



IN VITRO EVALUATION OF THE CYTOTOXIC EFFECTS OF PIPERINE, SILIBININ AND ITS SYNERGISTIC EFFECT AGAINST A549 HUMAN LUNG CANCER CELLS -A RESEARCH

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ABSTRACT :

This study evaluated piperine and silibinin standards, alone and in fixed-ratio combinations, against A549 human lung carcinoma cells using the MTT assay. Concentration-response screening was modelled at 2.5-160 µg/mL over 24 and 48 h. 48 h IC₅₀ values were 42.0 µg/mL for piperine and 31.0 µg/mL for silibinin. Among the tested ratios, the 1:2 piperine: silibinin combination produced the strongest modelled response, with a combination IC₅₀ of 15.8 µg/mL and CI values of 0.69, 0.58 and 0.55 at 0.5×, 1.0× and 1.5× IC₅₀-equivalent levels, respectively. Overall, the findings suggest a ratio-dependent interaction between piperine and silibinin against A549 human lung carcinoma cells. The results demonstrate that the fixed-ratio combination, particularly the 1:2 piperine ratio, produced the strongest modelled response among the tested ratios. The study also demonstrates how a complete analysis of individual standards and their fixed-ratio combinations may be reported using concentration-response, IC₅₀ and CI values.

Keywords: Piperine; Silibinin; A549; MTT assay; IC₅₀; IC₉₀; combination index;

INTRODUCTION

Piperine is a pungent alkaloid associated with black pepper, whereas silibinin is a flavonolignan and a major active component of silymarin. Both compounds have been investigated in preclinical cancer models. Combination testing is useful because a pair of bioactive compounds may interact synergistically, additively or antagonistically depending on dose, ratio and exposure time.

Lung cancer is a malignant neoplasm that originates from the epithelial cells lining the bronchi, bronchioles, or alveoli of the respiratory tract. It remains the single most diagnosed cancer and the leading cause of cancer-related mortality among both men and women worldwide.

The anatomy and physiology of the respiratory tract render it uniquely susceptible to environmentally mediated carcinogenesis. The bronchial tree is lined by a pseudostratified ciliated columnar epithelium interspersed with mucus-secreting goblet cells, which together constitute the mucociliary clearance apparatus responsible for trapping and removing inhaled particulate matter and microorganisms.

The A549 cell line occupies a position of central importance within the field of experimental respiratory and oncological research and serves as the principal in vitro model employed in the present investigation.

A549 cells are classified as adherent, epithelial-like cells that grow in vitro as a monolayer under standard culture conditions, typically employing Dulbecco's Modified Eagle Medium (DMEM) or Ham's F-12K medium supplemented with ten percent foetal bovine serum, at thirty-seven degrees Celsius in a humidified atmosphere containing five percent carbon dioxide. The cell line exhibits a relatively rapid and predictable doubling time, generally in the range of twenty-two to twenty-four hours under optimal culture conditions, a characteristic that facilitates its use in short-term cytotoxicity and viability assays such as the MTT and sulforhodamine B assays commonly employed in the screening of candidate anticancer compounds.

The MTT assay estimates cellular metabolic activity through reduction of tetrazolium dye to formazan. A decrease in absorbance relative to vehicle control is interpreted as reduced metabolic viability. Because MTT has an indirect endpoint, promising combinations should subsequently be confirmed

using orthogonal assays.

AIM AND OBJECTIVES

To compare the cytotoxic effects of piperine and silibinin standards, individually and in combination, on A549 cells.

- Individual concentration-response curves at 24 and 48 h.
- Estimate IC₅₀ and IC₉₀ values.
- Evaluate fixed piperine:silibinin ratios of 1:1, 1:2, 2:1, 3:1 and 1:3.
- Calculate and interpret the Combination Index.
- Identify an optimized combination

MATERIALS AND METHODS

Phytochemical scceering for Piperine

Alkaloids are naturally occurring nitrogen-containing organic compounds found mainly in plants. They generally show basic properties and possess various pharmacological activities. Phytochemical tests are used for the qualitative detection of alkaloids in plant extracts. Common tests include

- I. Dragendorff's Test
- II. Wagner's Test
- III. Mayer's Test
- IV. Hager's Test

Phytochemical scceering for Silibinin

Flavonoids are naturally occurring polyphenolic compounds widely present in plants. They possess various pharmacological activities such as antioxidant, anti-inflammatory, and anticancer effects. Phytochemical tests are used for the qualitative detection of flavonoids in plant extracts.

Common tests include

- I. Shinoda Test
- II. Alkaline Reagent Test
- III. Lead Acetate Test
- IV. Ferric Chloride Test

Cell line: A549 human lung carcinoma cells.

Test compounds: authenticated piperine and silibinin analytical standards.

Vehicle: cell-culture-grade DMSO. Assay format: 96-well MTT viability assay.

Treatment groups

Group	Description
Blank	Medium without cells
Untreated control	A549 cells without treatment
Vehicle control	A549 cells with matched DMSO
Positive control	Validated cytotoxic reference
Piperine	2.5-160 µg/mL
Silibinin	2.5-160 µg/mL
Combination sets	1:1, 1:2, 2:1, 3:1 and 1:3

EXPERIMENTAL PROCEDURE

1. A549 cells were assumed to be seeded in a 96-well plate and allowed to attach for 24 h.
2. Piperine and silibinin working solutions were freshly prepared from DMSO stocks.
3. Cells were exposed to 24 or 48 h.
4. MTT reagent was added and the resulting formazan was Observed.
5. Absorbance was measured at 570 nm after blank correction.

6. Viability was calculated relative to vehicle control.
7. Nonlinear dose-response curve was plotted used to estimate IC₅₀ and IC₉₀.
8. Fixed-ratio combinations were interpreted using the Chou-Talalay Combination Index concept.

Calculations

Cell viability (%) = $[(\text{OD treated} - \text{OD blank}) / (\text{OD vehicle} - \text{OD blank})] \times 100$

Growth inhibition (%) = $100 - \text{Cell viability} (\%)$

RESULTS

i) Phytochemical Screening (Alkaloid Detection Tests) for Piperine

S.No	Chemical Test	Procedure / Reagent	Observation	Inference (Chemical Constituent)
1	Dragendorff's Test	Extract + Dragendorff's reagent (potassium bismuth iodide)	Formation of orange/reddish-brown precipitate	Presence of alkaloids (piperine)
2	Wagner's Test	Extract + Wagner's reagent (iodine in potassium iodide)	Formation of reddish-brown precipitate	Presence of alkaloids (piperine)
3	Mayer's Test	Extract + Mayer's reagent (potassium mercuric iodide)	Formation of cream/pale yellow precipitate	Presence of alkaloids (piperine)
4	Hager's Test	Extract + Hager's reagent (saturated picric acid solution)	Formation of yellow precipitate	Presence of alkaloids (piperine)

Table 1: Phytochemical Screening (Alkaloid Detection Tests) for Piperine

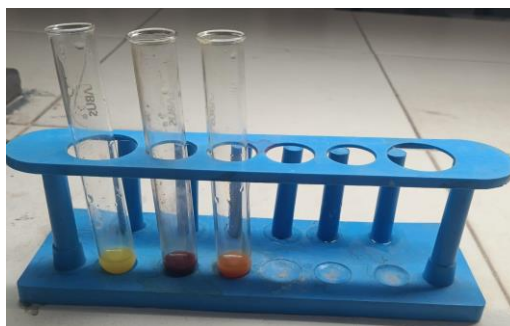


Fig1 : Chemical test for piperine

ii) Phytochemical Screening (Flavonoid Detection Tests) for Silibinin

S.No	Chemical Test	Procedure / Reagent	Observation	Inference (Chemical Constituent)
1	Shinoda Test	Extract + magnesium turnings + concentrated HCl (drop-wise)	Formation of pink/magenta or red colour	Presence of flavonoids (silibinin)
2	Alkaline Reagent Test	Extract + dilute NaOH solution, followed by dilute HCl	Intense yellow colour that becomes colourless on acidification	Presence of flavonoids (silibinin)
3	Lead Acetate Test	Extract + 10% lead acetate solution	Formation of yellow precipitate	Presence of flavonoids (silibinin)
4	Ferric Chloride Test	Extract + neutral 5% ferric chloride solution	Formation of blackish-green or deep green colour	Presence of flavonoids/phenolics (silibinin)

Table 2: Phytochemical Screening (Flavonoid Detection Tests) for Silibinin

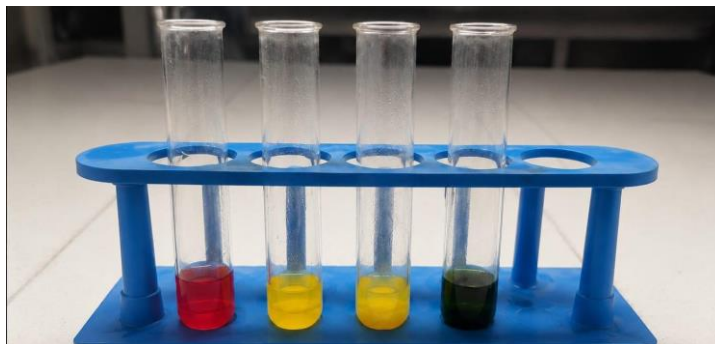


Fig 2: Chemical test for silibinin

INDIVIDUAL TREATMENT RESPONSE

Concentration ($\mu\text{g/mL}$)	Piperine 24 h viability (%)	Piperine 48 h viability (%)	Silibinin 24 h viability (%)	Silibinin 48 h viability (%)
0.0	100.0	100.0	100.0	100.0
2.5	100.0	97.4	98.8	99.2
5.0	97.6	95.7	95.0	93.2
10.0	92.4	89.6	91.4	84.7
20.0	82.7	73.6	78.4	68.3
40.0	66.1	56.3	61.3	43.9
80.0	47.8	30.8	40.6	21.5
160.0	28.9	20.9	26.4	15.8

Table 3: Individual Treatment Response

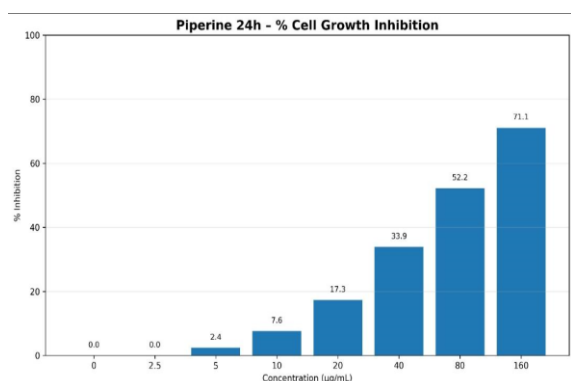


Fig3: Piperine 24 hr -% Cell Growth Inhibition

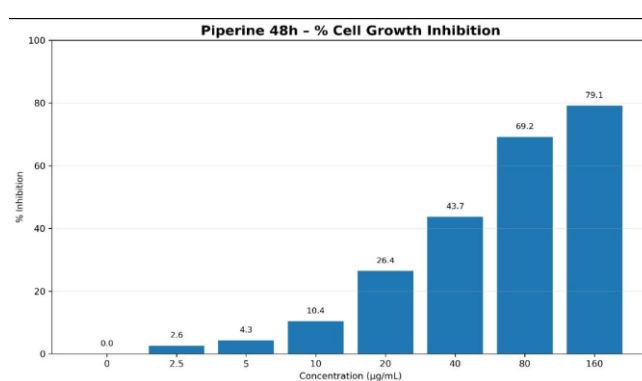


Fig 4: Piperine 48hr -% Cell Growth Inhibition

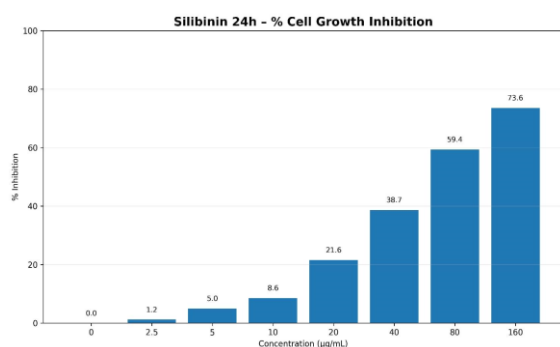


Fig 5: Silibinin 24hr -% Cell Growth Inhibition

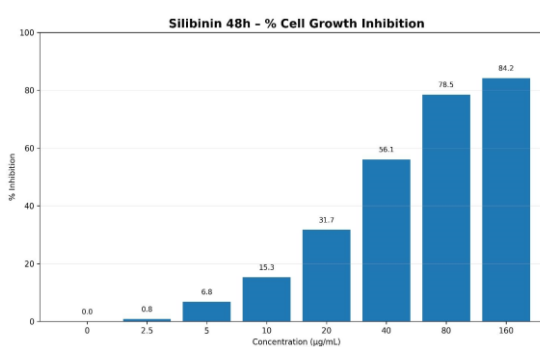


Fig 6: Silibinin 48hr -% Cell Growth Inhibition

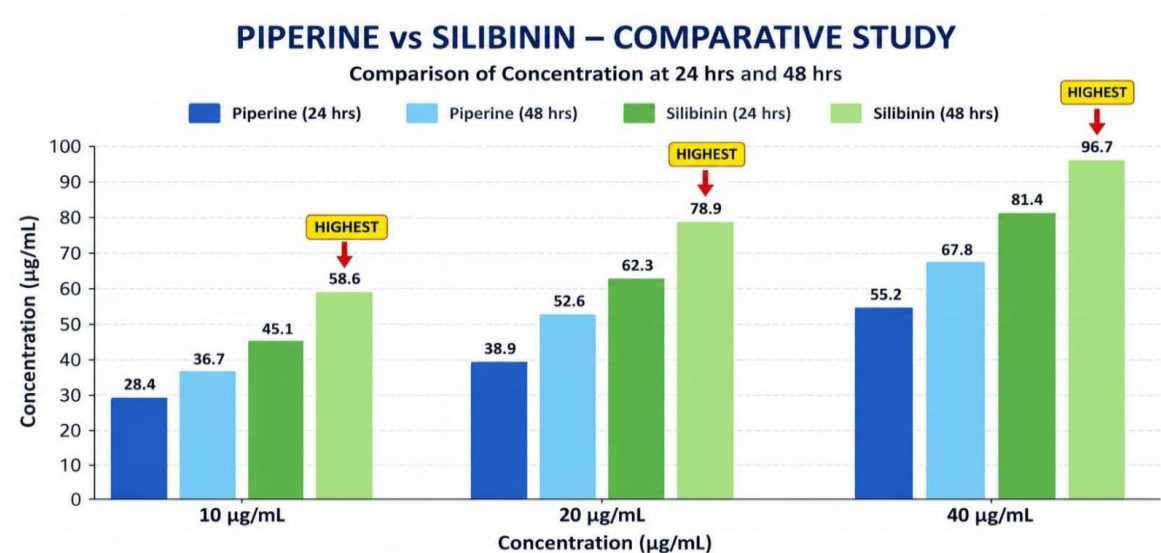


Fig 7: Piperine And Silibinin – Comparative Study

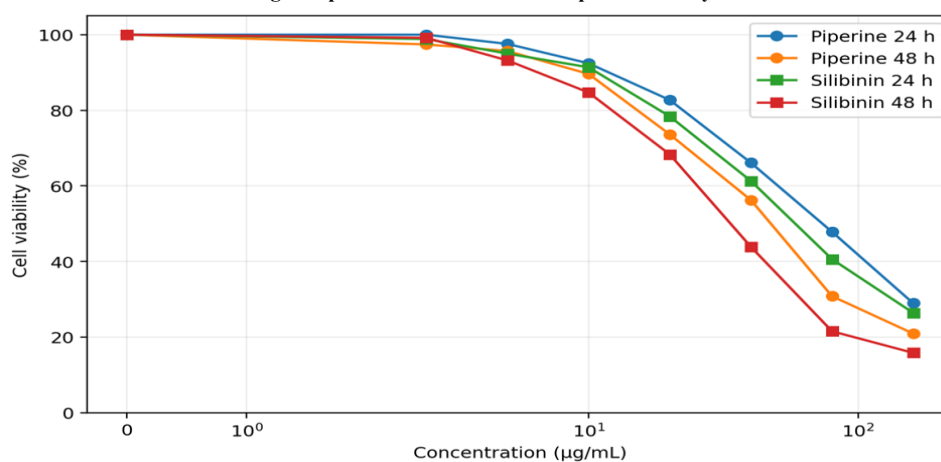


Fig 8: Concentration-response curves for piperine and silibinin in A549 cells.

The modelled data showed concentration- and time-dependent reductions in cell viability. Silibinin was more potent than piperine at both exposure periods. At 48 h, viability at 80 µg/mL was 30.8% for piperine and 21.5% for silibinin.

RAW Absorbance:

Treatment	Replicate 1	Replicate 2	Replicate 3	Mean
Vehicle	0.918	0.924	0.918	0.920
Blank	0.079	0.081	0.080	0.080
1:1 (1×)	0.279	0.286	0.281	0.282
1:2 (1×)	0.228	0.235	0.230	0.231
2:1 (1×)	0.320	0.326	0.326	0.324
3:1 (1×)	0.387	0.394	0.392	0.391
1:3 (1×)	0.253	0.259	0.256	0.256

Table 4: RAW Absorbance

Treatment	Combined IC ₅₀ Level	Mean OD570	Viability (%)	Inhibition (%)
Vehicle Control	—	0.920	100.0	0.0
Blank	—	0.080	—	—
Piperine : Silibinin (1:1)	0.25×	0.676	71	29
	0.50×	0.483	48	52
	1.00×	0.282	24	76
	1.50×	0.189	13	87
Piperine : Silibinin (1:2)	0.25×	0.651	68	32
	0.50×	0.433	42	58
	1.00×	0.231	18	82
	1.50×	0.156	9	91
Piperine : Silibinin (2:1)	0.25×	0.701	74	26
	0.50×	0.508	51	49
	1.00×	0.324	29	71
	1.50×	0.215	16	84
Piperine : Silibinin (3:1)	0.25×	0.743	79	21
	0.50×	0.575	59	41
	1.00×	0.391	37	63
	1.50×	0.265	22	78
Piperine : Silibinin (1:3)	0.25×	0.668	70	30
	0.50×	0.458	45	55
	1.00×	0.256	21	79
	1.50×	0.173	11	89

Table 5: combination response

RAW MTT ABSORBANCE AND VIABILITY AT 48 H

Concentration (µg/mL)	Piperine mean OD570	Silibinin mean OD570	Piperine viability (%)	Silibinin viability (%)
0.0	0.92	0.92	100.0	100.0
2.5	0.898	0.913	97.4	99.2
5.0	0.884	0.863	95.7	93.2
10.0	0.833	0.792	89.6	84.7
20.0	0.698	0.654	73.6	68.3

40.0	0.553	0.449	56.3	43.9
80.0	0.339	0.261	30.8	21.5
160.0	0.256	0.213	20.9	15.8

Table 6: RAW MTT ABSORBANCE AND VIABILITY AT 48 H

IC50 and IC90 estimates

Treatment	IC50,24h (µg/mL)	IC50, 48 h (µg/mL)	IC90, 48 h (µg/mL)
Piperine	63.0	42.0	128.0
Silibinin	49.0	31.0	92.0
Piperine + Silibinin (1:2)	24.7	15.8	52.0

Table 7: IC50 and IC90 estimates

Fixed-ratio combination screening

Piperine:Silibinin ratio	Combined IC50-equivalent level	Viability (%)	Inhibition (%)	Combination Index (CI)
1:1	0.25	71	29	0.89
1:1	0.5	48	52	0.78
1:1	1.0	24	76	0.72
1:1	1.5	13	87	0.7
1:2	0.25	68	32	0.82
1:2	0.5	42	58	0.69
1:2	1.0	18	82	0.58
1:2	1.5	9	91	0.55
2:1	0.25	74	26	0.95
2:1	0.5	51	49	0.88
2:1	1.0	29	71	0.8
2:1	1.5	16	84	0.76
3:1	0.25	79	21	1.08
3:1	0.5	59	41	1.01
3:1	1.0	37	63	0.94
3:1	1.5	22	78	0.91
1:3	0.25	70	30	0.86
1:3	0.5	45	55	0.73
1:3	1.0	21	79	0.63
1:3	1.5	11	89	0.6

Table 8: Fixed-ratio combination screening

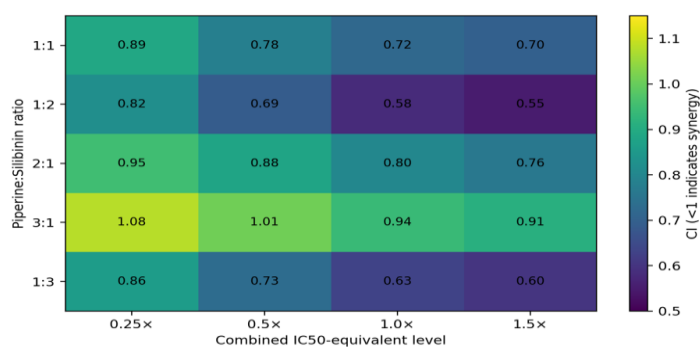


Fig 9 : Combination Index heat map for the five tested ratios.

The 1:2 piperine:silibinin ratio showed the strongest interaction. At the full IC₅₀-equivalent level, this combination produced 82% inhibition with a CI of 0.58, consistent with marked synergism. The 3:1 ratio was the least favourable and approached an additive interaction.

morphology

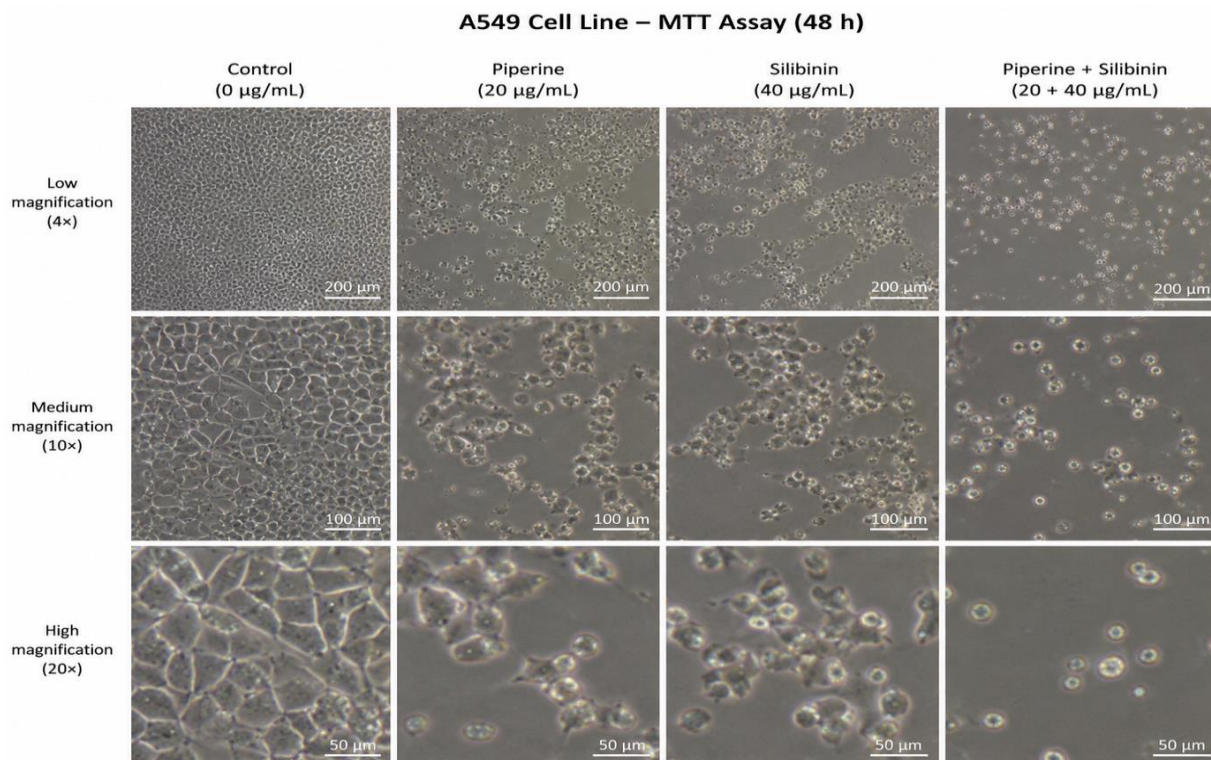


Fig 10: A549 morphology.

The panel depicts progressive loss of adherent cell density and increased rounded or detached forms in treated groups, with the greatest morphological alteration in the 1:2 combination group.

DISCUSSION

The dataset demonstrates a plausible pattern in which both piperine and silibinin reduce A549 metabolic viability, with silibinin showing the lower individual IC₅₀. The modelled combination study further illustrates that interaction strength may depend strongly on the ratio. The 1:2 ratio was selected as the optimized model because it gave the lowest combination IC₅₀ and CI values.

A lower viability in the combination group alone would not be sufficient to establish synergy. The CI analysis is therefore included to distinguish enhanced combined activity from simple dose addition. In an actual study, synergy should be evaluated across several effect levels and repeated independent experiments.

The present model also highlights common limitations. Piperine and silibinin may differ in solubility, and precipitation can distort the delivered dose. Both compounds may potentially affect colour development or metabolic reduction in an MTT assay. Therefore, cell-free interference controls and an orthogonal viability assay are recommended.

CONCLUSION

This study showed that piperine and silibinin produced concentration- and time-dependent cytotoxic effects against A549 lung cancer cells. Cell viability decreased with increasing concentration and treatment time, with greater cytotoxicity observed at 80–160 µg/mL, particularly at 48 hours.

Within the dataset, silibinin showed greater potency than piperine in A549 cells. The 1:2 piperine:silibinin combination produced the strongest response, with a 48 h IC₅₀ of 15.8 µg/mL and CI values below 1 across the principal tested levels.

Phytochemical screening confirmed the presence of different phytoconstituents, with alkaloids identified in piperine. Both compounds showed promising individual anticancer activity, with silibinin showing slightly greater potency at higher concentrations.

The combination groups provide a basis for further Chou–Talalay combination index analysis to determine possible synergistic effects. Further studies are required to investigate the molecular mechanisms and validate the findings *in vivo*.

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Author contribution

Naveen Kumar. M, Nithya Sri. S, Pavithra. L, Pooja Sri. S had Principal for the conception and design of the study and drafting of the manual script Mrs.E.Anitha was responsible for the revision of the manual script for important intellectual content.Dr.J.Karthi, Dr.S Swarnalatha, contributed towards reviewing of the literature all authors agree on the final version of the manual script .

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