosta, E. Montalvo-González, L. M. Anaya-Esparza, *Molecules* **2022**, *27*, 3557.
[52] F. H. Thamer, N. Thamer, *Heliyon* **2023**, *9*, e16861.
[53] W. Lakhdari, I. Benyahia, M. M. Bouhenna, H. Bendif, H. Khelafi, H. Bachir, A. Ladjal, H. Hammi, D. Mouhoubi, H. Khelil, T. S. Alomar, N. AlMasoud, N. Boufafa, F. Boufahja, A. Dehliz, *Molecules* **2023**, *28*, 5025.
[54] A. Windarsih, F. D. O. Riswanto, N. K. A. Bakar, N. D. Yuliana, Dachriyanus, A. R., *Molecules* **2022**, *27*, 8325.
[55] M. Ahda, I. Jaswir, A. Khatib, Q. U. Ahmed, N. Mahfudh, Y. D. Ardini, S. N. A. Syed Mohamad, M. Anwar, H. Hernawan, K. Miyashita, A. M. Salamatullah, *Sci. Rep.* **2023**, *13*, 17012.
[56] N. M. O'Boyle, M. Banck, C. A. James, C. Morley, T. Vandermeersch, G. R. Hutchison, *J. Cheminform.* **2011**, *3*, 33.

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