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(RESEARCH ARTICLE)

EFFECT OF PHYTOCHEMICALS FROM HEXANE AND METHANOLIC BARK EXTRACTS OF *BETULA UTILIS* D. DON. ON CYTOTOXICITY AND ON APOPTOSIS-, PROLIFERATION-, AND INFLAMMATION-RELATED GENE EXPRESSION IN HUMAN U251 GLIOBLASTOMA CELLS: AN IN VITRO EXPERIMENTAL STUDY

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ABSTRACT

Background: Glioblastoma is the most common and aggressive primary malignant brain tumor in adults, and current chemotherapy offers limited benefit, which sustains interest in plant-derived cytotoxic agents. *Betula utilis* D. Don (Himalayan silver birch) is used traditionally for inflammatory and neoplastic conditions and contains cytotoxic triterpenoids, but its effects on glioblastoma cells have not been characterized.

Objective: To compare the cytotoxicity of hexane and methanol bark extracts of *B. utilis* against U251 human glioblastoma cells and to examine associated changes in apoptosis-, proliferation- and inflammation-related gene expression.

Methods: In this in vitro experimental study, U251 cells were treated with 25–200 µg/mL of each extract Viability was measured by the MTT assay in triplicate, and IC_{50} values were derived. We quantified BAX, BCL2, CDK4, MKI67, IL6, and PTGS2 expression by SYBR Green RT-qPCR ($2^{-\Delta\Delta Ct}$ method; *RPL13A* as reference) in three biological replicates. IC_{50} values were compared by two-way ANOVA.

Results: The methanol extract was more cytotoxic ($IC_{50} \approx 140$ µg/mL) than the hexane extract ($IC_{50} \approx 360$ µg/mL). Solvent, concentration level and their interaction all had significant effects ($P < 0.001$). Extract treatment markedly

increased *BAX* expression (up to approximately 90-fold) and significantly decreased *BCL2*, *CDK4*, *IL6*, *MKI67* and *PTGS2* ($P < 0.001$), with *MKI67* showing the greatest suppression.

Conclusion: *B. utilis* bark extracts, particularly the methanol extract, were cytotoxic to U251 cells, and this was accompanied by transcriptional changes consistent with intrinsic apoptosis, reduced proliferation and attenuated inflammatory signaling. These preliminary findings justify bioassay-guided fractionation and protein-level validation.

Keywords: Betula Utilis; Glioblastoma; U251; Cytotoxicity; Apoptosis; BAX/BCL2; RT-Qpcr; Natural Products

1 INTRODUCTION

Glioblastoma, classified as IDH-wildtype, CNS WHO grade 4 in the current WHO classification [1], is the most common and aggressive primary malignant brain tumor in adults. It is characterized by rapid proliferation, marked invasiveness and a poor prognosis despite maximal safe resection followed by radiotherapy and temozolomide chemotherapy; in the pivotal randomized trial, median survival with this regimen was 14.6 months [2]. The disease is almost universally fatal after recurrence [3,4]. The limited efficacy and considerable toxicity of existing cytotoxic drugs, together with the restrictive nature of the blood–brain barrier, sustain the search for new chemical scaffolds, including plant-derived molecules capable of inducing selective apoptosis in glioma cells [3].

Natural products and their derivatives remain an important source of anticancer drug leads [5]. *Betula utilis* D. Don (Himalayan silver birch; family Betulaceae) is a high-altitude Himalayan tree whose bark has long been used in traditional medicine for inflammatory, dermatological and neoplastic conditions [6]. Phytochemical studies of the bark have identified pentacyclic triterpenoids, including betulin, betulinic acid, lupeol, ursolic acid, oleanolic acid and β-amyrin, several of which show tumor-cell-selective cytotoxic and pro-apoptotic activity in other cancer models [6,7]. In a bioassay-guided study, a methanolic extract of *B. utilis* bark was fractionated and screened against nine human cancer cell lines; the ethyl acetate fraction was among the most potent, and ursolic acid showed selectivity for breast cancer cells over non-tumorigenic breast epithelial cells [7].

Related birch triterpenoids suggest that glioma cells may be susceptible. Betulinic acid, a lupane-type triterpenoid from birch bark, is cytotoxic to a range of tumor cell lines [8,9]. In glioma models it has been reported to inhibit proliferation, to increase BAX and decrease BCL-2 levels [10], and to enhance radiosensitivity and temozolomide-induced cytotoxicity [9,11]. Crude birch bark extracts, in which betulin predominates, have also been compared with purified betulin and betulinic acid for cytotoxicity in carcinoma cell lines [12]. Whether crude bark extracts of *B. utilis* act against central nervous system tumors such as glioblastoma has not, to our knowledge, been examined.

Apoptosis in glioma cells is governed largely by the BCL-2 protein family, in which pro-apoptotic effectors such as BAX oppose pro-survival members such as BCL-2 to determine commitment to mitochondrial (intrinsic) apoptosis [13]. In U251 cells, the relative expression of Bax and Bcl-XL determines whether Bax overexpression results in apoptotic or necrotic death [14], and plant-derived compounds that raise the Bax:Bcl-2 ratio and modulate cell-cycle regulators inhibit U251 proliferation [15]. Proliferation can be monitored through *CDK4*, which drives G1-to-S progression, and *MKI67* (Ki-67), a nuclear proliferation antigen of established prognostic relevance in glioblastoma [16]. Inflammatory mediators also contribute to glioma biology: IL-6 expression rises with glioma malignancy grade and the *IL6* gene is amplified in a subset of glioblastomas [17,18], and cyclooxygenase-2 (encoded by *PTGS2*) has been explored as a therapeutic target [3]. A coordinated rise in the *BAX*:*BCL2* ratio together with decreases in proliferation and inflammation markers is therefore generally interpreted as transcriptional evidence of an anti-tumor, pro-apoptotic response.

The present study therefore aimed (i) to compare the cytotoxicity of hexane and methanol bark extracts of *B. utilis* against U251 human glioblastoma cells using the MTT assay, and (ii) to characterize the accompanying transcriptional response of apoptosis (*BAX*, *BCL2*), proliferation (*CDK4*, *MKI67*) and inflammation (*IL6*, *PTGS2*) markers by RT-qPCR, in order to provide preliminary mechanistic evidence for the anti-glioma potential of this traditionally used Himalayan bark.

2 MATERIAL AND METHODS

2.1 Study design and setting

This was a controlled, laboratory-based in vitro experimental study (Figure 1). The independent variables were extraction solvent (hexane or methanol) and extract concentration (25, 50, 100 and 200 µg/mL). The dependent variables were U251 cell viability, expressed as IC_{50}, and the relative expression of six target genes, assessed using RT-qPCR.. The process starts with a control group, an untreated brain cancer cell line and treatment groups. Cells were

treated with increasing concentrations of phytochemical extract obtained using methanol and hexane at 25 µg/ml, 50µg/ml, 100 µg/ml, and 200 µg/ml for 24, 48, and 72 hours. Untreated cells served as the control. Plant material was collected from Mustang District, Nepal, in October 2024. The laboratory experiments, including extract preparation, cytotoxicity assessment, and gene expression analysis, were conducted at Annapurna Research Centre, Maitighar, Kathmandu, Nepal, between Jan. 2026 and July 2026.

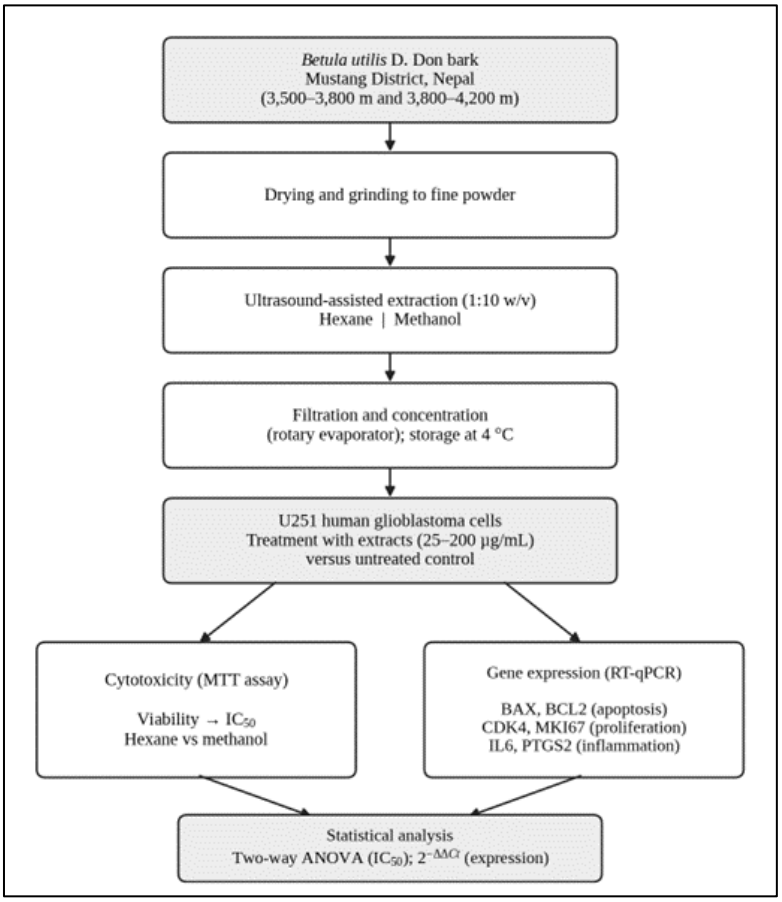


Figure 1: Study workflow. Bark of *Betula utilis* was extracted with hexane and methanol and applied to U251 glioblastoma cells to measure cytotoxicity (MTT assay; IC_{50}) and the expression of apoptosis-, proliferation- and inflammation-related genes (RT-qPCR).

2.2 Plant material and extract preparation

Bark samples of Betula utilis D. Don were collected from naturally occurring populations in Mustang District, Gandaki Province, Nepal, during October 2024. Sampling covered 14 locations along an altitudinal gradient of 3,647–4,140 m above sea level. Three healthy, mature and disease-free trees were selected from each location, resulting in a total of 42 individual trees. Trees were selected at least 20–30 m apart, and bark samples were collected approximately 1 m above ground level. Botanical identification was confirmed by qualified taxonomists at the National Herbarium and Plant Laboratories (KATH), Godawari, Lalitpur, Nepal, where voucher specimens were deposited for permanent preservation and future reference.

Dried Betula utilis D. Don bark was ground into a fine powder using a mechanical grinder and stored in airtight amber glass containers at 4°C until extraction. The powdered samples were extracted separately with hexane and methanol at a sample-to-solvent ratio of 1:10 (w/v) using an ultrasonic extractor (Biobase UC-30A). The extracts were filtered, concentrated to dryness under reduced pressure using a rotary evaporator (Biobase), and stored at 4°C until further use. For cell assays, extract stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted with culture medium to obtain final concentrations of 25, 50, 100 and 200 µg/mL.

2.3 Cell line

The human glioblastoma cell line U251 was obtained from the Department of Neurosurgery, Kagoshima University, Japan. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.2.4 Cytotoxicity assay (MTT).

Cytotoxicity was assessed using the MTT colorimetric assay [19] with minor modifications. Cells were seeded at 4×10^4 cells per well in 96-well flat-bottomed plates, allowed to adhere for 24 h, and treated with hexane or methanol extracts at 25, 50, 100 or 200 µg/mL for 24, 48 and 72 h. Each condition were tested in triplicate alongside untreated controls. After treatment, the supernatant was discarded, and 150 µL of medium containing 20 µL of MTT solution was added to each well and incubated for 4 h. Formazan crystals were dissolved in 100 µL of DMSO, and absorbance was measured at 595 nm with a reference wavelength of 630 nm. Cell viability was calculated relative to untreated controls, and IC_{50} values were determined for each extract and treatment condition.

2.4 RNA extraction, cDNA synthesis and RT-qPCR

For gene expression analysis, U251 cells were treated with *B. utilis* bark extracts from the highest (4,140 m) and lowest (3,647 m) collection altitudes; confirm extract type (hexane or methanol), treatment concentration and exposure time, and the identity of the two treatment groups (TCH and TBH), and compared with untreated controls. Total RNA was isolated using TRIzol reagent followed by the Direct-zol RNA MiniPrep Kit (Zymo Research, USA) according to the manufacturer's protocol. RNA concentration and purity were determined spectrophotometrically (NanoDrop), and only samples with an A_{260}/A_{280} ratio of 1.8–2.0 were used. First-strand cDNA was synthesized from 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed on an Applied Biosystems real-time PCR system using SYBR Green master mix (Thermo Scientific) and gene-specific primers for BAX, BCL2, CDK4, IL6, MKI67 and PTGS2. RPL13A served as the reference gene. Three independent biological replicates were processed per condition, and all reactions were run in technical triplicate. Relative expression was calculated by the comparative $2^{-\Delta\Delta Ct}$ method [20] and expressed relative to the untreated control (set to 1).

2.5 Statistical analysis

IC50 values were compared by two-way analysis of variance (ANOVA) with extraction solvent (hexane, methanol) and concentration level (25, 50, 100, 200 µg/mL) as fixed factors, including their interaction. Pairwise comparisons was used for gene expression; biological replicates were the experimental unit and Student's t-test was applied for comparisons between two groups, while one-way ANOVA followed by an appropriate post hoc multiple-comparison test was used for comparisons among multiple treatment groups. Data are presented as mean ± SD, and $P < 0.05$ was considered statistically significant. Analyses were performed in R (version 4.5.1) and Python (version 3.14.0).

3 RESULTS

3.1 Cytotoxic activity against U251 cells

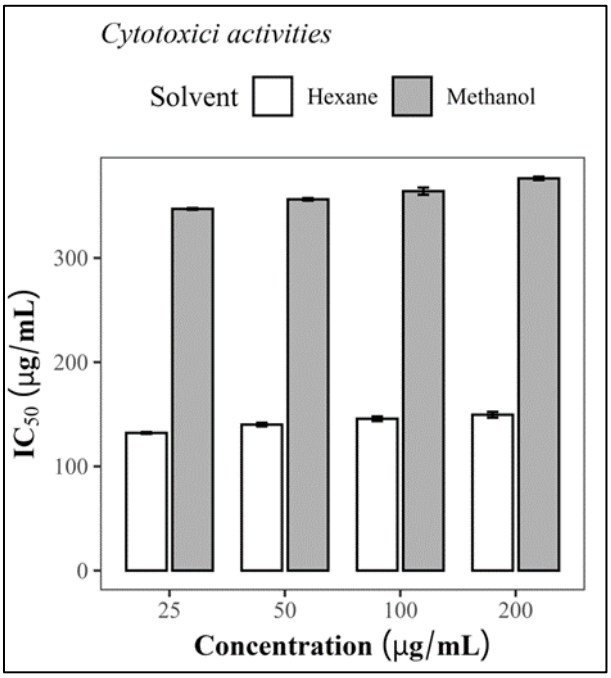


Figure 2: IC_{50} (µg/mL) of hexane and methanol *B. utilis* bark extracts against U251 cells across the four concentration levels tested. Bars show mean ± SEM

Both extracts reduced the viability of U251 glioblastoma cells, but the methanol extract was markedly more potent. Across the four concentration levels tested, IC_{50} values of the methanol extract were approximately 130–150 µg/mL,

compared with approximately 345–375 µg/mL for the hexane extract (Figure 2; Table 1). Pooled across levels, the mean IC_{50} of the methanol extract (≈ 140 µg/mL) was less than half that of the hexane extract (≈ 360 µg/mL), and the two extracts fell into distinct post hoc groups (Figure 3). IC_{50} values varied only modestly across the four concentration levels, and the gap between the two solvents remained wide throughout.

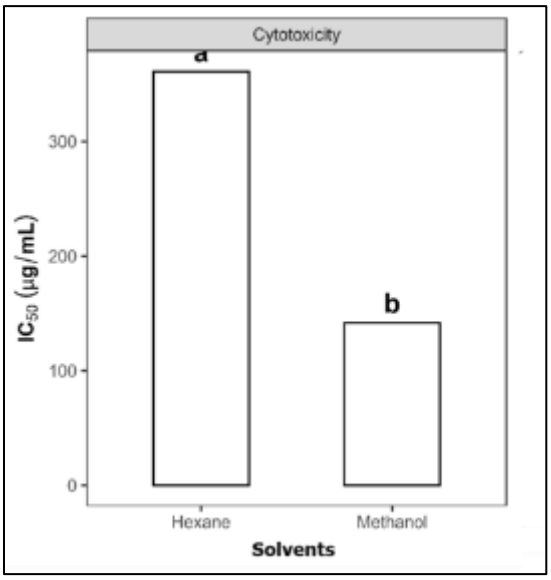


Figure 3: Mean IC_{50} (µg/mL) of hexane and methanol *B. utilis* bark extracts against U251 cells. Bars not sharing a letter (a, b) differ significantly on post hoc comparison.

Table 1: Cytotoxicity (IC_{50}) of *B. utilis* bark extracts against U251 cells.

Extract	IC_{50} range across concentration levels (µg/mL)	Pooled mean IC_{50} (µg/mL)	Post hoc group
Hexane	≈ 345–375	≈ 360	a
Methanol	≈ 130–150	≈ 140	b

Values are approximate. Groups not sharing a letter differ significantly.

Two-way ANOVA showed that solvent was the dominant source of variation in IC_{50} ($F_{1,16}$ = 71,723.6, P < 0.001). Concentration level ($F_{3,16}$ = 147.3, P < 0.001) and the solvent × concentration interaction ($F_{3,16}$ = 10.6, P < 0.001) were also significant, indicating that the advantage of the methanol extract over the hexane extract was not uniform across concentration levels (Table 2).

Table 2: Two-way ANOVA of IC_{50} by extraction solvent and concentration level.

Source of variation	df	Sum of squares	Mean square	F value	P value	Sig.
Solvent	1	288,112	288,112	71,723.58	< 0.001	
Concentration	3	1,775	592	147.29	< 0.001	
Solvent × Concentration	3	128	43	10.59	< 0.001	
Residuals	16	64	4	—	—	—

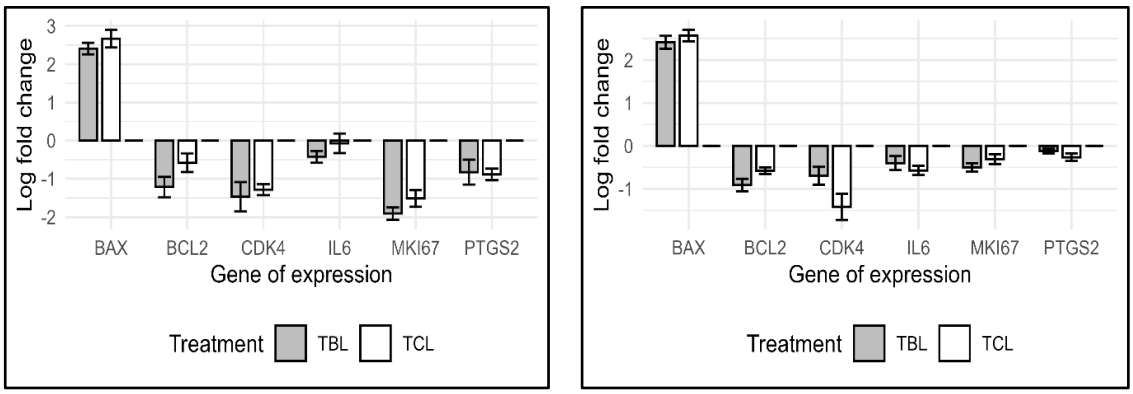
df, degrees of freedom; Sig., significance (*** P < 0.001).

3.2 Expression of apoptosis-, proliferation- and inflammation-related genes

Extract treatment was accompanied by a consistent shift in the expression of all six target genes relative to untreated control cells (Figure 4; Table 3). The pro-apoptotic gene *BAX* was strongly upregulated in both extract-treated groups, by approximately 90-fold in one group and approximately 40-fold in the other (Figure 4A). verify whether BAX expression values (~90 and ~40) represent linear fold change or $\log_2$ fold change, and provide the gene-specific P values for each treatment group; the thesis reports only an overall treatment effect of P < 0.001. In contrast, the anti-apoptotic gene *BCL2* and the proliferation- and inflammation-associated genes *CDK4*, *IL6*, *MKI67* and *PTGS2* were all significantly downregulated (P < 0.001 for each comparison; Figure 4B). *MKI67* showed near-complete suppression in both groups;

BCL2, *CDK4* and *IL6* were reduced to a lesser but still significant extent; and *PTGS2* showed the most variable response between the two groups.

When all six genes were plotted on a common $\log_2$ scale (Figure 4C), *BAX* was the only upregulated gene in both groups, and *MKI67* showed by far the largest decrease. This pattern of concurrent *BAX* induction and suppression of *BCL2*, *CDK4*, *MKI67*, *IL6* and *PTGS2* is consistent with activation of the intrinsic apoptotic pathway together with reduced proliferative and inflammatory signaling.



U20S cell line (A) U251 cell line (B)

Figure 4: Relative expression of *BAX*, *BCL2*, *CDK4*, *IL6*, *MKI67* and *PTGS2* in U251 cells treated with *B. utilis* bark extract relative to untreated control (set to 1). (A) *BAX*. (B) *BCL2*, *CDK4*, *IL6*, *MKI67* and *PTGS2*. (C) All six genes on a common $\log_2$ scale.

Data are mean ± SEM of three biological replicates.

Table 3: Summary of gene expression changes in extract-treated U251 cells relative to untreated control.

Gene	Biological role	Direction of change	Reported magnitude / significance
BAX	Pro-apoptotic effector	Increased	≈ 90- and ≈ 40-fold in the two treated groups
BCL2	Anti-apoptotic	Decreased	$P < 0.001$
CDK4	G1-to-S cell-cycle progression	Decreased	$P < 0.001$
MKI67	Proliferation marker	Decreased (near-complete suppression)	$P < 0.001$; largest decrease
IL6	Pro-inflammatory cytokine	Decreased	$P < 0.001$
PTGS2	Inflammation (COX-2)	Decreased	$P < 0.001$; most variable between groups

4 DISCUSSION

4.1 Principal findings

This study provides preliminary in vitro evidence that hexane and methanol bark extracts of *B. utilis* reduce the viability of U251 human glioblastoma cells in a solvent-dependent manner, and that this effect coincides with a coordinated shift in apoptosis-, proliferation- and inflammation-related gene expression. The methanol extract was roughly 2.5-fold more potent than the hexane extract (IC_{50} ≈ 140 versus ≈ 360 µg/mL).

4.2 Solvent-dependent cytotoxicity

The greater potency of the methanol extract is in line with earlier work on *B. utilis* bark, in which a methanolic extract fractionated by solvent polarity yielded an ethyl acetate fraction that was among the most active across nine cancer cell lines, and in which ursolic acid and other triterpenoids were isolated [7]. Hexane preferentially extracts non-polar, lipophilic constituents, whereas methanol additionally recovers more polar phenolics, flavonoids and glycosylated

triterpenoids. The solvent-dependent difference observed here may therefore reflect a higher content of polar cytotoxic constituents in the methanol extract. This interpretation remains provisional, because individual phytochemicals were neither isolated nor quantified.

An IC_{50} of about 140 μg/mL indicates moderate potency, as expected for a crude, unfractionated preparation. For comparison, betulinic acid has been reported to increase apoptosis in U87MG and A172 glioblastoma cells at 8.75 μg/mL [10], although that study used purified compound, different cell lines and a different end point. Such comparisons suggest that bioassay-guided fractionation could concentrate the active constituents of the bark.

4.3 Apoptosis-related gene expression

The marked increase in *BAX* together with the decrease in *BCL2* is consistent with a shift toward the mitochondrial (intrinsic) apoptotic pathway, in which the balance between pro-apoptotic and pro-survival BCL-2 family members sets the threshold for cell death [13]. A similar pattern, with increased Bax and decreased Bcl-2 and survivin, was reported at the protein level in U87MG and A172 glioblastoma cells treated with betulinic acid [10], and betulinic acid has been described as inducing apoptosis and enhancing temozolomide cytotoxicity in glioblastoma models [11]. In U251 cells specifically, the relative level of Bax and Bcl-XL determines whether cells die by apoptosis or necrosis [14], and Moringa-derived isothiocyanate quinazolinone derivatives raise the Bax:Bcl-2 ratio and induce apoptosis in this cell line [15]. The magnitude of *BAX* induction (up to approximately 90-fold) suggests a strong transcriptional response; however, mRNA changes alone do not establish apoptosis, and confirmation by Western blotting, Annexin V/propidium iodide flow cytometry or caspase activity assays is required.

4.4 Proliferation- and inflammation-related gene expression

The near-complete suppression of *MKI67*, a standard marker of proliferating cells with prognostic relevance in glioblastoma [16], indicates reduced proliferative activity in treated cells. The concurrent decline in *CDK4*, a kinase central to G1-to-S transition, is compatible with impaired cell-cycle progression, in keeping with reports that plant-derived compounds combine Bax:Bcl-2 modulation with suppression of cell-cycle regulators in U251 cells [15]. Cell-cycle arrest was not measured directly here. A comparable combination of reduced proliferation and invasion with induction of apoptosis has been reported in U251 cells treated with the flavonoid glycoside vitexin [21], supporting the plausibility of a plant-derived agent producing this phenotype in glioblastoma cells.

The reduction in *IL6* and *PTGS2* is also of interest, because IL-6 expression rises with glioma malignancy grade [17,18] and COX-2 has been explored as a therapeutic target in glioma [3]. These data suggest that the extract may attenuate pro-tumorigenic inflammatory signaling. However, transcript levels of many genes fall in cells that are dying or have stopped proliferating, so the present design cannot distinguish a specific anti-inflammatory effect from a secondary consequence of reduced cell viability; dedicated assays, such as measurement of secreted IL-6 or prostaglandin E_2, would be needed.

Limitations

Several limitations should be considered. First, gene expression was assessed at the mRNA level only, without protein-level or functional apoptosis assays, so the transcriptional changes are indicative rather than conclusive evidence of apoptosis. Second, all data derive from a single glioblastoma cell line and no non-tumor cell control was included, so selectivity for tumor over normal cells cannot be inferred. Third, the extracts were crude and chemically uncharacterized, and the constituents responsible for the activity are unknown. Fourth, the IC_{50} of the hexane extract exceeded the highest concentration tested (200 μg/mL) and is therefore an extrapolated estimate. Fifth, the treatment conditions used for gene expression analysis (extract, dose and exposure time) require full definition. Finally, all findings are in vitro, and in vivo validation, including consideration of blood–brain barrier penetration, is needed before any therapeutic inference can be drawn.

Future directions

Priorities for further work are bioassay-guided fractionation with chemical profiling of the active fractions; confirmation of apoptosis and cell-cycle effects at the protein and functional levels; testing in additional glioma cell lines alongside non-tumor astrocytes; and evaluation of combinations with temozolomide.

Abbreviations

Abbreviation	Definition
BAX	BCL2-associated X protein; pro-apoptotic effector of the intrinsic (mitochondrial) apoptotic pathway

BCL2	B-cell lymphoma 2; anti-apoptotic (pro-survival) member of the BCL-2 protein family
CDK4	Cyclin-dependent kinase 4; regulates G1-to-S phase cell-cycle progression
IL6	Interleukin-6; pro-inflammatory cytokine implicated in glioma progression
MKI67	Marker of proliferation Ki-67; nuclear antigen expressed in proliferating cells
PTGS2	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2, COX-2)
RPL13A	Ribosomal protein L13a; reference (housekeeping) gene
ANOVA	Analysis of variance
Ct	Threshold cycle in quantitative PCR
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
IC_{50}	Half-maximal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
WHO	World Health Organization

5 CONCLUSION

The methanol bark extract of *B. utilis* was more cytotoxic to U251 glioblastoma cells than the hexane extract, and this activity was accompanied by gene expression changes consistent with intrinsic apoptosis and reduced proliferative and inflammatory signaling. These preliminary findings support further bioassay-guided fractionation and protein-level validation of *B. utilis* as a source of anti-glioma leads.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

The authors declare no conflict of interest

Statement of ethical approval

Use this as a draft, but do not claim that no institutional approval was required until the approval certificate and collection permissions have been checked

Author contributions

Sunita Koirala: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Writing - Original Draft, Writing -Review & Editing. Himal Luitel: Conceptualization, Supervision. Dipak Paudel: Conceptualization, Methodology, Investigation.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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