RESEARCH ARTICLE

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Arsenic-Based Homeopathic Preparations Induce Metabolic Disruption and Reactive Oxygen Species-Mediated Cell Death in Glioblastoma Multiforme

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Abstract

Background: Glioblastoma multiforme (GBM) is a highly aggressive brain cancer with a poor median survival rate. There is an urgent need for effective and affordable anti-cancer agents for GBM treatment. In this context, arsenic-based homeopathic preparations may serve as promising therapeutic candidates. **Objectives:** This study aimed to evaluate the efficacy of *Arsenicum iodatum* and *Arsenicum album*-induced cytotoxicity in GBM cells and to investigate the underlying mechanisms of action in the U-87-MG and LN-229 cell lines. **Results:** Treatment with varying concentrations of *Arsenicum iodatum* and *Arsenicum album* resulted in dose- and time-dependent inhibition of GBM cells growth in U87-MG and LN-229. These preparations induced distinct morphological changes and cell death in both GBM cell lines. Gas chromatography-mass spectrometry (GC-MS)-based metabolomics revealed significant alterations in the key metabolic pathways. A total of 107 metabolites were quantified. Univariate analysis identified 73 and 30 significantly altered metabolites in *Arsenicum album*-treated U-87-MG and LN-229 cells, respectively. Meanwhile, U-87 showed 69 and LN-229 showed 50 significantly affected metabolites in the *Arsenicum iodatum*-treated groups. In GBM cells treated with *Arsenicum album* and *Arsenicum iodatum*, glycine and serine, which are involved in redox balance, were altered, while branched-chain amino acids (valine, leucine, isoleucine)- essential for protein synthesis and mTOR signaling- were downregulated. Changes were also observed in nucleotide sugar, purine, and nicotinate/nicotinamide metabolism. The findings suggest that both agents cause strong metabolic disruptions, potentially contributing to their anti-cancer effects. Biochemical assays confirmed increased reactive oxygen species (ROS) levels and decreased mitochondrial membrane potential following treatment with these arsenic based homeopathic preparation. **Conclusion:** *Arsenicum iodatum* and *Arsenicum album* exhibit growth-inhibitory effects on GBM cells, likely through metabolic disruption and ROS-mediated cell death. Further studies are warranted to elucidate the precise mechanisms of cell death and to evaluate their efficacy and safety *in vivo.*

Keywords: Glioblastoma Multiforme- Gas Chromatography-Mass spectrometry- Metabolomics- Cell death

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Introduction

Glioblastoma multiforme (GBM) is among the most malignant brain tumors with the poorest prognosis, has a median survival time of 14.6 months and a 5-year survival rate of <10% [1]. GBM is a WHO grade 4 Glioma and the primary tumor of glial cells that form the tissue that surrounds and protects other nerve cells found within the brain and spinal cord [2]. The WHO defines GBM as neuro-epithelial cancer of the central nervous system (CNS) with two different variants: large cell glioblastoma and gliosarcoma. The worldwide incidence of GBM is <10% per 100,000 persons [3]. These tumors are often aggressive and infiltrate surrounding brain tissue. Despite progress in neurosurgery of brain tumors, complete resection of GBM is not possible due to its highly invasive nature to the surrounding brain tissue [4]. The potential effects of radiotherapy on GBM are limited by the associated toxicity to normal tissues [5]. They are the most commonly occurring tumors of the CNS, which account for almost 80% of all malignant primary tumors of brain [6]. In addition, the efficiency of chemotherapy

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is also limited, as chemotherapeutic agents are unable to efficiently cross the Blood Brain Barrier, while glioma cells also have a high tendency to develop resistance against chemotherapeutic agents [7].

Arsenic has significant medicinal properties and used as a therapeutic agent for more than 2400 years [8]. Homeopathic medicines may be produced from healing substances, neutral substances, or highly toxic substances like arsenic [9, 10]. *Arsenicum iodatum* is a homeopathic Medicine that is chemically prepared from metallic arsenic and iodine. Arsenic's anti leukemic activity was first reported in the 1878 from Boston city hospital [11, 12]. Weak preparations of this metal have a history of medicinal use for respiratory health and during fever. Arsenic trioxide (As_2O_3), a traditional Chinese medicine, inhibits growth and promotes apoptosis in many different cancer cell types. It has been proven to be highly effective against hematologic malignancies, such as acute promyelocytic leukemia (APL) [13, 14]. Moreover, promising preclinical activity of As2O3 against malignancies other than APL was noted, such as myeloid leukemia, lymphoma, lymphocytic leukemia, and solid tumor cell lines of prostate, cervix, bladder, ovary, colon, stomach, and esophagus [11]. As Arsenic has shown its anti-proliferative and Programmed Cell Death (PCD) inducing properties in various cancers, Role of *Arsenicum iodatum* in cancer has not been explored. Previous studies reported *Arsenicum album* has significant anti-proliferative and apoptotic potential against breast cancer cells [15]. Similar, antiproliferative effect of *Arsenicum album* is reported on leukocyte but till date no studies were performed on GBM [16]. Therefore, the potential of *Arsenicum iodatum* and *Arsenicum album*, two arsenic-based homeopathic formulations, warrants investigation in GBM treatment.

Metabolic perturbations are the major hallmark of cancer cell transformation and growth. The upregulation of metabolic pathways are known to be active in cancer which fulfil ever increasing energy demands of fast proliferating cancer cells and at the same time provide necessary building blocks supporting cancer cell growth [17]. High-throughput and sensitive analytical tools are offering new insights into cellular physiology. Metabolomics, a rapidly emerging field, involves the study of metabolic profiles during various cellular states. Metabolomic analysis provides critical mechanistic insights into upregulated and downregulated pathways, identifying metabolite-based drug targets [18].

Till date mechanism of action of *Arsenicum iodatum*-induced apoptosis is not clearly defined. In-depth understanding of mechanism is essential to further categorize the *Arsenicum iodatum* as an independent chemotherapeutic agent or facilitator molecule in conjunction with other chemotherapeutic agents. Prognosis remains poor for most patients although treated with the most effective available therapies are surgery, radiation and chemotherapy [19]. The available treatment modality of GBM have its limits: serious side effect and high dose levels required for the response. Therefore, novel therapeutic strategies to eliminate invasive tumor cells without damaging the normal brain parenchyma are urgently required. Therefore, we tested the efficacy of the Arsenic based homeopathic preparation in GBM cells. We focused on the antiproliferative effects of these drugs and demonstrate their effects via targeting metabolic pathways and antioxidant defense of cancer cells.

Materials and Methods

Materials

The human GBM cell lines U-87 MG and LN-229 were obtained from National Center for Cell Sciences (Pune, India). The cell lines were tested to be free from Mycoplasma. *Arsenicum iodatum* and *Arsenicum album* (6C, 12 C and 30 C potencies) were procured from Hapco (Kolkata, India). HPLC grade Acetonitrile and Methanol were obtained from Sisco Research Laboratories (India). The ultrapure HPLC grade water procured from Thermo scientific. Internal standard D-ribitol and Pyridine were purchased from Sigma Aldrich (St. Louis, MO, USA). Methoxylamine hydrochloride was purchased from Merck (Darmstadt, Germany). Alkane standard mixture contains C10-C40, 50 mg/l in n-hexane was obtained from Supelco (Bellefonte, PA, USA). N-methyl-N (trimethylsilyl) trifluoroamide (MSTFA)+ 1% TMCS was purchased from Sisco Research Laboratories (India).

Cell Viability Assay

Human glioma cell lines LN-229 and U87-MG (4×10^3 cells/well) were seeded in 96-well plates and cultured under standard conditions. LN-229 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), and U87-MG cells in α-Minimum Essential Medium (α-MEM), each supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 hours of incubation, culture medium was replaced with a 200 µl of fresh medium. Effects of *Arsenicum iodatum* and *Arsenicum album* on GBM cell lines were determined after exposure of various potencies (6C, 12C, 30C). 5µl drug of each potency was used at different time interval (24 h, 48 h, and 72 h) and 5 µl ethanol used as vehicle. Subsequently, 10 µL of MTT (M6494, Invitrogen, USA) working solution (5 mg/mL in phosphate buffer solution) was added to each well and the plate was incubated for 4 hours at 37 °C in a CO_2 incubator. The medium was then aspirated, and the formed formazan crystals were solubilised by adding 50 µL of DMSO per well for 30 min at 37 °C in a CO2 incubator. The intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader (Spectrmax paradigm, Molecular devices, USA) at 570 nm [20].

Metabolite Extraction

LN-229 and U87-MG cells were seeded in 6-well plates at a density of 3×10^5 cells per well. Upon reaching approximately 70% confluence, the cells were treated with 50 µL of *Arsenicum iodatum* (6C) and *Arsenicum album* (6C) potency for 72 hours. After that, two million cells per sample were collected in 350 µL of chilled methanol (MeOH). The samples were then snap frozen in liquid

nitrogen and vortexed for 2 minutes. After thawing at 37°C, the samples were centrifuged at 14,000×g for 10 minutes at 4°C. The supernatant was carefully transferred to a new centrifuge tube. The freeze-thaw extraction process was repeated by adding an additional 400 µL of chilled MeOH. The samples were vortexed, then centrifuged again at 14000×g for 10 minutes at 4°C, and the supernatant was transferred to a fresh tube. Finally, the samples were mixed with 250 µL of ice-cold water and subjected to another freeze-thaw cycle. After the final centrifugation at 14000×g for 10 minutes, the supernatants were collected and subjected to vacuum centrifugal evaporation to remove the solvent. To the dried extracts, 20 µL of methoxyamine hydrochloride (MOX) reagent (Merck, USA, 89803) (20 mg/mL in pyridine) was added. The samples were incubated in a shaking dry bath at 60°C and 1100 rpm for 90 minutes to allow for the methoximation of carbonyl compounds. After this incubation, 80 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (SRL, India) was added to the samples for silylation. The derivatization reaction continued for 60 minutes in the Thermomixer at 60°C. The samples were injected in the GC-MS (Thermo Trace 1300 gas chromatography and TSQ 8000 mass spectrometry) system, and a pooled QC and alkane standard (Supelco, USA) were run every time after 10 samples for checking system suitability, stability and retention indices calculation respectively as described previously [21].

Data Processing and metabolites annotation

Baseline filtering and calibration, Peak picking, deconvolution, spectral alignment, and metabolite annotation were performed using MS-DIAL version 4.90. The raw data obtained from the electron ionisation mass spectrometry (EI-MS) analysis were processed to identify and classify metabolites. For data annotations, we utilized publicly accessible spectral libraries for compound identification by comparing the mass spectral patterns to various EI spectral libraries. Metabolite identification followed the Metabolomics Standards Initiative (MSI) criteria: Level 2 (identification based on matching spectra with reference databases) and Level 3 (putative identification based on spectral similarity to known compounds within a chemical class) [22].

Cytotoxicity Assay

LN-229 and U87-MG cells were seeded in 96-well plates at a density of 4×10^3 cells per well. Cells were treated with different concentrations of *Arsenicum iodatum* and *Arsenicum album* (6C, 12C, and 30C) by adding 5 µL of drug from each potency to each well containing 100µl media, and incubation was continued for 48 h. After treatment, both adherent and floating cells were collected, washed, and resuspended in phosphate-buffered saline (PBS). Cell viability was assessed using a Coulter counter after staining with trypan blue, where non-viable cells selectively absorbed the dye and were excluded from viable counts. A significant reduction in cell viability was observed in both GBM cell lines following treatment with the homeopathic formulations [23].

Measurement of mitochondrial ΔΨm

LN-229 and U87-MG cells were seeded in 6-well plates at a density of 3×10^5 cells per well in 2 ml medium. After cell attachment, cultures were treated with 50 µL of *Arsenicum iodatum* (6C) and *Arsenicum album* (6C) potency for 24 hours. Intracellular mitochondrial ΔΨ m was measured using the ratiometric dye JC1. Thirty minutes before the cells were harvested, JC-1(2 µM) was added directly to the culture medium at 37ºC in the dark. After labeling, the cells were harvested, pelleted, and resuspended in PBS and analyzed immediately by flow cytometry (FACSlyrics, BD biosciences) [24].

ROS assay

Intracellular reactive oxygen species (ROS) levels were assessed using the DCF-DA assay. In brief, LN-229 and U87-MG cells were seeded in 6-well plates at a density of 3×10^5 cells per well and treated with 50 µL of *Arsenicum iodatum* or *Arsenicum album* (6C) for 24 h. One hour prior to harvesting, 10 µM DCF-DA was added directly to the culture medium and incubated at 37 °C in the dark. Following incubation, cells were collected, washed with PBS, and analyzed immediately on a BD FACSLyric flow cytometer equipped with a 488 nm excitation laser. The fluorescence intensity of 2′,7′-dichlorofluorescein (DCF), generated by intracellular oxidation of the probe, was quantified as a measure of ROS production.

Statistical analysis

All the experiments were performed adopting a completely randomized design with minimum 3 replicates. Data were presented as mean ± standard deviation (SD). Significant changes were analyzed using a student's t-test using GraphPad Prism 5.

The metabolomic experiments was conducted following a completely randomized design with three replications. Prior to performing multivariate and univariate analyses, the MS-DIAL extracted intensities was normalized using ribitol as the internal standard, subjected to Log10 transformation, and Pareto scaling, all through the open-source software MetaboAnalyst 6.0 (Pang et al., 2024). Following the normalization, univariate analysis was conducted using the student's t-test ($P \leq 0.05$), significant metabolites were represented in heatmap. Further, in addition to p- value ≤ 0.05 a Fold Change analysis (FC) with a threshold of 2 is applied to identify only metabolites that altered more than 2-fold. The results was graphically be represented using a Volcano plot P value ≤ 0.05 and FC > 2.

Subsequently, supervised Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), to explore and visualize metabolic alterations across different experimental groups. In the model's score plots, the Hotelling's T2 region, represented as an ellipse, corresponds to the 95% confidence interval for the variation modeled. The Variable Importance in Projection (VIP) scores highlight the contribution of each variable to the OPLS-DA model, with variables exhibiting VIP scores above 1 considered significant for differentiating

between groups. To avoid overfitting, the OPLS-DA model was validated using a permutation test (with 100 permutations), considering the model valid when the empirical p-values for Q2 and R2Y was ≤ 0.05 and when Q2 and R2Y values close to 1. Finally, enrichment analysis was performed through a Metaboanalyst tool using the SMPDB pathway-based library.

Results

Cytotoxicity of Arsenicum iodatum and Arsenicum album in GBM Cells

The LN-229 and U-87 GBM cells were treated with *Arsenicum iodatum* (6C) and *Arsenicum album* (6C) potency for 24 and 48 hours and images of untreated control cells appeared confluent and with epithelial like morphology, mostly elongated and spindle-shaped. Treated cells were less confluent and some cells showed rounding and shrinkage, suggesting cytotoxic damage indicating extensive cell death (Figure 1a & 1b). To assess the cytotoxic effects of *Arsenicum album* and *Arsenicum iodatum* at potencies 6C, 12C, and 30C, LN-229 and U87-MG GBM cell lines were treated with each formulation for 24 h, 48 h, and 72 h. Cell viability was measured using the MTT assay. After 24 h treatment both *Arsenicum album* and *Arsenicum iodatum* showed a modest reduction in cell viability, with 30C potency demonstrating a more pronounced effect compared to 6C and 12C potency. Post 48 hour of treatment, the cytotoxic effects were more evident at all potencies, with significant decreases in viability observed particularly with 12C and 30C potencies of *Arsenicum iodatum*. A marked reduction in cell viability was observed across all treatment groups after 72h of treatment. Both *Arsenicum album* and *Arsenicum iodatum* at 30C exhibited the highest cytotoxic activity, significantly reducing cell viability compared to untreated controls. Similar trends were observed in LN-229 cells (Figure 1c & 1d).

Overall, the data suggest that both *Arsenicum album* and *Arsenicum iodatum* induce cytotoxicity in GBM cell lines, with increasing potency and longer exposure times leading to greater reductions in cell viability. Among the two, *Arsenicum iodatum* generally demonstrated slightly higher cytotoxic effects, particularly at 30C potency.

Metabolic changes characterization in *Arsenicum album* and *Arsenicum iodatum* treated GBM cells: Metabolomic analysis enabled the relative quantification of 107 putatively annotated metabolites from a total of 2,484 shared unknown EI-MS features. First, we performed univariate statistical analysis on annotated metabolites and T-test were used to identify differentially accumulated metabolites on *Arsenicum album* treatment in U-87 and LN-229 cells. IN T-test, a total of 73 significantly altered metabolites were identified in U-87 whereas 30 metabolites were reported in LN-229 (p-value <0.05). Out of these altered metabolites 5 have negative t stat value whereas 68 have positive t stat value that represent increased accumulation and decreased accumulation of these metabolites in *Arsenicum album* treated U-87 cells respectively (Supplementary data 1). In LN-229 have 28 metabolites have negative and 2 metabolites have positive t stat value. All the altered metabolites were represented in the heatmap where red colour shows increased accumulation of metabolites whereas blue colour is represented decreased accumulation of metabolites in respective group (Figures 2a and 2b). Colour intensity in heatmap is directly proportional to relative peak intensity of that metabolites. Further, *Arsenicum iodatum* treated U-87 shows 69 significantly altered metabolites, out of which 63 metabolites have positive t-stat value with decreased accumulation while 6 metabolites have negative t stat value that shows increased accumulation of corresponding metabolites. Similarly, in LN-229, out of 50 significantly impacted metabolites 40 have negative t-stat value and 10 have positive t stat value that represent higher and lower levels of these metabolites respectively on iodatum treatment (Figures 3a and 3b).

Further, we performed volcano plot analysis in *Arsenicum album* and *Arsenicum iodatum* treated U-87 and LN-229 cells to identify the metabolites that have log 2 FC > 2.0 and p-value <0.05. Volcano plot analysis showed all the altered metabolites that were obtained in U-87 and LN-229 treated with *Arsenicum album* have fold change of more than 2 (Figures 2c and 2d). Similarly, all the metabolites altered on *Arsenicum iodatum* treatment also have change of more than 2-fold. This suggest that *Arsenicum iodatum* and album have strong metabolic impacts that induce cancer cell death (Figure 3c and 3d) (Supplementary Data 1).

Multivariate statistical analysis to identify important metabolic patterns corresponds to *Arsenicum album* and *Arsenicum iodatum* treatment: We performed orthogonal partial least square discriminant analysis (OPLS-DA) as a part of multivariate statistical analysis in *Arsenicum album* treated U-87 and LN-229 cells. Score plot of OPLS-DA shows clear separation between treatment and control group. The T score of OPLS-DA score plot was 52.9% and orthogonal T score is 20.3% in U-87 cells. While, OPLS-DA of LN-229 cells shows T score of 26.4% and orthogonal T score of 25.6 % (Figure 4a and 4b). Similarly, *Arsenicum iodatum* treated with U-87 and LN-229 cells have clear OPLS-DA separation. *Arsenicum iodatum* treated U-87 have T-score of 50.8 while orthogonal T score of 16.8%. Score plot of LN-229 treated with *Arsenicum iodatum* showed T-score 41.6 and Orthogonal T-score value 25.8% (Figures 5a and 5b). To identify the important metabolites that play important role in group separation VIP plot of U-87 and LN-229 identified a total of 58 and 48 metabolites in *Arsenicum album* treated group with score greater than 1.0 (Figures 4c and 4d). Moreover, VIP plot of *Arsenicum iodatum* shows 58 metabolites of U-87 and 49 features in LN-229 had VIP score greater than 1.0 (Figures 5c and 5d). The model of study was validated to avoid overfitting through a permutation test characterized by significant ($p \leq 0.05$) R2 (0.996) and Q2 (0.939) for U-87 as well as ($p \leq 0.05$) R2 (0.995) and Q2 (0.777) for LN-229 (Figures 4e and 4f). Model validation based on permutation test of 100 suggested significance and reproducibility of data ($p \leq 0.05$) R2 (0.997) and Q2 (0.92) for U-87 as well as ($p \leq 0.05$) R2 (0.993) and Q2 (0.963) for LN-229 treated with *Arsenicum iodatum* (Figures 5e and 5f).

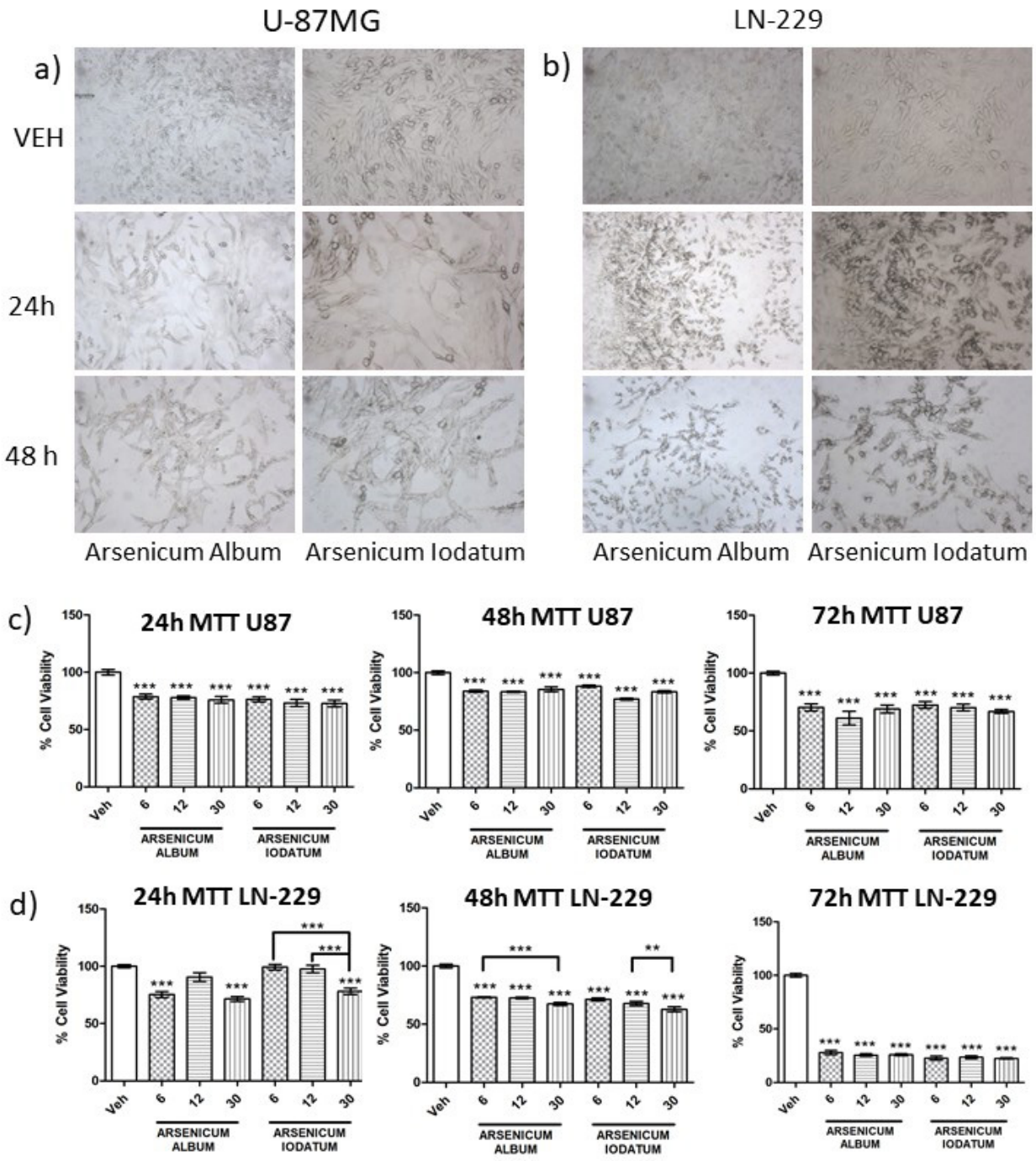

Figure 1. Antiproliferative Activity of Arsenic-based Homeopathic Drugs *Arsenicum iodatum* and *Arsenicum album* in GBM Cells: Arsenicum iodatum and Arsenicum album alter the morphology of GBM cells. U87-MG (a) and LN-229 (b) cells were cultured in 6-well plates at the specified density and incubated with Arsenicum iodatum and Arsenicum album at 6C potencies for 24 and 48 hours, or left untreated. Representative images of light microscopy at 40x magnification demonstrate that drug treatment induces significant morphological alterations in both cell lines. Cell viability assay on U87-MG and LN-229 cell lines: Cells were seeded in 96-well plates and treated with arsenic compounds for 24, 48, and 72 hours. (c) MTT assay after 24, 48, and 72 hours of treatment with Arsenicum album (6C, 12C, 30C) and Arsenicum iodatum (6C, 12C, 30C) in U87-MG cells. (d) MTT assay after 24, 48, and 72 hours of treatment with Arsenicum album (6C, 12C, 30C) and Arsenicum iodatum (6C, 12C, 30C) in LN-229 cells. Data represent mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus untreated.

Identifying enriched metabolic pathways in Arsenicum iodatum and Arsenicum album induced cell death in GBM cells

To identify physiologically relevant metabolic patterns, enriched pathways were analyzed using MetaboAnalyst 6.0 with reference to the SMPDB database. Comparative metabolomic profiling of *Arsenicum album* and *Arsenicum iodatum*-treated groups versus controls revealed the top 25 significantly enriched metabolic pathways ($p \leq 0.05$). These metabolic pathways

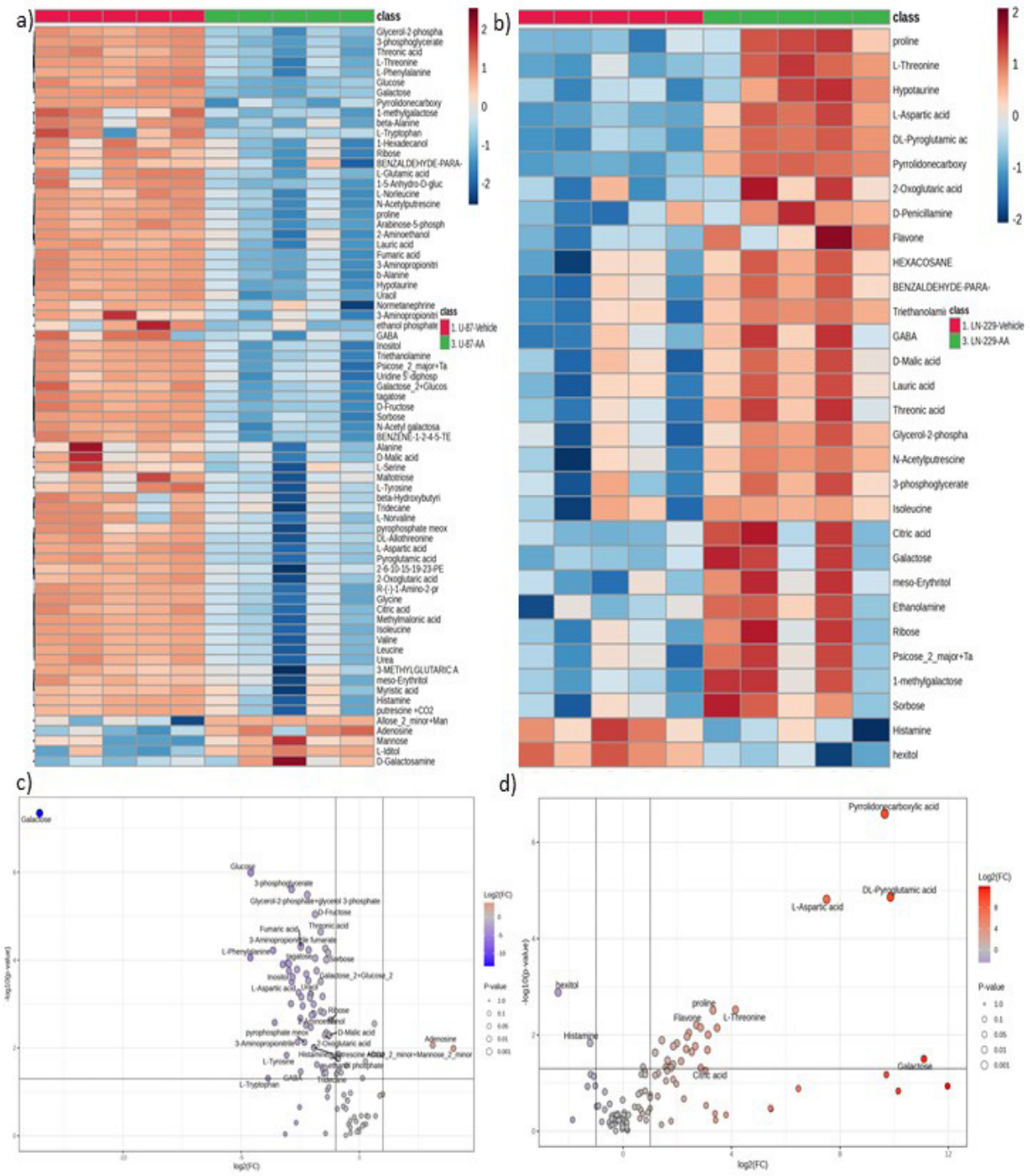

Figure 2. Metabolic Alterations in *Arsenicum iodatum* and *Arsenicum album* Treated U87 and LN-229 Cells based on GC/MS Measurements. Relative metabolite abundance is represented using a color scale, where blue indicates lower abundance and red indicates higher abundance. (a) Heatmap showing representative significantly altered metabolites in U87 cells upon Arsenicum album treatment ($p \leq 0.05$). (b) Heatmap showing significantly altered metabolite accumulation in LN-229 cells upon Arsenicum album treatment ($p \leq 0.05$). (c) Volcano plot depicting the $\log_2$ fold change in metabolite concentrations between control and Arsenicum album-treated U87 cells. (d) Volcano plot of LN-229 cells showing differentially regulated metabolites in Arsenicum album treated group ($\log_2FC \geq 2.0$ and $p \leq 0.05$; $n = 3$).

participated in carbohydrate, amino acids, lipid and nucleotide metabolism (Supplementary Figure 1). The overview of some of the important metabolic pathways crucial for cancer cell proliferation and accumulation of corresponding metabolites directly correlated with cell survival. Beta alanine, tyrosine, threonine, glycine, lysine, serine, arginine, proline, cysteine, methionine, valine, leucine and isoleucine were enriched amino

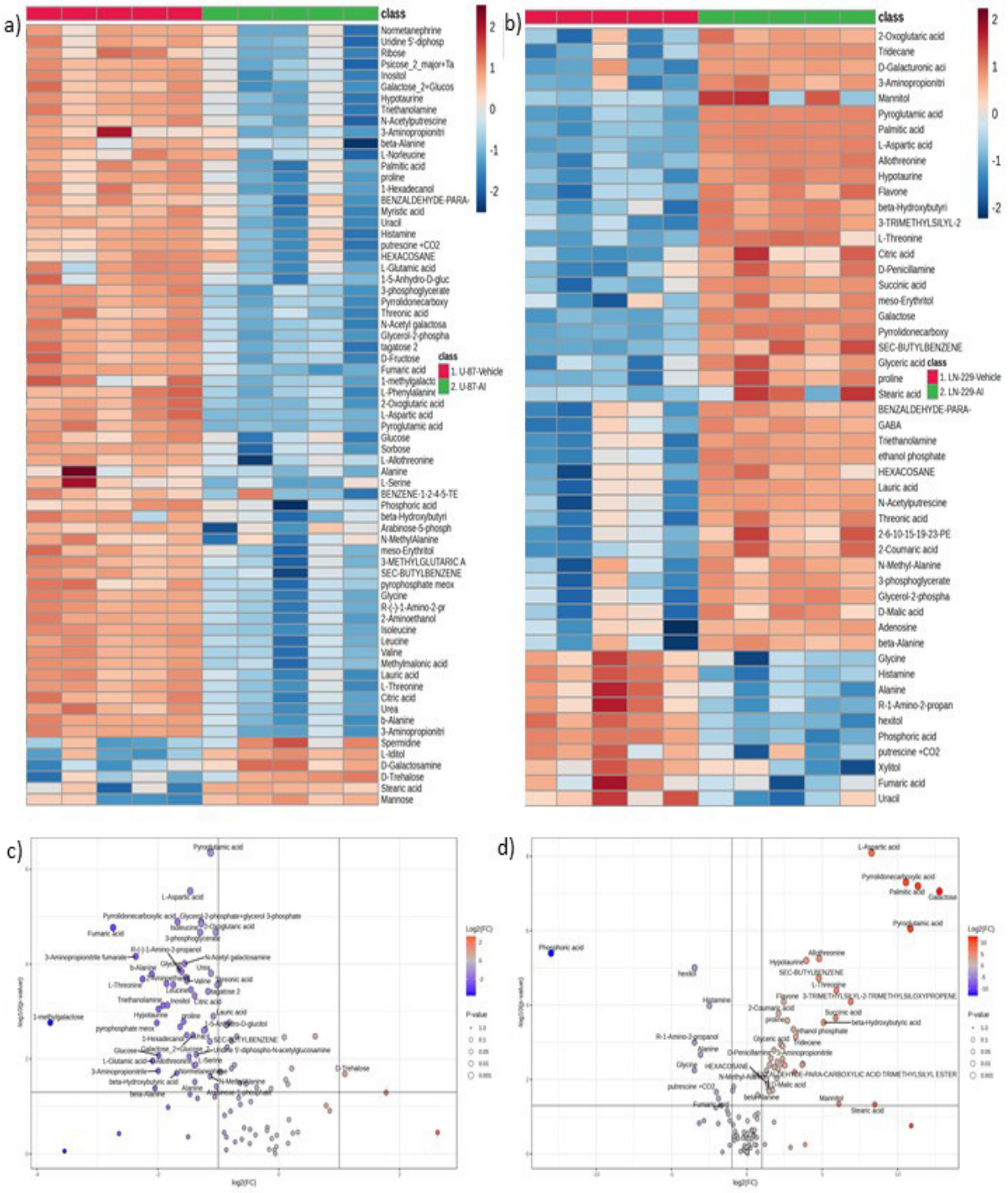

Figure 3. Hierarchical Clustering of *Arsenicum iodatum*-treated U87 and LN-229 Cells based on GC/MS Measurements of Metabolite Concentrations. (a) U-87 treated with Arsenicum iodatum (b) Arsenicum iodatum treated LN-229 (p-value ≤0.05). Volcano plot depicting the $\log_2$ fold change in metabolite concentrations between Control and Arsenicum album-treated U-87 GBM cells (c) and LN229 GBM cells (d) (log2 (FC) ≥ 2.0 and p-value ≤0.05).

acid metabolic pathways in treatment group. Nucleotide sugar metabolism, purine and their associate nicotinate, nicotinamide metabolism were also identified in enriched and altered pathway. Moreover, pathways associated with antioxidant siganling glutathione metabolism, glycine, serine and cysteine metabolism were reported downregulated in the *Arsenicum iodatum* and *Arsenicum album* treated group (Supplementary Figure 1a, 1b, 1c and 1d). Glycine and serine metabolism is the common metabolic pathways that was down regulated in both the cell line and drug treated group. It suggests that *Arsenicum iodatum* and album significantly impact and hinder antioxidant defense mechanism and promote cell death. Altered metabolic pathways along with their corresponding metabolites are listed in Supplementary Tables (1, 2, 3 and 4).

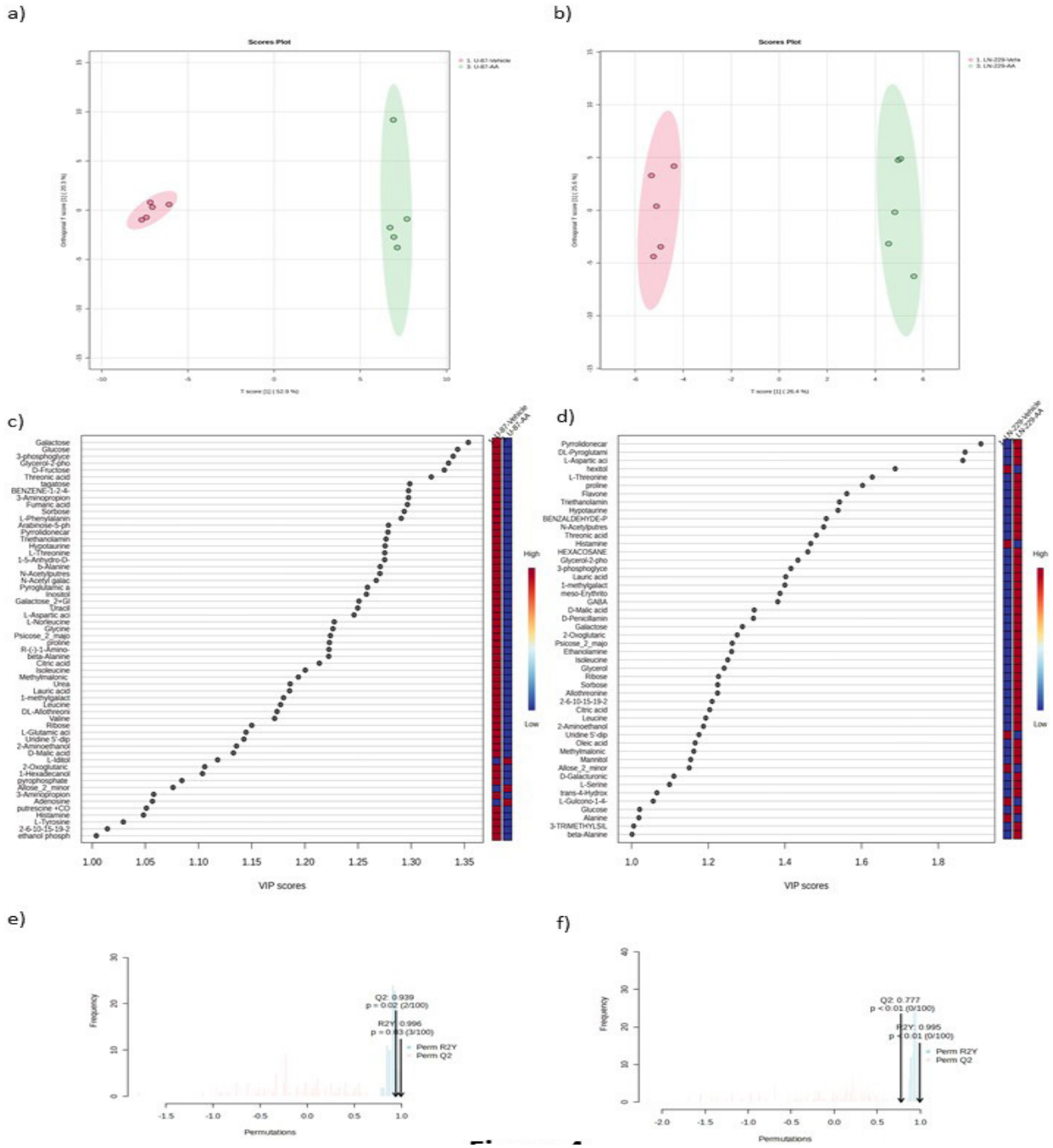

Figure 4. (a) OPLS-DA scores plot of *Arsenicum album* treated U87 cells (b) Score plot of Arsenicum album-treated LN-229 cells. Each point represents an individual sample, and the ellipses indicate the 95% confidence intervals for each group. (c) Variable importance in projection (VIP) score plot showing the top metabolite features (VIP score > 1.0) identified from OPLS-DA analysis in U-87 cells treated with Arsenicum album. (d) The VIP scores were derived from metabolite profiling data comparing control and Arsenicum album-treated LN-229 cells, highlighting the most influential variables contributing to group separation (VIP score > 1.0). (e) Model overview of the OPLS-DA models for the metabolite dataset in U-87 cells. (f) Permutation test applied for overview depicting the observed and cross-validated R2Y and Q2 coefficient in LN-229 cells.

Arsenicum Iodaum and Arsenicum album induces cell death in GBM cell lines via loss of mitochondrial membrane potential

The cytotoxic effects of *Arsenicum album* and *Arsenicum iodatum* were evaluated on GBM cell lines U87-MG and LN-229 using the trypan blue exclusion assay. The results showed a significant decrease in cell viability in both U87-MG and LN-229 cell lines following treatment with *Arsenicum album* and *Arsenicum iodatum*. The reduction in viability was potency-dependent, with the 30C preparations generally exhibiting the most pronounced cytotoxic effects (Supplementary Figure 2a and 2b).

To investigate whether *Arsenicum album* and *Arsenicum iodatum* induce cell death through a mitochondria-mediated pathway, changes in Δψm were assessed in U87-MG and LN-229 human glioma cell lines following treatment with 6C potency of each drug.

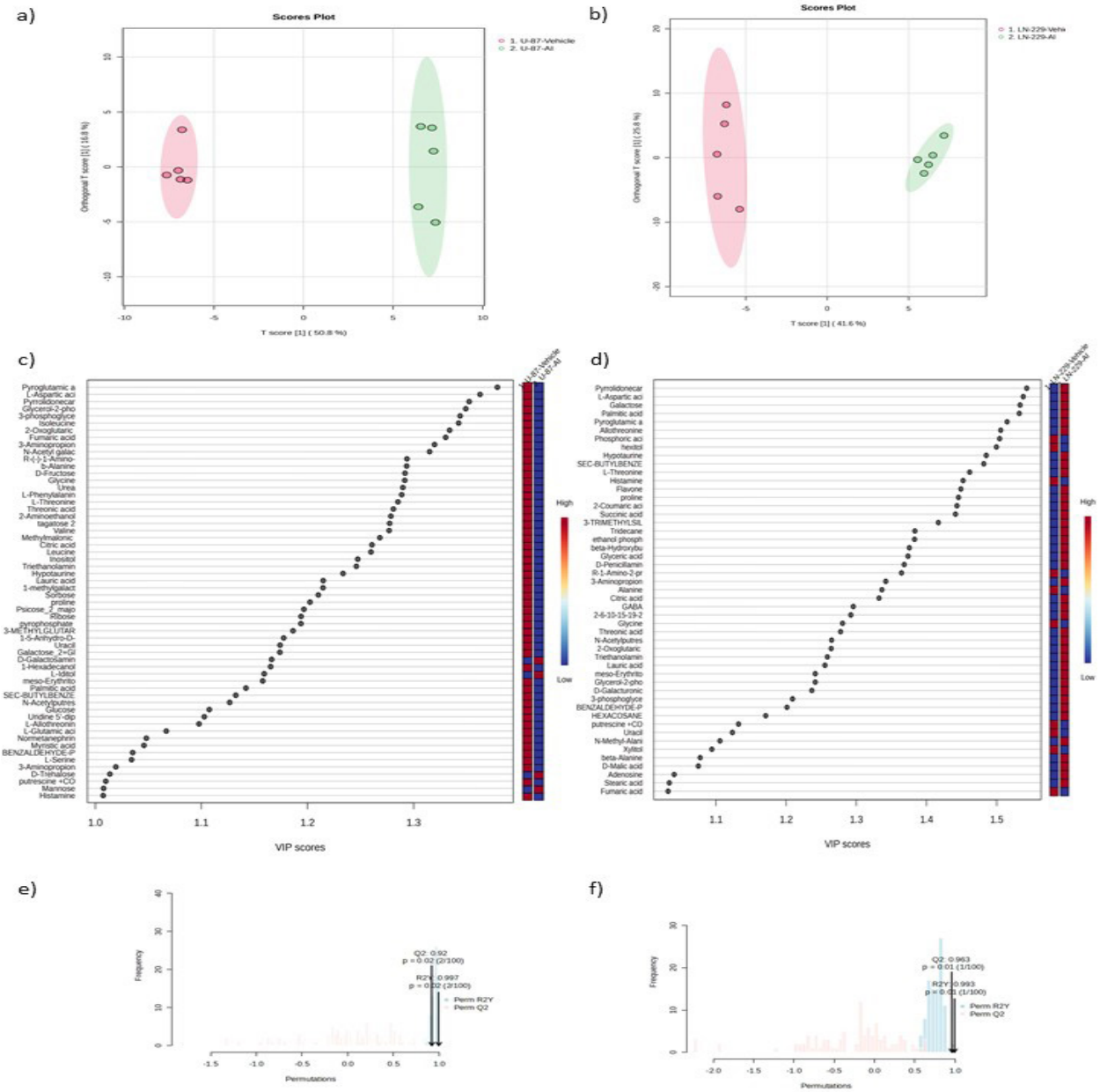

Figure 5. Multivariate Statistical Analysis in *Arsenicum iodatum* Treated Cells. (a) OPLS-DA scores plot of U87 cells (b) Score plot of LN-229 cells. (c) Variable importance in projection (VIP) plot showing the top metabolite features (VIP score > 1.0) identified from OPLS-DA analysis in U-87 cells. (d) The VIP plot were derived from metabolite profiling data comparing control and treated LN-229 cells (VIP score > 1.0). (e) Permutation test for OPLS-DA models in U-87 cells. (f) Permutation test results in LN-229 cells.

Loss of Δψm was monitored using a fluorescent dye JC-1, where an increase in green fluorescence indicates depolarization of the mitochondrial membrane. Treatment with both *Arsenicum album* and *Arsenicum iodatum* at 6C resulted in a significant increase in green fluorescence in both cell lines, indicating a rapid dissipation of Δψm. This loss of Δψm is a hallmark of early apoptotic signaling and suggests that mitochondrial dysfunction is involved in arsenic-induced cytotoxicity. The increase in fluorescence was more pronounced with *Arsenicum iodatum*, further supporting its comparatively higher cytotoxic potential observed in earlier viability assays. These results confirm the involvement of the intrinsic mitochondria mediated cell death pathway in arsenic-induced cytotoxicity in GBM cells (Supplementary Figure 2c and 2d).

Reactive oxygen species levels in *Arsenicum iodatum* and *Arsenicum album* treated cells: To evaluate the effect of *Arsenicum iodatum* and *Arsenicum album* (6C) on oxidative stress, we quantified intracellular ROS levels in U-87 and LN-229 glioblastoma cell lines using DCFDA-based flow cytometry. Our analysis revealed a significant increase in ROS levels in both cell lines upon treatment with *Arsenicum iodatum* and *Arsenicum album* (6C) compared to untreated controls. These findings indicate that both homeopathic preparations induce oxidative stress in GBM cells, potentially contributing to their cytotoxic effects (Supplementary Figure 3).

Discussion

In this study, Arsenic preparations successfully inhibited proliferation and induced cell death of malignant glioma cell lines. The metabolomic profiling of U-87 and LN-229 GBM cell lines treated with *Arsenicum album* and *Arsenicum iodatum* revealed significant metabolic alterations. Among 2,484 extracted EI-MS features, 107 metabolites were quantified. Univariate analysis identified 73 significantly altered metabolites in *Arsenicum album*-treated U-87 cells and 30 in LN-229 ($p < 0.05$). In U-87, 68 metabolites were up-accumulated, whereas LN-229 exhibited predominant decreased-accumulation (28 metabolites). Heatmap visualizations supported these findings, with color intensity indicating relative metabolite abundance. Similarly, *Arsenicum iodatum* treatment resulted in 69 and 50 altered metabolites in U-87 and LN-229 cells respectively, with a majority showing significant fold changes (log2FC > 2) in volcano plots. Multivariate OPLS-DA analyses demonstrated clear separation between treated and control groups, with VIP scores identifying 58 and 48 important metabolites in *Arsenicum album*-treated U-87 and LN-229 cells, respectively. The models were robust, as confirmed by permutation tests ($R^2 > 0.99$, $Q^2 > 0.77$, $p \leq 0.05$), ensuring reliability and biological relevance. These findings suggest that both homeopathic agents induce significant metabolic disruptions, potentially contributing to anti-cancer effects via altered metabolite abundance profiles.

Mitochondria play several essential roles, including energy production and cell signaling, which are tightly linked to cellular metabolism [25]. Cancer cells undergo significant metabolic reprogramming to meet the demands of rapid proliferation, survival, and adaptation to hypoxic tumor microenvironment [26]. In this study, treatment with *Arsenicum iodatum* and *Arsenicum album* induced substantial metabolic perturbations, particularly impacting amino acid and nucleotide metabolism, as well as antioxidant defense systems.

Among the amino acid metabolic pathways, beta-alanine, tyrosine, threonine, glycine, lysine, serine, arginine, proline, cysteine, methionine, valine, leucine, and isoleucine were found to have lower accumulation in the treatment groups. These amino acids play diverse roles in supporting cancer metabolism. For instance, glycine and serine are essential for one-carbon metabolism, a critical process for nucleotide synthesis and redox balance, and are often upregulated in rapidly proliferating cells [27]. Similarly, branched-chain amino acids like valine, leucine, and isoleucine contribute to protein synthesis and mTOR signaling, which are central to tumor growth [28].

Alterations were also observed in nucleotide sugar metabolism and purine metabolism, along with associated pathways such as nicotinate and nicotinamide metabolism. These changes are critical since purines are required for DNA and RNA synthesis, and nicotinamide is a key component of NAD+/NADP+, coenzymes involved in redox reactions and energy metabolism [29]. The disruption of these pathways upon treatment suggests a reduction in proliferative capacity and energy homeostasis in cancer cells.

Interestingly, antioxidant signaling pathways, including precursor of glutathione metabolism e.g. glycine, serine, and cysteine metabolism, were notably downregulated in the *Arsenicum iodatum* and *Arsenicum album* treated groups. Glutathione plays a central role in quantifying ROS and maintaining redox balance in cancer cells. Its synthesis relies heavily on cysteine availability and is supported by glycine and glutamate metabolism [30]. The observed downregulation of these pathways implies a compromised antioxidant defense, potentially rendering cells more susceptible to oxidative stress and promoting cell death.

Arsenic exposure is known to impair mitochondrial function by damaging mitochondrial integrity and halting biogenesis events that occur upstream of mitochondria-dependent apoptosis [31]. This process involves elevated ROS levels, which in turn cause oxidative stress and DNA damage, leading to cytotoxicity [32]. Our observations align with known mechanisms of anticancer drug-induced apoptosis. Importantly, oxidative stress results not only from increased ROS but also from the depletion of antioxidant defense systems, including thiols and enzymatic ROS scavengers [33].

Globally, an increasing number of individuals are turning to homeopathic medicines due to their perceived efficacy and minimal side effects [34]. Our study highlights the potential of *Arsenicum iodatum* and *Arsenicum album* as therapeutic agents or adjuvants for GBM, addressing unmet medical needs. Incorporating these molecules into nanocarrier-based delivery systems represents a promising approach—enhancing drug targeting, improving bioavailability, and minimizing off-target effects. This strategy ensures more efficient drug internalization into cancer cells while reducing systemic toxicity.

In conclusion, arsenic-based homeopathic preparations successfully inhibited proliferation and induced cell death in malignant glioma cell lines. Our findings support earlier studies on arsenic compounds in other cancers. We observed the relative efficacy and specificity of *Arsenicum iodatum* and *Arsenicum album*, and identified a novel drug delivery approach for targeting brain tumors. These results suggest that clinically safe concentrations of these compounds may serve as potential therapeutic agents for treating malignant gliomas.

Author Contribution Statement

All authors contributed equally in this study.

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Credit authorship contribution statement

AS conceptualized the idea of the manuscript. AS supervised the experiments, and writing-review and editing. AP performed experimental investigation, data curation, formal analysis, writing-original draft, writing-review and editing and validation. CK and MST performed experimental investigation, data curation, formal analysis and writing-review and editing. KP performed experimental investigation and data analysis. AKG was involved in supervising experimental design. NC supervised the study, experimental investigation and validated the manuscript. All authors concur with the final version of the manuscript.

Data availability statement

The datasets of this study are available in supplementary data.

If any scientific body approved it/ if it is part of an approved student thesis

No need of any scientific body approval because it is an invitro study. This data not a part of any student thesis.

Ethical approval

As it is invitro study no ethical clearance needed.

Any conflict of interest

Authors declare no conflict of interest.

Was the study registered in any registration dataset

The study was not registered in any trial registry, as it is an original in-vitro investigation and not a clinical trial, guideline, or meta-analysis.

Abbreviations

CNS: Central Nervous System
GBM: Glioblastoma Multiforme
HPLC: High Performance Liquid Chromatography
GC-MS: Gas Chromatography-Mass Spectrometry
OPLS-DA: Orthogonal Partial Least Square Discriminant Analysis
ROS: Reactive Oxygen Species
VIP: Variable Importance in Projection

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