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TERMITICIDAL AND BIOCHEMICAL ACTIVITY OF VITEX NEGUNDO LEAF EXTRACT AGAINST SUB-TERRANEAN TERMITE, ODONTOTERMES HORNI

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ABSTRACT
Subterranean termites are destructive pests causing heavy economic losses in agriculture, forestry and wooden buildings. The present study was carried out to evaluate the termiticidal potential of Vitex negundo L. leaf extract against Odontotermes horni by toxicity, phytochemical and biochemical analyses. Ethyl acetate leaf extract was prepared by Soxhlet extraction, and its toxicity was tested by the filter paper method. Probit analysis showed a concentration and time dependent toxicity with LC_{50} of 2.589% and LT_{50} of 23.10 h. The GC–MS analysis revealed 15 phytochemical constituents. Estragole (11.34%) was the major compound followed by linalool (2.60%), isoborneol (2.36%) and eucalyptol (1.89%). The extract showed 0.473 ± 0.003 mg GAE g-1 total phenolics and 2.29 ± 0.02 mg RE g-1 total flavonoids indicating the presence of biologically active secondary metabolites. Biochemical studies revealed that V. negundo treatment significantly decreased the protein content from 957.00 ± 7.76 to 932.00 ± 8.12 μg mL^{-1} and inhibited acetylcholinesterase (27.18%) and mixed-function oxidase activities (37.50%), suggesting disruption of neural transmission and detoxification pathways. The overall results suggested that the V. negundo has a prominent termiticidal activity which was due to its various phytochemical constituents and physiological effects on termites.

KEYWORD: Vitex negundo; Odontotermes horni; Botanical insecticide; GC–MS; Termiticidal activity; Detoxifying enzymes.

1. INTRODUCTION

Vitex negundo L. (Verbenaceae), commonly referred to as Nirgundi or the five-leaved chaste tree, is one of the most studied species within the Genus Vitex. Recent studies have highlighted V. negundo due to its unexplored metabolites, which demonstrate a range of biological activities, including antioxidant, antibacterial, and insecticidal effects. Termites are among the most problematic pests for plants, trees, and wooden structures, causing significant damage to crops and urban infrastructure. A variety of control strategies are implemented to protect buildings and crops from termite infestations. There has been growing interest among scientists in the chemical compounds and biological properties of pesticides derived from natural sources to mitigate the issues associated with synthetic chemicals. The pesticidal potential of V. negundo against insect pests of economic importance has been demonstrated in several studies. Leaf extracts and essential oils have been found to be highly toxic to mosquito larvae, stored-product insects, lepidopteran pests and phytophagous mites. The richness of terpenoids, flavonoids and other volatile constituents is mainly attributed to the insecticidal activity of the plant, which interferes with insect nervous and metabolic systems (Isman 2006). Recent studies also reported significant acaricidal and ovicidal activity of V. negundo leaf extracts against the tea red spider mite, Oligonychus coffeae (Ghosh et al., 2023) and larvicidal activity against Helicoverpa armigera (Matharu and Mehta 2024), pointing to its broad-spectrum pesticidal potential. Botanicals have been utilized for pest control for a long time. These compounds provide numerous environmental benefits. Nevertheless, their application during the 20th century has been quite limited compared to other biological control strategies for pests and diseases. Advances in the comprehension of plant allelochemical activity mechanisms present fresh opportunities for employing these substances in safeguarding crops. Therefore, the present study was carried out to evaluate the toxicity of V. negundo leaf extract in ethyl acetate against O. horni, characterize its phytochemical constituents using GC–MS analysis, and determine its effects on major detoxification enzymes. The findings of this study will contribute to the development of environmentally friendly botanical termiticides for integration into integrated termite management programmes.

2. MATERIALS AND METHODS

2.1. Collection of Leaves and Preparation of V. negundo Leaf Extract

Fresh leaves of V. negundo L. were collected from the surroundings of Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The collected leaves were thoroughly washed under running tap water and shade-dried at room temperature for 7–10 days. The dried leaves were ground into a fine powder using a stainless-steel blender and stored in airtight zipper bags at 4°C until extraction. Thirty grams of dried leaf powder was placed in a cellulose thimble of a Soxhlet extraction apparatus (Techno Pvt. Ltd., India), and 300 mL of ethyl acetate was added to the round-bottom flask. Extraction was performed for 10–12 h at the boiling point of ethyl acetate (approximately 77°C). The extract was filtered and concentrated under reduced pressure using a rotary vacuum evaporator (Heidolph, Germany) at 45°C for approximately 30 min to obtain the crude extract. The concentrated extract was stored at 4°C until further use in bioassays and phytochemical analyses (Gundakanal et al., 2024).

2.2. Studies on Toxicity of V. negundo Leaf Extract against O. horni

The toxicity of the ethyl acetate leaf extract of V. negundo was evaluated under laboratory conditions using the filter paper method (Acda 2014) against O.horni. Different concentrations of the crude extract were prepared by dissolving the extract in ethyl acetate. Whatman No. 1 filter paper discs were placed in Petri dishes and uniformly treated with the respective concentrations of the extract for approximately 30 s, followed by air-drying for 10 min at room temperature. Based on preliminary screening experiments, the extract was tested at concentrations ranging from 0.25% to 7.00%. Ten healthy worker termites were introduced into each Petri dish, and each treatment was replicated three times. Control filter papers were treated with solvent alone without the plant extract. The experimental units were maintained in a growth chamber at 25 ± 2°C and 85 ± 5% relative humidity. Termite mortality was assessed after 24 h of exposure. Individuals that failed to respond to gentle probing were considered dead. Mortality data were corrected using Abbott's formula (Abbott 1925). The median lethal concentration (LC_{50}) and median lethal time (LT_{50}) values were estimated by probit analysis (Finney 1971).

2.3. Studies on impact of V. negundo on protein and detoxifying enzyme activity

2.3.1. Preparation of whole body homogenates

Enzyme extracts of each population were prepared by homogenizing the whole body of worker of both treated (LC_{30}) and untreated termites in 1.5 ml of 0.1 M ice-cold sodium phosphate buffer. The homogenized workers were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was centrifuged again at 10,000 rpm for 15 min at 4 °C.

2.3.2. Assessement of total proteins, AchE and MFO

The activities of total protein, Acetylcholineesterase (AChE), mixed function oxidase (MFO) were assessed in the enzyme samples. The total protein content was determined using the Coomassie Brilliant Blue G-250 dye-binding method, with bovine serum albumin (BSA) serving as the standard (Bradford 1976). The optical density (OD) was measured at 595 nm using a spectrophotometer (Eppendorf AG, Germany).

AChE activity was calculated using the colorimetric method (Ikezawa and Taguchi 1981). In the reaction mixture 0.1 ml enzyme extract, 2.6 ml phosphate buffer (0.1 M, pH 7.5), 0.1 ml acetylcholine iodide substrate, and 0.1 ml DTNB reagent were added. After incubation for 10 min at room temperature, absorbance was recorded at 412 nm using a spectrophotometer and was described as nmol/min/mg protein (Nasser et al. 2024).

MFO activity was determined as given by Yasoob et al. (2018) using p-nitroanisole as substrate. The reaction mixture included 100 µl enzyme extract, 1 ml of 50 mM p-nitroanisole and 1.7 ml of 50 mM Tris-HCl buffer and pre-incubated at 34 °C for 3 min. The reaction was initiated by adding of 200 µl of 10 mM NADPH and further incubated at 30 °C for 10 min. The formation of p-nitrophenol was followed at 405 nm with a spectrophotometer. The enzyme activity was expressed for p-nitrophenol formed by using an extinction coefficient of 3.32 mM^{-1} cm^{-1}.

2.4. GC-MS Analysis of Leaf Extracts of V. negundo

GC–MS analysis of V. negundo ethyl acetate leaf extract was carried out on an Agilent GC–MS system equipped with HP-5MS UI capillary column (Agilent Technologies, Germany). Helium was used as carrier gas, at a constant flow rate of 1 mL min-1. The extract was injected in splitless mode (1 µL) with an injector temperature of 250 °C. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV with a scan range of m/z 40–450. Individual phytochemical constituents were identified by comparing their retention times and mass spectra with those available in the National Institute of Standards and Technology (NIST 14) mass spectral library (Gomathi et al., 2015).

2.5. Molecular Docking

The amino acid sequence of cytochrome monooxygenase from Odontotermes sp. was retrieved from NCBI and modeled in SWISS-MODEL for homology-based 3D structure prediction (Waterhouse et al., 2018). The model was evaluated using SWISS-MODEL's structural quality parameters and the backbone φ and ψ angles in the Ramachandran plot. Blind molecular docking of linalool, which was retrieved from PubChem, was then carried out with CB-Dock2. CB-Dock2 prepared the receptor and ligands, detected potential binding cavities, and conducted docking with AutoDock Vina (Liu et al., 2022). The top-ranked pose for each ligand was chosen based on the most negative docking score and analyzed for binding-pocket interactions.

2.6. Statistical Analysis

All experiments were carried out in a completely randomized design (CRD) with three replications. Mortality data were corrected by applying the formula of Abbott (Abbott, 1925). Median lethal concentration (LC_{50}), median lethal time (LT_{50}), 95% fiducial limits, chi-square (χ^2) values, regression equations and coefficients of determination (R^2) were estimated using probit analysis by Finney's method (1971). Data are presented as mean

± standard deviation (SD). The biochemical data (protein, acetylcholinesterase and mixed function oxidase activities) and mortality percentages were analyzed using one-way analysis of variance (ANOVA) and treatment means were separated by Tukey's honestly significant difference (HSD) test at 5% probability. The coefficient of variation (CV), critical difference (CD) at 5% and probability (p) values were calculated to test for statistical significance at $P < 0.05$. Statistical analyses were performed using IBM SPSS Statistics version 26.0.

3. RESULTS

3.1. Studies on Toxicity of V. negundo Leaf Extract against O. horni

The median lethal concentration (LC_{50}) and median lethal time (LT_{50}) of V. negundo leaf extract against O. horni were determined by probit analysis. The estimated LC_{50} value was 2.589% with 95% fiducial limits between 2.260% and 2.950%, which is the concentration needed to kill 50% of the termite population. The regression equation $y = 2.7322x + 3.8712$ with a high coefficient of determination ($R^2 = 0.99$) was fitted for the concentration–mortality relationship. The chi-square value ($\chi^2 = 15.10$) indicated a good fit of the probit regression model to the observed mortality data (Table 1). Similarly, probit analysis of the time-mortality response indicated an LT_{50} of 23.10 h, with 95% fiducial limits of 19.92–26.79 h (Table 2). The time–mortality relationship was signified by the regression equation $y = 2.9402x + 0.9908$, with an excellent coefficient of determination ($R^2 = 0.991$). The low chi-square value ($\chi^2 = 1.06$) additionally established the consistency and goodness of fit of the probit model, representing that V. negundo leaf extract exhibited toxicity against O. horni.

3.2. Studies on impact of V. negundo on protein and detoxifying enzymes activity

The effect of V. negundo leaf extract on protein content and activities of detoxifying enzymes of O. horni are depicted in Table 3. Total protein content of treated termites was 932.00 ± 8.12 µg mL-1 which was significantly lower than that of the untreated control (957.00 ± 7.76 µg mL-1) indicating adverse effect of the extract on the protein metabolism. Activities of AChE and MFO in O. horni after exposure to LC_{30} concentration of V. negundo leaf extract is presented in Table 4. V. negundo treated samples showed reduced activity of AChE corresponding to 27.18% inhibition compared to untreated control. Exposure to the extract reduced MFO activity by 37.0% similarly.

3.3. Metabolomic profiling of V. negundo

GC–MS analysis of V. negundo leaf extract showed 15 phytochemical constituents, primarily belonging to the classes of monoterpenes, oxygenated monoterpenes, and sesquiterpenes (Table 4; Fig.1). Among the identified compounds, estragole was the most abundant constituent (11.34% of total chromatographic peak area) followed by linalool (2.60%), isoborneol (2.36%) and eucalyptol (1.89%). Other compounds identified in a relatively lower percentage were D-limonene (0.69%), α-terpineol (0.56%), o-cymene (0.25%), bornyl acetate (0.25%), camphor (0.24%), camphene (0.18%), caryophyllene (0.16%), γ-terpinene (0.13%), β-clovene (0.08%), terpineol (0.04%) and α-phellandrene (0.01%). The present study revealed that the abundance of oxygenated monoterpenes and phenylpropanoids, especially estragole, linalool, isoborneol, and eucalyptol, suggested that these bioactive compounds may be responsible for the insecticidal activity of V. negundo leaf extract against O. horni (Fig. 2).

3.4. Molecular Docking

The modeled protein was verified using a Ramachandran plot, which showed that most residues of the modeled Odontotermes cytochrome monooxygenase occurred within favored or additionally allowed conformational regions, indicating acceptable backbone geometry for molecular docking. Linalool showed a docking score of -5.2 kcal mol^{-1} within a cavity by Ile4, Phe16, Leu17, Cys20, Phe63, Leu137, Ile140, Thr141, Ile142, and His143 (Fig 3).

4. DISCUSSION

The probit analysis of LC_{50} and LT_{50} values showed that ethyl acetate leaf extract of V. negundo was toxic to O. horni in a concentration- and time-dependent manner. Similar dose-dependent mortality has been widely reported for botanical insecticides, whose efficacy usually increases with increasing concentration and exposure period due to the cumulative action of multiple bioactive constituents (Isman, 2020). Similar toxicity has also been reported for Vitex essential oils against stored-product insects indicating that the toxic action of Vitex species is mediated through disruption of essential physiological processes and rapid knockdown effects (Sahaf et al., 2008). Also, botanical extracts have been identified as potential alternatives to synthetic termiticides due to their multiple modes of action, lower persistence in the environment and less risk of resistance development. (Verma et al., 2009).

The marked decrease in the total protein content of the treated termites may indicate disruption of protein metabolism possibly due to an increased protein degradation or increased utilization of amino acids to meet the energy demands for stress and detoxification. Similar decreases in protein levels have been reported for insects exposed to botanical insecticides and xenobiotics which reflect a physiological stress and metabolic adaptation (Després et al., 2007). The inhibition of AChE and MFO activities was not significantly different from that of the untreated control. However, the decreases in activity observed indicate that V. negundo may interfere with both neural transmission and detoxification processes. Acetylcholinesterase terminates cholinergic nerve impulses by hydrolyzing acetylcholine, and several monoterpenoids and constituents of essential oils have been reported to inhibit this enzyme, causing impaired nerve impulse transmission, paralysis, and insect mortality (Regnault-Roger et al., 2012; Yeom et al., 2015). Similarly, mixed-function oxidases, particularly cytochrome P450 monooxygenases, are involved in the detoxification of xenobiotics and plant secondary metabolites, and inhibition of these enzymes can decrease the ability of insects to metabolize toxic compounds (Li et al., 2007).

The phytochemical characterization by GC–MS revealed the extract to be rich in oxygenated monoterpenes and phenylpropanoids, estragole, linalool, isoborneol and eucalyptol being the major constituents. Previously, these compounds have been reported to have fumigant, repellent, insecticidal and neurotoxic activities against a variety of insect pests. The essential oils rich in linalool, eucalyptol, β-caryophyllene, and α-terpineol have been reported to act on the insect nervous and respiratory systems via multiple physiological targets (Dharmagadda et al., 2005; Zheng et al., 2009). Eucalyptol (1,8-cineole) was also found to possess strong fumigant and insecticidal activity against Tribolium confusum (Tine et al., 2023). Also, camphor, isoborneol, γ-terpinene, and phellandrene have been associated with neurotoxic and behavioral deterrent effects whereas o-cymene, bornyl acetate and camphene have been related to insect repellency and toxicity (Kordali et al., 2006; Ebadollahi et al., 2021). Hence the termiticidal activity observed in the present investigation is likely to be due to the combined action of these volatile constituents (Qasim et al., 2024).

The molecular docking study also supported the possible mode of action of V. negundo as it showed a favorable interaction between linalool and cytochrome monooxygenase of Odontotermes sp. with a docking score of −5.2 kcal mol^{-1}. Cytochrome P450 monooxygenases are one of the major detoxification enzyme systems in insects and are involved in the metabolism of synthetic insecticides and plant derived allelochemicals (Li et al., 2007). The interaction between linalool and the predicted binding cavity suggests that this compound may inhibit the catalytic function of cytochrome monooxygenase, resulting in a decrease in the detoxification capability of O. horni and an increase in the toxicity of the extract. Although molecular docking only gives computational evidence for ligand–protein interactions, the docking results, together with the observed toxicity and enzyme inhibition, suggest that linalool may be responsible for the insecticidal activity of V. negundo. Further biochemical validation of this proposed mechanism of action will require enzyme inhibition assays and gene expression studies.

5. CONCLUSION

The present study showed significant termiticidal activity of ethyl acetate leaf extract of V. negundo against O. horni suggesting its potential as an eco-friendly substitute to the conventional synthetic termiticides. The extract was highly concentrated and time-dependent in its toxicity as indicated by the LC_{50} and LT_{50} values calculated through probit analysis. GC–MS profiling revealed the presence of a wide range of bioactive phytochemicals, predominantly oxygenated monoterpenes and phenylpropanoids, with estragole, linalool, isoborneol and eucalyptol being the major constituents which may be responsible for the observed insecticidal activity. Further biochemical studies revealed a reduction in total protein level and inhibition of acetylcholinesterase and mixed-function oxidase activities. Thus, the extract may have an effect on neural transmission and detoxification mechanisms in termites. The results were supported by molecular docking that showed a good interaction between linalool and cytochrome monooxygenase that could be a possible molecular target for the insecticidal activity of the extract. To summarize, toxicity assessment, phytochemical profiling, biochemical analysis and molecular docking together provide convincing evidence for promising application of V. negundo as an eco-friendly botanical termiticide. The future studies should be focused on purification of the active constituents, validation thru enzyme inhibition and gene expression studies, formulation development, field evaluation and assessment of non-target safety to facilitate its use in integrated termite management programs.

Table 1: Probit analysis studies on contact toxicity of V. negundo leaf extract against O. horni (LC_{50})

LC_{50} (ppm)	95% Fiducial limit of LC_{50} (ppm)		χ2 value	Probit fitted regression equation	R^2
	LL	UL			
2.58900	2.2600	2.9500	15.1027	y =2.7322x + 3.8712	0.99

Values are mean ± SD. Different superscript letters within each extract indicate significant differences by Tukey HSD. CD, CV and p value are placed as the last three rows below.

Table 2: Probit analysis studies on toxicity time of V. negundo leaf extract against O. horni (LT_{50}).

Extracts	LT_{50} (hours)	95% Fiducial limit of LT_{50} (hours)		χ2 value	Probit fitted regression equation	R^2
		LL	UL			
V. negundo	23.099078	19.915334	26.791788	1.05523	y = 2.9402x + 0.9908	0.991

Values are mean ± SD. Different superscript letters within each extract indicate significant differences by Tukey HSD. CD, CV and p value are placed as the last three rows below.

Table 3: Protein and detoxifying enzyme activity of V. negundo treatment and control

Treatment	Protein activity (µg mL^{-1})	Protein inhibition (%)	AChE apparent activity	AChE inhibition (%)	MFO apparent activity	MFO inhibition (%)
V. negundo	932.00 ± 8.12	2.61	0.000150 ± 0.000080	27.18	0.000800 ± 0.000400	37.50
Control	957.00 ± 7.76	0.00	0.000206 ± 0.000223	0.00	0.001000 ± 0.000500 a	0.00

Table 4: GC-MS results of the extract V. negundo

COMPOUND NAME	RETENTION TIME	COMPOUND AREA	FORMULA	AREA PERCENT	MATCH FACTOR
Camphene	4.8320	177391.8	C10H16	0.183085	94.2
.alpha.-Phellandrene	5.7938	8410.7	C10H16	0.008681	84.0
o-Cymene	6.0183	244707.2	C10H14	0.252561	89.4
D-Limonene	6.0870	664049.3	C10H16	0.685361	78.6
Eucalyptol	6.1318	1828230	C10H18O	1.886904	91.8
.gamma.-Terpinene	6.5663	123597.2	C10H16	0.127564	93.8
Terpineol	7.0423	38853.5	C10H18O	0.0401	75.5
Linalool	7.1987	2519009	C10H18O	2.599853	99.4
Camphor	7.9433	235903.9	C10H16O	0.243475	95.3
Isoborneol	8.1380	2290805	C10H18O	2.364325	97.2
.alpha.-Terpineol	8.6467	546359.6	C10H18O	0.563894	95.7
Estragole	8.7423	10989213	C10H12O	11.3419	99.6
Bornyl acetate	10.0726	244406.7	C12H20O2	0.252251	94.9
Caryophyllene	11.9468	158711.9	C15H24	0.163806	94.5
.beta.-Clovene	11.9495	76350.5	C15H24	0.078801	62.5

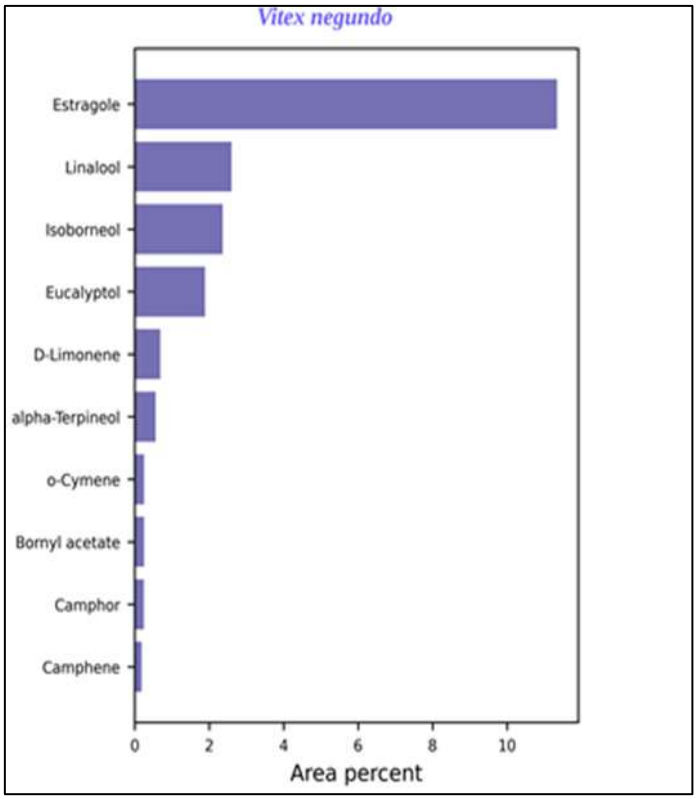

Fig 1: Representation of calculated area percent of top metabolites

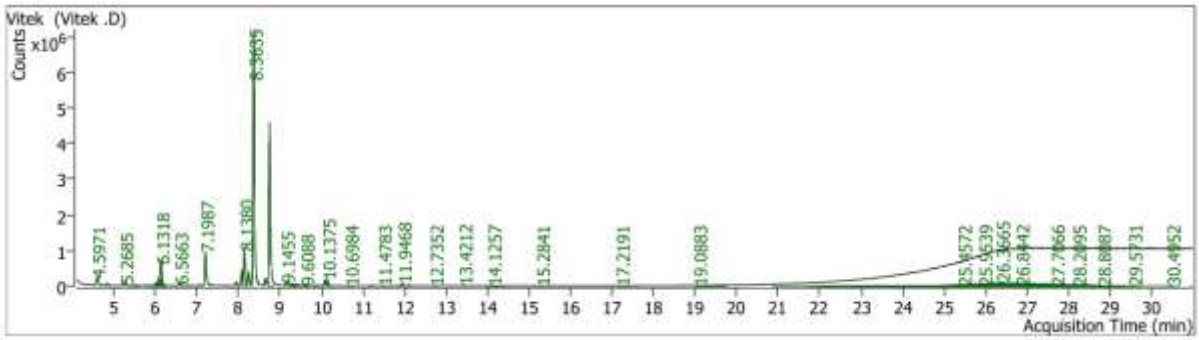

Fig. 2: Chromatogram of the GC-MS of V. negundo

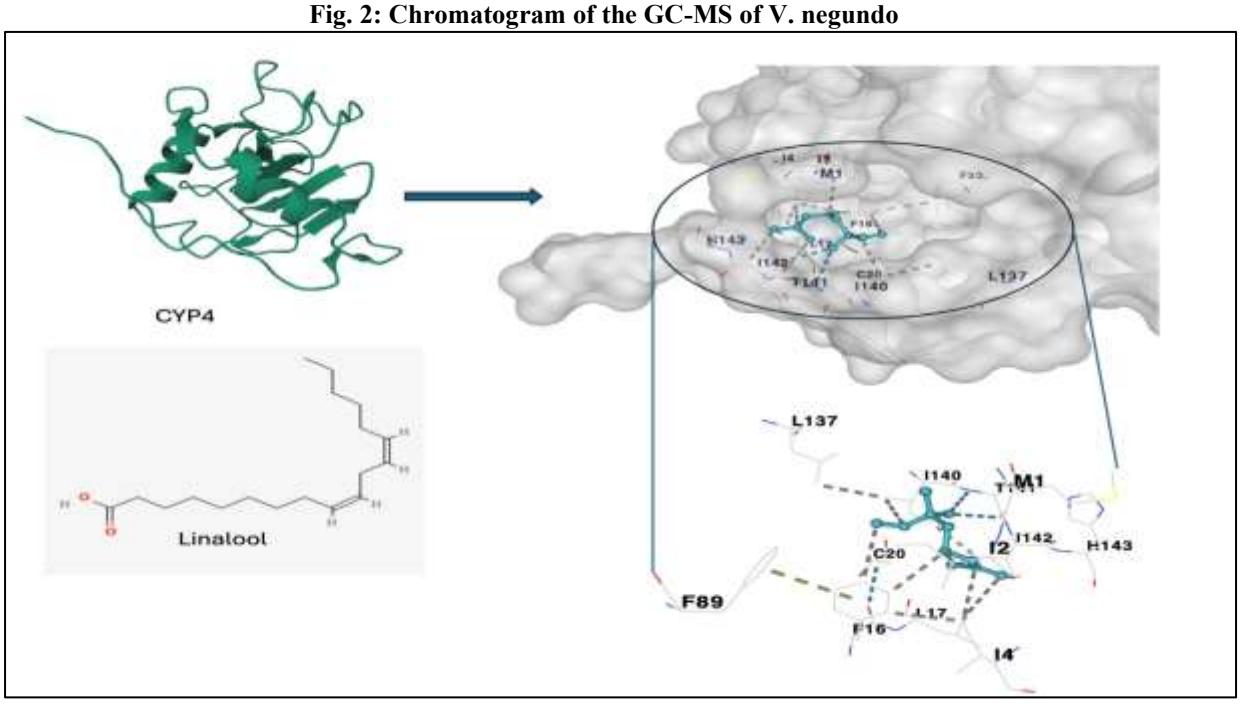

Fig. 3: Docking interaction between Cyt450 and ligand linalool

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Authors Contribution

SB and KP conceptualized, investigated, visualized and wrote the original manuscript. KP and MM validated, reviewed and edited the manuscript. KP, MM, DU validated, wrote, reviewed and approved the manuscript.

Declarations

Conflict of interest

The authors declare no conflict of interest**.**

Ethical approval, consent to participate, consent to publish

This declaration in not applicable

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