chemistry proceedings

MDPI

Proceeding Paper

In Vitro and In Silico Investigations on Natural Compounds with Predicted Activity against Neuroblastomas †

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† Presented at the 26th International Electronic Conference on Synthetic Organic Chemistry; Available online: https://ecsoc-26.sciforum.net.

Abstract: In the present study, the ability of an ethanolic extract from *Stokesia laevis* to inhibit the development of human glioblastoma cell line U87 has been investigated; cytotoxic activity has been estimated at 78%, by comparison the control cells, astrocyte cell line NHA, while IC50 values were 9.12 μg gallic acid ([GAE]) equivalents per 1 mL extract for U87, and 24.17 μg gallic acid ([GAE]) equivalents per 1 mL extract for NHA, respectively. Docking simulation using the active compounds in *Stokesia aster*, by comparison to curcumin, on the Bcl-2 anti-apoptotic protein target has revealed the following order in the magnitude of the docking score: native ligand > curcumin > luteolin-7-O-glucoside > luteolin-8-O-glucoside > luteolin, with RMSD between 0.01 and 0.93. Since the access to neuroblastomas is not restricted by the blood-brain barrier, and selective cytotoxicity has been demonstrated, higher on tumor cells than the normal control astrocytes, the use of *Stokesia aster* ethanolic extracts and luteolin derivates as an alternative approach in neuroblastomas is to be considered.

Keywords: stokesia extract; anti-proliferative activity; glioblastoma U87; docking studies; BCL-2

Citation: Pirvu, L.C.; Neagu, G.; Çığ, Ö.; Albulescu, A.; Pintilie, L.; Stefaniu, A. In Vitro and In Silico Investigations on Natural Compounds with Predicted Activity against Neuroblastomas. *Chem. Proc.* **2022**, *4*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s): Julio A. Seijas

Published: 15 November 2022

1. Introduction

Glioblastoma is one of the most common and aggressive types of malignant brain tumors affecting the central nervous system in adults; the current therapeutic approaches usually involve surgical intervention, chemo, and radiotherapy, but its invasive nature and high resistance to conventional therapies raise major health concerns [1]. Neuroblastoma is another form of cancer affecting nervous tissue, but also some other body tissues such as the lung, liver, bone marrow, adrenal gland, etc.; neuroblastomas mostly occur in children and they start from the immature, developing cells namely neuroblasts, situated along the sympathetic nervous system in humans (SNS) [2]. Given the range of location and accessibility in the human body, when studying the antiproliferative effect of nutraceuticals and active vegetal compounds [3], their bioavailability, size, and polarity must be considered; thus, in the case of glioblastoma, the size and polarity of the compounds are the key factors of their selection. In the case of neuroblastoma, the search list can be expanded with larger and more hydrophilic compounds, since access to tumors is no longer restricted by the blood-brain barrier. Therefore, neuroblastomas theoretically can benefit from active compounds that normally reach the human circulatory system. Proving these, some recent in vitro studies using a transepithelial anti-neuroblastoma co-culture model system in which several plants diluted juices (kale, dandelion, lettuce, spinach) were applied to apical *Caco-2Bbe1* cells atop dividing *SH-SY5Y* neuroblastoma cells,

indicated inhibitory activity for kale and dandelion juices [4]. Concerning the potential active compounds, kaempferol in kale and luteolin in dandelion appear as the most feasible anti-neuroblastoma compounds. Curcumin, a dimer of ferulic acid, also was proved to be very active on the neuroblastoma tumor cell line N2a; curcumin inhibited ERK1/2 activation by phosphorylation, and the same time specifically induced *Bex* genes to involve PI3-kinase, JNK, and p53 associated pathways, resulting in the apoptosis of N2a neuroblastoma cells in a dose-dependent manner, with 100% cell death in 50 μM curcumin treatment [5]. The present study has proposed to investigate in vitro and in silico ability of an ethanolic extract from *Stokesia laevis* and corresponding active compounds, luteolin derivates, to inhibit the development of human glioblastoma. In vitro studies were done on tumor and normal brain (astrocyte) cell lines U87 and NHA, while in silico studies investigated the active compounds in stokesia on the anti-apoptotic protein Bcl-2 in comparison to curcumin.

2. Materials and Methods

Stokesia laevis, medium size powder from the aerial part, was extracted with 70% (*v*/*v*) ethanol, for 1 h at boiling temperature. The extractive solution was further prepared as standardized extract (named Slae26), with a content of 5 mg total phenols expressed as gallic acid equivalents ([GAE]) *per* 1 mL 40% (*v*/*v*) ethanol solution [6]. Slae26 was further prepared as a dilution series of 5, 10, 20, 30, 40, and 50 μg [GAE] per 1 mL sample; the same dilution series was prepared for the solvent sample, 40% ethanol; both series were used for pharmacological in vitro studies.

Pharmacological in vitro studies were done on the human tumor brain cell line U87 (glioblastoma) in comparison to normal human brain cell line NHA (astrocytes); the MTS test was carried out in two variants, cytotoxicity test and anti-proliferative test, as described in the Technical Bulletin of Promega Corporation CellTiter 96 AQueous One Solution Cell Proliferation Assay [7]. Practically, at approx. 70% (cytotoxicity test) and 30% (anti-proliferative test) confluence of the cells' cultures (U87 and NHA) Slae26 and 40% ethanol dilution series were applied and allowed to act for 24 and 48 h (h). The culture medium was then removed, and the cells were incubated with MTS solution, for another 2 h. Finally, the absorbance at 490 nm of the test sample (Slae26 series) in comparison with the control solvent sample (40% ethanol series), on each cell culture studied (U87 and NHA) was measured, using BMR-100 Microplate Reader (Boeco, Germany). The recorded values were used for both, cell' viability and cell' anti-proliferative activity estimation; the results can be presented as % of cell viability (see formula below) or simply optical density (O.D.) at 490 nm along the total phenolics' in samples (μg/mL).

$$\%\ cell\ viability = \frac{A490\ of\ treated\ cells}{A490\ of\ control\ cells} \times 100$$

The docking simulations have been performed using CLC Drug Discovery Workbench software on the anti-apoptotic protein Bcl-2 in complex with an acyl-sulfonamide-based ligand, PDB ID 2O2F [8]. The simulations protocol was previously validated [9]. The occurred intermolecular interactions reflect mainly the hydrogen bonding of the investigated ligands (curcumin, luteolin, luteolin-7-O-glucoside, luteolin-8-C-glucoside) in complex with Bcl-2 fragment, into the binding pocket of 36.35 $Å^3$. Results are given as docking score function and distance of the identified hydrogen bonds, as strength of the potentially formed complexes.

3. Results and Discussions

Figure 1 presents the results of the cytotoxicity study at 24 h, Slae26 dilution series versus solvent sample (40% ethanol) dilution series, tested on human tumor brain cell line U87 in comparison to normal human brain cell line NHA. Cytotoxicity diagram at 24 h indicates that the influence of the solvent sample (40% ethanol) upon the tumor cells U87 (blue line) and normal cells NHA (red line) is similar and of low intensity. Differently, the

Slae26 test sample induced a decrease in the viability of both types of brain cells in culture, more augmented in the case of human tumor brain cell line U87 (gray line) in comparison to normal brain cell line NHA (yellow line). Table 1 shows the cytotoxicity study results computed as a percentage of inhibitory activity upon the two brain cell lines, along with the total phenolics' concentration in the Slae26 dilution series. The dynamic of the inhibitory activity suggests Slae26 selective activity upon the brain cells at concentrations between 10 and 30 μg [GAE]/mL, and the possibility of decreasing the dilution in samples towards 5 μg/mL.

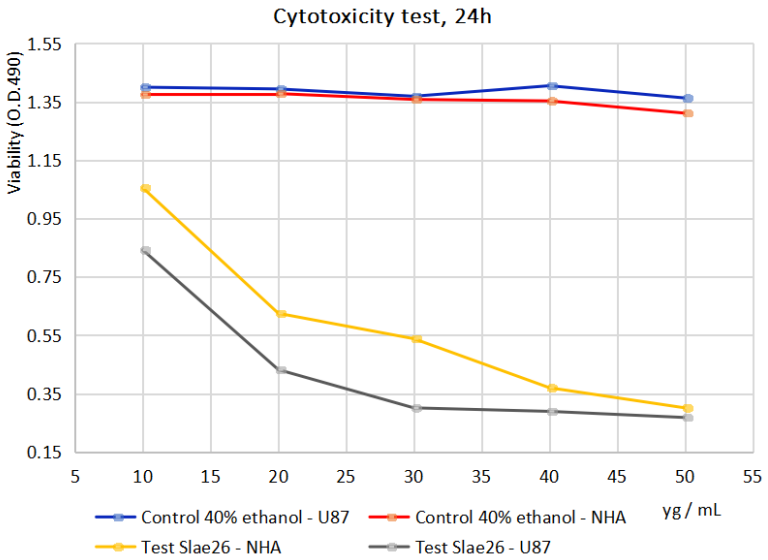

Figure 1. Slae26 dilution series effects on the viability of normal and tumor brain cell lines NHA and U87, compared with the solvent sample, 40% ethanol dilution series, respectively; mean values ± SD, n = 3.

Table 1. Cytotoxic activity of Slae26 on normal and tumor brain cells NHA and U87.

Cytotoxicity study/Cell line	Inhibitory activity (%) of Slae26 along the dilution series (μg [GAE]/mL sample)				
	10	20	30	40	50
NHA	23.27	54.64	60.38	72.65	77.04
U87	39.86	69.01	78.01	79.43	80.33

Figure 2 presents the results of the anti-proliferative study, at 24 h and 48 h respectively, Slae26 dilution series versus solvent sample (40% ethanol) dilution series, tested on human tumor brain cell line U87. As opposed to the cytotoxicity study, the anti-proliferative study has underlined the capacity of the ethanol solvent to induce alone the decrease of the viability of tumor brain cell line U87 in culture; there were registered independent activities at 24 h (blue line) and 48 h (red line) respectively, with a media of inhibitory activity along the dilution series at about 44%. Slae26 dilution series also induced augmented decreases of tumor brain cell U87 viability in culture, both at 24 h (gray line) and 48 h (red line) after treatment; average values for anti-proliferative, inhibitory activity upon the tumor brain cells U87 were computed at 60.62% in the first 24 h, and 35.28% in the next 24 h, with a maximum of 78% inhibitory activity.

Table 2 shows the results as percent of inhibition at 24 and 48 h respectively, along with the total phenolics' in the Slae26 dilution series.

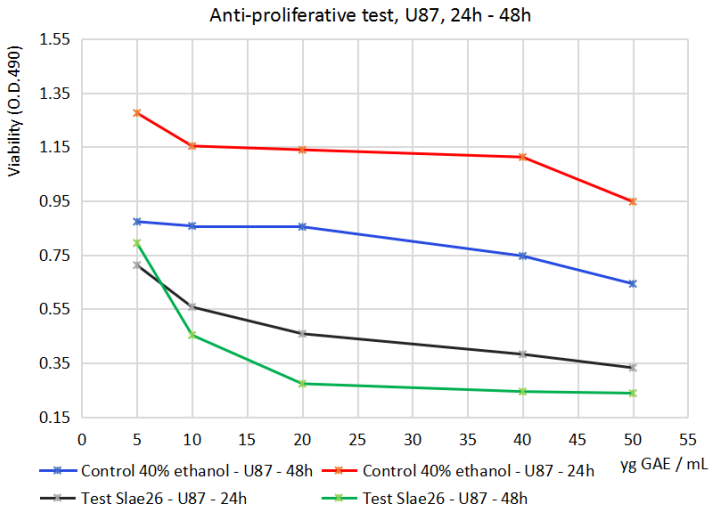

Figure 2. Slae26 dilution series effects on the viability of tumor brain cell line U87, at 24h and 48h, in comparison with the solvent sample, 40% ethanol dilution series, respectively; mean values ± SD, n = 3.

Table 2. Anti-proliferative activity of Slae26 on tumor brain cell line U87 (24 h, 48 h).

Anti-Proliferative Study/24–48 h	Inhibitory Activity (%) of Slae26 along the Dilution Series (µg [GAE]/mL Sample)				
	5	**10**	**20**	**40**	**50**
U87 - 24 h	18.44	35.01	46.37	48.79	48.36
U87 - 48 h	37.73	60.71	76.03	78.06	78.01

Figure 3 reveals the resulted docking scores for the studied compounds, as a measure of the stability of their complexes with Bcl-2 protein, and Figure 4 illustrates the hydrogen bond interactions formed by curcumin, the structure revealing the greatest binding affinity, exhibiting docking score −63,88. Interactions are occurring with PHE109 and VAL130 amino acid residues on the A chain.

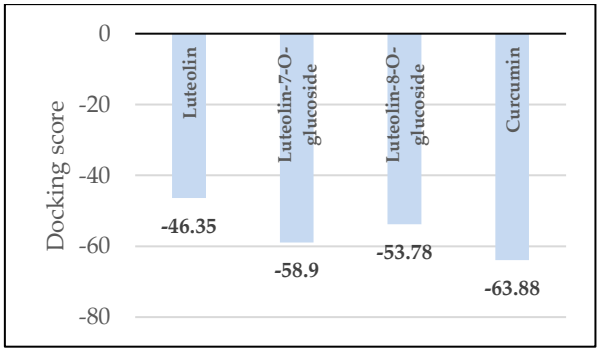

Figure 3. Docking results for investigated structures.

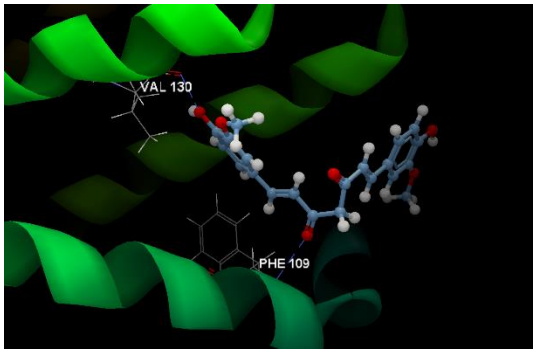

Figure 4. Hydrogen binding of curcumin within the binding pocket.

4. Conclusions

The active compounds in *Stokesia laevis* are luteolin, luteolin-7-O-glucoside and luteolin-8-C-glucoside. Judging the results of pharmacological in vitro studies on Slae26 standardized extract, dilution series from 5 to 50 μg [GAE]/mL sample, it resulted in IC50 values of 9.12 μg [GAE]/mL extract for human tumor brain cell line U87, and 24.17 μg [GAE]/mL extract for human normal brain cell line NHA, and an anti-proliferative activity on the tumor cell line U87 up to 78%. The opportunity to consider *Stokesia laevis* extracts and the main active compounds in ethanolic extracts (luteolin derivates) in further applications across-the-board glioblastoma has been revealed. The use of *Stokesia lae*vis extracts and the main active compounds from ethanolic extracts such as luteolin derivates on glioblastoma cell lines shows promise for future applications. The results of dicking approach can be forward supplemented with studies on other, more specific targets. Also considering the previous studies [5] on *Stokesia laevis* ethanolic extracts (e.g., on human colon cancer cell line Caco-2/IC_{50} = 36 μg GAE/mL extract, human tumor breast cell line BT20/IC_{50} = 42 μg GAE/mL extract, and on murine melanoma cell line B16/IC_{50} = 39 μg GAE/mL extract), which showed a positive outcome, the extension to in vivo level, would be an insightful approach.

Author Contributions: Conceptualization, L.C.P.; methodology, G.N., A.A. and A.S.; formal analysis, L.C.P. and O.G..; investigation, L.P., and O.G.; resources, L.C.P. and A.S.; writing—original draft preparation, L.C.P.; writing—review and editing, A.S.; visualization, L.P. and A.S.; supervision, L.C.P. and A.S.; project administration, L.C.P. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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