Screening Natural Compound Extracts for Potential Anti-cancer Activity on Glioblastoma Cell Line U251MG

By

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Thesis BTEC 9000A-F (27 units)
Submitted to Flinders University
for the degree of

Master of Biotechnology

College of Medicine and Public Health

21 December 2021

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LIST OF ABBREVIATED TERMS

2D- 2 dimensional

3D- 3 dimensional

ANOVA- Analysis of variance

CGH- Comparative genomic hybridisation

CKI- Compound Kushen Injection

CNS- Central nervous system

CO_2- Carbon dioxide

CPT- Camptothecins

CSC- Cancer stem cell

DMEM- Dulbecco's modified eagle medium

DMSO- Dimethyl sulfoxide

DYRK- dual specificity tyrosine regulated kinase

ECM- Extra cellular matrix

EGF- epidermal growth factor

EGFR- Epidermal growth factor receptor

FAK- Focal adhesion kinase

FBS- Foetal bovine serum

GA- Gallic acid

GABRA1- Gamma- aminobutyric acid receptor subunit alpha-1

GBM- Glioblastoma multiforme

GRIDD- Griffith Institute for Drug Discovery

HGF- Hepatocyte growth factor

HIF-1α- Hypoxia- inducible factor 1 alpha

IC_{50}- Half maximal inhibitory concentration

mGlu- metabotropic glutamate receptor

NEFL- Neurofilament light gene

NF1- Neurofibromatosis-1

OS- Overall survival

PBS- Phosphate buffer saline

PDGF- Platelet derived growth factor

PDGFRA- Platelet derived growth factor receptor alpha

PTEN- Phosphatase and tensin homologue

SYT1- Synaptotagmin-1

TCM- Traditional Chinese Medicine

TMZ- Temozolomide

UV- Ultraviolet

VEGF- Vascular endothelial growth factor

WHO- World health organisation

XA- Xanthurenic acid

CLINICAL NOMENCLATURE

Invasion- Process by which motile cancer cells penetrate directly through the extra cellular matrix into surrounding tissues (Hammarström, 1999).

Metastasis/migration- Mechanism through which cancer cells from the primary tumour detach themselves and establish a secondary tumour at a different part of the body (Hapach et al., 2019).

Tumour microenvironment- A dynamic environment around the tumour which constantly interacts with the tumour and promotes tumour progression. Microenvironment typically constitutes of immune cells, blood vessels surrounding the tumour, signalling molecules and fibroblasts (Whiteside, 2008).

Adjuvant therapy- Use of therapeutic agents following primary treatment of cancer (i.e., chemotherapy, surgery, or radiation therapy) to maximise the effectiveness of the primary treatment or reduce the risk of recurrence (Tse et al., 2021)

ABSTRACT

Glioblastoma multiforme is the most malignant and aggressive form of brain cancer in adults. Its highly invasive nature results in poor prognoses and even with the best treatment, median survival of patients is 15 months. Current treatments involving surgical resection, radiotherapy and temozolomide (TMZ) have remained unchanged since 2005. Bioactive compounds extracted from plants, fungi and marine invertebrates have been a source of natural compounds used to treat many diseases and pathologies.

This study investigated the potential of root extracts of a traditional Chinese medicinal herb *Sophora flavescens* (Kushen) and a library of natural compound extract fractions from NatureBank in inhibiting glioblastoma cell invasion using U251MG cells. In the first aim, transwell invasion assay used to screen NatureBank compound extract library identified twenty compounds that inhibited invasion of U251MG cells (invasion percentages from 6.5% to 53%). In the second aim, all the twenty compounds were tested for cytotoxicity at different concentrations (0.03 to 1 µM). No cytotoxicity was detected for any of the compounds. Four compounds (3-Methylbut-3-enyl-O-a-L-rhamno-pyranosyl-(1-6)-O-b-D-glucopyranoside; Xanthurenic acid and two novel compounds) were selected for further study. Half maximal inhibitory concentrations (IC_{50}) of these compounds ranged from 0.24 to 0.43 µM. In the third aim, root extracts of Kushen were tested for their effect on limiting invasion of U251MG cells. A concentration of 50 µg/ml was effective in inhibiting invasion of cells by 57%.

The outcomes of this study are the identification of 20 natural compounds that were effective in limiting glioblastoma invasion. Further work needs to be conducted to identify the mode of action of these compounds.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Aparna Bindu

21st December 2021

ACKNOWLEDGEMENTS

I would like to express my humble gratitude to my supervisors Dr. Sunita Ramesh and Prof. Andrea Yool for the help and support provided throughout for the successful completion of my project. I would like to thank A/Prof. Munish Puri, the topic co-ordinator for organising the topic and all the support provided. I would also like to thank Alanah Varricchio and Hayden Gillings for their help and support.

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INTRODUCTION

Glioma refers to all forms of tumours that originate from glial cells. Their general characteristics include uncontrollable cell proliferation, diffuse infiltration, predilection towards necrosis, intense angiogenesis, lower rate of apoptosis and high genomic instability (Claes et al., 2007). According to World Health Organisation's (WHO) classification, there are four different types of gliomas. These are astrocytic tumour grades I, II, III and IV(Glioblastoma) (Anjum et al., 2017). Grade IV glioma or Glioblastoma Multiforme (GBM) is the most aggressive tumour of the Central Nervous System. GBM is widely known for its poor prognosis and lower patient survival rates. It is also the most common out of the four types in humans. In 2018, it was the 10th most common cause of death by cancer in Australia (Cancer Australia, 2018). While around 60% of brain tumours are de-novo (primary), 40% of them are malignant tumours (Anjum et al., 2017). The median survival rate for GBM ranges from 5 to 15 months after initial diagnosis. The 5-year survival rate ranges from 0-5%, i.e., only 5% of the patients diagnosed with GBM live for 5 years (Tran and Rosenthal, 2010).

About 5% of patients inherit the risk of the disease whereas all the other cases arise sporadically without any specific association with heredity (Farrell and Plotkin, 2007). Most of these malignant tumours occur as a result of sequential accumulation of genetic mutations. Abnormal regulation of growth factor signalling pathways also adds up to cause these malignant tumours. Growth factors/ molecules like vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF) regulate these sporadic proliferations (Alifieris and Trafaris, 2015).

Classification of GBM

The WHO classification of different grades of gliomas is mostly based on histology and immunohistochemistry. Out of the WHO classification, oligodendrogliomas (Grade II) have more favourable prognosis than glioblastoma as they are more chemo sensitive (Burger, 2002). However, apart from cases with distinct histological features, high grade gliomas show very little cellular differentiation. Therefore, characterizing GBM with histological features can lead to incorrect diagnosis. Every tumour varies from the other to great extent making diagnosis of such non-classical cases risky and controversial. This makes the accuracy and reproducibility of these non-classical diagnoses questionable (Nutt et.al., 2003; Burger, 2002). Such high level of interpersonal variability calls for a different basis of classification of these tumours which would result in effective prognosis.

Based on genetic and biological differences, glioblastomas can be divided into two types, primary and secondary glioblastomas. Primary glioblastomas are usually found in people older than 50 years

and are caused mainly by mutations and amplification of EGFR. Loss of heterozygosity of chromosome 10q, deletion of phosphatase and tensin homologue of chromosome 10 (PTEN) and p16 deletion are also some of the other reasons for cause of primary glioblastoma (Wen and Kesari, 2008). Secondary glioblastomas are lesser prevalent and are mostly detected in younger patients as compared to primary. They manifest as anaplastic astrocytomas and transform into glioblastomas (secondary) with time. Secondary glioblastomas are characterised by mutations in tumour suppressor gene p53 and by overexpression of PDGFR (Watanabe et.al., 1996). Even though they are genetically different, primary, and secondary glioblastoma are morphologically similar and indistinguishable. They respond similarly to conventional therapy but may respond differently to targeted molecular therapy.

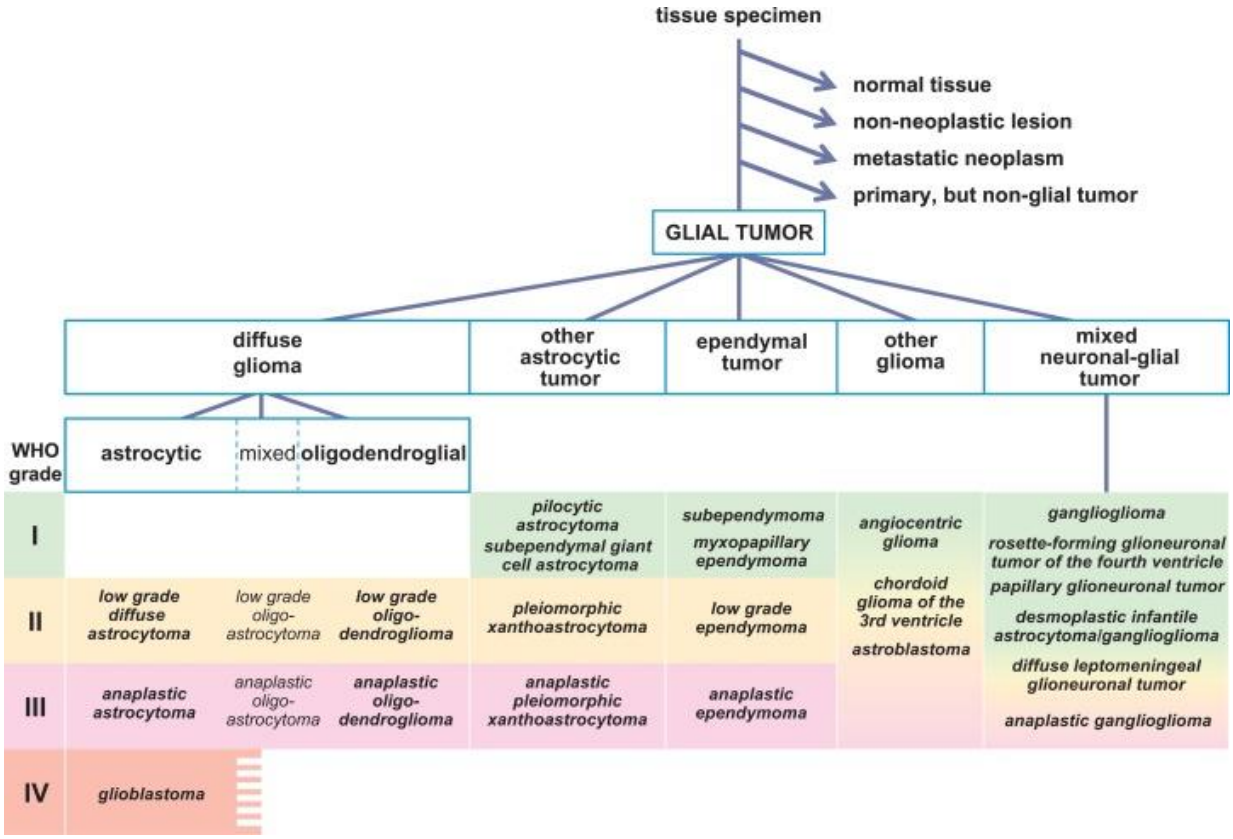

Figure 1: GBM classification by World Health Organisation based on histology (Perry and Wesseling, 2016). Reprinted with permission from Elsevier Books.

In their effort to better understand and classify GBM cells, researchers around the globe have been working on high dimensional profiling studies, gene expression studies (Nutt et al., 2003; Freije et al., 2004). Features such as gene expression signatures (such as EGFR overexpression), somatic mutations and DNA copy number studies are believed to be more efficient in predicting patient outcomes as compared to histology alone (Persano et al., 2013). Verhaak et. al., 2010, classified GBM based on somatic mutations, gene expression and DNA copy numbers. This led to classifying GBM as neural, proneural, classical and mesenchymal subtypes.

The neural subtype is characterised typically by expression of neuronal markers such as Neurofilament light gene (NEFL), Gamma-aminobutyric acid receptor subunit alpha-1 (GABRA1), Synaptotagmin 1 (SYT1). A key characteristic of proneural subtype was found as focal amplification of the chromosomal locus 4q12 which houses platelet derived growth factor receptor alpha gene (PDGFRA) and thereby a higher expression of PDGFRA. The mesenchymal subtype is defined by deletion of regions of chromosome 17 in only one of the loci or abnormal expression of the neurofibromatosis 1 gene (NF1) in general. The Classical subtype can be identified by chromosome 7 amplification together with chromosome 10 loss and four-fold high expression of EGFR gene (Verhaak et. al., 2010). Identification of these specific gene expression patterns in different subtypes can be potentially used to target specific subset of tumour cell types in a heterogenous tumour mass. However, no such treatment strategy is available currently.

Heterogeneity in GBM

Like other tumours, GBM is also highly heterogeneous. Studies as early as 1981 have reported remarkable differences in karyotype among freshly isolated clinical specimens (Bonavia et al., 1981). A study by Wilkstrand et al. in 1983 in a commercial cell line including 8 of its single cell derived clones showed heterogeneity in tumour associated antigens. Inter tumour heterogeneity can be described as two tumours, either within the same patient or in different patients behave or respond differently to therapy. Intra tumour heterogeneity occurs when cells within the same tumour behave or respond differently to a drug (Anjum et al., 2017). This heterogeneity often results in cancers with varying morphologies, growth rates and most importantly, their response to therapeutic drugs (Yung et al., 1982).

Intratumor heterogeneity may arise from clonal expansion of GBM cells during tumour progression. Another possible reason for heterogeneity within a tumour could be selective pressure resulting from factors like therapy (Steiber et al., 2014). Increasing evidence suggests that intra-tumoral heterogeneity arises from a genetic level. The role of Epidermal Growth factor Receptor variant III (EGFRvIII) in the progression of GBM is very well studied. EGFRvIII was found to influence normal cells in the surrounding by stimulating different paracrine mechanisms and promotes tumour growth (Nishikawa et al., 2004). However, these mutant receptors are found only in a subpopulation of tumour cells (Inda et al., 2010). Area-specific clonal diversity in GBM was confirmed by FISH analysis (Snuderl et al., 2011) and by comparative genomic hybridisation (CGH) and expression profiling (Sottoriva et al., 2013). This level of variation within a tumour mass makes it challenging to devise an effective standard treatment strategy for GBM. This also suggests that treating GBM should be a synergistic approach rather than targeting single subset of molecular markers.

Cancer stem cells in GBM

A high level of heterogeneity in GBM led to the search for a particular cell type to target and treat GBM. This led to the discovery of multipotent cancer stem cells (CSCs). Glioblastoma multiforme was one of the cancers in which cancer stem cells were identified (Facchino et al., 2011). The discovery of cancer stem cells has been a milestone in understanding the pathogenesis of the disease. There are multipotent neural stem cells in the Central Nervous System (CNS), which can proliferate, differentiate, and self-renew—identifying such cells led to the hypothesis that some of these transformed cells, which are precisely like a somatic stem cell, could behave as a tumour initiating cell or a cancer stem cell (CSC) (Facchino et al., 2011). CSCs have also been identified as the reason behind tumour reversal and relapse. CSCs express a membrane glycoprotein called CD133, which is well characterised and is used as a biomarker for isolating CSCs (Singh et al., 2004). CD133 activates the Wnt pathway, leading to increased expression of VEGF-A and Interleukin-8 (IL-8). This leads to increase in angiogenesis (one of the key mechanisms that drives cancer progression by forming new blood vessels) and thereby accelerating the growth rate of these cells (Barzegar Behrooz et al., 2019). Though CD133 has been very useful in isolating CSCs, it has become evident in recent years that they cannot be used as a marker for isolating CSCs in different patients as CSC phenotypes vary from patient to patient.

Cancer progression is regulated in multiple ways. A high growth rate of cells, as mentioned above, leads to a decrease in the availability of oxygen in cells leading to hypoxia. Hypoxia increases the expression of hypoxia-inducible factor 1-α (HIF-1α) in cancerous cells by stimulating glycolysis. Metabolism of the cell is elevated by increasing the glucose uptake and fermenting the glucose into lactate (Warburg effect), which has been identified as a characteristic of cancer cells (Liberty and Locasale, 2016).

There have been two different hypotheses to account for the heterogeneity centred around the "stemness" of cells. The classical approach defined by the 'stochastic model' hypothesises that all cells in the tumour possess the same tumorigenicity, and any of them can potentially act as tumour initiating cells. This theory predicts that these cells might undergo epigenetic changes resulting from their microenvironment and progresses by naturally selecting the best candidate for proliferation (Dalerba et al., 2007). Another predicted possibility is that of a 'hierarchical model', where only a specific subset of cells within the tumour possesses the ability to self-renew and proliferate without limits into different cell types to form a heterogeneous tumour mass. A considerable amount of experimental data has backed the latter concept. This hypothesis implied the existence of a particular set of cells similar to normal adult stem cells, which were later called cancer stem-like cells or CSCs, that on transplanting can give rise to a new bulk of tumours with terminally differentiated cells. CSCs

are classified into a superior class which precedes the terminally differentiated cells, thus leading to a hierarchy within the tumour (Visvader and Lindeman, 2008). However, recent studies in this fast-evolving field point towards different CSC pools in the same tumour mass, variability of CSC phenotype within and across tumours, and the potential of non-CSCs to morph into CSCs (Visvader and Lindeman, 2012). Altogether, this reveals the highly dynamic nature of CSCs in GBM. It is also possible that CSCs do not function as suggested in either of the hypothesised models but rather suit both hypotheses. CSCs were initially pursued as a possible therapeutic target for GBM, but as they vary significantly within a tumour, CSCs cannot be used as a primary target to treat GBM. In the event of using CSCs as a target for therapy, a personalised approach should be devised to specifically target different CSC pools.

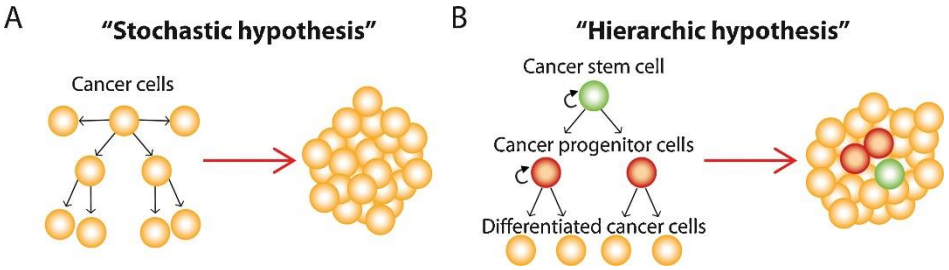

Figure 2: Different models of cancer progression (Visvader and Lindeman, 2012). Reprinted with permission from Cell Stem Cell.

A. The stochastic hypothesis suggests that all cells in the tumour mass have the same tumorigenic potential.

B. The hierarchic hypothesis suggests that cancer stem cells possess a unique ability to self-renew and terminally differentiate.

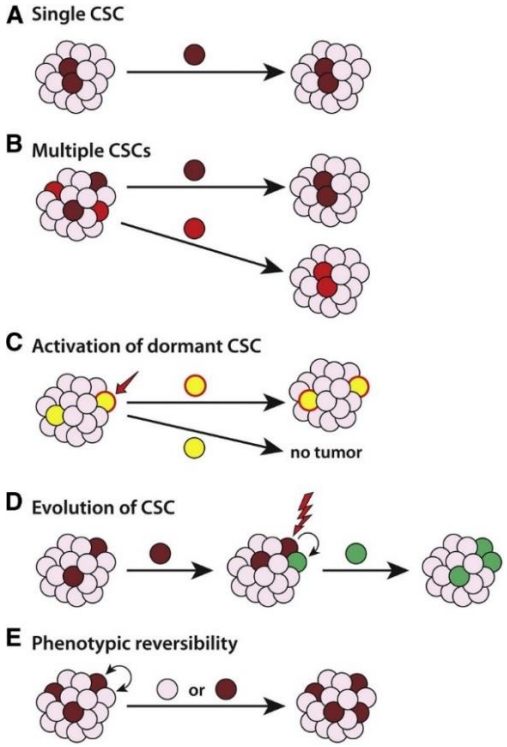

Figure 3: Different possible ways in which Cancer Stem Cells contribute to tumour heterogeneity (Visvader and Lindeman, 2012). Reprinted with permission from Cell Stem Cell.

A) Only one set of CSC present in the tumour with the ability to generate a tumour mass.

B) Multiple subpopulations of CSC's present within a tumour, where both possess the ability to regenerate.

C) Dormant for a long time (yellow with red border) CSC can be activated leading to recurrence long after therapy.

D) Already existing CSC acquires epigenetic mutation resulting in a more aggressive variant with the ability to continue tumour progression.

E) Unstable CSC phenotype, results in reversing of CSC cell surface markers.

Cell motility in cancerous and non-cancerous cells

Cell motility is a major event which enables tissue development, homeostasis, immune response as well as in cancer progression and metastasis (Friedl and Bröcker, 2000). Movement of cells can be in 2-dimension (2-D) or 3-dimension (3-D). Motion of cells towards a ligand coated surface without crossing any matrix barriers is considered as 2-D or haptokinetic motion of cells while movement of cells across a matrix (for instance, extra cellular matrix (ECM)) is considered 3-D motion of cells (Friedl and Bröcker, 2000). The 2-D cell movement occurs as a result of a series of adhesion and deadhesion processes. The three major events in this process are (1) protrusion of the leading edge and receptor mediated adhesion to surface, (2) contraction of the cell body, (3) detachment of the trailing edge. These sequential events were studied in non-neoplastic cells such as fibroblasts, keratinocytes and myoblasts (Abercrombie et al., 1977; Loffenburger and Horwitz, 1996). However, extended studies on cancerous cells showed that they use similar methods to migrate to different parts of our body and to invade surrounding tissues (Friedl and Wolf, 2003).

Cell migration or invasion involves not only a number of biochemical signalling but also numerous events of biomechanical remodelling and phenotypic changes. The ECM acts as a substrate for cellular adhesion as well as a barrier for the advancing cell body (Friedl and Wolf, 2003). As the first step in migrating, a moving cell becomes polarised and elongated leading to formation of an elongated protrusion called lamellipoda/filipoda/pseudopod or invadopod (Adams, 2001). These extensions grow and touch ECM initiating binding with the help of trans membrane adhesion molecules (mostly belonging to integrin family). Integrins couple with actin cytoskeleton to form locally enriched focal adhesion complexes which helps the cell to glide forward. 3-D movement of the cells across ECM follows similar stages with an addition of proteolytic remodelling of ECM after the lamellipoda attachment to the ECM (Ohuchi et al., 1997). Although cellular protrusions that help in the process of migration can differ in morphology, they are all composed of actin and different sets of signalling proteins (Friedl and Wolf, 2003).

The initial polarisation and elongation of cells is facilitated by polymerisation of actin and their arrangment into actin filaments (Loffenburger and Horwitz, 1996). As these protrusions grow and touch the ECM, transmembrane receptors, particularly those belonging to integrin family are activated and binding to ECM is initiated (Hynes, 2002). Integrins then couple with actin cytoskeleton with the help of adapter proteins to form a dense cluster called focal complex. These focal complexes

then grow and stabilise themselves to form a focal contact (Burridge and Chrzanowska-Wodnicka, 1996). Integrins that take part in the focal contact formation can vary depending on the cell type and ECM substrate. Different non-integrin receptors like CD-44, CD-26, surface proteoglycans and other immunoglobulin superfamily members also take part in signalling cell motility (Maaser et al., 1999). The onset of adhesion receptors towards the ECM binding site activates surface proteases to the site of ECM binding. This leads to degradation of ECM components which are close to the focal complex (focal proteolysis). As ECM degradation progress, the moving cell propels towards the ECM and gains required space for it to migrate through the matrix structure (Friedl et al., 1997).

Current treatment strategies and challenges

Numerous studies have been done for understanding the pathogenesis of malignant gliomas. These studies have helped in developing effective strategies for the intervention of disease progression. However, the complete mechanism of pathogenesis is still not completely understood. Therefore, currently available treatment methods are not always completely successful in curing GBM.

Current treatment strategies for GBM include surgical resection, chemotherapy, radiotherapy, and treatment with temozolomide (TMZ). Treating GBM also involves clinical management of symptoms like cerebral oedema, seizures, infections, cognitive dysfunction, venous thromboembolism. Traditionally, after successful resection of the tumour, patients undergo radiation therapy five times a week for five to six weeks, along with oral administration of temozolomide daily. Recurrence can be observed as soon as 6.9 months after initial diagnosis. Tumour recurrence is usually treated with re-irradiation if eligible. Due to ethical constraints, re-operating is not an option for many patients and therefore clinical trial participation remains the most viable option (Alifieris and Trafaris, 2015; Wen and Kesari, 2008). While surgical resection removes most of the tumour mass, a potent subset of tumour cells would have already moved across the visible tumour boundary and would lead to malignancy (Ghosh et al., 2018). Radiation therapy has shown considerable improvement in survival rates; however, radiation-induced DNA damage, necrosis, neuronal damage, and insensitivity (radioresistance) of some GBM types are some of the limitations of this mode of treatment. Extreme proliferation of cells within the tumour leads to lesser availability of oxygen within the tumour as compared to the surrounding cortex, leading to hypoxia. Hypoxia is often attributed for radioresistance of tumours (Torrisi et al., 2020). Treatment with TMZ has been the most successful in conjunction with surgery and radiation (Stupp et al., 2005).

Temozolomide is an oral alkylating agent that has 96-100% bioavailability. It is successful in diffusing through the blood-brain barrier into the central nervous system. Starting dosage of TMZ is from 75mg/m^2 daily for six weeks, along with regional radiotherapy at the site of operation. This is

followed by a concomitant dose of 150mg/m^2 orally for five days and bumped up to 200mg/m^2 if well-tolerated in 28-day cycles (Chinot et al., 2001; Villano et al., 2009). Currently, this is the preferred treatment mode considering the comparable survival benefits that it provides (median overall survival (OS) 14.6 months, 2-year survival 26.5%) (Chen et al., 2013). However, high doses of TMZ are required for the desired effect, which often leads to cytotoxicity in non-cancerous cells. Furthermore, prolonged usage of TMZ can make the tumour resistant to the drug and leaves the patient with no more options.

Other drugs currently available for treating GBM include chloroethylating agents such as carmustine and lomustine. Carmustine coated wafers are implanted in the surgical cavity which are designed to release carmustine slowly over a period of 2-3 weeks. Though this has shown significant improvement in patient survival rates, terrible side effects like seizures, difficulty in wound healing, intra cranial infection have been observed in many patients (Iacob and Dinca, 2009). For patients that do not respond to TMZ (often offered as a first line of therapy), a second line of cytotoxic agents like carboplatin, etoposide, oxaliplatin, irinotecan is conventionally used. There are other agents which include monoclonal antibodies that target angiogenic growth factors and inhibitors that target specific kinases, which are more often offered as an experimental approach in chemotherapy (Iacob and Dinca, 2009). Even though moderately successful options of treatments are available for treating GBM and there are several ongoing research for new intervention strategies, most of them are focused on cell death. Out of the vast number of papers published upto 2019, only 11% focused on limiting the motility of cells (Yool and Ramesh, 2020).

Recurrence or relapse of the tumour is another significant threat in treating GBM. Even after surgical resection followed by chemo and radiotherapy, there have been reports of recurrence of tumour mass (Ghosh et al., 2018). The brain being a terminally differentiated organ, the rate of regeneration is very slow or nearly zero. As a result, highly invasive techniques cannot be used in treating GBM. This could later lead to recurrence if GBM cells invade into surrounding regions of the brain. It is also possible that GBM cells invade different functional areas of the brain, making it difficult for surgeons to operate as there is a risk losing the patient's cognitive processes (Sanai and Berger, 2008). Tumour heterogeneity is yet another problem when it comes to treating malignant gliomas. Different cells in the same tumour with different genotypic and phenotypic expression patterns might react differently to therapy, leading to failure of treatment and recurrence of tumour (Persano et al., 2013). The diffuse nature of the tumour is a significant feature of GBM. Diffuse gliomas are characterised by extensive infiltration of tumour cells into the dense network of neurons or neuronal processes known as neuropil (Claes et al., 2007). Cancer stem cells, as discussed in the previous section, poses an obvious challenge in treating glioblastoma. Furthermore, CSCs are known to have a dormant period or a

period of inactivity during which they behave like other cancer cells. They can be triggered by different micro-environmental factors or chemotherapy to revert into CSCs (Moore and Lyle, 2010). Even though many studies are many ongoing studies for finding an effective way to treat glioblastoma, a significant gap in the knowledge exists around limiting cell motility while the primary mass is being treated.

Pharmacological agents that prevent cancer cell migration especially using natural extracts is a major area that has not been explored to their full potential. Nevertheless, ample amounts of anticancer agents are used in the clinic, either natural or derived from a natural source. Major chemotherapy drugs currently in use like vincristine, irinotecan, etoposide, paclitaxel are natural compounds derived from plants. Moreover, there are other classes of compounds like taxanes, alkaloids, were identified as potential anticancer agents (Nobili et al., 2009). Natural products have opened up a promising arena of prospective adjuvants when applied with TMZ have shown potential in treating cancer. Ginsenoside from the herbal plant ginseng which shows anti-tumour and anti-angiogenic effect (Sun et al., 2016). The bioactive in green tea (epigallocatechin gallate) which inhibits viability and migration (Zhang et al., 2015) are some of the promising compounds among many others.

Traditional Chinese Medicine (TCM) is a practice that has been around for thousands of years and is still followed not only in China but also in many parts around the world. Camptothecins (CPT) are one of the major contributions from TCM with regards to treating cancer. CPT is a natural alkaloid extracted from the bark of *Camptotheca acuminata* otherwise known as Chinese happy tree (Wall et al., 1966). Though CPT showed promising results early on, severe bladder toxicity associated with it blighted the scope. However, chemical structure manipulation of CPT gave rise to two potent analogues topotecan and irinotecan, they are specific inhibitors of topoisomerase I enzyme which helps in DNA unwinding before replication and transcription (Mann, 2002; Martino et al., 2017). Both the analogues were cleared for clinical use and irinotecan is one of the second -tier drugs used in treating GBM (Iacob and Dinca, 2009). A recent study by Nourmohammadi et al., 2019, shows that a Chinese traditional medicinal compound, Compound Kushen Injection (CKI), has been very effective in limiting invasion, proliferation and gene expression in a range of different types of cancer cell lines. This compound has been chemically refined, fractionated and dose-dependent studies have been carried out. A rather exciting outcome of this study was that this activity was not a result of a single component. Instead, it was the combination of different identified fractions which was responsible for the effect. Furthermore, it was also found that the effective combination was different for different cancer types. Therefore, this suggests that combinatorial therapy using such natural compounds alongside current medication could prove much more effective in treating glioblastoma.

Cancer is a complex disease. There are multiple pathways through which the disease progress. For the last few decades, treatment of cancer mainly focused on limiting the proliferation of cancerous cells. However, new strategies (such as CAR T-cell therapy) followed as studies expanded and painted a more comprehensive picture of the disease progression. Most of the treatment methods available today focus on arresting cell cycle activities or selectively targeting specific receptor complexes to stop cellular proliferation. However, these strategies fail to address one of the significant properties of cancer cells, invasiveness. Invasiveness is mainly the reason GBM recurs, and recurrence is the primary reason for treatment failure. Therefore, treating cancer should follow a synergistic approach rather than targeting a single molecular target. Targeting a single molecular target and using another agent to limit the invasiveness of the cancerous cells could stop the proliferation as well as prevent cancer cells from invading the surrounding healthy tissues leading to possibly lower chances of recurrence. As seen in recent years, concomitant or adjuvant therapy with a different drug and chemotherapy is more effective compared to treatment with only chemotherapy. So, treating GBM with an adjuvant that limits invasiveness together with chemotherapy could help tackle recurrence.

Rationale and techniques followed in the study

As described in the previous sections, cancer is a complex and dynamic disease. Therefore, tackling such a disease would require a multipronged approach. This study utilises natural compounds from NatureBank, a compound library by Griffith Institute of Drug Discovery (GRIDD). Compounds in this library have been extracted from native Australian plants, animals, fungi and marine invertebrates. The extracts provided by NatureBank which are used in this study are purified fractions from plants, animals, fungi or marine invertebrates.

The study by Nourmohammadi et al. on CKI revealed interesting findings with regards to limiting GBM invasion and migration. CKI is a potent mixture prepared from *Sophora flavescens* (Kushen) and *Heterosmilax chinensis*. At the start of the project, it was proposed by the supervisors to use CKI to further the work done by Nourmohammadi et al. by focusing only on the action of CKI on GBM cell line (U-251 MG). However, due to Covid-19 and political climate associated with it, sourcing CKI from China was not possible. Sourcing the roots of *Sophora flavescens* was still a viable option and therefore it was consensually decided to study the effect of crude Kushen extracts on GBM invasion and migration.

The transwell migration assay which was used in this study utilises the ability of a cell to migrate across a chemotactic gradient through a physical barrier (Justus et al., 2014). Coating the transwell membrane with a matrix (Matrigel® in this case) mimics extracellular matrix and can help in

investigating cell invasion. Cells are added to the Matrigel coated membrane along with growth media containing lower serum concentration. These membrane inserts containing cells are then placed into bottom wells of the transwell plate containing growth media with a higher serum concentration. This creates a chemotactic gradient allowing cells to migrate towards the bottom chamber. Cells that successfully invade through the added matrix are trapped within the membrane and the matrix coating. Those cells that do not invade are removed from the top and the membrane is fixed stained and washed to visualise the cells that successfully invaded.

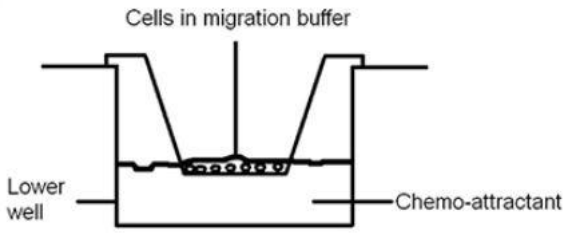

Figure 4: Transwell membrane insert during tranwell invasion assay. Reprinted with permission from JoVE.

Hypotheses and aims of the study

This study aims to investigate the effect of natural compounds in inhibiting the invasion of glioblastoma cells. This study hypothesises that natural compounds like those from Griffith University's Nature Bank and Chinese traditional medicinal herb Kushen (Sophora flavescens) effectively inhibit the invasion of glioblastoma cells across the extracellular matrix. NatureBank compounds include extracts from various Australian plants, fungi and marine invertebrates. The aims of the study are:

(1) To screen compounds from natural compound library, NatureBank to identify their anti-cancerous effect on glioblastoma cells using U-251MG cell lines.

(2) To test cytotoxicity and identify the minimum inhibitory concentration of the identified compounds.

(3) To investigate the inhibitory effect of crude Kushen extract on the invasion of U251-MG cell lines.

MATERIALS AND METHODS

Cell line used

There are multiple genotypic and phenotypic cell models available to study GBM. This study utilised U251-MG cell lines and evaluated the effect of different natural compounds in limiting the invasiveness of U251-MG cells. U251-MG cells are slowly proliferating, phenotypically distinct (as compared to other model cell lines such as U-87) commercial cell lines possessing intrinsic resistance to TMZ. They belong to the mesenchymal molecular subtype (Motaln et al., 2015).

The U251-MG cell line purchased from CellBank Australia was kindly provided by Prof. Andrea Yool from the University of Adelaide. Cells used in transwell invasion assays, cytotoxicity assays and dose response assays belonged to passages 12 to 14. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS) and were maintained at 37°C with 5% CO_2.

NatureBank Compounds

A small subset of 500 compounds (from a library of 20000 natural extracts) were obtained from Griffith Institute of Drug Discovery's NatureBank compound library with an MTA in place with Flinders University. Out of these 500 compounds, 88 of them were screened as a part of this project. 1µL of each compound at 5mM concentration was received from NatureBank for initial screening. They were resuspended in DMSO and were used at a final concentration of 0.9 or 1µM in transwell invasion assays (refer appendix for detailed dilution volumes).

Kushen roots harvesting and processing

Kushen roots were sourced from the Genomics Centre, University of Adelaide, Waite Campus (kindly provided by A.Prof. Kenneth Chalmers). The roots provided were of plants that traditionally grows in 9 different geographical locations in China (plants were grown in Genomic Centre, Waite campus, Adelaide University) (Schultz et al., 2021). Collected Kushen roots were washed multiple times to remove any soil, or solid impurities adhered on to them. Washed roots were pat dried with a tissue and were packed in a paper bag. These paper bags were then left in a 37°C drying incubator for 3 months. Though there were 9 different plant lines available only two lines, Line 2 from Inner Mongolia and Line 4 from Hebei province were used for experiments as the other lines did not have enough plant material to process.

Once the roots were thoroughly dried, they were cut into small pieces and were ground into a fine powder using a spice grinder. This fine powder was then transferred into pre-weighed flasks. Each of

the flasks were weighed for a second time to record their weights while containing the powdered root material. Dry weights of the roots were calculated using the pre-weighed and post-weighed values. Root extraction was carried out using two solvents, water, and ethanol. Once the dry weights of samples were calculated, solvent volume corresponding to ten times of the dry weight was added to respective flasks. These flasks where then heated at 60°C for 80 minutes and were stirred occasionally. After 80 minutes of heating, they were left to cool down and then were aliquoted out into two different 50ml falcon tubes. Falcon tubes were then centrifuged at 4500 rpm for ten minutes and the supernatant was then aliquoted out into 2ml pre-weighed Eppendorf tubes. These Eppendorf tubes were left for two days on a heating block at 70°C with the lids open to let the solvent evaporate completely. Once the solvent was completely evaporated from the tubes, they were weighed again to calculate the actual weight of the residue left in them. The ethanol extracted samples were resuspended in DMSO and the water extracted samples were resuspended in water to obtain 1.5, 2, 3.12, 6.25, 12.5, 25 and 50 µg of dried sample/ml of solvent (DMSO or water).

Maintaining U251-MG cells in culture media

All cell culture techniques were carried out in a laminar hood under aseptic conditions. All the equipment used for cell culture inside the laminar hood was sterilised with 80% alcohol. Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS was used to culture U251-MG cells in T75 flasks. They were maintained at 37°C with 5% CO_2. Cells were maintained as adherent monolayers and their confluency was monitored twice a week using Leica microsystems DMi1 microscope. Once the cells reach a confluency of 80-90% they were passaged or used for experiments.

Passaging or subculturing involved aspirating the culture medium and washing the cells with 1x Phosphate Buffer Saline (PBS). PBS was apirated after washing. The cells were then trypsinised with 1x Trysin/EDTA solution (10% of culture volume) for 2-3 minutes. Following typsinisation, the cell solution was neutralised with 10% FBS, DMEM media. Cells were then gently pipetted two to three times to ensure complete resuspension. After resuspending the cells, a volume that would result in at least 30% confluency in the new flask was calculated and pipetted out (calculations provided in appendix). This volume was added to a fresh T175 culture flask. The flasks were then topped up to 30 ml, with fresh culture media.

Reanimation and cryopreservation of U251-MG cells

Two vials of cryogenic preserved cells were obtained from University of Adelaide at the start of the experiments. In order to reanimate these cells, tubes from the obtained stock were removed from the freezer and were allowed to thaw on wet ice until all the contents became liquid. Thawed contents were then transferred to a 15ml centrifuge tube, and approximately 4ml of DMEM (10% FBS) was

added to it. Tubes are then centrifuged at 120g for 5 minutes at room temperature. Next, the resulting supernatant was carefully removed, and the cells were resuspended in 5ml of DMEM (10% FBS). Finally, 5ml of the resulting cell solution was transferred into a fresh flask (T175) and was topped up with appropriate amounts of DMEM (10% FBS).

Simultaneously, cryogenic stocks of cells were prepared using cells grown to 90- 95% confluency for further use and were maintained at appropriate temperatures (-80°C for continuing culture and in liquid nitrogen for long term storage). The spent media from the flask was removed, and the cells were washed with 1x PBS. After aspirating PBS, cells were trypsinised with 1x trypsin/EDTA for 2-3 minutes, following which they were neutralised with DMEM (10%FBS). 10µl of this solution was used to perform a cell count on a haemocytometer. A resuspension volume is calculated based on the cell count to obtain approximately 4×10^4 cells per ml. The rest of the cell suspension was centrifuged at 4500 rpm for 5 minutes at room temperature. The supernatant was aspirated, and the cells were resuspended in the calculated resuspension volume of complete DMEM (calculation provided in the appendix). 500µl of this cell suspension was transferred into a cryovial to which 500µl of freezing mix was added (refer to appendix for components). These vials were stored at -80°C overnight and were later moved to liquid nitrogen storage tanks (-196°C).

Transwell invasion assay

24-welled transwell inserts from Corning® (6.5mm, 8µm pore size) were used to perform transwell invasion assay. Top layer of the removable inserts was coated with Matrigel (Sigma-Aldrich) (1 in 40 dilution) which mimics the ECM and acts as a physical barrier for the cells. Inserts coated with Matrigel were left overnight to dry and were rehydrated with 50µl of serum free media one hour prior to the experiment. Cells were grown to 40% confluency with 10% FBS, DMEM media and were then starved with 2% FBS media 24 hours prior to the treatment with NatureBank compounds. Cells were then detached and resuspended in serum free media to obtain approximately 60000 cells in 10ml (refer appendix for calculations). 70µl of the cell suspension made with respective compounds (NatureBank or Kushen extracts) or vehicle control were added to the top inserts (120µl in total with rehydration volume). Bottom wells of the plate were filled with 600µl of growth media with 10% FBS creating a chemotactic gradient along with respective compounds or vehicle control to create a. Cells were then incubated for 4.5 hours at 37^{o}C and 5% CO_2. After the incubation period, non-invasive cells were removed from the top using a cotton swab and the inserts were fixed using 70% ethanol for 7 minutes. Fixed inserts were then stained using 0.2% w/v crystal violet solution (Justus et al., 2014) for 12 minutes. Five images of each insert were taken under 10x magnification covering the entire surface area of the insert with minimal overlap between each image. The average cell

numbers were then calculated by counting the invaded cells in each of the five 5 images. The average values were normalised to that from the vehicle controls and was expressed as percentage invasion.

$$\%Invasion = \frac{Average\ cell\ count\ for\ a\ treatment}{Average\ cell\ count\ of\ control} \times 100$$

For the initial screening of NatureBank compounds, invasion assays were performed as described above. 1.2µL (0.9mM) of each compound or vehicle control were added to both top and bottom mixes resulting in a concentration of 1µM on top inserts and 0.9µM in bottom wells. DMSO was used as a vehicle control for screening.

Resuspended Kushen extracts at 2µg dried root extract/ml of solvent were used to perform invasion assay as described. 1µL (2mg/ml) of each extract was added to the top mix resulting in 2µg/ml final concentration. 1.2µL (2mg/ml) of each of the extracts were added to the bottom mixes to result in 2µg/ml final concentration. The same process was repeated for a final concentration of 50µg of dried root extract/ml of solvent to study the inhibitory effect on cell invasion. For ethanol extracted samples resuspended in DMSO, DMSO was used as vehicle control. Water extracted samples resuspended in water used water as vehicle control.

Invasion assays were carried out using different doses of identified NatureBank compounds from the preliminary screening, to determine an effective inhibitory concentration. Different concentrations, specifically 1µM, 0.5 µM, 0.3 µM, 0.1 µM and 0.03 µM were used to evaluate the response and generate a dose response curve. Stocks of different concentrations were prepared (resuspension volumes provided in appendix). 1µL and 1.2µL of each compound were added to top and bottom mixes respectively to reach different final concentrations (1µM, 0.5 µM, 0.3 µM, 0.1 µM and 0.03 µM). DMSO was used as vehicle control for dose response experiments.

Cytotoxicity assay with NatureBank compounds

Cytotoxicity was assessed by using the Alamar Blue assay (O'Brien et al., 2000). First, cells were detached using trypsin/EDTA solution and were resuspended in DMEM with 10% FBS. Following this, the cells were seeded on a 96-welled plate with approximately 700-800 cells/well. These plates were then incubated at 37°C with 5% CO_2 for 48 hours. After 48 hours, media was removed from all the wells, replaced with 100ul of the respective treatments, and incubated at the same conditions for 4.5 hours. Finally, treatments were replaced with 100ul of Alamar Blue solution (1:10 with warm DMEM (10% FBS)) after 4.5 hours of incubation. To assess cell viability, fluorescence readings were taken (FLUOstar Omega, BMG Labtech) every 45 minutes until 90 minutes. Cells with DMSO were treated as vehicle control, and growth media with no cells were considered negative control, used for

background colour correction. The fluorescence values were normalised with reference to control and were expressed as percentage of cell viability based on signal intensity.

Treatments included 4 different concentrations of different NatureBank compounds which were 1µM, 0.3µM, 0.1µM and 0.03µM. The same procedure was repeated for identifying cytotoxicity of Kushen extracts by using different concentrations of Kushen extracts. The concentrations that were trialled included 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.15 µg/ml, 2 µg/ml and 1.5µg/ml.

Statistical analysis

Statistical analysis was carried out using software Graphpad Prism version 9.2.0. All the experiments carried out consisted of at least three independent biological replicates (n=3) except for initial screening of NatureBank compounds using transwell invasion assay (n=2). All the results are expressed as mean and error bars represent standard deviation. One-way ANOVA was carried out to determine significance and $p<0.005$ was considered to be significant. Specific p-values and exact replicate numbers are provided in figure legends.

RESULTS

Screening NatureBank compounds for inhibition of invasiveness using transwell invasion assay

77 compounds from a 96-well plate received from NatureBank were screened to find compounds that inhibited invasion. While some of the compounds amplified the invasiveness of U251 cells, some of them successfully inhibited invasion. Compounds which amplified the invasiveness were not studied further as they did not limit invasion by definition. Compounds with invasion percentages less than 60% in both cell lines (U251 and U87) were selected and further studied to identify any cytotoxicity associated with them. A dose response was also carried out to determine the half maximal inhibitory concentration of specific compounds which are described in detail in following subsections. The invasion percentages varied from as low as 6.5% to as high as 476% (Figure 5).

	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	-	-	-	-	-	-	-	-	-	-
B	SN598	SN608	SN612	SN617	SN621	SN622	SN630	SN637	SN638	SN310	SN311	DMSO
C	SN312	SN313	SN316	SN317	SN318	SN322	SN323	SN324	SN325	SN326	SN327	DMSO
D	SN328	SN329	SN330	SN331	SN332	SN333	SN334	SN337	SN338	SN339	SN340	DMSO
E	SN341	SN342	SN343	SN344	SN345	SN346	SN347	SN348	SN349	SN367	SN392	DMSO
F	SN396	SN399	SN405	SN408	SN409	SN548	SN550	SN594	SN599	SN607	SN314	DMSO
G	SN315	SN319	SN321	SN376	SN389	SN398	SN119	SN120	SN122	SN123	SN093	DMSO
H	SN094	SN095	SN096	SN097	SN098	SN100	SN101	SN102	SN103	SN104	SN106	DMSO

Table 1: Plate layout for the screened plate of compounds received from NatureBank

Screening of the above-mentioned compounds (Table 1) identified three compounds which inhibited invasion in U251-MG cell lines, which were SN096- 6.5%, SN104- 31%, and SN594- 8.5% (values indicate percentage of cells that invaded through the membrane- invasion percentages) (Figure 5). Another study by an honour's student, in which the same compounds were screened using U-87 (another model cell line for GBM (neural subtype)) cell line led to identification of compound SN098 which showed 53% invasion in U251-MG cell lines and 42% inU87-MG. This study was conducted in collaboration with a research group from the University of Adelaide. Another plate of compounds received from NatureBank were screened at the University of Adelaide on U87MG and U251MG cell lines. This screening resulted in identifying 16 compounds which showed inhibition in both the cell lines (Figure 6).

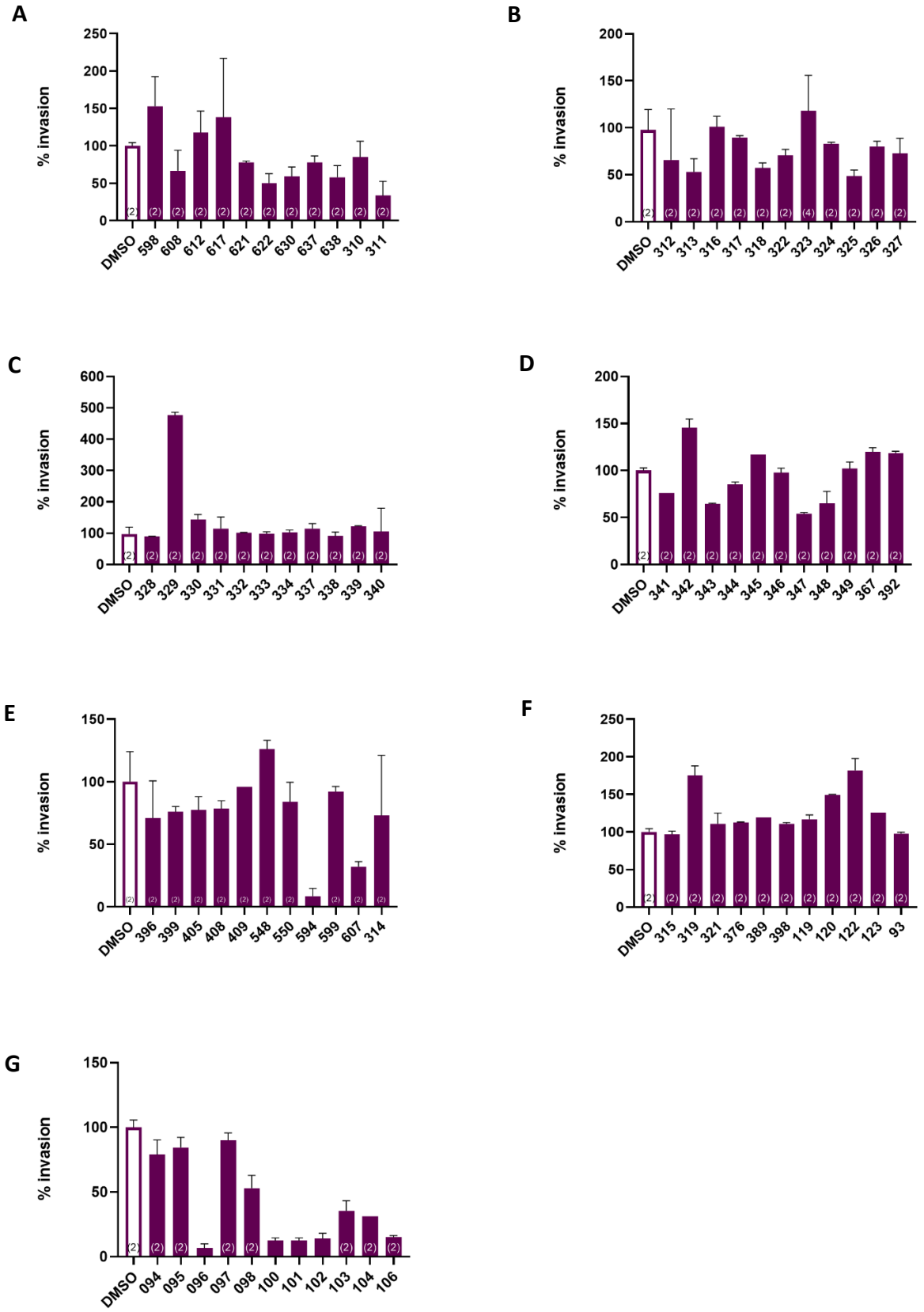

Figure 5**: Cell counts expressed as invasion percentage with respect to DMSO controls after treating with different NatureBank compounds on U251-MG cell line.** A-G invasion percentages of each NatureBank compound in along with DMSO controls. Error bars represent standard deviation, n=2.

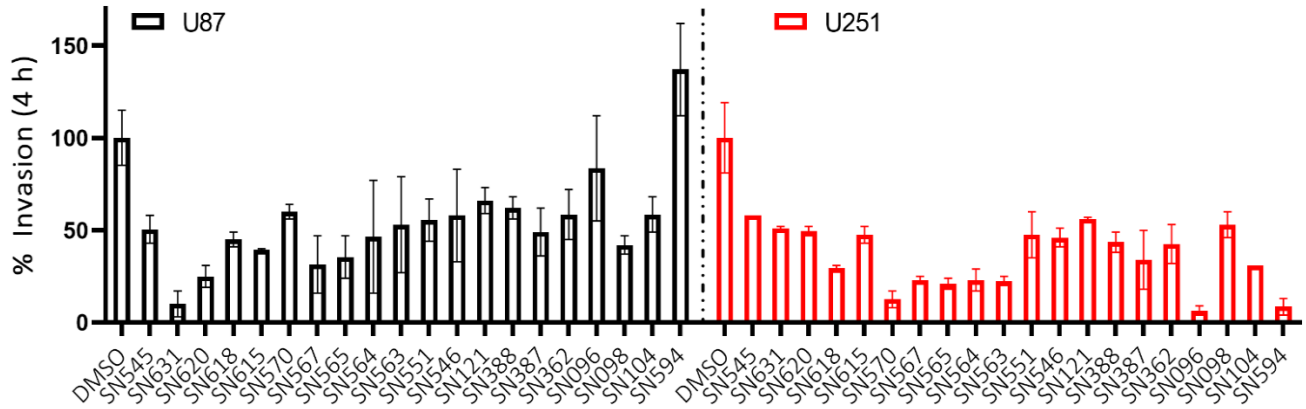

Figure 6: A comparison of the identified compounds between U251MG and U87MG. Invasion percentages of 20 NautreBank compounds identified from the initial screening. Invasion percentages for the identified compounds represented in black for U87MG cell line (provided by collaborators) and in red for U251MG cell line (data for SN098, SN096, SN104 and SN594 generated in this project, rest of the data provided by collaborators). Error bars represent standard deviation.

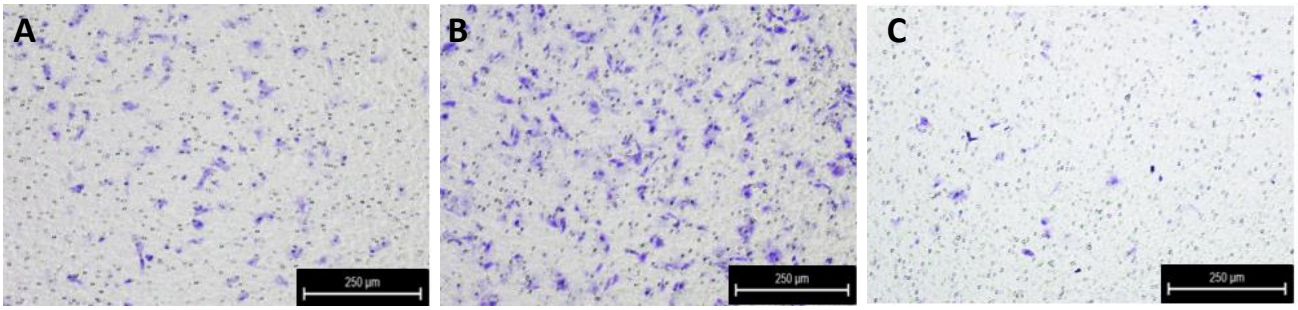

Figure 7: U251-MG cells fixed with 70% ethanol and stained with 0.2% crystal violet under microscope (10x magnification)

A) DMSO control

B) Transwell filter treated with SN319, one of the compounds that potentiated invasiveness

C) Transwell filter treated with SN096, one of the compounds that inhibited invasion.

Testing cytotoxicity of the identified NatureBank compounds on U251-MG cell line

All the 20 compounds identified from the initial screening were tested for cytotoxicity using Alamar Blue reagent. Different concentrations of each compound, 1µM, 0.3 µM, 0.1 µM,0.03 µM, were tested for cytotoxicity. None of the identified compounds were cytotoxic in any of the tested concentrations.

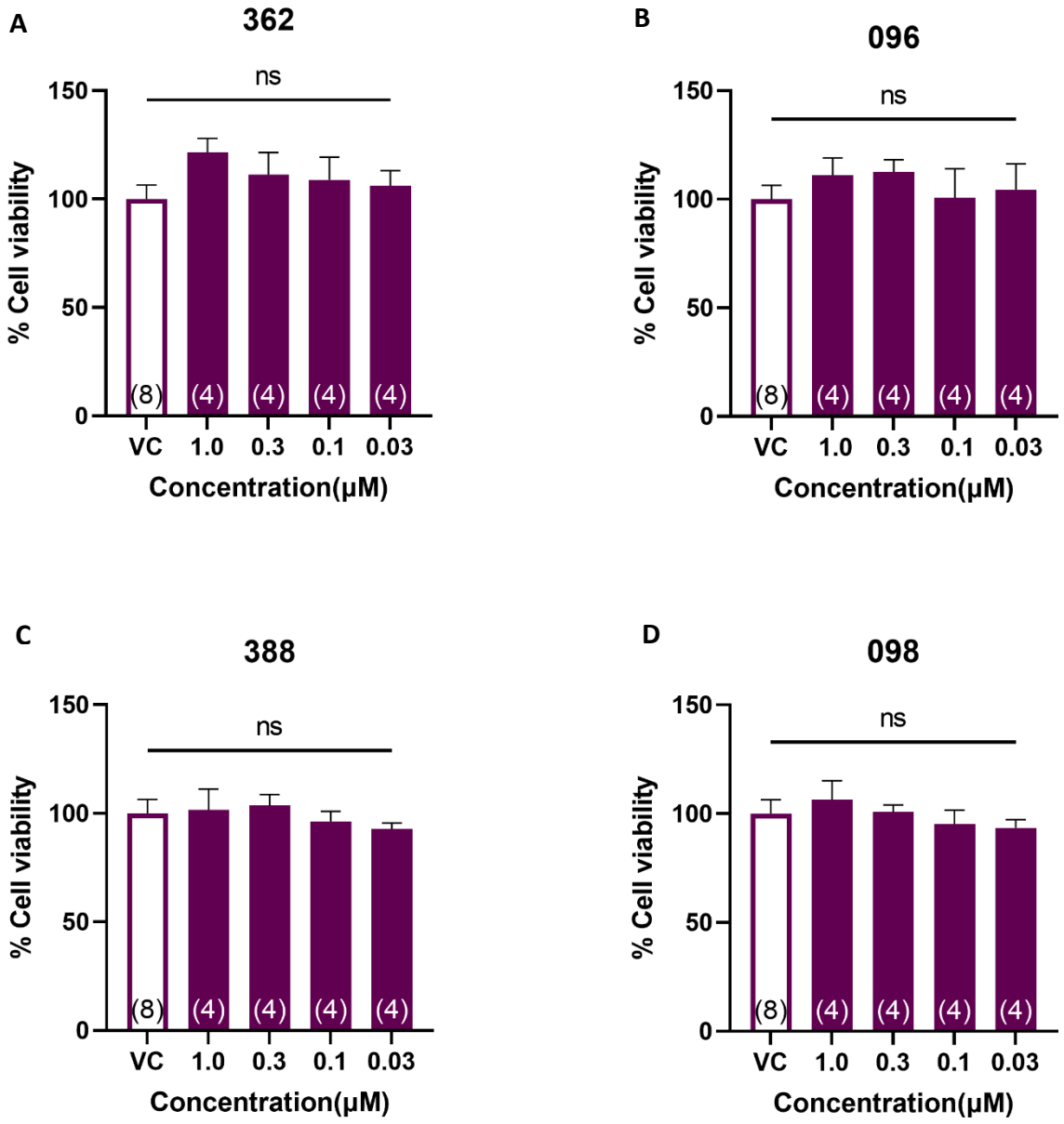

Figure 8: Cell viability assessed using Alamar Blue assay to identify any cytotoxic effect of the identified compounds. Different concentrations were tested (0.03, 0.1, 0.3, 1.0μM) for compounds SN362 (A), SN096 (B), SN388 (C), SN098 (D). n=8 for DMSO controls, n=4 for all treatments, ns= no significance. Error bars represent standard deviation.

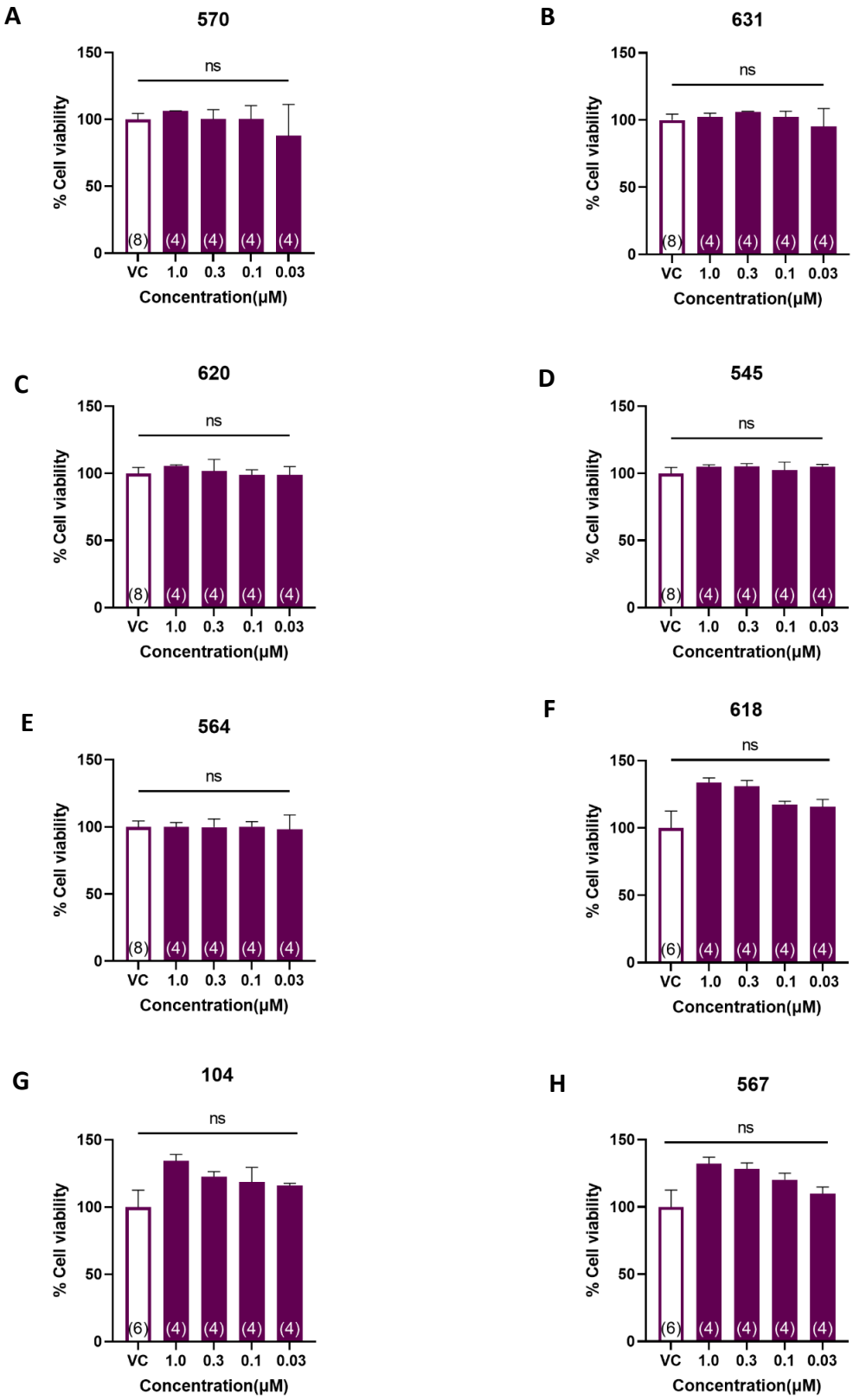

Figure 9: Cell viability assessed using Alamar Blue assay to identify any cytotoxic effect of the identified compounds. Different concentrations were tested (0.03, 0.1, 0.3, 1.0µM) for compounds SN510 (A), SN631 (B), SN620 (C), SN545 (D), SN564 (E), SN618 (F), SN104 (G), SN567 (H). n=8 for DMSO controls, n=4 for all treatments, ns= no significance. Error bars represent standard deviation.

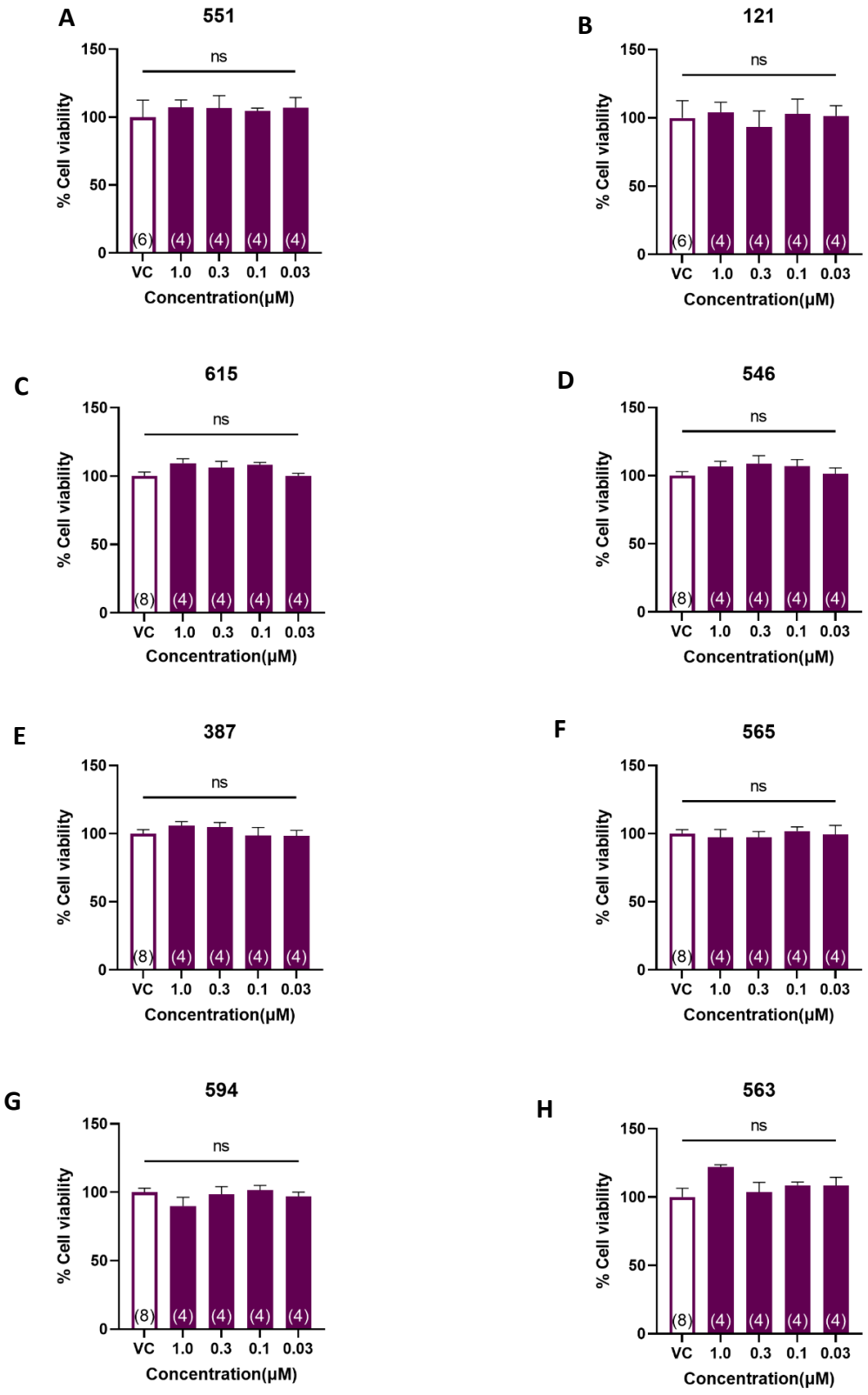

Figure 10:Cell viability assessed using Alamar Blue assay to identify any cytotoxic effect of the identified compounds. Different concentrations were tested (0.03, 0.1, 0.3, 1.0μM) for compounds SN551 (A), SN121 (B), SN615 (C), SN546 (D), SN387 (E), SN565 (F), SN594 (G), SN563 (H). n=8 for DMSO controls, n=4 for all treatments, ns= no significance. Error bars represent standard deviation.

Further, these results were presented to NatureBank by the research team revealing the chemical names and structures of the identified compounds. Table 2 provides chemical names corresponding to the SN codes for 20 identified compounds.

SN code	Trivial Name (if available)
SN545	Sauristolactam / Saurolactam
SN631	Xanthurenic acid
SN620	Enterocarpam II
SN618	3-Methylbut-3-enyl-O-a-L-rhamno-pyranosyl-(1-6)-O-b-D-glucopyranoside
SN567	Xanthurenic acid
SN565	RAD natural product
SN564	RAD natural product
SN563	RAD natural product
SN551	Caelestine
SN546	Caldensine
SN121	Gallic acid
SN388	Methyl ether of pestalactam A
SN387	Pestalactam A
SN362	Natural product
SN096	Davis compound (under MTA)
SN098	Davis compound (under MTA)
SN594	Ellipticine
SN104	Polyandrocarpamine synthetic derivative

Table 2**: SN codes and corresponding trivial names of the compounds as identified by NatureBank.** RAD refers to Rohan Davis library

Dose response and half maximal inhibitory concentrations of four of the identified compounds

From the 20 identified compounds four of them (SN618, SN631, SN565 and SN362) were selected for dose response experiments. Transwell assays were performed with different concentrations (1 µM, 0.5 µM, 0.3 µM, 0.1 µM and 0.03 µM) of the four different compounds. Only four compounds were included in this experiment in the interest of availability of time and the compounds themselves. Data from both cell lines U251-MG and U87-MG were considered while selecting the four compounds for further analysis. Log of concentration was plotted against invasion% to obtain a semi-log plot which depicts the dose dependent response on invasion. From the dose response curve half maximal inhibitory concentrations (IC_{50}) for respective compounds were identified. Different IC_{50} identified were, 0.24 µM for SN618, 0.41 µM for SN362, 0.25 µM for SN631 and 0.43 µM for SN565 (Figure 11).

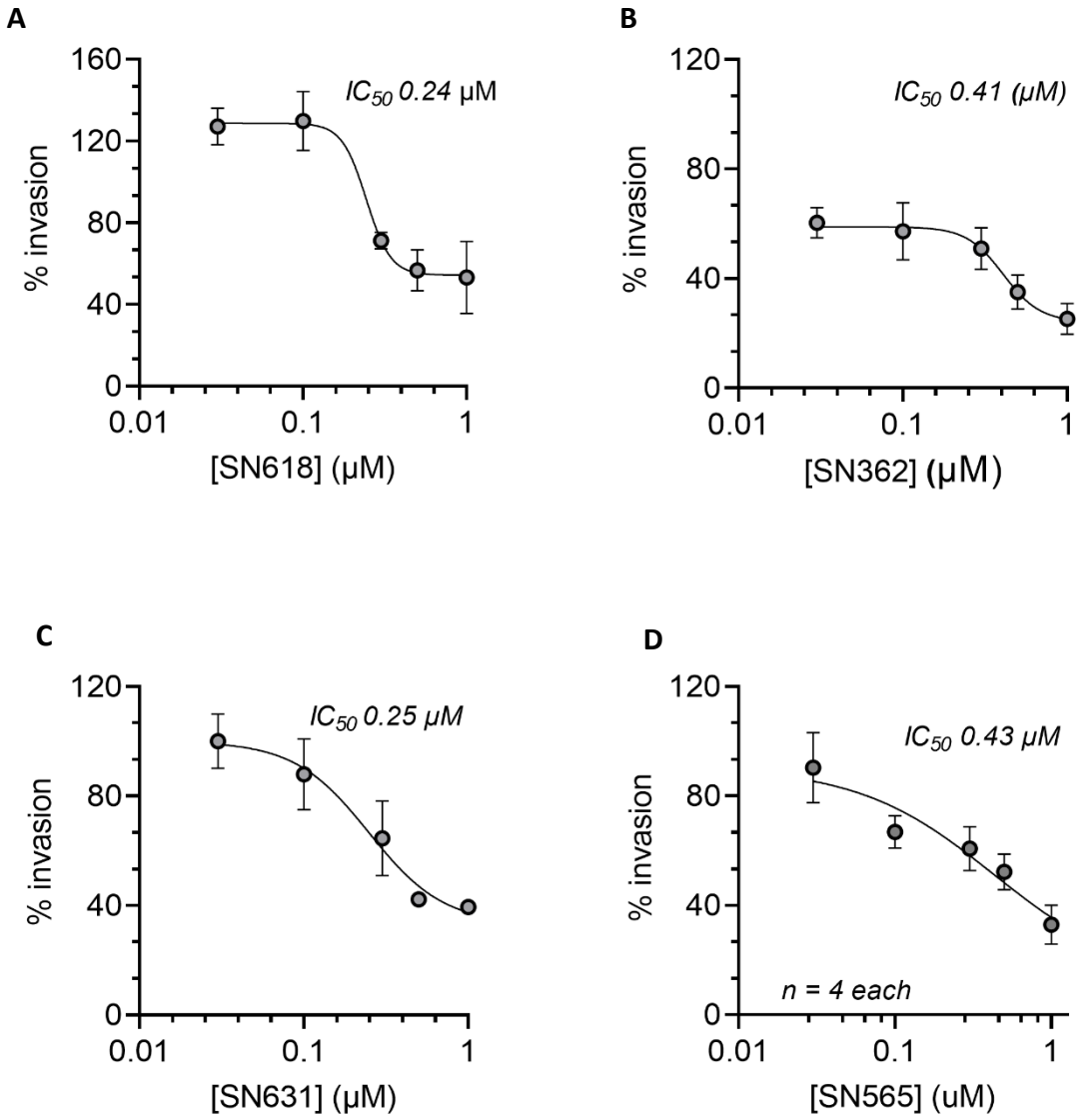

Figure 11:Dose response curves for compounds SN618, SN362, SN631, SN565. A) SN618- IC_{50} 0.24µM, B) SN362- IC_{50} 0.41µM, C) SN631- IC_{50} 0.25µM, D) SN565- IC_{50} 0.43µM. n=4 for all treatments. Error bars represent standard deviation.

Evaluating cytotoxicity of prepared Kushen root extracts on U251-MG cell line

Kushen root extracts were prepared with ethanol and water and were resuspended in DMSO and water respectively for testing cytotoxicity using Alamar Blue. Different concentrations 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml, 3.12µg/ml, 2µg/ml, 1.5µg/ml were tested to identify any cytotoxic effect they might have. After treatment, cells were 100% viable, implying no cytotoxicity at any of the tested concentrations (Figure 12).

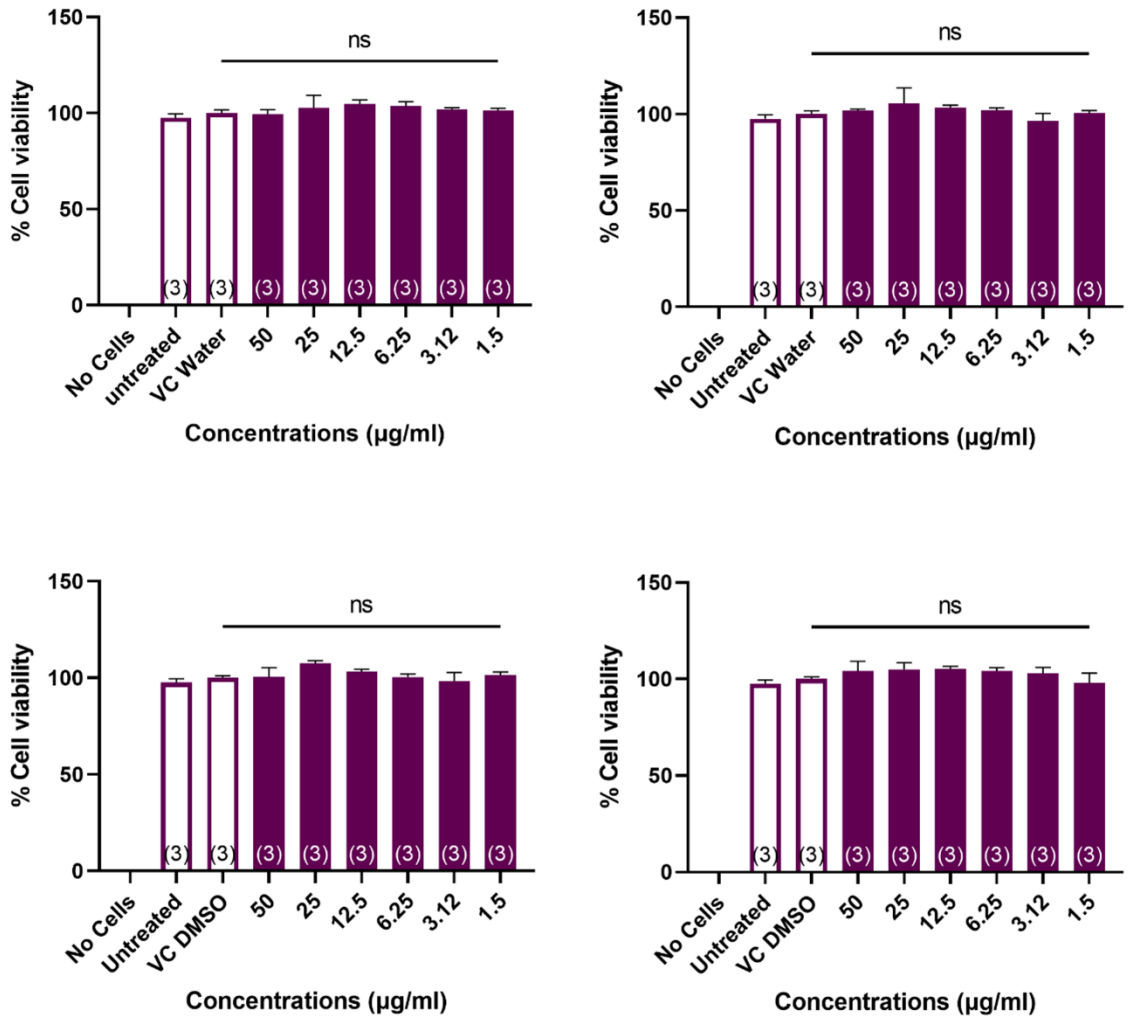

Figure 12: Cell viability assessed by Alamar Blue assay for different Kushen extracts prepared. A) Line 2 water extracted samples, B) Line 4 water extracted samples, C) Line 2 ethanol extracted samples, D) Line 4 ethanol extracted samples. n=3 for all treatments, ns= no significance.

Testing the prepared extracts for any inhibitory action on invasion of U251-MG cell line using transwell invasion assay

Transwell invasion assay was performed with a 2 µg/ml extracts to detect any inhibitory effect (Figure 13). However, no significant inhibition of invasion was observed at this concentration with either ethanol extracted, or water extracted samples. Controls were treated with DMSO along with ethanol extracted samples resuspended in DMSO. Controls were treated with water along with water extracted samples resuspended in water.

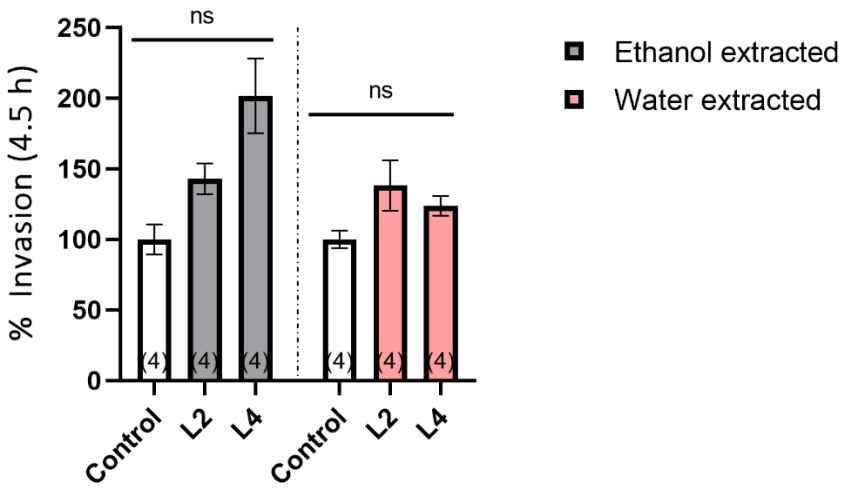

Figure 13: Kushen root extracts at 2µg/ml for transwell assays to identify any inhibitory action on invasion of U251-MG cells. Ethanol extracted samples resuspended in DMSO of Line 2 and Line 4 Kushen roots depicted in grey along with control (DMSO), water extracted samples resuspended in water of Line 2 and Line 4 Kushen roots depicted in orange along with control (water). n=4 for all treatments, ns= no significance. Error bars represent standard deviation.

Further, 50 µg/ml concentration of the extracts were used in transwell invasion assays for identifying any inhibitory effect on invasion (Figure 14). Roots of Line 4 Kushen plant extracted with ethanol and resuspended in DMSO allowed invasion of 57.5% (p= 0.006) while inhibiting 42.5% of cells similar extracts from Line 2 Kushen did not inhibit invasion. For Kushen roots extracted with water and resuspended in water, extracts of Line 2 Kushen allowed invasion of 73.81% (p=0.02) while inhibiting 26.19% of cells. Similar extracts from Line 4 Kushen allowed invasion of 81.14% and inhibited only 18.86% of cells.

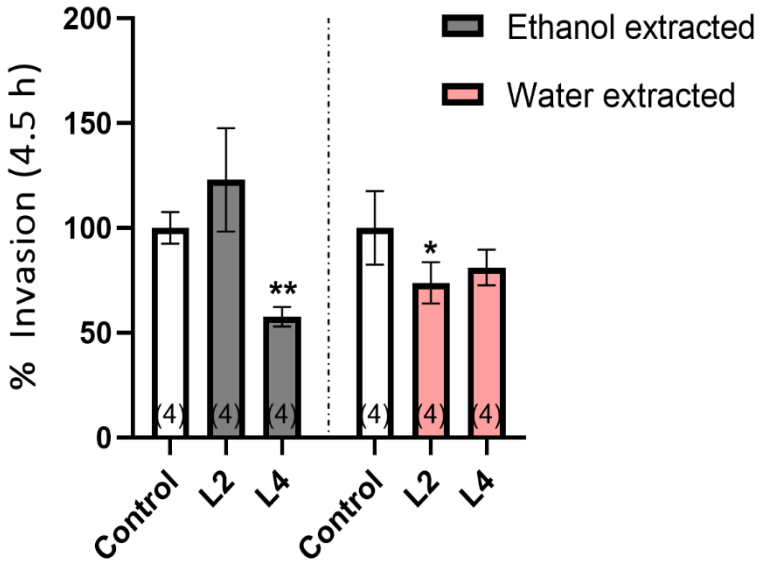

Figure 14:: Kushen root extracts at 50µg/ml for transwell assays to identify any inhibitory action on invasion of U251-MG cells. Ethanol extracted samples resuspended in DMSO of Line 2 and Line 4 Kushen roots depicted in grey along with control (DMSO), water extracted samples resuspended in water of Line 2 and Line 4 Kushen roots depicted in orange along with control (water). n=4 for all treatments. Error bars represent standard deviation. **p=0.006, *p=0.02

DISCUSSION

Summary of findings

Glioblastoma, like other cancers, is a complex disease regulated and driven by multiple factors such as invasiveness of the cancer cells, adaptive metabolism, and molecular signalling (Garcia et al., 2021). Tumour recurrence, diffuse nature of tumours, presence of cancer stem cells (CSCs), tumour heterogeneity, resistance to chemo and radiotherapy are some of the major challenges in treating GBM. These challenges collectively call for new treatment strategies. This study aimed to explore some of the natural extract fractions provided by NatureBank and extracts prepared from the roots of Traditional Chinese Medicinal herb Kushen (*Sophora flavescens)*, for their inhibitory action on GBM invasiveness. Screening of compounds from NatureBank led to identification of 20 compounds that inhibited GBM invasion in U251 cells (Figure 6), without being cytotoxic to the cells (Figures 8, 9, and 10). Further investigation of four of the identified compounds showed IC_{50} values less than 0.5µM (SN618- 0.24µM, SN362- 0.41µM, SN631- 0.25µM and SN565- 0.43µM) (Figure 11). Only four of the identified compounds were further pursued in the interest of availability of time and availability of compounds themselves. Kushen root extracts inhibited GBM invasion at a concentration of 50µg/ml (Figure 14). This proves the hypothesis that natural compounds can effectively inhibit glioblastoma invasion.

NatureBank compounds decoded: discussion on the identified compounds based on available literature

While 14 of these compounds have already been identified and classified, 6 of them are still under investigation (Table 2). Compounds SN545, SN631, SN620, SN618, SN567, SN551, SN546, SN121, SN388, SN387, SN594 and SN104 were identified as saurolactam (SN545); xanthurenic acid (SN631 and SN567), enterocarpam II (SN620), beta- D-glucopyranoside (SN618), caelestine (SN551), caldensine (SN546), gallic acid (SN121), methyl ether of pestalactam A (SN388), pestalactam A (SN387), elliptisine (SN594) and polyandrocarpamine derivative (SN104). Other compoundsSN563, SN564, SN565, SN362, SN096, SN098 and SN104 have not been chemically identified.

Compound SN545 was identified as Sauristolactam/ saurolactam, an aristolactam (Table 2). Aristolactams are alkaloids that have gained popularity for their array of biological properties including anti-inflammatory and neuroprotective (Choi et al., 2009; Sauristolactam, 2021). A study by Li et al. in 2015 showed that saurolactam inhibits proliferation, migration, and invasion of human osteosarcoma cells. This study found that saurolactam inhibited proliferation and decreased colony formation in MG-63 and HOS (human osteosarcoma cell lines) cell lines. Administering saurolactam at 25mg/kg of body weight over a 21-day period was found to dramatically reduce the growth of MG-

63 xenografts in nude mice. Another study in 2011 by Oh et al. revealed the anti-tumor activity of a sauristolactam derivative on human breast cancer cell line MDA-MB-231, due to its inhibitory action on EGFR (Oh et al., 2011). EGFR over expression is well studied in GBM and is typically associated with tumour progression. Activation of EGFR by epidermal growth factor (EGF) leads to induction of a cascade of downstream signalling pathways that eventually results in DNA synthesis and cellular proliferation (Oda et al., 2005). This study showed that sauristolactam is an inhibitor of GBM invasion by allowing only 58% of U251 cells to invade through ECM (Figure 6). The crucial role of EGFR in GBM and the literature on anticancer activity of sauristolactam on other cancer cell lines as discussed above makes it a candidate for further investigation. Any influence that sauristolactam may have on EGFR expression should be investigated. Further studies should be carried to identify the mode of action of this compound.

Compound SN631 and SN121 were identified as Xanthurenic acid (XA) (Table 2). XA is naturally found in brain as it is a metabolite of the tryptophan oxidation pathway (Gobaille et al., 2008). Tryptophan oxidation pathway is a major biochemical pathway through which many neurotoxins and neuroprotective compounds are produced in brain (Gobaille et al., 2008). From previous studies XA is known to have cytotoxic effect on senile cataract and some infectious diseases such as yellow fever and malaria and triggers apoptotic cell death at 10µM concentration in human lens epithelial cells (Malina et al., 2002). The IC_{50} of SN631 from this study was found to be 0.25 µM (Figure 11c) and was not cytotoxic to the cells even at the highest tested concentration (1 µM) (Figure 9b).

XA have also been identified as metabotropic glutamate receptor (mGlu) ligand (Copeland et al., 2013). Glutamate is an excitatory neurotransmitter in the CNS and is known to enhance proliferation and migration in GBM cells. Glutamate excitotoxicity leads to death of surrounding brain tissues and provides more space for GBM to grow (Corsi et al., 2019). Lyons et al., 2007 in their study have shown that autocrine glutamate signalling promotes glioma cell invasion by activating glutamate receptors like AMPA-Rs on neighbouring cells or on themselves (Lyons et al., 2007). Glutamate plays an important role in glioblastoma invasion as glutamate mediated Ca^{2+} oscillation is requirement for neuronal migration. Glutamate is released by glioma cells in exchange for cystine which is used in synthesis of GSH, a cellular antioxidant. GSH protects the tumour from reactive oxygen species thereby enabling glioma growth. A study conducted in 2008 by Sontheimer showed decreased growth and invasion of GBM when glutamate release from the tumour mass was indirectly impeded by pharmacological agents (Sontheimer, 2008). Metabotropic glutamate receptors can inhibit presynaptic neurotransmission thereby decreasing synaptic excitability (Sladeczek et al., 1993). Therefore, it is possible that XA being a metabotropic glutamate receptor ligand, could decrease glutamate excitotoxicity and thereby inhibit invasion.

Further, stimulating neuroblastoma cells by XA showed excitatory responses that led to increased cytosolic calcium ion levels suggesting the role of XA as a neuromodulator or neurotransmitter (Taleb et al., 2012). XA is also known to be able to penetrate the blood brain barrier into the brain freely which is a vital property as many molecules fail to penetrate the blood brain barrier (Gobaille et al., 2008). As XA is a metabolite found in brain, all the available studies discuss the effect of endogenous XA on mammalian brain cells and effects of exogenous XA application remains unknown (Copeland et al., 2018; Fazio et al., 2015; Taleb et al., 2012). Therefore, it is not possible to conclude based on the evidence from this study that XA could be a potential adjuvant in treating GBM. Further studies should be done in elucidating the method of action of XA in inhibiting invasion.

Compound SN620 is identified as enterocarpam II, which is an aristolactam (an alkaloid), like sauristolactam (SN545) (Table 2). Enterocarpam III showed cytotoxicity on liver cancer cell line HepG2 and mammalian breast cancer cell line MDA-MB-231 with IC_{50}s 26µM and 51.3µM respectively. 24-hour treatment with enterocarpam III led to induction of apoptosis through mitochondrial or intrinsic pathways with the activation of capsase-9 (Banjerdpongchai et al., 2015). Caldensine (SN546) is also an aristolactam with antiparasitic activity (Table 2). Antiparasitic activity of this compound was evaluated in chloroquine sensitive *P. falciparum* and the IC_{50} was identified as 25µM (Levrier et al., 2013). Caelestine (SN551) is a naturally occurring brominated quinoline carboxylic acid. This compound was evaluated for cytotoxicity at 100µM on three different mammalian cell lines (MCF-7 (breast cancer cell line), MM96L (melanoma cell line), NFF (neonatal foreskin fibroblast cells)) and was found to have only minimal cytotoxic effects at the tested concentration (Yin et al., 2010).

SN121 was identified as Gallic acid (GA) (Table 2). GA is a phenolic acid and has previously shown anticancer activity in small cell lung cancer cell line (Wang et al., 2016). The study by Wang et al., on SCLC H446 cells showed that GA possesses anticancer activity and acts synergistically with cisplatin to enhance its anticancer activity through the ROS dependent mitochondrial apoptotic markers. Another study in 2015 by Weng et al., on human oral cancer cells (SCC-4) revealed the role of GA in inducing DNA damage and inhibiting DNA repair thereby causing cell death in SCC-4 cells (Weng et al., 2015). GA was shown to induce apoptosis in human pancreatic cells in a dose and time dependent manner with IC_{50}s 102.3 and 135.2µM respectively after 48 hours of treatment (Liu et al., 2012). It was also found to potentiate the action of carboplatin/paclitaxel combination treatment for human cervical cancer by initiating apoptosis (Aborehab and Osama, 2019). Literature clearly describes the role of GA in inducing apoptosis in multiple cancer cell lines however there is no insight on GA inhibiting invasion in any cancer cell line. Therefore, studies focusing on mode of action of GA in inhibiting cell invasion is a potential aspect that needs to be explored further.

Compounds SN387 and SN388 were identified as pestalactam A and methyl ether of pestalactam A respectively. While there are not many studies recorded on either of these compounds, pestalactam A was found to have moderate cytotoxicity on breast cancer cell line MCF-7 in a primary study where the compound was first identified (Davis et al., 2010). Cytotoxic activity of the compound was evaluated at a concentration of 100µM. Antimalarial activity of pestalactam A was tested in the same study and the results indicate potential antimalarial activity at a concentration of 25µM. It is rather interesting to find that 100µM of the compound showed only moderate cytotoxicity on mammalian breast cancer cells however the same compound at a much lesser concentration of 1µM was potent enough to inhibit glioblastoma invasion by almost 50% (Figure 6).

SN594 was identified as ellipticine (Table 2). Ellipticine is an alkaloid and is its role as a DNA intercalator has led to further investigations of its potential anticancer activity. This activity has been studied and supported in multiple cancer types including leukemia, breast cancer, lung cancer, colon cancer and glioblastoma (Russell et al., 2014; Stiborová et al., 2011; Ruckdeschel et al., 1992; Xu et al., 2008). Ellipticine was identified as a pro-drug as it ismetabolised by the body after administration into a pharmacologically active compound (Stiborová et al., 2012). Since it is a pro-drug, the pharmacological efficiency and toxicity of ellipticine is found to depend on its activation (Stiborová et al., 2011). It is known to arrest cell cycle progression by regulating the expression of cyclin B1 and cdc2, two important proteins involved in cell division and induce apoptosis (Kuo et al., 2005).

In glioblastoma cell lines ellipticine was seen to activate p53 pathway which is a stress signalling pathway in humans. In U87-MG cell line ellipticine caused an early (G0/G1) cell cycle arrest whilst in U373 cell line the cell cycle arrest occurred at S and G2/M phase (Martinkova et al., 2010). As it is a DNA intercalator, ellipticine inhibits the binding of topoisomerase II, a key enzyme in replication, to DNA thereby preventing cell division and leading to DNA damage suggesting its role as a mutagen. In addition, it is also known to form ellipticine-DNA adducts leading to cytotoxicity (Stiborová et al., 2011). However, there has been no mention of ellipticine inhibiting invasion of cancer cells. As ellipticine have multiple modes of action and since most of the studies have focused on cytotoxicity for evaluating anticancer effect of the compound, it is not very surprising that the inhibition of invasion by ellipticine is not studied. Therefore, it is worth further investigating the possible action of ellipticine in inhibiting the invasion of glioblastoma cells.

SN104 was identified as polyandrocarpamine (Table 2). Polyandrocarpamines are known to be potent kinase inhibitors (Loaëc et al., 2017). Kinases are vital class of proteins which regulate many functions like proliferation, cancer cell signalling and angiogenesis in cancer cells. According to the Loaëc et al., study in 2017, polyandrocaroamines show significant selective inhibitory effect on dual

specificity tyrosine regulated kinases (DYRKs) (Loaëc et al., 2017). DYRKs are reported to be involved in cancer-related events like altered metabolism, increased growth signalling, escaping apoptosis and migration and tissue invasion (Boni et al., 2020). DYRK inhibition in primary glioblastoma cells and neural progenitor cells was shown to reduce EGFR dependent growth and self-renewal capacity of normal and cancer cells (Pozo et al., 2013). Kinases actively participate in cancer cell invasion. For instance, focal adhesion kinases or FAKs play a significant role in integrin signalling (Tamura et al., 1999) and thereby directly relate to GBM cell invasion. Therefore, the possibility of this kinase inhibitor inhibiting GBM invasion is reasonable and should be further pursued to find its possible mechanism of action.

SN618 was identified as 3-methylbut-3-enyl-O-a-L-rhamno-pyranosyl-(1-6)-O-b-D-glucopyranoside (Table 2). Etoposide which is currently used as a second-tier treatment agent for GBM is also a beta-D-glucopyranoside (Kingston, 1989). Etoposide inhibits topoisomerase II binding to the DNA and leads to cell cycle arrest and in turn cell death (Montecucco et al., 2015). The compound identified in this study being a glucopyranoside could possibly have a closely related mode of action which is responsible for its inhibitory nature on glioblastoma cell line U251MG. The IC_{50} of the compound was found to be 0.24µM in this study. Though there is no literature available to compare, the identified IC_{50} value being in sub micromolar level suggests that it is potent in inhibiting invasion in glioblastoma (Figure 11a).

Extracts from *Sophora flavescens* root inhibit invasion of U251-MG cells

Compound Kushen Injection (CKI) is being used in China since past three decades in combination with chemotherapeutic agents like oxaliplatin in treating gastric, liver, and non- small cell lung cancer (Aung et al., 2017). It is an extract from two TCM plants, *Radix Sophorae flavescentis* and *Rhizoma Smilacis Glabrae*. It is known to contain flavonoids, alkaloids, saccharides, and organic acids (Aung et al., 2017), however, no single compound has been identified responsible for its anticancer action (Nourmohammadi et al., 2019). This study evaluated the potential of root extracts made from *Sophora flavescens* in inhibiting GBM invasion. Root extracts at 2µg/ml concentration was not seen to have inhibitory properties when compared to corresponding controls (Figure 13). A higher concentration, 50µg/ml extracts showed inhibitory action (57%) on invasiveness of U251 cells (Figure 14). Dried roots of Line 4 Kushen plants extracted in ethanol and resuspended in DMSO showed higher inhibition of invasion (57.5%) as compared to Line 2 ethanol extracts resuspended in DMSO which did not inhibit invasion (122.87%). In case of water extracted samples resuspended in water, the invasion percentages were comparable for Line 4 (81.14%) and Line 2 (73.81%) (Figure 14). Overall, extracts from Line 4 shows higher activity than extracts from Line 2 (Figure 13 and figure 14). CKI traditionally used in combination with chemotherapeutic agents in treatment of gastric, liver, and non-

small cell lung carcinoma traditionally is a highly purified mixture extract and is directly administered as an injection to patients. Whereas this study utilised crude extracts prepared from roots of the Kushen plant which could account for the minor inhibitory action observed even at a higher concentration of 50µg/ml. Moreover, Nourmohammadi et al., study shows that it is not one compound responsible for the observed properties of CKI but rather it is the different fractions together that are responsible for the anticancer activity of CKI (Nourmohammadi et al., 2019). As the extracts prepared in this study used only one of the component plants root it is not surprising to see that inhibition of invasion is not as promising as compared to the original compound (CKI) (Figure 13 and figure 14).

Conclusion

Natural compound extracts including NatureBank compounds and Kushen root extracts identified in this study could be pursued further as potential drugs in treating GBM. The early, undetected invasion of GBM cells into surrounding tissues and leading to recurrence even after several rounds of chemotherapy is a major challenge in treating glioblastoma. The ability of compounds identified in this study to inhibit invasion without being cytotoxic to the cells can prove beneficial in treating glioblastoma as these compounds would inhibit glioblastoma cell invasion. Using agents that inhibit invasion in combination with standard chemotherapeutic agents could therefore be the right strategy to prevent tumour recurrence. This could be especially important in treating glioblastoma since brain is a terminally differentiated organ and therefore restricts the use of highly invasive surgical resection strategies as it would risk losing patient's cognitive skills. Further investigation needs to be carried out on each of the identified compounds to elucidate their mechanism of action, target molecules and any off-target effects that they might have.

Limitations of current study

Current study investigated the effect of different natural compounds in inhibiting the invasion of glioblastoma cell line U251. This study was carried out in a short span of time (9 months). The overall delay in shipping due to the global pandemic affected the study. Delivery of cell culture reagents and the compounds from NatureBank were delayed and which delayed the start of experiments. Initially, it was decided to evaluate the inhibitory action of the original CKI on U251 cells and progress further by evaluating the gene expression levels. However, that did not take off, as sourcing CKI from China was not a viable option considering current circumstances and shipping delays.

Transwell invasion assays were the main technique followed in this study to identify inhibitory action of compounds. These assays use transwell invasion plates that come with special inserts. These inserts are not usually reused once they are fixed and stained. The shortage of transwell plates and shipping delays affected the pace of experiments. Reusing the inserts without ensuring complete removal of

cells from previous experiment can lead to increase in cell count and false negatives. An article by Kato et al., 2021 stated that it is safe to reuse transwell inserts once they are trypsinised and sterilised by ethanol and UV light (refer appendix for detailed repurposing protocol). A similar protocol was followed to successfully clean the inserts and the same was visualised under microscope to ensure that there were no remaining residues from the previous experiments.

The cells stained after transwell assay are imaged and counted manually to express them as invasion percentages with respect to the corresponding controls. Even though, efforts were made to use different software like Fuji ImageJ to automatically count the cells, these efforts failed as the morphology of the cells and prominent Matrigel pores increased the noise and made it impossible to count these cells using any existing cell counting plugins in ImageJ. Therefore, there is a higher chance of counter bias and human error. Utilising other approaches like blinded experiments can be beneficial to mitigate this error and ensure the precision of the results. However, transwell insert assays are still considered the gold standard for invasion experiments.

Future direction

Drug discovery is often a very long process which typically takes 10-12 years for an identified compound to be tested, tried, and formulated into an effective drug. Main steps in drug discovery pipeline include basic research, lead discovery, pre-clinical trials, clinical trials and finally filing to be approved as a drug (Hughes et al., 2011). This study was a preliminary effort to identify lead/leads for identification of compounds that could potentially prevent the invasion of GBM cells. Further studies must be carried out in identifying potential target/targets on which these compounds are acting and their mechanism of action. This can be done by extracting the mRNA from cells which have been treated by respective compounds and using qPCR to study expression levels of different genes that are crucial in glioblastoma invasion.

As tumour heterogeneity is a well-known challenge in treatment of glioblastoma, conducting preclinical experiments in multiple GBM cell lines will prove beneficial. As commercial cell lines do not completely mimic tumours *in-vivo*, many aspects like influence of tumour microenvironment and cross talk between different cell types present within the tumour cannot be fully understood from them. Primary cell are cells derived from biopsy samples which are cultured preserving their epigenetic characteristics. They can be cultured for only a specific amount of time. Conducting the same study on primary cells would lead to better understanding of the action of the identified compounds *in-vivo*. Simultaneously, murine xenograft models could also be utilised to study any off-target effects and toxicity that the compounds may cause once the compound is inside a living system.

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APPENDICES

Resuspending NatureBank compounds for initial screening

1µl of compounds at 5mM concentration was received from NatureBank in a 96-welled plate. Last column (12^{th}) of every plate contained DMSO at the same concentration and volume as that of the compounds, to be used as a vehicle control. Compounds were resuspended by adding 4.5µl of DMSO to each well resulting in a final concentration of 0.9mM.

C_1=5mM, V_1= 1µL; V_2=5.5µL

$C_1V_1 = C_2V_2$

$C_2 = (C_1V_1)/ V_2$

C_2= (5mM x 1µL)/5.5µL = 0.9mM

Resuspending dried Kushen samples for experiments

Dried Kushen samples extracted in ethanol was resuspended in DMSO and samples extracted in water were resuspended in water. A 2mg/ml stock was made from an ethanol extracted dried sample that weighed 20mg. 1ml DMSO was added to it to make a 20mg/ml stock. 100µL of this was diluted with 900µl of DMSO to get 2mg/ml stock.

C_1=20mg/ml, C_2= 1mL; V_2=5.5µL

$C_1V_1 = C_2V_2$

$V_1 = (C_2V_2)/ C_1$

V_1= (2mg/ml x 1mL)/20mg/ml = 0.1ml = 100µL

Similarly, water extracted sample that weighed 60mg was resuspended in 1ml water to obtain 60mg/ml stock. 300µL of this was added to 700µL of water to get a final concentration of 2mg/ml. The same was followed with an ethanol extracted 50mg dried sample and a water extracted 50mg sample to obtain a final concentration of 50mg/ml. Stocks of different concentrations used in cytotoxicity assay (25mg/ml, 12.5 mg/ml, 6.25mg/ml, 3.12mg/ml, 1.5mg/ml) was made following the same calculation.

Resuspension volume calculation for transwell invasion assay

Detached cells from the culture flask were counted using a haemocytometer. 10µL of the cell suspension was loaded on the haemocytometer and was counted under the microscope at 10x magnification. Resuspension volume is calculated as shown in the example below.

Average cell count = 72 cells in 10µL

Cell density in 10ml of cell suspension = 72,000 cells/10ml

Required number of cells for performing assay = 60,000 cells/10ml

Resuspension volume = (72,000 x 10)/ 60,000 = 12ml

10ml of the original cell suspension was centrifuged at 4500rpm and the supernatant was discarded. The cell pellet thus obtained was resuspended in 12ml of serum free DMEM media.

Resuspending U251-MG cells for cryo-preservation

Cells were grown to 90% confluency before they were used for making cryo-preserved stocks. Cells were detached from the growth flask by trypsinisation. 10µL of the cell suspension was used to count the number of cells on a haemocytometer. While preparing cryogenic stock of cells, the aim was to have $4x10^4$ cells/ml. Example calculation given below.

Average cell count = 176 cells in 10µL

Cell density in 1ml of cell suspension = 17600 cells/ml

Cell density in 5ml of cell suspension = 17600 x 5 = 88000 cells in 5ml

5ml of this cell suspension was centrifuged at 4500 rpm for 5 minutes and resuspended in 2ml of complete DMEM (DMEM with no added penicillin/streptomycin, glutamax) to obtain approximately $4x10^4$ cells/ml. 500µl of this cell suspension was pipetted into a cryo vial to which 500µL freezing mix was added (freezing mix composition given below).

	Name of component	Volume of component (ml)
1.	Complete DMEM	5.5
2.	DMSO	2
3.	FBS	2.5
	Total	10

Table 3: Components of freezing mix for cryogenic preservation of cells.

Resuspending NatureBank compounds for dose response experiments

Identifies compounds from initial screening was obtained as powders in 10mM concentration. For dose response experiments different concentrations like 1mM, 0.5mM, 0.3mM, 0.1mM, 0.03mM were to be prepared. Example calculation shown below.

C_1=10mM, C_2= 1mM; V_2=5ml

$C_1V_1 = C_2V_2$

$V_1 = (C_2V_2)/ C_1$

V_1= (1mM x 5ml)/10mM = 0.5ml = 0.5g

Therefore, 0.5g of the obtained powder of the respective compound was dissolved in 5ml of DMSO to obtain 5ml of 1mM stock solution of the respective compound. This stock was used to make stocks of other concentrations.

For preparing 2ml of 0.5mM stock solution, C_1=1mM; C_2= 0.5mM; V_2=2ml

$V_1 = (C_2V_2)/ C_1$ = 1ml (1ml of 1mM stock in 1ml of DMSO).

Similarly, volume needed was calculated for 0.3mM, 0.1mM and 0.03mM as 0.6ml, 0.2ml and 0.06ml respectively and were made up to 2ml with DMSO.

Repurposing used inserts for experiments

Used inserts were trypsin treated to detach the cells present on them from previous experiment following which they were submerged in ethanol and were sterilized under UV light. 600µL of 2x trypsin/EDTA was used in bottom wells and the inserts were submerged by adding 100µL of 2x trypsin/EDTA on top. Plates were then incubated for 34 hours at 37°C. After 34 hours, trypsin/EDTA

solution was removed, and the inserts were submerged in 600µL of 80% ethanol in the bottom well and 100µL of 80% ethanol on top for 10 minutes. After aspirating ethanol from the wells, inserts were air dried and were sterilised under UV light for 5 minutes on each side. Sterilised inserts were moved to a sterile 24-well plate and were stored in an airtight sterile container until further use.