

Phytochemical and pharmacological evaluation of *Vanda tricolor* from Gumitir, East Java, Indonesia

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Abstract. Ulum FB, Putri YA, Farhan AM, Setyati D, Rajamani R, Sugiharto B, Su'udi M. 2025. *Phytochemical and pharmacological evaluation of Vanda tricolor from Gumitir, East Java, Indonesia. Biodiversitas 26: 4783-4792.* *Vanda tricolor* an epiphytic orchid native to the coffee agroforest of Gumitir, East Java, Indonesia, represents a promising source of pharmacologically active compounds. This study aimed to characterize the bioactive constituents of *V. tricolor* and evaluate their antioxidant, antimicrobial, and anticancer potential. Methanolic leaf extracts were analyzed using gas chromatography-mass spectrometry (GC-MS) for metabolite profiling. Its biological activities were assessed through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, agar well diffusion method, and molecular docking against the antiapoptotic Bcl-2 protein. GC-MS analysis identified 48 metabolites, with 4-butylphenol (7.57%) as the predominant compound. The extract demonstrated very strong antioxidant activity (IC₅₀: 37.25 ppm), along with moderate antibacterial effects against *Staphylococcus aureus* and *Escherichia coli*; however, it showed no growth inhibition against *Salmonella typhi*. Molecular docking revealed that 4-butylphenol exhibited the highest binding affinity toward Bcl-2 (-5.50 kcal/mol), indicating its potential as a lead compound for anticancer therapy. These findings represent the first integrated phytochemical and pharmacological profile of *V. tricolor* from Gumitir, underscoring its value as a source of natural bioactive agents. The results also provide a scientific basis for further exploration of orchids in drug discovery and contribute to the conservation of this underutilized native species.

Keywords: Antimicrobial activity, antioxidant activity, GC-MS analysis, molecular docking, *Vanda tricolor*

INTRODUCTION

Indonesia is widely recognized as one of the world's mega-biodiverse countries, hosting a vast range of flora and fauna due to its archipelagic geography and tropical climate. The nation harbors approximately 20,000 species of flowering plants, 4,000 species of ferns, and over 400 species of palms, which are distributed across a mosaic of ecosystems from lowland rainforests to montane regions (Kusmana and Hikmat 2015). Among these, orchids (family Orchidaceae) constitute one of the most diverse and ecologically significant plant groups. Indonesia alone is estimated to have more than 6,000 orchid species, accounting for nearly a quarter of the global orchid flora, which comprises roughly 26,000 species (Wati et al. 2023). Despite their aesthetic and ecological importance, orchids have also gained attention for their phytochemical and pharmacological properties.

Numerous genera, such as *Dendrobium*, *Cymbidium*, *Coelogyne*, *Anoectochilus*, and *Vanda*, have been studied for their medicinal properties, with reports of antioxidant, antimicrobial, and anti-inflammatory activities (Gantait et al. 2021). Key metabolites isolated from medicinal orchids include anthocyanins, bibenzyl derivatives, phenanthrenes, and related stilbenoids that underpin activities such as COX-1/COX-2 inhibition and antioxidant effects (Gantait

et al. 2021). Traditional sources also document extensive therapeutic use; for example, roots of *Vanda roxburghii* are applied for rheumatism, with recent work indicating reduced acute inflammation, aligning with broader ethnomedicinal employment across China and Asia (Shengji and Zhiwei 2018). However, despite these ethnomedicinal claims, the scientific characterization of bioactive compounds, especially from wild Indonesian *Vanda* species, remains fragmentary.

The Gumitir region in East Java, Indonesia, offers a unique ecological setting where coffee agroforests, formerly montane forests, retain remnant canopy trees for erosion control and shade provision (Yuliasmara 2017). These semi-natural systems support high floristic diversity, including medicinal and epiphytic species (Ulum et al. 2023; Rizqoni et al. 2024; Setyati et al. 2024). One such species is *Vanda tricolor*, a locally dominant epiphyte identified through DNA barcoding and previously noted for its bioactive potential (Setyati et al. 2021; Su'udi et al. 2022). Epiphytic orchids, such as *V. tricolor*, face habitat loss due to the expansion of agroforestry, underscoring the urgency of documenting their phytochemicals to support the conservation of Indonesian orchids.

Earlier investigations have revealed the presence of volatile compounds in *V. tricolor*, including eugenol, nerolidol, and methyl cinnamate (Darmasiwi et al. 2015). Notably, eugenol is recognized for its ability to scavenge

free radicals (Khan et al. 2019). Setyati et al. (2021) further confirmed the presence of flavonoids and alkaloids, indicating the potential antioxidant properties of *V. tricolor* leaf extracts. While these findings are promising, existing studies primarily rely on limited chemical screening and lack integrative biological validation. Few studies have employed comprehensive metabolite profiling combined with functional bioassays and in silico analysis. GC-MS profiling of the related epiphytic orchid *Vanda tessellata* identified 33 root-derived compounds, several of which were associated with significant antibacterial and antifungal activities; however, no anticancer activity was detected (Ramana et al. 2020). Integrating metabolite profiling with antioxidant, antimicrobial, and cytotoxicity assays enables a direct connection between chemical composition and pharmacological function. At the same time, molecular docking simulations can predict ligand-target interactions, offer mechanistic insights and guide early-stage drug discovery (Sait et al. 2025).

Despite the recognized bioactivity of *V. tricolor*, no previous research has combined Gas Chromatography-Mass Spectrometry (GC-MS), in vitro bioassays, and molecular docking to comprehensively elucidate its pharmacological potential. Such an integrative framework is essential for simultaneously validating compound identity, confirming functional bioactivity, and prioritizing lead molecules for drug development. This study addresses the critical gap by conducting a multi-tiered investigation of the methanolic leaf extract of *V. tricolor*, encompassing GC-MS metabolite profiling, antioxidant and antibacterial assays, and molecular docking simulations targeting the antiapoptotic Bcl-2 protein. By connecting phytochemical composition to specific biological activities, this approach not only

enhances understanding of *V. tricolor*'s pharmacological repertoire but also substantiates its potential as a promising source of natural therapeutic agents.

MATERIALS AND METHODS

Study area

Leaf samples of *Vanda tricolor* were collected from a coffee agroforest within the montane landscape of Gumatir, Jember, East Java, Indonesia (Figure 1). This semi-natural agroecosystem harbors remnant canopy trees and supports diverse epiphytic flora, including native orchids such as *V. tricolor* (Setyati et al. 2021). Identification of the species was conducted based on morphological characteristics following regional orchid identification keys, and further confirmed by an orchidologist at the National Research and Innovation Agency (BRIN) (Susilowati 2018) and through DNA barcoding (Suudi et al. 2023). A voucher specimen was prepared and deposited at the Herbarium of the Biology Department, Universitas Jember, under the accession number JR 0000001797.

Plant material collection

The collection complied with the guidelines of the East Java Forestry Office (Permit No. 35X/803/PI/2017). The collected specimens were gently detached from their epiphytic substrates, rinsed thoroughly with clean water to remove surface debris, and subsequently air-dried at ambient room temperature (25-27°C) for 24 hours. The dried leaves were then stored in clean paper envelopes before extraction.

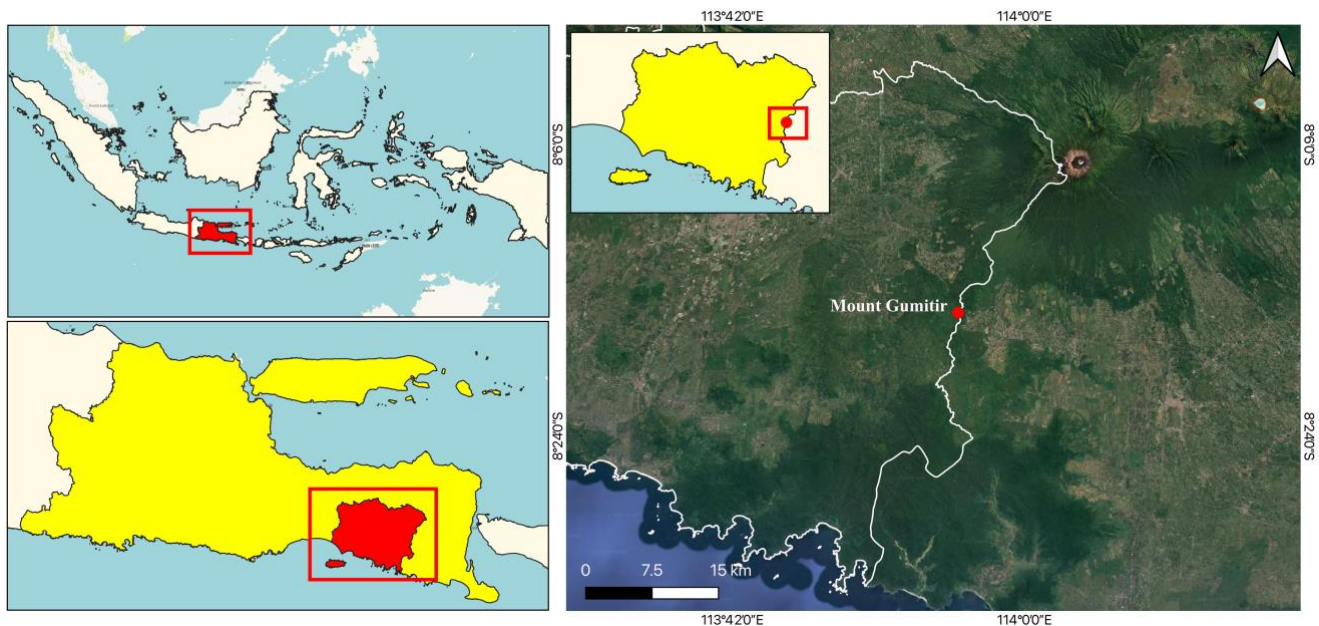


Figure 1. Research location at Mount Gumatir, Jember District, East Java, Indonesia (8°15'37.55"S, 113°57'1.62"E)

Extraction

Extraction was conducted using maceration and methanol as a solvent, following the protocol described by Amalini et al. (2025). Briefly, 30 g of air-dried leaf material was immersed in 300 mL of 96% methanol (1:10 w/v) and left under static conditions at room temperature for 72 hours, with manual stirring every 24 hours to enhance metabolite diffusion. The mixture was then filtered through 20 µm pore-size paper, and the filtrate was concentrated under reduced pressure at 60°C using a rotary evaporator for approximately 40 minutes. The crude extract was stored in sterile amber vials at 4°C until analysis.

GC-MS analysis

The crude methanolic extract of *V. tricolor* was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis using a Shimadzu QP-2010 Plus system equipped with a semi-polar Rtx-50 capillary column (30 m × 0.25 mm × 0.25 µm). The injection port temperature was maintained at 290°C, while the mass spectrometer detector operated at 280°C. The oven temperature was initially maintained at 80°C for 10 minutes, then ramped to 230°C at a rate of 5°C/min, and then held isothermally at 230°C for an additional 10 minutes. Helium served as the carrier gas at a constant flow rate of 1.53 mL/min. One microliter of the extract was injected in split mode. The resulting chromatographic peaks were analyzed based on retention time (RT) and mass spectral fragmentation patterns. Compound identification was achieved by comparing the spectra with those in the WILEY 7.LIB library database, using both retention index and spectral similarity scores as criteria for compound confirmation (Amalini et al. 2025).

Antimicrobial assay

The antibacterial efficacy of the methanolic leaf extract of *V. tricolor* was evaluated using the agar well diffusion technique, following the protocol described by Balouiri et al. (2016), with slight modifications to optimize reproducibility. The antimicrobial assay targeted three clinically relevant bacterial strains: *Staphylococcus aureus* (Gram-positive bacteria), *Salmonella typhi*, and *Escherichia coli* (both Gram-negative). Before inoculation, all bacterial strains were sub-cultured and incubated under aerobic conditions until they reached the mid-logarithmic phase, as described by Kim et al. (2017) for standardized growth phase determination. Ciprofloxacin (0.1% w/v) was employed as a positive control to benchmark antibacterial potency, whereas absolute methanol served as the negative control to rule out solvent-related inhibition. The plant extract was tested at five concentration levels (10%, 20%, 30%, 40%, and 50% v/v). 1 mL aliquot of each bacterial suspension was homogeneously mixed with molten nutrient agar (NA) and poured into sterile Petri dishes. Once solidified, 5 mm diameter wells were aseptically bored into the agar using a sterile cork borer, and each well was filled with 20 µL of either the plant extract or control solution. All treatments were carried out in triplicate to ensure statistical robustness. Following 24 hours of incubation at 37°C, antimicrobial activity was assessed by measuring the diameter of the

inhibition zones (in mm) formed around each well. The results were interpreted based on inhibition zone diameters, classified as strong (>20 mm), moderate (5-20 mm), or weak (<5 mm), as defined by Ouchari et al. (2019). While this diffusion-based assay provides preliminary insight into antimicrobial efficacy, the current study did not determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). This limitation is acknowledged, and the current results are intended to provide an initial screening of antibacterial potential.

Antioxidant activity assay

The antioxidant capacity of the methanolic extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. A 0.1 mM DPPH stock solution was prepared by dissolving 2 mg of DPPH in 50 mL of methanol. The maximum absorbance wavelength (λ_{max}) of 517 nm was determined by scanning the solution across the 400-800 nm range using a UV-Vis spectrophotometer. Ascorbic acid (vitamin C) served as the positive control. A 1000 ppm vitamin C stock solution was prepared by dissolving 25 mg of vitamin C in 25 mL of methanol and vortexing to achieve uniformity. Serial dilutions (100, 200, 300, and 400 ppm) were prepared for the extract and the standard. For each assay, 0.3 mL of sample solution was mixed with 1.2 mL of DPPH solution and incubated in the dark at room temperature for 30 minutes. After incubation, 1.5 mL of the reaction mixture was transferred to a quartz cuvette, and absorbance was recorded at 517 nm. Each treatment was conducted in triplicate. The percentage inhibition of DPPH radicals was calculated using the formula:

$$\text{Inhibition (\%)} = [(Ab_{\text{control}} - Ab_{\text{sample}}) / Ab_{\text{control}}] \times 100$$

Where, Ab_{control} and Ab_{sample} represent the absorbance of the control and test samples, respectively. The IC_{50} value, defined as the concentration required to scavenge 50% of DPPH radicals, was determined by plotting the inhibition percentage against concentration and applying linear regression analysis in Microsoft Excel (Amalini et al. 2025). The IC_{50} values were interpreted according to Zamzani and Triadisti (2021), with values classified as very strong (<50 ppm), strong (50-100 ppm), moderate (100-150 ppm), or weak (150-200 ppm).

In silico analysis

Protein preparation

The three-dimensional crystal structure of the human B-cell lymphoma 2 (Bcl-2) protein was retrieved from the Protein Data Bank (PDB ID: 8HTS). All heteroatoms, water molecules, and co-crystallized ligands were removed using PyMOL (v2.5) to eliminate non-essential components that may interfere with binding site accessibility. The protein structure was subsequently protonated to satisfy valency and optimized to relieve steric clashes using the Swiss-PDB Viewer. This preprocessing ensured a geometry-refined target for accurate molecular docking simulations (Abd-Ghani et al. 2020; Pugazhenthil et al. 2023).

Ligand preparation

Based on the GC-MS profile of *Vanda tricolor*, these three compounds -2-(2-Hydroxypropoxy) propan-1-ol, 4-Butylphenol, and Acetic acid- were taken for the in silico molecular docking studies to determine the biological properties of the plant. The three ligands -2-(2-Hydroxypropoxy) propan-1-ol (PubChem ID: 32881), 4-Butylphenol (PubChem ID: 15420), and Acetic acid (PubChem ID: 176), were obtained from the PubChem database in SDF format. These structures were converted into the required PDB format using the OpenBabel software, followed by geometry optimization to ensure an accurate 3D conformation for docking (Lokesh et al. 2023).

Molecular docking

AutoDock 4.2 was used to perform molecular docking using the Lamarckian genetic process. Protein preparation involved removing water molecules, adding polar hydrogens, and assigning Kollman charges. Torsion angle optimization was done on ligands, and a docking grid (12Å×12Å×12Å) was established. LigPlot+ and BIOVIA Discovery Studio Visualizer were used to examine potential binding sites, binding energy, Van der Waals interactions, hydrogen bonds, their distances, and interacting amino acid residues (Roy et al. 2020; Lokesh et al. 2023).

Data analysis

Phytochemical constituents detected via GC-MS were interpreted based on their retention times, relative peak intensities, and mass spectral fragmentation patterns. Compounds were identified through spectral matching with the WILEY 7.LIB database. The pharmacological profiling of bioactive compounds was then cross-validated by querying multiple chemical databases, including the NIST Mass Spectral Library, PubChem, ChemSpider, and literature sources (Duryat et al. 2025). For antioxidant and antimicrobial assays, results were obtained from triplicate replicates (n: 3). Since only three replicates were used, no inferential statistical tests (e.g., ANOVA) were performed, and results are presented as mean values to provide descriptive insights into the bioactivity trends (Ulum et al. 2023; Amalini et al. 2025).

RESULTS AND DISCUSSION

Phytochemical diversity

Gas Chromatography-Mass Spectrometry analysis of the methanolic leaf extract of *Vanda tricolor* revealed 48 distinct phytoconstituents, underscoring its chemical complexity (Figure 2). Of these, 11 compounds were classified as major constituents, each comprising more than 4% of the total peak area. Their identities and reported biological activities, primarily antioxidant and antimicrobial properties, are detailed in Table 1. Additionally, 6 minor constituents (each contributing <4% peak area) were detected, including 3 phenolics and 3 terpenoids, which have also been previously implicated in redox modulation and antimicrobial defense mechanisms (Supplementary Table 1).

The most abundant compound identified was phenol, 4-butyl- (7.57%), a phenolic derivative known for its potent antioxidant activity (Shakira et al. 2022). Alcohol-based metabolites such as 1-propanol, 2-(2-hydroxypropoxy)- (6.70%) and 2-propanol, 1,1'-oxybis- (4.27%) demonstrated both antioxidant and antimicrobial properties (Chirumamilla et al. 2022), while acetic acid (6.09%) contributed antimicrobial effects (Meiliana et al. 2022). Notably, two phenolic compounds, phenol, 2,6-dimethoxy-4-(2-propenyl)- (6.03%) and chavicol (4.42%), are recognized for their antioxidant and antifungal activities (Setyati et al. 2024; Sarma et al. 2018). Additionally, 3 ketones, ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)- (5.36%), acetovanillone (4.16%), and 2-nonanone (4.36%), have been reported to exhibit anticancer, anti-inflammatory, or analgesic activities (Gangadharan et al. 2024; Subramanian et al. 2020). Other notable compounds included 1,6-anhydro-β-D-glucopyranose (5.23%), which possesses anti-inflammatory potential (Azizov et al. 2022), 5-hydroxymethylfurfural (5.01%) from the furan group known to have antioxidant activity (Unno et al. 2024), and pentadecanoic acid methyl ester (4.06%), a saturated fatty acid ester exhibiting antibacterial properties (Fadzir et al. 2018).

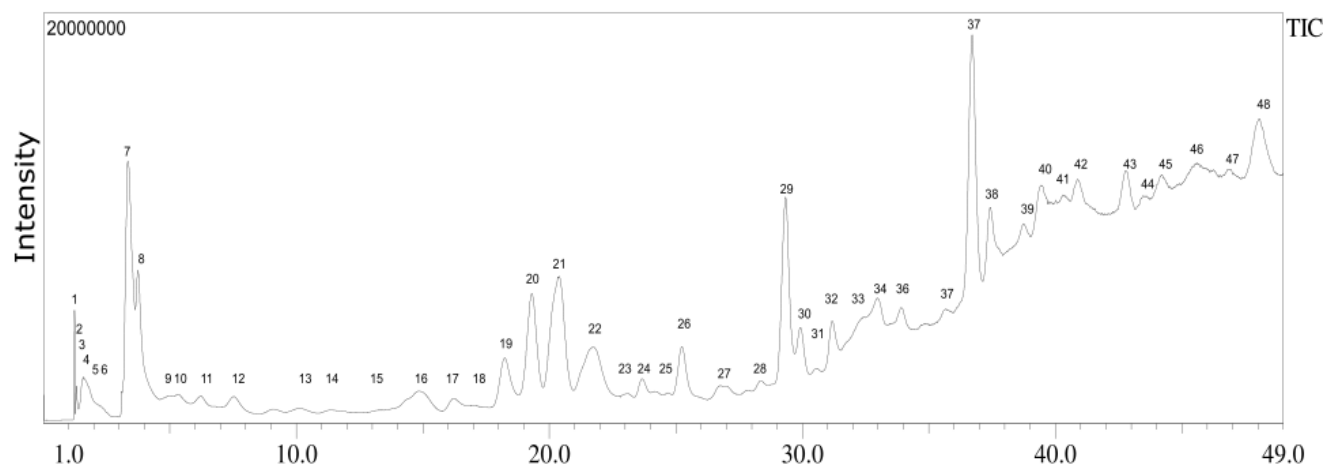


Figure 2. GC-MS chromatogram of *Vanda tricolor*

Table 1. Phytochemical profile of *Vanda tricolor* identified by GC-MS and bioactive potencies

Peak number	Area (%)	Compound	Group	Bioactivity
37	7.57	Phenol, 4-butyl-	Phenol	Antioxidant (Shakira et al. 2022)
21	6.70	1-Propanol, 2-(2-hydroxypropoxy)-	Alcohol	Antioxidant, antimicrobial (Chirumamilla et al. 2022)
7	6.09	Acetic acid	Organic acid	Antimicrobial (Meiliana et al. 2022)
42	6.03	Phenol, 2,6-dimethoxy-4-(2-propenyl)-	Phenol	Antioxidant (Setyati et al. 2024)
46	5.36	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	Ketone	Anticancer (Gangadharan et al. 2024)
40	5.23	1,6-anhydro-beta-d-glucopyranos	Monosaccharide	Antioxidant, anti-inflammatory (Azizov et al. 2022)
29	5.01	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Furan	Antioxidant (Unno et al. 2024)
33	4.42	Chavicol	Phenol	Antioxidant, antifungal (Sarma et al. 2018)
22	4.36	2-Nonanone	Ketone	Anti-inflammatory, non-central analgesic, antirheumatic agent (Subramanian et al. 2020)
20	4.27	2-Propanol, 1,1'-oxybis-	Alcohol	Antioxidant, antimicrobial (Chirumamilla et al. 2022)
39	4.16	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)-	Ketone	Anticancer (Gangadharan et al. 2024)
43	4.06	Pentadecanoic acid, 14-methyl-, methyl ester	Sat. Fatty acid methyl ester	Antibacterial (Fadzir et al. 2018)

GC-MS analyses of other *Vanda* species have revealed a rich presence of bioactive compounds with potential medicinal applications. For example, *Vanda tessellata* root extracts have been found to contain anti-inflammatory and anticancer phytoconstituents such as phytol, hexadecanoic acid, and eugenol, which support its traditional therapeutic use (Ramana et al. 2020). Similarly, studies on *Vanda spathulata* and *Vanda cristata* identified diverse compounds, including fatty acid esters and pyranones known for their antioxidant, antimicrobial, and cytoprotective properties (Rani et al. 2021; Sharma et al. 2022).

Phenolics are well-recognized for their potent antioxidant activity, primarily mediated by hydroxyl groups on their aromatic rings, which facilitate radical scavenging, electron donation, and metal ion chelation (Lv et al. 2021; Roshanpour et al. 2021). Additionally, their antimicrobial actions involve disrupting bacterial cell wall integrity, inhibiting DNA replication, and interfering with various enzyme systems, resulting in broad-spectrum cellular damage (Lobiuc et al. 2023). Although present in low abundance, these trace-level compounds can exhibit significant bioactivity, either independently or through synergistic interactions, warranting their inclusion in early-stage metabolite profiling to avoid overlooking key therapeutic leads (Atanasov et al. 2021; Amalini et al. 2025). It is particularly crucial for downstream applications such as bioactivity-guided fractionation and untargeted metabolomics. Moreover, metabolite profiles can be influenced by various factors, including solvent polarity, extraction technique, plant maturity, and environmental variables (Patel et al. 2021; Salam et al. 2023). Among the phenolics, phenol, 2,6-dimethoxy- (1.24%) and eugenol (0.09%) were reported to possess antioxidant activity (Maulina and Nurtahara 2020; Ulanowska and Olas 2021), while phenol, 4-ethenyl-2-methoxy- (1.20%) exhibited antifungal, anti-inflammatory, and antispasmodic effects (Clarke 2008).

In parallel, terpenoids, biosynthesized from isoprene units, are recognized for their wide-ranging biological effects, including antioxidant, antimicrobial, anti-inflammatory, and cytoprotective actions (Azalia et al. 2023). Within this group, dihydro-3-methylene-5-methyl-2-furanone (0.09%) demonstrated strong antioxidant activity (Fei et al. 2023), while 1-cyclohexanone, 2-formyl-6-isopropyl-3-methyl- (0.28%) exhibited antimicrobial potential (Nguyen et al. 2019). Notably, the presence of these terpenoids alongside phenolic constituents may contribute to synergistic bioactivity. The synergistic mechanism among natural compounds occurs when their combined effects exceed the sum of their individual actions, often through complementary mechanisms such as enhanced cellular uptake, multi-target engagement, and improved metabolic stability (Rajčević et al. 2022).

Antimicrobial activity

The methanolic leaf extract of *V. tricolor* possessed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. But no inhibitory effect was observed against *Salmonella typhi*, as indicated by the absence of inhibition zones (Table 2). The extract produced inhibition zones of 1.16 cm, 0.92 cm, and 0.73 cm at concentrations of 100%, 50%, and 25%, respectively, against *S. aureus*, demonstrating a dose-dependent response. The inhibition zone was detected at 100% and 50% concentrations, with diameters of 1.16 cm and 0.46 cm, respectively, against *E. coli*. No inhibition was recorded at 25%. In contrast, *S. typhi* showed complete resistance at all tested concentrations, suggesting a higher level of intrinsic defense. The positive control (ciprofloxacin 0.1%) consistently produced large inhibition zones across all test strains, with zones of 3.85 cm (*S. aureus*), 6.32 cm (*E. coli*), and 3.48 cm (*S. typhi*), thereby validating the efficacy of the assay. No inhibitory activity was observed in the negative control (methanol), confirming the absence of solvent-related antimicrobial effects.

Table 2. Antibacterial activity of *Vanda tricolor* methanolic extract

Microbe	Concentration (%)	Inhibition zone±SD (cm)
<i>Staphylococcus aureus</i>	100	1.16±0.74
	50	0.92±0.67
	25	0.73±0.31
<i>Salmonella typhi</i>	Ciprofloxacin 0.1	3.85±0.59
	Methanol 100	0.00
	100	0.00
	50	0.00
	25	0.00
<i>Escherichia coli</i>	Ciprofloxacin 0.1	3.48±1.32
	Methanol 100	0.00
	100	1.16±22
	50	0.46±0.65
	25	0.00
	Ciprofloxacin 0.1	6.32±0.59
	Methanol 100	0.00

GC-MS analysis of the methanolic leaf extract of *V. tricolor* identified 13 compounds with reported antimicrobial activities. These include acetic acid (ethanoic acid), a broad-spectrum antimicrobial known to destabilize microbial membranes (Meiliana et al. 2022), and chavicol, a phenolic compound with documented antifungal properties (Sarma et al. 2018). Additionally, dipropylene glycol (2-propanol, 1,1'-oxybis-) has demonstrated antimicrobial properties, particularly in cosmetic formulations, serving as both a penetration enhancer and a preservative agent (Chirumamilla et al. 2022). Long-chain saturated fatty acids and their esters, such as pentadecanoic acid, 14-methyl-, methyl ester, and hexadecanoic acid (palmitic acid), are also present in substantial quantities. These compounds exhibit antibacterial activity by integrating into microbial lipid bilayers, disrupting membrane integrity, and modulating bacterial enzymatic pathways (Fadzir et al. 2018). Other notable antimicrobial metabolites include 4-methyl-2,5-dimethoxybenzaldehyde, which possesses both bacteriostatic and anti-inflammatory properties, and acetol (2-propanone, 1-hydroxy-), an organic intermediate known for its antibacterial and antifungal potential (Muriady et al. 2022). Additional minor constituents with antimicrobial activity include phenol, 4-ethenyl-2-methoxy-, 1,4-dioxane-2,6-dione (diglycolli), and 1-cyclohexanone, 2-formyl-6-isopropyl-, all of which contribute to the overall antimicrobial profile. 2-Cyclopentene-1,4-dione and 2-butanone, 1-(acetyloxy)- were also detected and have been previously linked to inhibitory effects on both Gram-positive and Gram-negative pathogens (Sevcikova et al. 2014; Muriady et al. 2022).

The presence of these 13 bioactive metabolites provides a compelling chemical explanation for the moderate antibacterial activity observed in the agar well diffusion assay, particularly against *S. aureus* and *E. coli*. The lack of activity against *S. typhi*, however, may be attributed to its intrinsic resistance mechanisms. *S. typhi* employs active efflux systems such as AcrAB-TolC, passive outer membrane exclusion due to its lipopolysaccharide (LPS) layer, and possibly enzymatic detoxification pathways, each contributing

to reduced intracellular accumulation of antimicrobial agents. These multidrug-resistant properties limit the efficacy of many phytochemicals, especially in the absence of adjuvants that inhibit efflux pumps or enhance membrane permeability (Punchihewage-Don et al. 2024; Zhydetski et al. 2024). Compared to *Liparis resupinata* from the same Gunitir region (Ulum et al. 2023), which exhibits broader antimicrobial activity, including growth inhibition against *S. typhi*, *V. tricolor* showed selective activity, effectively inhibiting *S. aureus* and *E. coli* but not *S. typhi*, likely due to differences in phytochemical composition. *L. resupinata* was rich in fatty acids such as tetradecanoic acid and 9-octadecenoic acid that are known as membrane-disruptive. *V. tricolor* contained a more diverse profile of phenolic and alcohol-based compounds, including 4-butylphenol and chavicol, which may act through oxidative stress mechanisms and protein-target interactions.

Despite its widespread use, the agar well diffusion assay presents inherent limitations in evaluating plant-based antimicrobial extracts, particularly those rich in nonpolar compounds. Terpenoids and fatty acids, commonly found in *V. tricolor*, exhibit poor aqueous solubility and limited agar diffusion, often resulting in an underestimation of their actual bioactivity (Djarot et al. 2025; Romano et al. 2025). Previous investigations on *Piper crocatum*, *Teucrium polium*, and *Napoleona imperialis* demonstrated that although these extracts produced minimal inhibition zones in agar diffusion assays, their true potency was revealed by broth microdilution and time-kill studies, which showed significantly higher antimicrobial activity (Odeyemi and Enweani-Nwokelo 2024; Javadpour et al. 2025). Moreover, broth-based methods offer greater quantitative reliability and are considered the gold standard for antimicrobial susceptibility testing (Haley et al. 2024; Singh et al. 2025). Thus, the antibacterial potential of *V. tricolor*, especially its lipophilic constituents, may be substantially underestimated by diffusion-based techniques and warrants reassessment using more sensitive in vitro models.

Antioxidant activity

The antioxidant potential of the methanolic leaf extract of *V. tricolor*, as determined by the DPPH radical scavenging assay, was classified as strong, with an average IC₅₀ value of 37.25 ppm (Table 3) (Zamzani and Triadisti 2021). The positive control ascorbic acid had an IC₅₀ of 6.65 ppm, consistent with its well-established redox activity in biological systems (Yimcharoen et al. 2019). The DPPH radical scavenging activity of metabolites from *V. roxburghii* showed that gigantol exhibited the strongest antioxidant activity (IC₅₀: 4.16±0.37 ppm), followed by dihydroconiferyl dihydro-p-coumarate (IC₅₀: 12.16±0.55 ppm) and vanillin (IC₅₀: 19.64±0.73 ppm), and syringaldehyde exhibited the weakest effect (Ahmed et al. 2021). The observed antioxidant potential of *V. tricolor* is consistent with its GC-MS profile, which contains abundant phenolic and terpenoid compounds that function through hydrogen atom transfer (HAT), single electron transfer (SET), metal ion chelation, and modulation of oxidative stress signalling pathways (Leopoldini et al. 2011).

Table 3. Antioxidant activity of *Vanda tricolor* leave extract

Concentration (ppm)	% Inhibition	Regression value	IC ₅₀ (ppm)
80	83.11		
60	81.20	$y = 0.7908x + 25.607$	30.85
40	58.24	$R^2 = 0.918$	
20	38.05		
80	76.58		
60	72.78	$y = 0.8239x + 15.467$	41.91
40	47.08	$R^2 = 0.9379$	
20	30.22		
80	82.27		
60	76.32	$y = 0.8987x + 15.285$	38.63
40	51.75	$R^2 = 0.9515$	
20	30.55		
Average			37.13

The GC-MS profiling of *V. tricolor* methanolic leaf extract revealed a chemically diverse spectrum of antioxidant-related compounds. Among the most abundant were phenolic compounds, including phenol, 4-butyl-, chavicol, phenol, 2,6-dimethoxy-4-(2-propenyl)-, and phenol, 2-methoxy-4-(2-propenyl)-. These molecules are well-characterized for their capacity to neutralize reactive oxygen species through hydrogen atom donation and single-electron transfer mechanisms (Mavric-Scholze et al. 2024). In addition, sugar-derived antioxidants such as 1,6-anhydro-β-D-glucopyranose and 5-hydroxymethylfurfural (5-HMF) were also detected, suggesting possible involvement of Maillard-reaction pathways in oxidative stress modulation (Periasamy et al. 2024). Fatty acids, such as hexadecanoic acid (palmitic acid), along with dipropylene glycol and 2,5-furandione derivatives (e.g., citraconic acid), contribute to antioxidant defense through mechanisms, including lipid peroxidation inhibition, metal ion chelation, and redox buffering (Fadzir et al. 2018).

Several metabolites, such as chavicol, possess multifunctional properties, and 6,6-dimethyl-9-methyliden-

2,5,10-undecatrien-1-ol has been implicated in enzyme inhibition, potentially enhancing cellular protection against oxidative damage (Fadzir et al. 2018; Sarma et al. 2018; Chirumamilla et al. 2022). Halogenated and fluorinated compounds (e.g., 1-chloro-1-fluoroethane, fluoroacetylene), although in trace amounts, may introduce subtle redox-modulating effects, though their exact roles remain unclear (Begum and Narasimha 2022). Finally, the detection of oxygenated heterocycles, such as 4H-pyran-4-one, 3-hydroxy-2-methyl- and unsaturated fatty acids, further reinforces the extract's antioxidant complexity. These compounds act through complementary pathways, including free radical scavenging, singlet oxygen quenching, and the upregulation of antioxidant enzymes (Wang et al. 2021; Huchzermeyer et al. 2022).

Molecular docking

The molecular docking of three compounds against the Bcl-2 protein (PDB ID: 8HTS) revealed varying levels of binding affinity and interaction (Figure 3). Among them, 4-butylphenol exhibited the most favorable binding with a binding energy of -5.50 kcal/mol and an estimated inhibition constant (K_i) of 92.90 μM, and ligand interacted with the protein formed two hydrogen bonds with Arg146(A) and Asp140(A), at a distance of 2.90 and 2.91 Å, and interacted with other residues such as Glu136(A), Leu137(A), Phe138(A), and Arg139(A), indicating a stable interaction within the binding pocket. In contrast, 2-(2-hydroxypropoxy)-1-propanol showed a weaker binding affinity, with a binding energy of -3.08 kcal/mol and a higher K_i of 5.55 mM, and formed 3 hydrogen bonds in the residues of Leu119(A) and Ser116(A) at a distance of 3.14, 2.78, and 2.53 Å. Similarly, acetic acid displayed a low binding energy of -3.41 kcal/mol and a K_i of 3.19 mM, indicating poor binding affinity. It formed 2 hydrogen bonds with Arg183(A) and His184(A) at a distance of 2.92 and 2.95 Å (Table 4). Therefore, based on the results, 4-butylphenol is the most promising candidate due to its stronger binding energy, favorable hydrogen bonding, and micromolar-level inhibition constant.

Table 4. The docking scores, represented as E total, for the interaction between the Bcl-2 protein and the selected ligands

Compound	Binding energy (kcal/mol)	Estimated inhibition constant, K _i	H-bond amino acids	Bonding	Distance (Å)	Other amino acids
2-(2-Hydroxypropoxy)-1-propanol	-3.08	5.55 mM	Leu119(A)	N:O	3.14	Leu121(A), Arg164(A), Glu160(A), His120(A), Gln118(A), Ser117(A)
			Ser116(A)	O:O	2.78	
				O:O	2.53	
4-Butylphenol	-5.50	92.90 μM	Arg146(A)	N:O	2.91	Glu136(A), Leu137(A), Phe138(A), Arg139(A)
			Asp140(A)	N:O	2.90	
Acetic Acid	-3.41	3.19 mM	Arg183(A)	N:O	2.92	-
			His184(A)	N:O	2.95	

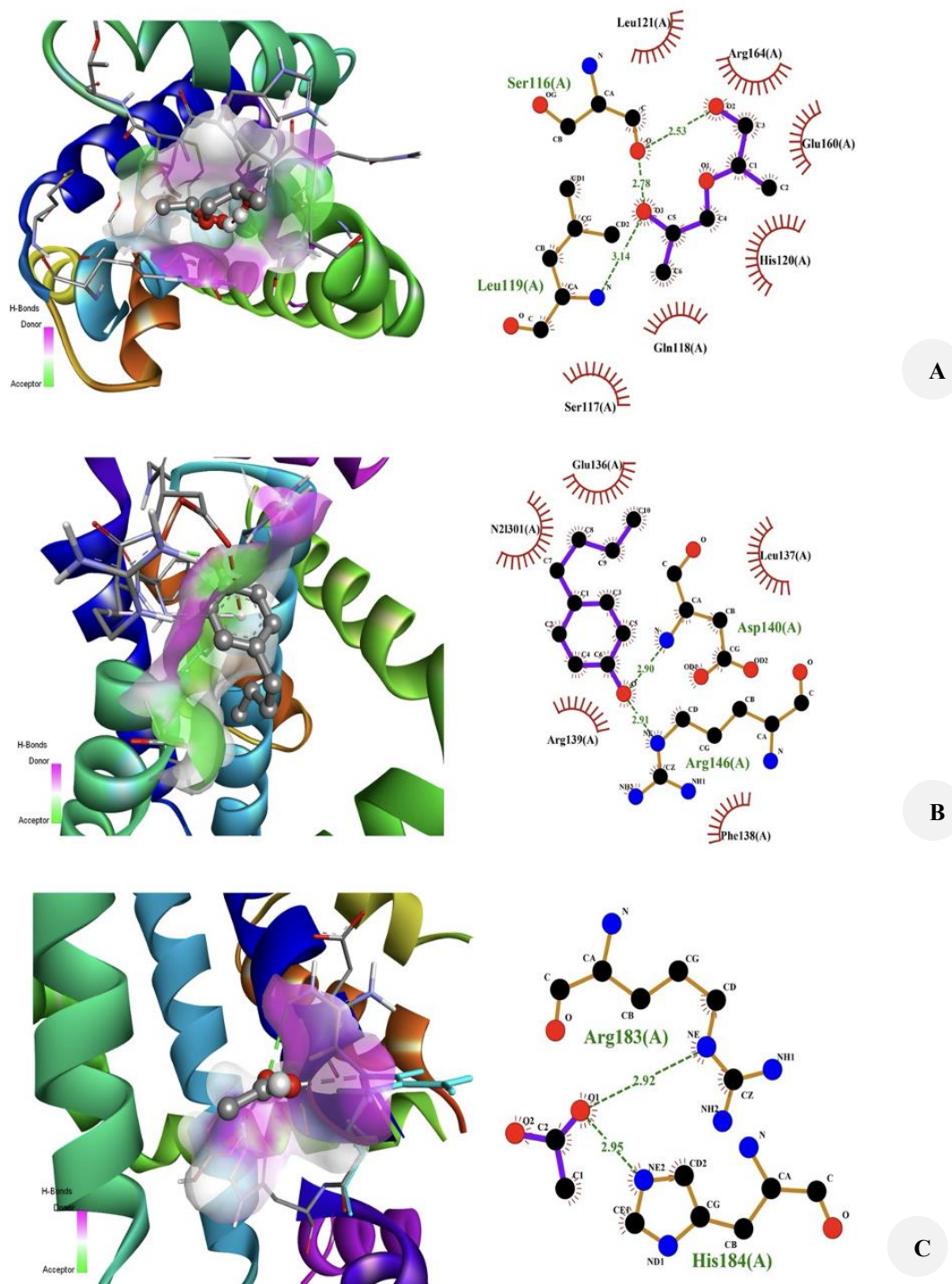


Figure 3. 3D and 2D intermolecular interactions between selective inhibitors. A. 2-(2-Hydroxypropoxy) propan-1-ol and Bcl-2 protein, B. 4-Butylphenol and Bcl-2 protein, C. Acetic Acid and Bcl-2 protein

In conclusion, this study presents the first comprehensive analysis of the phytochemical composition and the potential pharmacological activity of *V. tricolor* from the Gumitir region. GC-MS analysis revealed 48 secondary metabolites, with 4-butylphenol identified as a dominant compound exhibiting notable antioxidant and anticancer potential, as determined by molecular docking. Antibacterial

evaluations demonstrated moderate, concentration-dependent inhibition against *S. aureus* and *E. coli*, attributed to synergistic interactions among phenolics and terpenoids. The strong antioxidant potential and moderate antibacterial activity suggest therapeutic relevance, particularly for compounds such as 4-butylphenol, which showed high affinity for the antiapoptotic protein Bcl-2 in molecular

docking. These findings support the potential of *V. tricolor* as a source of lead compounds for the development of natural drugs. 4-Butylphenol may be investigated in vivo for its potential anticancer properties due to its strong binding affinity to the Bcl-2 protein. Conservation strategies for *V. tricolor* in Gunitir should prioritize habitat protection and sustainable harvesting to safeguard its long-term availability. Further research is required, including bioassay-guided fractionation, in vivo pharmacological testing, toxicity profiling, and ecological sustainability assessments, to validate the efficacy, safety, and applicability of its bioactive constituents in pharmaceutical contexts.

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Supplementary Table 1. Complete phytochemical profile of *V. tricolor* identified by GC-MS and bioactive potencies

Peak Number	Area (%)	Compound	Group	Bioactivity
37	7,57	Phenol, 4-butyl-	Phenol	Antioxidant (Shakira et al. 2022)
21	6.70	1-Propanol, 2-(2-hydroxypropoxy)- (CAS) 2-(2-	Alcohol	Antioxidant, antimicrobial (Chirumamilla et al. 2022)
7	6.09	Acetic acid (CAS) Ethylic acid	Organic acid	Antimicrobial (Meiliana et al. 2022)
42	6.03	Phenol, 2,6-dimethoxy-4-(2-propenyl)- (CAS) 4-allyl-2,6-Phenol	Phenol	Antioxidant (Setyati et al. 2024)
46	5.36	Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl	Ketone	Anticancer (Gangadharan et al. 2024)
40	5.23	1,6-anhydro-beta-d-glucopyranos	Monosaccharide	Antioxidant and anti-inflammatory (Azizov et al.2022)
29	5.01	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	Furan	Antioxidant (Unno et al. 2024)
33	4.42	Chavicol	Phenol	Antioxidant and antifungal (Sarma et al. 2018)
22	4.36	2-Nonanone (CAS) methyl heptyl ketone	Ketone	Anti-inflammatory, non-central analgesic, antirheumatic agent (Subramanian et al. 2020)
20	4.27	2-Propanol, 1,1'-oxybis- (CAS) dipropylene glycol	Alcohol	Antioxidant, antimicrobial (Chirumamilla et al. 2022)
39	4.16	Ethanone, 1-(4-hydroxy-3-methoxyphenyl)- (CAS) acetovanillone	Ketone	Anticancer (Gangadharan et al. 2024)
43	4.06	Pentadecanoic acid, 14-methyl-, methyl ester	Sat. Fatty acid methyl ester	Antibacterial (Fadzir et al. 2018)
48	3.55	Hexadecanoic acid (CAS) Palmitic acid	fatty acid	Antioxidant, antimicrobial, anti-inflammatory, hepatoprotective (Fadzir et al. 2018)
38	3.52	4-methyl-2,5-dimethoxybenzalde	Benzaldehyde	Antimicrobial, antioxidant, anti-inflammatory, analgesic (Chirumamilla et al. 2022)
41	3.44	6,6-dimethyl-9-methyliden-2,5,10-U	Ketone	Antioxidant and enzyme activity inhibitor (Jaradat 2021)
8	3.25	2-propanone, 1-hydroxy- (CAS) Acetol	Ketone	Antibacterial and antifungal (Muriady et al. 2022)
45	2.87	L-serine, O-(phenylmethyl)-	amino acid	-
19	2.10	Ethanol, 2,2'-oxybis- (CAS) diethylene glycol	Alcohol	Antimicrobe (Dugan et al. 2024)
26	2.01	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	Pyranone	Antioxidant (Yu et al. 2009)
16	1.67	1,3-cyclopentanedione, 4-butyl- (CAS) 4-N-B	Ketone	-
47	1.59	Oxiranecarboxylic acid, 3-methyl-3-phenyl-, e	Ester	Anti-inflammatory, antioxidant (Yu et al. 2009)
44	1.56	2,4-Hexadienedioic acid, 3,4-diethyl, dimethyl ester, (Z,Z)-	Ester	Antioxidant, cholesterol-lowering, hemolysis inhibitor (Suryowati et al. 2015)
34	1.39	methyl ester of 5-nitro-2-oxocyclooctancarboxylic	Ester	-
32	1.24	Phenol, 2,6-dimethoxy- (CAS) 2,6-dimethoxy	Phenol	Antioxidant (Maulina and Nurtahara 2020)
30	1.20	Phenol, 4-ethenyl-2-methoxy-	Phenol	Antifungal, anti-inflammatory, antispasmodic (Clarke 2008)
4	1.10	Carbamic acid, monoammonium salt (CAS) A	inorganic nitrogen	Key compound in urea synthesis (Komarayati et al. 2018)
24	0.75	Oxetane, 2-propyl- (CAS) 2-N-propyl-OX	Ether	Antioxidant (Doner et al. 2023)
27	0.58	2-Propanamine, N-methyl-N-nitroso- (CAS) Isopropyl Methyl Nitrosamine	Nitrosamine	-
17	0.53	2,5-furandione, 3-methyl- (CAS) Citraconic acid	Furan	Antioxidant (Chaves et al. 2012)
11	0.48	2-furancarboxaldehyde (CAS) furfural	Furan	Anti-inflammatory (Obaidullah et al. 2022)
23	0.39	4H-pyran-4-one, 3-hydroxy-2-methyl- (CAS)	Pyrone	Antioxidant, antimicrobial, antifungal (Uyanik et al. 2009)
3	0.36	1,4-dioxane-2,6-dione (CAS) diglycolli	Ether	Antifungal (Fei et al. 2023)
1	0.33	Cyclopropane, 1,1-dibromo-2-chloro-	cyclopropane	Antioxidant (Batubara et al. 2022)

		2-fluoro-		
25	0.33	1-P-menthen-8-yl acetate	Terpenoid	-
36	0.28	1-Cyclohexanone, 2-formyl-6-isopropyl-3-methyl-	Terpenoid	Antimicrobial (Nguyen et al. 2019)
13	0.28	2-furanmethanol (CAS) furfuryl alcohol	Furan	Carcinogenic and allergenic (Kobzar et al. 2023)
10	0.23	Propanoic acid, 2-oxo-, methyl ester (CAS) Methyl pyruvate	Ester	-
12	0.21	2-heptanone (CAS) heptan-2-one		-
18	0.20	Methacrylic acid, 3-methyl-2-methylene-3-butenyl ester		-
28	0.20	2-Pentene, 4,4'-oxybis- (CAS) ether, DI-3-P		-
2	0.19	Ethane, 1-chloro-1-fluoro- (CAS) 1-chloro-1-	Artefact	Antioxidant (Begum and Narasimha 2022)
14	0.18	2-cyclopentene-1,4-dione	Ketone	Antifungal and antibacterial (Sevcikova et al. 2014)
6	0.16	Pentanal (CAS) n-pentanal	Aldehyde	-
5	0.15	Ethyne, fluoro (CAS) fluoroacetylene	Alkyne	Antioxidant (Ramos et al. 2012)
35	0.15	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	unsaturated fatty acid	Antioxidant and anticancer (Elwekeel et al. 2023)
15	0.09	Dihydro-3-methylene-5-methyl-2-furanone	Terpenoid	High antioxidant activity (Fei et al. 2023)
31	0.09	Phenol, 2-methoxy-4-(2-propenyl)- (CAS) Eugenol	Phenol	Antioxidant (Ulanowska and Olas 2021)
9	0.08	2-butanone, 1-(acetyloxy)- (CAS) 1-acetoxy-	Ketone	Antimicrobial (Muriady et al. 2022)