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Preliminary Cytotoxic Activity of *Sutherlandia frutescens* and *Carpobrotus edulis* on Malignant glioblastoma Cells

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ABSTRACT

Glioblastoma (GBM) is the most common and aggressive intracranial tumour with limited therapeutic options due to their high tumour vasculature and invasiveness. Treatment of GBM includes surgery, followed by chemotherapy and radiotherapy. Despite these treatment options, tumour relapse is still a major issue hence the need for cheap and effective treatment strategies. South Africa is endowed with numerous medicinal plants including *Sutherlandia frutescens* (*S. frutescens*) and *Carpobrotus edulis* (*C.edulis*), which has been previously reported for their antioxidant and neuroprotective activities. Accordingly, this study was designed to investigate the cytotoxicity of both medicinal plants (*S. frutescens* and *C. edulis*) in malignant human GBM cells U251 and U87. The cytotoxic activity was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay while the effect of extracts on colony formation and survival was determined using clonogenic assay. Results show that both *S. frutescens* and *C .edulis* induced cytotoxicity in both GBM cells and inhibit colony formation in U251 cells. These findings show that extracts from these plants may be useful in the treatment of GBM and thus requires further investigations into their mechanisms of action as well as isolation of bioactive components responsible for these activities.

Keywords: *Sutherlandia frutescens, Carpobrotus edulis,* Glioblastoma, Cytotoxicity, Colony formation.

Introduction

Glioblastoma (GBM) is the most aggressive type of brain tumour with limited treatment options and comprises about 16% of all primary brain and central nervous system tumours.[1] Malignant gliomas are highly vascularized and invasive neoplasms and due to their invasive properties, they are nearly impossible to resect completely in surgery.[2] The likelihood of tumour survival amongst adults is slim as tumours drastically reduce life expectancy.[3] The standard treatment of gliomas consists of surgery plus radiotherapy which is eventually followed by chemotherapy.[4] Despite these treatment modalities, the median survival of GBM patients beyond one year remains daunting as tumour relapse often occurs.[5,6] This dismal prognosis warrants continued intensive investigation of cheap and effective therapeutic options capable of reducing the burden of GBM.

Complementary and alternative medicines (CAM) is a choice of treatment for over 80% of the world's population.[7] Botanical medicines which are a form of CAM contain several active ingredients which sometimes show similar molecular targets as pharmaceutical drugs.[8, 9] Thus, herbal and botanical medicines represent an enormous medicinal resource at a fraction of the cost of conventional medicine. Many drugs used for the treatment of cancer have been discovered from medicinal plants.[10] Notable examples are vincristine, irinotecan, etoposide and paclitaxel which are presently in use as chemotherapeutic drugs for cancer.[11]

Sutherlandia frutescens (*unwele* in Zulu; *kankerbos* in Afrikaans; cancer bush in English), a member of the pea family, is a shrub with attractive flowers used traditionally by South Africans to treat a variety of maladies. [12] Its medicinal use is alleged to have originated from the Khoi and Nama people; who used the plant decoctions externally to wash wounds and internally for fevers, stomach problems and a variety of other ailments including internal cancers.[12-16] Its ethanolic extract has been reported to induce a concentration-dependent anti-proliferative activity on human tumours including the SNO oesophageal cancer and MCF-7 breast cancer cell lines.[17,18] Similarly, its aqueous extract was also reported to induce cytotoxicity in both MCF-7 and MDAMB 231 breast cancer cell lines as well as in HL60 and Jurkat leukaemia cells.[18-22] More so, *S. frutescens* has been reported to show anti-cancer activity in prostate cancer as it induced cytotoxicity in human prostate cancer cells DU-145, PC3, LNCaP and mouse prostate cancer cell, TRAMP-C2.[20,23] It also induced apoptosis and inhibited the PI3K-kinase cell survival pathway in CaCo2 colon cancer cells.[24] More recently, our laboratory also reported that *S. frutescens* induced cytotoxicity and apoptosis in SKNBE(2) and SH-SY5Y neuroblastoma cells via the accumulation of intracellular reactive oxygen species (ROS) and depolarization of mitochondria membrane potential (MMP).[25] The toxicity of *S. frutescens* leaf powder has been studied in both adult vervet monkeys and humans and no toxic or other side effects were observed.[26]

Carpobrotus edulis L. (*C. edulis*) is a medicinal and edible succulent plant native to the coast of South Africa. It has different common names: sour fig, Cape fig, Hottentots fig (English); "*ghoenavy*", "*hottentotsvy*", "*kaapsevy*", (Afrikaans); "*ikhambi-lamabulawo*" and "*umgongozi*" (Zulu)

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and "*igcukuma*" in Xhosa communities.[27] The leaves of *C. edulis* is used traditionally to treat HIV/AIDS in the Eastern Cape region of South Africa.[28] Reports indicate that the *in vitro* biological activities of *C. edulis* include antioxidant, immune modulating, antimicrobial and anticholinesterase effects.[27, 29-33] Its anti-cancer activity was first reported when *C. edulis* inhibited P-glycoprotein, which is known to promote drug resistance in mouse lymphoma as well as antiproliferative activities in HCT116 colorectal carcinoma cells.[32, 34]

One of the hallmarks of cancer is the rapid proliferation of cells due to oncogenic factors, thus, inhibition of cell proliferation is critical in the development of an anti-cancer agent.[35] More so, there is a knowledge gap on the anti-cancer activities of these medicinal plants (*S. frutescens* and *C.edulis*) in glioblastoma. Hence, this study was designed to investigate the anti-proliferative activities of *S. frutescens* and *C. edulis* in U251 and U87 malignant GBM cells.

Materials and Methods

Preparation of S. frutescens aqueous extract

The dried powdered leaves of *S. frutescens* R. BR. [family: Fabaceae] was procured from Big Tree Health Products (Fish Hoek, South Africa), while fresh leaves of *C. edulis* [family: Aizoaceae] were collected from the nature reserve of the University of the Western Cape (33.9335° S, 18.6280° E), Cape Town, South Africa and identified at the Department of Biodiversity and Conservation Biology. Moisture content was reduced by air-drying and the dried leaves blended to powder. Thereafter, 1 kg of the powdered leaves of each plant was soaked overnight in 10 L of boiled water for extraction to occur and the resultant extract was filtered with Whatman filter paper. Following this, the filtrates obtained were freeze-dried and stored at -20°C for future use. On each day of our experiments, aliquot portions of the dried crude aqueous extracts were weighed and dissolved in PBS (phosphate buffered saline, Lonza Group Ltd. Verviers, Belgium) to give a stock solution from which further dilutions in media were made for the treatment of cells.

Cell lines and culture conditions.

The World Health Organisation stage IV human malignant glioblastoma cells U251 and U87 used for this study were maintained in monolayer using Dulbecco Modified Eagles Medium supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza Group Ltd. Verviers, Belgium). Cells were grown in a humidified incubator at 37°C with 5% CO_2 as well as 95% air and cell growth media were routinely changed every two to three days. Sub-culturing of cells was performed when they attained 80% confluency using a solution of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium).

Cytotoxicity assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Roche, USA) was used to determine cytotoxicity following the manufacturer's instructions. Briefly, U251 and U87 glioblastoma cells were seeded at a density of 4000 cells per well in 96-well cell culture plates and left overnight for attachment. Thereafter, cells were treated with increasing concentrations of *S. frutescens* and *C. edulis* (0 to 5 mg/ml) for 48 hours and wells without extract treatment were used as controls. Thereafter, 10 µL of the MTT solution (5 mg/mL) was added to each well and cells were incubated for additional 4 hours at 37°C, followed by the addition of 100 µL of the solubilisation buffer (10% SDS in 0.01 M HCl). Plates were then left overnight at 37°C and absorbance of each well was read at 570 nm using a BMG Labtech Omega® POLARStar multimodal plate reader. Cell viability was expressed as a percentage of control cells and the maximal inhibitory concentration (IC_{50}) was obtained using sigmoidal curve from GraphPad Prism6 (GraphPad software, San Diego, CA, USA). All experiments were performed in triplicates.

Clonogenic assay

Clonogenic assay was conducted to investigate the effect of plant extracts on cell proliferation and survival. U251 cells were plated at appropriate densities in 60 mm dishes and left overnight for attachment before treatment with two different concentrations of the extracts (half the IC_{50} and IC_{50}) for 24 hours. After treatments, cells were collected by trypsinization, counted and re-plated in 35 mm dishes at a density of 500 cells per dish. The cells were then incubated for no more than fourteen days with routine media changes to allow for colony formation. At the end of the experiment, colonies were washed in PBS, fixed with methanol and glacial acetic acid solution (3:1) and stained with 0.5% crystal violet stain. Images of the dishes were taken and areas covered by colonies were calculated using Image J software and expressed as a percentage of control which was set to a hundred percent.

Statistical analysis

Data generated from this study are expressed as means ± standard error of means (SEM) of three independent experiments and analyzed using the GraphPad Prism 6 software. Significance of difference was calculated using one way ANOVA with the level of significance set at $P \leq 0.05$.

Results and Discussion

S. frutescens and C. edulis induce cytotoxicity in malignant glioblastoma cells.

To establish the cytotoxicity of *S. frutescens* and *C. edulis* on U87 and U251 GBM cells, the MTT cell viability assay was performed. Cells were treated and exposed to concentrations of extracts (0 to 5 mg/mL) for 48 h. The results show that *S. frutescens* induced a concentration-dependent cytotoxicity in U251 and U87 cell lines with IC_{50} values of 2.51 and 1.44 mg/mL, respectively (Figure 1). A similar outcome was evident in the cells treated with *C. edulis* though with higher IC_{50} values of 3.32 and 2.96 mg/mL for U251 and U87 cells, respectively (Figure 2). Data generated from this study is consistent with previous findings which show that *S. frutescens* and *C. edulis* induce antiproliferative effects in cancer cells.[17,21,32,34] The cytotoxicity observed in these study could be in part as a result of the chemical constituents of the extracts tested. For *S. frutescens*, it has been reported that the plant is rich in amino acids including l-arginine and l-canavanine as well as other components like pinitol, flavonol glycosides and triterpenoid saponins which have been demonstrated to possess anti-cancer activity.[12,36-38] Similarly, *C. edulis* has been reported to contain lots of antioxidants, impede protein glycation as well as cell proliferation with inhibition of the P-glycoprotein efflux pump as a mechanism of action.[32, 34] Together, these results show that *S. frutescens* and *C. edulis* inhibits proliferation of malignant glioblastoma cells and most importantly, the U87 cells were more sensitive due to the lower IC_{50} values obtained.

S. frutescens and C. edulis inhibit colony formation and survival in glioblastoma cells

Clonogenicity is reported to be a hallmark of transformed as well as malignant cell types and it is routinely evaluated by a clonogenic assay which assesses the efficacy of novel compounds in anti-cancer drug discovery.[39,40] It also ascertains the reproductive capacity of cells to form colonies when cells are seeded in very low densities after exposure to anti-cancer agent. A colony is regarded as an aggregate of 50 cells.[41] To determine the impact of *S. frutescens* and *C. edulis* on cell survival and clonogenicity, a clonogenic assay was performed. Figures 3A and B show that *S. frutescens* and *C. edulis* reduced the ability of U251 cells to form colonies at both the ½IC_{50} and IC_{50} concentrations. Further, when the colony area was quantified, both extracts show a significant concentration-dependent decrease in colonies when compared to control. Indeed, the percentage colony area for ½IC_{50} and IC_{50} concentrations of *S. frutescens* was 31.2% and 6.9%, respectively, while that of *C. edulis* was 75.3% and 53.1%, respectively. Our findings are consistent with previous reports for some plant extracts and other existing brain tumour therapies known to inhibit colony formation in glioblastoma cells.[42-45] Altogether, these results demonstrated that both extracts of *S. frutescens* and *C. edulis* impeded colony formation as well as survival in the U251 glioblastoma and this activity was better demonstrated in cells treated with *S. frutescens*.

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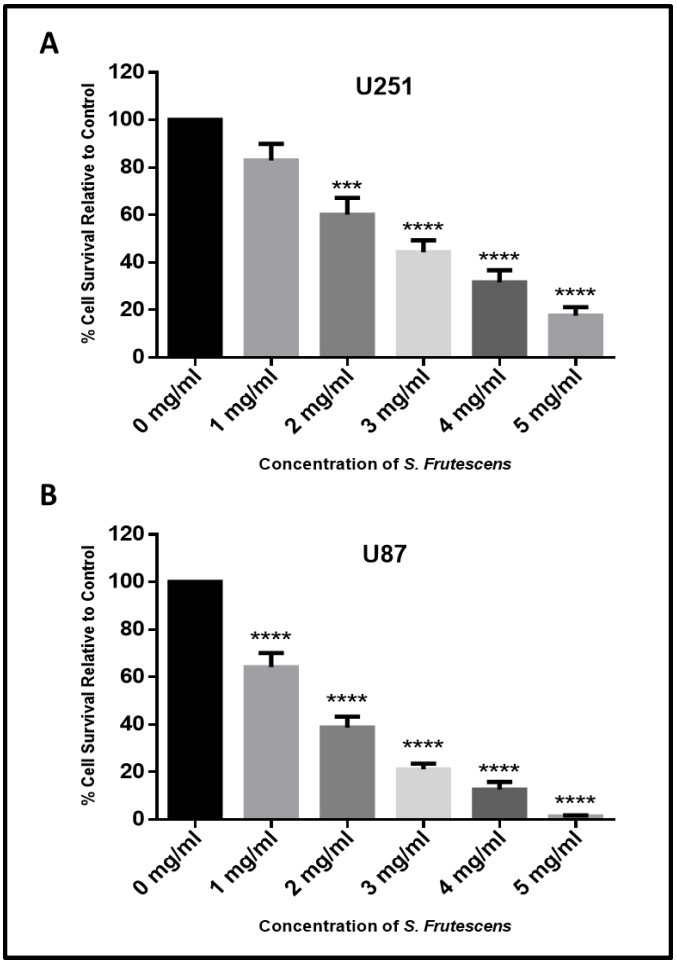

Figure 1: *S. frutescens* induces cytotoxicity in glioblastoma cells. *S. frutescens* induced concentration-dependent cytotoxicity in (A) U251 and (B) U87. Each bar represent means ± SEM.

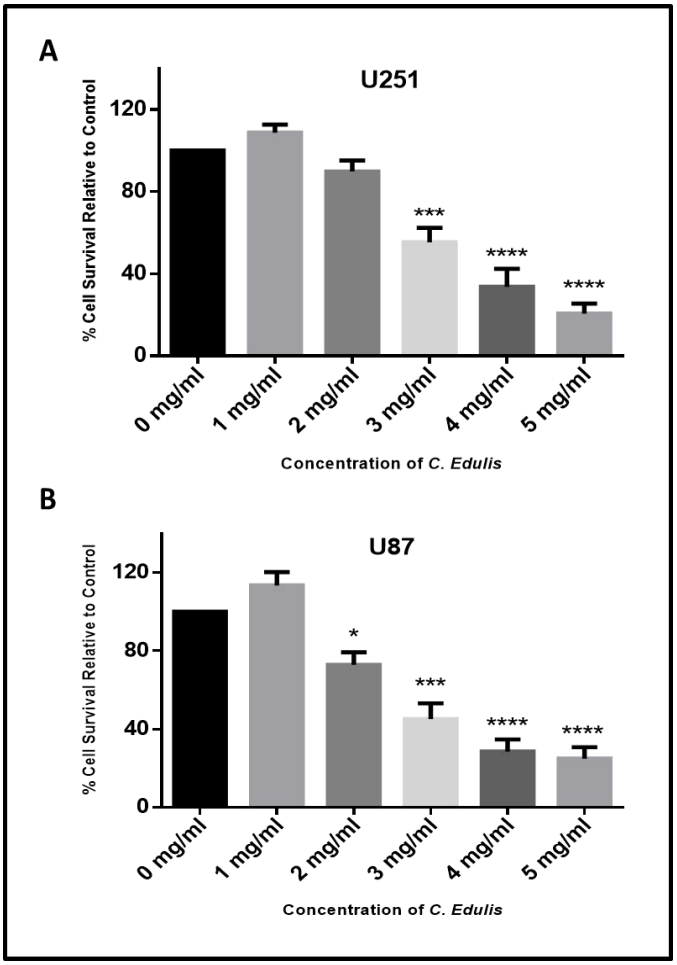

Figure 2: *C. edulis* induces cytotoxicity in glioblastoma cells. Cytotoxicity in (A) U251 and (B) U87. Each bar represent means ± SEM.

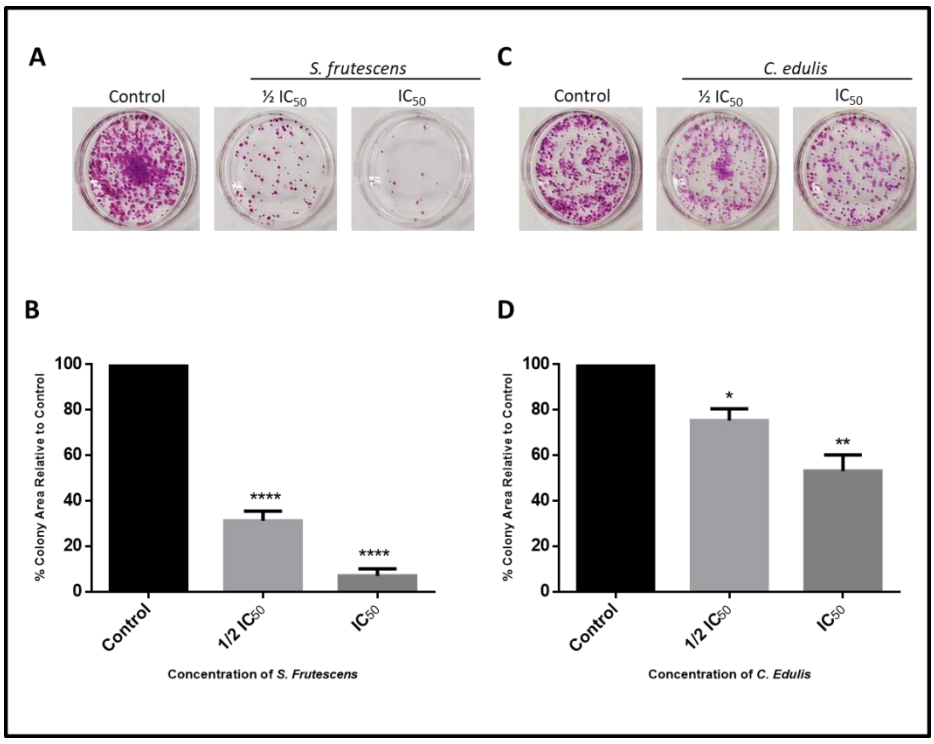

Figure 3: *S. frutescens* and *C. edulis* inhibit colony formation in U251 glioblastoma cells. (A) and (B) shows the reduction in colonies in U251 cells after staining with crystal violet. (C) and (D) shows the quantification of colony area using Image J. Each bar represent means ± SEM of three independent experiments.

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Conclusion

In the present study, the anti-proliferative activity of aqueous extract of *S. frutescens* and *C. edulis* was evaluated in U251 and U87 malignant glioblastoma cell lines. Our findings show that in both cell lines tested, extracts of *S. frutescens* and *C. edulis* inhibited cell proliferation. A possible explanation for this activity could be as a result of the chemical composition of these extracts which have been reported to show anti-cancer activity.[32,36-38] Further investigations are required to elucidate the molecular mechanisms of action responsible for the anti-cancer activity of these extracts.

Conflict of Interest

The authors declare that there is no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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