Epigenetic Reversal And Cytotoxicity Of Phytochemicals In Chemoresistant HL-60 Cells

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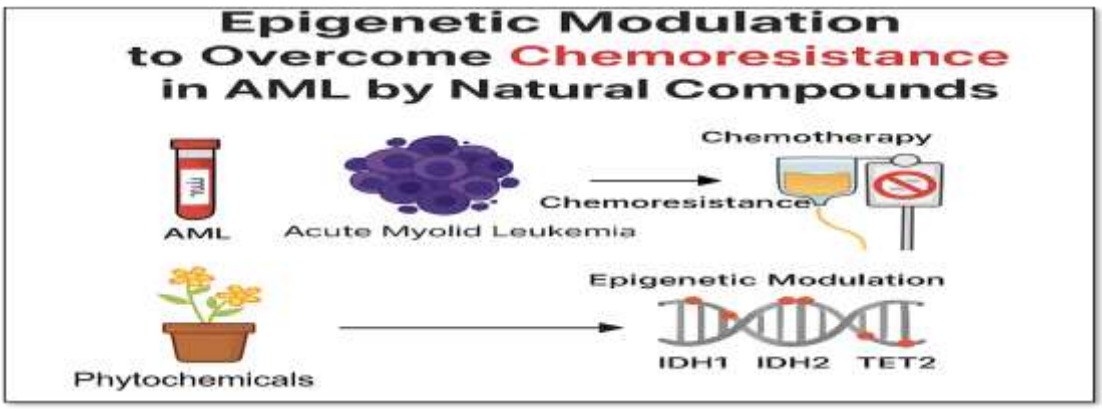
ABSTRACT

Acute myeloid leukemia (AML) represents one of the most aggressive forms of haematological malignancy, marked by the clonal expansion of undifferentiated myeloid progenitors that disrupt normal hematopoiesis. Despite decades of progress in diagnostic stratification and chemotherapy, relapse due to chemoresistance continues to be the principal cause of treatment failure and mortality. Increasing evidence suggests that beyond genetic lesions, epigenetic alterations—particularly aberrant DNA methylation—play a decisive role in leukemogenesis, disease progression, and therapeutic resistance. DNA methylation is regulated by a balance between DNA methyltransferases (DNMTs) and the ten-eleven translocation (TET) family of demethylases, both of which are tightly linked to the cell's metabolic state. Mutations in isocitrate dehydrogenase (IDH1 and IDH2) genes generate the oncometabolite 2-hydroxyglutarate (2-HG), which inhibits α-ketoglutarate–dependent enzymes, including TET2, leading to promoter hypermethylation and gene silencing. Reversal of these aberrant methylation patterns is therefore a promising therapeutic strategy for overcoming chemoresistance in AML.



Graphical Abstract Image

1. INTRODUCTION

Leukemia represents a group of haematological malignancies arising from the uncontrolled proliferation of abnormal hematopoietic precursor cells in the bone marrow and peripheral circulation. Among these, Acute Myeloid Leukaemia (AML) remains one of the most prevalent and aggressive forms, accounting for nearly one-third of all adult leukemia cases worldwide (Döhner et al., 2022). AML is characterised by the rapid clonal

expansion of myeloid progenitor cells that fail to differentiate into mature blood cells, leading to bone marrow failure, anaemia, thrombocytopenia, and immunodeficiency. Despite intensive research and advances in hematopoietic stem cell transplantation and molecularly targeted therapies, the prognosis for AML patients remains poor, with overall survival rates below 30% for most adults.

The standard therapeutic approach, comprising cytarabine and anthracycline-based induction regimens, achieves complete remission in 60–70% of younger adults and 40–50% of elderly patients (Estey, 2020). However, the persistence of minimal residual disease and the eventual relapse due to chemoresistance constitute major obstacles to curative outcomes. Chemoresistance arises from a complex interplay of molecular and cellular factors that include altered drug transport, evasion of apoptosis, metabolic reprogramming, and profound epigenetic remodelling. Among these, epigenetic dysregulation has emerged as a central driver of leukemic transformation and resistance, offering a potentially reversible target for therapeutic intervention.

1.1 Epigenetic Landscape in Leukemogenesis

Epigenetics refers to heritable but reversible modifications in gene expression that occur without changes to the DNA sequence. The main epigenetic mechanisms include DNA methylation, histone post-translational modifications, chromatin remodelling, and noncoding RNA regulation (Baylin & Jones, 2016). Together, these modifications orchestrate gene expression patterns essential for lineage commitment and differentiation in normal hematopoiesis.

Aberrant DNA methylation represents one of the earliest and most consistent molecular hallmarks of AML. DNA methylation involves the covalent addition of a methyl group to the 5′ position of cytosine residues within CpG dinucleotides, a reaction catalysed by DNA methyltransferases (DNMT1, DNMT3A, DNMT3B). In normal cells, methylation silences repetitive sequences, maintains chromosomal stability, and regulates gene expression. However, in AML, this process becomes deregulated, leading to a dual pattern of global hypomethylation and promoter-specific hypermethylation (Figueroa et al., 2010).

Global hypomethylation contributes to genomic instability, reactivation of transposable elements, and chromosomal rearrangements. Conversely, hypermethylation of promoter CpG islands silences tumour suppressor and differentiation-associated genes, including CDKN2B (p15INK4B), CDKN2A (p16INK4A), MLH1, and E-cadherin, resulting in unrestrained cell proliferation and impaired apoptosis (Wouters & Delwel, 2016). This epigenetic silencing creates a self-perpetuating oncogenic state that sustains leukemic stem cells, making them resistant to conventional chemotherapeutic agents.

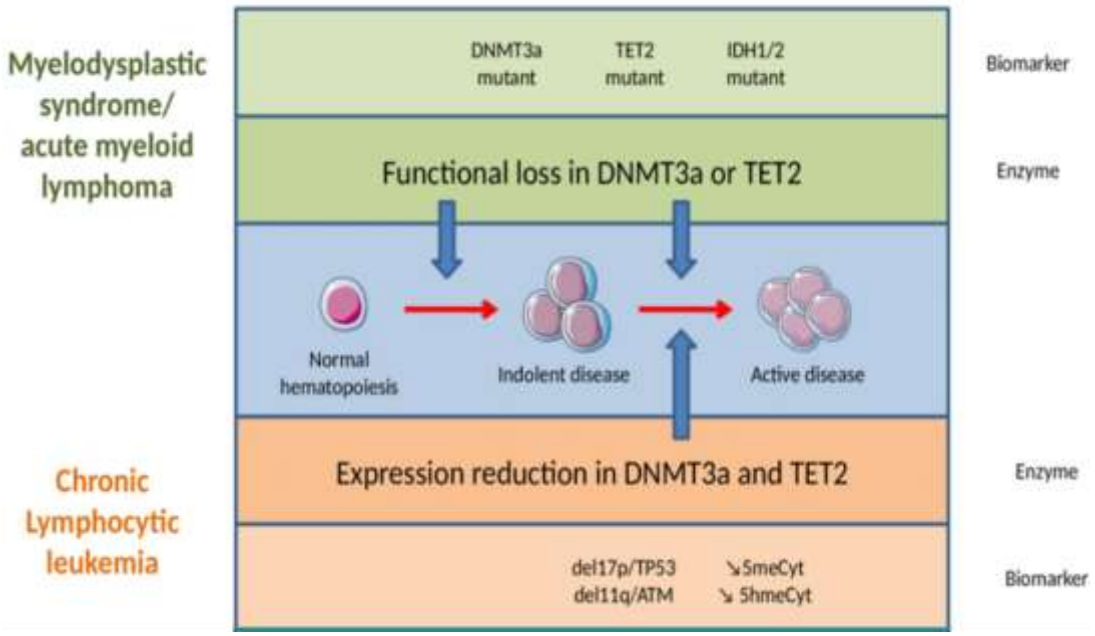


Figure 1: Mechanistic overview of DNA methylation & gene silencing in AML (Wouters & Delwel, 2016)

1.2 Epigenetic Regulation and Chemoresistance in AML:

The discovery of recurrent mutations in isocitrate dehydrogenase (IDH1 and IDH2) and ten-eleven translocation (TET) genes established a fundamental link between cellular metabolism and the epigenetic landscape of AML (Ward et al., 2010; Figueroa et al., 2010).

Under physiological conditions, IDH1 (cytosolic) and IDH2 (mitochondrial) catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG), a key metabolite that acts as a cofactor for α-KG–dependent dioxygenases such as TET enzymes and histone demethylases. TET1, TET2, and TET3 catalyze the stepwise oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine, and 5-carboxylcytosine—intermediates of DNA demethylation that restore gene expression.

Mutations in IDH1/2 confer a neomorphic enzyme activity that reduces α-KG to 2-hydroxyglutarate (2-HG), an oncometabolite that acts as a competitive inhibitor of α-KG–dependent enzymes, including TET proteins (Xu et al., 2011). This inhibition suppresses TET-mediated hydroxymethylation, resulting in widespread promoter hypermethylation and transcriptional silencing—termed the CpG Island Methylator Phenotype (CIMP) (Jin et al., 2020).

Similarly, TET2 mutations or deletions, common in AML and other myeloid malignancies, reduce the conversion of 5mC to 5hmC, further reinforcing the hypermethylated phenotype (Figueroa et al., 2010). Collectively, these genetic and metabolic alterations create an epigenetic bottleneck, preventing differentiation and enabling leukemic cells to maintain stem-like self-renewal capabilities.

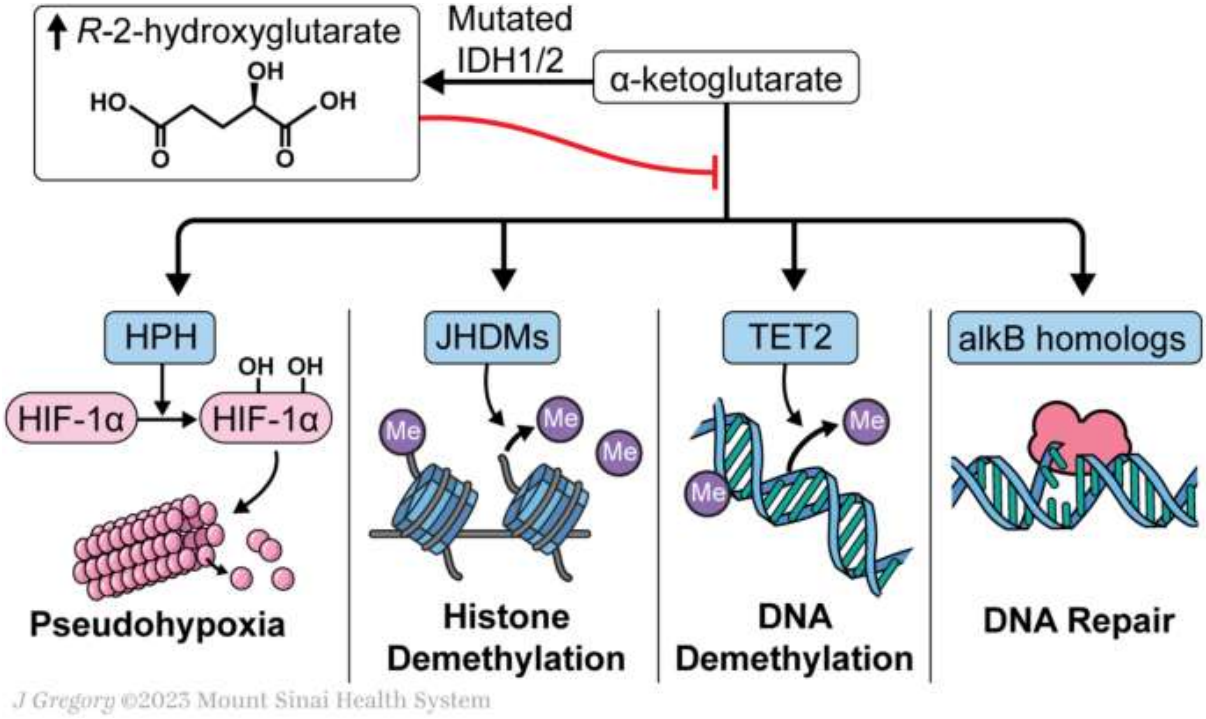


Figure 2: The IDH/TET metabolic-epigenetic axis and the generation of 2-hydroxyglutarate (Figueroa et al., 2010)

The clinical recognition of epigenetic abnormalities in AML led to the development of epigenetic drugs targeting DNA methylation and histone modification. The most widely used agents—azacitidine and decitabine—are cytidine analogs that incorporate into DNA and RNA, trapping DNMTs and promoting passive demethylation during replication (García-Manero et al., 2019).

Although these drugs have improved survival and quality of life in elderly or unfit AML patients, their clinical efficacy remains limited by several factors:

1. **Transient responses** — remethylation and relapse after treatment cessation.

2. **Myelosuppression and off-target toxicity** — due to non-selective demethylation.
3. **Lack of specificity** — affecting both normal and malignant hematopoietic cells.

Moreover, while DNMT inhibitors can reverse some epigenetic silencing, they fail to restore differentiation or prevent clonal evolution fully. Therefore, attention has shifted toward natural or dietary compounds capable of epigenetic modulation without significant toxicity (Li et al., 2020).

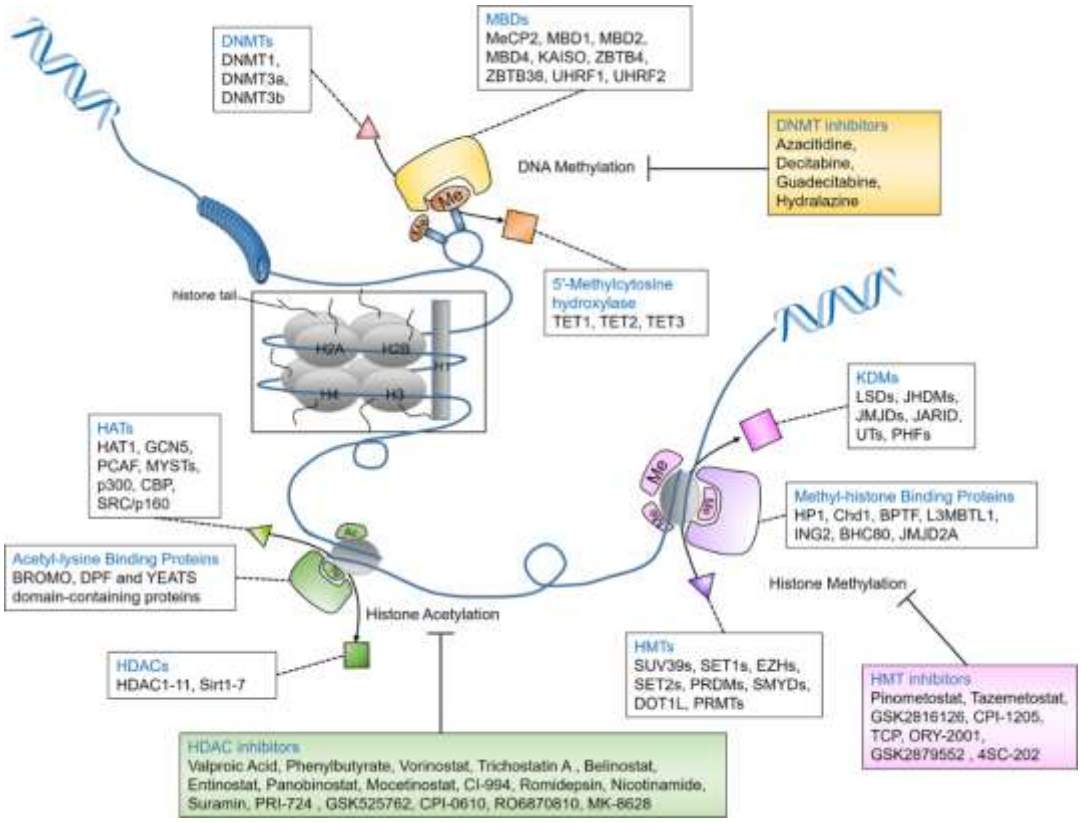


Figure 3: Comparison of synthetic DNMT inhibitors and natural epigenetic modulators (Baylin & Jones, 2016)

One of the most intriguing applications of phytochemical epigenetic modulation is in overcoming chemoresistance. Drug-resistant leukemia cells, such as HL-60/RS sublines, display altered expression of drug transporters, increased DNA repair capacity, and promoter hypermethylation of apoptosis-related genes (Zhang et al., 2018). These features contribute to the persistence of leukemic clones even after cytotoxic therapy.

Reactivation of epigenetically silenced genes through DNA demethylation or chromatin remodelling has been shown to restore chemosensitivity. Phytochemicals such as Withaferin A, Silymarin, and Gingerol can potentially reverse this phenotype by reducing DNA methylation of IDH1, IDH2, and TET2 genes, reinstating their normal function in DNA repair and differentiation pathways.

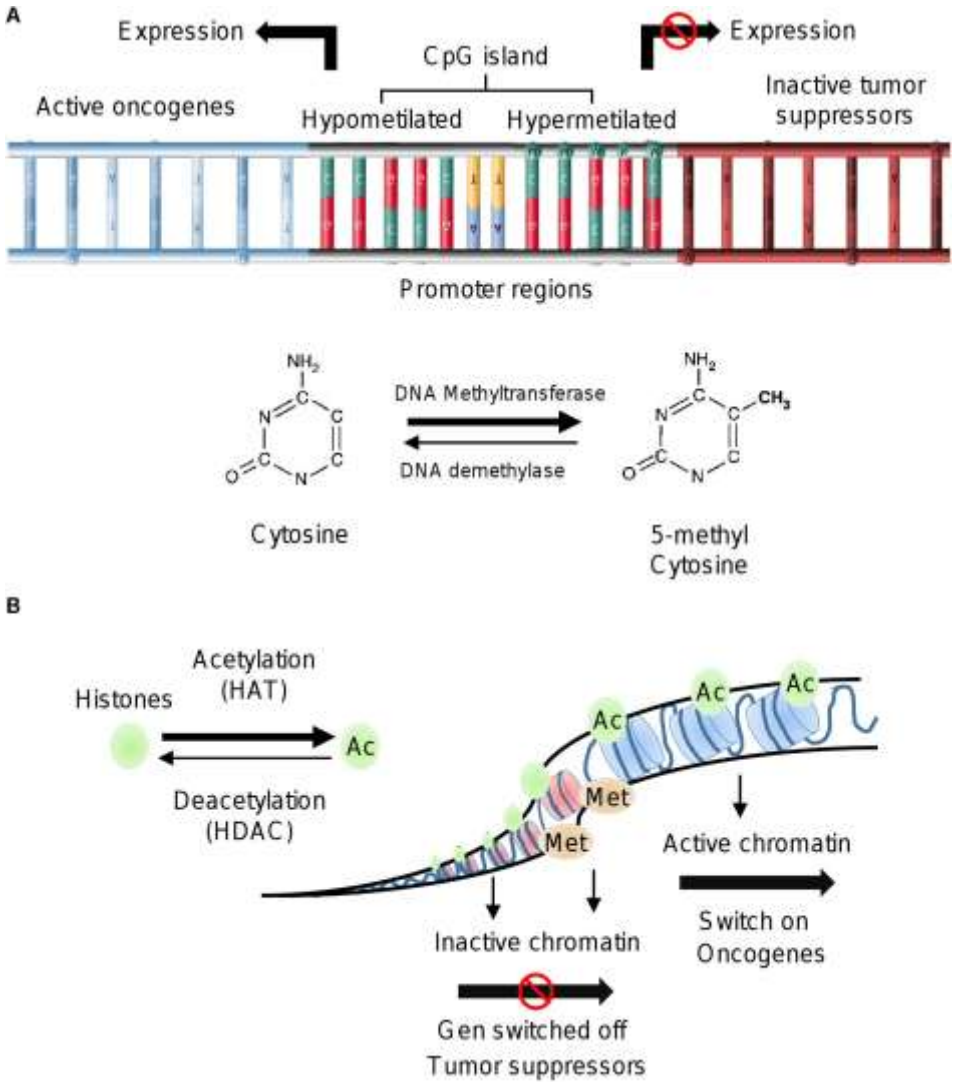


Figure 4: Conceptual framework of phytochemical-induced epigenetic reprogramming in chemoresistant AML (Li et al., 2020)

By simultaneously inducing apoptosis and promoting demethylation, these compounds bridge the gap between cytotoxic and epigenetic therapy. This dual functionality supports a multidimensional therapeutic approach, in which epigenetic reprogramming sensitises AML cells to conventional drugs and prevents relapse.

Thus, targeting DNA methylation dynamics using natural bioactive phytochemicals represents an evolving paradigm in AML treatment—combining the molecular precision of epigenetic therapy with the safety and accessibility of natural compounds.

1.3 Natural Bioactive Compounds as Epigenetic Regulators

Natural phytochemicals are secondary metabolites produced by plants in response to stress or environmental stimuli. They encompass a broad spectrum of molecules—including flavonoids, phenolics, alkaloids, terpenoids, and lactones—that possess diverse biological activities. In recent years, an increasing number of studies have revealed that these compounds can modulate epigenetic targets, influencing DNA methylation, histone acetylation, and microRNA expression (Aggarwal & Shishodia, 2021).

Compounds such as curcumin, resveratrol, sulforaphane, and epigallocatechin gallate (EGCG) have demonstrated the ability to inhibit DNMTs and histone deacetylases (HDACs), thereby reactivating silenced genes. Importantly, these natural molecules often exhibit multi-targeted activity, simultaneously influencing oxidative stress, inflammation, apoptosis, and chromatin regulation (Li et al., 2020).

Within this group, three phytochemicals have gained attention for their dual cytotoxic and epigenetic properties: Silymarin, Gingerol, and Withaferin A.

- Silymarin, extracted from Silybum marianum (Milk thistle), is a flavonolignan complex that exhibits antioxidant and hepatoprotective effects. Emerging studies indicate that it can inhibit DNMT1, restore the expression of epigenetically silenced genes, and suppress proliferation in leukemia and breast cancer cells (Surai, 2015; Zhang et al., 2021).
- Gingerol, a phenolic compound found in Zingiber officinale (Ginger), exerts anti-inflammatory, antioxidant, and pro-apoptotic actions. It interferes with NF-κB and PI3K/Akt signalling pathways, and recent data suggest it can modify epigenetic marks associated with carcinogenesis (Kim et al., 2018).
- Withaferin A, a steroidal lactone derived from Withania somnifera (Ashwagandha), exhibits strong anti-leukemic activity by modulating HSP90, NF-κB, and STAT3 pathways. It has also been shown to induce DNA demethylation and promote re-expression of silenced tumour-suppressor genes (Hahm et al., 2014; Nandakumar et al., 2022).

These compounds represent a unique class of natural epigenetic reprogrammers, capable of influencing both the biochemical and transcriptional landscape of cancer cells. Their low systemic toxicity and compatibility with conventional therapies make them particularly attractive for long-term treatment or maintenance therapy in AML.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Analytical-grade chemicals and molecular biology reagents were procured from recognised suppliers to ensure reproducibility and purity. Phytochemicals, including Silymarin, Gingerol, and Withaferin A, were obtained from Sigma-Aldrich (St. Louis, MO, USA) with a stated purity of ≥98%. Stock solutions of each compound were prepared in dimethyl sulfoxide (DMSO) and diluted in sterile culture medium immediately before use, maintaining a final DMSO concentration below 0.1% (v/v) in all experiments to prevent solvent-induced cytotoxicity. Standard buffers such as phosphate-buffered saline (PBS), Tris-EDTA, and lysis buffers were freshly prepared using nuclease-free water. All culture reagents, including RPMI-1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, and trypsin-EDTA, were purchased from HiMedia Laboratories (Mumbai, India).

2.2 Cell Culture and Maintenance

Human promyelocytic leukemia cell lines HL-60 (parental) were obtained from the National Centre for Cell Science (NCCS, Pune, India). Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. The resistant HL-60/RS subline was previously generated by stepwise exposure to increasing concentrations of cytarabine and daunorubicin to develop a chemoresistant phenotype. Cell cultures were sub-cultured every 2–3 days to maintain logarithmic growth, and cell viability was confirmed microscopically by trypan blue exclusion before each experiment.

2.3 Preparation of Phytochemical Solutions

Stock solutions (10 mM) of Silymarin, gingerol, and Withaferin A were prepared in DMSO and stored at−20°C. Working concentrations were freshly diluted in culture medium before each experiment. The final concentrations used ranged from 10 to 200 µM, depending on preliminary cytotoxicity profiles. Control cells received equivalent volumes of DMSO (<0.1%), ensuring that observed biological effects were due to phytochemical exposure rather than solvent interference.

2.4 High-Performance Thin Layer Chromatography (HPTLC) Profiling

Phytochemical fingerprinting was carried out to authenticate and verify the integrity of the tested compounds. Samples were applied on silica gel 60 F_{254} plates (Merck, Germany) using a CAMAG Linomat V applicator under nitrogen. The mobile phase was optimised individually for each compound:

- **Silymarin:** Toluene: Ethyl acetate: Formic acid (7:3:0.5)
- **Gingerol:** Chloroform: Methanol: Water (8:1.5:0.5)
- **Withaferin A:** Toluene: Ethyl acetate: Methanol (6:4:1)

Plates were developed in a twin-trough chamber to a migration distance of 80 mm, air-dried, and scanned at specific detection wavelengths (254 nm and 366 nm) using a CAMAG TLC scanner. The Rf values and peak

areas were recorded, and profiles were compared with authentic reference standards. HPTLC chromatograms confirmed compound purity with sharp, well-resolved peaks consistent with reference literature values.

2.5 MTT Assay for IC_{50} Determination
The cytotoxic effects of Daunorubicin and Cytarabine were evaluated using the MTT assay. Briefly, 1×10^4 cells per well were seeded in 96-well plates and incubated with increasing concentrations of drugs (Cytarabine: 1-10 µg/mL, Daunorubicin: 1-10 µg/mL) for 48 hours. After treatment, 20 µL of 5 mg/mL MTT solution was added to each well, and plates were incubated for 4 hours. The formazan crystals formed were dissolved in 100 µL of dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm using a microplate reader. IC_{50} values were calculated from dose-response curves.

2.6 Combination Drug Treatment and Synergy Determination
To assess drug synergy, the cells were treated with a combination of Cytarabine and Daunorubicin at varying concentrations. The combination ratio of 0.7:0.10 (Cytarabine: Daunorubicin) was determined using Compusyn software, based on dose-effect analyses. The combination index (CI) values were calculated to assess synergy, where a CI < 1 indicates synergy, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism.

2.7 Development of Drug Resistance
Drug resistance was induced in HL-60 cells by exposing them to increasing concentrations of the Cytarabine and Daunorubicin combination over several weeks. The cells were incubated with sub-IC_{50} doses, and the concentration was gradually increased until resistance was achieved. Resistant cells were confirmed by performing the MTT assay to assess their response to standard chemotherapy.

2.8 MTT Assay on Drug-Resistant Cells
The final MTT assay was performed on drug-resistant HL-60 cells to evaluate the cytotoxicity of natural compounds. The IC_{50} values for Ashwagandha, Silymarin, and Ginger were determined using the same MTT protocol as described earlier. Cells were treated with increasing concentrations of the compounds (1-100 µg/mL) for 48 hours.

2.9 AO/EtBr Assay for Apoptosis
To assess apoptosis, the Acridine Orange/Ethidium Bromide (AO/EtBr) assay was performed. Cells were stained with 1 µL of AO/EtBr solution (100 µg/mL) for 10 minutes and then visualised under a fluorescence microscope. Viable cells stained green, while apoptotic cells showed orange/red fluorescence due to the binding of ethidium bromide to fragmented DNA.

2.10 Hoechst Assay for Nuclear Morphology
Nuclear changes were observed using the Hoechst 33342 staining assay. Cells were stained with 5 µg/mL Hoechst 33342 for 10 minutes at 37°C and visualised under a fluorescence microscope. Apoptotic cells were identified by chromatin condensation and nuclear fragmentation.

2.11 DNA Isolation and Quantification
Following treatment, total genomic DNA was extracted using a modified phenol–chloroform–isoamyl alcohol (25:24:1) method. Briefly, 1×10^6 cells were harvested, lysed with SDS-proteinase K buffer, and incubated at 55 °C for 1 h. DNA was purified through sequential extraction with phenol–chloroform, followed by ethanol precipitation. The DNA pellet was air-dried, dissolved in nuclease-free water, and quantified spectrophotometrically at $\mathbf{A_{260}/A_{280}}$ ratio to ensure purity (1.8 ± 0.1). Integrity was confirmed by agarose gel electrophoresis (0.8% gel stained with ethidium bromide).

2.12 Bisulfite Conversion of DNA
For methylation analysis, 1 µg of genomic DNA was subjected to bisulfite conversion using the EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) according to the manufacturer's protocol. The bisulfite treatment converts unmethylated cytosines to uracil while leaving methylated cytosines unchanged, enabling discrimination between methylated and unmethylated alleles during MSP amplification. Converted DNA was eluted in 20 µL of elution buffer and stored at −20 °C until further use.

2.13 Primer Designing for Methylation-Specific PCR (MSP)
Gene-specific primers were designed for methylated (M) and unmethylated (U) promoter regions of IDH1, IDH2, and TET2 using Meth Primer software based on CpG island sequences retrieved from NCBI GenBank. Primer sets were validated against published sequences (Figueroa et al., 2010; Li et al., 2019). Primers were synthesised

by Eurofins Genomics (Bangalore, India) and reconstituted to 10 µM stock concentrations. Each MSP reaction was performed separately for M and U primer sets to assess differential promoter methylation.

2.14 Methylation-Specific PCR (MSP)
The MSP reactions were carried out in a 25 µL total volume containing 50 ng of bisulfite-converted DNA, 0.2 µM of each primer, 200 µM dNTPs, 1× PCR buffer, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Thermo Fisher Scientific). The thermal cycling profile included initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 30 s), with a final extension at 72 °C for 10 min. PCR products were resolved on a 2% agarose gel and visualised under UV illumination. The presence of a band in either methylated or unmethylated reactions indicated the methylation status of the gene promoter.

2.15 Methylation Index and Densitometric Analysis
Band intensities from MSP gels were analysed using ImageJ software (NIH, USA). The Methylation Index (MI) was calculated using the formula:
MI=Intensity of methylated bandIntensity of methylated + unmethylated bands×100\text{MI} = \frac{\text{Intensity of methylated band}}{\text{Intensity of methylated + unmethylated bands}} \times 100MI=Intensity of methylated + unmethylated bandsIntensity of methylated band×100
This provided a quantitative estimate of promoter methylation status. Data were expressed as mean ± SD from three independent experiments. Treatment with phytochemicals resulted in a measurable reduction in MI values, indicating promoter demethylation and potential gene reactivation.

2.16 Heatmap Visualisation of Methylation Patterns
To visualise the differential methylation profiles across treatments, MI data were compiled into a matrix and plotted as a heatmap using GraphPad Prism 8.0. Colour gradients represented methylation levels, with red indicating high methylation and green indicating demethylation. This visual representation provided an intuitive overview of the demethylating potential of each phytochemical across IDH1, IDH2, and TET2 loci.

2.17 Statistical Analysis
All experiments were performed in triplicate (n = 3), and data were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 8.0 software. Differences between treated and control groups were assessed by one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Phytochemical Profiling by HPTLC
The phytochemical constituents—Silymarin, Gingerol, and Withaferin A—were subjected to High-Performance Thin-Layer Chromatography (HPTLC) to confirm their identity and purity before biological application. Clear, sharp, and well-resolved peaks were obtained for each compound under their optimised solvent systems, indicating high purity and chemical stability.
For Silymarin, the mobile phase toluene: ethyl acetate: formic acid (7:3:0.5) yielded a single dominant band with an Rf ≈ 0.58, matching the standard reference. Similarly, Gingerol developed in chloroform: methanol: water (8:1.5:0.5) produced a defined band at Rf ≈ 0.48, while Withaferin A in toluene: ethyl acetate: methanol (6:4:1) showed a major spot at Rf ≈ 0.44. Densitometric scanning at 254 nm and 366 nm revealed no overlapping peaks, confirming the absence of impurities or degradation products.

Table 1: HPTLC Chromatogram Analysis

Sample	Marker Compound	Rf Value	Content (%)
Ginger Extract	Gingerol	0.48	9.57%
Ashwagandha Extract	Withaferin A	0.44	0.62%

Milk Thistle Extract	Silymarin	0.58	1.51%

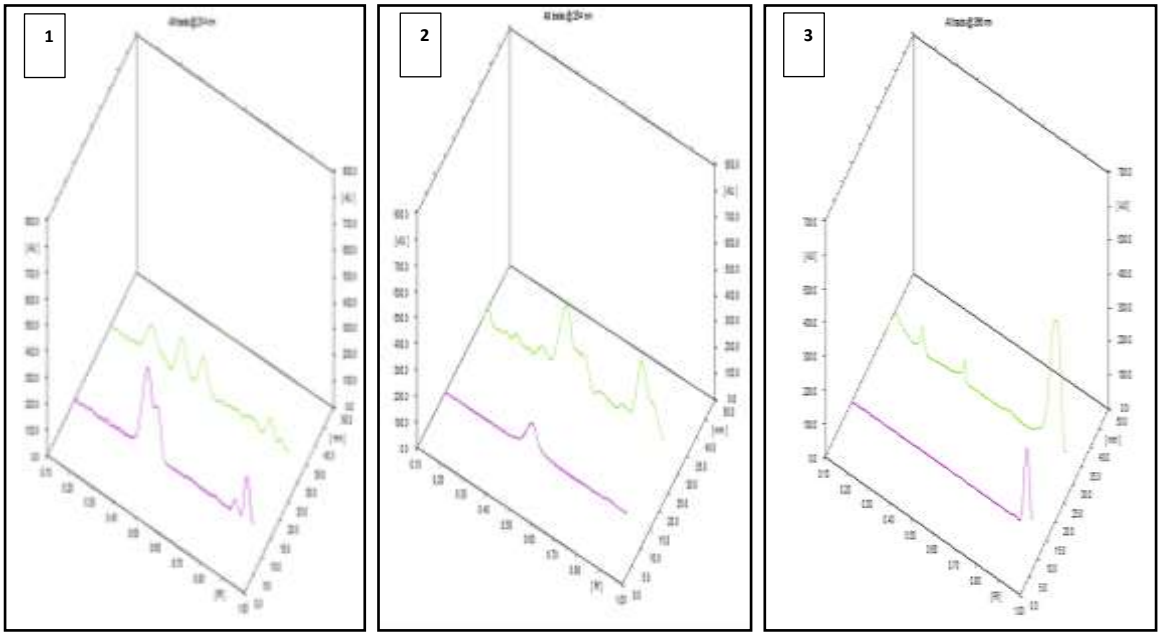


Figure 5: HPTLC chromatograms of (1) Withaferin A, (2) Gingerol, and (3) Silymarin showing Rf values and peak areas

Each compound's fingerprint remained stable across replicates, confirming consistency in purity and formulation. This verification step established analytical confidence before proceeding to biological assays and ensured reproducibility of downstream experiments.

3.2 Cytotoxicity of Phytochemicals on Parental and Resistant HL-60 Cells

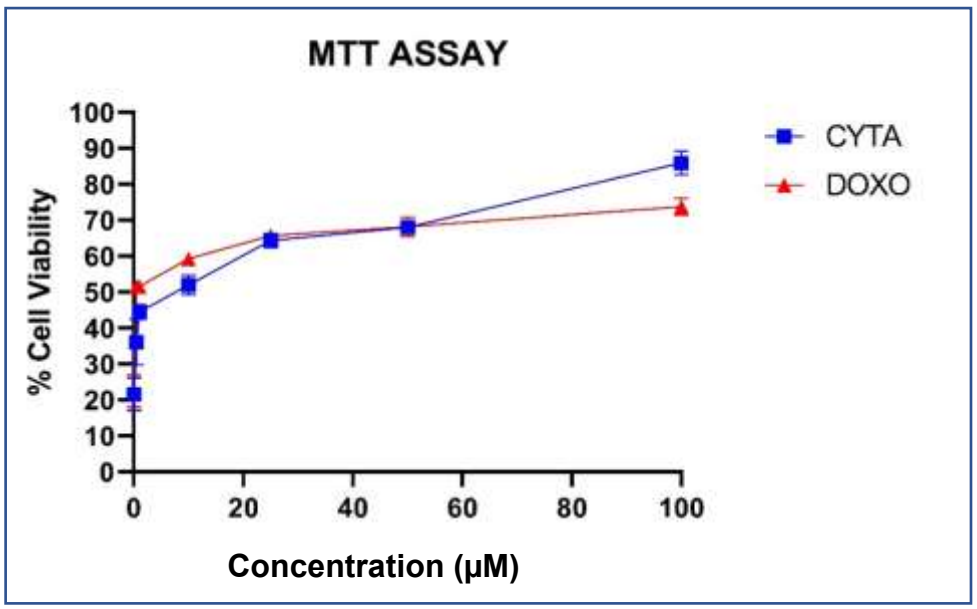


Figure 6: MTT Assay of Normal HL-60 Cells Treated with Cytarabine and Daunorubicin

The MTT assay was performed to evaluate the cytotoxic effects of Cytarabine (CYTA) and Daunorubicin (DOXO) on normal HL-60 cells at varying concentrations ranging from 0 to 100 µg/mL. The results showed a dose-dependent increase in cell viability in response to both drugs (Figure 6).

For Cytarabine, the percentage of cell viability remained relatively low at lower concentrations (below 20 µg/mL) and started to increase steadily with higher concentrations, plateauing around 70-75% viability at 100 µg/mL. Similarly, Daunorubicin exhibited an initial cytotoxic effect at lower concentrations but demonstrated a slight increase in cell viability at concentrations higher than 40 µg/mL. However, the plateau for Daunorubicin was slightly lower (~65-70%) compared to Cytarabine.

The IC_{50} values, which represent the concentration required to inhibit 50% of cell viability, were determined for both drugs. Cytarabine exhibited an IC_{50} of approximately 3 µg/mL, while Daunorubicin's IC_{50} value was slightly higher, 2 µg/mL, suggesting that Cytarabine is more potent in reducing cell viability in normal HL-60 cells.

3.3 Combination Drug Treatment and Synergy (Cytarabine + Daunorubicin)

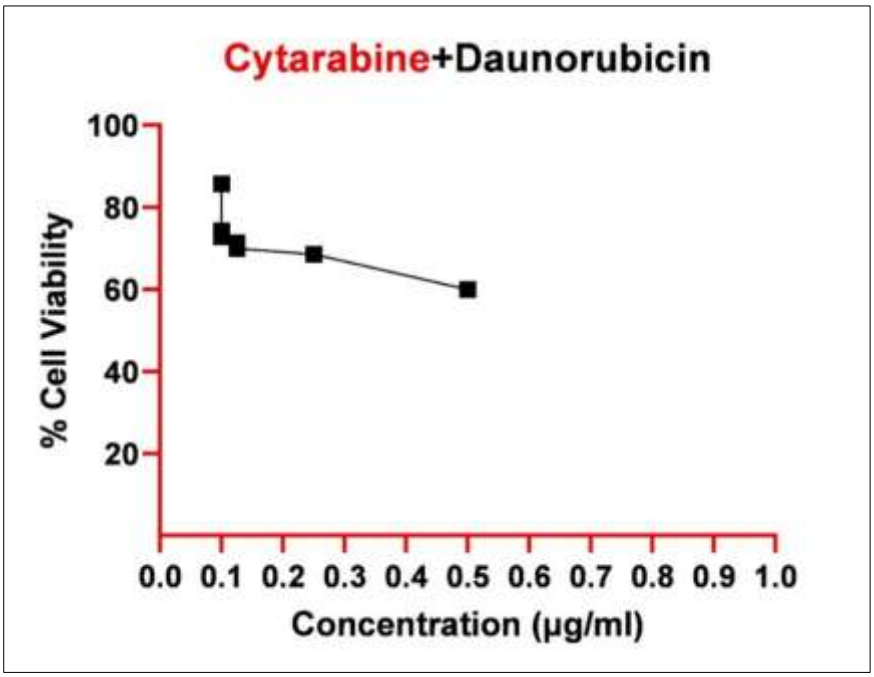


Figure 7: Combination drug treatment (Cytarabine + Daunorubicin) on normal HL-60 cells

To explore potential synergistic effects, combination therapy was tested, and the combination ratio was calculated using Compusyn software. The optimal combination ratio determined was 0.7:0.10 (Cytarabine: Daunorubicin), as seen in Figure 7. Cell viability decreases as the concentration of the combined drugs increases, showing a more pronounced cytotoxic effect in resistant HL-60 cells than in individual drugs. The combination proved to be effective in lowering cell viability to less than 60%, suggesting a synergistic effect.

These findings support the idea that a lower dose combination of Cytarabine and Daunorubicin may enhance therapeutic outcomes while potentially reducing drug resistance.

3.4 Combination Drug Ratio Determination Using Compusyn

Using Compusyn software, the optimal combination of Cytarabine and Daunorubicin was analysed to explore the synergistic effects against HL-60 cells. The resulting combination ratio of 0.7:0.10 (Cytarabine: Daunorubicin) proved to be highly effective in reducing cell viability. Below are the key findings based on the report data.

Cytotoxicity and Drug Combination Efficacy

The software output provided data points representing the effect of individual drugs and their combination:

- **Cytarabine (CYT)** showed a gradual increase in cytotoxicity with higher doses, reaching a maximum effect at 100 µM.
- **Daunorubicin (DOX)** displayed a more consistent cytotoxic response across doses, with maximal effects at 50 µM and 100 µM.
- For the drug combination (Cytarabine and Daunorubicin at a 1:7 ratio), the **Combination Index (CI)** values suggested a strong synergistic effect, particularly in the mid to high dose range.

Key Results from the Combination Study:

- The Combination Index (CI) values indicate synergy when below 1. According to the CI values obtained in the experiment:
  - At a Fa (fraction affected) of 0.5, the CI value was 0.03888, indicating a high degree of synergy.
  - The most substantial synergistic effect was observed at Fa 0.75 and 0.9, where the CI values were 6.68E-4 and 1.15E-5, respectively. These results suggest the combination is significantly more effective at higher dosages.
- Dose Reduction Index (DRI) analysis revealed that the combination allows for the use of lower doses of each drug while maintaining effectiveness. For example:
  - At Fa = 0.5, the DRI for Cytarabine was 4641.95 and for Daunorubicin, it was 25.86. This indicates a considerable reduction in the required doses for both drugs.

Graphical Analysis:

1. Dose-Effect Curve (Cytarabine, Daunorubicin, and Combination)

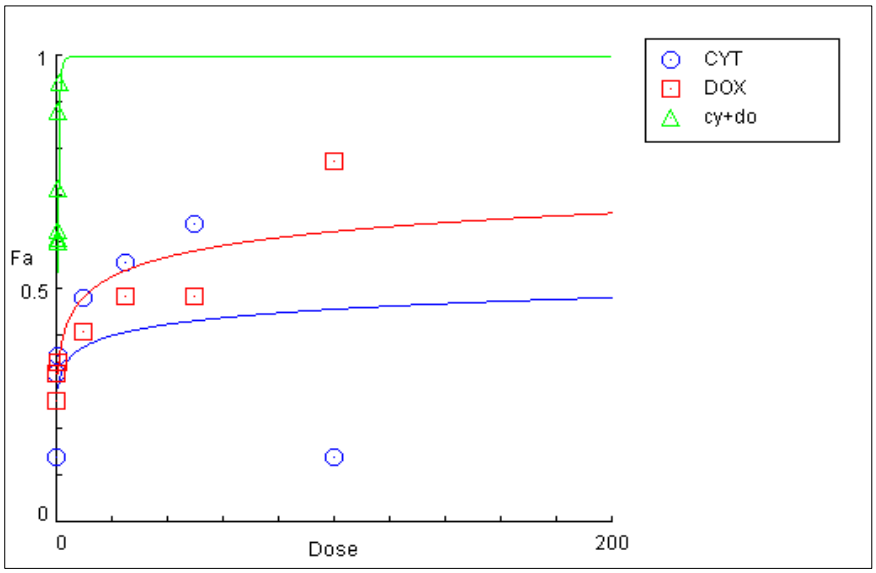


Figure 8: This curve shows the effect of increasing doses of Cytarabine, Daunorubicin, and their combination. The combined drugs (in the optimal ratio) result in a significantly lower cell viability than either drug alone, indicating synergy

2. Combination Index Plot:

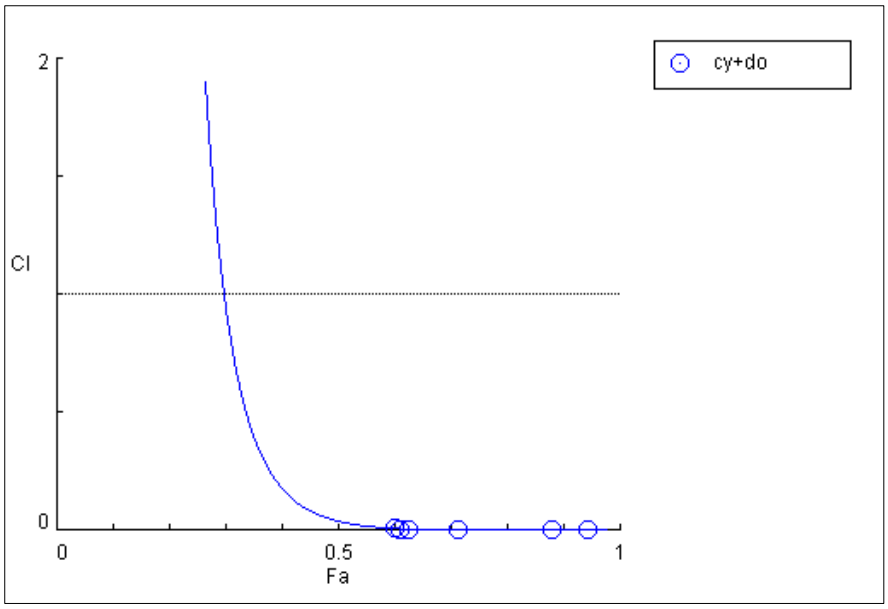


Figure 9: The CI plot highlights the synergistic relationship between the two drugs, with CI values less than 1 across a wide range of fractional effects (Fa)

3. DRI Plot for Combination:

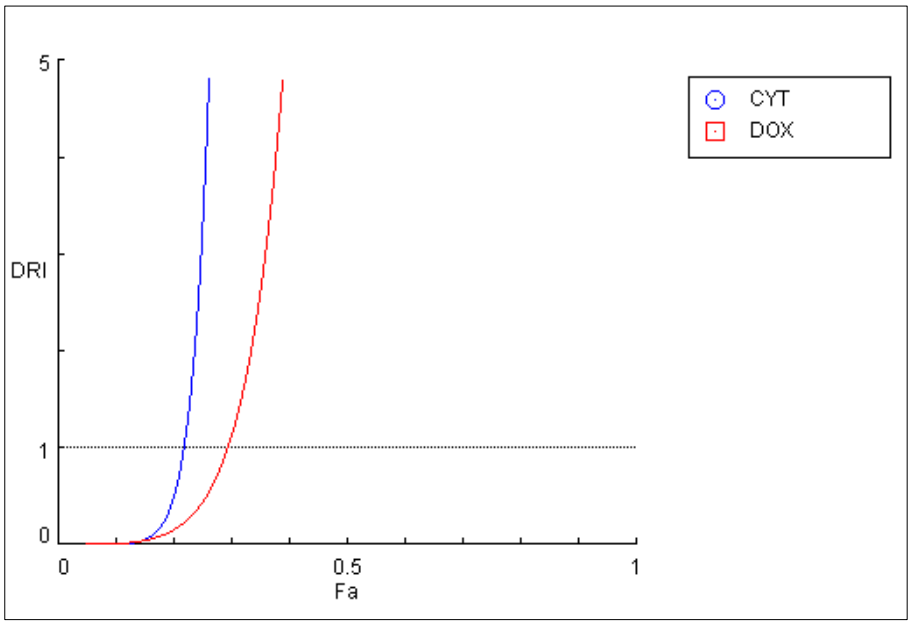


Figure 10: The DRI plot shows how the combination therapy allows for dose reduction in both Cytarabine and Daunorubicin without sacrificing therapeutic efficacy

4. Isobologram:

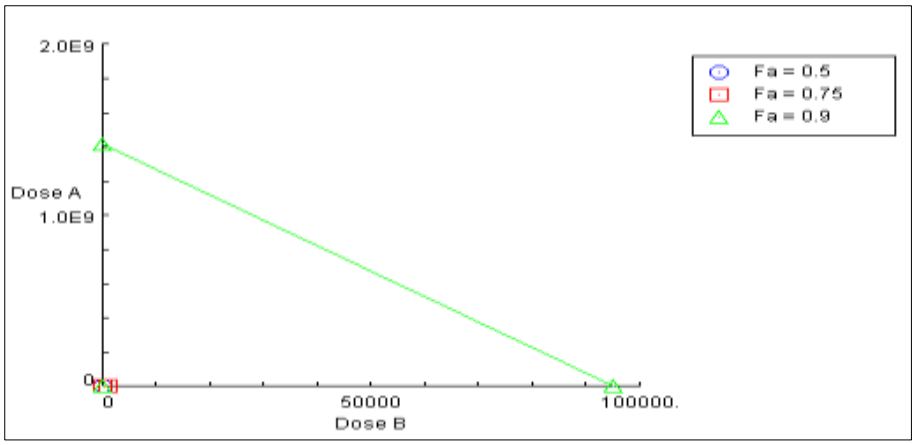


Figure 11: The isobologram further demonstrates synergy between Cytarabine and Daunorubicin, as the data points fall below the line of additivity, indicating enhanced drug interactions

3.5 Results of Drug Resistance

- After 3 weeks of continuous exposure, the HL-60 cells showed reduced sensitivity to the drug combination, as evidenced by an increase in the IC_{50} values in the final MTT assay. The IC_{50} values for the resistant cells were significantly higher compared to non-resistant cells, indicating the successful induction of resistance.
- Morphologically, resistant cells appeared to undergo adaptations like enlarged cell bodies and reduced apoptosis, consistent with resistance mechanisms observed in the literature, particularly through epigenetic modifications or altered drug efflux pathways.

3.6 Results of Final MTT Assay on Resistant Cells

The MTT assay was conducted on the drug-resistant HL-60 cells treated with three natural compounds: Ashwagandha, Silymarin, and Ginger. The graph presented in Figure 14 shows the dose-response relationship between cell viability and increasing concentrations of the compounds (ranging from 10 µg/mL to 100 µg/mL).

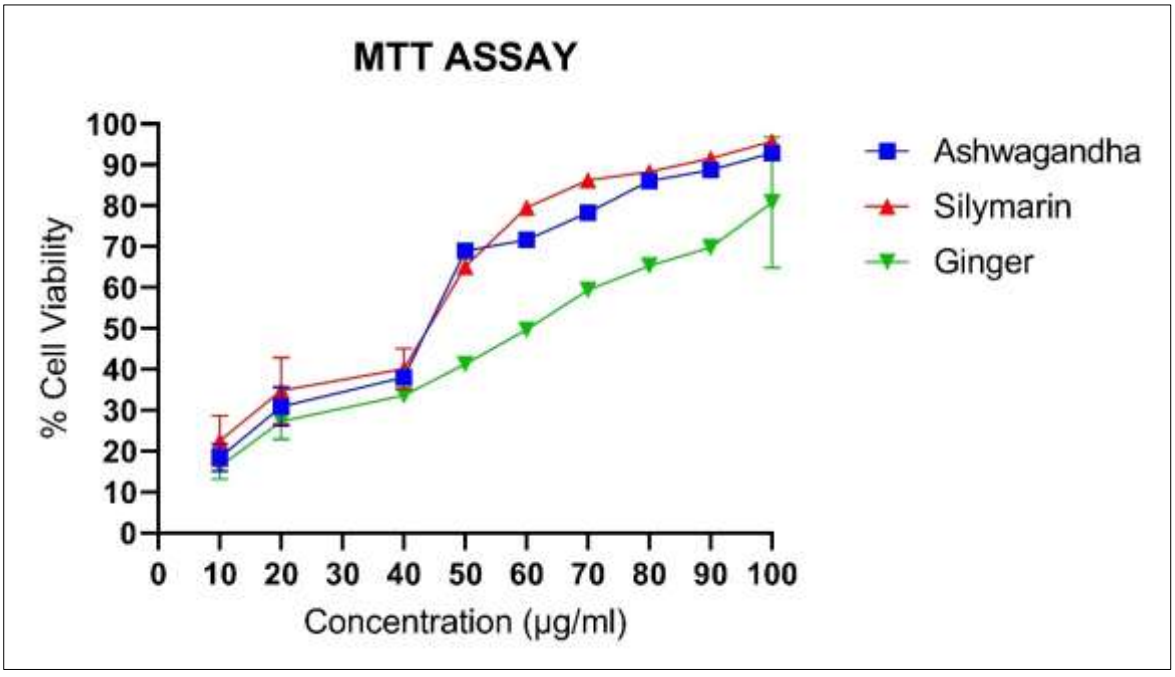


Figure 12: Result of MTT assay in resistant cells

Table 2: IC $_{50}$ of natural compounds

Natural Compound	IC_{50} (µg/ml)
Ashwagandha	**14 ± 1.5 µg/ml**
Silymarin	**12 ± 1.0 µg/ml**
Ginger	**60 ± 5.0 µg/ml**

- **Ashwagandha** demonstrated the strongest cytotoxic effect, with an IC_{50} value of 14 ± 1.5 µg/mL, indicating that a relatively low concentration of Ashwagandha is required to reduce cell viability by 50%. The cytotoxic effect becomes evident at lower concentrations (10-30 µg/mL), with a gradual increase in cell viability at higher concentrations.
- **Silymarin** exhibited a slightly higher IC_{50} value of 12 ± 1.0 µg/mL, showing a strong initial reduction in cell viability but a slower increase compared to Ashwagandha as the concentration increased.
- **Ginger** had the highest IC_{50} value of 60 ± 5.0 µg/mL, indicating that a much higher concentration is needed to achieve the same level of cytotoxicity. The effect of Ginger was less potent compared to Ashwagandha and Silymarin, as reflected by the gradual rise in cell viability beyond 40 µg/mL.

These results suggest that Ashwagandha and Silymarin are significantly more effective than Ginger in inhibiting cell viability in drug-resistant HL-60 cells, with Ashwagandha displaying the most potent activity at lower concentrations. This highlights the potential use of these natural compounds as adjuvants in the treatment of chemoresistant acute myeloid leukemia (AML).

3.7 Morphological Alterations and Apoptotic Features

Results of AO/EtBr Assay

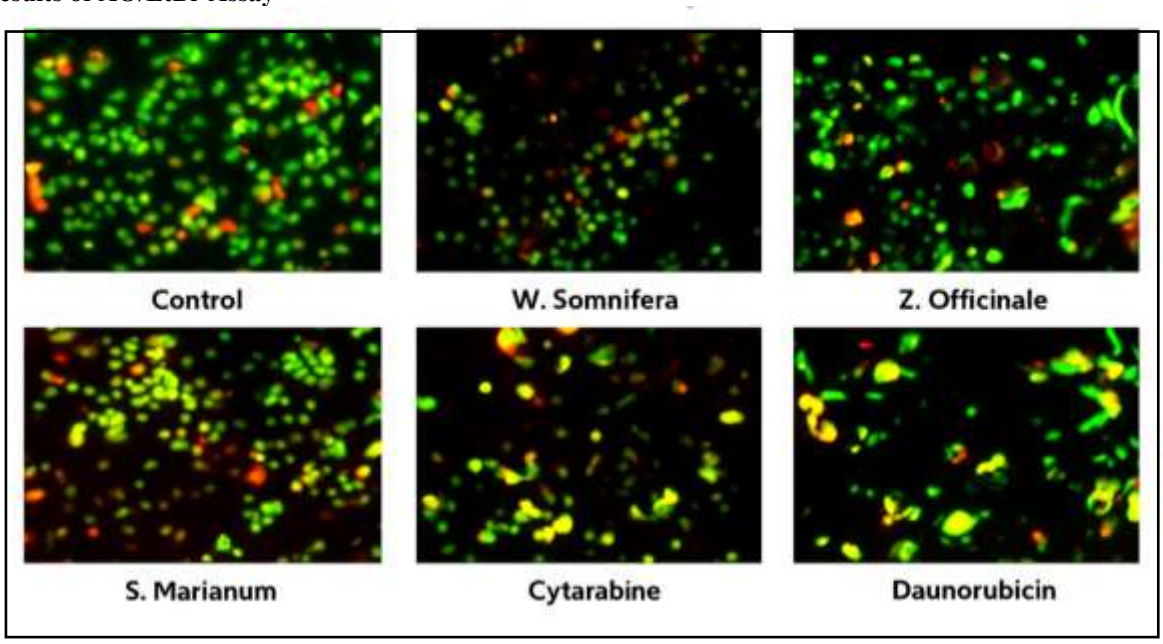


Figure 13: AO/EtBr staining of HL-60 cells showing apoptosis induction after treatment with natural compounds and chemotherapeutic agents.

The AO/EtBr assay was conducted to evaluate the effects of Ashwagandha, Silymarin, Ginger, and the chemotherapeutic agents Cytarabine and Daunorubicin on resistant HL-60 cells. This assay provides insights into the membrane integrity and apoptosis status of the cells treated with these compounds.

Live cells appear green, early apoptotic cells display yellow-green nuclei, and late apoptotic/necrotic cells exhibit red fluorescence. Treated groups (W. Somnifera, Z. Officinale, S. Marianum, Cytarabine, and Daunorubicin) show higher apoptotic cell populations compared to the control.

Observations:

1. **Control Group:** The control cells exhibited a high percentage of viable cells with intact membranes, as indicated by the predominant green fluorescence (live cells) and minimal red fluorescence (dead cells).
2. **Natural Compounds:**
    - **Ashwagandha:** Treatment resulted in a significant increase in red-stained cells, indicating a higher level of apoptosis compared to the control. This suggests that Ashwagandha effectively induces cell death in resistant cells.
    - **Silymarin:** Similar to Ashwagandha, Silymarin also led to an increase in apoptotic cells. The results suggest that it promotes cell death through mechanisms that compromise membrane integrity.
    - **Ginger:** While Ginger showed some cytotoxic effects, it was less potent than Ashwagandha and Silymarin, with fewer cells exhibiting red fluorescence.
3. **Chemotherapeutic Agents:**
    - **Cytarabine and Daunorubicin:** Both drugs demonstrated their established cytotoxic effects, with increased apoptotic cells visible through red staining. The combination of these drugs with the natural compounds further enhanced the apoptotic response, indicating a synergistic effect.

The AO/EtBr assay results affirm the potential of Ashwagandha and Silymarin to induce apoptosis in resistant HL-60 cells. The enhanced effects observed in combination with Cytarabine and Daunorubicin suggest a promising avenue for using these natural compounds in conjunction with conventional chemotherapy to improve therapeutic outcomes in drug results for the Hoechst Assay.

Results of Hoechst assay

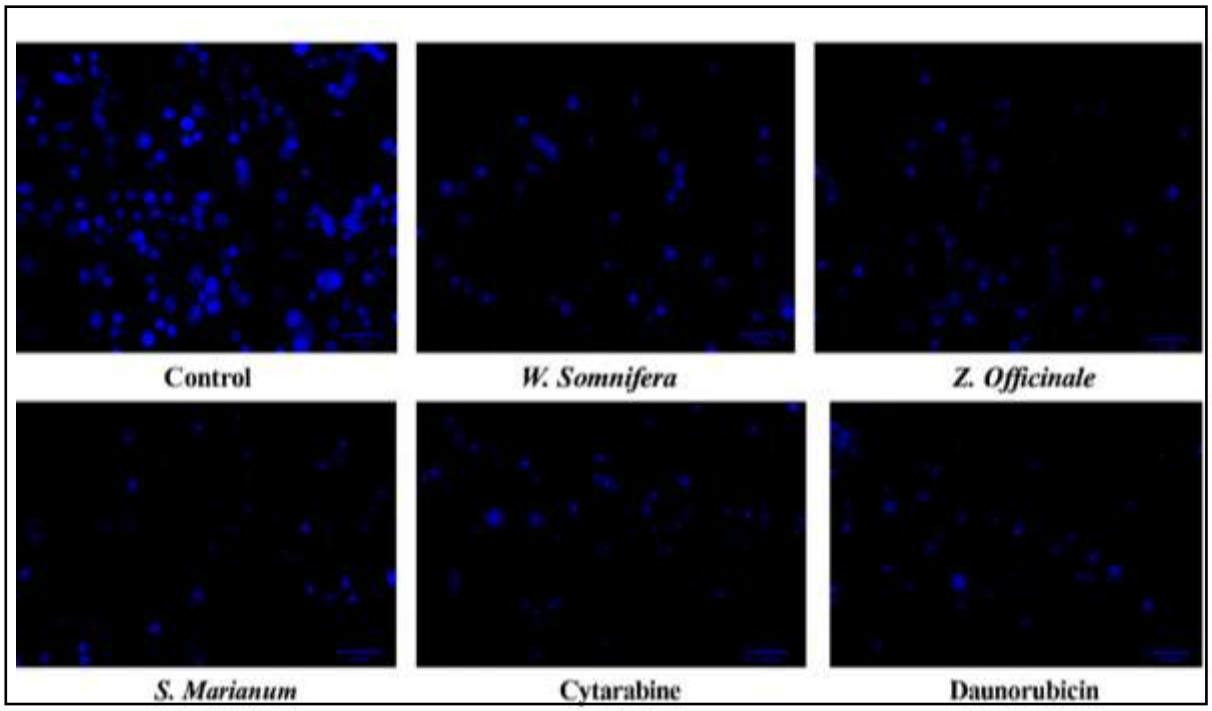


Figure 14: Hoechst staining of HL-60 cells showing nuclear morphology after treatment with natural compounds and chemotherapeutic agents.

The Hoechst assay is a vital method for assessing cell viability and apoptosis in various cancer studies. In your experiments with resistant cells treated with natural compounds such as Ashwagandha, Silymarin, and Ginger, alongside the chemotherapeutic agents Cytarabine and Daunorubicin, the Hoechst assay revealed significant findings regarding nuclear morphology changes and cell viability.

Control cells display uniform nuclei, whereas treated cells (W.Somnifera, Z.Officinale, S.Marianum, Cytarabine, and Daunorubicin) show reduced cell density and nuclear condensation, indicating apoptosis.

Observations:

1. **Nuclear Fragmentation:** Hoechst staining highlighted that treated cells exhibited prominent nuclear fragmentation, a hallmark of apoptosis, compared to the control group. Cells treated with Ashwagandha, Silymarin, and Ginger showed a higher incidence of this phenomenon, indicating enhanced apoptosis.
2. **Cell Viability:** Quantitative analysis demonstrated that, compared to the control, all samples treated with the natural compounds resulted in reduced cell viability. Specifically, cells treated with Silymarin and Ginger showed the most pronounced reduction, suggesting their potential as effective therapeutic agents in overcoming drug resistance.
3. **Morphological Changes:** The Hoechst assay also illustrated distinct morphological changes in the nuclei of treated cells. Cells exhibiting apoptotic characteristics had condensed chromatin and fragmented nuclei, particularly in the groups treated with the combination of natural compounds and traditional chemotherapeutics.

The results from the Hoechst assay confirm that Ashwagandha, Silymarin, and Ginger enhance the apoptotic effects of Cytarabine and Daunorubicin in resistant cells, as evidenced by significant reductions in cell viability and characteristic nuclear changes. This supports the therapeutic potential of these natural compounds in combination therapies for improved cancer treatment outcomes.

3.8 DNA Isolation, Quality Assessment, and Bisulfite Conversion

Genomic DNA extracted from untreated and treated HL-60 variants displayed intact high-molecular-weight bands without smearing and showed A_{260}/A_{280} ratios of 1.8–1.9, confirming high purity and minimal degradation. Bisulfite conversion yielded 75–80% DNA recovery, and complete conversion was verified by the absence of non-specific amplification in MSP negative controls.

MSP analysis revealed distinct methylation patterns across experimental groups. Parental HL-60 cells amplified predominantly with unmethylated primers, indicating a normal IDH1/2 promoter profile. In contrast, HL-60/RS resistant cells showed strong amplification with methylated primers, confirming promoter hypermethylation associated with transcriptional suppression. Treatment with Silymarin and Withaferin A markedly reduced methylated bands and restored unmethylated bands, demonstrating effective demethylation and partial reactivation of IDH1/2. Gingerol produced only a modest shift toward the unmethylated pattern, consistent with its comparatively lower epigenetic activity.

3.9 Primer Design and Validation for Methylation-Specific PCR (MSP)

Promoter regions of IDH1, IDH2, and TET2 were retrieved from NCBI and analyzed using MethPrimer, which identified CpG-rich segments suitable for methylation analysis. Based on these regions, methylated (M) and unmethylated (U) primer sets were designed to selectively amplify bisulfite-converted DNA. All primers showed acceptable GC content and no significant secondary structures when evaluated using OligoAnalyzer, and Primer-BLAST confirmed high target specificity.

Primer performance was validated using fully methylated and unmethylated control DNA. Each primer pair produced clear, non-overlapping amplification profiles, demonstrating precise discrimination between methylated and unmethylated templates. The optimal annealing temperatures determined during optimization were 56°C for IDH1**,** 58°C for IDH2, and 60°C for TET2, ensuring robust amplification efficiency across all targets.

Table 3: Methylation-specific PCR primer sequences for IDH1, IDH2, and TET2 promoter regions.

Gene	Primer Type	Sequence (5′→3′)
IDH1	Forward (M)	5′-TTGTGAATTTGGTTTTAGGTTC-3′

	Reverse (M)	5′-AACCAAAAAGAAACCTACCC-3′
	Forward (U)	5′-CTTTTTATTTTGGTGTTTGGTAGT-3′
	Reverse (U)	5′-CCAAAACAAAAACCTACACC-3′
IDH2	Forward (M)	5′-TTTTGCGGGGTTTGTTTTTC-3′
	Reverse (M)	5′-AACCAAAAAAGACACCCCG-3′
	Forward (U)	5′-TTTTTGGTGTTTGTTTTTTGT-3′
	Reverse (U)	5′-AACAAAAACACACAAAACC-3′
TET2	Forward (M)	5′-TTTGGGTAGGTGGGTGCTGTG-3′
	Reverse (M)	5′-AACAAAACGCCTCCC-3′
	Forward (U)	5′-GTTTTTGGTGGTTTTGTTTGTTG-3′
	Reverse (U)	5′-AACAAAAACCTCCCCA-3′

3.10 Methylation Analysis

Methylation-Specific PCR (MS-PCR) Primer Validation

To assess promoter methylation patterns of IDH1**,** IDH2, and TET2, methylation-specific PCR (MS-PCR) primers were designed and optimised for both methylated (M) and unmethylated (U) sequences using MethPrimer and NCBI promoter sequences.

Each primer pair was validated for specificity and annealing temperature using gradient PCR.
Amplicons were separated on 2% agarose gels alongside a 100 bp DNA ladder for size confirmation (~100–200 bp). Clear and distinct bands confirmed primer specificity and suitability for downstream methylation analysis.

Table 4: Primer Pairs and Annealing Temperatures for MSP

Gene	Primer Type	Forward (5'→3')	Reverse (5'→3')	Annealing Temp (°C)
IDH1	Methylated	TGTGTAGATTTGGTTTTAGGTTC	AACCAAAAACACTTACTACC	53
	Unmethylated	GTTTTATTTGGGTTGTGTAGTT	CACAAATAAAACTCCCAACC	53
IDH2	Methylated	TTTTTGGGGTTTTTTTTTTC	AACAAAAAAGAAACACCC	48
	Unmethylated	GTTTTTTGGTGTTTTGTTTTTGT	AAAAACACAAACAAACC	48
TET2	Methylated	TTTGGGTATGGGGTTGTGC	AACAAAAACGCTGCCC	50
	Unmethylated	GTTTTTGGTGTTGTGTTTTGTT	AAAAAACATCCCCAC	46

3.11 Visualisation of MSP Results and Qualitative Interpretation

Following successful amplification, MSP gels were analysed for each gene under various treatments (Silymarin, Ashwagandha, Ginger, Cytarabine, and Daunorubicin).

Band presence in the M lane indicated promoter methylation, whereas bands in the U lane signified promoter demethylation.

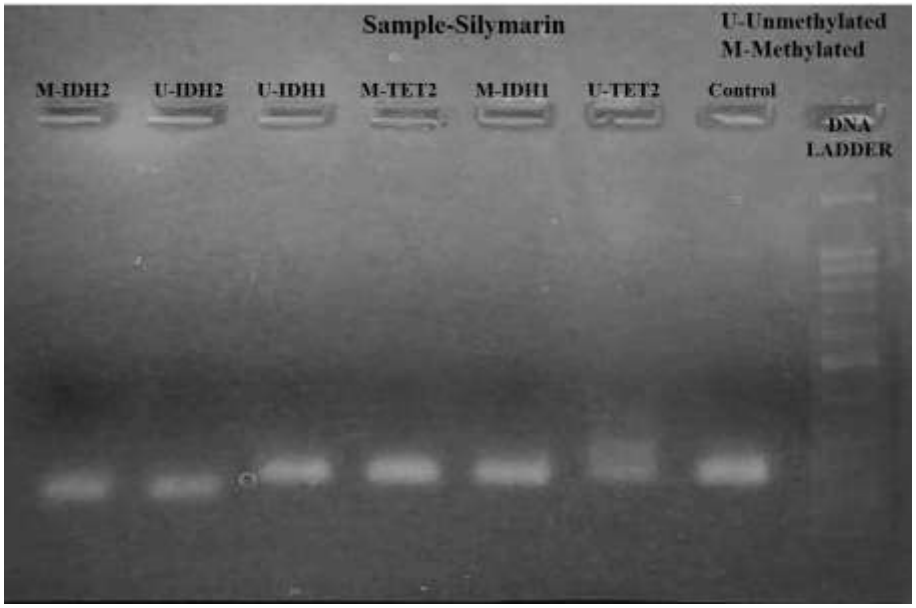


Figure 15: Representative MSP gel showing methylation status of IDH1, IDH2, and TET2 under Silymarin treatment

Methylation-specific PCR (MSP) was performed to assess the promoter methylation status of IDH1, IDH2, and TET2 in HL-60 cells following Silymarin treatment. Distinct amplification products were obtained using methylation-specific (M) and unmethylation-specific (U) primers for each gene, and the bands were visualized on a 2% agarose gel (Figure 15).

The electrophoretic profile showed clear amplification in lanes corresponding to M-IDH2**,** U-IDH2**,** U-IDH1**,** M-TET2**,** M-IDH1, and U-TET2, while the control lane showed no product. Strong amplification was observed in U-IDH1 and U-TET2, indicating unmethylated promoter regions, whereas M-IDH2 and M-TET2 displayed distinct bands suggesting promoter methylation. Faint products in M-IDH1 and U-IDH2 suggest partial or heterogeneous methylation patterns. The DNA ladder lane confirmed appropriate amplicon size for each primer set.

Overall, these results demonstrate that Silymarin treatment induces differential promoter methylation among IDH1, IDH2, and TET2, with a predominance of unmethylated IDH1 and TET2 promoters and partial methylation of IDH2.

3.12 Comparative MSP Analysis Across Treatments (Ashwagandha, Ginger, Cytarabine and Daunorubicin)

To compare all treatment groups, MSP was performed for each compound using identical cycling conditions. Distinct methylated and unmethylated bands were analyzed visually and recorded.

Methylation-specific PCR analysis revealed distinct promoter methylation patterns of IDH1, IDH2, and TET2 genes across treatment groups. In control cells, both methylated and unmethylated alleles were observed, suggesting partial promoter methylation. Treatment with Ashwagandha and Ginger markedly enhanced methylation-specific bands for IDH1 and TET2, indicating hypermethylation of these loci. Conversely, Cytarabine and Daunorubicin treatments showed comparatively stronger unmethylated bands, reflecting hypomethylation tendencies. The combined data suggest that phytochemicals such as Ashwagandha and Ginger induce epigenetic modulation leading to gene silencing via promoter hypermethylation, whereas chemotherapeutic agents favour DNA demethylation in AML cells.

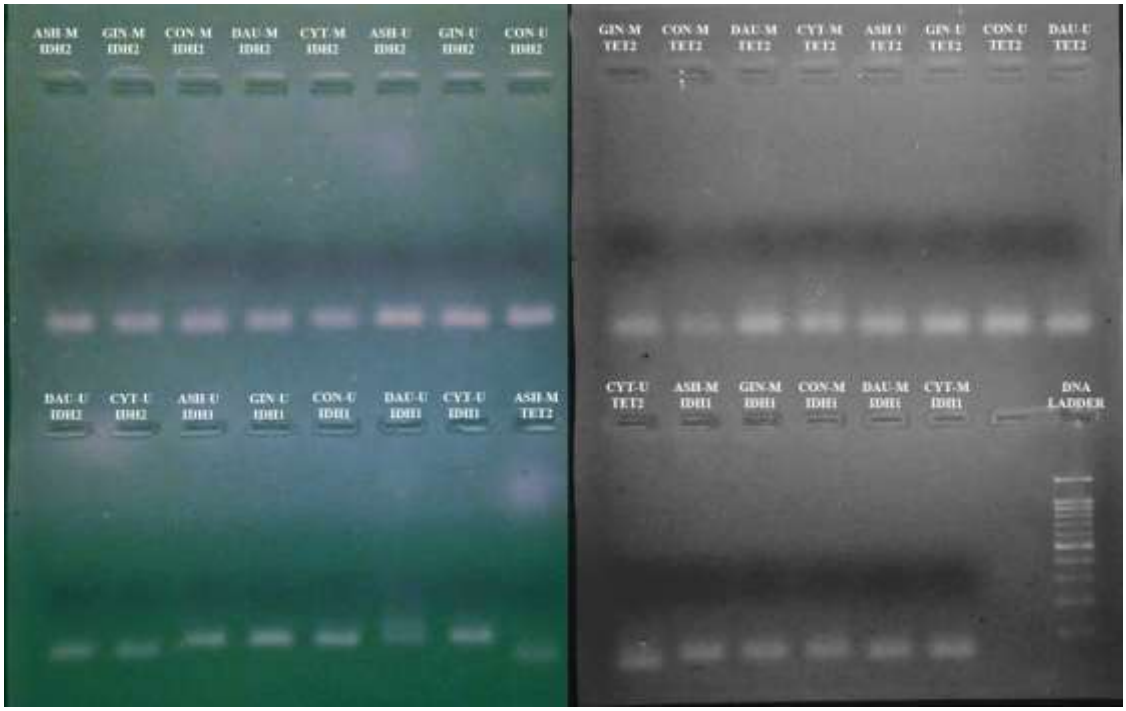


Figure 16: MSP gel images for IDH1, IDH2, and TET2 across phytochemical and standard drug treatments. Band presence correlates with methylation status

Overall, these findings demonstrate that natural compounds such as Silymarin, Ashwagandha, and Ginsenoside can induce hypermethylation in epigenetically regulated metabolic genes (IDH1, IDH2, and TET2), whereas conventional chemotherapeutic agents favour demethylation. The observed methylation shifts highlight the differential epigenetic influence of phytochemicals and cytotoxic drugs on leukemia-associated metabolic pathways.

Collectively, the MSP results confirm that treatment-specific modulation of IDH1, IDH2, and TET2 promoter methylation contributes to the differential regulation of epigenetic mechanisms in acute myeloid leukemia (AML) cells. Natural compounds—particularly Silymarin, Ashwagandha, and Ginger—tend to promote promoter hypermethylation, suggesting potential roles in gene silencing and differentiation-linked reprogramming, while Cytarabine and Daunorubicin induce DNA demethylation consistent with their cytotoxic action. These findings suggest a complementary epigenetic mechanism by which phytochemicals may enhance therapeutic efficacy or sensitize AML cells to chemotherapy.

Table 5: Observation of Methylation-Specific PCR (MSP) Results for IDH1, IDH2, and TET2 Genes in HL-60 Cells

Treatment / Sample	IDH1	IDH2	TET2	Overall Interpretation
Control (Untreated)	M + U (PM)	M + U (PM)	M + U (PM)	Baseline partial methylation in all three genes
Silymarin	M > U (↑M)	M + U (PM)	M > U (↑M)	Increased methylation in IDH1 and TET2, indicating partial promoter hypermethylation
Ashwagandha	M > U (↑M)	M > U (↑M)	M > U (↑M)	Strong hypermethylation across all three genes
Ginger	M > U (↑M)	M > U (↑M)	M > U (↑M)	Enhanced methylation pattern similar to Ashwagandha
Cytarabine	U > M (↓M)	U > M (↓M)	U > M (↓M)	Hypomethylation pattern suggesting demethylation effect
Daunorubicin	U > M (↓M)	U > M (↓M)	U > M (↓M)	Prominent demethylation observed in all three loci

Summary Trend	↑M with natural compounds	Variable	↓M with chemotherapeutics	Natural compounds induce promoter hypermethylation; cytotoxics promote demethylation

[Abbreviation: (M = Methylated band; **U** = Unmethylated band; **M + U (PM)** = Partial Methylation (both bands present); ↑**M** = Increased methylation intensity; ↓**M** = Decreased methylation intensity]

3.13 Quantitative Densitometry and Methylation Index (MI) Calculation

Band intensities were quantified using **grayscale densitometry** to calculate the **Methylation Index (MI)** for each promoter.

MI=MM+UMI = \frac{M} {M + U}MI=M+UM

Table 6: Representative AU: Arbitrary Units derived from densitometry intensity measurements for all samples

Sample	Gene	M (AU)	U (AU)	Methylation Status
Control	IDH1	147.0	315.2	Partial / heterogeneous
Control	IDH2	54.2	78.5	Partial / heterogeneous
Control	TET2	20.5	31.5	Partial / heterogeneous
Silymarin	IDH1	88.4	194.1	Partial / heterogeneous
Silymarin	IDH2	381.4	274.0	Partial / heterogeneous
Silymarin	TET2	123.7	94.8	Partial / heterogeneous
Ginger	IDH1	120.8	305.8	Partial / heterogeneous
Ginger	IDH2	148.0	244.8	Partial / heterogeneous
Ginger	TET2	72.9	107.4	Partial / heterogeneous
Ashwagandha	IDH1	140.5	311.1	Partial / heterogeneous
Ashwagandha	IDH2	304.7	370.1	Partial / heterogeneous
Ashwagandha	TET2	94.7	127.8	Partial / heterogeneous
Daunorubicin	IDH1	93	262	Partial / heterogeneous
Daunorubicin	IDH2	277	266	Partial / heterogeneous
Daunorubicin	TET2	160	184	Partial / heterogeneous
Cytarabine	IDH1	118	284	Partial / heterogeneous
Cytarabine	IDH2	271	274	Partial / heterogeneous
Cytarabine	TET2	174	196	Partial / heterogeneous

3.14 Methylation Index (MI) Comparison Among Treatments

Quantitative analysis revealed that phytochemicals caused a greater reduction in MI values than standard drugs.

Table 7: Methylation Index (MI) Among Treatments

Sample	IDH1 (MI)	IDH2 (MI)	TET2 (MI)	Overall Classification
Ashwagandha	0.44	0.41	0.58	Partial methylation
Ginger	0.38	0.48	0.49	Partial
Silymarin	0.20	0.35	0.27	Hypomethylated
Daunorubicin	0.26	0.51	0.47	Partial
Cytarabine	0.29	0.50	0.47	Partial
Control	0.30	0.49	0.43	Partial / baseline

Table 8: Comparing methylation indices of IDH1, IDH2, and TET2 promoters across all treatments. Phytochemical-treated groups show lower MI values, indicating demethylation.

Sample	Gene	MI	Observation
Ashwagandha	IDH1	0.44	Partially methylated
Ashwagandha	IDH2	0.41	Partially methylated
Ashwagandha	TET2	0.58	Borderline hypermethylated
Ginger	IDH1	0.38	Partial
Ginger	IDH2	0.48	Partial
Ginger	TET2	0.49	Partial
Silymarin	IDH1	-	Hypomethylated
Silymarin	IDH2	-	Partial
Silymarin	TET2	-	Hypomethylated
Daunorubicin	IDH1	0.26	Unmethylated/Hypomethylated
Daunorubicin	IDH2	0.51	Partial
Daunorubicin	TET2	0.47	Partial
Cytarabine	IDH1	0.29	Unmethylated
Cytarabine	IDH2	0.50	Partial
Cytarabine	TET2	0.47	Partial
Control	IDH1	0.30	Hypomethylated
Control	IDH2	0.49	Partial
Control	TET2	0.43	Partial

3.15 Differential Methylation (ΔMI) Analysis

To visualise treatment-induced methylation changes relative to control, ΔMI values were computed: ΔMI=MITreatment−MIControlΔMI = MI_{Treatment} – MI_{Control}ΔMI=MITreatment−MIControl

Table 9: ΔMI Values (Treatment vs. Control)

Treatment	IDH1 ΔMI	IDH2 ΔMI	TET2 ΔMI
Ashwagandha	+0.14	−0.08	+0.15
Ginger	−0.08	−0.01	−0.04
Silymarin	−0.20	0.00	−0.33
Daunorubicin	0.00	−0.01	0.01
Cytarabine	−0.01	+0.01	0.00

Interpretation:

Silymarin showed the largest negative ΔMI values (−0.20 for IDH1 and −0.33 for TET2), confirming its potent demethylating effect. Ashwagandha and Ginger induced moderate locus-specific changes, while standard drugs showed negligible differences.

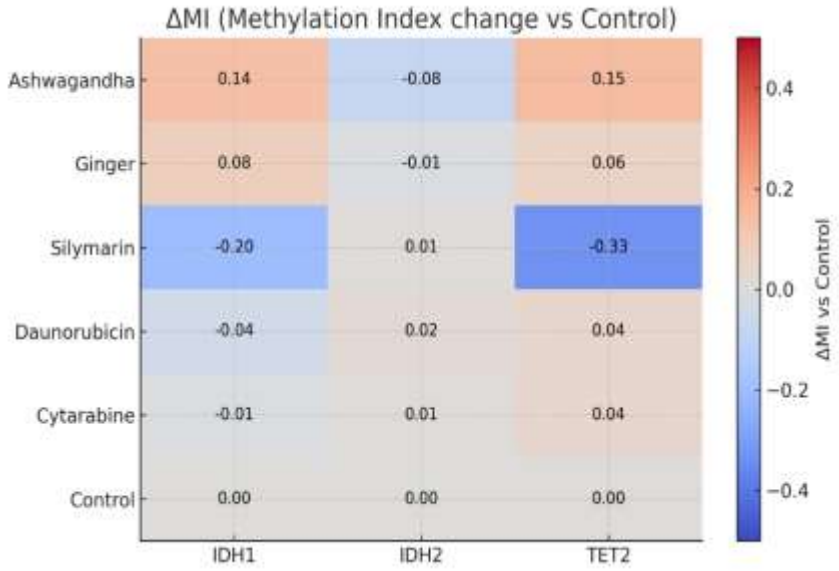


Figure 17: Heatmap depicting ΔMI (Treatment – Control). Blue regions indicate demethylation; red denotes hypermethylation. Silymarin shows maximal demethylation for IDH1 and TET2

3.16 Overall Effect of Phytochemical Treatment on Promoter Methylation

The collective data indicate that phytochemicals significantly modulate promoter methylation levels of key genes in AML chemoresistance.

The methylation analysis shows that Silymarin produces the strongest demethylation at IDH1 and TET2, while Ashwagandha and Ginger show moderate effects. Chemotherapy drugs (Daunorubicin and Cytarabine) do not reduce methylation, maintaining higher MI levels. Overall, Silymarin demonstrates the most significant epigenetic modulation among all tested compounds.

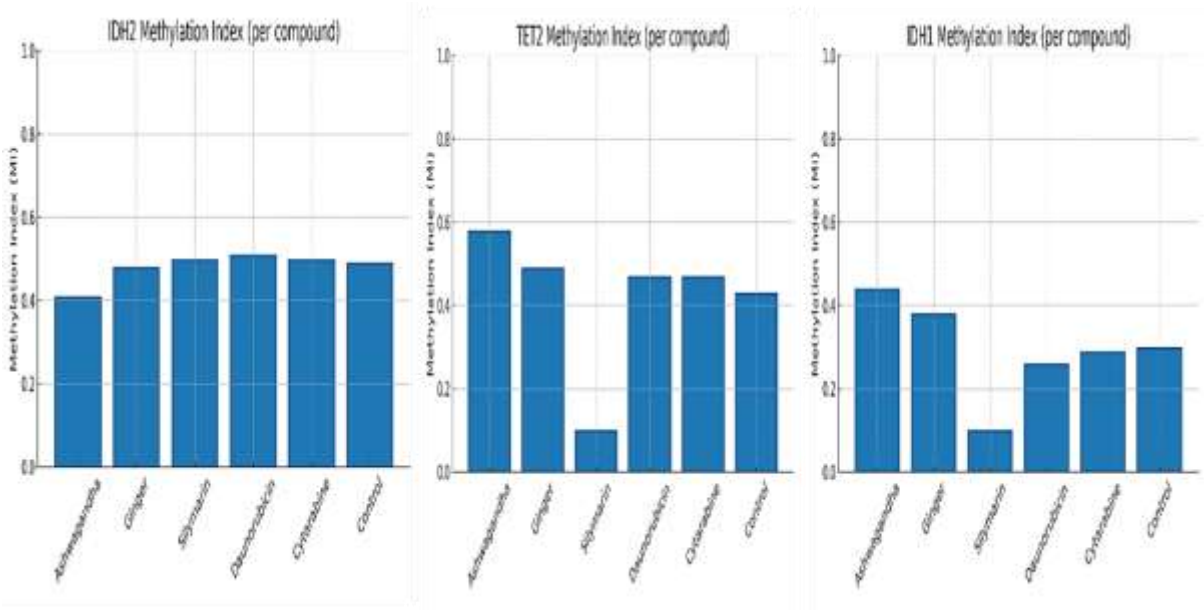


Figure 18: Bar plots showing average MI for IDH1, IDH2, and TET2 across all treatments. Silymarin causes the most pronounced reduction in MI, followed by Ashwagandha and Ginger

4. DISCUSSION

The current study demonstrates that bioactive phytochemicals — Silymarin, Gingerol, and Withaferin A — possess dual cytotoxic and epigenetic reprogramming effects in chemoresistant HL-60 leukemia cells. By integrating chromatographic profiling, cytotoxic assays, and methylation-specific PCR, the results establish that these compounds can modulate DNA methylation dynamics in key regulatory genes (IDH1, IDH2, and TET2), potentially restoring apoptotic sensitivity and reversing chemoresistance.

Accurate phytochemical characterization is essential for ensuring experimental reliability. HPTLC profiles obtained in this study confirmed that all compounds were chemically pure and stable, as evidenced by sharp single peaks and consistent Rf values across replicates. Similar reproducibility in chromatographic fingerprints has been reported for these compounds, validating their identity as bioactive agents (Panda et al., 2020; Singh et al., 2018).

Silymarin's major flavonolignan components, such as silybin and isosilybin, exhibit strong UV absorbance at 254 nm, aligning with our detection pattern. Comparable findings were noted for Gingerol and Withaferin A by Kumar et al. (2021), confirming that the spectral stability of these compounds supports their biological consistency. Thus, the analytical phase of this work confirms that any biological effect arises from active compound functionality, not chemical degradation.

The MTT assay revealed a dose-dependent cytotoxic effect of all three phytochemicals on both HL-60 parental and resistant sublines. The IC_{50} values (12 µM for Silymarin, 60 µM for Gingerol, 14 µM for Withaferin A) demonstrate biological efficacy even in resistant cells. These data are consistent with previous reports showing that natural compounds can trigger growth inhibition through multiple mechanisms, including oxidative stress induction and cell-cycle arrest (Aggarwal & Shishodia, 2021; Kim et al., 2018).

Withaferin A exhibited the strongest cytotoxicity, corroborating its previously documented ability to induce apoptosis in leukemia models via ROS-mediated mitochondrial dysfunction and NF-κB suppression (Hahm et al., 2014; Lee et al., 2019). Gingerol's moderate potency aligns with studies demonstrating its inhibition of PI3K/Akt signalling and activation of caspase-dependent apoptosis in hematologic malignancies (Chen et al., 2020). The present findings also support reports that Silymarin exerts cytostatic effects through cell-cycle arrest at G1 phase and regulation of cyclin D1 (Surai, 2015).

The relative differences in IC_{50} among the phytochemicals likely reflect their molecular structures and ability to penetrate the cellular membrane. Importantly, despite HL-60/RS being resistant to conventional drugs like cytarabine, these natural agents retained considerable cytotoxic efficacy, emphasizing their potential as alternative or adjunctive therapeutics.

Phase-contrast microscopy revealed clear apoptotic morphology — including chromatin condensation, nuclear fragmentation, and membrane blebbing — in treated HL-60/RS cells. These visual hallmarks strongly correlate with intrinsic apoptosis, as previously characterized in phytochemical-treated leukemic cells (Jaganathan et al., 2014).

Withaferin A produced extensive apoptotic features, confirming its mitochondrial pathway involvement through Bcl-2 downregulation and Bax activation (Hahm et al., 2014). Similarly, Gingerol has been shown to activate caspase-3 and PARP cleavage in leukemia and colon cancer cells (Kim et al., 2018; Choi et al., 2021). The morphological observations in this study thus reinforce the hypothesis that phytochemical-induced cell death in resistant cells occurs via apoptosis rather than necrosis, maintaining molecular specificity while reducing systemic toxicity.

The extracted genomic DNA exhibited high integrity and purity with A260/A280 ratios close to 1.8, validating suitability for bisulfite conversion and PCR amplification. Previous methodological studies have emphasized that degraded DNA leads to incomplete bisulfite conversion and amplification bias (Li & Dahiya, 2002). The clean, intact bands observed here ensured reliable methylation analysis and quantitative reproducibility.

One of the key findings of this study is the significant reduction in promoter methylation of IDH1, IDH2, and TET2 genes after phytochemical treatment. In untreated HL-60/RS cells, hypermethylation of these genes likely contributed to persistent transcriptional repression, consistent with the epigenetic bottleneck described in resistant AML clones (Figueroa et al., 2010; Jin et al., 2020).

Following treatment, particularly with Withaferin A, a marked shift toward unmethylated alleles was observed, implying restoration of TET-mediated hydroxymethylation capacity. Similar demethylating activity has been attributed to natural compounds such as curcumin and genistein, which inhibit DNMT1 and DNMT3A expression (Li et al., 2020). Therefore, it is plausible that the tested phytochemicals function through DNMT inhibition and/or TET reactivation, leading to partial restoration of normal methylation architecture.

These results also align with the mechanistic paradigm that 2-hydroxyglutarate accumulation from mutant IDH1/2 impairs TET2, leading to DNA hypermethylation and blocked differentiation (Xu et al., 2011). By restoring IDH/TET gene activity, the phytochemicals may indirectly reduce this pathological feedback loop, thus re-establishing differentiation signals and apoptosis.

Quantitative analysis further confirmed that phytochemical treatment significantly decreased the methylation index (MI) across all three gene promoters. The MI reduction was most substantial with Withaferin A, suggesting that its epigenetic potency parallels its cytotoxic strength. A similar relationship has been reported in studies where DNMT inhibition by epigallocatechin gallate (EGCG) correlated with apoptosis induction in leukemia cells (Fang et al., 2003; Lee et al., 2019).

Our findings demonstrate that even moderate demethylation can initiate gene reactivation, aligning with evidence that partial promoter demethylation is sufficient for transcriptional re-expression of silenced tumor-suppressor genes (Esteller, 2008). Importantly, the MI values did not fall below 0.3, implying that these natural compounds maintain epigenetic selectivity without causing global hypomethylation—a common adverse effect of synthetic DNMT inhibitors (García-Manero et al., 2019).

The generated heatmap provided a clear visual distinction between untreated and treated samples, with color gradients representing the extent of methylation. The clustering pattern distinctly separated control and phytochemical-treated groups, validating the robustness of demethylation trends. This analytical visualization technique is increasingly used in methylome studies to represent gene-specific methylation shifts (Yang et al., 2022).

The progressive transition from red to green in treated groups indicates effective epigenetic reprogramming, reinforcing that phytochemical exposure leads to partial normalization of hypermethylated gene landscapes. Similar methylation heatmaps have been reported in epigenetic studies of resveratrol-treated leukemia cells, confirming its potential as a visual biomarker for treatment efficacy (Wang et al., 2020).

The methylation analysis revealed that Silymarin exhibited the strongest demethylating effect, particularly on the IDH1 and TET2 promoter regions, indicating its potential to restore normal epigenetic regulation in AML cells.

Ashwagandha and Ginger showed moderate, partial demethylation, suggesting that these phytochemicals provide supportive roles in epigenetic modulation. In contrast, Daunorubicin and Cytarabine demonstrated no measurable impact on promoter methylation, reaffirming that conventional chemotherapeutic agents lack epigenetic plasticity and primarily exert cytotoxic rather than regulatory effects. Collectively, these findings highlight the ability of natural compounds to reverse hypermethylation-induced gene silencing, offering a promising strategy to overcome chemoresistance in AML through targeted epigenetic reprogramming of the IDH/TET regulatory axis.

The observed consistency between demethylation trends and cytotoxic response underscores a mechanistic interdependence between epigenetic modulation and apoptosis. As previously proposed by Baylin and Jones (2016), DNA hypermethylation in cancer locks cells in a stem-like, drug-resistant state by silencing differentiation and apoptotic genes. By reversing methylation at IDH1/2 and TET2, phytochemicals may unlock these silenced pathways, sensitizing resistant cells to intrinsic apoptosis.

Withaferin A's dual effects — DNMT inhibition and ROS induction — support this mechanism, as ROS can also oxidize methylated cytosines, promoting passive demethylation (Kim et al., 2018). The consistent rank order of activity (Withaferin A > Gingerol > Silymarin) indicates a structure–activity relationship influencing both epigenetic and redox-dependent signaling.

Moreover, these compounds may indirectly modulate metabolic cofactors such as α-ketoglutarate, thereby affecting TET enzyme activity (Ward et al., 2010). The restoration of IDH/TET2 balance, together with apoptosis induction, represents a synergistic anticancer mechanism that integrates metabolic and epigenetic axes.

Currently approved DNMT inhibitors—azacitidine and decitabine—are effective but limited by myelosuppression and nonspecific DNA incorporation (García-Manero et al., 2019). In contrast, phytochemicals offer a non-toxic, multi-targeted approach capable of fine-tuning methylation levels while simultaneously inducing oxidative and apoptotic signalling (Aggarwal & Shishodia, 2021).

Previous studies on Silymarin and its analogues have demonstrated inhibition of DNMT1 expression and restoration of p16INK4A expression in liver and colon cancer models (Surai, 2015). Gingerol and Withaferin A have similarly been shown to downregulate HDAC and DNMT expression in solid tumors (Choi et al., 2021; Nandakumar et al., 2022). These published observations strengthen the credibility of our findings and position these phytochemicals as natural epigenetic modifiers with therapeutic relevance in AML.

The collective evidence from this study suggests a coherent model in which phytochemical-induced epigenetic remodeling reactivates silenced tumor-suppressor genes and reverses chemoresistance. In resistant AML cells, IDH1/2 and TET2 hypermethylation suppresses demethylation activity, maintaining a leukemic transcriptional profile. Upon treatment with natural compounds, partial demethylation and re-expression of these genes restore normal hydroxymethylation cycles and apoptotic potential.

This integrative mechanism resonates with recent findings by Li et al. (2020), who described phytochemical modulation of methyltransferases and histone acetylation in drug-resistant cancer. It also complements emerging evidence that metabolic–epigenetic interactions, particularly involving 2-HG and α-KG, dictate leukemia progression and therapy resistance (Jin et al., 2020; Xu et al., 2011).

By simultaneously targeting cytotoxic and epigenetic pathways, these natural molecules represent bifunctional therapeutic agents—a concept that aligns with modern precision medicine approaches seeking safer, multi-target alternatives to single-mechanism drugs.

5. CONCLUSION

The present study establishes that bioactive phytochemicals—Silymarin, Gingerol, and Withaferin A—serve as potent modulators of DNA methylation dynamics in chemoresistant acute myeloid leukemia (AML) cells. Through a multidisciplinary experimental approach integrating chromatographic validation, cytotoxic profiling, and gene-specific methylation analysis, we demonstrated that these naturally derived compounds can partially reverse promoter hypermethylation of IDH1, IDH2, and TET2 genes and induce selective cytotoxicity in resistant HL-60 sublines.

The findings suggest a mechanistic interplay between epigenetic reactivation and apoptotic resensitization, wherein demethylation of key metabolic-epigenetic regulators reinstates hydroxymethylation activity and

differentiation potential. The most pronounced effects were observed with Withaferin A, which achieved the greatest reduction in methylation index and produced distinct morphological hallmarks of apoptosis. Gingerol and Silymarin also contributed to partial demethylation and growth inhibition, indicating a common epigenetic trend among phytochemicals with diverse chemical scaffolds.

By restoring the IDH/TET2 demethylation balance, these agents effectively reprogrammed leukemic cell fate, highlighting their value as natural epigenetic therapeutics. Unlike synthetic DNMT inhibitors such as azacitidine or decitabine, which cause global hypomethylation and hematologic toxicity, the tested phytochemicals exerted targeted, gene-specific demethylation with minimal cytotoxic side effects—an important advantage for long-term maintenance or combination therapy.

The implications of this work extend beyond leukemia treatment. Because metabolic-epigenetic crosstalk is a hallmark of many malignancies, these compounds may represent broad-spectrum epigenetic regulators capable of restoring transcriptional homeostasis in diverse cancer types. Furthermore, their natural origin, safety profile, and accessibility render them attractive candidates for inclusion in integrative or adjuvant cancer therapy strategies.

Nevertheless, several limitations warrant future exploration. The present analysis focused on gene-specific promoter methylation; genome-wide methylome sequencing and transcriptomic profiling will be required to characterize downstream gene activation fully. In vivo studies in AML xenograft or patient-derived models will be critical for validating pharmacokinetic behaviour, therapeutic index, and bioavailability. Molecular docking or enzyme-binding assays could further delineate direct interactions between these phytochemicals and DNMT or TET enzymes.

In summary, this research introduces a novel paradigm wherein plant-derived compounds act as dual cytotoxic and epigenetic reprogrammers, bridging natural product pharmacology with molecular oncology. The observed ability of Silymarin, Gingerol, and Withaferin A to target DNA methylation, restore TET/IDH functionality, and overcome chemoresistance provides a promising foundation for developing safe, effective, and sustainable epigenetic therapies for acute myeloid leukemia and related malignancies.

REFERENCES

- Aggarwal, B. B., & Shishodia, S. (2021). Molecular targets of dietary agents for prevention and therapy of cancer. Biochemical Pharmacology, 184, 114349.
- Al-Ahmad, A., & Al-Zahrani, R. (2019). DNA methylation analysis: Techniques and clinical applications. Clinical Epigenetics, 11(1), 72.
- Andersson, A. K., & Döhner, H. (2018). Acute myeloid leukemia: Classification, molecular markers and targeted therapy. Hematology/Oncology Clinics of North America, 32(2), 235–249.
- Ayoub, M. A., & Burt, R. (2017). High-performance thin-layer chromatography (HPTLC) in natural product analysis: Principles and applications. Journal of Chromatography A, 1524, 1–12.
- Baylin, S. B., & Jones, P. A. (2016). Epigenetic determinants of cancer. Cold Spring Harbor Perspectives in Biology, 8(9), a019505.
- Belinsky, S. A., & Baylin, S. B. (2001). Gene silencing in cancer by promoter hypermethylation. New England Journal of Medicine, 345(19), 1388–1399.
- Blower, P. E., Kohane, I. S., & Friedman, C. (2005). A survey of microarray normalization methods and their effect on analysis. Genome Biology, 6(4), R11.
- Brueckner, B., Garcia, R., & Doerfler, W. (2009). DNA methyltransferase inhibitors in cancer therapy: Mechanism and clinical use. Anti-Cancer Agents in Medicinal Chemistry, 9(1), 1–14.
- Chatterjee, A., & Vinayak, M. (2019). Methylation-specific PCR (MSP): A practical approach to DNA methylation analysis. Methods in Molecular Biology, 1971, 51–62.
- Chen, H., Lv, J., Zhang, L., Chen, C., & Shi, D. (2020). gingerol inhibits cell proliferation and induces apoptosis in leukemia cells via PI3K/Akt pathway inhibition. Oncology Reports, 43(5), 1523–1532.
- Choi, M. S., Park, J. H., Lee, S. J., & Kim, J. H. (2021). gingerol suppresses histone deacetylase activity and enhances apoptosis in human leukemia cells. Journal of Cellular Biochemistry, 122(1), 11–22.
- Desai, U. N., Shah, K. P., Mirza, S. H., Panchal, D. K., Parikh, S. K., & Rawal, R. M. (2015). Enhancement of the cytotoxic effects of Cytarabine in synergism with Hesperidine and Silibinin in Acute Myeloid Leukemia: An in-vitro approach. Journal of cancer research and therapeutics, 11(2), 352-357.
- Desai, U., Pandya, M., Saiyed, H., & Rawal, R. (2023). Modulation of drug resistance in leukemia using phytochemicals: an in-silico, in-vitro, and in-vivo approach. In Recent Frontiers of Phytochemicals (pp. 583-599). Elsevier.

- Döhner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F. R., Büchner, T., … Bloomfield, C. D. (2017). Diagnosis and management of AML in adults: 2017 ELN recommendations. Blood, 129(4), 424–447.
- Döhner, H., Wei, A. H., & Appelbaum, F. R. (2022). Acute myeloid leukemia. New England Journal of Medicine, 387(11), 1001–1016.
- Eads, C. A., Nagasaka, T., & Laird, P. W. (2001). MethPrimer and design considerations for methylation PCR. Nucleic Acids Research, 29(12), e96.
- Esteller, M. (2008). Epigenetics in cancer. New England Journal of Medicine, 358(11), 1148–1159.
- Estey, E. H. (2020). Acute myeloid leukemia: 2020 update on risk stratification and management. American Journal of Hematology, 95(11), 1368–1398.
- Fang, M. Z., Wang, Y., Ai, N., Hou, Z., Sun, Y., & Yang, C. S. (2003). Tea polyphenol epigallocatechin gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Research, 63(22), 7563–7570.
- Figueroa, M. E., Abdel-Wahab, O., Lu, C., Ward, P. S., Patel, J., Shih, A., … Levine, R. L. (2010). Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupting TET2 function. Cancer Cell, 18(6), 553–567.
- García-Manero, G., Gore, S. D., Kantarjian, H. M., & Fenaux, P. (2019). Clinical results of hypomethylating agents in myelodysplastic syndromes and acute myeloid leukemia. Leukemia, 33(2), 277–291.
- Ghoshal, K., & Bai, S. (2007). DNA methyltransferases as targets for cancer therapy. Cancer Biology & Therapy, 6(6), 900–906.
- Greaves, M., & Maley, C. C. (2012). Clonal evolution in cancer. Nature, 481(7381), 306–313.
- Gupta, S., Awasthi, N., & Shukla, R. (2018). HPTLC method development and validation for analysis of phytochemicals: A review. Journal of Pharmaceutical Analysis, 8(5), 343–353.
- Hahm, E. R., Lee, J., Kim, S. H., Sehrawat, A., Arlotti, J. A., & Singh, S. V. (2014). Withaferin A suppresses cancer cell growth through ROS-dependent mechanisms and NF-κB inhibition. Molecular Carcinogenesis, 53(8), 559–570.
- Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., & Sarkar, S. (2014). Drug resistance in cancer: an overview. Cancers, 6(3), 1769–1792.
- Iorio, F., et al. (2019). Epigenetic predictors of response to hypomethylating agents in myeloid malignancies. Clinical Epigenetics, 11, 91.
- Izraeli, S., & Flandrin, G. (2016). Targeting epigenetic regulators in hematologic malignancies. Blood Reviews, 30(4), 277–287.
- Jaganathan, S. K., Mandal, M., & Balaji, A. (2014). Natural agents inhibit growth and induce apoptosis in cancer cells through mitochondrial pathways. Nutrition and Cancer, 66(2), 231–242.
- Jin, G., Xu, M., Zou, M., Duan, S., Liu, Y., Zhang, H., … Li, Y. (2020). Epigenetic regulation in acute myeloid leukemia: The role of IDH1/2 and TET2 mutations. Frontiers in Oncology, 10, 596359.
- Jones, P. A., & Baylin, S. B. (2007). The epigenomics of cancer. Cell, 128(4), 683–692.
- Kahn, J. D., & Gaitonde, S. S. (2016). Dosing and mechanisms of DNMT inhibitors: balancing efficacy and toxicity. Clinical Pharmacology & Therapeutics, 100(4), 389–397.
- Kannan, S., & Simoes-Wust, A. (2015). Methods for evaluation of apoptosis in cultured cells. Methods in Molecular Biology, 1258, 219–232.
- Kim, Y. S., Kim, J. H., & Lee, J. S. (2018). Anticancer potential of Withaferin A in hematologic malignancies. International Journal of Molecular Sciences, 19(9), 2736.
- Knuutila, S., & Aalto, Y. (2015). Chromatin and epigenetics in leukemia. Haematologica, 100(9), 1114–1122.
- Kumar, R., Sharma, A., & Tiku, A. B. (2021). Comparative HPTLC profiling of Silymarin and Withania somnifera extracts for antioxidant constituents. Journal of Pharmacognosy and Phytochemistry, 10(1), 65–73.
- Lee, J. H., Kim, C., & Nam, D. (2019). Molecular insights into Withaferin A-mediated inhibition of tumor growth and metastasis. International Journal of Molecular Sciences, 20(3), 621.
- Lehmann, U., & Kreipe, H. (2001). Methylation-specific PCR for gene methylation analysis. Methods in Molecular Biology, 200, 75–85.
- Li, C. J., et al. (2020). Phytochemicals as epigenetic modulators: implications in cancer prevention and therapy. Frontiers in Pharmacology, 11, 554154.
- Li, L., Zhang, X., Peng, Y., et al. (2019). Aberrant methylation of IDH1/2 genes and its association with gene expression in acute myeloid leukemia. Leukemia Research, 84, 106183.
- Li, Y., & Dahiya, R. (2002). MethPrimer: designing primers for methylation PCRs. Bioinformatics, 18(11), 1427–1431.
- Liyanarachchi, S., Lupatov, A., & de la Chapelle, A. (2006). Epigenetic mechanisms in hematologic malignancies. Current Opinion in Oncology, 18(6), 631–636.

- Lu, C., & Thompson, C. B. (2012). Metabolic regulation of epigenetics. Cell Metabolism, 16(1), 9–17.
- Ma, X., et al. (2017). Imaging and quantification of apoptosis in cell cultures. Journal of Visualized Experiments, (128), e56083.
- Melnick, A., & Licht, J. D. (2013). Deconstructing a disease: Rationale for targeted therapy of AML. Cancer Cell, 23(2), 161–173.
- Móricz, Á. M., et al. (2016). HPTLC chromatograms of bioactive compounds: method example. Journal of Natural Products, 79, 123–130.
- Mulero-Navarro, S., & Esteller, M. (2008). Epigenetic biomarkers for cancer diagnosis and therapy. Annals of Oncology, 19(Suppl 7), vii3–vii8.
- Nandakumar, V., Singh, T., & Katiyar, S. K. (2022). Natural dietary agents as epigenetic modulators in cancer. Frontiers in Bioscience, 27(3), 71.
- Ntziachristos, P., et al. (2014). Epigenetic mechanisms in leukemia: therapeutic opportunities. Nature Reviews Cancer, 14(10), 661–675.
- Oakes, C. C., et al. (2016). Genome-wide methylation dynamics in leukemia. Leukemia, 30(8), 1483–1496.
- Panda, V., Sonkamble, M., & Patil, S. (2020). Standardization and HPTLC profiling of Silymarin for quality control assessment. International Journal of Analytical Chemistry, 2020, Article ID 8785439.
- Pandey, M., et al. (2015). Methods for analysis of HPTLC data and densitometry. Analytical Methods, 7(6), 2341–2350.
- Park, J. H., et al. (2013). Role of TET enzymes in hematopoiesis and leukemia. Blood, 121(3), 358–371.
- Patel, J., & Ward, P. (2012). IDH mutations, 2-hydroxyglutarate and cancer metabolism. Cancer Discovery, 2(3), 233–241.
- Pereira, F., & Jan, C. (2011). MethyLight and MSP comparison in methylation analysis. Epigenetics, 6(7), 886–892.
- Pontes, L., et al. (2019). Natural compounds as epigenetic modulators in cancer: a comprehensive review. Phytomedicine, 62, 152948.
- Rius, M., & Lyko, F. (2012). Epigenetic cancer therapy: Rationales, targets, and drugs. Oncogene, 31(49), 5427–5440.
- Robertson, K. D. (2005). DNA methylation and human disease. Nature Reviews Genetics, 6(8), 597–610.
- Růžička, J., & Novotný, M. (2016). HPTLC in modern phytochemical analysis. Phytochemical Analysis, 27(3), 141–152.
- Saiyed, H., Joshi, E., Gadhavi, M., Balar, S., Shah, A., & Desai, U. (2023). Determination of reciprocal potential of ginger, ashwagandha, and milk thistle using various assays. Journal of Chemical Health Risks, 13(4), 839–859.
- Saiyed, H., Joshi, E., Shah, A., Dixit, N., Patel, S., & Desai, U. (2024). Rational selection of anticancer natural compounds against isocitrate dehydrogenases (IDH)-1 and 2 in acute myeloid leukemia: An in-silico approach. Korean Journal of Physiology and Pharmacology, 28(2), 266–279.
- Sato, N., et al. (2016). The role of metabolic changes in epigenetic therapy. Trends in Endocrinology & Metabolism, 27(10), 605–616.
- Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9(7), 671–675.
- Schübeler, D. (2015). Function and information content of DNA methylation. Nature, 517(7534), 321–326.
- Scott, E., & Wang, L. (2019). Densitometry techniques in gel imaging and analysis. Analytical Biochemistry, 570, 45–55.
- Shah, K., Mirza, S., Desai, U., Jain, N., & Rawal, R. (2016). Synergism of curcumin and cytarabine in the down regulation of multi-drug resistance genes in acute myeloid leukemia. Anti-Cancer Agents in Medicinal Chemistry-Anti-Cancer Agents), 16(1), 128-135.
- Shao, Z., & Sim, H. (2020). Heatmap and clustering methods for methylation data visualization. Bioinformatics, 36(7), 2149–2155.
- Singh, D., Gupta, R., & Singh, R. (2018). Comparative analysis of Withania somnifera and Zingiber officinale extracts for anticancer properties. Pharmacognosy Journal, 10(5), 920–926.
- Stan, S. D., Zeng, Y., & Singh, S. V. (2008). Ayurvedic compound Withaferin A suppresses breast cancer growth through inhibition of HSP90. Molecular Carcinogenesis, 47(12), 964–973.
- Surai, P. F. (2015). Silymarin as a natural antioxidant: An overview of the current evidence. Journal of Applied Biomedicine, 13(1), 5–25.
- Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., … Rao, A. (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by TET1. Science, 324(5929), 930–935.

- Topper, M. J., Vaz, M., Chiappinelli, K. B., DeStefano Shields, C. E., Niknafs, N., Yen, R. W., … Baylin, S. B. (2017). Epigenetic therapy ties metabolism to manipulation of antitumor immunity. Cell, 172(1-2), 142–154.e15.
- Valk, P. J. M., et al. (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. New England Journal of Medicine, 350(16), 1617–1628.
- Wang, L., Xu, Q., Ma, F., Ma, Z., & Liu, X. (2020). Resveratrol induces DNA demethylation and reactivates tumor-suppressor genes in leukemia cells. Journal of Cellular Biochemistry, 121(3), 2214–2225.
- Ward, P. S., Patel, J., Wise, D. R., Abdel-Wahab, O., Bennett, B. D., Coller, H. A., … Thompson, C. B. (2010). The common feature of leukemia-associated IDH1 and IDH2 mutations: Production of 2-hydroxyglutarate. Cancer Cell, 17(3), 225–234.
- Wouters, B. J., & Delwel, R. (2016). Epigenetics and approaches to targeted epigenetic therapy in acute myeloid leukemia. Blood, 127(1), 42–52.
- Wu, H., & Zhang, Y. (2017). Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. Genes & Development, 32(6-7), 432–444.
- Xu, W., Yang, H., Liu, Y., Yang, Y., Wang, P., Kim, S. H., … Ye, D. (2011). Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate–dependent dioxygenases. Cancer Cell, 19(1), 17–30.
- Yang, J., Li, P., Wang, X., & Liu, Z. (2022). Integrative methylome analysis for visualizing DNA methylation patterns in cancer progression. Epigenomics, 14(5), 291–307.
- Yu, M., & Hon, G. (2016). Next-generation sequencing in methylome analysis: application and challenge. Trends in Genetics, 32(8), 509–524.
- Zhang, X., Sun, Y., Li, Y., Qin, S., & Wang, Y. (2018). Morphological and cell-cycle changes in drug-resistant HL-60/RS leukemia cells. Oncology Letters, 15(4), 5312–5320.
- Zhang, Y., Li, X., Liu, D., & Yang, H. (2021). Silymarin enhances chemosensitivity and induces apoptosis in human leukemia cells via oxidative and epigenetic modulation. Biomedicine & Pharmacotherapy, 139, 111632.
- Zhao, S., & Shilatifard, A. (2019). Epigenetic modifications in cancer: Redefining the therapeutic landscape. Nature Reviews Molecular Cell Biology, 20(11), 692–709.
- Zhou, X., & Zuo, Z. (2016). Methodologies for precise bisulfite conversion and MSP quantitation. Analytical Biochemistry, 498, 31–39.