

ORA- Experimental Research

In Vitro Evaluation of *Amritabhallataka Paka* on Cell Viability and Apoptosis in U87MG Glioblastoma Cells and Cytocompatibility in L929 Fibroblasts

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ABSTRACT:

Background: Glioblastoma is an aggressive primary brain tumour with poor prognosis due to recurrence and treatment resistance. *Amritabhallataka Paka* (AP), an Ayurvedic formulation containing *Semecarpus anacardium* L.f. (*Bhallataka*), has limited experimental evidence in glioblastoma models. **Objective:** To evaluate the effects of AP on U87MG glioblastoma cell viability and apoptosis, assess its cytocompatibility in L929 mouse fibroblasts, and compare its effects with temozolomide (TMZ) and their combination. **Methods:** U87MG cells were treated with AP, TMZ and AP+TMZ, while L929 fibroblasts received AP and TMZ. Cell viability was assessed using the MTT assay at six concentrations in three independent experiments with technical replicates. IC₅₀ values were estimated using nonlinear dose-response regression. Apoptosis in U87MG cells was evaluated using Annexin V-FITC/propidium iodide flow cytometry in three independent experiments. Cellular morphology was examined using phase-contrast microscopy. **Results:** AP produced a concentration-dependent reduction in U87MG cell viability. The IC₅₀ values were 334.85 µg/mL for AP, 249.42 µg/mL for TMZ and 327.20 µg/mL for AP+TMZ. In L929 fibroblasts, AP maintained 71–87% viability across tested concentrations. TMZ-treated L929 cells showed 99.5% viability at 15.6 µg/mL, with reduced viability at concentrations ≥31.25 µg/mL. Total apoptosis in U87MG cells was highest with AP+TMZ (25.15 ± 1.80%), followed by TMZ (14.71 ± 1.20%), AP (11.85 ± 1.75%) and untreated controls (3.26 ± 0.50%). **Conclusion:** AP reduced U87MG cell viability while maintaining comparatively high L929 fibroblast viability under the tested conditions. AP+TMZ produced higher total apoptosis than either treatment alone. However, synergistic or enhanced combination efficacy cannot be established without formal drug-interaction analysis.

KEYWORDS: *Amritabhallataka Paka*, Apoptosis, Glioblastoma, In Vitro Techniques, L929, MTT Assay, *Semecarpus anacardium* L.f., U87MG.

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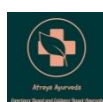
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1. INTRODUCTION

As per the estimation of Global cancer observatory (GLOBOCAN) there were 19.3 million new cancer cases for the year 2020 worldwide where India ranked third after China and USA. In 2040 the cancer cases in India are estimated to increase 2.08 million representing an increase of 57.5 percent from 2020. [1] In 2021 brain cancer represented 2.5% of all malignancies and was responsible for 2.5% of death worldwide. The survival rate for brain cancer is comparatively lower than that of other cancers due to challenges and difficulty in treating intracranial tumors. [2] Although brain cancer is rare, but brain cancer due to its anatomical complexity of CNS, presence of blood brain barrier, poor prognosis makes it difficult to manage clinically. [3]

Gliomas are the most prevalent primary brain tumors originating from glial cells. Astrocytomas, ependymoma, oligodendrogliomas, mixed gliomas are included in Gliomas. The most aggressive, recurring form of astrocytoma in adults is Glioblastoma multiforme (GBM) accounting for nearly 60% of adult brain tumors. Regardless of advancements in surgery, radiotherapy, chemotherapy, glioblastoma continues to have poor prognosis with median survival limited to approximately 14-15 months post diagnosis. Resistance and recurrence remain significant setbacks in the treatment of glioblastoma. [4] The U87MG glioblastoma cell is widely employed in vitro experimental model for screening and evaluating potential therapeutic agents against glioblastoma. [5]

Temozolomide (TMZ) is a standard chemotherapeutic used in combination with surgery and radiotherapy for treatment of glioblastoma. Although TMZ is generally well tolerated, its prolonged use may lead to adverse effects such as hepatotoxicity, and nearly half of patients develop resistance during treatment. [6] Therefore, additional experimental approaches require scientific evaluation in glioblastoma models.

In Ayurveda, cancer-like conditions are broadly correlated with concepts such as *arbuda* (tumor), and *granthi* (nodule)

described in classical texts. [7] Among the medicinal plants described in Ayurveda, *Semecarpus anacardium* Linn. f. known as *Bhallataka* has Rasayana property and demonstrates antioxidant, anti-inflammatory, antimicrobial and CNS stimulating properties. Phytochemical analysis of *Semecarpus anacardium* Linn. f. has shown bioactive compounds such as Alkaloids, Flavonoids, Phenols and Bioflavonoids. [8] Previous experimental studies have reported biological and cytotoxic effects of *Semecarpus anacardium* Linn. f. in different experimental models. [9] *Amritabhallataka paka* is an Ayurvedic formulation prepared from *Semecarpus anacardium* Linn. f. having rejuvenating and cytotoxic properties. [10-13]

In recent years particularly Ayurveda, in Complementary and Alternative Medicine (CAM) has gained recognition as a supportive care in cancer treatment. [14] Although experimental studies have reported biological and cytotoxic effects of *Semecarpus anacardium* Linn. f and *Bhallataka*-formulations in different models, evidence regarding *Amritabhallataka Paka* in glioblastoma remains limited. Hence, the present study aimed to evaluate the effect of *Amritabhallataka Paka* on U87MG glioblastoma cell viability using MTT assay, its cytocompatibility in L929 mouse fibroblasts using MTT assay, and its apoptosis effects in U87MG cells using Annexin V-FITC/PI flow cytometry.

2. MATERIALS AND METHODS

Collection and authentication of raw drug materials: The nuts of *Semecarpus anacardium* Linn. f. were procured from KLE Society's Ayurveda Pharmacy, Belagavi on 27 September 2024. Authentication of the *Semecarpus anacardium* Linn.f. was carried out on 28 September 2024 at the Ayurveda, Siddha, Unani Drug Testing Laboratory, a part of the Central Research Facility (CRF) at Shri B.M.K Ayurveda Mahavidyalaya, Belagavi, Karnataka with CRF code- CRF/Auth/526/2024 and Voucher specimen number- BMK/CRF/420/2024-25. Cow milk, Cow ghee was procured from a local dairy and was evaluated at Ayush Drug Testing Laboratory with Reference No-CRF/FG/32/2025 and Reference No-CRF/RM/66/2025-26 respectively. Sugar was

procured from the local market and evaluated under Reference No- CRF/FG/25/2025-2026

Drug Preparation: As *Bhallataka* is mentioned under Schedule E of Drug and Cosmetic Act, it requires proper processing before administration. [15] *Bhallataka* fruits which sink in water were chosen as per classical guidelines. [16] To reduce its oil content, the *Bhallataka* was processed using brick powder. [17] The purified *Bhallataka* fruits were used in preparation of *Bhallataka decoction*.

For the preparation of *Amritabhallataka Paka*, 370 g of purified *Bhallataka*, 1480 mL of Potable water, 1480 mL of cow milk, 370 mL of cow ghee and 370 g of sugar were used. The process was started by mixing one part of *Bhallataka* with four parts of potable water, heating it on low flame until the volume was reduced to one fourth. At the same time cow milk was heated in a separate stainless-steel vessel. After the milk reached boiling point, the prepared *Bhallataka decoction* was added and boiled until the volume again reduced to one fourth. The cow ghee was added to the formulation, and the mixture was boiled further. After the completion of ghee-processing stage, sugar was added, and the preparation was continued heating until the characteristic classical end point features of the preparation were attained. [10] The *Amritabhallataka Paka* was strictly prepared according to classical guidelines [18] in the Department of Rasashastra and Bhaishajya kalpana, KAHER'S shri BMK Ayurveda Mahavidyalaya, Belagavi Karnataka. The final yield of *Amritabhallataka Paka* was 960 g. The prepared formulation was designated as Experimental Batch No.1, cooled and stored in an airtight container at room temperature for further evaluation.



Figure 1: Prepared *Amritabhallataka Paka* used for in-vitro evaluation.

Analytical evaluation of formulation: Before initiation of study, the prepared formulation *Amritabhallataka Paka* was analyzed for its quality in Ayush approved Drug testing Laboratory at CRF, BMK Ayurveda Mahavidyalaya, Belgaum, Karnataka with Reference No-CRF/FG/317/2025-26. The formulation was evaluated for organoleptic characteristics, physicochemical parameters and microbial quality. The same experimental batch was subsequently used for cell-line experiments.

Procurement and authentication of cell lines: The U87MG glioblastoma cell line was procured from National Centre for Cell science NCCS, Pune, India in 2025 (Request No. 1144/2025-26) and was supplied at passage P49. The NCCS repository report documented mycoplasma status as "not detected". Authentication was performed using 16-locus short Tandem Repeat (STR) profiling with American Type Culture Collection (ATCC) standards which showed 93% match.

The L929 mouse fibroblast cell line was procured from NCCS, Pune, India in 2023 (Request No.535/2023-24) and was supplied at passage P47. The NCCS repository record documented cells as adherent and mycoplasma as "not detected".

Maintenance of cell lines: The U87MG glioblastoma cells and L929 mouse fibroblast cells were maintained under aseptic conditions at KAHER'S Dr. Prabhakar Kore Basic Science Research Centre, Belagavi, Karnataka. Subculturing was done using complete media Dulbecco's Modified Eagle Medium (DMEM) 89 mL, fetal bovine serum (FBS) 10 mL, antibiotic solution 1 mL. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. [19] and incubated until 85% confluency. As per the standard protocol Trypsinization was done using trypsin (TCL007, Lot No. 536691) as per standard protocols.

Reagents:

Cell culture:

1. Dulbecco's Modified Eagle Medium (DMEM) with low glucose-HiMedia, Ref. AL250A

2. Fetal bovine serum (FBS) - HiMedia, Ref.RM10432, Lot No.573421

3. Antibiotic-Antimycotic 100X solution- HiMedia, Ref. A002, Lot No.5392281)

4. TACS Annexin V-FITC Apoptosis Detection kit (R & D Systems) Cat No- 4830-01-K

- Annexin V-FITC V; Use 5µL per test.
- Propidium Iodide (PI); Use 2 µL per test.
- 10X Annexin V Binding Buffer:0.1 M NaOH (pH 7.4), 1.4 M NaCl, 25 Mm CaCl₂.
- 1X Working Solution, dilute 1 part of the 10X Annexin V Binding Buffer to 9 parts of distilled water.

5. 1 X Phosphate-buffered saline (PBS; HiMedia)

6. 0.1% dimethyl sulfoxide (DMSO),

7. 5ml sterile centrifuge and FACS tubes- Tarsons, India.

8. Temozolomide- GLS Pharma Ltd., India, Batch No. TZCB2417G

9. DxFLEX Flow Cytometer, (Beckman Coulter, USA)

MTT Cell-Viability Assay
2.7.1 Principle: 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl-2H-tetrazolium bromide is commonly known as MTT reagent which is used for assessing cell viability and proliferation. [20] MTT reagent is a mono-tetrazolium salt composed of three aromatic rings including two phenyl moieties and one thiazolyl, surrounding a positively charged tetrazole ring containing four nitrogen atoms. When MTT undergoes reduction, there is breakdown of the tetrazole ring and the formation of violet-blue water-insoluble dye. This conversion occurs in metabolically active cells as MTT reagent can penetrate both the cell membrane and the inner membrane of mitochondria due to its positive charge and lipophilic structure. [21]

Procedure: U87MG glioblastoma and L929 mouse fibroblast cells were seeded in 96-flat bottoms well plate at approximately 5×10^3 cells/well and 1×10^4 cells/well respectively, and allowed to attach for 24 h at 37°C in a humidified atmosphere containing 5% CO₂.

In a single treatment group, the test compounds of *Amritabhallataka Paka* (AP) and Temozolomide (TMZ) were subjected to serial dilution from 500, 250, 125, 62.5, 31.25 and 15.6 µg/mL. [22] The final concentration of 0.1% dimethyl sulfoxide (DMSO) was used in the treatment preparations. The untreated cells were used as the negative control group.

In the U87MG combination treatment group, AP and TMZ were combined with the obtained IC₅₀ values of 334.85 µg/mL and 249.42 µg/mL respectively. Later the resulting combination preparation of AP +TMZ was serially diluted throughout dilution series with same proportion of AP and TMZ with their respective IC₅₀ concentration; the plotted concentration values represent the total concentration of the combined mixture. After 24 h, the supernatant of test compound and Temozolomide was removed and cells were washed twice with phosphate-buffered saline. They were incubated in complete media for 24 h in 5% CO incubator (recovery phase). After 24 h of incubation 50 µL of MTT reagent was added in media and the plates were wrapped in aluminium foil. Formazan crystals were dissolved using 100µL DMSO and 25µL glycine buffer after carefully removing supernatant. Using the spectrophotometer absorbance was measured at 570 nm.

The MTT assay was independently repeated three times (n=3). Each concentration was tested in three technical replicate wells within each independent experiment. The technical replicates within each experiment were averaged before statistical analysis.

Cell viability (%) was calculated by dividing the optical density (OD) of the test by the OD of control multiplied by 100. [20]

Apoptosis Assay

Principle: Apoptotic cell death was assessed in this study using Annexin V-FITC/Propidium Iodide (PI). The effectiveness of apoptosis assay depends on plasma membrane integrity and permeability. [23]

Procedure: U87MG cells were seeded in 6-well flat bottom plates containing coverslips and incubated overnight at

37°C in a humidified atmosphere containing 5% CO₂ to facilitate cell attachment. The cells were treated with IC₅₀ concentration of *Amritabhallataka Paka* (AP), Temozolomide (TMZ) and the combination (AP+TMZ). The concentrations used for Apoptosis were 334.85 µg/mL, 249.42 µg/mL, and 327.20 µg/mL respectively. The untreated U87MG cells were considered as the control group. After treatment, they were washed twice with Phosphate-buffered saline (PBS). Centrifugation was done at 500 x g for 5 minutes at 4°C. After removing the supernatant, the cell pellets were suspended in ice cold 1x binding buffer (1 x 10⁶ cells/ML). 5 µL of TACS Annexin V-FITC and 2 µL of propidium iodide (PI) were mixed and added to the suspension. The tubes were incubated in dark on ice for 15 min and 400 µL binding buffer was added and mixed. Using flow cytometry within 30 minutes the stained cells were analyzed and data analysis was done using FlowJo X version 10.0.7 software. [24]

Cells were classified according to Annexin V/PI staining as viable (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and dead (Annexin V-/PI+). The apoptosis assay was repeated independently three times (n=3), and quantitative results were expressed as mean ± standard deviation (SD).

Statistical analysis: Data are presented as mean ± SD from three independent experiments (n=3). Within each independent experiment, technical replicate wells were averaged before statistical analysis. U87MG and L929 MTT data were analyzed separately using two-way ANOVA with factor as treatment and concentration, including the treatment × concentration interaction, followed by Sidak multiple comparison test between treatment groups at corresponding concentrations.

IC₅₀ values were estimated using four-parameter log-logistic (4PL) nonlinear dose response regression model and are reported with 95% confidence intervals and goodness of fit (R²). Cell viability (%) was considered the response variable and drug concentration (µg/mL) the explanatory variable. The model parameters were the upper asymptote, lower

asymptote, Hill slope and midpoint concentration (IC₅₀). The lower asymptote was constrained to zero and for each treatment separate models were fitted. Statistical analyses were performed using R software and Graphpad Prism Version 11.0.0

Total Apoptosis (early + late apoptosis) was compared among treatment groups using one-way ANOVA followed by Sidak multiple comparison test.

A two-sided P-value < 0.05 was considered statistically significant.

3. RESULTS

Analytical evaluation of Amritabhallataka Paka: The tested batch of *Amritabhallataka Paka* is semi-solid in form, light brown in color, sweet in taste, and rich and characteristic in odor. The physicochemical analysis showed loss on drying of 2.223%, fat content of 32.299%, and reducing sugar content of 13.317%. Microbial quality- control testing showed absence of *E. coli*, *S. aureus*, *P. aeruginosa*, and *S. abony*. No growth was seen in Total bacterial and fungal counts. The analytical report concluded that the sample was of standard quality.

Effect of AP, TMZ and AP + TMZ on U87MG cell viability: In U87MG cells, AP, TMZ and AP +TMZ showed concentration dependent reduction in cell viability across the tested range. Mean cell viability values for AP were 82.00 ± 2.90%, 71.00 ± 0.80%, 67.00 ± 3.00%, 62.00± 2.30%, 58.00 ± 1.30% and 42.00 ± 2.10% at 15.6,31.25,62.5,125, 250 and 500 µg/mL, respectively. The corresponding mean cell viability for TMZ were 65.00 ± 2.10 %, 56.00 ± 1.70 %, 57.00 ± 1.40%,58.00± 1.20%, 52.00 ± 1.60%, and 43.00 ± 3.30%, while AP + TMZ showed 82.00 ± 2.20%,73.00 ± 1.90%,71.00 ± 1.70 %, 62.00 ± 2.30%, 55.00 ± 1.70% and 43.00 ± 2.80 % viability respectively.

Two-way ANOVA showed significant effects of concentration (F(5,36)=262.18, p < 0.0001), treatment (F(2,36)=104.44, p<0.0001), and the treatment × concentration interaction (F(10,36)=12.36, p<0.0001). Sidak multiple-comparisons testing showed significant differences between AP and TMZ at 15.6,31.25, 62.5 and 250 µg/mL.

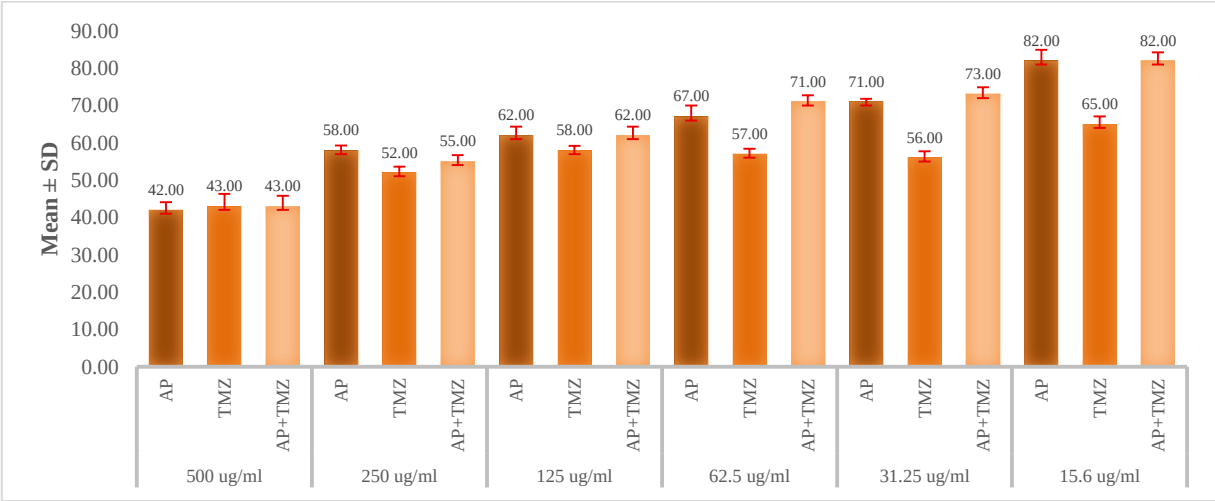


Figure 2. Effect of AP, TMZ and AP +TMZ on U87MG cell viability across the tested concentrations. Data are presented as mean ±SD from three independent experiments (n=3). Sidak adjusted pairwise p values are provided in [Table 2](#).

Table 1: Effect of treatment and Concentration on U87MG Cell Viability by Two-Way ANOVA.

Source of Variation	DF	Sum of Square	Mean Sum of Square	F-Statistic	P-Value
Concentration	5	5899	1179.80	262.18	< 0.0001
Treatment	2	940	470.00	104.44	< 0.0001
Interaction Effect	10	556	55.60	12.36	< 0.0001
Residual Effect	36	162	4.50	-	-
Total	53	7557	-	-	-

Table 2: Pairwise Comparison Between U87MG Treatment Groups at each concentration using Sidak-Adjusted multiple comparisons

Treatment	Concentration (U87MG)					
	15.6 µg/mL	31.25 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
AP - (AP+TMZ)	1.0000	0.5876	0.0781	1.0000	0.2507	0.9189
AP - TMZ	< 0.0001	< 0.0001	< 0.0001	0.0781	0.0042	0.9189
(AP+TMZ) - TMZ	< 0.0001	< 0.0001	< 0.0001	0.0781	0.2507	1.0000

Effect of AP and TMZ on L929 cell viability: In L929 cells, AP maintained cell viability between 71.0% and 87.0% across the tested concentrations, whereas marked reductions in cell viability were observed with the TMZ at higher concentrations. Mean cell viability values for AP were 87.00 ± 3.50%, 84.00 ± 3.10%, 76.80 ± 2.00%, 74.50 ± 2.50%, 72.20 ± 1.40% and 71.00 ± 1.50% at 15.6, 31.25, 62.5, 125,250 and 500 µg/mL, respectively. The corresponding values for TMZ were 99.50 ± 2.50%, 35.80 ± 1.50%,22.80 ± 1.20%, 16.50 ± 1.80%,12.00 ± 1.10% and 9.00 ± 1.20%. Thus,

marked reduction in TMZ treated L929 viability was observed at concentrations ≥31.25 µg/mL, whereas viability remained high at 15.6 µg/mL. Two-way ANOVA showed significant effects of concentration (F(5,24) =93.761, p<0.0001), treatment (F (1,24) = 6888.61, p<0.0001) and concentration × treatment interaction (F (5,24) =5.364, p=0.00189). Sidak test showed significant differences between AP and TMZ at all tested concentrations (p <0.0001).

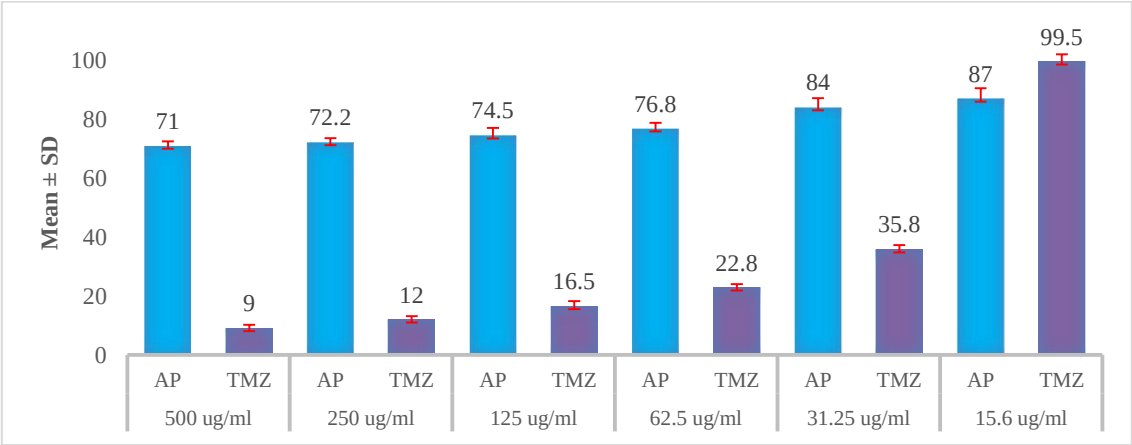


Figure 3: Effect of AP and TMZ on L929 mouse fibroblast cell viability across the tested concentrations. Data are presented as mean $\pm$ SD from three independent experiments (n=3). Sidak adjusted pairwise p values are provided in Table 4.

Table 3: Effect of treatment and concentration on L929 Cell Viability by Two-Way ANOVA.

Source of Variation	DF	Sum of Squares	Mean Square	F-Statistic	P-Value
Concentration	5	2007	401	93.761	< 0.0001
Treatment	1	29487	29487	6888.61	< 0.0001
Interaction Effect	5	115	23	5.364	0.00189
Residual Effect	24	103	4	-	-
Total	35	31712	-	-	-

Table 4: Pairwise Comparisons between AP and TMZ at each L929 concentration using Sidak adjusted multiple comparisons

Treatment	Concentration (L929)					
	15.6 μ g/mL	31.25 μ g/mL	62.5 μ g/mL	125 μ g/mL	250 μ g/mL	500 μ g/mL
AP - TMZ	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Nonlinear dose-response regression and IC₅₀ values:

Nonlinear dose-response regression using a four-parameter log-logistic model was used to calculate IC₅₀ values.

In U87MG cells, the IC₅₀ of AP was 334.85 μ g/mL (95% CI: 261.79-407.91; R²=0.92), the IC₅₀ of TMZ was 249.42 μ g/mL (95% CI: 144.83-354.01; R²=0.74), and the IC₅₀ of AP+TMZ was 327.20 μ g/mL (95% CI:261.54-392.87; R² =0.96). The U87MG TMZ model had a comparatively weaker goodness of fit (R²=0.74) than AP (0.92) and AP +TMZ (0.96).

In L929 cells, AP did not reach 50% inhibition within the tested concentration and so reported as IC₅₀ > 500 μ g/ML (95% CI: NA; R²=0.8865). TMZ showed an IC₅₀ of 25.29 μ g/ML (95% CI: 15.60-39.46; R2=0.9417)

Table 5: Dose-response parameters and IC₅₀ values for U87MG cells treated with AP, TMZ, and their combination.

Treatment	IC ₅₀ (μ g/mL)	95% CI (μ g/mL)	R ²
AP	334.85	(261.79 - 407.91)	0.92
TMZ	249.42	(144.83 - 354.01)	0.74
AP+TMZ	327.20	(261.54 - 392.87)	0.96

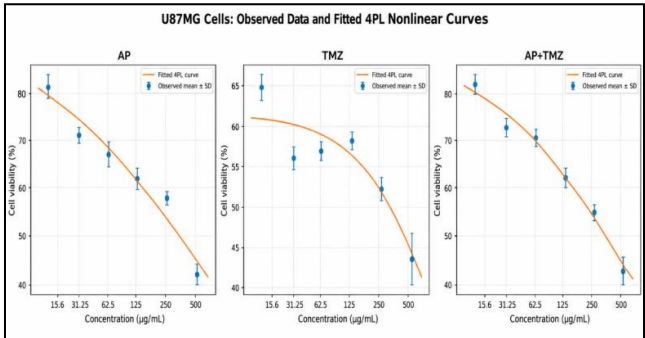


Figure 4- Fitted 4PL concentration-response curves for AP, TMZ and AP+TMZ in U87MG cells. Data are mean $\pm$ SD from three

independent experiments (n=3). R² values were 0.92, 0.74 and 0.96, respectively.

Table 6: Dose-response parameters and IC₅₀ values for L929 cells treated with AP and TMZ.

Treatment	IC ₅₀ (µg/mL)	95% CI (µg/mL)	R ²
AP	>500	NA	0.8865
TMZ	25.29	15.60 – 39.46	0.9417

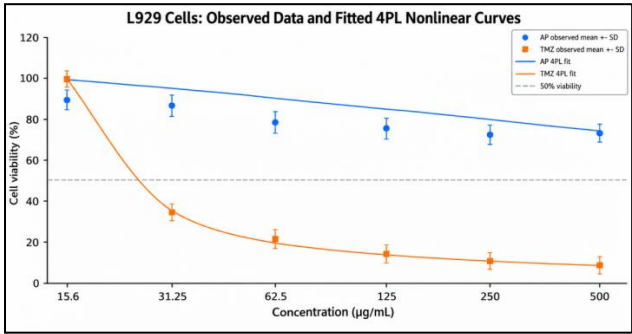


Figure 5- Fitted 4PL concentration-response curves for AP and TMZ in L929 mouse fibroblasts. Data are mean ± SD from three independent experiments (n=3). AP did not reach IC₅₀ within the tested range (>500 µg/mL).

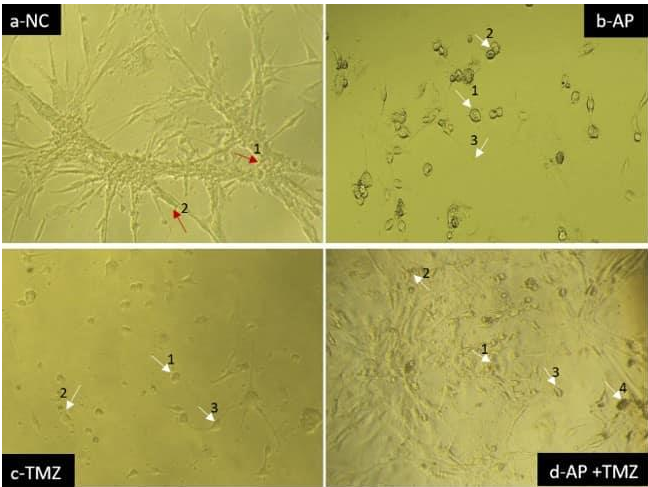


Figure 6: Representative phase contrast microscopic images of U87MG cells at 40× magnification following treatment with AP, TMZ and AP+TMZ (white arrows) compared with untreated control (red arrows)

- (a)-NC: 1. normal adherent cells 2. cellular process
- (b)-AP: 1. Floating round cell 2. shrunken cell 3. detached floating cell
- (c)-TMZ: 1. 2. detached cell 3. Loss of cellular process
- (d)-AP + TMZ: 1. round cell 2. detached cell 3. shrunken cell 4. morphological damage to cell

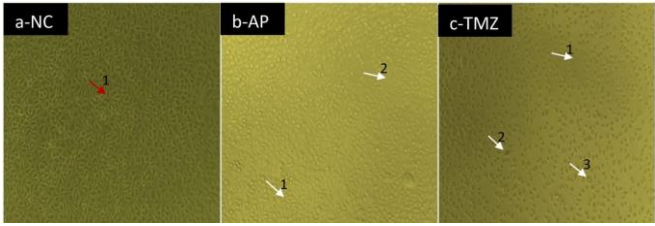


Figure 7: Representative phase contrast microscopic images of L929 mouse fibroblasts at 40× magnification following treatment with AP and TMZ (white arrows) compared with untreated control (red arrow)

- (a)-NC: 1. Normal spindle shaped morphology
- (b)-AP: 1. Most of the cells maintained fibroblast morphology 2. Reduced cell density
- (c)- TMZ: 1. Reduced cell density 2. Shrunken cell 3. Change in normal spindle arrangement

Apoptosis analysis in U87MG cells: Total Apoptosis was calculated as early apoptosis plus late apoptosis, which differed significantly among the treatment groups. The mean total apoptosis values were 3.26 ± 0.50% in the negative control group, 11.85 ± 1.75% in the AP group, 14.71 ± 1.20% in the TMZ group and 25.15 ± 1.80% in the AP + TMZ group.

One-way ANOVA showed a significant difference among groups (F=380.50, p< 0.0001). Sidak multiple -comparisons test showed significant differences for AP vs NC (p < 0.0001), TMZ vs NC (p < 0.0001), AP+TMZ vs NC (p<0.0001), TMZ vs AP (p=0.0229), AP+TMZ vs AP (p< 0.0001) and AP + TMZ vs TMZ (p<0.0001).

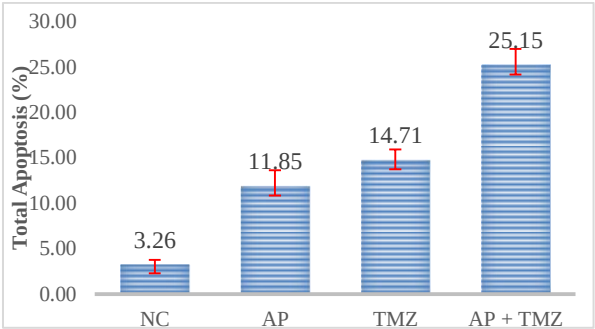


Figure 8- Total apoptosis (early +late apoptosis) in U87MG cells following treatment with AP, TMZ, AP+TMZ. Data are presented as mean ± SD from three independent experiments (n=3). Statistical comparisons were performed using one-way ANOVA followed by Sidak-adjusted pairwise comparisons; adjusted P values are reported in Table 8.

Table 7: Comparison of Total Apoptosis (%) among treatment groups by one-way ANOVA.

Source of Variation	DF	Sum of Squares	Mean Square	F-Statistic	P-Value
Treatment	3	760.20	253.41	380.50	< 0.0001
Residual Effect	8	5.30	0.67	-	-
Total	11	765.50	-	-	-

Table 8: Pairwise Comparison of Apoptosis by Sidak multiple comparisons Test.

Treatment	NC	AP	TMZ	AP + TMZ
NC	-	-	-	-
AP	< 0.0001	-	-	-
TMZ	< 0.0001	0.0229	-	-
AP + TMZ	< 0.0001	< 0.0001	< 0.0001	-

4. DISCUSSION

Discussion on probable mode of action of *Amritabhallataka Paka* according to Ayurvedic and Contemporary Perspective:

Amritabhallataka paka, is a classical Ayurvedic formulation prepared from *Purified Bhallataka* (*Semecarpus anacardium* Linn. f) along with other ingredients such as *godugdha* (cow milk), *ghrita* (cow ghee) and sugar. The formulation is described to have *Madhura Rasa*, *Laghu-snigdha guna*, *Madhura Vipaka*, and *Sheeta virya*. *Madhur vipaka* and *snigdha guna* of a *Bhallataka* contribute to *Rasayana* effects by improving the nutritional value of *Rasa*, which enhances the qualities of *Dhatus*. [10] Previous experimental studies have reported protective effects of flavonoid constituents isolated from *Semecarpus anacardium* Linn. f; however, such mechanisms were not investigated in the present study. [25] *Bhallataka* possesses cytotoxic properties and is used in the management of various conditions such as skin diseases, diabetes and piles. [10] Preparations made from *Bhallataka* are also indicated in *gulma* (abdominal tumor), *pleeha roga* (spleen disorders), *hridroga* (heart diseases). [26]

Discussion on recent studies: Previous GC-MS analysis of *Bhallataka* oil, has revealed the presence of approximately 30 chemical compounds responsible for its pharmacological properties. [27] The pericarp of (*Semecarpus anacardium*

Linn. f.) contains 90% anacardic acid, which demonstrated cytotoxic and enzyme modulating activities in experimental studies. [28, 29] Kumar et al. reported protective effects of flavonoid constituents isolated from *Semecarpus anacardium* Linn. f against hydrogen peroxide-induced cytotoxicity in lung and liver cell models. [28] Milk-derived components such as lactoferrin have also been investigated in experimental glioblastoma models. These previous observations provide a possible biological background for further investigation of *Bhallataka* containing formulations. However, the present study did not directly measure phytochemical constituents, cyclooxygenase activity, oxidative stress markers, molecular apoptotic pathways or intracellular signaling mechanisms. Therefore, such mechanisms can be considered only as literature-derived hypothesis and cannot be attributed to *Amritabhallataka Paka* on the basis of the present experiment. [30] The contribution of individual milk-derived constituents to the effects observed with *Amritabhallataka Paka* was not evaluated in the present study and cannot be inferred from the current findings.

Discussion on Temozolomide: In U87MG cells, TMZ showed lower cell viability values than AP across the evaluated concentration range, with statistically significant difference at 15.6, 31.25, 62.5 and 250 µg/mL. The nonlinear regression-derived IC₅₀ of TMZ was 249.42 µg/mL (95% CI: 144.83-354.01; R²=0.74), which was lower than corresponding point estimate for AP (334.85 µg/mL). These findings indicate comparatively greater cytotoxic potency of TMZ than AP in the U87MG MTT assay under the tested conditions.

In L929 mouse fibroblasts, TMZ showed 99.5% viability at 15.6 µg/mL, followed by reductions in viability at

concentration ≥ 31.25 $\mu\text{g/mL}$. Therefore the result demonstrates dose dependent effects of TMZ in the L929 fibroblast model but it can't be interpreted as evidence of toxicity to normal human tissues, because L929 cells are mouse fibroblasts and are not a disease relevant non-malignant human neural counterpart.

In the apoptosis assay, total apoptosis with TMZ was $14.71 \pm 1.20\%$, which was higher than AP ($11.85 \pm 1.75\%$) but lower than AP+TMZ ($25.15 \pm 1.80\%$). However, the higher apoptotic fraction observed with AP+TMZ does not establish enhanced TMZ efficacy or synergy because formal drug-interaction analysis was not performed.

Discussion on MTT assay: In U87MG cells, AP produced a dose-dependent reduction in cell viability across the tested range. AP viability decreased from 82% at 15.6 $\mu\text{g/mL}$ to 42% at 500 $\mu\text{g/mL}$. The regression derived IC_{50} AP was 334.85 $\mu\text{g/mL}$. These findings demonstrate a measurable dose-dependent effect of AP on U87MG cell viability under the present in-vitro conditions.

The AP+TMZ group also demonstrated dose dependent changes in U87MG viability. However, AP + TMZ was not significantly different from AP at any of the tested concentrations, and the combination did not consistently produce lower viability than TMZ alone. The regression derived IC_{50} of AP +TMZ was 327.20 $\mu\text{g/mL}$ (95% CI: 261.54 - 392.87 ; $R^2=0.96$), which was close to AP estimate of 334.85 $\mu\text{g/mL}$. The U87MG TMZ model had a comparatively weaker goodness of fit ($R^2=0.74$) than AP ($R^2=0.92$) and AP +TMZ ($R^2=0.96$). Therefore, its IC_{50} estimate should be interpreted cautiously.

In L929 fibroblasts, AP maintained viability between 71% and 87% throughout the tested concentration range and did not reach 50% inhibition at concentration up to 500 $\mu\text{g/mL}$. In contrast, TMZ showed marked reductions in viability at concentrations ≥ 31.25 $\mu\text{g/mL}$, although viability remained high at the lowest concentration of 15.6 $\mu\text{g/mL}$. These observations support comparatively high cytocompatibility of AP within this specific mouse fibroblast model.

However, the L929 findings cannot establish tumor selectivity, cyto-protection or safety for normal human tissues because only one mouse fibroblast cell line was evaluated and it is not a non-malignant human neural counterpart to U87MG cells. A valid selectivity index with confidence intervals was also not established in the present study.

Discussion on Apoptosis assay: Annexin V-FITC/PI flow cytometric analysis demonstrated treatment associated differences in apoptotic cells. Total apoptosis was lowest in the negative control group ($3.26 \pm 0.50\%$), followed by AP ($11.85 \pm 1.75\%$) and TMZ ($14.71 \pm 1.20\%$), and was highest in the AP +TMZ ($25.15 \pm 1.80\%$). The overall group difference was statistically significant, and the AP+TMZ group differed significantly from both AP and TMZ.

The higher total apoptosis observed in the AP+ TMZ group indicates that the combination influenced apoptotic cell populations under the tested experimental conditions. However, these findings should not be interpreted as evidence of enhanced therapeutic efficacy or synergistic interaction. In the MTT assay, AP + TMZ did not demonstrate consistently greater reduction in cell viability than TMZ alone, and formal drug-interaction or combination index analysis was not performed.

Therefore, synergistic, additive or antagonistic interaction between AP and TMZ cannot be concluded from the present data. Similarly, the present experiment does not establish that AP enhances the clinical efficacy of TMZ or supports its use as an adjunct to chemotherapy.

Previous studies of *Bhallataka Rasayana* and *Semecarpus anacardium* Linn. f preparations have reported cytotoxic activity in MCF-7 and HT-29 cells. [10] These studies support continued experimental investigation of *Bhallataka*-containing formulations but do not establish anticancer efficacy of *Amritabhallataka Paka* in humans.

Overall, the present findings demonstrated that *Amritabhallataka Paka* produced a dose dependent reduction in U87MG cell viability and maintained comparatively high viability in the L929 mouse fibroblast

model. Treatment associated differences in total apoptotic cell populations were also observed. Further studies using additional glioblastoma cell lines, disease relevant non-malignant human neural cells, molecular mechanistic assays and formal drug interaction analysis are required to clarify the biological significance of these findings.

Limitations of the study: The study was limited to a single glioblastoma cell line, which limits generalization across glioblastoma models. The L929 mouse fibroblast cell line was used as a general cytocompatibility comparator but is not a disease relevant non-malignant human neural counterpart. The exact experimental passage range after receipt and the exact duration of cell maintenance before experimentation were not separately documented. A concurrent matched 0.1% DMSO vehicle-control group was not included. In flow cytometry, number of flow-cytometric events acquired, compensation procedure, unstained and single-stain controls, and exact quadrant setting strategy were not available for reporting. Formal diagnostic testing of ANOVA including normality of residuals and homogeneity of variance, was not documented. Molecular mechanism and signaling pathways underlying the observed effects were not investigated. A formal selectivity index with confidence intervals was not established and formal drug-interaction analysis was not performed. Therefore, tumor selectivity, synergistic interaction, clinical safety, therapeutic efficacy or protection against chemotherapy associated toxicity cannot be concluded from the present study.

5. CONCLUSION

Under the present in vitro conditions, *Amritabhallataka Paka* showed dose dependent reduction in U87MG glioblastoma cell viability. AP showed a higher IC₅₀ point estimate than Temozolomide and maintained comparatively high viability in L929 mouse fibroblasts. The AP + TMZ group showed a higher total apoptosis than the individual treatment groups in the flow cytometric analysis. However, the MTT assay did not demonstrate superior cytotoxicity of the AP+TMZ compared with TMZ alone, and formal drug

interaction analysis was not performed. Therefore, synergistic or enhanced combination efficacy, tumor selectivity, clinical safety and adjunctive therapeutic benefits cannot be concluded. Further studies using additional glioblastoma models, relevant non-malignant human neural cells, molecular assays and formal drug interaction analysis are required.

List of Abbreviations

AP-*Amritabhallataka Paka*; TMZ-Temozolomide
ATCC-American Type Culture Collection
U87MG-U87MG glioblastoma cell line; L929-L929 mouse fibroblast cell line
MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM-Dulbecco's Modified Eagle Medium
FBS-Fetal Bovine Serum; PBS-Phosphate Buffered Saline
DMSO-Dimethyl sulfoxide; PI-Propidium Iodide
STR-Short Tandem Repeat; NCCS-National Centre for Cell science
IC₅₀ -Half-Maximal Inhibitory Concentration
SD-Standard Deviation; CI-Confidence Interval
ANOVA-Analysis of Variance

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