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RESEARCH ARTICLE

Investigating the antiproliferative properties of *Amaryllidaceae* plant species and their bioactive compounds on brain tumour cell lines

Sylvester I. Omoruyi[1,2] · Tanya N. Augustine[1] · Lawrence Mabasa[3,6] · Ahmed A. Hussein[4] · Vuyo Mavumengwana[5]

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Abstract

Glioblastoma multiforme is considered the most aggressive type of brain tumour due to its highly invasive properties that make complete surgical resection almost impossible and treatment very challenging. The current treatment for glioblastoma involves surgery followed by radiotherapy and chemotherapy. Despite these treatment options, tumour recurrence and toxicity from the chemotherapeutic agents remain problematic, which calls for novel treatment approaches. In this study, we investigate the antiproliferative activities of three *Amaryllidaceae* plant species, *Crossyne flava*, *Amaryllis belladonna*, and *Boophone haemanthiodes,* as well as their isolated bioactive compounds on U87 and U251 glioblastoma cell lines, with H9C2 cardiac myocyte used as a normal cell line. The effect of plant extracts and compounds on cell viability and long-term survival was determined using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] and clonogenic assay, respectively. Additionally, the ATP levels and apoptosis-inducing potential of the plant extracts and compounds were determined using the Promega Mitochondrial ToxGlo™ and Caspase-Glo™ 3/7 assay kits, respectively. The results reveal that both plant extracts and compounds induce cytotoxicity in glioblastoma cell lines, and the extracts also inhibit the long-term survival of U87 and U251 cells. The extracts were also selective to the cancer cells when the selectivity index was calculated. Furthermore, the plant extracts and compounds inhibited ATP production in the cancer cells, while induction of apoptosis was only evident in the compound-treated cells. Overall, our findings suggest that the *Amaryllidaceae* plant family could be a rich source of botanicals and phytochemicals that might be effective against glioblastoma.

Keywords *Amaryllidaceae* · Glioblastoma · Antiproliferative · Cytotoxicity · Apoptosis

Introduction

Glioblastoma is the most aggressive of all primary malignant brain tumours in adults, and they invade deep into the surrounding brain, so completely removing all the cancerous tissues during surgery is impossible (Louis et al. 2007; Wen and Kesari 2008; Mahajan et al. 2015). Glioblastoma cells are rapidly proliferating cells, making them difficult to treat, and their high proliferation rate and invasiveness are sustained by increased demand for ATP (Pavlova and Thompson 2016). This is further supported by metabolic changes characterized by the Warburg effect, where cancer cells

✉ Sylvester I. Omoruyi
sylvester.omoruyi@wits.ac.za

1 School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Parktown, Johannesburg 2193, South Africa

2 Department of Global Health, African Cancer Institute, Stellenbosch University, Tygerberg, Cape Town 7505, South Africa

3 Biomedical Research and Innovation Platform, South African Medical Research Council, Francie Van Zijl Dr, Tygerberg, Cape Town 7505, South Africa

4 Department of Chemistry, Cape Peninsula University of Technology, Symphony Road, Bellville 7530, South Africa

5 Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research, Centre for Tuberculosis Research,, Stellenbosch University, Cape Town 7505, South Africa

6 Department of Medical Physiology, Stellenbosch University, Tygerberg, Cape Town 7505, South Africa

switch to glycolysis over oxidative phosphorylation, even in the presence of an adequate oxygen supply (Vander Heiden et al. 2009). Though this process is relatively less effective in terms of ATP yield, it provides intermediates necessary for biosynthetic pathways critical to tumour progression(Lunt and Vander Heiden 2011; Agnihotri and Zadeh 2015; Cortes Ballen et al. 2024). In addition, ATP is actively released into the tumour microenvironment by glioblastoma cells, orchestrating cell signalling and immune evasion, culminating in increased cell proliferation and bypassing apoptosis (Garcia et al. 2021). Thus, interventions aimed at reducing intracellular ATP levels, such as inhibiting nutrient uptake (e.g., glucose or glutamine), can trigger energy crises in glioblastoma cells, resulting in cell death, either in the form of apoptosis or necrosis (Garcia et al. 2021; Yang et al. 2022).

The current treatment for glioblastoma involves surgery, followed by radiotherapy and chemotherapy with temozolomide, but even with these treatments, patients may only survive for about 15 months on average from diagnosis (Ferraris et al. 2020; Thakkar et al. 2014). The survival rate after 5 years is less than 5%, and tumour recurrence is a significant challenge (Chang et al. 2016). Clinical trials for developing new drugs have shown a poor success rate. This can be attributed in part to the lack of relevant preclinical models, the presence of the blood–brain barrier and the unique tumour microenvironment of brain tumour tissues, which could render some chemotherapeutic agents less potent as well as systemic toxicities that may arise from the usage of some anticancer agents (Aldape et al. 2019; Rominiyi et al. 2019). It is crucial to note that many patients across Sub-Saharan Africa cannot access or afford surgery, chemo- and radiotherapy. Additionally, treatments that might benefit patients in various traditional African healthcare settings are rarely studied. Therefore, there is an urgent unmet need to investigate new therapeutic options that might be effective and affordable for treating glioblastoma.

The use of plants and herbs as medicine has been documented for over 5000 years (Fridlender et al. 2015; Ozioma and Chinwe, 2019). It's interesting to note that reports suggest that 75–90% of people in underdeveloped and developing countries rely on traditional medicine for treating various ailments, including cardiovascular, renal, and neurodegenerative diseases (Fridlender et al. 2015; Aryan 2018; Ozioma and Chinwe, 2019). Medicinal plants have also been found to be useful in treating various types of cancer, due to their antineoplastic and apoptosis-inducing effects (Roy et al. 2018; Rezadoost et al. 2019; Verma and Singh 2020). Researchers have isolated bioactive ingredients from numerous plant species to further validate their importance; some compounds are currently being used for cancer treatment or in clinical trials to test their efficacy (Greenwell and Rahman 2015; Gezici and Şekeroğlu 2019; Baradaran Rahimi et al. 2020). For instance, anticancer agents vincristine, paclitaxel, epigallocatechin-3-gallate, and noscapine were first isolated from *Catharanthus roseus, Taxus brevifolia*, green tea, and *Papaver somniferum*, respectively, and are now synthetically produced (Chen et al. 2015; Zhang and Kanakkanthara 2020; Škubník et al. 2021; Yin et al. 2021).

The *Amaryllidaceae* plant family is widely recognised and has gained attention in medicinal plant research due to its diverse usage in treating ailments and diseases, such as wound infections, infertility, malaria, and cancer (Nair and Van Staden 2013; Nair et al. 2017). With approximately 1000 species, these plants are distributed worldwide but are predominantly found in South America, the Mediterranean, and Southern Africa. Notably, some species, like *Boophone* and *Brunsvigia,* are unique to South Africa (Meerow and Snijman 1998; Nair and Van Staden 2014). The plant family has been studied for their potential as anticancer agents, and ongoing research aims to identify the bioactive compounds responsible for their anticancer activities (Nair and Van Staden 2021b). In particular, *Lycoris radiata*, *Hymenocallis littoralis*, *Amaryllis belladonna L.*, *Boophone disticha*, and *Crinum delagoense* have gained attention for their usage in managing cancer both within and outside of South Africa (Graham et al. 2000; Caamal-Fuentes et al. 2011; Nair and Van Staden 2013) and in vitro have validated their traditional use (Jokhadze et al. 2007; Isbilen et al. 2018; Omoruyi et al. 2021b). Strikingly, *Amaryllidaceae* plants are known for their unique alkaloids. Remarkable amongst them is galantamine, which was approved by the United States Food and Drugs Administration for the treatment of Alzheimer's disease (Heinrich and Teoh 2004). Moreover, like the plant species, isolated alkaloids from the plants have been documented to possess anticancer potentials (Zupko et al. 2009; Havelek et al. 2014; Masi et al. 2019). Despite some species being studied for their anticancer properties, there is a lack of data on the activities of *C. flava, B. haemanthoides, and A. belladonna* against brain tumours. Therefore, this study provides evidence of the antiproliferative activities of the bulbs of the three plant species and isolated compounds in the U87 and U251 glioblastoma cell lines.

Materials and methods

Collection and identification of plant material

Crossyne flava, *B. haemanthoides* and *A. belladonna* were collected from the Karoo Desert National Botanical Garden in Worcester, Western Cape Province, South Africa. After collection, identification and authentication were done by a plant biologist, Professor Christopher Cupido, Botany Department, the University of Fort Hare, and voucher specimens were deposited in the Giffen Herbarium of the University of Fort Hare (UFH). The accession numbers were

UFH 2020-4-20, UFH 2020-4-11 and UFH 2020-3-01 for *C. flava*, *B. haemanthoides* and *A. belladonna*, respectively.

Preparation of plant material and isolation of compounds

The preparation of plant materials and isolation of compounds were performed at the Natural Products Chemistry Team laboratory, Department of Chemistry, Cape Peninsula University of Technology. Briefly, fresh bulbs weighing about 200 g each of *C. flava*, *B. haemanthoides* and *A. belladonna* were homogenised separately and extracted with methanol for two days at room temperature, and the extracts were filtered afterwards. The resulting extracts were filtered and evaporated under reduced pressure at 45 °C. The solvent-free methanolic extracts were stored at 4 °C until further use. For more detailed information on the processes used to isolate and identify compounds from these plants, please see previously published studies (Ibrakaw et al. 2020; Omoruyi et al. 2021a). Buphanisine and epibuphanisine isolated from *C. flava* were previously designated compound **3** and compound **4,** respectively (Omoruyi et al. 2021a). Similarly, distichamine and 1α,3α-diacetylnerbowdine isolated from *B. haemanthiodes* were previously designated compound **1** and compound **2,** respectively (Ibrakaw et al. 2020).

Cell culture and treatments

The U251 and U87 human glioblastoma cells and the H9C2 rat cardiomyocytes were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM, Gibco, Life Technologies Corporation, Paisley, UK), supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza Group Ltd., Verviers, Belgium). Cell lines were maintained in an incubator with a temperature set at 37 °C and a CO_2 concentration of 5% with a routine change of cell growth medium every three days. Once cells were about 70–80% confluent, they were sub-cultured or split for experiments. To treat the cells, stock solutions of the extracts were prepared at a concentration of 40 mg/mL in Dimethyl sulfoxide (DMSO, Sigma Aldrich, St Louis, USA), and this was further diluted in medium to 1 mg/mL concentrations. The final concentrations of 20, 40, 60, 80 and 100 µg/mL were obtained by further diluting the 1 mg/mL concentrations in the cell growth medium. For the compounds, 10 mg/mL stock solutions were prepared in DMSO and further diluted to 1 mg/mL in cell growth medium from which final concentrations of 2.5, 5, 7.5 and 10 µg/mL were obtained. As a positive control, cells were treated with cisplatin (Sigma Aldrich, St Louis, USA) with similar concentrations as the total plant extracts.

Determination of cell viability

To ascertain the effect plant extracts and isolated compounds have on the viability of U87 and U251 cells, the MTT (Sigma Aldrich, St Louis, USA) assays were performed. Briefly, 5×10^3 U87 and U251 cells were seeded per well in a 96-well plate and incubated for 24 h. Once cells were attached, the expended culture medium was replaced with fresh medium containing 20, 40, 60, 80 and 100 µg/mL of either plant extracts, the positive control or 2.5, 5, 7.5 and 10 µg/mL of the four compounds. Cells treated with the same amount of DMSO in the highest concentration of the extract or compound served as vehicle control. Treatment was for 48 h, and after that, 10 µL of the MTT solution (Sigma-Aldrich, St Louis, USA) was added to each well and incubated for 4 h. After incubation, the resultant formazan formed was solubilised with DMSO and absorbance was read at 560 nm with the Promega GloMax® explorer multimode microplate reader. The obtained absorbance values were analysed and represented as percentages of the control and sigmoidal plots on GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA), were used to determine the maximal inhibitory concentration required to kill 50% of the cells (IC_{50}).

Clonogenic assay

The clonogenic assay was conducted using a previously established protocol (Omoruyi et al. 2020). The U87 and U251 cells were cultured at a density of 1.7×10^5 cells in 60 mm dishes and incubated overnight to allow cellular adherence before exposure to half the IC_{50} or IC_{50} of the three plant extracts for 48 h. The vehicle-treated cells were used as control. Once treatment time elapsed, cells were collected by trypsinisation, counted, and regrown in 35 mm dishes at a density of 500 cells/dish in 2 mL of growth medium. Cells were monitored for not more than fourteen days to allow colony formation, and the growth medium was changed every three days. At the termination of experiments, the colonies formed were fixed using a solution of methanol and glacial acetic acid (3:1) and stained with 0.5% crystal violet stain (Sigma-Aldrich, St Louis, USA). Stained colonies were photographed using a digital camera. Furthermore, the areas covered by colonies in the dishes were determined using the colony area plugin on ImageJ (Guzmán et al. 2014). Calculated colony areas were expressed as a percentage of the control-treated cells.

Cell morphology

To investigate morphological changes in the U87 and U251 cells following treatment with the IC_{50} of plant extracts, the Olympus IX51 inverted light microscope with a 10X objective

lens was used, and images were captured using the Olympus CellSens Imaging Software.

Adenosine triphosphate detection assay

The ATP levels in the cells were determined using the Mitochondrial ToxGlo™ assay kit (Promega, Madison, USA). To begin the assay, U87 and U251 cells were seeded into white-walled 96-well plates at a seeding density of 5×10^3 cells per well and left overnight for adherence. After this, the cell growth medium was removed, and the cells were exposed to half the IC_{50} and IC_{50} of plant extracts and compounds for 48 h. At the end of the treatment duration, the assay followed the manufacturer's instructions by adding 100 µL of the ATP detection reagent to each well. After incubation for 30 min at 37 °C, the Promega GloMax® explorer multimode microplate reader was used to read luminescence, expressed as percentages of control.

Detection of apoptosis

To determine the pro-apoptotic potential of the treatments on the U87 and U251 cells, the Caspase-Glo™ 3/7 assay kit (Promega, Madison, USA) was used. Cells were seeded in white-walled 96-well plates using a seeding density of 5×10^3 cells per well and left overnight for adherence. After that, the expended culture medium was removed, and cells were exposed to half the IC_{50} or IC_{50} of plant extracts and compounds for 48 h. After treatment, the assay was performed according to the manufacturer's instructions by adding 100 µL of caspase 3/7 assay detection solution to each well and incubating for 30 min. Using the Promega GloMax® explorer multimode microplate, the luminescence read, and the values obtained were expressed as a fold of control.

Statistical analysis

The results of this study are presented as the mean values ± standard error of the mean (SEM) from three independent experiments. The data was analysed using the GraphPad Prism 6 software, and the Two-Way ANOVA data analysis tool was used to compare the treatment groups with the control. The significance level was set at $P \leq 0.05$, and Dunnett's multiple comparisons test was used to determine the statistical significance.

Results

Amaryllidaceae plant extracts and compounds inhibit the proliferation of glioblastoma cells

To determine the effects of the plant extracts and compounds on the proliferation of the U251 and U87 glioblastoma cells, MTT assays were performed after treating cells with increasing concentrations of plant extracts and compounds. Figure 1 shows that all plant extracts had inhibitory effects on cell proliferation, which was mainly concentration-dependent except for the U87 cells treated with *C. flava* (Fig. 1A). From the IC_{50} values obtained, it was evident that the U87 and U251 cells were more sensitive to *A. belladonna* extract as the IC_{50} values were lower than those obtained for the *C. flava* and *B. haemanthiodes* (Table 1). Furthermore, the cisplatin-treated cells (positive control) showed more sensitivity, indicating that cisplatin was more cytotoxic under the concentrations tested (Fig. 1D). In addition, the bioactive compounds were also investigated for their antiproliferative activities on the glioblastoma cells, by subjecting the cells to concentrations ranging from 2.5 to 10 µg/mL. Figure 2 shows that the compounds also reduced the viability of the cells, and this was more in the cells treated with 20 µg/mL (highest concentration) and the IC_{50} values obtained for the compounds are shown in Table 1.

To determine the selectivity of the extracts and compounds to cancer cells, the H9C2 cardiomyocytes were used as a normal cell line. The cells were exposed to similar concentrations used for the glioblastoma cells. Figures 1 and 2 show that the extracts and compounds reduced the viability of H9C2 cells but with higher IC_{50} values in the extract-treated cells compared to the cancer cells (Table 1). A selectivity index (SI), which is the ratio of IC_{50} in normal cells to that of cancer cells, was calculated to determine which extracts and compounds were more selective. *A. belladonna* was more selective among the plant extracts, while the compounds showed no selectivity, except for the U251 cells treated with epibuphanisine (Table 1). While it is difficult to establish the structure–activity relationship due to the small number of compounds and the close IC_{50} values, compounds like buphanisine (**3**) and ebipuphanisine (**4**) have similar structures; the only difference is the orientation of the methoxy group at C-3. The cytotoxicity of **4** is higher than **3** against U87 and U251, and lower against H9C2. On the other hand, compounds **1** and **2** differ in the substitution pattern at C-1, C2 and C3. Compound **2** has two acetyl groups at C1 and C3 and showed higher toxicity than **1**, which may be due to the increasing lipophilicity of the structure. Together, these results demonstrate that

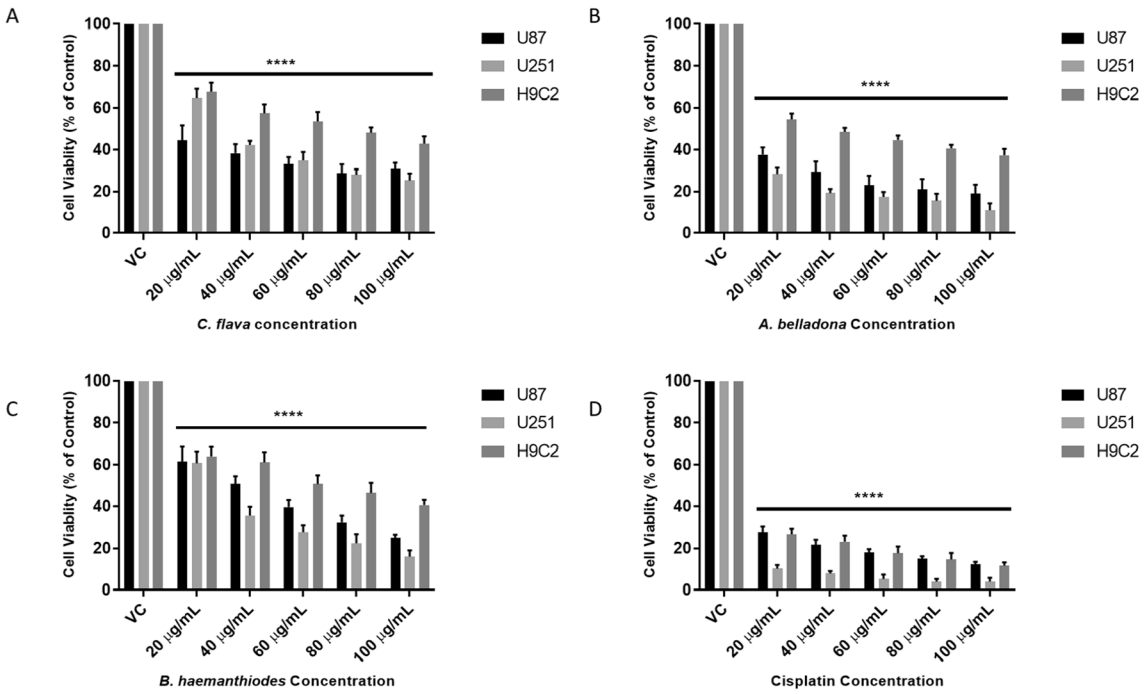

Fig. 1 Effects of plant extracts on the viability of U87, U251 and H9C2 cells. The cells were treated with *C. flava* (**A**), *A. belladonna* (**B**), *B. haemanthiodes* (**C**) and cisplatin (**D**) for 48 h. The viability of the cells was measured using MTT assays, and the results were obtained from at least three independent experiments. The bars in the graph represent the mean ± SEM. The significance of differences was denoted with **** ($p < 0.0001$) when treated cells were compared to the control

Table 1 Summary of IC_{50} values and selectivity index of plant extracts and compounds

Plant Extract/Compound	Maximal Inhibitory Concentration (IC_{50})/Selectivity Index (SI)	Cell Line		
		U87	U251	H9C2
C. flava	IC_{50} (µg/mL)	11.84	32.99	68.22
	SI	5.76	2.06	
A. belladona	IC_{50} (µg/mL)	8.56	4.55	32.82
	SI	3.83	7.25	
B. haemanthiodes	IC_{50} (µg/mL)	36.48	27.27	62.23
	SI	1.71	2.28	
Cisplatin	IC_{50} (µg/mL)	4.00	0.76	3.91
	SI	0.98	5.14	
1α,3α-diacetylnerbowdine	IC_{50} (µg/mL)	5.88	4.36	7.00
	SI	1.19	1.61	
Distichamine	IC_{50} (µg/mL)	7.19	5.37	9.95
	SI	1.38	1.85	
Buphanisine	IC_{50} (µg/mL)	6.86	7.51	6.54
	SI	0,95	0.87	
Epibuphanisine	IC_{50} (µg/mL)	3.84	6.26	11.5
	SI	2.99	1.84	

Amaryllidaceae plant extracts and compounds reduced the viability of the U87 and U251 glioblastoma cells, and the plant extracts were selective to cancer cells when compared to both cisplatin and the compounds. Only two

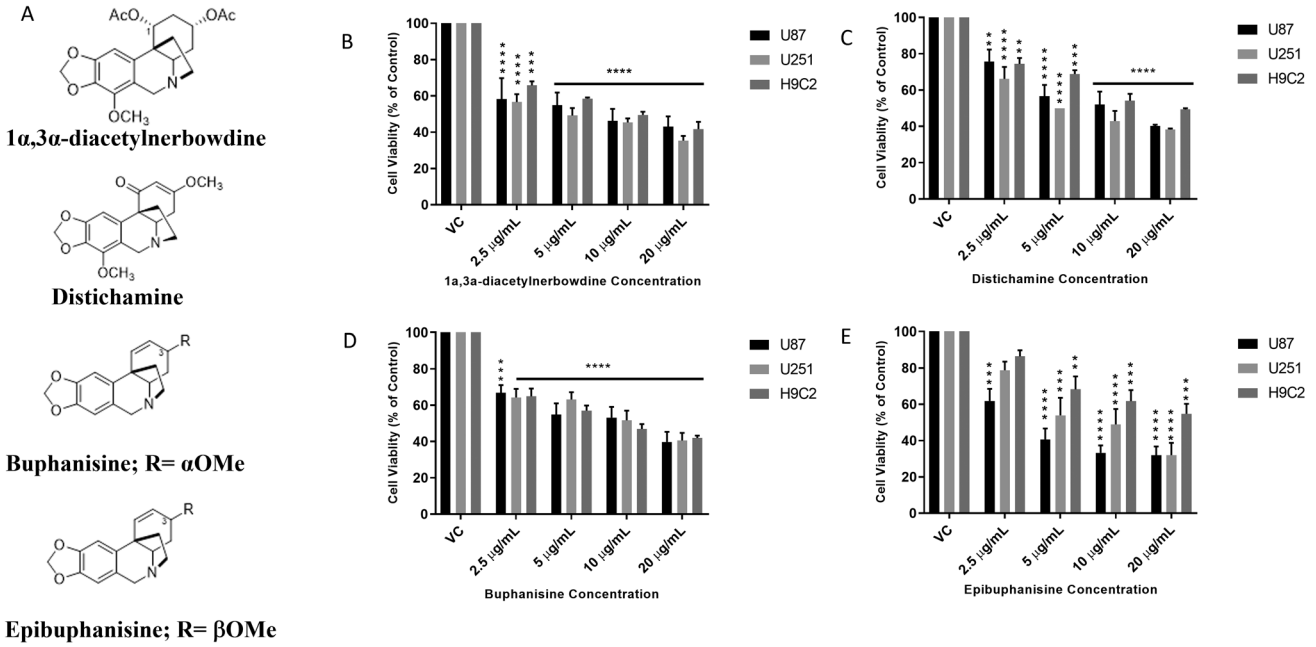

Fig. 2 Effects of plant extracts on the viability of U87, U251 and H9C2 cells. Chemical structure of compounds (**A**). The cells were treated with 1α,3α-diacetylnerbowdine (**B**), Disitichamine (**C**), Buphanisine (**D**) and Epibuphanisine (**E**) for 48 h. The viability of the cells was measured using MTT assays, and the results were obtained from at least three independent experiments. The bars in the graph represent the mean ± SEM. The significance of differences was denoted with ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$) when treated cells were compared to the control

compounds, 1α,3α-diacetylnerbowdine and distichamine, isolated from *B. haemanthiodes* were investigated further.

Impact of *Amaryllidaceae* plant extracts on colony formation

The clonogenic or colony formation assay was used to determine whether plant extracts could suppress the long-term survival of U87 and U251 glioblastoma cells. This in vitro assay checks if an anticancer agent can limit tumour cell survival after treatment. The assay relies on the ability of a single cell to divide and grow into colonies (a colony consists of at least 50 cells) after brief exposure to cytotoxic agents or radiation (Franken et al. 2006). In Fig. 3A, it is evident that the treated U87 and U251 cells with half the IC_{50} or IC_{50} of the plant extracts showed a decrease in the number of colonies formed compared to the control cells. When colony areas were quantified and expressed as a percentage of the control-treated cells, colony areas for cells treated with half the IC_{50} and IC_{50} of *C. flava* were 3.7 and 2.2% for the U87 cells and 36.7 and 22.4% for the U251 cells respectively (Fig. 3B). Similarly, the cells treated with half the IC_{50} and IC_{50} of *A. belladonna* had percentage colony areas at 1.7 and 0.7% for the U87 cells and 15.7 and 13.7% for the U251 cells respectively (Fig. 3C). As seeing the stained dishes, *B. haemanthiodes* also showed a reduction in the areas covered by colonies for the U87 cells, which had 7.8 and 3.1% and 91.4 and 56.3% for half the IC50 and IC50, respectively (Fig. 3D).

Effect of *Amaryllidaceae* plant extracts on cell morphology

To observe how plant extracts affect cell morphology, the glioblastoma cells were treated with the IC_{50} of the plant extracts. Figure 4 shows changes in the morphology and density of the U87 and U251 cells. When compared to control, the morphological changes in the extract-treated cells are typical of cells experiencing programmed cell death or not dividing. Some of the changes include shrinkage and reduction in cell size, as well as the loss of cellular projections, as seen in the U87 cells.

Amaryllidaceae plant extracts and compounds inhibit cellular ATP levels

Cellular energy levels are crucial for the survival of cells, and ATP is a critical component in maintaining these levels (Deng et al. 2021). To investigate if *Amaryllidaceae* plant extracts and compounds affect cellular energy/ATP levels, we treated U87 and U251 cells with half the IC_{50} and IC_{50} of plant extracts for 48 h. Our findings in Fig. 5 show that treatment with the extracts and compounds compared to control led to a significant decrease in cellular

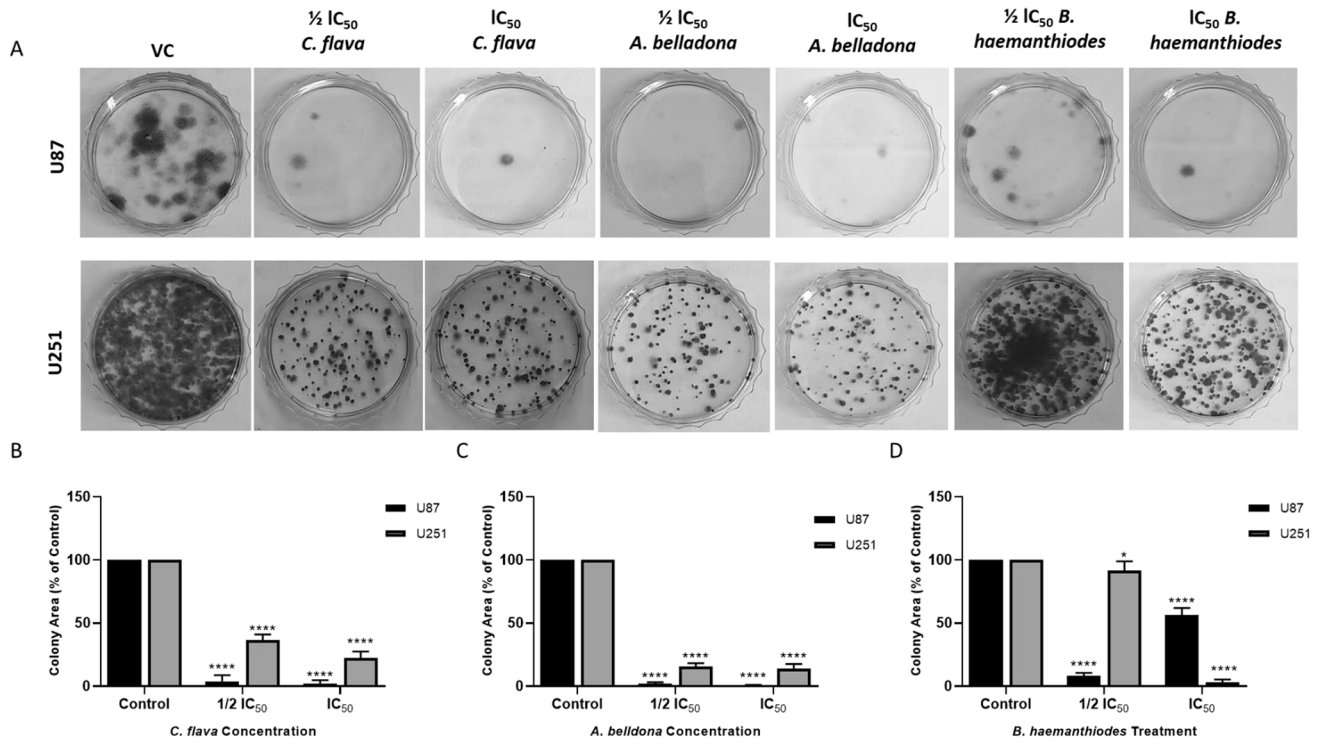

Fig. 3 Effect of *Amaryllidaceae* plant extracts on colony formation in glioblastoma cells. **A** The U87 and U251 cells were treated with either half the IC_{50} or IC_{50} of the plant extracts for 48 h, and thereafter, 500 cells were grown in 35 mm dishes for fourteen days and stained with crystal violet. **B**–**D** Quantified colonies showing percentage colony area for each treatment with respect to control. Bars on graphs represent means ± SEM. The significance of differences was denoted with * ($p < 0.005$), **** ($p < 0.0001$) when treated cells were compared to the control

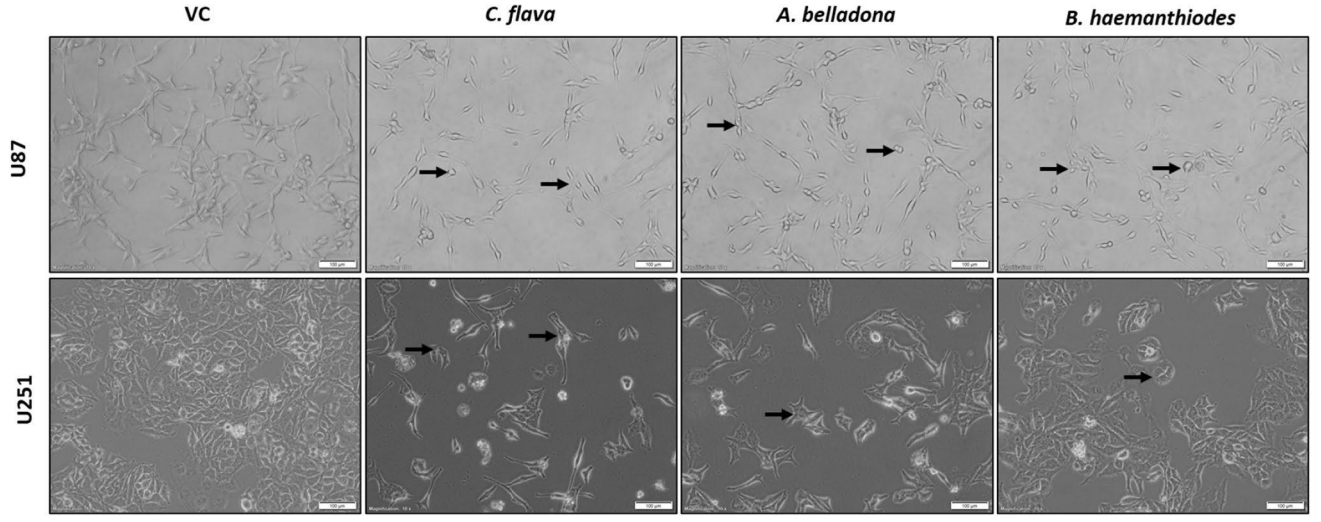

Fig. 4 Effect of *C. flava, A. belladonna* and *B. haemanthiodes* extracts on cellular morphology. U87 and U251 cells exposed to IC_{50} values of *C. flava, A. belladonna* and *B. haemanthiodes* for 48 h, and images were obtained with an Olympus microscope with the 10X objective lens. Scale bar = 100 µm. Morphological changes, which include shrinkage and reduction of the cell size as well as loss of cellular projections in the U87 cells, are indicated by arrows

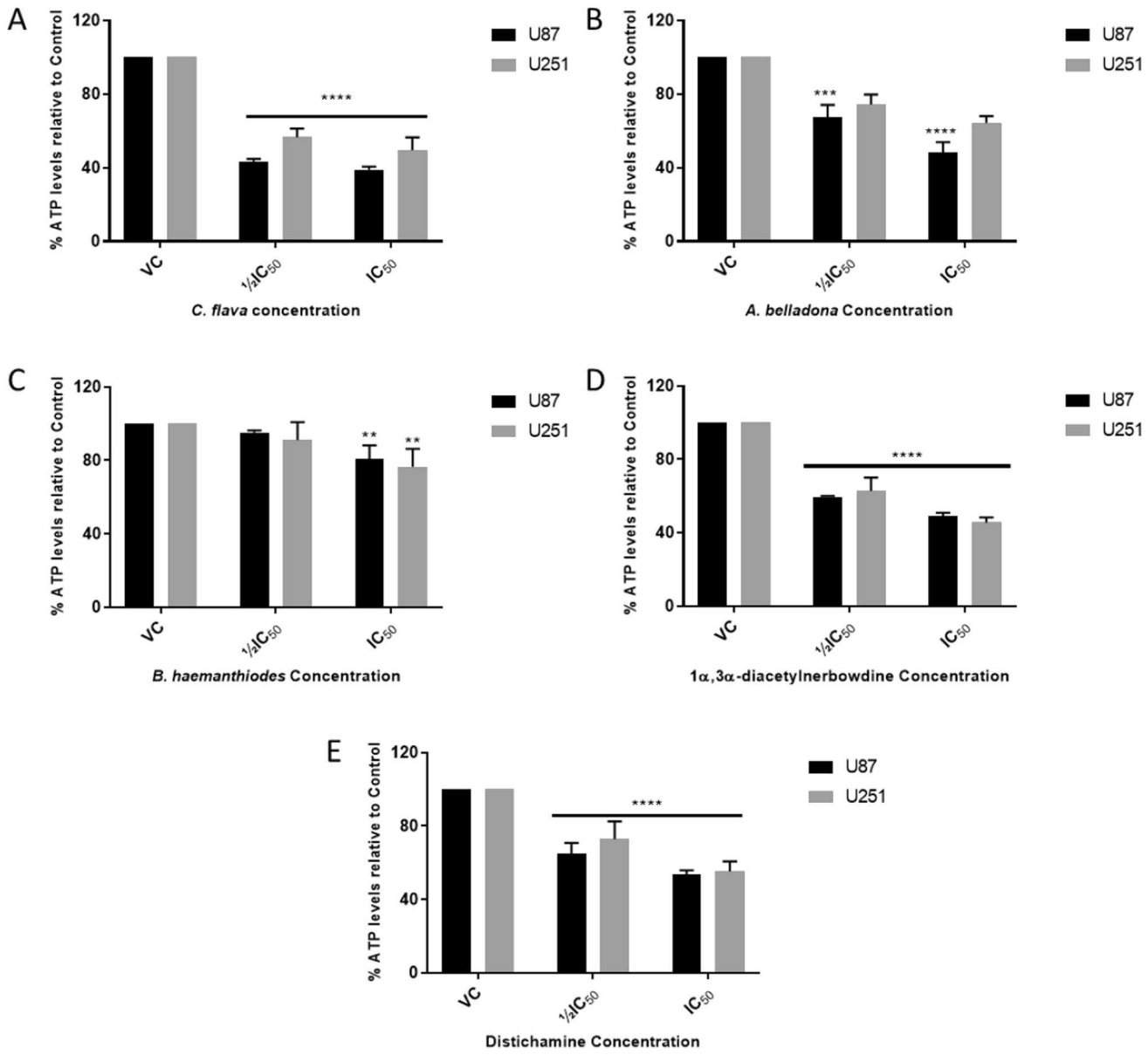

Fig. 5 *Amaryllidaceae* plant extracts and compounds attenuate ATP depletion in glioblastoma cells. The U87 and U251 cells were exposed to half of the IC_{50} and IC_{50} of *C. flava* (**A**), *A. belladonna* (**B**), *B. haemanthiodes* (**C**), 1α,3α-diacetylnerbowdine (**D**) and Distichamine (**E**), for 48 h and the vehicle-treated cells served as controls. The ATP levels were expressed as a percentage of control, and the bars in the figures represent the mean percentage ± SEM of the data obtained from triplicate experiments. The statistical significance of the difference is indicated with ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$) when the treated cells were compared to the control cells

ATP levels, except for the U87 and U251 cells treated with half the IC_{50} of *B. haemanthiodes*. Notably, this reduction was concentration-dependent, as IC_{50}-treated cells had lower ATP levels than cells treated with half the IC_{50}. These results suggest that the plant extracts and compounds alter cellular energy levels, potentially contributing to the reduced cell viability observed.

Effect of *Amaryllidaceae* plant extracts and compounds on apoptosis

To determine if extracts and compounds from the *Amaryllidaceae* plant family can induce apoptosis, glioblastoma cells were treated with half the IC_{50} and IC_{50} of extracts and compounds for 48 h, and the caspase 3/7 glo assay was used to measure apoptosis. The results revealed that while

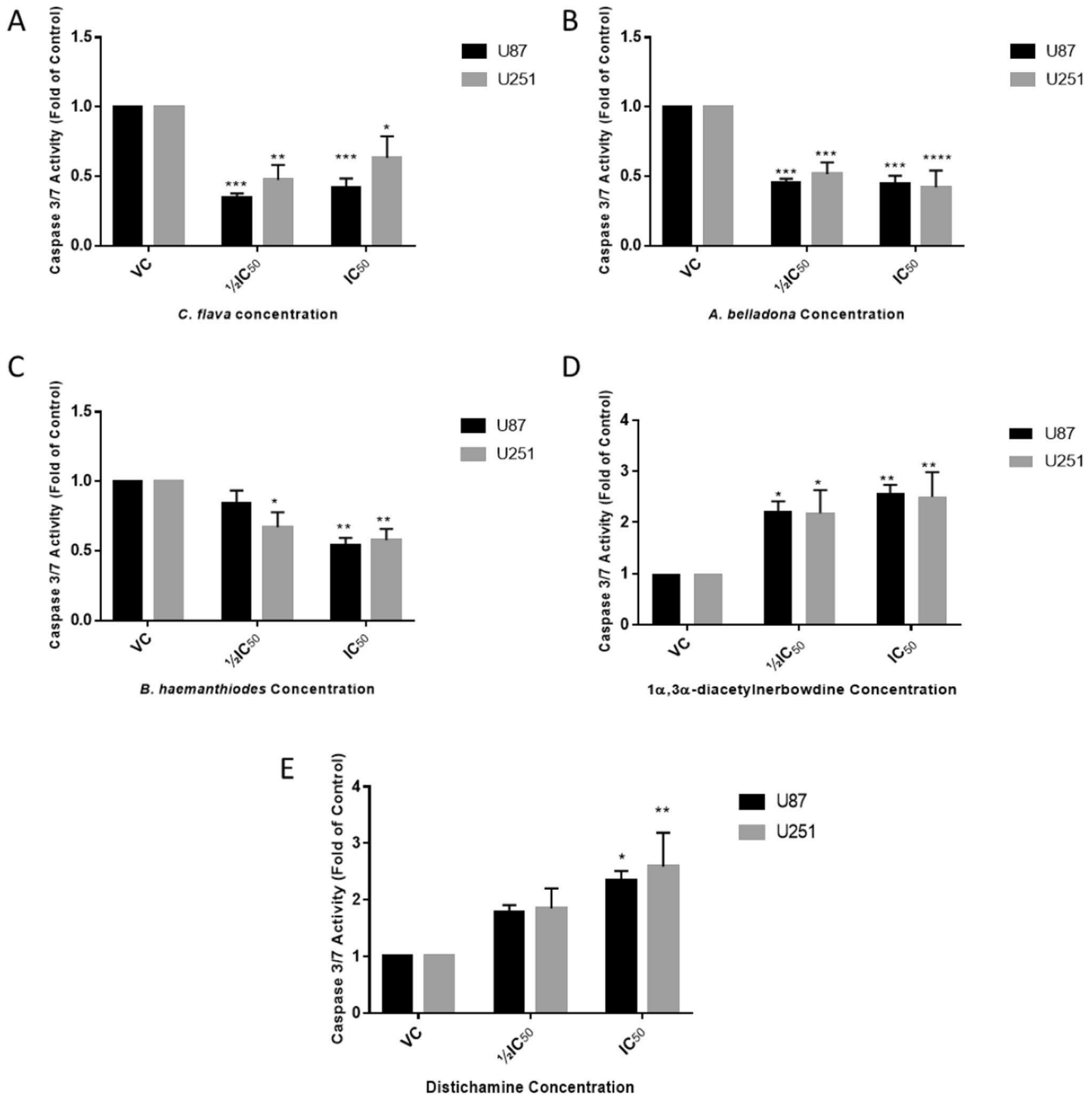

Fig. 6 Impact of *Amaryllidaceae* plant extracts and compounds on Caspase 3/7 activities in glioblastoma cells. The U87 and U251 cells were exposed to half of the IC_{50} and IC_{50} of *C. flava* (**A**), *A. belladonna* (**B**), *B. haemanthiodes* (**C**), 1α,3α-diacetylnerbowdine (**D**) and Distichamine (**E**), for 48 h and the vehicle-treated cells served as controls. Caspase 3/7 activities were expressed as fold of control, and figures show bars with the mean fold change ± SEM of data generated from triplicate experiments. The statistical significance of the difference is indicated with * ($p \leq 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) when treated cells were compared to control

treatment with plant extracts led to a decrease in caspase 3/7 activity, treatment with half the IC_{50} and IC_{50} concentrations of the compounds caused a significant increase in caspase 3/7 activity (Fig. 6). The U87 cells treated with 1α,3α-diacetylnerbowdine at half the IC_{50} and IC_{50} concentrations showed a 2.22-fold and 2.57-fold increase in caspase 3/7 activity, respectively, while the U251 cells showed a 2.17-fold and 2.49-fold increase under the same conditions. Similarly, distichamine treatment led to a 1.77-fold and 2.34-fold increase in caspase 3/7 activity for the U87 cells at half the IC_{50} and IC_{50} concentrations, respectively, and a 1.85-fold and 2.59-fold increase for the U251 cells. These findings

suggest that only the compounds triggered increased caspase 3/7 activity in the cells under these treatment conditions.

Discussion

In this study, extracts of *C. flava*, *A. belladonna* and *B. haemanthiodes* were investigated for their antiproliferative activities in malignant glioblastoma cells. Also investigated were isolated compounds epibuphanisine and buphanisine from *C. flava* as well as 1α,3α-diacetylnerbowdine and distichamine from *B. haemanthiodes*. The findings showed that the extracts from *Amaryllidaceae* plants induced cytotoxicity in the glioblastoma cells, demonstrated by decreased cell viability, altered cellular morphology, and reduced colony formation. Indeed, the IC_{50} values obtained for the glioblastoma cell lines tested ranged from 4.55 to 36.48 µg/mL for the extracts, while those of the compounds were between 3.84 and 7.19 µg/mL. Therefore, these extracts can be considered promising according to the American National Cancer Institute (NCI) benchmark IC_{50} for plant extracts that are deemed cytotoxic and suitable for further purification, which is less than 30 µg/mL (Suffness 1990; Canga et al. 2022). However, only the IC_{50} obtained for U251 cells exposed to *C. flava* and the U87 cells treated with *B. haemanthiodes* went slightly above the benchmark. Contrary to the benchmark set by the NCI, another study reported that plant extracts with IC_{50} values up to 100 µg/mL could also be termed cytotoxic and might be potential sources of active anticancer agents (Ayoub et al. 2014). Moreover, the NCI also classified cytotoxic compounds as highly active if they have an IC_{50} of less than 20 µg/mL (Anywar et al. 2022). The IC_{50} values obtained in this study were less than 10 g/mL, so the compounds investigated could be considered highly active. These findings are in line with previous research, which has shown that *Amaryllidaceae* plants and their isolated compounds (*Amaryllidaceae* alkaloids) have cytotoxic potential in several cancer cells, including brain tumour cells cell (Nair et al. 2013, 2016; Nair and Van Staden 2021a; Omoruyi et al. 2021b). In particular, distichamine investigated in this study was reported to exhibit in vitro cytotoxicity in various human cancer cell lines such as HeLa (cervical), CEM (T lymphoblast), K562 (erythroleukemia), MCF-7 (breast cancer) and G-361 (IC50 2.2–14.7 µM) (Nair et al. 2012b; He et al. 2015). Similarly, buphanisine was also shown to be cytotoxic when tested against a panel of human cancer cell lines including the U373 glioblastoma cells (Nair et al. 2012a). Furthermore, medicinal plants from Georgian *Amaryllidaceae* have also been shown to induce cytotoxicity in Hela cervical, HCT-116 colon carcinoma, and HL-60 acute myeloid leukaemia cancer cells, with IC_{50} ranging from 8 to 58.2 µg/mL (Jokhadze et al. 2007).

It is important for an anticancer agent to exhibit cytotoxicity towards cancer cells while sparing normal or non-tumorigenic cells. The degree of selectivity of an anticancer agent towards cancer cells is known as the selectivity index. A selectivity index of 2 or higher is considered to be favourable. (Koch et al. 2005; Badisa et al. 2009). In this study, the H9C2 cardiac myocytes were used as the non-tumorigenic cell line owing to the cardiotoxicity associated with some anticancer agents (Florescu et al. 2013; Chaulin et al. 2020; Briasoulis et al. 2022). Findings from this study show that compared to cisplatin, which was not selective, *C. flava, A. belladonna* and *B. haemanthiodes* showed selectivity to cancer cells as the selectivity indices were greater than 2. However, the isolated compounds tested were not selective, and their efficacy as anticancer agents may be limited in their current form. They could be adapted for use in nanotechnology by functionalising them with peptides specific to surface receptors of cancer cells or as scaffolds for developing novel drugs (O'brien et al. 2004; Geisberg and Sawyer 2010).

As a result of the cytotoxicity induced by medicinal plants and natural products, the energy levels in cells can be affected, leading to a depletion of ATP, which is produced in the mitochondria (Bianchi et al. 2018; Winitchaikul et al. 2021). As the mitochondria serve as the primary energy source for cells, they are a promising target for cancer therapy (Dong and Neuzil 2014; Vasan et al. 2020). Many natural products have been shown to inhibit ATP synthesis and impede the activity of ATP synthase and other complexes involved in the mitochondrial electron transport chain, leading to programmed cell death (Yang et al. 2020; Wang et al. 2021). Moreover, natural products such as terpenoids, flavonoids, coumarins, alkaloids, and quinones have also been implicated in programmed cell death in cancer cells through the mitochondrial pathway (Choi et al. 2008; Xu et al. 2015; Chen et al. 2023). In this study, our findings revealed that *C. flava*, A. *belladonna*, *B. haemanthiodes*, and the compounds investigated inhibited ATP generation in the cells, indicating mitochondrial toxicity. Although we cannot speculate on which mitochondrial complexes were specifically targeted, research has shown that natural products may inhibit four complexes: NADH-ubiquinone reductase (complex I), succinate-ubiquinone reductase (complex II), ubiquinol-cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV) (Vyas et al. 2016; Yang et al. 2020). It is important to note that Complex V (ATP synthase), which is a mitochondrial target for cancer, works with the other four complexes to finalise the oxidative phosphorylation process that leads to the production of ATP (Yang et al. 2020; Wang et al. 2021).

Impaired cellular energy levels can trigger programmed cell death (Eguchi et al. 1997; Xu et al. 2020). In this study, we investigated apoptosis as a potential mode of cell death by analysing the levels of caspases 3/7, which

are downstream of the extrinsic or intrinsic apoptotic pathway activated by caspase 8 and caspase 9, respectively (Mccomb et al. 2019). Our findings indicate that the plant extracts did not induce apoptosis, as evidenced by the reduced or no change in the levels of caspase 3/7 activities after 48 h. However, the compounds had increased levels of caspase 3/7 activities under all treatment conditions. Since the compounds were not selective, their use in their current form cannot be justified. These findings suggest that apoptosis may not be a mode of cell death induced by the extracts as, unlike the compounds, the plant extracts contain other ingredients. Therefore, it is worth exploring other forms of programmed cell death that may be associated with the activities of these extracts.

Conclusion

This study highlights the antiproliferative activities of three plant species, *C. flava*, *A. belladonna* and *B. haemanthiodes*, which belong to the *Amaryllidaceae* family. The study also reported the antiproliferative activities of four bioactive compounds, two isolated from *C. flava* and the other two from *B. haemanthiodes*. The experiments were carried out on the U87 and U251 malignant glioblastoma cell lines. The results show that the extracts selectively induced cytotoxicity in the glioblastoma cells, while the non-cancerous H9C2 cardiac myocytes showed less cytotoxicity when compared to the glioblastoma cells. However, the compounds investigated also induced cytotoxicity but were not selective. Regarding the mechanism of action, the extracts and compounds were found to inhibit ATP production. However, only the compounds induced apoptosis. Therefore, this study provides evidence that *Amaryllidaceae* plant species and their bioactive compounds may be useful in treating glioblastoma. This study also offers a basis for further isolation of bioactive compounds from these plant species to identify compounds that will be more selective to cancer cells.

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Data availability Data presented are available upon request from the authors.

Declarations

Ethical approval This article does not contain any studies involving animals performed by any of the authors. This article does not contain any studies involving human participants performed by any of the authors. However, ethics waiver for this study was obtained from the Wits Human Research Ethics Committee (HREC)- W-CBP-221024-02.

Conflict of interest The authors declare that they have no competing interest.

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