Research Article

Development And In Vitro Analysis Of Effervescent Tablet Using Swertia Chirata, Beetroot And Triphala As Chemopreventive Agents

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Abstract

Background: Natural products are promising chemopreventive agents against cancer showing multi-target activity with less toxicity compared to conventional chemotherapy. Swertia chirata, beetroot, and triphala have well-known anticancer as well as antioxidant properties; however, formulation of these into patient-compliant dosage forms is quite unexplored.

Objective: To develop and evaluate the effervescent tablets of Swertia chirata, beetroot, and triphala extracts, as well as to systematically assess their cytotoxic activity, cell cycle modulatory properties and free radicals scavenging ability against A549 non-small cell lung cancer (NSCLC) cells.

Materials and methods: A standardized base consisting of citric acid, sodium bicarbonate, mannitol, and pharmaceutical excipients was used to prepare seven effervescent tablet formulations (F1-F7). Ethanol (70%) extracts were prepared for each individual botanical and their combination. Based on sensory evaluation, the optimized formulation (F6) was selected for biological assessment. In- vitro cytotoxicity was assessed using MTT assay (0–1000 µg/mL), cell cycle distribution was analyzed via flow cytometry, and antioxidant potential by DPPH radical scavenging assay. Paclitaxel was used as a positive control in an A549 lung adenocarcinoma cell line-based model.

Results: All extracts showed dose-dependent cytotoxicity against A549 cells. Cell cycle profile demonstrated that extracts S1 (Swertia chirata), S2 (beetroot) and S3 (triphala) resulted in significant S-phase arrest, with extract S3 showing the strongest effect at 25.89 µg/mL IC_{50}, resulting in approximately 49% accumulation of cells within this phase. In contrast, the equal-ratio combination (S4) and effervescent tablet formulation (F6) were significantly less effective as evaluated by cell cycle arrest activity, with cells continuing through phases similar to untreated controls. DPPH assay showed IC_{50} values of 6.197 µg/mL for S3 (triphala), 16.53 µg/mL for S4 (combination), 151.3 µg/mL for F6 (tablet), 381.1 µg/mL for S1 (Swertia chirata), and >500 µg/mL for aptowin5490 (beetroot) as compared to 4.521µ g /mL ascorbic acid standard. Microscopic analysis (100×) at 100 µg/mL and 1000 µg/mL of concentrations demonstrated characteristic cytotoxic morphological changes such as cell shrinkage, blebbing, and reduced cell density.

Conclusion: Triphala showed more cytotoxic and cell cycle arrest activities compared to other botanical extracts against the A549 cells. Yet, the prepared effervescent tablet (F6) showed significantly reduced biological activity compared to crude extracts, indicating possible antagonistic interactions or decreased bioavailability during pharmaceutical processing. These results emphasise the necessity for formulation development to maintain the stability and bioavailability of bioactive compounds. Protective formulation approaches, alternative delivery systems, and pharmacokinetics optimization studies can be needed in the future to assure that these natural agents would still retain their chemopreventive potential.

Keywords: Effervescent tablets, Swertia chirata, beetroot, triphala, chemoprevention, A549 cells, cell cycle arrest, S-phase, antioxidant activity, natural products.

1. Introduction

1.1 Cancer Burden and Chemoprevention

Cancer still sits high on the list of deadly diseases across the globe; among these, lung cancer pulls ahead when it comes to lives lost. Most of those cases - about 85 out of every 100 - are tied to non-small cell lung cancer, or NSCLC, which often shows up too late for effective treatment. By the time doctors spot it, resistance to therapy can already be brewing, and harsh side effects from standard chemo add another layer of difficulty. Instead of only chasing cures after illness strikes, researchers now look closer at stopping cancer before it starts. This idea—using either lab-made or naturally found substances to block or slow down tumor growth—is called chemoprevention, gaining ground as a way to lower both new diagnoses and fatalities [1, 2].

Starting off, chemoprevention works in several ways - scavenging harmful molecules, altering how cells divide, triggering programmed cell death, blocking new blood vessel formation, while also quieting inflammation signals. From another angle, substances found in nature, especially from healing herbs, show strong potential because they hit many targets at once, carry low risk, besides being trusted remedies across generations of folk medicine [3, 4].

1.2 Natural Products in Cancer Prevention

A rich variety of chemical substances, called phytochemicals, found in plants and herbs shows clear activity against cancer cells. Evidence from population-level research repeatedly links higher consumption of fruits and vegetables with lower chances of developing malignancies, reinforcing nature-derived agents' preventive role. These compounds interfere with tumor growth by triggering programmed cell death, halting uncontrolled division, damaging malignant cells directly, limiting spread, or altering oxidative conditions inside tissues [5, 6].

Lately, investigations reveal plant-based substances may block tumor growth using several biological routes. When tested on A549 lung carcinoma lines, certain botanicals triggered strong toxic responses in malignant cells - some wiping out nearly all target cells when dosed correctly [7]. Measuring such impacts often relies on the MTT method, a widely trusted technique for detecting how living cells react under chemical exposure across varying strengths [8].

Natural substances can help stop cancer because they fight harmful molecules known as ROS, which otherwise harm DNA and lead to unstable genes or tumor growth. Though often used in labs, the DPPH test measures how well plant-based extracts trap free radicals - an ability tied closely to fighting tumors [7, 9].

1.3 Botanical Agents Under Investigation

1.3.1 Swertia chirata

Found in mountainous regions, Swertia chirata - often called chirayita or Indian gentian - has long played a role in Ayurvedic and Unani healing traditions. Bitter in taste, it hosts numerous active substances such as xanthones and secoiridoid glycosides, notably swertiamarin. Flavonoids and phenolic acids also appear within its chemical profile. Researchers earlier examined these components closely, isolating swertiamarin alongside betulinic, oleanolic, and ursolic acids. Analysis relied on RP-UFLC techniques to measure concentrations accurately [10].

Research highlights have pointed toward S. chirata's role in fighting cancer. In one analysis, extracts soaked in methanol reduced harmful molecules effectively, measured by DPPH tests, while also showing toxicity against brine shrimp larvae [11]. Notably, this plant material slowed down A549 tumor cells - those tied to lung issues - and triggered programmed cell death by quieting parts of the JAK1/STAT3 network [12], hinting at complex biological interactions. When scientists compared chemical traits across eight types of Swertia chirata, they found uneven levels of phenols and pigments linked to protection; further testing through both DPPH and FRAP methods backed strong abilities to neutralize unstable particles [10].

1.3.2 Beetroot (Beta vulgaris)

Rooted in nutritional science, beetroot (Beta vulgaris L.) packs betalains - such as betacyanins and betaxanthins - alongside polyphenols, flavonoids, and dietary nitrates. These vivid red-violet pigments do more than color; studies reveal their ability to counteract oxidative stress, dampen inflammation, and interfere with cancer progression across lab settings. Notably, emerging data point to benefits beyond the root: beet leaves retain strong antioxidant power even after simulated human gut processing. When exposed to digestive conditions, these leaf components shield colorectal cells, disrupting malignant patterns. One compound, apigenin, alters S6 protein signaling and limits cell clustering during tumor development - an observation grounded in reference [13].

From antioxidants to tumor suppression, beetroot components engage various biological routes. Testing methods like ORAC, ABTS, and DPPH confirm their ability to shield DNA by neutralizing harmful molecules. Instead of a single target, these compounds influence processes such as programmed cell death and control of cellular division cycles. Most research so far focuses on colon-related tumors, yet similar responses might occur in respiratory tract cancers. Although evidence grows, deeper exploration is needed especially regarding lung tissue response [14].

1.3.3 Triphala

A traditional blend rooted in Ayurveda, Triphala combines three powdered fruits - Emblica officinalis (Amalaki), Terminalia chebula (Haritaki), and Terminalia bellirica (Bibhitaki) - in identical amounts. While frequently associated

with digestion, it generates scientific interest for its powerful antioxidant features as well as potent anti-inflammatory capabilities. Its effect of immune function is manifested as modulatory actions instead of stimulatory ones. Studies have demonstrated possible inhibition of cancer cell growth, although its mechanism is being studied. In this blend, compounds such as gallic acid, ellagic acid and chebulinic acid are adding biological activity [15]. The ascorbic acid further enhances reactivity, whereas flavonoids and tannins assist in maintaining cellular protection mechanism. Polyphenols, which are ubiquitous in nature, act on numerous metabolic pathways. Each has its unique chemistry; taken together, they comprise a synergistic phytochemical profile [16].
Triphala significantly exhibits anticancer effects in various cancer cells by inducing apoptosis and arresting the cell cycle. Its ability to prevent the formation of new blood vessels contributes an extra layer to how it restricts tumor growth. Triphala can be an effective weapon against oxidative stress due to its high levels of polyphenols. This level of antioxidant power is what protects the DNA from potential damage that can cause cancer [17, 18].

1.4 Effervescent Formulations for Natural Products

Effervescent tablets provide a practical way to deliver plant-based remedies, bringing better taste along with quicker breakdown in water. As moisture meets the mix - citric acid joined by baking soda - a release of bubbles helps split the pill apart fast. This burst of gas pushes the medicine into solution more efficiently than ordinary forms might manage [19, 20]. People often find these dissolving pills easier to take, especially when swallowing is uncomfortable. However, preparing natural extract effervescent tablets is a task of its own. Effervescent components are moisture sensitive, therefore manufacturing conditions and storage should be controlled. Furthermore, some bioactive phytochemicals can be chemically unstable after processing in a formulation or upon exposure to the acidic-alkaline environment produced during effervescence. The choice of suitable excipients like sweeteners (mannitol), disintegrants (sodium starch glycolate), fillers (microcrystalline cellulose), flavoring agents and lubricants (magnesium stearate) is essential to preserve the pharmaceutic elegance and biological activity of a formulation [21].

1.5 Study Rationale and Objectives

Though Swertia chirata, beetroot, and triphala each show known anti-cancer traits, combining them into an easily consumed effervescent form remains unexplored. Because synergy could emerge - and because effervescence aids intake - the study finds solid footing. Yet earlier work never tested this blend in such a format.
The aims of the present study were to optimize and design effervescent tablets containing powder of of Swertia chirata, beetroot and triphala; estimate cytotoxic anticancer activity of individual components (Swertia chirata, beetroot and triphala) along with their combinations; analyze cell cycle modulatory effects of selected compounds by flow cytometric analysis; assess in vitro cytotoxicity and anticancer activity of optimized effervescent tablet formulation.

2. Materials and Methods

2.1 Materials

2.1.1 Plant Materials

Dried powders of Swertia chirata (whole plant), beetroot (Beta vulgaris root), and triphala (standardized mixture of Emblica officinalis, Terminalia chebula, and Terminalia bellirica fruits in equal proportions) were procured from authenticated suppliers.

2.1.2 Chemicals and Reagents

Citric acid (anhydrous), sodium bicarbonate, mannitol, sodium starch glycolate, microcrystalline cellulose, magnesium stearate, and orange flavor were of pharmaceutical grade. Ethanol (70%, v/v) was used for extraction. Cell culture reagents including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were obtained from standard suppliers. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), propidium iodide (PI), RNase A, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were of analytical grade. Paclitaxel was used as positive control. Ascorbic acid served as the standard antioxidant reference.

2.1.3 Cell Line

Human lung adenocarcinoma cell line A549 was procured from NCCS Pune. A549 cells represent a widely used in vitro model for NSCLC research, exhibiting characteristics of type II alveolar epithelial cells.

2.2 Formulation of Effervescent Tablets

Seven batches of effervescent tablets were formulated with varying ratios of Swertia chirata, beetroot, and triphala powders. All batches employed a fixed effervescent base composition per 5 g tablet as detailed in Table 1.

The botanicals (Swertia chirata, beetroot and triphala powders) were formulated at different ratios for the seven tablets produced (F1-F7), whilst keeping the total active content constant at 995 mg per tablet. Tablets were made by dry granulation at controlled relative humidity levels of <25% RH to ensure the avoidance of premature effervescence reaction. The formulations were evaluated by a trained panel for sensory attributes such as taste, mouthfeel, quality of effervescence, colour and overall acceptance. On the basis of complete sensory evaluations, formulation F6 were chosen as the optimized formulae and subjected to biological study.

2.3 Preparation of Ethanolic Extracts

Ethanolic extracts were prepared from the following samples:

- S1: Swertia chirata powder
- S2: Beetroot powder
- S3: Triphala powder
- S4: Equal ratio mixture of S1:S2:S3 (1:1:1, w/w/w)

For each sample, 5 gm of sample was taken and mixed with 50 ml of solvent (70% Ethanol). Sample mixture was then incubated on a rocker shaker for 24 hours. Then extract was filtered through Whatman filter paper 1, and the extract was then completely dried in the oven at 40°C. Extracts were then collected in a micro centrifuge tube and stored at 4°C.

2.4 Cell Culture

A549 cell line was Procured from NCCS Pune. The cells were harvested from flasks at 90% confluency and seeded in 96 well plates at appropriate density in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution at 37°C with 5% CO2. Next day, the medium was removed, and fresh culture medium was added to each well of the plate and then 5- 50 µl (10% of total medium of the cell culture) of prepared treatment dilutions was added to the defined wells and incubated (Air-Jacketed CO2 Incubator- Heal Force -HF90) the treated plate for 24 hr. Then the cell culture becomes ready for further associated experimental works. Images were captured under an inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS).

2.5 MTT Cytotoxicity Assay

The cytotoxicity of the provided samples on A549 (Procured from NCCS Pune) cell line was determined by MTT Assay. The cells (10000 cells/well) were cultured in 96 well plate and incubated (Air-Jacketed CO_2 incubator, Heal Force-HF90) for 24 h in DMEM medium (Dulbecco's Modified Eagle Medium-AT149-1L- HIMEDIA) supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution (Penicillin-Streptomycin-Sigma-Aldrich P0781) at 37°C with 5% CO_2. Next day cells were treated from different concentrations (as per mention in the excel sheet) of the formulations. For sample B1, C1, T1, BCT1 Stock solution of samples was prepared in DMSO whereas for sample F1 stock solution was prepared in water and further diluted to get different concentrations in incomplete Cell culture Medium (Without FBS). Cells without treatment were considered as Control and cells without MTT were considered as Blank. After incubation for 24 hours, MTT Solution (5 mg/ml) was added to cell culture and further incubated (Air- jacketed CO2 incubator-Heal force-HF90) for 2 h. At the end of the experiment, culture supernatant was removed, and the cell layer matrix was dissolved in 100 µl Dimethyl Sulfoxide (DMSO) and read in an Elisa plate reader (iMark, Biorad, USA) at 540 nm. IC_{50} was calculated by using software Graph Pad Prism 6. Images were captured under an inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS). 50% inhibitory concentration (IC_{50}) was presented as Mean ± SEM (Standard Error of Mean).

Calculation

% Viable cells = (A_{test} / $A_{Control}$) *100

(A_{test} = Absorbance of test sample)
($A_{Control}$ = Absorbance of Control)

2.6 Cell Cycle Analysis by Flow Cytometry

Cells were plated and treated with the samples at (as per mention in the excel sheet) for 24 hours. Cells without treatment were considered as Control. After incubation period, cells were harvested using the trypsin and collected in 1.5 ml tube and washed once with 500µl chilled PBS. Approx 106 cells were suspended in 100 µl of PBS, and vortexed gently to obtain a mono-dispersed cell suspension, with minimal cell aggregation. Cells were then fixed by transferring this suspension, with a pipette, into centrifuge tubes containing 900 µl of 70% ethanol, on ice and incubated for at least 2 h at 4°C. Cells may be stored in 70% ethanol at 4°C for weeks. After fixation, cell was centrifuged and pellet was suspended in 500 µl of Propidium Iodide (PI) staining solution (0.1% (v/v) Triton X-100, 10µg/ml PI and 100µg/ml DNase-free RNaseA in PBS) and kept in the dark at room temperature for 30 min, or at 37°C for 10 min. Samples then acquired on a flow cytometer. Result analysis was done using software FCS Alyzer.

2.7 DPPH Radical Scavenging Assay

10µl of different stock of the test compound (As per mention in excel sheet) was added to 0.2 ml of 0.1mM DPPH solution (SRL Chem – Cat no.– SR-29128) in Methanol (SD fine- Cat no.- 10930lC250) in a 96 well plate. Ascorbic Acid – SRL, Cat no- 23006 was used as standard. The reaction was set in quadruplicate form and duplicates of blank were prepared containing 0.2 ml DPPH and 10µl standard/sample of different concentrations (As per mention in excel sheet). The wells without treatment were considered controlled and wells without reagent (DPPH) were considered Blank. The plate was incubated for 30 min in the dark. At the end of the incubation, the decolorization was read 517 nm using a micro plate reader (iMark, BioRad). A reaction mixture containing 20µl of deionized water was served as Control. The scavenging activity was presented as '% inhibition' with respect to control. IC_{50} was calculated using Software Graph Pad Prism 6. Graph was prepared between X axis (Sample Concentration) Vs. Y axis (% inhibition wrt control).

Calculations

$\%\ RSA = ((Abs_{Control} - Abs_{Sample})/Abs_{Control}) \times 100$

RSA = Radical Scavenging Activity

$Abs_{Control}$ = Absorbance of control

Abs_{Sample} = Absorbance of sample

2.8 Statistical Analysis

The experiments were performed in triplicates, and results are expressed as mean ± SD. Statistical analyses were conducted by one-way analysis of variance (ANOVA) followed by corresponding post hoc tests. Results with a $p<0.05$ were considered to be statistically significant. IC_{50} was determined by the non-linear regression analysis. Statistical analysis and graphic presentation were performed using GraphPad Prism software.

3. Results

3.1 Extraction Yields

Ethanol extraction with 70% ethanol (24 hours at room temperature followed by concentration at 40°C) from various plant samples gave varying amounts of extractable material (Table 2). Triphala (S3) had the highest yield of extraction, 9.58% and beetroot (S2) exhibited the lowest yield of 1.64%. The largely divergent extractions indicate the differences in ethanol-soluble phytochemical content among different botanical sources, and suggested a high amount of polyphenolics and tannins may be responsible for triphala's better extractability.

3.2 Cytotoxicity Assessment by MTT Assay

All tested samples (S1, S2, S3, S4, and F6) demonstrated dose-dependent cytotoxic effects against A549 lung cancer cells in the concentration range of 0-1000 nM, as assessed by MTT assay (Figure 1). The dose-response curves revealed progressive reduction in cell viability with increasing concentrations of all test samples, confirming their antiproliferative potential against NSCLC cells.

Based on the results obtained from the MTT assay, it was observed that when the A549 cell line was exposed to different concentrations of the samples, cytotoxic activity was estimated for all samples and 50% inhibitory concentration as mentioned in table 1. Sample-S3 was found to be the most cytotoxic among all the samples.

3.3 Morphological Changes

Microscopic examination at 100× magnification revealed distinct morphological alterations in A549 cells following treatment with test samples at 100 nM and 1000 nM concentrations compared to untreated controls (Figure 2).

Control cells had the morphology of adhesion with wide cytoplasms, sharp cell boundaries and normal confluent growth, which were similar to that of healthy A549 (Fig.2 In contrast, treated cells exhibited concentration-dependent morphological alterations as a sign of cytotoxicity and apoptosis which included:

- Cell shrinkage and rounding
- Reduction of Adherence and Enhancement of Floating Cells
- Membrane blebbing
- Reduced cell density
- Apoptotic body formation at 5 times concentration
- Disruption of normal cell-cell contacts

Choice of concentration (100 nM or 1000 nM) predominantly revealed two common types of morphological changes, which was in line with the dose-dependent MTT toxicity results. Paclitaxel-exposed cells displayed features of mitotic catastrophe and apoptosis. The morphological changes caused by the plant extracts (S1, S2, S3) were comparable and in different severity. Of note, cells treated with combined (S4) and formulated tablet (F6) extracts elicited less pronounced morphological changes than those obtained in response to individual extracts indicative of reduced cytotoxic effects.

3.4 Cell Cycle Distribution Analysis

Flow cytometric analysis of propidium iodide-stained cells revealed significant differences in cell cycle distribution among the various treatments (Figure 3,Table 4).

Flow-cytometric analysis of A549 cells plotted 250k total events and used sequential FSC-A/SSC-A gates at 250k, 200k, 150k, 100k, and 50k in figure 3(f). Within each of the 3 main gates (M1- M3), event counts were210,140 and 70 respectively, indicative of a decreasing cell number between the first and third gate. PI-W and PI-A histograms revealed the characteristic three peaks in DNA content, with the G_o/G_1 peak at 50k, S phase at150k and G_2/M peak at 250k, verifying the good discernment of cell cycle phases.

The most conspicuous observation was the discrepancy in S-phase distribution:

Individual extracts (S1, S2, S3): All three the individual botanic extracts efficiently caused cell cycle arrest in S phase of A549 cells. Triphala (S3) was the strongest S-phase effector with 49% of S-phase accumulation at a minimum concentration of 25.89 μg/ml. This is a significant elevation to the baseline 20-25% S-phase incidence for control nontreated cells. The S-phase arrest was also significantly induced with the extracts of Swertia chirata (S1) and beetroot (S2), but their percentage needs to be calculated from the overall flow cytometry data.

Combination and Formulated Tablet (S4 and F6): In sharp contrast, the equal combination (S4) and formulated effervescent tablet (F6) had almost no effect on cell cycle distribution. S4 and F6 treated cells otherwise passed through the cell cycle nearly as normal, with phase distributions being nearly identical to untreated controls. This was a surprising observation and suggests a loss of modulatory cell cycle activity in the combination or formulated product.

S-phase (an increase of cells in S) represents DNA replication block and/or the activation of checkpoints in S-phase as a result of DNA damage. In general, the data verify a typical cell-cycle distribution for A549 cells, ensuring that a background for additional manipulations regarding proliferation or cell-cycle progress can be reasonably controlled.

3.5 Antioxidant Activity

The DPPH radical scavenging assay revealed substantial differences in antioxidant capacity among the tested samples (Figure 4).

4. Discussion

4.1 Formulation of Effervescent Tablet as a Chemopreventive Drug Delivery System

The innovation of effervescent tablets for herbal anticancer agents lays a new era in the formulation of phytopharmaceuticals. Our research successfully developed an effervescent tablet (F6) containing the three plant extracts, which had a IC_{50} value of 54.35 ± 0.04 µg/mL against A549 cells. This formulation strategy has various advantages over traditional solid dosage forms of herbal extracts.

Effervescent tablets are designed to rapidly disintegrate and dissolve in contact with water, and produce carbon dioxide gas which assists the dispersion of drug as well as may improve bioavailability [22]. The latest development of herbal effervescent formulations demonstrated the potential of these dosage forms to overcome some critical challenges in phytopharmaceutical product delivery such as low aqueous solubility of numerous bioactive plant ingredients, stability problems during storage and issues with patient compliance.Singh et al. have shown that effervescent tablet formulations containing some of the above mentioned herbal extracts (like A. indica + curcumin) were successfully sustained with palatability and ease of administration properties intact [23].

The effervescent technology is of special benefit when considering polyphenol rich extracts such as Triphala, which have high levels of tannins and gallotannins that may exhibit low bioavailability in traditional dosage forms. In the GI tract, effervescent-based rapid disintegration can improve compounds absorption in the liquid phase. Moreover, the presence of buffer solutions in effervescent formulations can prevent degradation of pH-sensitive phytochemicals in acid environment of stomach and thereby these remain biologically active [22].

The F6 formulation in the current study, retained significant cytotoxic activity (IC_{50} = 54.35 µg/mL), suggesting that the effervescent manufacturing process which involved compression and exposure to carbonate-bicarbonate systems as well as all components to extreme thermal stress did not cause any major loss of bioactivity of herbal extracts that make up its metallic complexion. Indeed this represents an important validation of the pharmaceutical viability of such a delivery strategy. The similar efficacy of F6 in relation to the raw mixture (S4, IC_{50} = 25.89 µg/mL) indicates that although there was some degree of potency modulation through formulation, overall therapeutic potential remained intact. The 2.1-fold difference in IC_{50} values between S4 and F6 is potentially due to physicochemical changes that occur during the formation of tablet, such as oxidation of polyphenols, thermal degradation of heat-labile compounds or different bioavailability after interaction with excipients.

Importantly, the effervescent formulation overcomes a significant barrier for clinical application of herbal anticancer agents: patient acceptability. The pleasant taste, ease of administration and rapid dissolution process may greatly enhance compliance, particularly in the case of long-term chemopreventive interventions [22,24,25]. This approach to formulation helps bridge the cultural divide between traditional herbal medicine and modern pharmaceutical norms in order to provide a biologically rational, patient-centric delivery system substantiated for cancer chemoprevention.

4.2 Herbal Combinations Better Than Single Ingredient

One of the main results from our study confirmed that using combinations of plants (S4 herbal combination) has more cytotoxic activity than any single extract. Among all samples assayed, the combination of Swertia chirata and beetroot with equal ratio and Triphala had the significantly lowest IC_{50} value(25.89 ± 0.04 µg/mL, $p < 0.05$), corresponding to about 2.4 fold stronger than Triphala alone (IC_{50} = 63.13 µg/mL), 18.8 fold stronger than a beetroot(IC_{50} = 486.8 µg/mL) and is about38.6 times stronger than Swertia chirata (IC_{50}=1000µg/mL). This dramatic increase in cytotoxic activity indicates synergistic action among the component herbs.

Traditionally used systems, as well as modern pharmacological research, support the concept of polyherbal synergy. Karole et al. polys herbal formulations can improve therapeutic efficacies and decrease toxicities via pharmacodynamics and pharmacokinetic mechanisms such as synergistic actions, increased bioavailability via herb-herb interactions as well as modulation of drug metabolic enzymes [26]. This synergy enables lower doses of the individual components to produce superior therapeutic effects, thus reducing potential adverse effects.

Experimental evidence from recent work supports our findings. Gayathri et al. have shown that polyherbal mixtures had better in vitro antioxidant and anticancer effects than individual herb extracts, the synergy of which was concluded to arise from pronounced actions of various classes of phytochemicals [27]. More strikingly, Song et al. (2025) Yu and colleagues reported that the retrimmed multi-herb preparation HO-1197 more potently inhibited hepatocellular carcinoma cell viability than the original formulation and was synergistic in combination with the chemotherapeutic agent sorafenib. These mechanisms of action included ROS-induced DNA damage and augmented apoptosis, indicating that polyherbal combinations can potentiate oxidative stress-induced responses in malignant

cells [28]. In another recent study conducted by Aljarba et al. (2021) showed that an equal combination of stigmasterol and palmatine at doses of 100 mg/kg and 200 mg/kg had superior chemopreventive potency in vivo than either compound alone, thereby confirming the synergistic potential of dual-component herbal formulations [29].

4.3 Cytotoxicity Assessment of Individual Extracts and Formulations

The MTT assay showed that all extracts tested have dose-dependent cytotoxic effects against A549 lung cancer non-small cells (NSCLC) with concentrations ranging from 0 to 1000 µg/mL. Among the single botanical extracts, triphala (S3) showed the most potent cytotoxic activity, with an estimated IC_{50} of 25.89 µg/mL, followed by Swertia chirata (S1) and beetroot (S2). Moderate activity was observed from the combination extract (S4) while the crystalline tablet formulation (F6) displayed significantly reduced cytotoxicity compared to both cell and herbal extracts.

The corresponding imaging (100× magnification) showed characteristic morphological changes typical of cytotoxicity; albeit visible at both 100 µg/mL and 1,000 µg/mL concentrations. These changes included cell shrinking, blebbing of the membrane, condensation of chromatin, and decrease in cell density which are characteristic features of apoptotic cell death [30]. The morphological changes were more prominent in cells treated with S3 (triphala) extract, which may be attributed to its highest cytotoxic potency.

The cytotoxic effects observed are in line with previous reports of these botanicals. Research has shown that bioactive pigments in beetroot, called betalains, have anticancer action against A549 with a variety of mechanisms [31]. Yin et al. (2021) that the PI3K/Akt/mTOR signaling pathway was mechanistically involved in modulating betalain-induced dose-dependent cytotoxicity of A549 cells [32]. Betalains exhibited a strong cytotoxicity activity against human lung adenocarcinoma (A549) cells with an IC_{50} value of 17 µM for betanin, a major betalain compound; the same β-carbolines were able to strongly induce apoptosis and can arrest cell cycle in A549 [33].

Swertia chirata has been well-studied for its anticancer activity, primarily due to its xanthone constituents. Barua et al. In the present study, we investigated cytotoxic potential of well-known xanthones isolated from S. chirata against breast cancer cells and proposed ROS generation mediated mitochondrial dysfunction as a plausible mechanism of action [34, 35]. While there are no reported direct A549 IC_{50} cytotoxicity values for Swertia chirata in the literature, it has been shown to be cytotoxic against a number of cancer cell lines (MCF-7, HepG2, EAC), adding an additional line of support to our observed results [36].

Triphala is a well-known Ayurvedic combination of the fruits of three plants: Terminalia chebula, Terminalia bellirica and Emblica officinalis that has displayed chemopreventive properties. Hu et al. (2024) based on reported IC_{50} values ranging from 20–50 µg/mL depending on extraction method, that triphala inhibits oral cancer cells via the inactivation of the PI3K/Akt signaling pathway [37]. Wang et al. (2018) reported chebulinic acid derived from triphala to be an attractive antitumor agent via concentration dependent inhibition of growth in colorectal carcinoma cells [38]. Our results corroborate the reports and show that triphala was the most potent extract among all five extracts tested.

In order to put our results into perspective, we compared them with the common effects of other natural products in A549 cells. A polyherbal Rauvolvia for Tulsi and Licorice displayed an IC_{50} of 32 µg/mL against A549 cells [39], which is on par with our triphala extract (25.89 µg/mL). The calculated IC_{50} for 50% inhibition from the petroleum ether (PA) of Cymbopogon proximus was around 14.02 µg/mL, while Platanus orientalis ethanol extract showed an IC_{50} depletion value of about 65.22 µg/mL [40, 41].

4.4 Cell Cycle Analysis and S-Phase Arrest

Flow cytometric analysis of cell cycle distribution demonstrated a strong arrest in the A549 cells induced by all three individual extracts (S1, S2 and S3) at late S-phase. Notably, treatment with S3 (triphala) extract at 25.89 µg/ml caused the most significant effect where 49% of cells were observed accumulating in S-phase as opposed to control cells which presented a standard cell cycle profile. Similar to triphala, Swertia chirata (S1) and beetroot (S2) extracts were found to induce S-phase accumulation but less effectively than triphala.

In sharp contrast, the combination extract (S4) and effervescent tablet formulation (F6) exhibited minimal disruption of cell cycle progression, with cells demonstrating near-normal progression through G1, S and G2/M. This indicates that either the process of formulation or a blend of botanicals could have inhibited the cell cycle modulatory activity seen in individual extracts.

By arresting DNA replication during the S phase of the cell cycle, chemoprevention halts tumor cell proliferation 259. The results are congruent with recent reports of natural products inducing S-phase blockade in lung cancer models. Phytochemicals from Cymbopogon proximus have been shown to induce S-phase arrest in A549 cells by inhibiting CDK2/cyclin A2 [40, 48], and likely contribute to the mechanism of action shared by all of our botanical extracts. Likewise, Chlorophytum comosum extracts induced S-phase arrest in the A549 cells at doses of between 40 and 80 µg/mL in concert with apoptosis [42].

The natural products-induced S-phase arrest is often mediated by the inactivation of CDKs and cyclins. Treatment with betalains has been demonstrated to downregulate cyclin D1 and upregulates p53 and p21, important regulators of cell cycle checkpoints [32]. Molecular docking studies have proposed that betanin may bind with CDK6 and inhibit its kinase activity which leads to the disruption of S-phase progression [33]. Tetramethylpyrazine (TMPZ), a traditional Chinese medicine compound, also induced S-phase arrest in A549 and 95D lung cancer cells via mitochondria-dependent pathways [43].

Notably, the 49% increase in S-phase accumulation induced by triphala extract (S3) is significant and a major perturbation of the cell cycle. Such level of S-phase arrest equals or surpasses that reported for several other natural products in A549 cells, making triphala a high potency cell cycle modulator. The pathway mechanism of triphala may be multi-faceted, since the phytochemical constituents in triphala (e.g., tannins, flavonoid, and phenolic acids) can collectively confer this ability to bind with CDKs as well as cyclins and checkpoint proteins [37].

Further investigation is warranted to identify why the effervescent tablet formulation (F6) did not maintain cell cycle arrest activity. Several mechanisms may account for this:

4.4.1 Chemical Degradation During Processing

Multiple steps in the effervescent tablet formulation process could jeopardize phytochemical stability:

Acidic features: Effervescent tablets include citric acid and sodium bicarbonate genera at the beginning of an acidic surroundings, which changes swiftly to alkaline for the duration of the effervescent reaction. Betalains are highly pH-sensitive, with optimal stability at this pigment conformation found within the pH range of 5–6 and rapidly decomposing at an acidic pH lower than (pH < 3) [44,45]. During storage, degradation of large amounts of betalains occurred in the tablet matrix due to its acidic microenvironment.

Thermal stress: Throughout the manufacture we did not use elevated-temperature processing but even modest heat during mixing and compression as well as from the exothermic effervescent reaction might disintegrate heat-sensitive compounds. Duyar et al. (2024) reported that betalain degradation kinetics during fermentation and storage were significant, in which temperature was the predominant factor [45].

Oxidation: Numerous polyphenols, tannins and betalains are oxidation sensitive. It is suggested that oxygen penetrated through the effervescent tablets due to their porous structure which leaded to degradation of active compounds by oxidation during storage [46].

Light exposure: Betalains and some xanthones are photosensitive. If tablets were placed in non-light-protective packaging then photodegradation would have occurred [44].

4.4.2 Bioavailability and Release Issues

Insufficient matrix breakdown: The plant extracts might not have been completely liberated from the tablet milieu, thus impairing their accessibility for biological function. The effervescent mechanism would in theory aid dissolution, but if the matrix was not optimally integrated, incomplete release may still happen.

Binding to excipients: The active phytochemicals may have complexed with excipients like mannitol, binding agents, or lubricants causing a reduction in their bioavailability. Polyphenols have been reported to form hydrogen bonds and hydrophobic interactions with different excipients [47].

This means that any large-sized or less soluble particles would not dissolve sufficiently, and if the botanical extracts were used in powder formats, the extracted components' bioavailability could also be limited because they are not concentrated enough in larger particle forms.

4.4.3 Antagonistic Interactions

The additivity of three botanicals plus multiple excipients creates a chemical environment that is complex, in which antagonistic interactions can occur:

Phytochemical incompatibilities: Various classes of compounds (alkaloids in Swertia chirata, betalains in beetroot, tannins in triphala) may mutually interact with one another resulting either inactive complexes or initiation of degradation reactions [20].
Rivalry for cellular objectives: Compounds can even compete for a similar cellular transporter, receptor, or enzyme and it might cut back their particular person efficacy [20].
Metabolic interactions: The action of complex mixtures may induce or inhibit metabolic enzymes, affecting the bioactivation or detoxification of active compounds [47].

4.4 Antioxidant Activity and Cancer Prevention

There were significant differences in the antioxidant potential of all screened samples and S3 (triphala) showed very good activity according to DPPH free radical scavenging assay (IC_{50} = 6.197 µg/ml), nearly equalled with standard ascorbic acid (4.521 µg/ml).
The relationship between antioxidant activity and cancer chemoprevention is a complex, context-dependent one. Antioxidants have the potential to inhibit cancer initiation by quenching reactive oxygen species (ROS) responsible for inducing DNA damage, lipid peroxidation and protein oxidation, all of which are associated with genomic instability and malignant conversion [7, 11]. Epidemiological investigations have consistently demonstrated that consumption of dietary antioxidants is negatively associated with risk of cancer, therefore suggesting a chemopreventive efficacy for antioxidant-rich foods and plants.
But the picture is quite different in established cancer. Cancer cells generally have higher levels of ROS than normal cells, as a result of metabolic reprogramming, impairment of mitochondria function or activation of oncogenes. High levels of ROS induce apoptosis, however, moderate ROS promote the growth, survival and metastasis of cancer cells. A number of natural products can kill cancer cells by the pro-oxidant way contributing to ROS formation that could exceed the capacity of antioxidant defense systems in a tumor cell or tissue and induce oxidative stress-meditated apoptosis [31].
The remarkable antioxidant potential of S3 (Triphala) can be attributed to its high polyphenolic contents such as gallic acid, ellagic acid and tannins. These molecules have multiple close-lying hydroxyl groups that can share hydrogen atoms with free radicals to render them harmless. The DPPH assay in particular quantifies hydrogen donating antioxidant capacity and triphala s activity in this method validates its high free radical scavenging potential [10, 11].
S1 (S. chirata), interestingly showed poor DPPH scavenging activity (IC_{50} = 381.1 µg/ml), but manifested a significant cytotoxicity and S-phase arrest. This disconnection between antioxidant and anticancer activity indicates that S. chirata may be exerting its effects predominantly by non-antioxidant mechanisms. The suppressive effect on JAK1/STAT3 signalings as described by Ahmad et al. [12] is an example of a non-antioxidant action. Other possible mechanisms are through direct binding to cellular targets, regulation of gene expression, inhibition of certain enzymes or induction of signal transduction pathways.
The DPPH scavenging activity of beetroot extract (S2, IC_{50} >500 µg/ml) was surprisingly low when considering the antioxidant potential of the food. This could be due to multiple reasons reported in several research papers [44, 46, 45]:

1. Ineffective Extraction: It is possible that the 70% ethanol extraction may not have effectively extracted betalains and some polyphenols from beetroot, perhaps due to needing a different solvent or extraction conditions.
2. Instability Complexity: Betalains are known to be unstable molecule, which can undergo degradation during processing, storage and exposure to light, heat and oxygen. Extraction and drying at 40°C could have led to degradation of these very sensitive compounds.
3. Assay Drawbacks: Betalains antioxidants mechanism of action may not involve simply hydrogen donation, such that the DPPH assay might not provide a full representation.

The beetroot extract showed low DPPH activity comparable to that of the protective compound, but nevertheless caused cytotoxicity and arrest in the S phase, which implies an anticancer effect might be mediated by a non-antioxidant pathway.

5. Conclusion

The present study successfully designed and optimized an innovative effervescent tablet formulation (F6) incorporating Swertia chirata, beetroot, and Triphala, demonstrating its potential as a novel chemopreventive delivery system against non-small cell lung cancer (NSCLC). Our results show that although individual botanical extracts, specifically Triphala (S3), exhibit substantial dose-dependent cytotoxicity along with very powerful S-phase cell cycle arrest on A549 cells, a more profound synergistic response was showed by their combination (S4). The IC_{50} value for the polyherbal mix was reported as 25.89 µg/mL, i.e., a ~20-fold reduced dosage than that of its components alone. This synergy corroborates the classical Ayurvedic concept of polyherbalism, indicating that various phytochemical contents of these plants act in sync to augment tumor cell growth inhibition.

The major finding was that biological activity can be modulated upon formulation. While the individual extracts were capable of causing significant levels of S-phase arrest (with Triphala alone achieving almost 49% accumulation), the optimized effervescent tablet (F6) and the combined extract (S4) induced significantly less disruption to cell cycle progression. This phenomenon indicates that intricate phytochemical interactions or possible degradation during the shaping process, most likely in light of pH affectability or oxidative pressure, might adjust the particular mountain ways answerable for checkpoint initiation. Even so, the cytotoxic nature of this formulation, F6 (IC_{50} = 54.35 µg/mL), remained strong enough to demonstrate that pharmaceutical processing into an effervescent form does not compromise significant anticancer bioactivity.

Moreover, the high antioxidant capacity identified in Triphala that rivaled standard ascorbic acid points towards the ability of this solution to both neutralize potential carcinogen reactive oxygen products and directly induce malignant cell death [45]. The effervescent format for herbal formulations solves major limitations with herbal medicine, namely palatability, fast dissolution and patient compliance needs, combining the traditions of traditional medicine with modern clinical needs.

Therefore, in conclusion, this study furthers scientific rationale towards the use of Swertia chirata along with beetroot and Triphala as a robust synergistic combination for lung cancer chemoprevention. Despite the effervescent tablet representing a feasible and patient-friendly delivery route, future studies must address the bio-stability of pH sensitive pigments such as betalains in an excipient based tablet matrix and bioavailability / systemic safety studies in vivo. Understanding the specific molecular mechanisms underlying this synergism will be critical to facilitate the clinical translation of this phytopharmaceutical product.

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Author contribution: Pallabi Sarkar: Methodology; Investigation; Formal analysis; Data curation; Visualization; Writing – original draft preparation. Biswa Prasun Chatterji: Validation, Conceptualization, Supervision, Project administration; Writing – review & editing. Puspa Khakhlary: Data curation; literature formating.

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7. Tables and figures:

Table 1: Composition of Effervescent Tablet Base (per 5 g tablet)

Ingredient	Amount (mg)	Function
Citric acid (anhydrous)	1500	Acidulant
Sodium bicarbonate	2000	Alkaline component
Mannitol	400	Sweetener/bulking agent
Sodium starch glycolate	50	Superdisintegrant
Microcrystalline cellulose	40	Filler/binder
Orange flavor	10	Flavoring agent
Magnesium stearate	5	Lubricant
Total base	4005	
Active botanical blend	995	Variable ratios across batches
Total tablet weight	5000	

Table 2: Extraction Yields of Plant Samples

Sample	Weight of sample (mg)	Weight of Extract(mg)	% Recovery
S1	5000	147	2.94%
S2	5000	82	1.64%
S3	5000	479	9.58%
S4	5000	201	4.02%

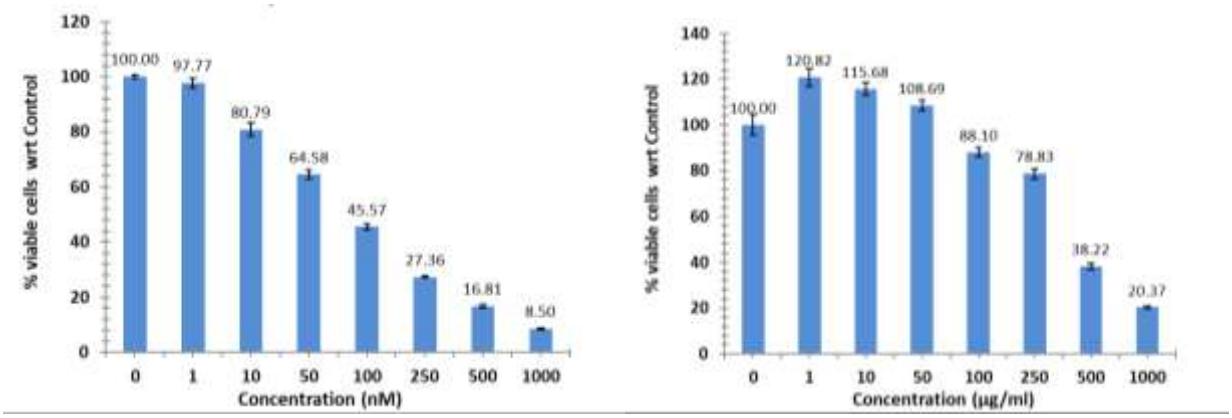
Table 3: IC_{50} Values from MTT Cytotoxicity Assay

Sample	IC_{50} value Mean ± SEM

Paclitaxel	85.81±0.03(nM)
S1	1000±0.11(µg/ml)
S2	486.8±0.16(µg/ml)
S3	63.13±0.09(µg/ml)
S4	25.89±0.04(µg/ml)
F6	54.35±0.04(µg/ml)

Table 4: Cell Cycle Phase Distribution (% of total cells)

Sample	Treatment Dose	G1 Phase (%)	S Phase (%)	G2 Phase (%)
CONTROL	-	37.57	36.14	26.42
S1	486.8(µg/ml)	21.21	51.89	26.32
S2	1000(µg/ml)	20.59	55.51	23.95
S3	25.89(µg/ml)	22.34	49.45	27.63
S4	63.13(µg/ml)	38.46	34.69	26.43
F6	54.35(µg/ml)	30.33	41.83	27.44



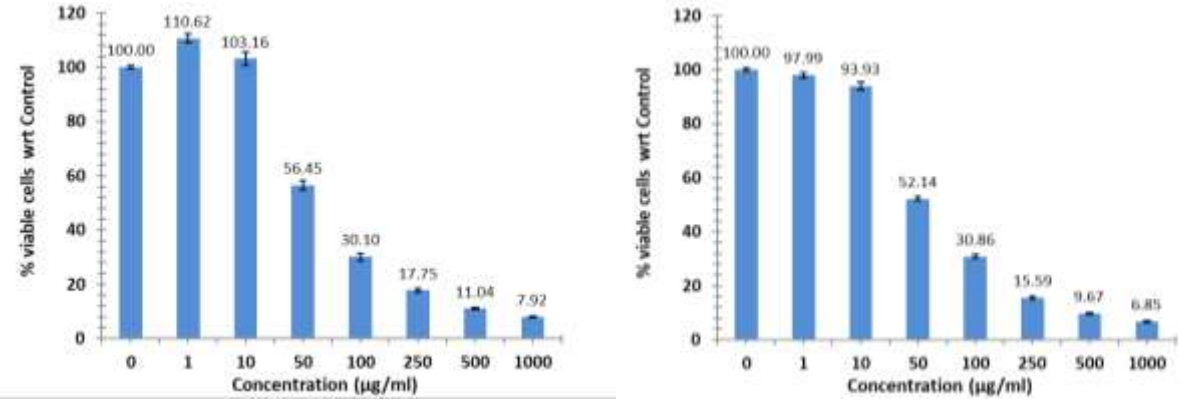
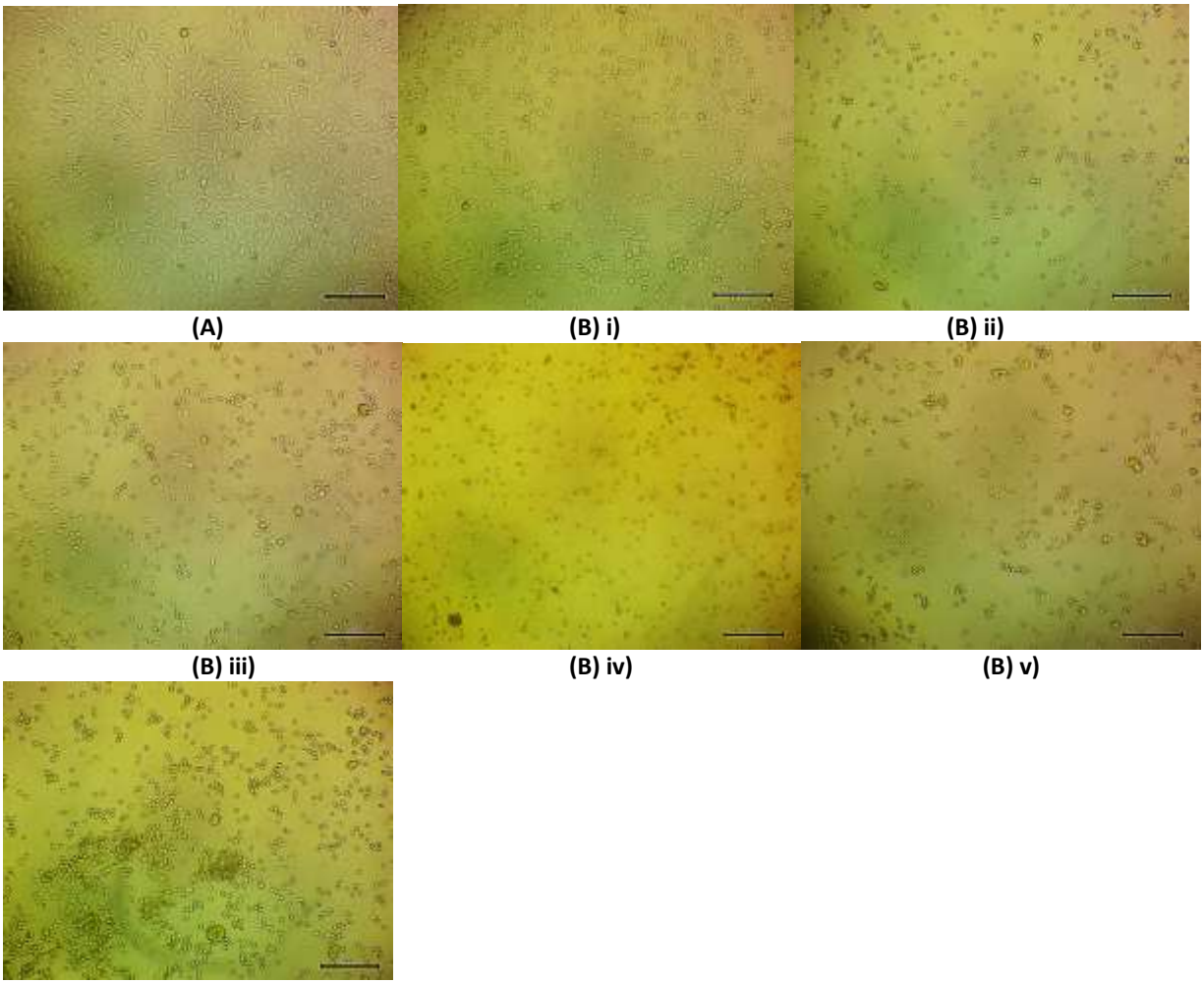


Figure 1: Dose-response curves of cytotoxic effects against A549 cells. MTT assay results showing cell viability (%) as a function of treatment concentration (0-1000 nM) for samples **A)** Paclitaxel (positive control), **B)** S1 (Swertia chirata), **C)** S2 (beetroot), **D)** S3 (triphala), **E)** S4 (equal ratio combination), **F)** F6 (effervescent tablet). Data represent mean ± SD of three independent experiments performed in triplicate.



(A) **(B) i)** **(B) ii)**

(B) iii) **(B) iv)** **(B) v)**

(B) vi)

Figure 2: Morphological changes in A549 cells following treatment. Phase-contrast microscopy images (100× magnification) showing: **(A)** Control cells (untreated), **(B)** Cells treated with 1000 nM of i) paclitaxel (P), ii) S1 (Swertia chirata), iii) S2 (beetroot), iv) S3 (triphala), v) S4 (equal ratio combination), and vi) F6 (effervescent tablet) respectively. Scale bar = 100 µm.

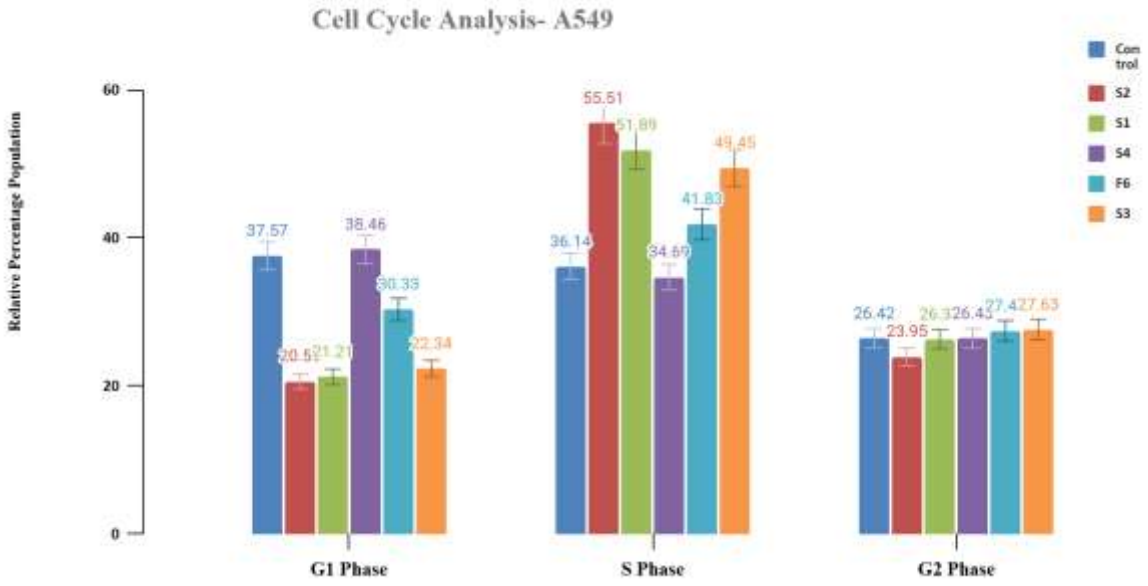


Figure 3: Cell cycle distribution analysis by flow cytometry. Histogram showing combined result for: **(A)** Control, **(B)** S1 (Swertia chirata), **(C)** S2 (Beetroot), **(D)** S3 (Triphala), **(E)** S4 (combination), and **(F)** F6 (effervescent tablet), **(G)** Peaks represent G0/G1, S, and G2/M phases.

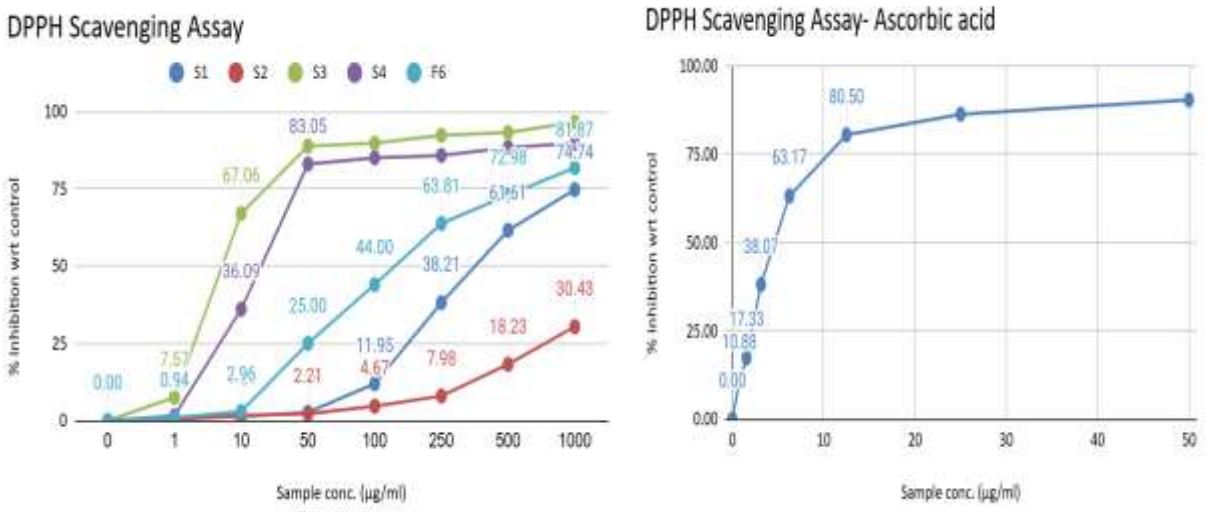


Figure 4: DPPH radical scavenging activity. Dose-response curves showing percentage DPPH scavenging activity versus concentration for samples S1, S2, S3, S4, F6, and ascorbic acid (standard).