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Phytochemical Characterisation and *In Vitro* Anticancer Potential of *Combretum indicum* Leaf Aqueous Extract Against A549 Cells

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Abstract

Background: Lung cancer is a major cause of cancer-related morbidity and mortality worldwide. Plant-derived therapeutics have attracted attention as safer, multitarget candidates for cancer management. **Aim:** This study evaluated the phytochemical profile, antioxidant activity, selective cytotoxicity, and docking-based anti-cancer potential of the aqueous leaf extract of *Combretum indicum* in A549 cells. **Methods:** Qualitative and quantitative phytochemical analyses were performed, followed by antioxidant assays [2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, nitric oxide, lipid peroxidation, and Ferric Reducing Antioxidant Power (FRAP)], Gas Chromatography-Mass Spectrometry (GC-MS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays in A549 and Vero cells, and molecular docking against Epidermal Growth Factor Receptor (EGFR), Anaplastic Lymphoma Kinase (ALK), C-Ros Oncogene 1 Receptor Tyrosine Kinase (ROS1), Phosphatidylinositol 3-Kinase (PI3K), and Mesenchymal-Epithelial Transition Receptor (MET). **Results:** The extract contained alkaloids, flavonoids, tannins, carbohydrates, proteins, and sterols. Flavonoids and tannins were present at high levels. *C. indicum*- Aqueous Leaf Extract (CI-ALE) showed concentration-dependent antioxidant activity and selective cytotoxicity against A549 cells, with an IC_{50} of 163.11 µg/mL. More than 80% of Vero cells remained viable at the highest tested concentration. GC-MS detected 38 compounds; phytol, n-hexadecanoic acid, octahydro-2(1H)-quinoline, and stigmasterol were prominent. Docking analysis showed that stigmasterol had the strongest binding affinity across major non-small cell lung cancer targets. Conclusion: The aqueous leaf extract of *C. indicum* demonstrated promising antioxidant and anti-cancer potential. Further mechanistic and *in vivo* evaluation is warranted.

Major Findings: CI-ALE showed selective cytotoxicity toward A549 cells, strong antioxidant activity, and multi-target docking interactions. Stigmasterol emerged as the most promising lead phytoconstituent against EGFR, ALK, ROS1, PI3K, and MET.

Keywords: A549 Cells, Anti-cancer Activity, Antioxidant Activity, *Combretum indicum*, Molecular Docking, Non-Small Cell Lung Cancer

Abbreviations: EGFR – Epidermal Growth Factor Receptor; ALK – Anaplastic Lymphoma Kinase; ROS1 – ROS Proto-Oncogene 1 Receptor Tyrosine Kinase; PI3K – Phosphoinositide 3-Kinase; MET – MET Proto-Oncogene Receptor Tyrosine.

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1. Introduction

Cancer remains a leading cause of death and contributes to significant social, psychological, and economic burden for patients and their families worldwide. The World Health Organisation (WHO) and Global Cancer Observatory report that cancer accounts for almost one in six deaths worldwide, with about 19.9 million new cases and 9.7 million deaths recorded in 2022[1]. Unlike many other chronic diseases, cancer is marked

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by unhindered cell growth, the ability to evade normal regulatory mechanisms, and genomic instability, and is likely to spread to other organs. These challenging features highlight the need for treatments that are both more effective and safer for patients. There are different types of cancer, including carcinoma, leukaemia, lymphoma, myeloma, sarcoma, melanoma, and brain tumours. Among these is lung cancer, which remains a significant health concern given its prevalence and impact[2].

Lung cancer represents a mounting public health crisis in India, according to the latest GLOBOCAN 2022 estimates from the WHO's International Agency for Research on Cancer (IARC). Lung cancer is the third leading cause of cancer fatality after oral and breast cancers. According to the Indian Council of Medical Research (ICMR) report[2], incidence rates are substantially higher in males than in females. Non-Small Cell Lung Cancer (NSCLC) is a kind of lung cancer, which makes up about 85% of cases. NSCLC includes large cell carcinoma, squamous cell carcinoma, and adenocarcinoma, with adenocarcinoma being the most common. Cigarette smoking is the leading risk factor and contributes to roughly 85–90 % of cases. Still, a significant number of NSCLC cases, especially in women, occur in people who have never smoked. In sharp contrast, a considerable proportion of lung cancer in Indian women occurs in non-smokers. It is increasingly linked to chronic exposure to indoor air pollution from biomass fuel combustion, asbestos, and occupational hazards, which are common in female non-smoking patients of Asian ethnicity[3].

Conventional cancer treatments primarily rely on surgery, radiotherapy, and chemotherapy. Among these, chemotherapy remains the cornerstone for advanced and metastatic disease due to its systemic action through multiple mechanisms, including generation of Reactive Oxygen Species (ROS), cell cycle arrest, DNA damage, induction of apoptosis, Epithelial-Mesenchymal Transition (EMT), angiogenesis, oncogenic signalling pathways (PI3K/AKT/mTOR, EGFR, and RTK), and necrosis[4]. However, its clinical efficacy is severely compromised by non-specific cytotoxicity, poor tumour penetration, acquired drug resistance, and dose-limited adverse effects such as myelosuppression, cardiotoxicity, nephrotoxicity, and neurotoxicity. These limitations have renewed global interest in phytomedicine, which has been an integral component of traditional healing systems, including Indian Ayurveda, traditional Chinese medicine, African, and Southeast Asian ethnomedicine for millennia. According to the National Cancer Institute (NCI), more than 3000 plant species have been documented to possess anti-cancer properties. Despite certain limitations identified by the Food and Drug Administration (FDA), such as high cost, rapid development of drug resistance, restricted use to patients with specific gene alterations, and limited availability in many regions for cancer therapy, plant-based therapeutic approaches remain eco-friendly, cost-effective, and stand out as an effective alternative. Extracts from various plant species, including *Aristolochia ringens, Barleria grandiflora, Tecoma stans*[6], *Taxus brevifolia*, milk thistle and *Catharanthus roseus*[7] have been reported to express anti-cancer activity in A549 cell lines.

Combretum indicum (L.) DeFilipps, commonly known as Rangoon Creeper, Chinese honeysuckle, or *MadhuMalathi*, is a vigorous woody vine belonging to the family Combretaceae, with year-round availability throughout tropical Asia and traditionally used in Ayurvedic, Chinese, and Southeast Asian medicine[5-8]. Various parts of the plant, particularly the leaves, flowers, seeds, and fruits, have been employed ethnomedicinally for the treatment of inflammation, rheumatism, parasitic infections, fever, and as a general tonic. Phytochemical investigations have revealed the presence of diverse bioactive secondary metabolites, including flavonoids, triterpenoids, lignans, phenolics, and tannins. Several studies have reported promising biological activities of *C. indicum* (CI) extracts, notably antioxidant[9], anti-inflammatory[10], antimicrobial[11], antipyretic[12], anthelmintic[13], immunomodulator[14], hypolipidemic[15], antidiabetic[16], and anticancer activities against Esophageal Adenocarcinoma (EAC9), Hepatoblastoma G2 (HepG2)[17], Prostate Cancer (PC), and Lymph Node Carcinoma of the Prostate (LNCaP)[18]. However, the anti-cancer potential of CI-ALE, with detailed *in vitro* and *in silico* studies targeting lung carcinoma, remains largely unexplored.

Keeping these in view, the present study is designed to evaluate the anticancer activity of CI-ALE against A549 human lung carcinoma cells, with particular focus on its possible computational interactions with

key oncogenic Receptor Tyrosine Kinases (RTK) and downstream signalling pathways frequently altered in non-small cell lung cancer. To achieve this, we first performed qualitative and quantitative phytochemical screening of the CI-ALE, followed by detailed metabolite profiling using GC-MS. The antioxidant activity of CI-ALE was evaluated through a battery of *in vitro* assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), FRAP, Lipid Peroxidation (LPO), Superoxide, and Nitric Oxide (NO). Finally, EGFR, ALK, ROS1, Phosphatidylinositol-3-Kinase (PI3K), and MET were selected for molecular docking studies based on their roles as driver oncogenes, their pathogenesis, and their therapeutic management in NSCLC. The collective findings from these phytochemical, biological, and computational investigations are expected to provide scientific evidence for the traditional use of CI-ALE and highlight its prospects as a source of novel lead compounds for targeted lung cancer therapy.

2. Materials and Methods

2.1 Materials

All chemicals and reagents used were of analytical grade and were sourced from HiMedia Laboratories, India, unless otherwise stated. Reagents for phytochemical and antioxidant assays included bromocresol green, Folin-Ciocalteu reagent, aluminium chloride, sodium nitrite, sodium carbonate, DPPH, thiobarbituric acid, trichloroacetic acid, phenazine methosulphate, nitroblue tetrazolium, sodium nitroprusside, and Griess reagent. For cell culture experiments, Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), penicillin-streptomycin, and MTT reagent were used. Cell lines were maintained in a humidified CO_2 incubator at 37°C with 5% CO_2. Absorbance was recorded using an Ultraviolet (UV)-visible spectrophotometer or microplate reader.

2.2 Plant Collection and Authentication

Mature specimens of *C. indicum* were sourced from a nursery in Coimbatore, Tamil Nadu. The plants were grown under controlled, disease-free, shaded conditions. The material was authenticated at the Botanical Survey of India (BSI), Coimbatore, and identified as *C. indicum* (L.) DeFilippis of the family *Combretaceae* (authentication no. BSI/SRC/5/23/2022/Tech./624).

2.3 Preparation of CI-ALE (*Combretum indicum*- Aqueous Leaf Extract)

Fresh leaves were harvested, washed thoroughly, shade-dried, powdered, and stored in airtight containers. For extraction, 5g of powdered leaf material was mixed with 100mL of double-distilled water. The mixture was continuously agitated at room temperature overnight. This process involved minor modifications to previously described methods for *C. indicum* leaf extraction[17]. The extract was filtered through Whatman No. 1 filter paper, concentrated, and stored at 4°C until use.

2.4 Qualitative Phytochemical Screening

Standard procedures described by Harborne were used to screen CI-ALE for major phytochemical groups, including alkaloids, flavonoids, phenolics, tannins, carbohydrates, proteins, sterols, saponins, and fixed oils[19].

2.5 Quantification of Phytochemicals

2.5.1 Alkaloid Estimation

Alkaloid content was quantified using the modified bromocresol green method[20]. Equal volumes of CI-ALE and 0.2M phosphate buffer (pH 4.2) were mixed and then combined with 1mL of 2N hydrochloric acid and 5mL of 0.1mM bromocresol green. After the addition of 3mL of chloroform, the yellow complex formed was measured at 430nm. Atropine served as the standard, and the results were expressed as mg atropine equivalents (mg AE/g dry extract). Assays were performed in triplicate.

2.5.2 Total Phenolic Content (TPC)

Phenolic content was measured by the Folin-Ciocalteu method[21]. A 0.2mL aliquot of CI-ALE was mixed with 0.8mL of distilled water and 0.4mL of 10% Folin-Ciocalteu reagent. After a 5-minute reaction period, 0.8mL of 10% sodium carbonate was added. The mixture was incubated in the dark for 1 hour, and absorbance was recorded at 700nm. Gallic acid was used as the calibration standard, and the results were reported as mg GAE/g dry extract.

2.5.3 Total Flavonoid Content (TFC)

Flavonoids were quantified using the aluminium chloride colourimetric method[22]. A mixture of 0.2mL

of CI-ALE and 2.3mL of distilled water was combined with 0.3mL of 5% sodium nitrite. After 5 minutes, 0.3mL of 10% aluminium chloride was added, followed by 2mL of 1M sodium hydroxide. The mixture was incubated in the dark for 40 minutes, and absorbance was measured at 520nm. Results were expressed as mg quercetin equivalents (mg QE/g dry extract).

2.5.4 Estimation of Tannins and Polyphenols

Tannin content was determined using the Folin-Ciocalteu method with Polyvinylpyrrolidone (PVP) to separate non-tannin polyphenols[23]. For total polyphenols, 1mL of extract was reacted with 1mL of Folin-Ciocalteu reagent and 2mL of 35% sodium carbonate after 5 minutes of incubation. Absorbance was recorded at 700nm after 1 hour in the dark. To quantify non-tannin polyphenols, 100mg of PVP was mixed with 1mL of CI-ALE, incubated, centrifuged, and the supernatant was treated similarly. Tannin content was calculated by subtracting non-tannin polyphenols from the total. Results were reported as mg tannic acid equivalents (mg TAE/g dry extract).

2.6 *In Vitro* Antioxidant Assays

2.6.1 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Free Radical Scavenging Activity

DPPH radical scavenging activity was determined as described previously[24]. CI-ALE (2–10 µg/mL) was mixed with 100µL of DPPH solution in methanol and incubated in the dark for 30 minutes. Absorbance was measured at 517nm, and ascorbic acid served as the reference standard.

2.6.2 Superoxide Radical Scavenging Assay

Superoxide scavenging activity was evaluated using the Nicotinamide Adenine Dinucleotide (NADH)-Phenazine Methosulphate (PMS)- Nitroblue Tetrazolium (NBT) system[25]. The reaction mixture contained phosphate buffer, NADH, NBT, PMS, and CI-ALE (20–100 µg/mL). After incubation for 5 minutes at 25°C, absorbance was recorded at 560nm. Ascorbic acid was used as the positive control.

2.6.3 Nitric Oxide (NO) Scavenging Assay

Nitric oxide scavenging activity was assessed according to the reported method[26]. Sodium nitroprusside (10mM) was incubated with CI-ALE (30–150 µg/mL) at room temperature for 1 hour. After incubation, an equal volume of Griess reagent was added, and the absorbance was read at 546nm. Curcumin served as the reference compound.

2.6.4 LPO Inhibition

A 10% egg yolk homogenate served as the lipid-rich medium. CI-ALE (50–250 µg/mL) was combined with 500µL of the homogenate and diluted to 1mL. Lipid peroxidation was initiated by adding 0.05mL of 0.07M $FeSO_4$ and incubating at 37°C for 1 hour. The reaction was stopped with a thiobarbituric acid: acetic acid: trichloroacetic acid solution and heated at 95°C for 60 minutes. After cooling, 5mL of butanol was added, the mixture was centrifuged, and the absorbance of the upper layer was measured at 532nm[27].

2.6.5 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP was assessed as described previously[28]. The ability of CI-ALE (20–100 µg/mL) to reduce Fe^{3+}-TPTZ to the blue-coloured Fe^{2+}-TPTZ complex was measured at 593nm. Results were compared with ascorbic acid.

2.7 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The phytochemical constituents of CI-ALE were profiled using a Shimadzu GC-MS QP2010 equipped with an electron ionisation source operated at 70eV and a Restek-5MS column (30m × 0.25mm, 0.25µm). Helium was used as the carrier gas at 1 mL/min. The injection volume was 1µL. The oven temperature was programmed from 50°C (1-minute hold) to 250°C at 10°C/minute (3-minute hold), followed by 280°C (5-minute hold). Mass spectra were acquired in the range of 35–500 m/z, and compounds were identified by comparison with the National Institute of Standards and Technology (NIST) library[29].

2.8 Anticancer Evaluation

2.8.1 Cell Lines and Culture Conditions

A549 human lung adenocarcinoma cells were selected as a representative non-small cell lung cancer model, whereas Vero cells (African green monkey kidney epithelial cells) were used as a non-cancerous mammalian cell line to assess selectivity. Both cell lines were procured from the National Centre for Cell Science (NCCS), Pune, India. Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37°C with

5% CO_2 and were passaged every 2 days according to standard cell-bank recommendations.

2.8.2 MTT Cytotoxicity Assay

Cell viability was evaluated by the MTT assay[30]. Cells were seeded in 96-well plates at 1×10^4 cells/well in 100µL of culture medium and allowed to attach overnight. The cells were then treated with CI-ALE at concentrations of 12.5–200 µg/mL. After the treatment period, 20µL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 hours. The resulting formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was measured at 570nm. Cell viability was calculated using the formula: Cell viability (%) = (absorbance of treated cells/absorbance of control cells) × 100. Percentage inhibition was calculated as 100 − cell viability. The IC_{50} value was determined from the dose-response curve by nonlinear regression analysis.

2.9 *In Silico* Analysis: Molecular Docking

Based on GC-MS findings, the major constituents hexadecane, 1-chloro-, octahydro-2(1H)-quinoline, n-hexadecanoic acid, phytol, and stigmasterol were selected for docking studies. Target proteins relevant to NSCLC, including EGFR (PDB ID: 2ITN), ALK (4ANL), ROS1 (4UXL), PI3K (4OVU), and MET (3DKC), were retrieved from the Protein Data Bank. Ligand preparation and docking were performed using AutoDock Vina with grid parameters defined separately for each target. Docking poses were visualised using PyMOL and Discovery Studio[31,32].

2.10 Statistical Analysis

All experiments were performed in triplicate, and the results are presented as Mean ± Standard Deviation (SD). IC_{50} values were derived from concentration-response plots. Statistical comparisons were performed where applicable, and $p < 0.05$ was considered statistically significant.

3. Results

3.1 Phytochemical Qualitative and Quantitative Analysis of CI-ALE

The phytochemical analysis of the CI-ALE revealed the presence of alkaloids, flavonoids, tannins, carbohydrates, proteins, and sterols, while saponins and oils were absent. In the quantitative analysis, the extract was found to contain flavonoids in the highest concentration (750 ± 2.3mg QE/g of dried extract), followed by tannins (263.2 ± 1.8mg TAE/g), alkaloids (85 ± 2.2mg AE/g), and phenols (73 ± 1.8mg GAE/g). These results confirm that the CI-ALE is rich in various bioactive compounds, particularly flavonoids.

3.2 *In Vitro* Antioxidant Activity

The *in vitro* antioxidant activity of the CI-ALE was evaluated using several standard assays, as shown in Figures 1. In the DPPH radical scavenging assay, CI-ALE exhibited a concentration-dependent increase in activity, with the scavenging percentage ranging from approximately 28% at 8 µg/mL to about 93% at 40 µg/mL (Figure 1A). Ascorbic acid, used as a standard, showed a similar trend, with activity rising from around 16% at 2 µg/mL to nearly 89% at 10 µg/mL (Figure 1B). In the superoxide radical scavenging assay, CI-ALE showed increasing scavenging activity from approximately 19% at 20 µg/mL to nearly 89% at 100 µg/mL (Figure 1C). Ascorbic acid again demonstrated strong activity, rising from about 13% to 90% across the tested concentrations (Figure 1D). The NO scavenging assay revealed that CI-ALE inhibited NO radicals in a dose-dependent manner, with activity ranging from 24% at 30 µg/mL to 92% at 150 µg/mL (Figure 1E), while curcumin, the reference, showed activity from 21% to 93% (Figure 1F). In the LPO inhibition assay, CI-ALE demonstrated a gradual increase in inhibition, reaching about 94% at 200 µg/mL (Figure 1G). Lipoic acid, the positive control, showed similar inhibition patterns, peaking at around 96% at the highest concentration tested (Figure 1H). Additionally, the FRAP assay showed a concentration-dependent increase in absorbance for CI-ALE, indicating enhanced reducing

Table 1. The IC_{50} values (in µg/mL) of the CI-ALE and their respective standards from the antioxidant assays

Assay	CI-ALE	Standard
	(µg/mL)	
DPPH scavenging	23.85 ± 2.3	Ascorbic acid: 5.93 ± 0.9
Superoxide scavenging	54.67 ± 3.4	Ascorbic acid: 56.68 ± 2.9
Nitric oxide scavenging	70.51 ± 2.4	Curcumin: 27.33 ± 1.8
Lipid peroxidation inhibition	92.89 ± 3.9	Lipoic acid: 66.27 ± 2.1

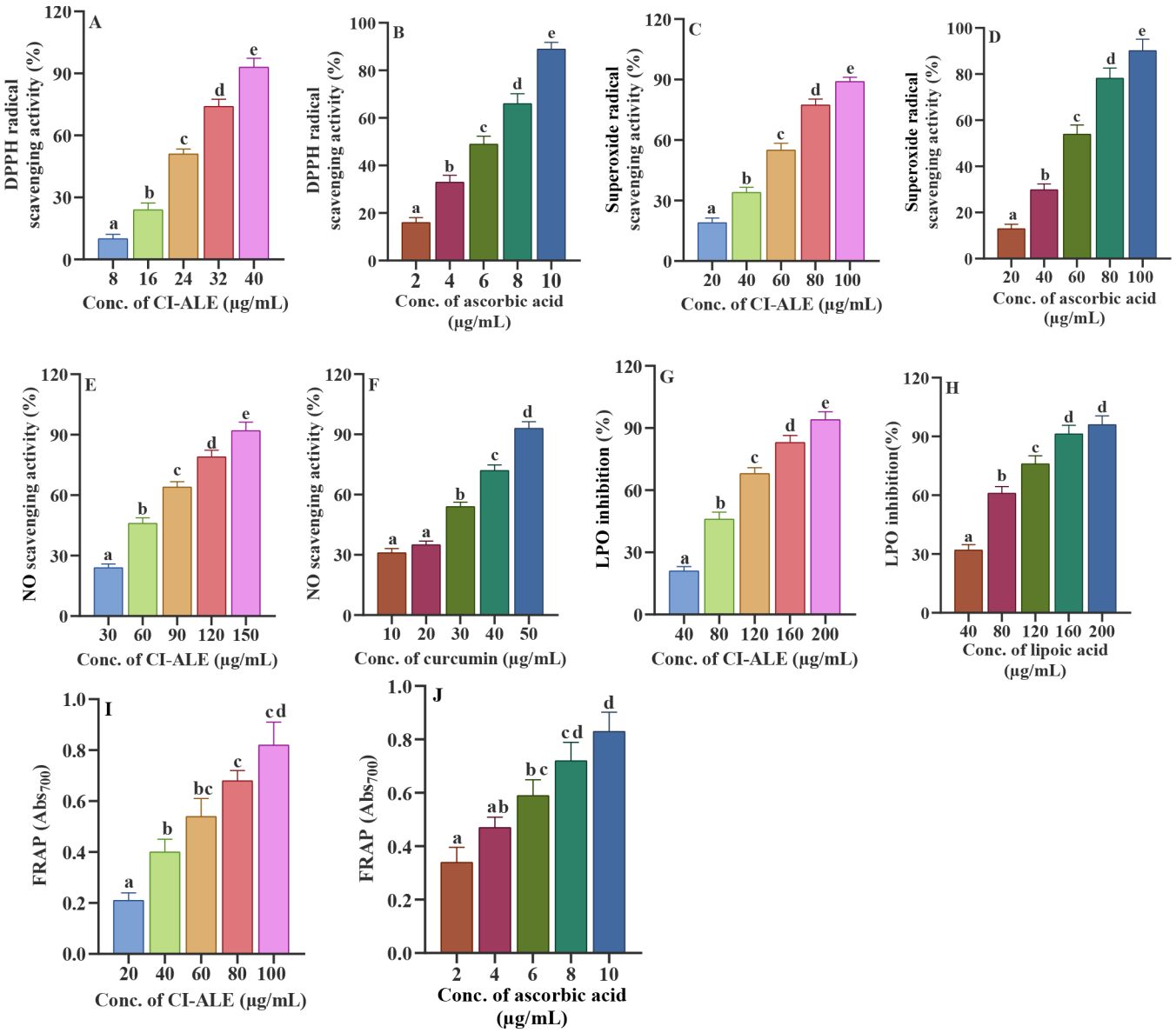


Figure 1. *In vitro* antioxidant activity of CI-ALE and standards. **(A, B).** DPPH radical scavenging activity; **(C, D).** Superoxide radical scavenging activity; **(E, F).** Nitric oxide radical scavenging activity; **(G, H).** Lipid peroxidation inhibition assay; **(I, J).** Ferric reducing antioxidant power (FRAP) assay. All values are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences ($p < 0.05$) among concentrations.

power. The absorbance at 700nm increased from 0.21 at 20 µg/mL to 0.82 at 100 µg/mL (Figure 1I). Ascorbic acid also exhibited a strong FRAP response with absorbance rising from 0.34 to 0.83 across 2–10 µg/mL (Figure 1J). The IC_{50} values of the CI-ALE and their respective standards from the antioxidant assays are given in Table 1.

3.3 GC-MS Analysis

The GC-MS chromatogram of CI-ALE is shown in Figure 2. A total of thirty-eight phytoconstituents were identified based on comparison with the NIST library, and their details, including retention time, molecular formula, molecular weight, and relative abundance, are provided in Table 2.

The analysis revealed a complex phytochemical profile composed predominantly of terpenoids, fatty acids, esters, alkaloids, phenolics, and phytosterols.

Among the identified constituents, phytol (17.37%) and n-hexadecanoic acid (14.10%) were found in the highest relative abundance. These were followed by

Table 2. Details of compounds identified through GS-MS analysis present in CI-ALE

RETENTION TIME (min)	COMPOUND	MOLECULAR FORMULA	MOLECULAR WEIGHT	AREA (%)
5.072	Cyclopentane-1-one,2-hydroxy	$C_6H_8O_2$	112.124	0.80
5.475	1-hexanol, 2-ethyl	$C_8H_{18}O$	130.23	0.44
6.003	2-pyrolin-1-ylethamine	$C_6H_{14}N_2$	114.19	0.51
7.105	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy	$C_6H_8H_4$	144.12	0.38
7.693	3-tetradecene, (z)-	$C_{14}H_{28}$	196.37	0.23
7.793	Benzofuran, 2, 3-dihydro	$C_9H_8O_3$	164.16	0.40
9.555	Pyrolidine, 1-(1-cyclohexen -1-y1)-	$C_{10}H_{17}N$	151.25	1.58
10.251	Hexadecane, 1-chloro-	$C_{16}H_{33}C1$	260.9	12.32
10.476	2-Bromopropionic acid, Octyl ester	$C_{11}H_{21}BrO_3$	265.19	0.83
11.130	Octadecane, 1-bromo-	$C_{18}H_{37}Br$	333.4	1.17
11.249	1, 2 –benzendicarboxylic acid, d	$C_8H_6O_4$	166.13	2.35
11.613	1-pentadecene	$C_{15}H_3O$	210.40	0.73
12.810	Octahydro-2(1H) –quinoline	$C_9H_{15}NO$	153.22	9.70
13.050	Mexiletine	$C_{11}H_{17}NO$	179.259	0.46
13.326	3-(2,2-dimethylhydrazino)-2-cyc	$C_2H_6N_2$	60.10	1.98
13.671	Tetradeconic acid	$C_{14}H_{28}O_2$	255.54	0.65
14.401	1-heptadecene	$C_{17}H_{34}$	238.45	0.43
15.191	2, 6, 10 – trimethyl, 14 – ethylene- 14 –f	$C_{18}H_{27}NO_5$	337.14	1.10
15.657	5,10- diethoxy – 2,3, 7, 8 – tetrahydro	$C_{14}H_{22}N_2O_2$	250.16	1.32
16.520	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O$	270.45	1.26
16.726	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	5.12
17.336	N-Hexadecanoic acid	$C_{16}H_{32}O$	256.4241	14.10
19.972	9,12,15-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	292.5	0.54
20.538	Phytol	$C_{20}H_{40}O$	296.53	17.37
20.765	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298.5	1.28
20.874	9,12,15-Octadecatrienoic acid, (Z, Z, Z)-	$C_{18}H_{30}O$	278.429	0.81
20.982	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282.5	1.20
21.584	Octadecanoic acid	$C_{18}H_{34}O_2$	284.48	0.63
27.706	Ethyl 5-oxo-2-pyrrolidinecarbo	$C_7H_{11}NO_3$	157.17	1.19
29.204	Hexadecenoic acid, 2-hydroxy-1-(hydroxy)	$C_{19}H_{38}O_4$	330.5	1.79
30.0776	1,2-benzenedicarboxylic acid	$C_8H_6O_4$	166.113	2.47
37.110	Squalene	$C_{30}H_{62}$	410.73	0.43
40.279	Hexadecanoic acid, tetradecyl ester	$C30H_{60}O_2$	452.79	0.68
41.506	Gamma. -Tocopherol	$C_{28}H_{48}O_2$	416.7	1.10
45.661	Stigmasta-5,22-dien-3-ol	$C_{31}H_{50}O_2$	454.7	1.11
46.702	Stigmasterol	$C_{29}H_{48}O$	412.7	7.33
47.147	Stigmast-5-en-3-ol, (3beta.)-	$C_{31}H_{50}O_2$	454.7	3. 12
48.175	Fucosterol	$C_{29}H_{48}O$	412.7	1.08

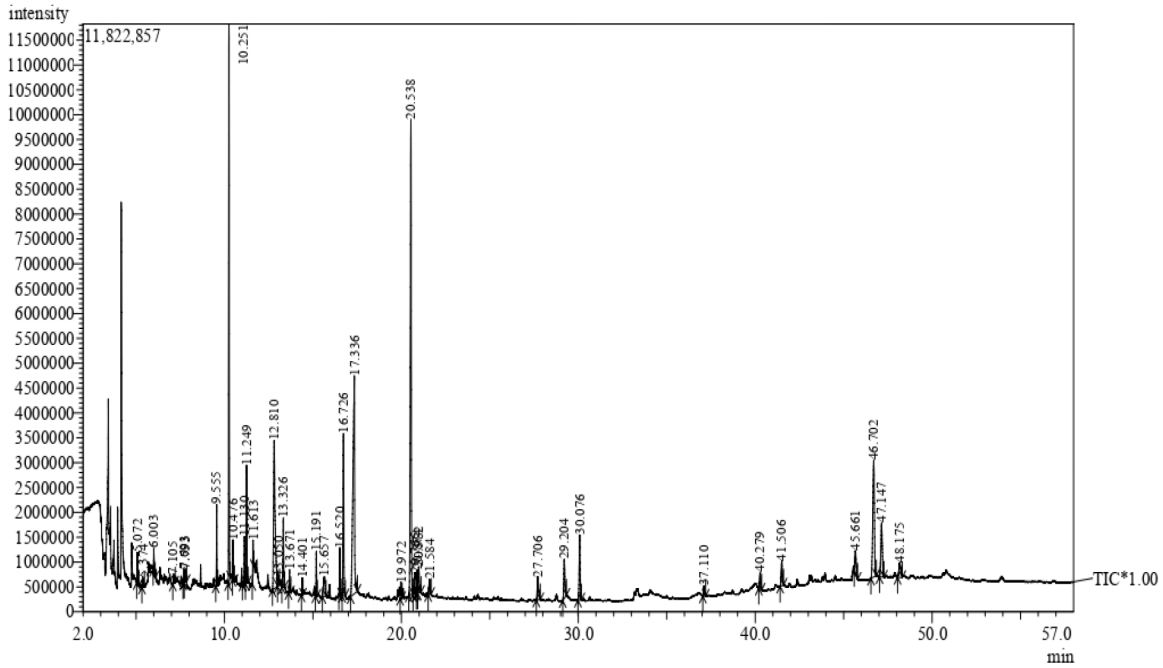


Figure 2. GC-MS chromatogram of CI-ALE. The total ion chromatogram (TIC) reveals the presence of 38 phytoconstituents in the *CI-ALE,* as identified using the NIST library.

hexadecane, 1-chloro- (12.32%) and octahydro-2(1H)-quinoline (9.70%), indicating a substantial presence of saturated hydrocarbons and nitrogenous heterocycles. Notably, stigmasterol (7.33%) and its structural analogues, stigmast-5-en-3-ol (3β) (3.12%) and stigmasta-5,22-dien-3-ol (1.11%), were also identified, highlighting the phytosterol richness of the CI-ALE.

Fatty acid derivatives were prevalent across the profile, including hexadecanoic acid, methyl ester (1.26%), octadecanoic acid, methyl ester (1.28%), 9,12,15-octadecatrienoic acid (both methyl ester and (Z, Z, Z) isomer), and octadec-9-enoic acid. Other notable constituents included gamma-tocopherol (1.10%), a known antioxidant, and squalene (0.43%), a triterpenoid precursor.

3.4 Effect of CI-ALE on Cell Viability

The cytotoxicity of CI-ALE was assessed using the MTT assay on A549 (human lung carcinoma) and Vero (normal kidney epithelial) cell lines across a concentration range of 12.5 to 200 µg/mL (Figure 3A). In A549 cells, CI-ALE exhibited a dose-dependent reduction in cell viability. At lower concentrations, the cell viability remained relatively high. A moderate decrease was observed at 50 µg/mL and 100 µg/mL. A significant reduction was recorded at 200 µg/mL (39% ± 2.8), indicating potential anti-cancer activity. The IC_{50} values of the CI-ALE against A549 cells were found to be 163.11 ± 6.8 µg/mL. In contrast, Vero cells maintained high viability across all tested concentrations. Even at the highest dose of 200 µg/mL, the viability remained at 88.14% ± 2.5, suggesting minimal cytotoxic effects on normal cells.

Morphological changes were further observed under a brightfield microscope (Figure 3B and 3C). Untreated A549 cells exhibited typical epithelial morphology with well-defined borders and dense monolayer formation. Upon treatment with CI-ALE, particularly at 100 and 200 µg/mL, A549 cells showed signs of cytotoxic stress, including cell shrinkage, membrane blebbing, detachment from the substrate, and reduced confluency, indicative of apoptotic or necrotic cell death. In contrast, Vero cells maintained their normal elongated morphology with minimal changes even at higher concentrations, supporting the selective cytotoxicity of CI-ALE towards cancer cells.

3.5 Molecular Docking Analysis

To explore the molecular basis of the anti-cancer potential of CI-ALE, major phytocompounds identified through GC-MS analysis were subjected to *in silico* docking against five lung cancer-associated proteins:

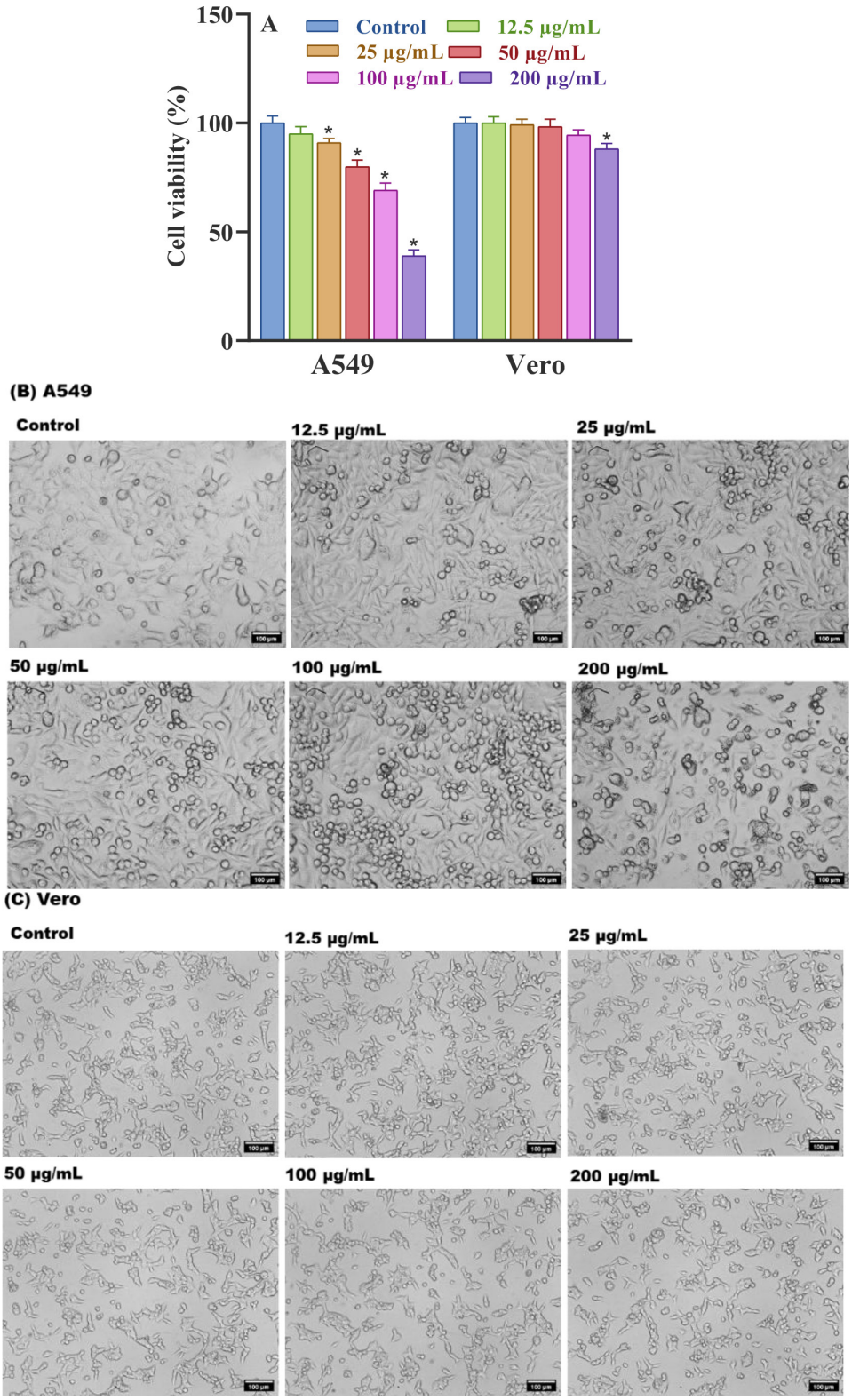


Figure 3. Effect of CI-ALE on cell viability and morphology. **(A).** Percentage cell viability of A549 and Vero cells treated with increasing concentrations of CI-ALE (12.5–200 µg/mL) for 24 h, assessed by MTT assay. Values represent mean ± SD (n = 3); *p < 0.05 *vs.* control; **(B, C).** Microscopic morphology of untreated A549 and Vero cells (control). Morphological changes in A549 and Vero cells treated with 200 µg/mL CI-ALE showing signs of cytotoxic stress, including cell shrinkage, rounding, and detachment in A549 cells, while Vero cells retained normal morphology.

Table 3. Docking scores of various compounds identified in CI-ALE with the target proteins of lung cancer

Ligand	ALK	MET	PI3K	EGFR	ROS1
	Dock Score (kcal/mol)				
Hexadecane, 1-chloro-	-4.0	-4.1	-5.0	-3.9	-4.0
N-Hexadecanoic acid	-4.4	-4.7	-5.0	-4.4	-4.6
Octahydro-2(1H)-quinolinone	-4.8	-5.3	-5.8	-5.8	-5.4
Phytol	-5.0	-4.3	-6.2	-4.8	-5.1
Stigmasterol	-8.8	-7.9	-8.8	-8.8	-8.0

Table 4. Details of the hydrogen bonding interaction and hydrophobic interaction of various compounds with different proteins

Complex		Hydrogen Bonding Interactions	Hydrophobic Interactions
	(Distance in Å)		
ALK	Phytol	Met1199 (2.8), Leu1122 (2.2), Lys1150 (2.9), Asp1270 (3.1), Asp1269 (2.7), Arg1253 (2.7)	Val1130 (4.4), Ala1148 (3.4), Val1180 (4.4), Ala1269 (4.7), Leu1122 (4.5)
	Stigmasterol	Ala1269 (2.7), Asp1270 (2.8), Val1130 (2.8), Leu1122 (2.1), Glu1132 (3.2), Met1199 (2.8), Ala1200 (2.7)	Ala1148n(4.5), Leu1256 (4.5), Val1130 (3.8), Leu1122 (4.0)
MET	Octahydro-2(1H)-quinoline	Lys1110 (3.1), Asp1222 (2.5), Gly1085 (3.0)	Val1092 (4.0)
	Stigmasterol	Pro1158 (2.2), Leu1157 (3.0), Asn1167 (2.6), Ile1084 (2.3), Arg1086 (2.6)	Val1092 (3.5), Ala1108 (4.7), Met1211 (4.3), Lys1110 (4.4)
PI3K	Phytol	Val851 (2.7), Ser854 (2.0), Glu849 (2.9), Tyr836 (3.0), Lys802 (2.8)	Tyr836 (3.5), Ile932 (4.0), Ile800 (4.5), Ile848 (4.6)
	Stigmasterol	Val851 (3.3), Ser854 (2.6), Tyr536 (2.3), Asp810 (3.0), Gly859 (3.5)	Ile932 (4.4), Ile800 (4.7), trp780 (4.7)
EGFR	Octahydro-2(1H)-quinoline	Cys775 (2.9), Met766 (2.6), Glu762 (3.1), Thr854 (2.1)	Leu844 (4.5)
	Stigmasterol	Asp855 (1.3), Thr854 (1.3), Phe856 (2.5), Lys745 (2.8), Leu718 (3.0), Pro794 (2.2), Met793 (2.5), Cys797 (1.5), Phe795 (1.2)	Leu844 (3.7), Val276 (4.5), Cys797 (3.2)
ROS1	Octahydro-2(1H)-quinoline	Met2029 (2.1), Val1959 (2.9)	Val1959 (4.1), Ala1978 (4.2)
	Stigmasterol	Glu2030 (2.7), Met2029 (2.7), Glu2101 (2.4), Asn2084 (2.6), Asp2033 (3.0), Leu1951 (2.5)	Leu1951 (3.9), Val1959 (4.0), Leu2086 (3.9)

ALK, MET, PI3K, EGFR, and ROS1. The docking scores are given in Table 3.

The details of interaction between ligands and proteins are given in Table 4, and hydrogen bonding interactions of the ligand with key amino acids of respective proteins are shown in Figure 4.

Among the tested ligands, stigmasterol consistently showed the highest binding affinity across all targets, suggesting a strong, stable interaction profile (Table 3). Its structural rigidity and presence of multiple functional groups facilitated hydrogen bonding and hydrophobic interactions within the active pockets of these proteins. For instance, stigmasterol formed key hydrogen bonds with catalytically significant residues in ALK (Asp1270, Met1199, and Glu1132) and EGFR (Cys797, Thr854, and Lys745), suggesting its potential to interfere with kinase activity at multiple regulatory nodes. The interaction with PI3K was particularly notable, as it engaged both catalytic and regulatory residues, including Tyr536 and Asp810, possibly contributing to the inhibition of downstream PI3K/AKT signalling. Phytol, another abundant metabolite, also demonstrated favourable docking, especially with PI3K and ALK. It showed effective accommodation within the hydrophobic cores of these kinases and established interactions with residues such as Glu849

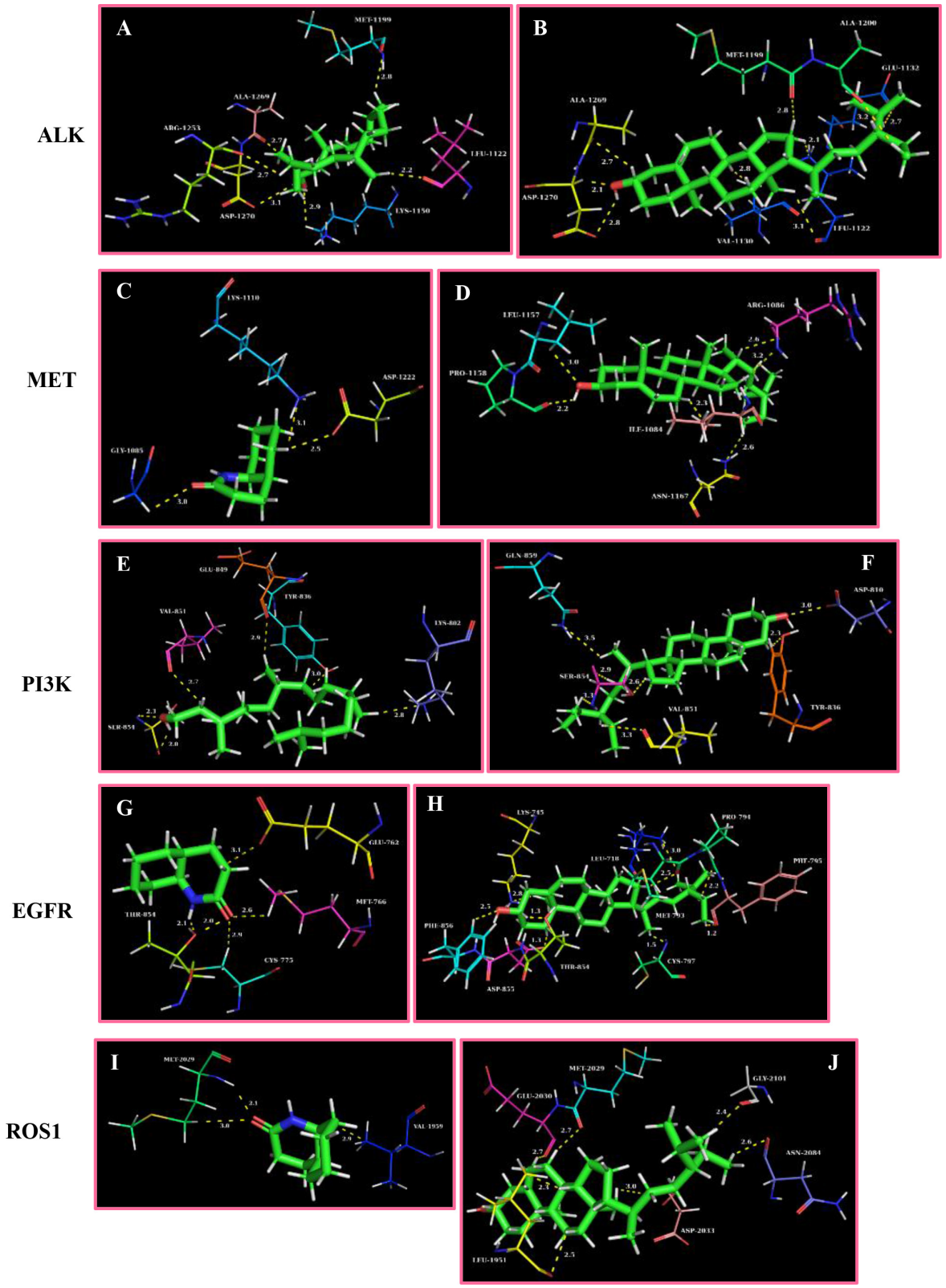


Figure 4. Molecular docking interaction of selected CI-ALE compounds with lung cancer target proteins. Docking poses of phytol **(A).** and stigmasterol **(B).** with ALK Octahydro-2(1H)-quinoline **(C)** and stigmasterol **(D).** with MET; phytol **(E).** and stigmasterol **(F).** with PI3K, Octahydro-2(1H)-quinoline **(G).** and stigmasterol **(H).** with EGFR and Octahydro-2(1H)-quinoline **(I).** and stigmasterol **(J).** with ROS1 proteins respectively, showing interaction within the active sites.

and Lys802 in PI3K and Asp1270 in ALK. Though less potent than stigmasterol, phytol's moderate binding affinities and broad receptor coverage imply additive or synergistic roles in multitarget inhibition. Octahydro-2(1H)-quinolinone exhibited moderate affinity across all targets and formed stable hydrogen bonds, particularly with MET, EGFR, and ROS1. Its interaction with MET included residues Lys1110 and Asp1222, which are crucial for ATP binding and activation, suggesting possible interference with receptor dimerisation or signal propagation. Similarly, in EGFR and ROS1, the ligand occupied functionally relevant regions of the catalytic cleft, forming hydrogen bonds with Glu762, Met2029, and other polar residues, thereby contributing to steric hindrance of substrate access. Hexadecane (1-chloro-) and n-hexadecanoic acid, though structurally simpler, displayed relatively weaker affinities and limited specific interactions. Their docking was largely driven by hydrophobic forces, with minimal hydrogen bonding. Their passive binding behaviour suggests a minor role in direct kinase inhibition but does not exclude indirect modulatory or membrane-related effects. The docking analysis highlights the multi-targeted nature of CI-ALE constituents, with stigmasterol and phytol standing out as key pharmacophores.

4. Discussion

Lung cancer is heterogeneous at the molecular and histological levels[33]. Identifying therapeutic agents for NSCLC is challenging. This study is the first to report the anti-cancer efficacy of CI-ALE against A549 cell line-based studies supported by antioxidant studies and validated through major oncogenic proteins computational interaction with CI-ALE compounds identified by GC-MS. A previous study,[13] reported the presence of steroids, proteins, phenols, tannins, alkaloids, flavonoids, and carbohydrates in CI extracts. The CI-ALE exhibit significantly higher quantity of flavonoids, 750 ± 2.3 mg QE/g of dried extract, compared to other metabolites, with the order of abundance being flavonoids > tannins (polyphenolic compounds with protein-binding capabilities) > alkaloids (nitrogen-containing compounds which can be bioactive) > and phenols (aromatic compounds with one or more hydroxyl groups, often showing antioxidant effects), possibly due to differences in polarity during extraction. These findings align with reports on several *Combretum* species, which are known to be rich in flavonoids and phenols, responsible for their antioxidant, antimicrobial, anti-inflammatory, and anti-cancer activities[9,34,35]. The current study results were consistent with previous reports[36]. Phytochemical studies[35,37] have further revealed that CI contains a wide range of bioactive compounds, including flavonoids, phenolics, steroids, terpenoids, aliphatic acids, and alkaloids, with numerous specific compounds isolated from its leaves, such as phenolic acids, flavonols, derivatives of quercetin, isorhamnetin, kaempferol, and myricetin as well as flavones, tannins, flavanones, and coumarins[9].

The major compounds identified via GC-MS analysis include hexadecane, phytol, n-Hexadecanoic acid, Quinoline, and stigmasterol in the CI-ALE, which have been reported to possess antimicrobial, anthelmintic, anti-inflammatory, antioxidant, and anti-cancer properties[13]. Among these, stigmasterol, a prominent plant-derived sterol identified in the CI-ALE, shows potent anti-cancer effects through inhibition of the Phosphoinositide 3 Kinase/Protein Kinase B/Mammalian Target of Rapamycin (PI3K/Akt/mTOR) and Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathways, modulation of apoptosis, and anti-angiogenic effects via suppression of Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) and Tumour Necrosis Factor Alpha (TNF-α)[38,39]. In NSCLC, dysregulation of these pathways is central to tumour cell proliferation, survival, and metastasis, highlighting stigmasterol as a multitarget therapeutic agent. Other bioactive compounds, such as phytol and octahydro-2(1H)-quinoline, further enhance the extract's anti-cancer potential. Phytol induces apoptosis through mitochondrial dysfunction, ROS generation and activation of caspases 8 and 9, accompanied by cleavage of poly (ADP-ribose) Polymerase (PARP)[40]. It may also promote autophagy and modulate lipid metabolism through PPARα activation, further supporting its antineoplastic potential, while suppressing aromatase expression through p38 Mitogen Activated Protein Kinase (MAPK) inhibition in estrogen-dependent cancers. Similarly, Octahydro-2(1H)-quinoline belongs to a class of nitrogen-containing heterocycles known for

inhibiting DNA replication and topoisomerase activity, mechanisms widely exploited in chemotherapeutic agents[41, 42].

CI-ALE exhibited strong, dose-dependent antioxidant activity that aligned closely with previous reports on aqueous extract of CI flowers and leaves[43-45], and the increased antioxidant activity can be directly linked to the high TPC and TFC as quantified in this study[46]. Phenolic compounds and flavonoids neutralize ROS through hydrogen atoms or electron donation, stabilise unpaired electrons, and chelate pro-oxidant metal ions, thereby preventing oxidative damage[47,48]. Tannins further inhibit LPO by interrupting peroxy radical propagation, thus preserving membrane integrity[49]. Key GC-MS identified constituents contributed significantly to the observed antioxidant effects. Phytol displayed robust radical scavenging activity, reduced LPO, and upregulated endogenous antioxidant defences by increasing the activity of enzymes such as Superoxide Dismutase (SOD), Catalase (CAT), and glutathione peroxidase (GPx) while stabilising intracellular Glutathione (GSH) levels, ultimately limiting oxidative DNA damage and slowing tumour progression[40,50]. Stigmasterol, a phytosterol similar in structure to cholesterol, exhibited potent antioxidant and anti-inflammatory effects by activating the Nrf2–Keap1 pathway, inducing cytoprotective enzymes, and suppressing COX-2 and iNOS[38,39]. Octahydro-2(1H)-quinoline, a nitrogen-containing heterocyclic compound, neutralises reactive oxygen and nitrogen species by chelating transition metals (Fe^{2+}, Cu^{2+}), thereby preventing hydroxyl radical formation via the Fenton reaction and modulating Nrf2 and NF-kB signalling[51]. Although n-hexadecanoic acid showed limited direct radical-scavenging activity, it may stabilise cell membranes and enhance the coexisting phytoconstituents' efficacy[38]. Extracts from *Combretum* species, including *Combretum punctatum* var *squamosum*, *Combretum duarteanum,* and *Combretum ovalifolium,* have consistently demonstrated potent DPPH, superoxide and NO scavenging capabilities[8,9,52,53]. The synergistic interplay among polyphenols, phytosterols, diterpenes, and heterocyclic compounds in CI-ALE amplifies its overall antioxidant potential, supporting its traditional medicinal use. Given the pivotal role of chronic oxidative and nitrosative stress in lung carcinogenesis, the multifaceted redox modulatory properties of CI-ALE provide a mechanistic rationale for its protective effects against oxidative stress-driven cancer progression[54].

CI-ALE was tested for cytotoxic activity by MTT assay in A549 cells, a well-established *in vitro* model for NSCLC research, due to its similarity to type II alveolar epithelial cells and its sensitivity to chemotherapy[55,56] and on Vero cells to evaluate selectivity. CI-ALE exhibited pronounced cytotoxicity against A549 cells with an IC_{50} value of 163.11 µg/mL, while maintaining greater than 80% cell viability at the highest tested concentration (200 µg/ml) used in the study, indicating selective anti-cancer activity consistent with previous findings on *Combretum sp*[57]. The observed cytotoxicity can be attributed to the synergistic action of multiple bioactive constituents identified in CI-ALE[54,58]. Quinoline derivatives, particularly octahydro-2(1H)-quinoline, inhibit topoisomerases and DNA replication, whereas related hydroquinoline structures suppress the invasive capabilities of cancer stem cells via downregulation of the CCL5 pathway[51,59]. N-Hexadecanoic acid (palmitic acid) has been implicated in mitochondrial apoptosis through the increased Bax/Bcl-2 ratio, upregulation of pro-apoptotic factors, such as caspase-3, caspase-9, Bax, and p53, along with the suppression of Bcl-2[60,61].

Overall, CI-ALE exerts multitargeted anti-cancer effects through induction of intrinsic and extrinsic apoptosis, cell cycle arrest, disruption of oncogenic signalling (PI3K/Akt/mTOR, NF-κB, STAT3), ROS generation, and suppression of metastasis-related pathways[55,62]. This broad mechanistic profile, combined with minimal toxicity toward normal cells and potential synergy with conventional chemotherapeutics, supports the ethnopharmacological use of CI and highlights its promise as an adjunct therapeutic agent in NSCLC management[63].

Molecular docking simulations offer valuable insights into ligand-target interactions by predicting the binding affinity and interaction profiles of phytochemicals with specific molecular targets involved in NSCLC development. Therefore, combining *in vitro* validation experiments with computational analysis is essential to reveal mechanistic details and confirm the therapeutic potential of CI-ALE. EGFR, ALK, ROS1, MET, and PI3K proteins are involved in signalling pathways that regulate cell proliferation, survival, migration, and resistance mechanisms, making

them highly appropriate targets for identifying new therapeutic agents from plant-derived compounds[33]. Mutations or rearrangements in PI3K/Akt/mTOR, MAPK, and JAK/STAT pathways activate critical oncogenic signalling events, including those that promote tumour growth, survival, angiogenesis, and resistance to conventional therapies[64].

The aberrant activation of RTK, such as EGFR and MET, plays a crucial role in the development and progression of lung cancer. EGFR overexpression and EGFR-activating mutations (exon 19 deletions and exon 21 L858R substitutions) occur in a significant proportion of cases and sensitise tumours to Epidermal Growth Factor (EGF), hyperactivating downstream pathways such as Erk1/2, PI3K–Akt, STAT3, and STAT5[65, 66]. Similarly, the MET receptor, overexpressed in a large proportion of NSCLC cases (61–75%), activates downstream signalling, particularly through ERBB3 and the PI3K/Akt pathway[67]. These findings highlight the importance of targeting multiple RTKs, including EGFR and c-Met, to effectively suppress tumour growth. Additional oncogenic drivers include ALK rearrangements (5% of NSCLC) and ROS1 fusions, both of which initially respond well to ALK inhibitors but often develop resistance over time[68-70].

In the current study, selected compounds were docked against Mutated EGFR and ALK receptors, with EGFR mutations being among the most prevalent oncogenic drivers in advanced NSCLC[65]. Stigmasterol exhibited the strongest binding affinities with docking scores of -8.8, -8.8, -8.0, -8.8, and -7.9 with the targets EGFR, ALK, ROS1, PI3K, and MET receptors, respectively. Its steroid-like framework appears to support stable hydrophobic and other non-covalent interactions within the receptor's binding pocket. The binding interactions of stigmasterol involve critical active site residues, such as Cys797, Asp855, Thr854, and Met793, within EGFR, which are essential for tyrosine kinase inhibition[39]. Previous Studies have shown that stigmasterol modulates the Akt/mTOR and JAK/STAT signalling pathways, and exhibits anti-angiogenic activity by downregulating tumour necrosis factor-α (TNF-α) and vascular endothelial growth factor receptor-2 (VEGFR-2)[38]. The multitarget activity of stigmasterol offers a means to overcome the single-target drug resistance recurrently encountered in clinical NSCLC therapy.

Quinoline and phytol showed moderate binding affinities. Quinoline, being a smaller heterocyclic molecule, shows a moderate level of binding affinity, likely because it can form important interactions such as hydrogen bonds and π–π stacking[41] Whereas phytol, with a long hydrocarbon chain and a single hydroxyl group, supports mixed hydrophobic and polar interactions. Hexadecane, 1-chloro-, and N-Hexadecanoic acid showed higher (less favourable) binding energies, indicating weaker interactions with the receptors. Even so, all the analysed compounds demonstrated noteworthy docking scores, suggesting that they may still hold potential as part of combination strategies for lung cancer treatment.

Despite the efficacy of targeted therapies, such as EGFR TKIs, ALK and ROS1 inhibitors, and emerging MET and PI3K inhibitors, they show potential activity; yet, growing drug resistance stresses the need for novel, multi-targeted agents. The multi-ligand, multitarget docking approach employed in the present study provides mechanistic insight into how bioactive phytochemicals from CI-ALE may simultaneously interact with multiple NSCLC targets, potentially offering synergistic effects and overcoming mono-target drug resistance. Altogether, the GC-MS profiling and *in silico* docking analyses provide valuable mechanistic evidence supporting the anti-cancer potential of CI-ALE against key NSCLC drivers. These findings validate its traditional use and encourage its further exploration through *in vivo* studies and clinical validation.

5. Conclusion

CI-ALE showed the presence of phytochemicals like flavonoids, phenols, tannins, and alkaloids. A higher concentration of flavonoids and tannins was found, contributing to significantly greater antioxidant activity compared to the positive controls. Around 38 compounds were identified using GC-MS, and among them, a few were reported to exhibit anti-cancer, anti-inflammatory, and antioxidant activities. The CI-ALE was tested for its anti-cancer potential using A549 (Human adenocarcinoma cell lines) and Vero (African green monkey kidney cell) lines, respectively, and the results were conclusive, supported by *in silico* docking studies performed with NSCLC-specific receptors. The docking results demonstrated stigmasterol

as a promising lead compound for further development as a multitarget inhibitor of EGFR, ALK, ROS1, PI3K, and MET receptors. These findings provide a foundation for further studies intended to develop effective anti-cancer therapeutics targeting these critical pathways. In the future, these compounds in the CI-ALE may be used to formulate new drugs for combinatorial therapy in cancer treatment.

6. References

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Author Contribution

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