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In-Silico and In-Vitro Evaluation of Polyherbal Formulation for Anticancer Activity Against Non-Small Cell Lung Cancer

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Abstract

Background: Non-small cell lung cancer (NSCLC) remains a major cause of cancer-related mortality. Plant-derived constituents are being investigated as sources of compounds with potential effects on cancer-cell viability and migration. This study evaluated a combined methanolic leaf extract prepared from Azadirachta indica, Ficus racemosa and Aegle marmelos (AFAE) using phytochemical screening, thin-layer chromatography (TLC), molecular docking and in-vitro assays.

Methods: Leaves were collected and authenticated, shade-dried, powdered and extracted using methanol by Soxhlet extraction. The combined extract was screened for major phytochemical groups and analysed by TLC. Quercetin was docked against fibroblast growth factor receptor 4 (FGFR4; reported PDB ID 5JKG) using CB-Dock 2/AutoDock Vina-based scoring. Cytotoxicity in A549 lung adenocarcinoma cells was assessed by MTT assay after 48 h. Cell migration was assessed by scratch/wound-healing assay, and cellular morphology was examined using acridine orange/ethidium bromide (AO/EtBr) staining.

Results: Phenols, tannins, glycosides and quinones were strongly detected (+++); flavonoids were moderately detected (++); coumarins, alkaloids and saponins were detected at lower qualitative levels (+). The TLC profile indicated flavonoid/phenolic constituents. Quercetin showed a docking score of −7.9 kcal/mol, compared with −8.4 kcal/mol for fisogatinib. AFAE reduced A549 cell viability in a concentration-dependent manner, from 98.45% at 12.5 µg/mL to approximately 19.66% at 200 µg/mL, with a reported IC_{50} of 119.21 µg/mL. At 48 h, wound closure was 78.31% in controls and 61.95% and 62.77% at 20 and 40 µg/mL, respectively. AO/EtBr staining showed changes consistent with apoptosis and membrane-compromised cell death.

Conclusion: The findings provide preliminary in-vitro and computational evidence of cytotoxic and antimigratory activity of AFAE against A549 cells. The formulation requires further chemical standardisation, mechanistic validation, toxicity testing and in-vivo evaluation before any therapeutic conclusions can be drawn.

Keywords: Polyherbal extract; NSCLC; A549; Azadirachta indica; Ficus racemosa; Aegle marmelos; molecular docking; MTT assay; apoptosis.

1. Introduction

Lung cancer is a heterogeneous malignancy characterised by uncontrolled growth of pulmonary cells, invasion of surrounding tissues and, in advanced disease, spread to distant organs. It is broadly classified into small cell lung cancer and non-small cell lung cancer. NSCLC accounts for most lung cancer cases and includes adenocarcinoma, squamous cell carcinoma and other less common histological subtypes. Disease outcome is influenced by stage, histology, molecular alterations and response to treatment.

Current management may include surgery, radiotherapy, chemotherapy, targeted therapy and immunotherapy. Molecularly guided treatment has improved options for selected patients, but tumour heterogeneity, acquired resistance and treatment-related adverse effects remain important challenges. These issues support continued research into new bioactive molecules and complementary discovery approaches, while established cancer therapies remain the standard of care.

Medicinal plants contain diverse secondary metabolites, including phenolic compounds, flavonoids, alkaloids, tannins, glycosides and terpenoid-related constituents. Some plant-derived compounds have been investigated for effects on cell proliferation, oxidative stress, apoptosis and migration. Combining extracts from multiple plants may expose cells to a chemically diverse mixture; however, additive or synergistic activity cannot be assumed and must be demonstrated experimentally.

Azadirachta indica (neem), Ficus racemosa (cluster fig) and Aegle marmelos (bael) have documented traditional uses and have been studied for a range of pharmacological properties. Their leaf constituents provide a rationale for preliminary screening of a combined extract. In this work, the combined extract is referred to as AFAE, representing the selected plant materials.

Fibroblast growth factor receptor 4 (FGFR4) is a receptor tyrosine kinase involved in cellular signalling. Altered growth-factor signalling has been investigated in cancer biology, including NSCLC. Molecular docking can provide a computational estimate of how a selected compound may fit a target binding site, but docking scores do not establish target inhibition, cellular mechanism or clinical efficacy.

1.1 Aim and objectives

The aim was to conduct a preliminary integrated evaluation of AFAE against A549 lung cancer cells. Specific objectives were to: (i) screen the combined extract for major phytochemical groups; (ii) profile the extract by TLC; (iii) evaluate the predicted interaction of quercetin with FGFR4; (iv) assess A549 cell viability by MTT assay; and (v) examine cell morphology/apoptosis and cell migration using AO/EtBr staining and a wound-healing assay.

2. Materials and Methods

2.1 Plant collection and authentication

Fresh leaves of Azadirachta indica, Ficus racemosa and Aegle marmelos were collected in Tiruchirappalli, Tamil Nadu, India. The source report states that the plant material was authenticated by a qualified taxonomist in the Department of Botany, National College (Autonomous), Tiruchirappalli. A voucher specimen was recorded as NCT/BOT/PI/CMK/26/0015.

2.2 Drying, powdering and extraction

The leaves were washed with water and shade-dried for approximately one to two weeks. The dried material was coarsely powdered using a grinder and stored in a sealed container in a cool, dark and dry place until extraction. The powdered material was subjected to Soxhlet extraction using methanol. The report describes 250 mL methanol at a 1:4 (w/v) plant-to-solvent ratio, extraction at approximately 65 °C for 72 h, followed by concentration using a rotary evaporator at 40–50 °C under reduced pressure. The resulting extract was stored in a desiccator for subsequent testing.

2.3 Preliminary phytochemical screening

Qualitative tests were conducted on the methanolic extract using standard phytochemical procedures. The report describes tests for alkaloids, saponins, tannins, phenolic compounds, flavonoids, coumarins, glycosides, quinones, volatile oils and sugars. Observations such as colour change, precipitate formation or persistent foam were interpreted as preliminary indications of the relevant phytochemical group. The notation +, ++ and +++ is reported as a qualitative indication and should not be interpreted as a quantitative measurement.

2.4 Thin-layer chromatography

TLC was performed using silica gel G plates. The report describes preparation and activation of plates at 110 °C for 30 min, application of extract at approximately 1 mg/mL, and chamber development using ethanol–water (2:1, v/v). After development, the plate was examined for separated spots and the retention factor (Rf) was calculated as the distance travelled by the analyte divided by the distance travelled by the solvent front. The observed profile was interpreted as indicating flavonoid/phenolic constituents.

2.5 Molecular docking

A computational docking assessment was carried out for quercetin against FGFR4 using the CB-Dock 2 server and a Vina-based scoring approach. The report identifies PDB ID 5JKG in the abstract/results table, while a discussion passage lists 5JKB; this discrepancy must be checked against the original docking files before journal submission. Fisogatinib was included as a reference FGFR4 inhibitor. More negative predicted binding energies indicate more favourable scores within the scoring model, but do not by themselves prove biological inhibition.

2. Materials and Methods (continued)

2.6 Cell culture and MTT cytotoxicity assay

A549 human lung adenocarcinoma cells were used for the in-vitro evaluation. The report identifies cell culture medium based on DMEM, supplemented with foetal bovine serum, and incubation under standard humidified conditions at 37 °C and 5% CO_2. For the MTT assay, cells were exposed to AFAE at 12.5, 25, 50, 100 and 200 µg/mL for 48 h. Following exposure, MTT reduction was used as an indicator of metabolically active cells, and absorbance was measured spectrophotometrically. Cell viability was expressed relative to the untreated control; percentage inhibition was calculated as 100 minus percentage viability. The reported IC_{50} was 119.21 µg/mL.

The source report provides average optical-density values with SEM and viability percentages, but the detailed replicate structure and complete absorbance protocol should be verified from laboratory records. For a publication-ready report, the exact cell seeding density, MTT concentration and incubation duration, solvent/vehicle control, wavelength, number of independent experiments and statistical method should be explicitly stated.

2.7 AO/EtBr dual-staining assay

Acridine orange/ethidium bromide dual staining was used to examine cellular morphology at the reported IC_{50}-related treatment concentration. Acridine orange can stain viable cells green, while ethidium bromide enters cells with compromised membrane integrity and produces orange-red fluorescence. The report describes observation of reduced cell density, chromatin condensation, membrane blebbing, nuclear fragmentation and orange-red staining in treated cells compared with predominantly green control cells. These morphological features are consistent with cell injury and apoptosis, but fluorescence images alone do not quantify the proportion of cells in each death category.

2.8 Wound-healing/scratch assay

The scratch assay was used to assess changes in A549 cell migration after treatment with AFAE at 20 and 40 µg/mL. A linear wound was made in a confluent cell monolayer, detached cells were removed by washing, and the cultures were incubated with test concentrations. Images were recorded at 0, 12, 24, 36 and 48 h. Wound closure was calculated as [(initial wound area − final wound area)/initial wound area] × 100. The report presents values as mean ± SD with n = 2.

2.9 Data handling and interpretation

Results are summarised descriptively using the values reported in the source document. Because the wound-healing assay reports n = 2 and the full statistical workflow is not provided, the findings are treated as exploratory. No unreported p-values or claims of statistical significance have been added. The experiments represent preclinical laboratory evidence and do not establish safety or efficacy in humans.

3. Results

3.1 Phytochemical screening

Qualitative screening indicated multiple phytochemical groups in the combined methanolic extract. Phenols, tannins, glycosides and quinones were recorded as strongly present; flavonoids were moderately

present; coumarins, alkaloids and saponins were recorded as present at lower qualitative levels. Volatile oils and sugars were recorded as not detected under the tests used.

Phytochemical group	Reported observation
Phenols	+++ (strongly detected)
Tannins	+++ (strongly detected)
Glycosides	+++ (strongly detected)
Quinones	+++ (strongly detected)
Flavonoids	++ (moderately detected)
Coumarins	+ (detected)
Alkaloids	+ (detected)
Saponins	+ (detected)
Volatile oils	− (not detected)
Sugars	− (not detected)

3.2 TLC profile

TLC analysis showed separated spots, and the source report associated a prominent spot with flavonoid/phenolic compounds. The report contains two Rf values in different sections (0.28 in the tabulated profile and approximately 0.38 in the narrative/figure description). The value should be confirmed from the original plate image and laboratory notebook before final publication.

3.3 Molecular docking

Ligand	Target	Reported Vina score (kcal/mol)	Interpretation
Fisogatinib	FGFR4; PDB ID reported as 5JKG	−8.4	More favourable predicted score
Quercetin	FGFR4; PDB ID reported as 5JKG	−7.9	Favourable predicted score, slightly weaker than reference

Quercetin showed a predicted binding score of −7.9 kcal/mol, compared with −8.4 kcal/mol for fisogatinib. The score difference suggests a somewhat less favourable predicted interaction for quercetin under the same docking setup. Docking should be interpreted as hypothesis-generating and requires confirmation through biochemical or cellular target-engagement studies.

3.4 MTT cytotoxicity assay

AFAE concentration (µg/mL)	Average OD ± SEM	Cell viability (%)	Inhibition (%)
Control	1.2585 ± 0.0045	100.00	0.00
12.5	1.293 ± 0.007	98.45	1.55
25	1.184 ± 0.012	94.08	5.92
50	0.954 ± 0.012	75.76	24.24
100	0.637 ± 0.016	50.61	49.39
200	0.248 ± 0.007	19.66	80.34

The extract produced a concentration-dependent reduction in cell viability over 48 h. The reported IC_{50} was 119.21 µg/mL.

3. Results (continued)

3.5 AO/EtBr staining

Control A549 cells predominantly showed green fluorescence and relatively intact nuclear morphology. In extract-treated cells, the report describes reduced cell density, increased orange-red fluorescence and morphological changes including chromatin condensation, membrane blebbing and nuclear fragmentation. These observations are consistent with a mixture of apoptotic changes and loss of membrane integrity. As no numerical cell counts or percentages for live, early apoptotic, late apoptotic and necrotic cells were reported, the outcome is presented qualitatively.

3.6 Wound-healing assay

Time	Control (%)	AFAE 20 µg/mL (%)	AFAE 40 µg/mL (%)
12 h	36.37 ± 4.81	37.50 ± 1.92	37.22 ± 5.87
24 h	55.17 ± 8.35	42.18 ± 3.36	42.11 ± 4.61
36 h	68.38 ± 2.10	57.91 ± 0.39	51.35 ± 0.72
48 h	78.31 ± 2.84	61.95 ± 2.98	62.77 ± 10.18

At 48 h, wound closure was lower in treated cultures than in untreated controls. The absolute difference from control was 16.36 percentage points at 20 µg/mL and 15.54 percentage points at 40 µg/mL. At 36 h, the 40 µg/mL group showed 51.35% closure compared with 68.38% in controls. These data suggest reduced wound closure under the tested conditions; because scratch closure can reflect both migration and proliferation, a migration-specific effect cannot be isolated without additional controls.

4. Discussion

This study combined qualitative phytochemical tests, TLC, computational docking and cell-based assays to explore the potential activity of AFAE against A549 cells. The detected phenolic and flavonoid groups provide a plausible basis for further chemical characterisation, but qualitative tests cannot identify individual molecules or establish their concentrations. TLC is similarly a preliminary profiling method and should be supplemented with validated analytical methods such as HPLC or LC–MS for standardisation.

The MTT data show a clear concentration-associated reduction in metabolic viability, with approximately half of control viability remaining at 100 µg/mL and a reported IC_{50} of 119.21 µg/mL. MTT measures cellular metabolic activity and is not a direct measure of cell number or a specific death mechanism. Confirmatory assays and testing against non-malignant lung cells are needed to determine selectivity and rule out non-specific cytotoxicity.

4. Discussion (continued)

The AO/EtBr observations are compatible with apoptotic and membrane-compromised cell death. However, qualitative fluorescence microscopy cannot by itself distinguish apoptosis from secondary necrosis with certainty. Quantitative flow cytometry, caspase activity, Annexin V-based assays or other orthogonal methods would strengthen the mechanistic interpretation.

The wound-healing data indicate lower closure in treated cells compared with controls. At 48 h, both tested concentrations reduced closure by roughly 16 percentage points relative to control. These findings are exploratory because the reported sample size is small (n = 2), variability at 40 µg/mL is substantial, and cytotoxicity may itself influence scratch closure. Future studies should use additional biological replicates and conditions that minimise confounding by cell death or proliferation.

Molecular docking predicted a quercetin–FGFR4 score of −7.9 kcal/mol, close to but less favourable than the reference fisogatinib score of −8.4 kcal/mol. This does not demonstrate that quercetin inhibits FGFR4 in cells or that FGFR4 mediates the observed effects of the whole extract. Target-specific biochemical testing, pathway readouts and appropriately designed controls are needed. The source report also contains inconsistencies in the receptor PDB identifier and TLC Rf value; these should be resolved before submission.

4.1 Study limitations

The work is limited by the use of a single cancer cell line, the absence of a reported non-cancerous cell comparator, limited replicate information, qualitative rather than quantitative phytochemical analysis, and the lack of in-vivo or pharmacokinetic data. The reported experiments do not establish that the three plants

act synergistically; the source discussion itself notes that an additive effect was not observed on mixing. Batch-to-batch standardisation and independent replication are also required.

5. Conclusion

The combined methanolic leaf extract of Azadirachta indica, Ficus racemosa and Aegle marmelos showed preliminary cytotoxic and antimigratory effects against A549 cells. Phytochemical screening detected several secondary-metabolite groups, quercetin displayed a predicted interaction with FGFR4, MTT results showed concentration-dependent loss of viability, and AO/EtBr staining revealed cellular changes compatible with cell death. Wound closure was reduced in treated cultures compared with controls. These findings justify further investigation but do not establish a clinically useful anticancer treatment. Future work should confirm extract composition, resolve reporting discrepancies, test normal-cell selectivity, improve replication and statistical analysis, validate the proposed mechanism, and conduct appropriate toxicity and in-vivo studies.

Declarations and publication notes

Ethics: Cell-line work is described; no human or animal experiments are reported in the supplied project document. Funding/conflict of interest: not stated in the source report and should be completed by the authors. Data availability: underlying raw data should be retained and made available according to the target journal's policy. Before submission, authors should verify all bibliographic details, PDB ID, TLC Rf, assay replicates, statistical methods and institutional author affiliations.

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