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*Original Article*

# Rationale and *in vitro* efficacy of *Ligustrum vulgare* hydroalcoholic extract for the treatment of brain tumors

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**Abstract:** The majority of drugs available for treating malignant diseases are based on natural products. *Ligustrum vulgare* extracts are used for prevention or treatment of several diseases, including cancer. Here, we investigated the effect of *Ligustrum vulgare* hydroalcoholic extract (LHAE) alone and in combination with temozolomide (TMZ) on three glioblastoma cell lines (GB1B, GB2B, GB8B) and one astrocytoma cell line (AC1B) *in vitro*. GC-MS analysis of LHAE revealed 28 compounds, most of them from the essential oil *Ligustrum vulgare* flowers. LHAE displayed cytotoxic effect against brain tumor cells, depending on cell type, drug concentration and period of treatment. Further, we found that the treatment with LHAE was more efficient when combining with a second agent TMZ. This study underlines the need for identification of new molecules able to kill brain tumor cells that would facilitate the development of better therapeutic approaches.

**Keywords:** *Ligustrum vulgare*, hydroalcoholic extract, temozolomide, brain tumors

## Introduction

Two types of chemotherapy are generally used in brain cancer: temozolomide (TMZ) and the combination of procarbazine, lomustine, and vincristine (PCV). The PCV therapy has been used for over 30 years to treat brain tumors, while temozolomide (TMZ) treatment is a newer therapeutic approach, used for the treatment of both low and high-grade astrocytic tumors [1].

Recent studies reported that more than 40% of glioblastoma patients have an alternated $O^6$-methylguanine-DNA methyl-transferase (MGMT) and isocitrate dehydrogenase 1, responding very well to alkylating cytostatics such TMZ [2]. However, the effect of TMZ on low-grade astrocytoma has been very little studied and the results are controversial [3, 4]. In a retrospective study by Taal *et al.*, the response to TMZ was evaluated in a cohort of 58 patients with low-grade astrocytoma, after the patients underwent surgery and radiotherapy. They found 67% progression-free survival (PFS) at six months and 25% at 12 months, with a tendency towards longer PFS for patients with mutated TP53. In contrast to glioblastoma patients, no difference was noted in the response to TMZ treatment between patients with a methylated- *vs.* unmethylated-MGMT promoter concerning PFS [4]. However, in a recent study by Houillier *et al.*, in addition to 1p-19q codeletion and isocitrate dehydrogenase (IDH) mutation, MGMT promoter methylation was correlated with a higher rate of response to TMZ. In their study, the authors evaluated the response to temozolomide by PFS and tumor size on successive magnetic resonance imaging (MRI) scans [3].

A range of cancer therapeutics from plants, such as combretastatin, isolated from the South African bush willow, *Combretum caffrum* [5]; paclitaxel, isolated from the Pacific yew tree, *Taxus brevifolia* and irinotecan, isolated from Asian tree *Camptotheca acuminata* [5, 6] have shown beneficial effects on cancer progression and less side-effects in many preclinical and clinical studies [7]. Cyclopamine, a com-

pound isolated from *Veratrum californicum*, corn lily that grows in the South San Francisco, California, USA, is a Sonic Hedgehog pathway antagonist with antineoplastic effect in several types of cancer, including brain tumors [8]. For these reasons, herbal medicines is believed to be safe and today more than 60% of cancer patients use alternative and complementary therapies [9].

In a recent report by Ćurčić *et al.* has shown that methanolic extract of *Ligustrum vulgare*, wild privet, a species native in Europe, Northern Africa and Southwestern Asia, efficiently killed human colon cancer cells [10].

The aim of this study was to determine the volatile compounds, total phenolic and flavonoid content of the *L. vulgare* hydroalcoholic extract and to analyze the effect of the extract on brain tumor cells. Using a panel of low-passage primary brain tumor cell lines, including three high-grade brain tumor cell lines derived from glioblastoma tumors and one low-grade brain tumor cell line derived from grade II astrocytoma tumor, we also analyzed the cytotoxic effect of LHAE alone or in combination with TMZ, the most common drug used to treat brain cancer.

## Materials and methods

### *Reagents and solvents*

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

### *Cell culture and cell treatment*

Early passage brain tumor cell cultures (GB1B, GB2B, GB8B and AC1B) used in this study were established from fresh samples of brain tumor tissue obtained from four patients diagnosed with brain tumors (three glioblastoma patients and one astrocytoma patient) at the "Bagdasar-Arseni" Emergency Hospital, Bucharest, Romania. The cell lines were established according to standard procedures [11]. The cells were cultured in MEM containing 10% FBS, 2 mM glutamine and antibiotic (100 IU/mL penicillin and 100 IU/mL streptomycin). The cells were grown in tissue culture flasks maintained in a 95% air/5% carbon dioxide atmosphere at 37°C in a humidified incubator. For experimental propose, cells were seeded in 96-well culture plates (0.5-1-3×$10^3$ cells/well) and treated with various concentrations of LHAE (10 µL, 100 µL), TMZ (10 µM, 100 µM) or combination of them for 7, 10 and 14 days. Drugs were refreshed with culture medium, every two days. Appropriate control groups with diluents only and blank control were included. The assay was done in triplicate or quadruplicate for each data point. All patients provided signed consent.

### *Liquid handling*

Automated dispensing of liquid reagents and media containing cells and drugs were performed with the epMotion 5070 instrument (Eppendorf, Hamburg, Germany). Fresh cell suspension was spotted onto the 96-well culture plates to make a final concentration of 1000-3000 cells per well (depending on experimental propose) and incubated for 24 hours in standard MEM 5% carbon dioxide under humidified conditions. The cells were then washed twice with 100 µL medium without serum, then 200 µL standard medium was added to each well and finally the cells were treated with various concentrations of LHAE, TMZ or combination of them. The cells were washed twice with 100 µL medium without serum and the drugs were refreshed every two days in standard medium. Appropriate control groups with diluents only and blank control were included. The assay was done in quadruplicate for each data point.

### *MTT cellular proliferation assay*

The antiproliferative effect of the treatment was examined using MTT assay. The assay is based upon the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells. Tests were conducted with 3000 cells/well, plated in 200 µL media in 96-well plates, with six replicates. At the end of each treatment time, 10 µL MTT reagent was added to each well, and then incubated for four hours at 37°C. After that, cells were lysed by addition of 100 µL solubilization buffer. Optical density was measured using a spectrophotometer at 595 nm.

### *Plant material*

From the *L. vulgare* species, the vegetal samples (flowers) were collected at the flowering

period, in April 2014, from Dâlga Forest, approximately 17 km from Craiova City, Dolj County, 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-1012013 code) are deposited in the Herbarium of the Department of Pharmacognosy & Phytotherapy, University of Medicine and Pharmacy of Craiova, Romania. The specimens were identified according to the Romanian Flora [12] and European Flora [13], and by comparison with authenticated vouchers from the Herbarium of "Alexandru Buia" Botanical Garden, University of Craiova, Romania.

*Preparation of hydroalcoholic extract*

Sample of accurately weighed, air-dried, and powdered flowers of *L. vulgare* species were macerated 14 days with diluted alcohol (70% ethanol) at room temperature, according to Romanian Pharmacopoeia, Xth edition (1993) [14]. The 20% hydroalcoholic extract (LHAE) was filtered and then stored in dark bottles in the refrigerator until use.

*GC-MS analysis*

LHAE was diluted with an *iso*-octane-toluene (9:1) mixture containing hexachlorobenzene (HCB) as internal standard to a volume of 2 µL. Analysis was performed on a Shimadzu QP 2010 gas chromatograph coupled with a mass spectrometer. A SLB 5 ms column (30 m length ×0.25 mm ID and film thickness of 0.25 µm) was used with He (Airgas) as carrier gas, at a flow rate of 1 mL/min. The column temperature ranged as follows: 40°C for 5 minutes, 40-200°C with 5°C/min. gradient, 200-300°C with 10°C/min. gradient, and 300°C, 10 minutes. One µL of sample was injected into the column, without splitting. Peaks with an intensity comparable to or greater than the internal standard (HCB) 0.02 µg/mL were analyzed. Shimadzu mass spectrometer detector has an electron-ionization ion source (electron impact) at 70 eV. Detector voltage was 1.22 kV. Ions with *m/z* 40-350 were scanned, at a scan rate of 1000 amu/s. The identification of peaks was performed using a mass-spectra library.

*Determination of total phenolic content*

Over 200 µL of LHAE and 2.5 mL of Folin-Ciocalteu reagent (diluted 1:10 with distilled water) were added. After four minutes, 2 mL of 75 g/L sodium carbonate solution were added. The reaction mixture was allowed to react for two hours at room temperature (23-25°C), in the dark, until spectrophotometric analysis [14]. Experiments were performed using a UV-VIS Double Beam UVD-3000 (Labomed, Inc., Culver City, California, USA) spectro-photometer. A standard curve was plotted using gallic acid as standard with concentrations ranging from 0 to 1000 mg/L. In a 100 mL volumetric flask was dissolved 0.5 g of gallic acid in 10-20 mL of 80% ethanol and then the flask was filled with water up to the mark. The obtained solution was stored at cold (4-8°C) prior to use within 48 hours. Of this solution, 0, 1, 2, 3, 5 and 10 mL were took in six 100 mL volumetric flasks, brought to final volume with distilled water afterwards. Solutions with 0, 50, 100, 150, 250, 500 and 1000 mg/L of gallic acid concentrations were obtained. The absorbance was measured at 765 nm. Results were expressed as mg gallic acid equivalents (GAE)/100 mL of LHAE. All measurements were performed in triplicate, calculating standard deviation.

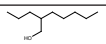
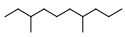
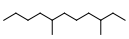
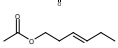
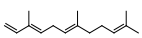
*Determination of total flavonoid content*

Determination of total flavonoids was performed using aluminum chloride 10% solution. The absorption maximum of the colored complexes was then assessed by reference to the calibration curve [14]. 0.5 mL of LHAE were taken and were mixed with 1.5 mL of 80% ethanol, 0.1 mL of aluminum chloride 10% solution, 0.1 mL of potassium acetate 1 M solution and 2.8 mL of distilled water. The sample was kept for 30 minutes at room temperature until spectrophotometric analysis was performed. The calibration curve was obtained using quercetin as standard (with concentrations ranging from 0 to 400 mg/L). Absorbance was measured at 415 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/100 mL of LHAE. All measurements were performed in triplicate, and standard deviation was calculated.

*Statistical analysis*

The statistical significance of the treatments in cell viability experiments was determined by Microsoft Excel's Student's paired *t*-Test, with one-tailed distributions. Each study was replicated in at least three independent experi-

**Table 1.** Compounds of LHAE identified using GC-MS technique

| Peak | Compound | M. F. | M | Structure | R. T. [min.] | Calculated K.I. | K.I.* | B. P. | % |
|---|---|---|---|---|---|---|---|---|---|
| 1. | 2-Propyl-1-heptanol | $C_{10}H_{22}O$ | 158 |  | 7.342 | 1194.05 | 1194 | 43 | 0.235 |
| 2. | *trans*-1,3-Dimethyl-cyclohexane | $C_8H_{16}$ | 112 |  | 7.525 | 842.03 | 842 | 55 | 5.438 |
| 3. | *n*-Butyl acetate | $C_6H_{12}O_2$ | 116 |  | 7.742 | 784.96 | 785 | 43 | 4.115 |
| 4. | 2,4-Dimethyl-hexane | $C_8H_{18}$ | 114 |  | 7.950 | 687.89 | 688 | 43 | 1.354 |
| 5. | 1-Methyl-2-methylene-cyclohexane | $C_8H_{14}$ | 110 |  | 8.133 | 847.01 | 847 | 67 | 4.235 |
| 6. | 1,4-Dimethyl-1-cyclohexene | $C_8H_{14}$ | 110 |  | 8.200 | 852.08 | 852 | 68 | 0.852 |
| 7. | *cis*-1,2-Dimethyl-cyclohexane | $C_8H_{16}$ | 112 |  | 8.267 | 843.95 | 842 | 55 | 3.567 |
| 8. | *n*-Propyl-cyclopentane | $C_8H_{16}$ | 112 |  | 8.317 | 859.04 | 859 | 69 | 2.885 |
| 9. | Ethyl-cyclohexane | $C_8H_{16}$ | 112 |  | 8.400 | 879.98 | 880 | 83 | 2.765 |
| 10. | 1,1,3-Trimethyl-cyclohexane | $C_9H_{18}$ | 126 |  | 8.517 | 915.11 | 915 | 111 | 1.412 |
| 11. | Ethylbenzene | $C_8H_{10}$ | 106 |  | 9.275 | 893.06 | 893 | 91 | 0.456 |
| 12. | 4-Hydroxy-3-hexanone | $C_6H_{12}O_2$ | 116 |  | 9.333 | 915.88 | 916 | 59 | 4.225 |
| 13. | 2,3,4-Trimethyl-hexane | $C_9H_{20}$ | 128 |  | 9.392 | 724.22 | 724 | 43 | 0.867 |
| 14. | Octahydropentalene (Bicyclo[3.3.0]octane) | $C_8H_{14}$ | 110 |  | 9.592 | 861.06 | 861 | 67 | 4.675 |
| 15. | *o*-Xylene | $C_8H_{10}$ | 106 |  | 10.425 | 908.99 | 907 | 91 | 0.735 |
| 16. | Benzaldehyde | $C_7H_6O$ | 106 |  | 13.000 | 982.21 | 982 | 106 | 0.275 |
| 17. | *β*-Pinene (6,6-Dimethyl-2-methylene-bicyclo[3.1.1]heptane) | $C_{10}H_{16}$ | 136 |  | 13.525 | 943.15 | 943 | 93 | 0.382 |
| 18. | *β*-Myrcene (7-Methyl-3-methylene-1,6-octadiene) | $C_{10}H_{16}$ | 136 |  | 13.950 | 958.06 | 958 | 93 | 0.225 |
| 19. | Sabinene {4-Methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane} | $C_{10}H_{16}$ | 136 |  | 14.625 | 896.95 | 897 | 93 | 0.095 |
| 20. | *D*-Limonene [1-Methyl-4-(1-methylethenyl)-cyclohexene] | $C_{10}H_{16}$ | 136 |  | 15.325 | 1018.03 | 1018 | 68 | 0.836 |
| 21. | 2,3,6-Trimethyl-heptane | $C_{10}H_{22}$ | 142 |  | 15.475 | 823.12 | 823 | 57 | 0.885 |
| 22. | 3,7-Dimethyl-decane | $C_{12}H_{26}$ | 170 |  | 16.175 | 1086.08 | 1086 | 57 | 0.483 |
| 23. | 3,7-Dimethyl-undecane | $C_{13}H_{28}$ | 184 |  | 17.658 | 1184.96 | 1185 | 57 | 0.354 |
| 24. | Nonanal (*n*-Nonaldehyde) | $C_9H_{18}O$ | 142 |  | 17.840 | 1104.03 | 1104 | 57 | 0.230 |
| 25. | 3-Methyl-4-heptanone | $C_8H_{16}O$ | 128 |  | 22.933 | 888.12 | 888 | 71 | 0.855 |
| 26. | (*Z*)-3-Hexen-1-ol acetate (*cis*-3-Hexenyl-1-acetate) | $C_8H_{14}O_2$ | 142 |  | 25.172 | 992.15 | 992 | 43 | 0.365 |
| 27. | 2,3,5-Trimethyl-decane | $C_{13}H_{28}$ | 184 |  | 27.910 | 1120.95 | 1121 | 57 | 3.975 |
| 28. | α-Farnesene (3,7,11-Trimethyl-1,3,6,10-dodecatetraene) | $C_{15}H_{24}$ | 204 |  | 29.025 | 1458.14 | 1458 | 93 | 1.362 |

M. F.-Molecular formula; M-Molecular weight; R. T.-Retention time [min.]; K. I.*-Kováts retention index from literature [19]; B. P.-Base peak m/e 100% (GC-MS spectrum); %-Relative content in area percent.

ments. Analysis of variance (ANOVA) and the *t*-test were performed for multiple comparisons to analyze the significance of differences between study groups, assuming the null hypothesis. $P<0.05$ values were considered statistically significant. All data are represented as mean ± standard deviation (SD).

## Results

### *Determination of terpenoids, total phenolic and flavonoid content of LHAE*

Both *in vitro* and *in vivo* experiments with extractive fractions (mainly water infusions) obtained from the leaves and/or fruits of *Ligustrum* species are distinguished by the some important pharmacodynamic actions: fungistatic [15], immunomodulatory and anti-inflammatory [16], antioxidant [17], antimutagenic, radical scavenging, diuretic and antihypertensive [18], antiproliferative and proapoptotic [10]. However, no data have been published in the literature on the chemical composition of *L. vulgare* flowers. In this study, the volatile compounds of the tincture were analyzed using GC-MS technique (**Table 1** and **Figure 1**). GC-MS analysis revealed 28 compounds with mass spectra and Kováts indices confirmation from the 45 separated com-

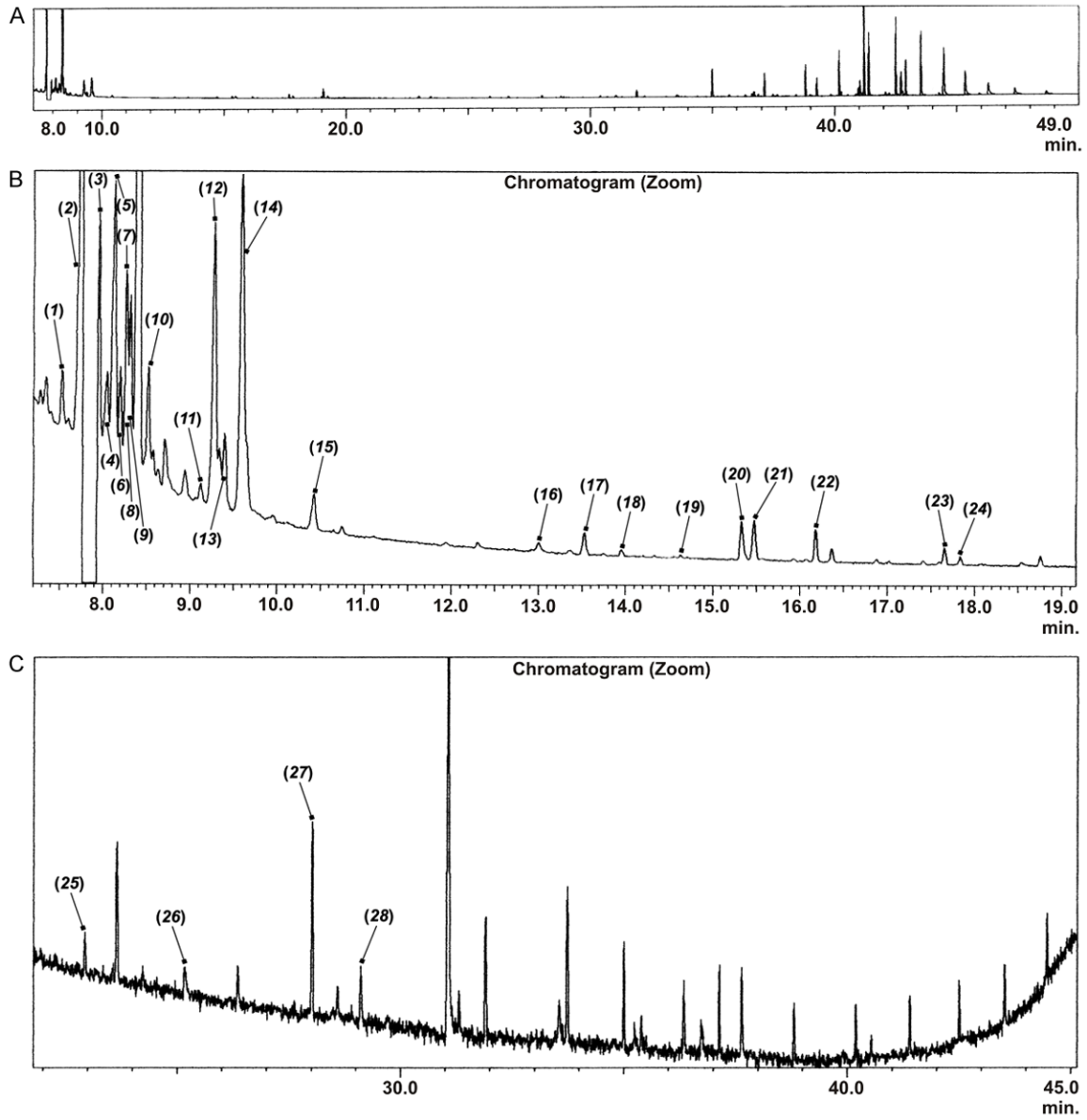



**Figure 1.** GC-MS chromatogram of separated compounds from *L. vulgare* flowers hydroalcoholic extract (LHAE). A. Full spectrum; B. Zoom in the range 7.2-19.2 min. For the identified compounds (*1*)-(*24*); C. Zoom in the range 21.8-45 min. For the identified compounds (*25*)-(*28*).

pounds, the most abundant being *trans*-1,3-dimethyl-cyclohexane (*2*) (5.44%), octahydropentalene (*14*) (4.68%), 1-methyl-2-methylene-cyclohexane (*5*) (4.24%), 4-hydroxy-3-hexanone (*12*) (4.23%), *n*-butyl acetate (*3*) (4.12%) and 2,3,5-trimethyl-decane (*27*) (3.98%), most of them from the essential oil of *L. vulgare* flowers. Small amounts of monoterpenoids, sesquiterpenoids and aromatic derivatives were also detected. Monoterpenoids are represented by 2-propyl-1-heptanol (*1*), *β*-pinene (*17*), *β*-myrcene (*18*), sabinene (*19*), *D*-limonene (*20*) and 2,3,6-trimethyl-heptane (*21*). *α*-Farnesene (*28*) was the only sesquiterpenoid compound detected. The aromatic derivatives composition of LHAE comprises ethylbenzene (*11*), *o*-xylene (*15*) and benzaldehyde (*16*).

Aluminum chloride forms stable coordination compounds with $C^4$-position of the keto group either $C^3$- or $C^5$-position, which binds hydroxyl groups in the case of flavones and flavonols. These complexes have maximum absorption at 415 nm. Instead, Folin-Ciocalteu reagent reacts

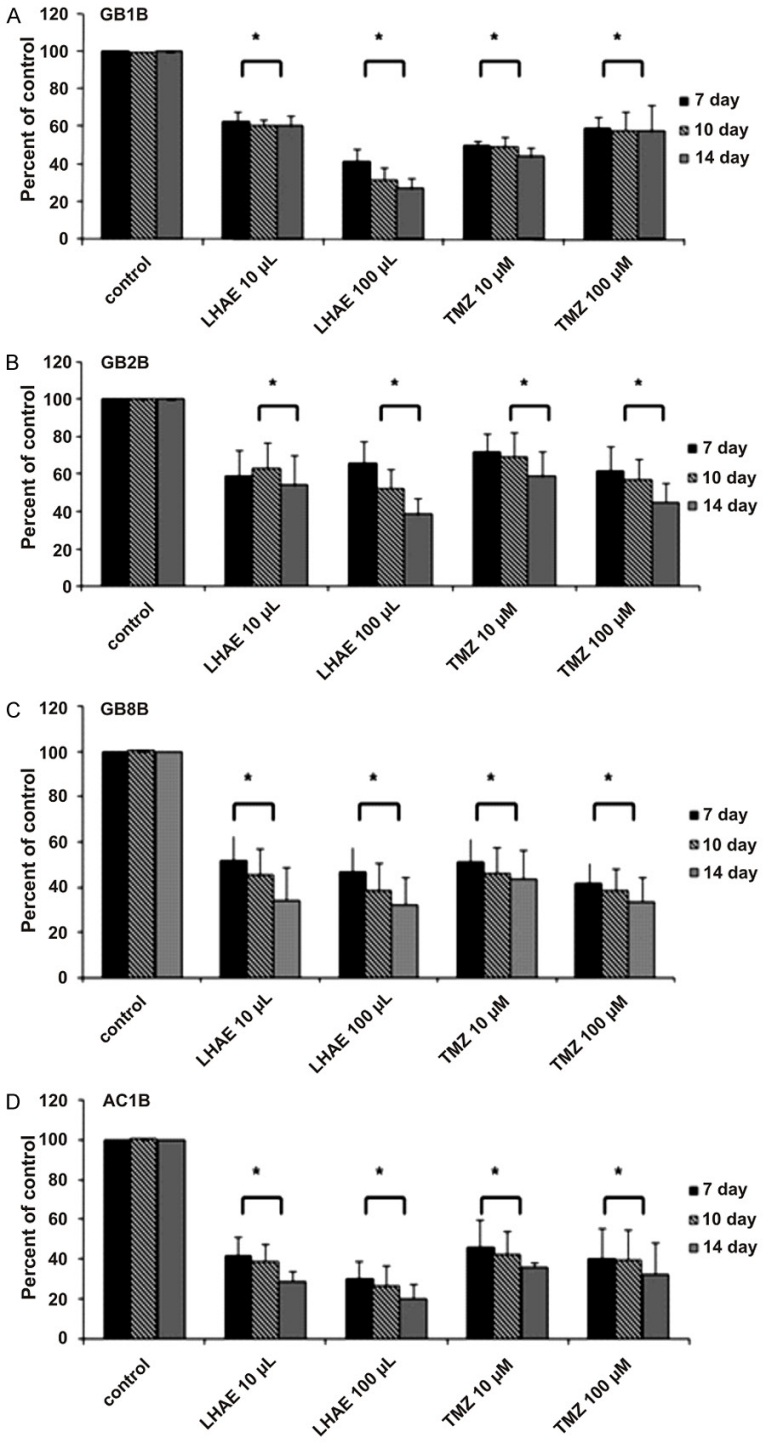



**Figure 2.** Effect of LHAE and TMZ on viability of GB1B cells (A), GB2B cells (B), GB8B cells (C), and AC1B cells (D). The cells were seeded in 96-well culture plates (1-10×$10^3$ cells/well) and treated with LHAE in a concentration of 10 and 100 µL; TMZ in a concentration of 10 and 100 µM. Cytotoxic effects of drugs were evaluated by MTT assay, 7 days, 10 days and 14 days after treatment. Results are expressed as percentage of control. Data are mean and standard error of three separate experiments. *P≤0.05 compared with the control. Statistically significant *P≤0.05 (combined treatment *vs.* LHAE, 7 days), **P≤0.05 (combined treatment *vs.* LHAE, 10 days), ***P≤0.05 (combined treatment *vs.* LHAE, 14 days).

with phenolic compounds, regardless of their structure, and forms blue coordinative compounds, with maximum absorption at 765 nm. For both determinations, a good linearity was obtained: calibration equation $y$=0.0038$x$, $R^2$=0.9982, for determination of polyphenols, and $y$=0.006$x$ + 0.026, $R^2$=0.9994, for total flavonoid content. For LHAE, we obtained a total content of phenolic compounds of 97.71929±0.56828 mg gallic acid equivalents (GAE)/100 mL tincture, and in the case of flavonoid content 56.88596± 1.45389 mg quercetin equivalents (QE)/100 mL tincture.

*The cytotoxic effect of LHAE on brain tumor cell lines*

In a study by Ćurčić *et al.*, it was reported that *L. vulgare* methanolic extract efficiently killed human colon cancer cells [10]. Here, we analyzed the effect of hydroalcoholic extract of *L. vulgare* on a panel of brain cancer cell lines *in vitro*. Our results showed that in GB1B, the treatment with 10 µL LHAE reduced cell viability with approximately 40%, irrespective of duration of exposure to drugs. Higher dose of LHAE (100 µL) induced time-dependent up to 14 days, decrease in survival of GB1B cells (41.5% at 7 days, 31.6% at 10 days and 27.2% at 14 days) (**Figure 2A**). The treatment of GB2B with 10 µL LHAE reduced the level of survival by approximately 40%, regardless of its duration. One hundred µL LHAE treatments for 7 days induced about 40% decrease in GB2B cell viability, while prolonged drug action for 10 and 14 days, decreased the cell viability by 62% (**Figure 2B**). Li-

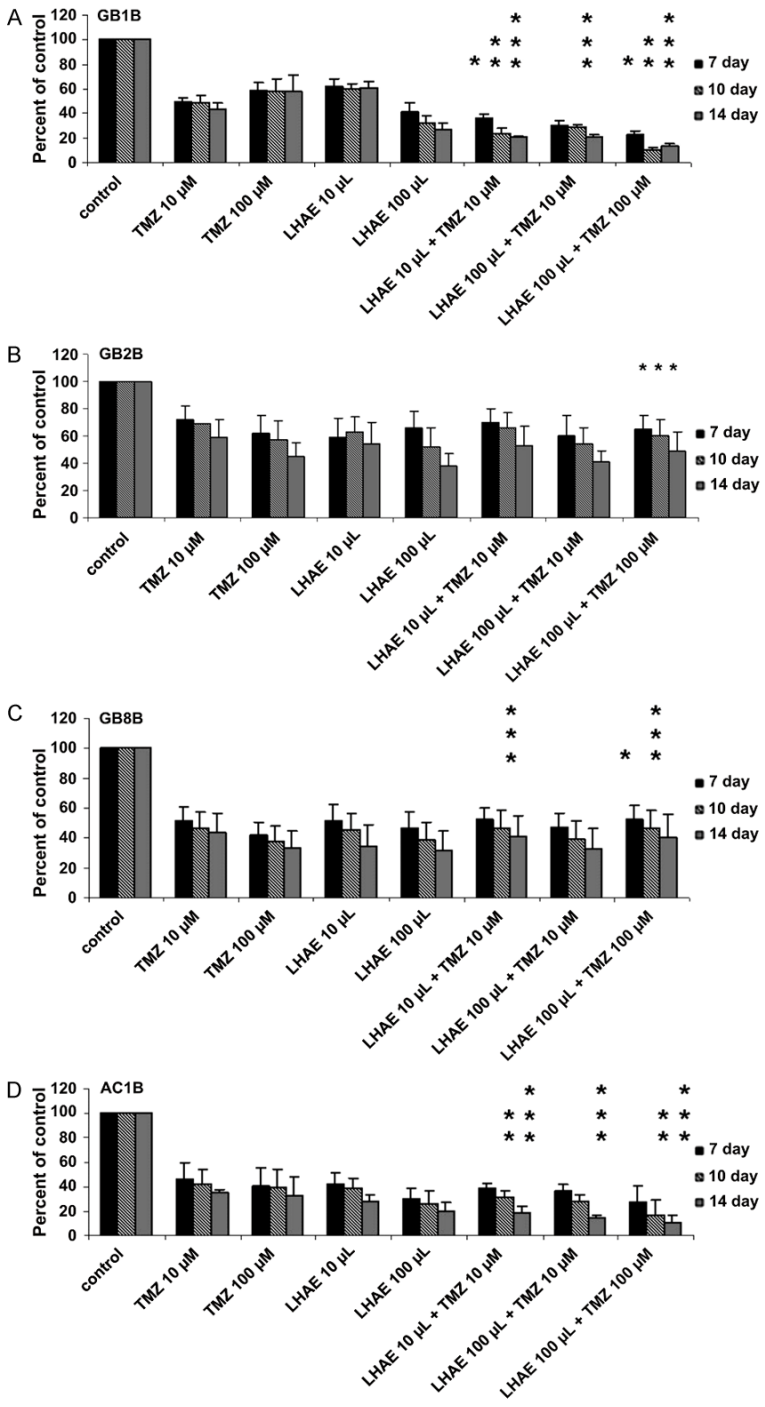



**Figure 3.** Effect of combined treatment (LHAE and TMZ) on viability of GB1B cells (A), GB2B cells (B), GB8B cells (C), and AC1B cells (D). Cells were seeded in 96-well culture plates ($10^4/10^3/10^2$ cells/well) and treated with LHAE in a concentration of 10 and 100 µL; the cytotoxic effects of the treatment were evaluated by MTT assay after 7 days, 10 days and 14 days. Results are expressed as percentage of control. Data are reported as the mean ± SD of three separate experiments. Statistically significant $^{*}P\leq0.05$ (combined treatment *vs.* LHAE, 7 days), $^{**}P\leq0.05$ (combined treatment *vs.* LHAE, 10 days), $^{***}P\leq0.05$ (combined treatment *vs.* LHAE, 14 days).

ttle growth suppression was observed in GB8B cells treated with higher dose of LHAE (100 µL) compared to the cells treated with 10 µL: 46.5% cell survival after 7 days treatment, 38.6% after 10 days treatment and 32% after 14 days treatment (**Figure 2C**). As showed in **Figure 2D**, exposure to 10 µL of LHAE for 7, 10 and 14 days induced a time-dependent decrease in survival of AC-1B cells (41.9%, 38.8% and 28.5% surviving cells, for the indicated time points). The treatment with 100 µL of LHAE resulted in 30.4%, 26% and 20.3% cell survival after 7 days, 10 days and 14 days, respectively. The response to anti-neoplastic agents often, but not always, correlated with cell proliferation rate.

*The cytotoxic effect of TMZ on brain tumor cell lines*

Temozolomide, also known as Temodal, Temodar and Temcad, is an alkylating cytotoxic agent routinely used a first-line therapy for glioblastoma and as a second-line therapy for astrocytoma. Treatment of GB1B cells with 10 µM TMZ reduced the level of cell survival to 50%, 49.1% and 43.8%, 7, 10 and 14 days after the treatment. Higher concentration of TMZ (100 µM) failed to cause a further reduction in cell growth, compared to 10 µM treatment (**Figure 2A**). In GB2B cells, administration of 10 µM TMZ for 7 days resulted in reduced cell survival to 72%. Prolonged treatment with TMZ for 10 and 14 days decreased the cell viability to 69% and 59%, respectively. When the GB2B

cells were treated with 100 μM TMZ, 51.9%, 57% and 45% reduction in cell viability was detected after 7, 10 and 14 days of treatment (**Figure 2B**). **Figure 2C** demonstrates that the treatment with TMZ alone also decreased cell viability in GB8B cells in a time dependent manner. The treatment of GB8B cells with 10 μM TMZ lowered cell viability to 51.4%, 7 days after the treatment, to 46.1%, 10 days after the treatment and to 43.3%, 14 days after the treatment. When the GB8B cells were treated with 100 μM TMZ, 41.9%, 38% and 33.5% cell survival was detected after 7, 10 and 14 days of treatment. At the time points indicated above, 10 μM TMZ treatment decreased AC1B cells viability to 45.6%, 41.9% and 35.8%. Administration of 100 μM TMZ resulted in 40.4% cell survival 7 days after the treatment, 39.2% cell survival 10 days after the treatment and 32.4% cell survival, 14 days after the treatment (**Figure 2D**). Thus, our results showed that the treatment with TMZ provided to be most efficient in GB8B and AC1B cells, when used in a highest concentration (100 μM).

*The cytotoxic effect of combined treatment with LHAE and TMZ on brain tumor cells*

Combining different compounds have been reported to enhance tumor cell death and to prolong survival of patients with malignant disease. In our investigation, concomitant treatment with LHAE and TMZ lowered the percentage of viable cells in GB1B cell line. LHAE 10 μL in combination with TMZ 10 μM decreased viability of the cells to 35.9% 7 days after treatment, 23.8% 10 days after treatment and 20.4% 14 days after treatment. The treatment with LHAE 100 μL and TMZ 10 μM reduced viability to 30.3% 7 days after treatment, 28.9% 10 days after treatment and 20.2% 14 days after treatment. Seven days treatment with TMZ 100 μM combined with LHAE 100 μL decrease cell viability to 22.7%, to 10.4% at 10 days treatment and to 13% at 14 days treatment (**Figure 3A**). GB2B cells were more resistant to the combined LHAE and TMZ treatment, the highest dose of combination (LHAE 100 μL in combination with TMZ) and the longest time treatment (14 days), cell GB2B cell viability decreased with about 45-50% (**Figure 3B**). When the GB8B cells were exposed to combined treatment with TMZ 10 μM and LHAE 10 μL, the percent cell survival detected was 52.3% at 7 days after treatment, 46.4% at 10 days after treatment and 40.9% at 14 days after treatment. Concomitant treatment with LHAE 100 μL and TMZ 10 μM resulted in 46.9% viability 7 days after treatment, 39.6% 10 days after treatment and 32.3% 14 days after treatment. The treatment with TMZ 100 μM combined with LHAE 100 μL for 7, 10 and 14 days resulted in cell viability decrease to 52.4%, 45.9% and 40.5%, respectively (**Figure 3C**). Treatment with LHAE (10 and 100 μL) in combination with TMZ 10 μM failed to induce more cytotoxic effect that the treatment with 100 μL LHAE alone, but the addition of 100 μM TMZ induced a decrease in AC1B cell survival: 26.6% cell survival after 7 days, 16.6% cell survival after 10 days and 10.1% cell survival after 14 days (**Figure 3D**).

## Discussion

Several reviews have highlighted the role of medicinal plants extracts in cancer treatment [20]. Here we found that LHAE displayed inhibition property against primary brain tumor cells *in vitro*. *Ligustrum* species were reported to contain different active molecules, such as: flavonoids, secoiridoids, mono- and triterpenoids, coumarins, polyphenol carboxylic acids and derivatives, lignans, proteins [21]. In our study, we also found that the alcoholic-based extract of *L. vulgare* contained phenolic compounds (flavonoids, polyphenol carboxylic acids and their derivatives). The highest amounts of phenolic compounds are extracted in ethanol than in other solvents (water, methanol, chloroform, hexane); our results are similar to those obtained for other herbal tinctures recognized for their antioxidant properties [22]. In addition, the hydroalcoholic mixture, also named tincture, is one of the most common formulations preserving the active principles for a long time. Our GC-MS results showed that LHAE contained 28 identified compounds, the most abundant being *trans*-1,3-dimethyl-cyclohexane, octahydropentalene, 1-methyl-2-methylene-cyclohexane, 4-hydroxy-3-hexanone, *n*-butyl acetate, and 2,3,5-trimethyl-decane.

Until now, the antiproliferative activity of the *L. vulgare* total extract was analyzed only in two types of cell cultures: HCT-116 cells (a human colon cancer cell line) [10] and HeLa cells (a human immortalized cervical cancer cell line) [23]. In both studies, the total unfractionated extracts were used to analyse the plant effect

on tumor cells. In concordance with these studies, we also found that total *L. vulgare* extract induced cytotoxicity in cancer cells.

For our investigations, we used four short-term cultures of primary brain tumors: three high-grade brain tumor cell lines, derived from glioblastoma tumors (GB1B, GB2B and GB8B) and one low-grade brain tumor cell line derived from grade II astrocytoma tumor (AC1B). Using cancer cell line as tumor model has been controversial; some cell lines provided to be very successful, whereas others were completely irrelevant [24]. Many scientists believe that failure of cell line models to mirror clinical setting is the prolonged cell culture, which may induce genetic changes [25]. Drug failures in the clinic may also be because preclinical models do not represent the heterogeneity that is observed in human tumors. Compared to established cell lines, low passage cell lines were reported to better preserve features of breast cancer [24]. At low passage, cancer cell lines are very heterogeneous and should better mimic the tumor heterogeneity *in vivo*, thus, they may have better value as tumor models. Therefore, in preclinical studies, it is maybe better to use short-term cell lines. The brain tumor cells used in this study have undergone three passages and then were cryoconserved. Adherent monolayer cells showed continuous growth and could recover from de-cryogenization.

TMZ is one of the most effective chemotherapeutic drugs against brain tumors, which is systemic administrated in primary and recurrent brain tumors and in brain metastasis [26]. Consistent with these reports, we also found that TMZ treatment was effective in inducing cell death in all brain tumor cell lines studied. To our surprise, we noticed again that low-grade astrocytic tumor (grade II astrocytoma) were more sensitive to TMZ drug than high-grade astrocytic tumors (glioblastoma).

The TMZ treatment has been very little studied in low-grade astrocytoma [1], where surgical resection and radiotherapy represent the standard treatment while chemotherapy is used only for disease recurrence. Our results showed that low-grade astrocytoma cells were sensitive to TMZ treatment but the mechanism that underlies the prodrug action remains to be determined.

Combining different compounds has been reported to enhance tumor cell death and to prolong survival of patients with brain cancer. Gliadel (carmustine wafer), a biodegradable polymer containing the alkylating agent *bis*-chloroethyl-nitrosourea (BCNU), in combination with radiation, chemotherapy and surgery, has been indicated for high-grade malignant glioma [27]. On the other hand, pre-clinical results suggested that medicinal plants in combination with TMZ were useful for cancer treatment, including brain tumors [28].

Brain tumors can be problematic to treat because this type of tumors contains different types of cell populations, each of them responding in different way to treatment. In addition, as many other neoplasms, brain cancer acquires resistance to anti-neoplastic drugs. Combining different drugs and therapeutic approaches seems to be a promising way to treat patients that no longer respond to conventional treatment. For example, the procarbazine, CCNU and vincristine (PCV) combination has been used for more than 25 years to treat astrocytic tumors but the treatment efficacy is still questionable.

Our results showed that LHAE had a clear impact on brain tumor cell viability but the combination of LHAE with TMZ resulted in amplified cytotoxic effect, rather than, as was expected. Even if, combined treatment was more potent that the single treatment more data are needed to support the drugs combination effectiveness.

Glioblastomas and astrocytomas are more resistant to chemotherapy than other types of brain tumors such as medulloblastomas, oligodendrogliomas, primary cerebral lymphoma or germ-cell tumors. The development of new therapeutic agents against brain tumors, particularly astrocytic tumors, is necessary to continue. Our results, together with recent reports showing that *L. vulgare* extract efficiently killed human cervical and colon cancer cells [10], indicate that *L. vulgare* plant extracts may represent a new approach in cancer therapy. However, examination of *L. vulgare* extract on several tumor types is necessary to demonstrate the generality of the plant extract cytotoxic effect on malignant cells.

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## Disclosure of conflict of interest

None.

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