ORIGINAL ARTICLE

LIGUSTRUM VULGARE HYDROALCOHOLIC EXTRACT INDUCES APOPTOTIC CELL DEATH IN HUMAN PRIMARY BRAIN TUMOUR CELLS

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Abstract

Glioblastomas and astrocytomas are neoplasms that arise from brain cells called astrocytes, tumours known to be more resistant to chemotherapy than other types of brain tumours. Medical research has turned its attention towards the field of natural products; these compounds have been used in cancer treatment and prevention for the last 50 years. The aim of this study was to investigate the effect of *Ligustrum vulgare* hydroalcoholic extract (LVHE) on the brain tumours cell viability. Our results showed that LVHE extract induced cell death in early passage low- and high-grade glioma cells by triggering caspase 3 activation. The effect of the LVHE in glioma cells was then compared to its effects on normal cells, using a Human Umbilical Cord-derived cell line for the assessment of LVHE effect on normal tissue. LVHE induced time- and dose-dependent decreases of the viability in glioma cells, causing apoptosis, as confirmed by caspase 3 activation. We also found that LVHE treatment was more cytotoxic in glioma cells then in normal cells.

Rezumat

Glioblastoamele şi astrocitoamele sunt neoplasme care provin din celulele cerebrale numite astrocite, tumori cunoscute ca fiind mai rezistente la chimioterapie decât alte tipuri de tumori cerebrale. Cercetările medicale şi-au îndreptat atenţia din ce în ce mai mult către domeniul compuşilor naturali, compuşi care au fost folosiţi în tratamentul şi prevenţia cancerului în ultimii 50 de ani. Obiectivul acestui studiu a fost să investigheze efectul extractului hidroalcoolic de *Ligustrum vulgare* (EHLV) asupra viabilităţii celulelor tumorale cerebrale. Rezultatele au arătat că EHLV induce moarte celulară în celulele de gliom de grad mic şi înalt, prin declanşarea activării caspazei 3. În vederea evaluării efectului EHLV asupra toxicităţii ţesutului normal, s-au utilizat linii celulare derivate din cordonul ombilical uman. EHLV induce o scădere a viabilităţii celulelor de gliom într-o manieră timp şi doză - dependentă, determinând apoptoză, confirmată prin activarea caspazei 3. În plus, tratamentul cu EHLV a avut citotoxicitate mai mare asupra celulelor de gliom comparativ cu celulele normale.

Keywords: *Ligustrum vulgare*, brain tumours, apoptosis, cell death

Introduction

Central nervous system (CNS) tumours are a heterogeneous group of tumours with different origins, prognoses and behaviours. According to their cell type, glioblastomas and astrocytomas are neoplasms that arise from brain cells called astrocytes. Glioblastomas and astrocytomas, are more resistant to chemotherapy than other types of brain tumours such as: medulloblastomas,

oligodendrogliomas, primary cerebral lymphoma or germ-cell tutors [25, 27, 30].

Radiotherapy and chemotherapy resistance is most commonly enhanced in brain tumour patients. The development of sophisticated anti-cancer drugs has changed the concept of chemotherapy to targeted therapy. These new drugs target molecular alterations in tumours and induce higher and more specific cell death; however, the effect of the targeted therapy is also temporary, because malignant cells develop quickly resistance to treatment [15, 24]. The failure in the treatment of cancer by using synthetic drugs increased the necessity to use medicinal plants, in order to offer the patient a more effective and less toxic treatment.

Many modern chemotherapeutics have derived from natural compounds. In fact, some of the most effective anticancer medications like taxanes, inhibitors of topoisomerases or *Vinca* alkaloids are extracted from plants [4, 25]. In brain tumours treatment, drugs like vincristine are already used in the adjuvant chemotherapy regimen PCV (procarbazine, lomustine and vincristine). There are also some natural compounds like: curcumin, catechins, isoflavones that proved their anti-tumour properties both *in vitro* and *in vivo* [2, 4].

Ligustrum vulgare (*Oleaceae* family) a plant usually found of the forests of Central and Southern Europe, is used in traditional medicine for various properties like: anti-inflammatory, antibacterial, antioxidant, hepatoprotective, antidiabetic, immunomodulatory [5, 6, 16, 20, 23, 33], but also cytotoxic and anti-mutagenic [17, 22].

A study published in 2012 reported that *Ligustrum vulgare* methanolic extract has antiproliferative and proapoptotic activities on human colon cancer cells either used alone or in combination with palladium complex [12]. However, its antiproliferative effects were never studied on brain tumour cells.

The aim of this study was to investigate the effect of *Ligustrum vulgare* hydroalcoholic extract on the brain tumour cell viability and to compare its effect on normal cells.

Materials and Methods

Reagents

Minimum essential medium (MEM), foetal bovine serum (FBS), penicillin/streptomycin antibiotics, trypsin where acquired from Gibco, USA. All other chemicals unless stated otherwise were purchased from Sigma, Germany.

Plant Material

Flowers from the *Ligustrum vulgare* species were collected in April 2013, from a South-Western region of Romania. No specific permissions were required for the location and the collection activity. The field studies did not involve endangered or protected species. Voucher specimens (LV–1012011 code) are deposited in the Herbarium of the Department of Pharmacognosy and Phytotherapy, University of Medicine and Pharmacy of Craiova, Romania. The specimens were identified according to the Romanian Flora [29] and European Flora [32] and by comparison with authenticated vouchers from the Herbarium of "Alexandru Buia" Botanical Garden, University of Craiova, Romania.

Ligustrum vulgare hydroalcoholic extract preparation

Samples of accurately weighed, air-dried and powdered flowers of *Ligustrum vulgare* species were macerated for 14 days with diluted alcohol (70% ethanol) at room temperature, according to Romanian Pharmacopoeia Xth edition [1]. The mixture of 20% hydroalcoholic extract was filtered and then stored in dark bottles in the refrigerator until use.

Cell culture techniques and maintenance of cell lines

Early passage glioma cell lines (GB1B, GB2B, GB8B and AC1B) were obtained from fresh tumour tissue fragments collected from patients diagnosed with brain tumours at the "Bagdasar-Arseni" Emergency Hospital, Bucharest, Romania. All patients provided signed consent forms indicating that they agreed to donate the tissue for the research study propose, when they were hospitalized. The cell lines were established according to standard procedures [27]. HUC1 cell line was established from human umbilical cord tissue, obtained after term natural births at the Emergency Hospital Craiova. The umbilical cord was washed three times with cold PBS and was cut in 3 cm fragments. Each fragment has been dissected and blood vessels and Wharton's jelly have been removed. The tissue was washed three times with culture medium, dissected in small pieces and incubated with collagenase IV, at a concentration of 1.5 mg/mL for 1 hour at 37°C, under continuous shaking. After the enzymatic dissociation, the sample was centrifuged at 400 g for 10 minutes; the cell pellet was re-suspended in 5 mL medium and again centrifuged at 400 g for 10 minutes. The cell pellet was then transferred in cell culture dishes and incubated in MEM, containing 10% FBS, 2 mM glutamine and antibiotic (100 IU/mL penicillin and 100 IU/mL streptomycin), at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. Cell cultures were amplified 2 to 3 passages from the initial biological material and then have been preserved at passage 3.

Cell culture treatment

For experimental purpose, cells were seeded in 96-well culture plates (0.5 - 1 - 3 × 10^3 cells/well) and treated with various concentrations of LVHE (10 µL, 100 µL) for 7, 10 and 14 days. Drugs were refreshed with culture medium, every two days. Appropriate control groups and blank control were included. The assay was performed in triplicate or quadruplicate for each data point.

Proliferation Assay

The antiproliferative effect of the treatment was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The assay is based upon the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells [34].

Apoptosis assay

Caspases are cysteine proteases, which exist as inactive pro-forms. By inducing apoptosis, they are cleaved to the active form. For experimental purposes, we used 10 cm Petri dishes. The cells treated with 100 µL LVHE for 48 hours, 72 hours or untreated cells were trypsinated and the cytosolic fraction was isolated at a concentration of 3×10^6 per sample. Apoptosis was analysed using ApoTarget Caspase-3 (CPP32) (Colorimetric Protease Assay kit, Assay kit using the manufacturer's recommendation (Invitrogen, Life Tehnologies, USA).

Statistical analysis

Each study was replicated in at least three independent experiments. Analysis of variance (ANOVA) and the t-test were used to analyse the significance of differences between study groups. $p < 0.05$ values were considered statistically significant. All data are represented as mean ± standard deviation (SD).

Results and Discussion

Ligustrum vulgare hydroalcoholic extract induced more cytotoxicity in glioma cells than in normal cells

Ligustrum vulgare methanolic extract was reported to induce cell death in human colon cancer cells [12]. In the present study, we used a hydroalcoholic extract of *Ligustrum vulgare* to study its cytotoxic effect on glioma cells. To test the extract effect, we used three high-grade glioma cell lines (glioblastoma cell lines: GB1B, GB2B, GB8B) and one low grade cell line (grade II astrocytoma: AC1B). A general restriction in experiments that use high passage cell lines is that they fail to reproduce the *in vivo* tumour heterogeneity. High passage tumour cell lines are reported to accumulate mutations that generate genetic changes, which have not been detected at earlier passages [14]. For this reason, in our study we used low passage glioma cell lines. Our results showed that LVHE induced cytotoxicity in glioma cells in a time and dose dependent manner. We observed that AC1B cells were more sensitive to LVHE than GB1B, GB2B, GB8B glioblastoma cells. We also investigated whether LVHE shows any differential effect on cancer and normal cells. Human stem cells are in the foreground as a preferable application for drug scanning, in order to reduce and replace animal testing. Here, we used a Human Umbilical Cord-derived cell line (HUC1) for the assessment of LVHE normal tissue toxicity.

The treatment with 10 µL LVHE for 7 days led to an enhanced cytotoxic activity on brain cancer cells compared to normal cells. The treatment was 30%, 40% and 50% statistically more toxic in GB1B, GB2B, GB8B and AC1B cells than in HUC1 cells ($p < 0.005$) (Figure 1). Increased concentrations of LVHE (100 µL) enhanced with 45% the cell death in GB1B cells, with 20% in GB2B cells, with 40% in GB8B cells and with 56% in AC1B cells, compared to normal HUC1 cells and the results were statistically significant ($p < 0.005$) (Figure 1).

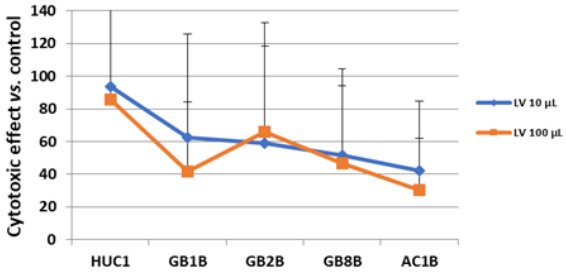

Figure 1.

Effect of 7 days LVHE treatment on viability of HUC1, GB1B, GB2B, GB8B and AC1B cells

*$p < 0.05$ (tumour cells *vs.* normal cells)

10 µL LVHE treatment for 10 days was 40% significantly more toxic in GB1B and GB2B cells compared with normal cells ($p < 0.005$), 55% more toxic in GB8B and 62% more toxic in AC1B cells, compared to HUC1 cells ($p < 0.005$) (Figure 2). The treatment with 100 µL LVHE for 10 days enhanced with 52%, 32%, 45% and 58% the cell death compared to HUC1 cells ($p < 0.005$) (Figure 2).

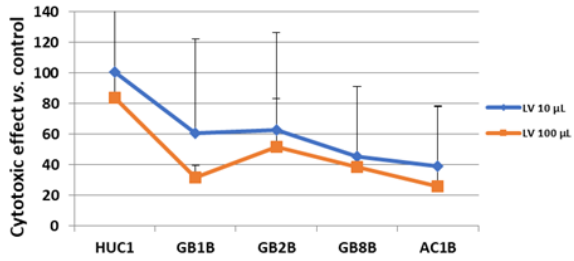

Figure 2.

Effect of 10 days LVHE treatment on viability of HUC1, GB1B, GB2B, GB8B and AC1B cells

*$p < 0.05$ (tumour cells *vs.* normal cells)

Prolonged treatment with LVHE 10 µL for 14 days induced 28%, 35%, 55% and 60% more cell death in GB1B, GB2B, GB8B and AC1B cells respectively, compared to HUC1 normal cells. The treatment with higher concentrations of LVHE (100 µL) for 14 days was also, more potent in inducing cyto-toxicity in brain cancer cells, compared to normal cells. The treatment was 37% more cytotoxic in GB1B, 26% more cytotoxic in GB2B, 57% more cytotoxic in

GB8B and 69% more cytotoxic in AC1B, compared to normal cells ($p < 0.005$) (Figure 3).

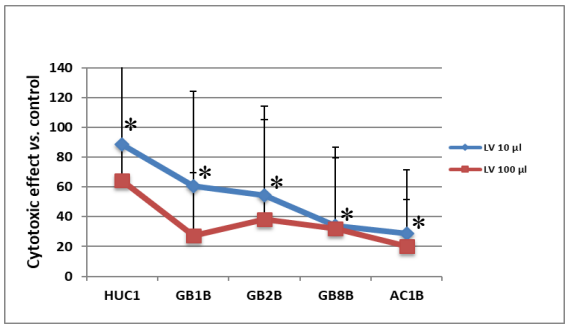

Figure 3.
Effect of 14 days LVHE treatment on viability of HUC1, GB1B, GB2B, GB8B and AC1B cells
*p < 0.05 (tumour cells *vs.* normal cells)

Thus, both 10 µL and 100 µL LVHE significantly induced ($p < 0.05$) a higher toxicity in brain tumour cells compared to normal cells, irrespective of the treatment duration.

Ligustrum vulgare hydroalcoholic extract induced caspase 3 activation in human glioma cells and normal cells

Ligustrum vulgare methanolic extract was reported to induce apoptosis in human colon cancer cells [12]. In the next step we intended to identify the caspases involved in LVHE-induced apoptosis. For this purpose, we measured the enzymatic activity of caspase 3, induced by LVHE at 48 and 72 hours.

The GB1B, GB2B, GB8B, AC1B and HUC-1 cells treated with 100 µL LVHE for 48 hours and 72 hours respectively or untreated cells were assayed for caspase 3 activation.

We found that in GB1B cells, the treatment with LVHE induced caspase 3 activation at 48 hours and remained active at 72 hours after drug administration (Figure 4). In GB2B and GB8B cells the treatment with 100 µL LVHE caspase 3 activation was detected only at 48 hours, while at 72 hours after the treatment caspase 3 decreased at the basic level (Figure 4). In AC1B cells caspase 3 activated at 48 hours after LVHE administration and increased at 72 hours after treatment (Figure 5), while in HUC-1 cells caspase 3 activated only at 72 hours after drug administration (Figure 6).

Astrocytic tumours accomplish more than 50% of all brain tumours and are classified as low-, intermediate- or high-grade glioma. Radiotherapy increases the overall survival for patients belonging to all tumour grades, although they cannot be cured by this therapy. Procarbazine, lomustine and vincristine (PCV) represent the standard chemotherapy combination used in the treatment of high grade glioma [7, 19]. For low-grade glioma patients, the standard treatment option is still disputed. For localized tumours, surgical resection is the most common treatment while radiotherapy is given to low grade glioma patients with partial resected initial tumours. PCV in combination with radiotherapy was reported to improve progression-free survival compared with radiation alone, in two clinical trials (a randomized study and a phase III study) conducted in patients with low grade gliomas [3, 30].

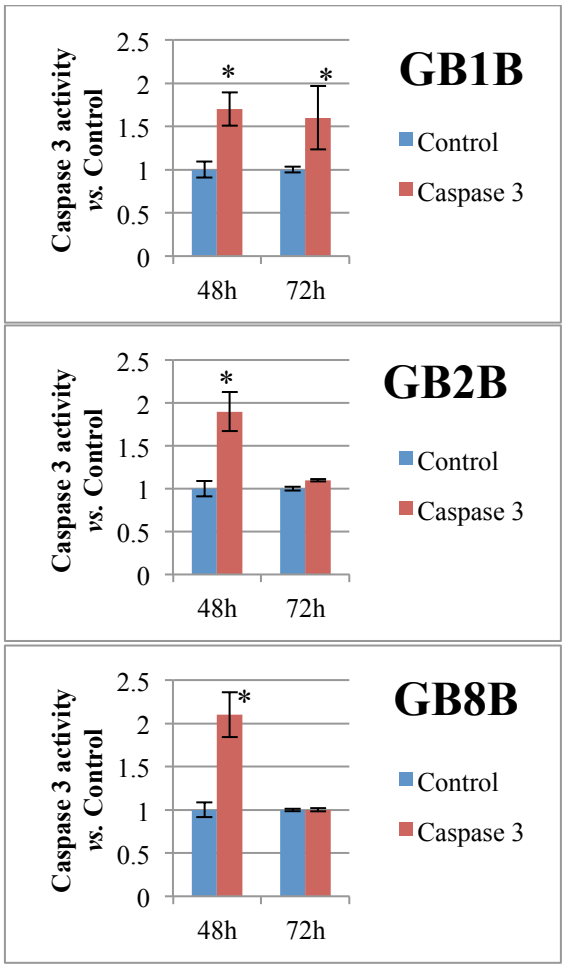

Figure 4.
The levels of caspase 3 induction by treatment with LVHE in GB1B, GB2B, GB8B glioblastoma cell lines
*p < 0.05 *vs* control group

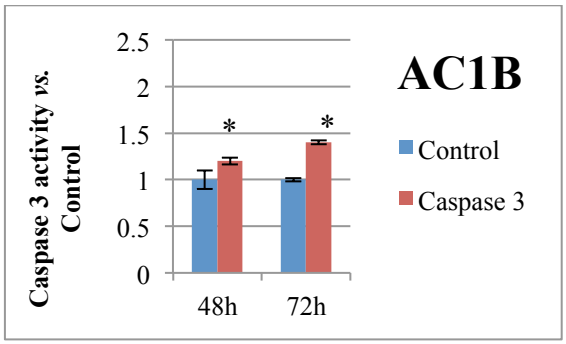

Figure 5.
The levels of caspase 3 induction by treatment with LVHE in AC1B glioblastoma cell lines
*p < 0.05 *vs* control group

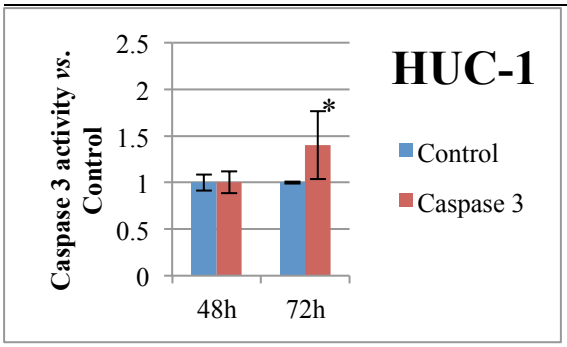

Figure 6.
The levels of caspase 3 induction by treatment with LVHE in HUC1 normal cell lines
*p < 0.05 *vs* control group

Several recent surveys showed that more than 50% of approved cancer drugs in the world are natural products or their analogues [11, 13]. Many of these natural drugs are routinely used in clinic to treat malignant diseases. Among them, vincristine, taxol, topotecan and irinotecan, etoposide is only a few known examples [10, 18].

Many promising new agents are in preclinical development. Ćurčić *et al.* reported that *Ligustrum vulgare* methanolic extract induced cell death in human colon cancer cells as single treatment and resulted in synergistic cell death in combination with palladium complex [12].

Our results showed that LVHE induced cell death in glioma cells in a time and dose dependent manner. AC1B, low grade astrocytoma cells, were more sensitive to LVHE treatment than GB1B, GB2B or GB8B glioblastoma cells.

The current interest in natural products to treat cancer can be attributed to a number of factors such as: several modern drugs are made from plants; natural products are used as single therapy but can also be used as adjuvants to overcome resistance to chemo- and radiotherapy, natural moieties have significantly fewer side effects than synthetic drugs. Here, we also found that LVHE produced less cytotoxic effects on normal cells compared to brain tumour cells. As a normal model, we developed Human Umbilical Cord-derived cell line (HUC-1), a common model used for drug scanning, in order to reduce and replace animal testing. Human glioma cells and especially malignant gliomas are characterized by resistance to pro-apoptotic stimuli [3, 8, 9]. The process of apoptosis is associated with caspase enzymes activation. Caspase 3 is a protease essential for normal brain development but also an executioner caspase involved in the apoptotic process. The protease is usually activated by several chemotherapeutics used for gliomas therapy. Some natural products like curcumin and quercetin are also reported to activate caspase 3 in brain tumour cells. In our study, we found that the treatment with LVHE induced caspase 3 activation in all glioma cell lines. However, we observed that the effect of the treatment was more pronounced in GB1B glioblastoma and AC1B astrocytoma cells, in which caspase 3 was activated at 48 hours and 72 hours after LVHE administration, while in GB2B and GB8B glioblastoma cells, caspase3 was activated only at 48 hours after the treatment. In normal human stem cells HUC-1, caspase 3 was activated only at 72 hours after drug administration.

Conclusions

The hydroalcoholic extract of *Ligustrum vulgare* flowers proved to have antiproliferative and pro-apoptotic effects on glioma cells and these effects were more pronounced in cancer cells when compared to normal human stem cells. This study may lead to further investigations of novel natural products that may have anti-neoplastic properties and are less aggressive with the healthy tissues.

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