



In vitro anti-cancer activity of a polyherbal preparation, VEDICINALS®9, against A549 human lung adenocarcinoma cells

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ABSTRACT

Purpose: Non-small cell lung cancer (NSCLC) is among the leading causes of morbidity and mortality worldwide. Despite the availability of several treatment options, the five-year survival rate of NSCLC is extremely low (<20%). This underlines the necessity of more effective therapeutic alternatives. In this context, plant-derived extracts and bioactive molecules extracted from plants, known collectively as phytochemicals, represent an extremely variegated source of bioactive compounds with potent anticancer potential. In the present study, we tested the *in vitro* anticancer activity of a polyherbal preparation, VEDICINALS®9, containing nine different bioactive principles extracted by medicinal plants.

Methods: The anticancer activity of VEDICINALS®9 was investigated by measuring its impact on A549 human NSCLC cell proliferation (MTT assay and trypan blue staining), migration (wound healing assay and transwell chamber assay) and by measuring the impact on the expression of cancer-related proteins (Human XL Oncology Protein Array).

Results: We show that VEDICINALS®9 at a concentration of 0.2% v/v has potent anticancer effect, significantly inhibiting A549 cell proliferation and migration. Mechanistically, this was achieved by downregulating the expression of proteins involved in cancer cell proliferation (Axl, FGF basic, enolase 2, progranulin, survivin) and migration (Dkk-1, cathepsins B and D, BCL-x, amphiregulin, CapG, u-plasminogen activator). Furthermore, treatment with VEDICINALS®9 resulted in increased expression of the oncosuppressor protein p53 and of the angiogenesis inhibitor endostatin.

Conclusions: Taken together, our results provide proof of principle of the potent anticancer activity of the polyherbal preparation VEDICINALS®9, highlighting its enormous potential as an alternative or adjuvant therapy for lung cancer.

List of abbreviation: NSCLC, Non-small cell lung cancer; SCLC, Small cell lung cancer; FGF, Fibroblast growth factor; FGFR, Fibroblast growth factor receptors; ENO2, Enolase 2; DKK-1, Dickkopf-1; MMPs, Matrix metalloproteinases; CapG, Macrophage-capping protein; P53, Tumor protein P53; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; ATCC, American Type Culture Collection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, Analysis of Variance; A549, Human lung adenocarcinoma cells.

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1. Introduction

Lung cancer is a leading cause of cancer death globally characterized by uncontrolled cell proliferation in the lung tissue. In the world, lung cancer is among the main causes of morbidity and mortality, as it represents one-fifth of all deaths associated to cancer [1], with 2.2 million cases diagnosed and 1.7 million deaths caused worldwide by lung cancer in 2020 [2]. Lung cancer is primarily classified as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common form of lung cancer, accounting for 85% of cases, and it is further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma subtypes [3]. The major risk factor of lung cancer is cigarette smoking, including both active smoking and passive smoke exposure [4].

Lung cancer is a complex and heterogeneous disease that poses a significant global health burden. During the multi-step process of cancer development, certain biological capabilities that are considered cancer hallmarks are acquired by malignant cells. These include uncontrolled and sustained proliferation, migration and invasion, angiogenesis, and metastasis [5]. The acquisition of these cancer hallmark is driven by the dysregulation of the expression of several effector proteins, promoting the development and progression of lung cancer [5]. Delving into the expression patterns and clinical significances of these proteins will further help to develop potential therapeutic interventions. Proteins that mediate the sustained proliferation of cancer cells include AXL, fibroblast growth factor (FGF), Enolase 2, progranulin, and survivin. AXL is a member of the TAM (Tyro3, AXL, Mer) family of receptor tyrosine kinases and it is primarily involved in various cellular processes, including cell survival, proliferation, migration, and invasion [6]. Studies utilizing gene expression profiling and immunohistochemistry have consistently demonstrated elevated expression of AXL in lung cancer tissues including adenocarcinoma [7,8] and squamous cell carcinomas [8] compared to normal lung tissues. Furthermore, preclinical studies using AXL inhibitors have demonstrated promising results in inhibiting tumor growth, metastasis, and enhancing the efficacy of existing treatments in lung cancer models [9,10].

The FGF family comprises a group of growth factors that play essential roles in embryonic development, tissue homeostasis, and angiogenesis, and the dysregulation of FGF signalling has been implicated in various cancers, including lung cancer [11]. Studies have shown the overexpression of FGF ligands, such as FGF2 and FGF9, in lung cancer tissues compared to normal lung tissues [12,13]. Additionally, upregulation of FGF receptors, particularly FGFR1, has been associated with adverse clinical outcomes in lung cancer patients [13]. Consistently, preclinical studies have demonstrated the efficacy of FGFR inhibitors in inhibiting tumor growth and enhancing the sensitivity of lung cancer cells to chemotherapy and targeted therapies [14,15].

Enolase 2 (ENO2), also known as neuron-specific enolase, is another cell proliferative enzyme which is mainly involved in glycolysis and energy metabolism [16]. While ENO2 is primarily expressed in neurons and neuroendocrine cells, its aberrant expression has been observed in various cancers, including lung cancer [17]. The upregulation of ENO2 in lung cancer has also been reported in adenocarcinoma A549 cells in vitro [18]. Given its involvement in energy metabolism and tumor progression, ENO2 also represents a potential therapeutic target in lung cancer treatment.

Progranulin is another secreted growth factor that is highly glycosylated and is involved in various biological processes, including cell proliferation, migration, and invasion [19]. Emerging evidence suggests that progranulin plays a multifaceted role in lung cancer, where its expression is dysregulated. Elevated expression of progranulin has been observed in male patients with lung adenocarcinoma, and its role has also been validated in vivo in a mouse xenograft model [20]. Similarly, survivin is a member of the inhibitor of apoptosis protein family, and it plays a crucial role in regulating cell survival and apoptosis [21]. High survivin expression has been correlated with reduced overall survival

and disease-free survival in lung cancer patients [22]. Additionally, preclinical studies have demonstrated the efficacy of survivin inhibitors in inhibiting tumor growth and enhancing the sensitivity of lung cancer cells to chemotherapy and targeted therapies [23,24].

Numerous proteins are involved in the regulation of the migratory capacity of lung cancer cells. DKK, or Dickkopf-1, is one of such proteins that plays a critical role in various cellular processes, including cell migration [25]. In the context of lung cancer, DKK has been found to be upregulated in serum, and its levels correlated with tumor-nodes-metastasis stage, lymph node involvement, and metastasis, suggesting a potential role for DKK in promoting cancer cell migration and invasion [26]. Studies have shown that DKK activates the Wnt signalling pathway, which is known to regulate cell migration and invasion, and the activation of the Wnt pathway by DKK leads to the transcriptional upregulation of target genes involved in promoting cancer cell migration [27].

Cathepsin B and cathepsin D are lysosomal proteases that have been implicated in various physiological and pathological processes, including cancer progression. Cathepsin B promotes cancer cell migration by degrading components of the extracellular matrix and activating signalling pathways involved in cell motility. It also plays a role in the activation of other proteases, such as matrix metalloproteinases (MMPs), which further contribute to cancer cell migration [28]. Cathepsin D, on the other hand, is involved in the proteolytic processing of various proteins, including growth factors and their receptors, that influence cancer cell migration and invasion [29].

BCLx is a member of the B-cell lymphoma 2 (BCL-2) family of proteins that regulate apoptosis, or programmed cell death [30]. Studies have shown that BCLx promotes lung cancer cell migration by regulating the expression and activity of MMPs. BCLx has been found to upregulate the expression of MMPs, such as MMP-2 and MMP-9, which are involved in degrading the extracellular matrix and facilitating cancer cell migration [31]. Furthermore, BCLx has been shown to interact with other proteins involved in cell migration, such as focal adhesion kinase, to promote cancer cell motility [31]. Another protein, Amphiregulin, is a member of the epidermal growth factor family of proteins. It promotes lung cancer cell migration and invasion by activating signalling pathways, such as the mitogen-activated protein kinase pathway and the phosphoinositide 3-kinase pathway [32]. Targeting any of the aforementioned proteins with the aim of inhibiting the pathways they activate may offer novel therapeutic approaches to inhibit lung cancer migration and metastasis.

Apart from the proliferative and migratory pathways, other proteins are involved in the regulation of cancer progression. One of these is p53, a nuclear phosphoprotein that acts as tumour suppressor. P53 is active at the transition between phases G1 and S and between phases G2 and M of the cell cycle, and it triggers the apoptosis of cancer cells [33]. Mutations in the gene encoding for p53 result in the dysregulation of apoptosis and are found in 60% of lung cancer cases [33,34]. Furthermore, endogenous angiogenesis inhibitors like endostatin, a specific inhibitor of endothelial cell proliferation and angiogenesis, have been found upregulated in the serum of non-small cell lung cancer patients compared to healthy individuals [35].

Common treatment modalities for lung cancer includes either single therapies or various combinations of surgical ablation, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. These therapies are often expensive, non-affordable and also associated with various side effects. [36] For this reason, identifying alternative therapies that are affordable with minimized side effects is highly essential for improving the quality of life of patients. Plant-based medicines, such as herbal remedies and/or active natural plant-derived compounds, represent one of such alternatives, and many studies have highlighted their potential in lung cancer treatment [37–39]. The current study investigates the therapeutic potential of VEDICINALS®9, a nutraceutical supplement which is composed of nine herbal-based bioactive compounds (baicalin, quercetin, luteolin, rutin, hesperidin, curcumin,

epigallocatechin-gallate, piperine, and glycyrrhizin) invented and marketed by Vedicinals India Private Limited. The objective of our study was to investigate the anticancer potential of VEDICINALS®9 in vitro using human lung adenocarcinoma cells line (A549).

2. Materials and methods

2.1. Cell Culture and VEDICINALS®9 treatment

A549 (ATCC, USA) were gifted by Prof. Alaina Ammit, Woolcock Institute of Medical Research, Sydney, AU. The cells were cultured in low-glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Merck, Australia), supplemented with 5% v/v heat-inactivated fetal bovine serum (Sigma-Merck, Australia), 1% v/v penicillin/streptomycin (Sigma-Merck, Australia), in a humidifier at 37 °C and supplied with 5% CO₂. The VEDICINALS®9 suspension was first dissolved in dimethyl sulfoxide (DMSO, Sigma-Merck, Australia) at 25% v/v concentration, due its tendency to form aggregates. The stock was diluted to the concentrations tested (0.05%, 0.1%, and 0.2%) in supplemented DMEM.

2.2. Cell viability assay (MTT assay)

To measure the antiproliferative effect of VEDICINALS®9, the MTT assay was performed as reported in a previous study [40]. Briefly, A549 cells were seeded at a density of 5000 cells/well/100 µL in a 96-well plate. After overnight attachment, cells were exposed to increasing concentrations of VEDICINALS®9 (0.05%, 0.1%, and 0.2%) or DMSO (corresponding to the VEDICINALS®9 0.2% concentration) for 24 h. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Merck, Australia) was added at a concentration of 0.5 mg/mL to the culture medium, and the cells were incubated for further 4 h at 37 °C to allow for the formation of the formazan crystals. The supernatant was removed, and the crystals were dissolved in 100 µL DMSO. The absorbance was read in a microplate reader (TECAN Infinite M1000) at 570 nm.

2.3. Cell viability assay – trypan blue staining

A549 cells were seeded at a density of 10000 cells/well/300 µL in 48-well plates. After overnight attachment, the cells were treated with 0.2% VEDICINALS®9 or DMSO for 24 h. The cells were then detached with trypsin-ethylenediaminetetraacetic acid solution (Sigma-Merck, Australia) and centrifuged at 500 g for 5 min. The cells were then mixed with a 0.4% trypan blue solution (ThermoFisher Scientific, Australia), and the live cells were counted using a light microscope.

2.4. Wound healing assay

The wound healing assay was performed as reported in a previous study [40], to determine the anti-migratory activity of the VEDICINALS®9 suspension. 300,000 cells/well/ 2 mL were seeded in 6-well plates and cultured until confluent. A scratch was then created in the cell monolayer using the tip of a sterile 200 µL pipette tip, followed by five washes with sterile PBS. Images were taken under a light microscope at time 0, then cells were treated with 0.2% VEDICINALS®9 or DMSO for 24 h. Pictures were taken after treatment, and the distance between the margins of the scratch was measured. The percentage wound closure was reported compared to the untreated control.

2.5. Transwell chamber assay

To determine the anti-migratory effect of the VEDICINALS®9 suspension, the transwell chamber assay was performed as reported in a previous study [39]. Transwell permeable membranes (6.5-mm insert 8-µm pore size polycarbonate membrane, Sigma-Merck, Australia) were used. 10,000 cells/well/200 µL were seeded onto each membrane, and

the membranes were placed in 600 µL DMEM. After attachment, the cells were treated with 0.2% VEDICINALS®9 or DMSO for 24 h, then the cells were allowed to migrate for a further 24 h after treatment. After this time, the cells that did not migrate through the transwell membrane were removed with cotton swabs, and the cells that migrated through the membrane were fixed with 10% formalin and subjected to hematoxylin/eosin staining. Finally, the cells that successfully migrated were counted upon acquisition of five random fields, in a light microscope at a magnification of 20X, counting the average cells per field of view.

2.6. Oncology protein array

The effect of the VEDICINALS®9 suspension on the expression of proteins related to cancer cell proliferation, migration, and invasion in A549 cells has been determined using a Human XL oncology array kit (R&D Systems, Minneapolis, USA), as reported in a previous study [41]. A549 cells were seeded at a density of 100,000 cells/well/2 mL in 6-well plates and left to attach. Successively, the cells were treated with 0.2% VEDICINALS®9 for 24 h. After treatment, the cells were washed three times with ice-cold PBS and lysed with 300 µL/well RIPA buffer (ThermoFisher Scientific, Australia) supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Protein concentration was determined using the bicinchoninic A/acid method (Pierce BCA kit, ThermoFisher Scientific). The cell lysates (300 µg protein/sample) were hybridized on each membrane, and the arrays were developed following the manufacturer's instructions. The chemiluminescent signal was acquired using a ChemiDoc MP imaging system (Bio-Rad, Hercules, USA). The pixel density of each spot was measured using ImageJ software v1.53 (Bethesda, USA).

2.7. Statistical analysis

Data are represented as mean ± standard error mean (SEM). To perform statistical analysis, GraphPad PRISM v9.3 software (San Diego, USA) was used. Statistical comparisons were performed via two-tailed Student's t-test or one-way ANOVA followed by Dunnett's t-test for pairwise comparisons, as reported in the figure legends. A P value < 0.05 was considered statistically significant for pairwise comparisons.

3. Results

3.1. Antiproliferative activity of VEDICINALS®9 against A549 cells

The effect of VEDICINALS®9 treatment on A549 cell proliferation is represented in Fig. 1a (MTT assay) and Fig. 1b (Trypan blue staining). Treatment of cells with VEDICINALS®9 at concentrations of 0.05%, 0.1%, and 0.2% v/v showed significant decline in cell proliferation to 55.3%, 53.8%, and 44.6% respectively in comparison to non-treated control cells via MTT assay (Fig. 1a). DMSO (0.2% v/v) treatment showed no significant change in A549 cell proliferation (Fig. 1a). Furthermore, the trypan blue staining of A549 cells subjected to VEDICINALS®9 treatment (0.2% v/v) validated the previous result, showing significant reduction of cell proliferation to 46.9% compared to the untreated control (Fig. 1b).

3.2. VEDICINALS®9 treatment suppresses the migration of A549 cells

VEDICINALS®9 extract was further evaluated by wound healing and transwell chamber assays to understand its effect on A549 cells migration. These results are shown in Fig. 2 (wound-healing assay) and Fig. 3 (transwell chamber assay). Fig. 2a shows the microscopic images of wound dimension variation at different time points for control (only media), DMSO in media, and VDC-D 0.2%-treated groups. After 24 h of treatment, the quantitative analysis revealed that the closure of the wound in the control and DMSO-treated groups was of 24.4% and 24.6%, respectively, whereas the wound closure observed in the VDC-D

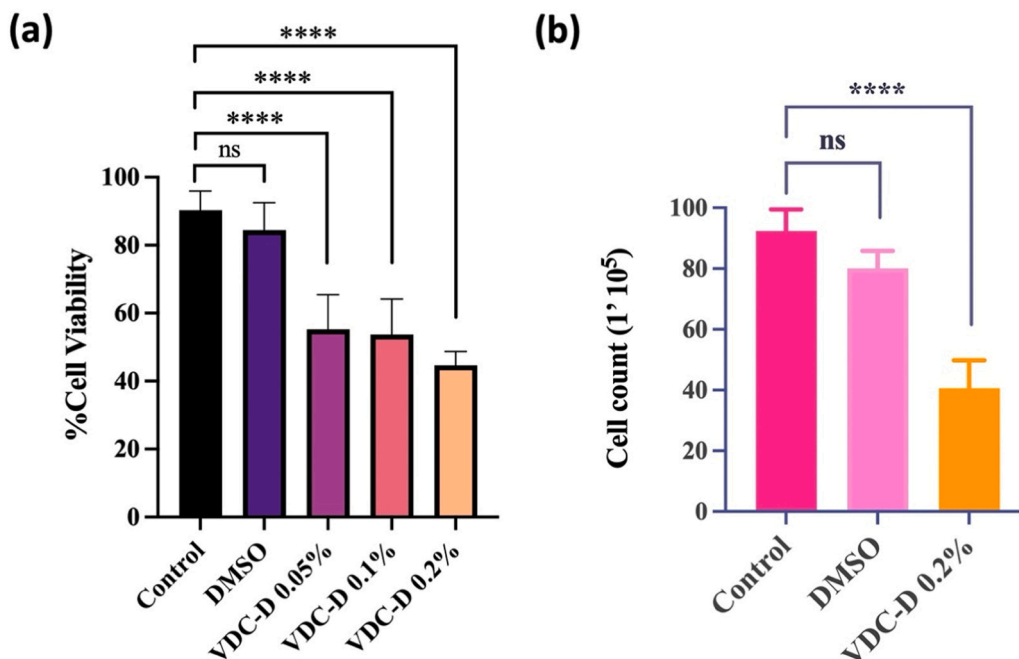


Fig. 1. Analysis of the effect of VEDICINALS®9 extract on A549 cells proliferation. (a) MTT assay showed significant decrease in cell viability with VEDICINALS®9 treatments compared to non-treated control cells (n = 4; **** = p < 0.0001). (b) Trypan blue staining showed significant reduction in cell counts with VDC-D 0.2% treatment compared to untreated control (n = 4; p < 0.0001). One-way ANOVA test.

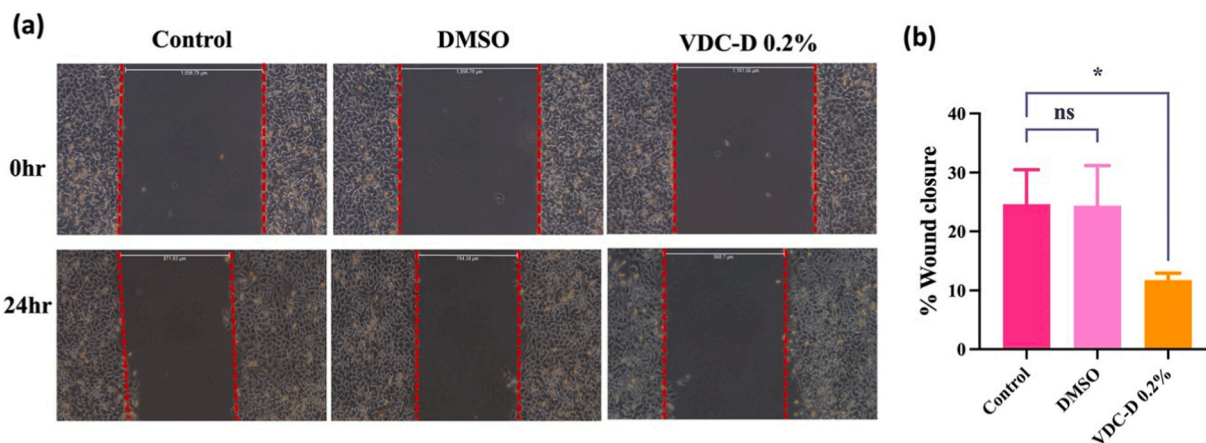


Fig. 2. Wound healing assay analysis of VEDICINALS®9 extract on A549 cells. (a) Microscopic image representation showing the change in wound dimensions at different timepoints with only DMSO and VDC-D (0.2%) treatment compared to non-treated control. Images were taken at 20X magnification. (b) Quantitative analysis showed the significant suppression of A549 cells wound closure with VDC-D 0.2% treatment after 24 h in comparison with control cells (n = 3; * = p < 0.05). One-way ANOVA test.

0.2%-treated cells was of 11.8%, corresponding to a significant 52.2% reduction of the extent of wound closure compared to the untreated control group (Fig. 2b). No significant difference in wound closure was determined between untreated control and DMSO-treated group (Fig. 2b).

These observations were validated with the transwell chamber assay as showed in Figs. 3a and 3b. VDC-D 0.2% treatment resulted in significant reduction of the A549 cells migration rate (cells/field) to 11.2 cells/field, which corresponded to a statistically significant 56.9% reduction of cell migration compared to the untreated control (26 cells/field, Fig. 3b). No statistically significant difference in cell migration was observed between the untreated control and the DMSO-treated group (25.6 cells/field, Fig. 3b).

3.3. Oncology protein array analysis

Oncological protein array evaluation was conducted to understand the various signaling cascade effected by VEDICINALS®9 extract exposure to A549 cells. Figs. 4, 5, 6a and b represent the relative expression of different proteins related to proliferation, migration, tumor suppression, and angiogenesis properties, respectively, in A549 cells subjected to treatment with VDC-D 0.2%. The quantitative analysis showed that treatment of A549 cells with VDC-D 0.2% resulted in the down-regulation of proliferation-related proteins such as Axl (74.7%), FGF basic (25.9%), Enolase2 (8.2%), Progranulin (19.7%), and Survivin (48.4%) compared to the untreated control (Fig. 4). The anti-migratory effect of VEDICINALS®9 extract was further correlated with the down-regulated expression of migration related proteins Dkk-1 (77.1%), Cathepsin B (25.6%), Cathepsin D (43.7%), BCL-x (60.9%),

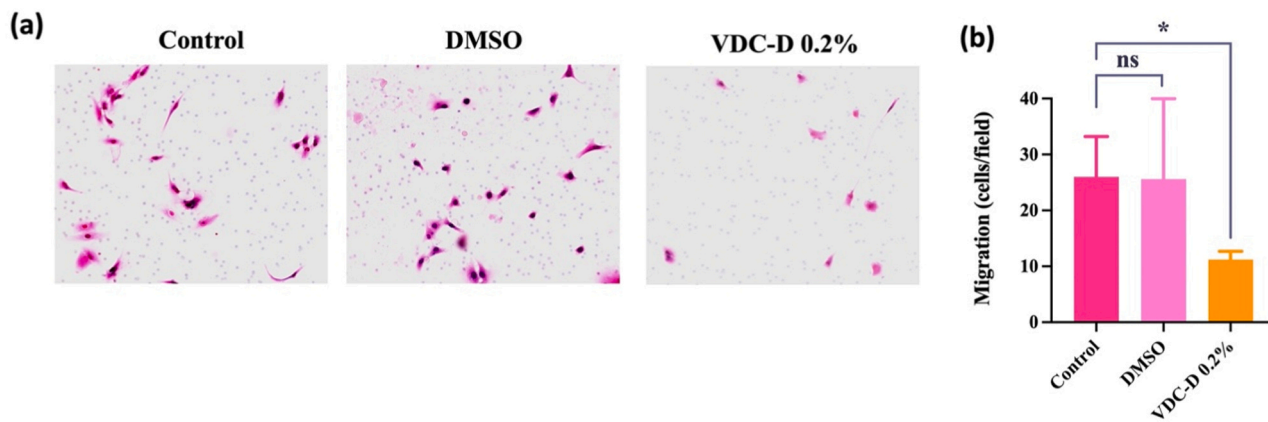


Fig. 3. Transwell chamber assay analysis of VEDICINALS®9 extracts on A549 cells. (a) Microscopic image representation showing the cell migration at different treatments compared with non-treated control. Images were taken at 20X magnification. (b) Quantitative analysis showed that A549 cells migration rate (cells/field) was significantly reduced with VDC-D 0.2% treatment for 24 h (n = 5; * = p < 0.05). One-way ANOVA test.

Proliferation pathway

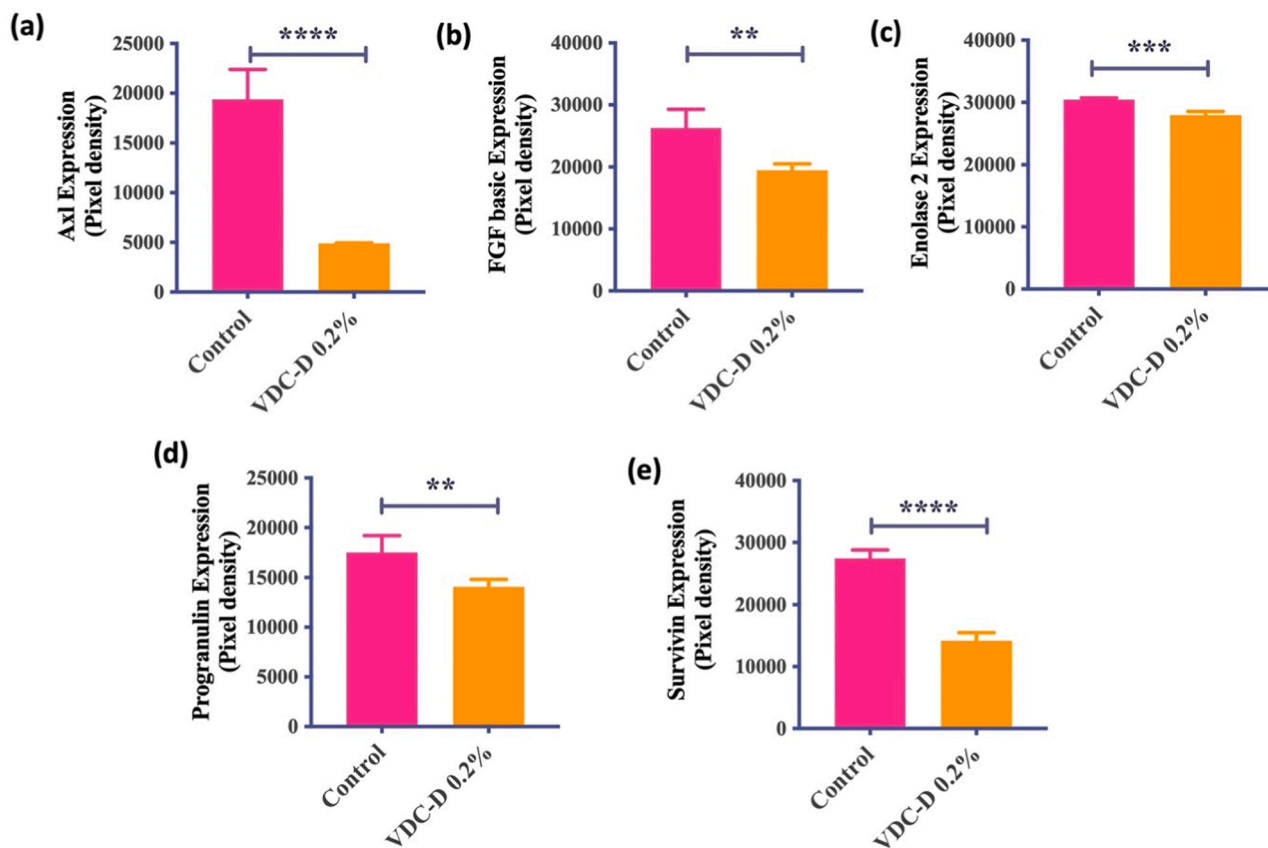


Fig. 4. Treatment of VEDICINALS®9 in A549 cells suppressed the proliferation pathway with downregulation in protein expression of (a) Axl, (b) FGF basic, (c) Enolase 2, (d) Progranulin, and (e) Survivin (n = 4, **p < 0.005; ***p < 0.0005; ****p < 0.0001). Two-tailed Student's t-test.

Amphiregulin (74.7%), macrophage-capping protein (CapG) (26.8%), and u-Plasminogen activator/Urokinase (28.7%) expression, compared to the untreated control (Fig. 5). Additionally, p53 and endostatin) expression was upregulated by 2.1 and 2.3-fold, respectively, upon VDC-D 0.2% treatment compared to untreated control (Fig. 6a and b, respectively).

4. Discussion

The currently available chemotherapy-based treatments for lung cancer are associated with very high costs, often not affordable by all patients, particularly within under-developed countries, and comes along with various side effects or adverse reactions. This has urged and motivated researchers to focus on alternative therapeutic strategies that could be cheaper and have little to no side effects compared to currently

Migration pathway

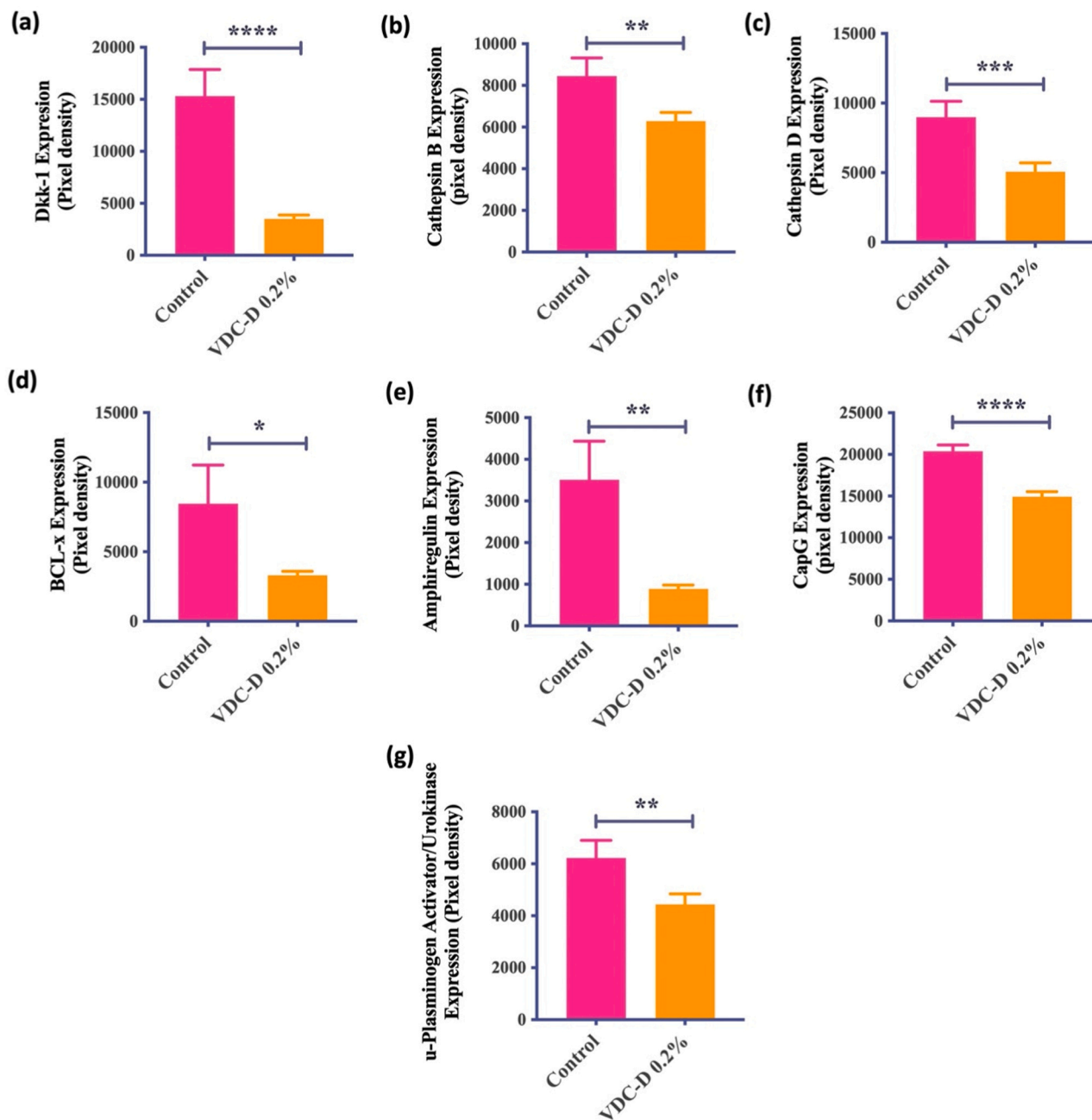


Fig. 5. VEDICINALS®9 treatment effected the migration properties in A549 cells by showing the decline in the expression of (a) Dkk-1, (b) Cathepsin B, (c) Cathepsin D, (d) BCL-x, (e) Amphiregulin, (f) CapG and (g) u-Plasminogen Activator/Urokinase (n = 4; *p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.0001). Two-tailed Student's t-test.

available chemotherapy. Indeed, the scientific study of various medicinal plants and their bioactive compounds could serve as a potential source of novel treatment options. These chemical moieties derived from herbal plants are well established for their variegated range of biological and therapeutic activities, which include anti-cancer activity [38, 41–47].

In the present study, we have revealed the potent anti-cancer activity of the VEDICINALS®9 formulation through in vitro experiments carried out in human lung adenocarcinoma A549 cells. The promising anti-cancer activity of this formulation was obtained through inhibition A549 proliferation, migration, and via the modulation of various proteins involved in cancer progression.

VEDICINALS®9 is invented and marketed by Vedicinals India Private Limited. It is worth noting that the composition of VEDICINALS®9 includes potent anti-cancer agents such as *Curcuma longa* L, *Camellia sinensis*, rutin, *Citrus reticulata*, *Scutellaria baicalensis* (as Baicalin 50%), *Arachis hypogaea* (as Luteolin 98%), quercetin powder, and *Piper nigrum* (as piperine). The rationale for choosing those medicinal plants or single compounds to formulate a polyherbal nutraceutical suspension is based on ours and others previous research findings, showing the potent anticancer activity of these plant extracts/compounds on A549 lung cancer cells. *Curcuma longa* and its potent compound curcumin have been shown to exert significant anticancer activity in most cancer types, including lung cancer [48]. Similarly, Rutin at a low concentration of

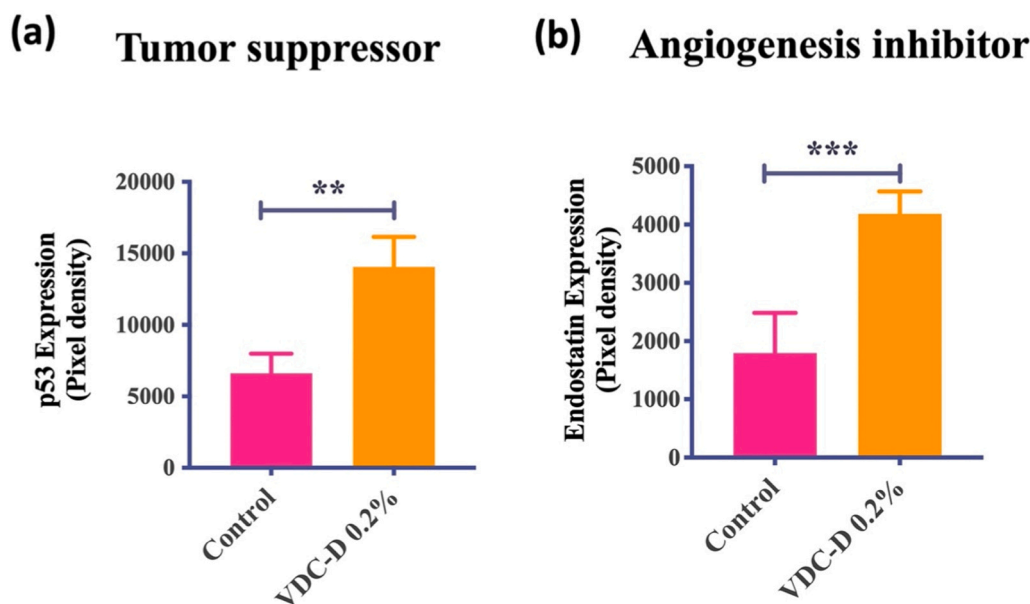


Fig. 6. Elevation of cancer suppressing agents with VEDICINALS®9 treatment in A549 cells showed by increased expression of (a) p53 as tumor suppressor and (b) Endostatin as angiogenesis inhibitor (n = 4; **p < 0.005; ***p < 0.0005). Two-tailed Student’s t-test.

20 mM was able to inhibit A549 cell proliferation and migration [39]. Baicalin extracted from *Scutellaria baicalensis* also possesses remarkable potential in inhibiting the proliferation and migration of the lung cancer cell lines A549 and H1299 [49] and even vascular smooth muscle cells [50]. *Camellia sinensis*, with its potent compound (–)-Epigallocatechin-3-gallate, is effective in inhibiting lung cancer cell progression by targeting key pathways such as MMPs (for cell invasion) and Wnt/β-catenin (for cell proliferation and induction of apoptosis) [51]. Similarly, another study reported the potent in vitro (A549 cells) and in vivo (nude mice) anti-lung cancer activity of *Citrus reticulata* peel oil. Quercetin has also been shown to have potent anticancer activity in vitro on A549 and H1299 cells [52]. Thus, our VEDICINALS®9 formulation is a combination of very effective and ideal anti-cancer agents.

To investigate the two key process of cancer progression; proliferation and migration/invasion, we employed commonly used experimental techniques. The MTT colorimetric cell proliferation assay (Fig. 1a) and trypan blue cell count (Fig. 1b) suggest that VEDICINALS®9 suspension [53] at a concentration of 0.2% v/v, decreased the cell viability by approximately 50%. However, there were no significant changes in A549 cell viability upon treatment with the vehicle DMSO (final concentration not exceeding 0.01%) used to dissolve VEDICINALS®9 suspension. With regards to cell migration and cell invasion, we employed wound healing (Figs. 2a and 2b) and transwell chamber assay (Figs. 3a and 3b). In both experiments, we observed a comparatively similar, significant reduction of A549 cells migration and invasion. From a mechanistic perspective, we investigated the proteins targeted by

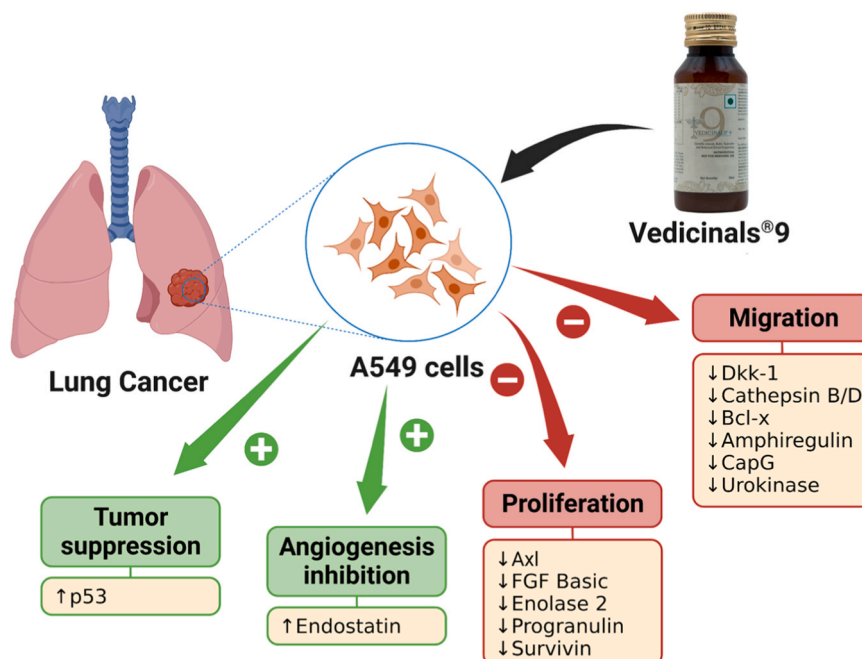


Fig. 7. Mechanism of action of VEDICINALS®9 against lung cancer. Image created with.

VEDICINALS®9 suspension to inhibit the proliferation and migration of A549 cells. Among various oncogenic protein included in the Human XL oncology protein array kit, VEDICINALS®9 was able to inhibit Axl, FGF basic, enolase 2, progranulin, and survivin, all proteins involved in cancer cell proliferation. Similarly, VEDICINALS®9 was able to inhibit Dkk-1, cathepsin B, cathepsin D, BCL-x, amphiregulin, CapG, and u-plasminogen activator/urokinase, all proteins involved in cancer cell migration. Interestingly, VEDICINALS®9 was able to upregulate the expression of the tumor suppressor protein p53 [54] and of the angiogenesis inhibitor endostatin [55]. Collectively, our results suggest a potent anti-cancer activity of VEDICINALS®9 against the A549 cell line, which is exerted by inhibiting the cell proliferation and migration (Fig. 7). Thus, the potent polyherbal VEDICINALS®9 formulation could serve as a better alternative for lung cancer management.

Despite the promising results provided by our study, there are some limitations as well that will serve as opportunities to expand the current study, as well as research platforms for other researchers for similar investigations. First of all, in the mechanistic characterization of the action of VEDICINALS®9, we have only shown the impact of the product on the expression of oncogenes at the protein level. However, it would be interesting to assess the mRNA expression of key genes such as Kristen rat sarcoma virus (*KRAS*), Phosphatase and tensin homolog (*PTEN*), Programmed-death ligand 1 (*PD-L1*), and *P53* to validate the findings of the present study and correlate gene and protein expression [56]. Furthermore, there is potential to expand our in vitro study to in vivo with various mice model of lung cancer such as cigarette smoke/cigarette carcinogen-exposed mice or genetically modified mice models [57]. Another interesting avenue would be the use of advanced lung-on-a-chip or microfluidics models [58,59], or 3D tumor spheroid models better representing the three-dimensional architecture of lung cancer [60], to further characterize the anti-lung cancer effect of VEDICINALS®9.

We also need to consider the potential disadvantage in the utilization of polyherbal extract that comes with potential cross-sensitivity (cross-reactivity) with other drugs or for specific patients. If a formulation contains a mixture of multiple single compounds, it's difficult to predict which chemical moiety is responsible for cross-reactivity. Similarly, it also makes it hard to determine which compounds is responsible for synergistic or antagonistic effects. Another problem is the lack of standardization that may make it problematic to get reproducible results and draw valid conclusions. Likewise, the challenges associated with allergenic potential and variability in patient response should be considered. These challenges must be carefully taken into consideration while selecting the composition of polyherbal formulations such as VEDICINALS®9. Nevertheless, our in vitro study suggests that, with further pre-clinical in vivo and clinical validation, VEDICINALS®9 could represent a promising therapeutic option in future for lung cancer.

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5. Conclusion

Our study was able to validate the in vitro anti-cancer activity of VEDICINALS®9 suspension via inhibiting of A549 cell proliferation and migration. The key proteins targeted by VEDICINALS®9 are: Axl, FGF basic, enolase 2, progranulin, and survivin involved in proliferation; Dkk-1, cathepsin B, cathepsin D, BCL-x, amphiregulin, CapG, and u-plasminogen activator/urokinase involved in migration; the tumor suppressor protein p53; and the angiogenesis inhibitor protein endostatin. We strongly believe that the potent anti-cancer activity is attributed by the ideal composition of various anticancer agents in our polyherbal and bioactive compounds-loaded formulation (VEDICINALS®9). Therefore, the VEDICINALS®9 suspension could be a promising candidate drug and dosage form for the development of novel therapies against lung cancer.

Ethical approval

Not applicable.

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CRediT authorship contribution statement

KD, KRP, and KAW designed research. KRP, RR and GDR conducted experiments. KRP, RR, GDR, VSRRA wrote the manuscript. KAW, GG, and SKS wrote and edited the manuscript. PMH, PS, GG and JG contributed to reagents or experimental requirements. KRP, RR analysed data. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Declaration of Competing Interest

This project is funded from VEDICINALS® International, India (A German-Indian BioTech Company). The VEDICINALS®9 formulation we have tested is a product of VEDICINALS®9International and more information about it can be found in their official website. <https://www.vedicinals.com/vedicinals-9/>.

Data Availability

The data relative to the present manuscript are available upon reasonable request to the corresponding author.

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