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ORIGINAL PAPER

CAN DANDELION'S PHENOLIC COMPOUNDS SUPPRESS GLIOBLASTOMA CELLS VIABILITY?

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Abstract

Introduction. Glioblastoma is one of the most aggressive malignant tumours of the central nervous system. Current therapies are not efficient enough to cure the disease. Thus, the identification of new substances with anti-glioblastoma potential, including those of natural origin such as *Taraxacum officinale*, is of utmost importance.
The objective of the studywas to evaluate the action of *Taraxacum officinale* FH Wigg (TO) extracts on glioblastoma cell line U-251 MG viability, correlated to total phenolic content (TPC) of the extracts.
Material and methods.TO leaf and root extracts were prepared with dimethyl sulfoxide (DMSO) and ethanol of different concentrations (20%, 50% and 80%). Glioblastoma U-251 MG cells viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT). The TPC was determined spectrophotometrically by Folin-Ciocalteu method.
Results. The highest antitumour activity (36.6±4.92%) was identified in case of TO leaves extracts made in ethanol of 50% (150,000 µg/L), as well as in case of

Résumé

Est-ce que les composés phénoliques du pissenlit peuvent supprimer la viabilité des cellules de glioblastome?

Introduction. Le glioblastome est l'une des tumeurs malignes les plus dangereuses du système nerveux central. Les thérapies actuelles n'offrent pas de remèdes qui traiteraient complètement la maladie. Ainsi, l'identification de nouvelles substances à potentiel anti-glioblastome, y compris celles d'origine naturelle comme *Taraxacum officinale*, est très importante.
L'objectif de l'étudeétait d'évaluer l'action des extraits de *Taraxacum officinale* FH Wigg (TO) sur la viabilité de la lignée cellulaire de glioblastome U-251 MG, corrélée au contenu phénolique total (TPC) des extraits.
Matériel et méthodes. Des extraits de feuilles et de racines de TO ont été préparés avec du diméthylsulfoxyde (DMSO) et de l'éthanol à différentes concentrations (20%, 50% et 80%). La viabilité des cellules de glioblastome U-251 MG a été évaluée par le test au

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leaves (44.98±5.21%) and roots (48.61±3.89%) extracts made with alcohol of 80% (respectively, 50,000 µg/L and 40,000 µg/L). The lowest antitumour activity (97.4±1.69%) was determined in case of roots extracts made with DMSO (1.86 µg/L). The TPC content varied from 2 to 38 mg TAE/mL. The maximum content of phenolic compounds was found in leaves extracted with ethanol. The increase of TPC content correlated with the decrease in the viability of tumour cells.

Conclusions. The extracts of *Taraxacum officinale* exhibit inhibitory effects on the viability of glioblastoma cells. The actions carried out depend on the type of extractant, its concentration, the part of the plant and the total content of phenols.

Keywords: *Taraxacum officinale*, glioblastoma, U-251 MG, MTT test, total phenolic content.

Abbreviations

TO = *Taraxacum officinale*
TPC = total phenolic content
CNS = central nervous system
DMSO = dimethyl sulfoxide
LDMSO = leaf extract with DMSO
RDMSO = root extract with DMSO
LEtOH = leaf extract with ethanol
REtOH = root extract with ethanol
Doxo = doxorubicin
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
FAK = focal adhesion kinase
TNF = tumour necrosis factor
ERK = extracellular signal-regulated kinase
MARPK = mitogen-activated protein kinase
DMEM = Dulbecco's Modified Eagle Medium
BSA = bovine serum albumin
ROS = reactive oxygen species
GRB2 = growth-factor receptor-bound protein-2
ERK1/2 = extracellular signal-regulated kinase 1/2
FAC = focal adhesion kinase
SRC = proto-oncogene tyrosine-protein kinase
DNA = deoxyribonucleic acid
PARP = poly (ADP-ribose) polymerases

bromure de 3-(4,5-diméthylthiazol-2-yl)-2,5-diphényltétrazolium (MTT). Le TPC a été déterminé par spectrophotométrie par la méthode de Folin-Ciocalteu.

Résultats. L'activité antitumorale la plus élevée (36.6 ± 4.92%) a été identifiée dans le cas des extraits de feuilles de TO fabriqués dans de l'éthanol à 50% (150,000 µg/L), ainsi que dans le cas des feuilles (44.98±5.21%) et des racines (48.61±3.89%) extraits à base d'alcool à 80% (respectivement 50,000 µg/L et 40,000 µg/L). L'activité antitumorale la plus faible (97.4±1.69%) a été déterminée dans le cas d'extraits de racines préparés avec du DMSO (1.86 µg/L). La teneur en TPC variait de 2 à 38 mg TAE/mL. La teneur maximale en composés phénoliques a été trouvée dans les feuilles extraites à l'éthanol. L'augmentation de la teneur en TPC est corrélée à la diminution de la viabilité des cellules tumorales.

Conclusions. Les extraits de *Taraxacum officinale* présentent un effet inhibiteur sur la viabilité des cellules de glioblastome. Les actions menées dépendent du type d'extractant, de sa concentration, de la partie de la plante et de la teneur totale en phénols.

Mots-clés: *Taraxacum officinale*, glioblastome, U-251 MG, test MTT, contenu phénolique total.

Introduction

The tumours of the central nervous system (CNS) are associated with significant morbidity and mortality. During 1975 – 2015, the five-year relative survival of patients with malignant brain tumours increased from 23% to 36%, excepting patients with glioblastoma, in whom this index increased only from 4% to 7%[1]. Glioblastoma is an aggressive type of neoplasm, which originates from astrocytes and accounts for 49% of all malignant tumours of the CNS. The actual treatment of glioblastoma includes surgical resection with simultaneous radiation and alkylation chemotherapy. However, almost all patients experience tumour progression with a median survival of less than 15 months[2].

Taraxacum officinale FH Wigg (TO) or Dandelion is well-known in traditional medicine for its therapeutic properties in diabetes mellitus type 2, blisters, spleen, and liver diseases. The chemical compounds

from TO are responsible for the antitumour activity of the plant[3]. It has been reported that the plant is rich in triterpenes, flavonoids, polyphenols, including cinnamic acid derivatives. There are many mechanisms by which TO exerts its antitumour action: inhibition of tumour cell proliferation, blocking the gelatinolytic activity of matrix metalloproteinase (MMP)-2 and MMP-9 through inhibition of focal adhesion kinase (FAK) and src (p-scr) signal transducers, inducing apoptosis through secretion of tumour necrosis factor (TNF) and interleukin-1 (IL-1), inhibition of extracellular signal-regulated kinase (ERK) activity, which is a major determinant of the mitogen-activated protein kinase (MARPK) pathway involved in cell survival, differentiation and cell growth[4]. Although there are many evidences about its antitumour effectiveness in different neoplasms, like breast and colorectal cancers, melanoma, leukaemia, there are few studies on the effect of TO on glial tumours[5-7].

The objective of the study was to evaluate the action of TO extracts on glioblastoma cell line U-251 MG viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and its correlation to total phenolic content (TPC) in the extracts.

Material and methods

Ethical issues. This study was approved by the Research Ethics Committee of the Nicolae Testemitanu State University of Medicine and Pharmacy, Chisinau, Republic of Moldova (approval no 3/ 11.02.2020).

Plant material and extracts preparation. The TO leaves and roots were harvested before flowering in May 2017 from a natural habitat of Republic of Moldova (47^0 4' 8" North, 28^0 40' 47" East). The raw material was desiccated in the laboratory at room temperature for two weeks and pulverized. The extracts were prepared by using ethanol (Luxfarmol, MD) of different concentration (20, 50 and 80%) and 0.1% DMSO (Merck, DE). The ratio biomass-to-solvent (mg/mL) was 10:1. The extraction was done for 24 hours. All extracts were filtered (Whatman nr.5, WHA1005090, Merck, DE) and stored at $+4^0$C. Aliquots of 200 µL were filtered again (Whatman nr.1, WHA10010155, Merck, DE), then filters were dried and weighted (RADWAG PS210/C/2, PL) for dried mass content calculation.

Cell culture and MTT test. The viability of human glioblastoma cell line U-251 MG (Cell Lines Service, DE) was evaluated by colorimetric MTT test. The experiment was performed according to Mosmann's method, which is based on succinate dehydrogenase activity of metabolically active mitochondria[8].

The glioblastoma cells were cultured in 5% CO_2 incubator conditions (Sanyo, JP). The Dulbecco's Modified Eagle Medium (DMEM) (Merck, DE) was supplemented with 4.5 g/L glucose, 2 mM glutamine, 10% bovine serum albumin (BSA) (Invitrogen, USA). The number of living cells was assessed by trypan blue 0.4% (Gibco, USA). The cells in logarithmic growth phase were seeded in 96-well plates ($5x10^3$/100 µL per well). After 3 hours of incubation (37°C, 5% CO_2), supernatant (90 µL) from each well was replaced with the same amount of tested compounds, followed by 24 hrs incubation. The clean extractant of the same concentration as that in tested group was added to the control wells. After incubation, 10 µL of MTT reagent (ATCC, USA) were added to each well and the plates were returned to the incubator. Periodically, the presence of the purple formazan precipitate was checked by microscopy (OLYMPUS CK40, JP). The detergent reagent (100 µL, ATCC, USA) was added to all wells when appeared a clearly visible precipitate. The covered plates were left in the dark for 4 hrs at room temperature. The optical absorbance was measured at 540 nm (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, USA BioTek, USA).

The TO action was compared to Doxorubicin (Doxo) solutions of different concentrations (1.28 – 10^5 µg/L). The results were reported as percent of viable cells which survived after incubation with the tested compounds. We applied the formula deduced by taking in consideration Karakas *et al.* recommendation[9].

$$\text{Viability (\%)} = \frac{OD_{specimen} - OD_{Blank}}{OD_{control} - OD_{Blank}} \times 100$$

where: $OD_{specimen}$ represents the optical density of tested specimens (cells + MTT + tested compounds); $OD_{control}$ is the optical density of control (cells + MTT + extractant); OD_{Blank} is the optical density of Blank (extractant of the same concentration).

Total phenolic content (TPC) assay. The TPC was determined spectrophotometrically at 765 nm (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek Instruments, USA) by Folin-Ciocalteu method, following the procedure described by Arabshahi-Delouee et al[10]. The method is based on the reduction by phenols of phosphotungstate-phosphomolybdate complex to blue reaction products. The concentration of TPC in the extracts was calculated using tannic acid equivalent (TAE). The standard tannic acid (95% purity, Thermo Scientific, USA) curve was prepared. The regression equation of the calibration

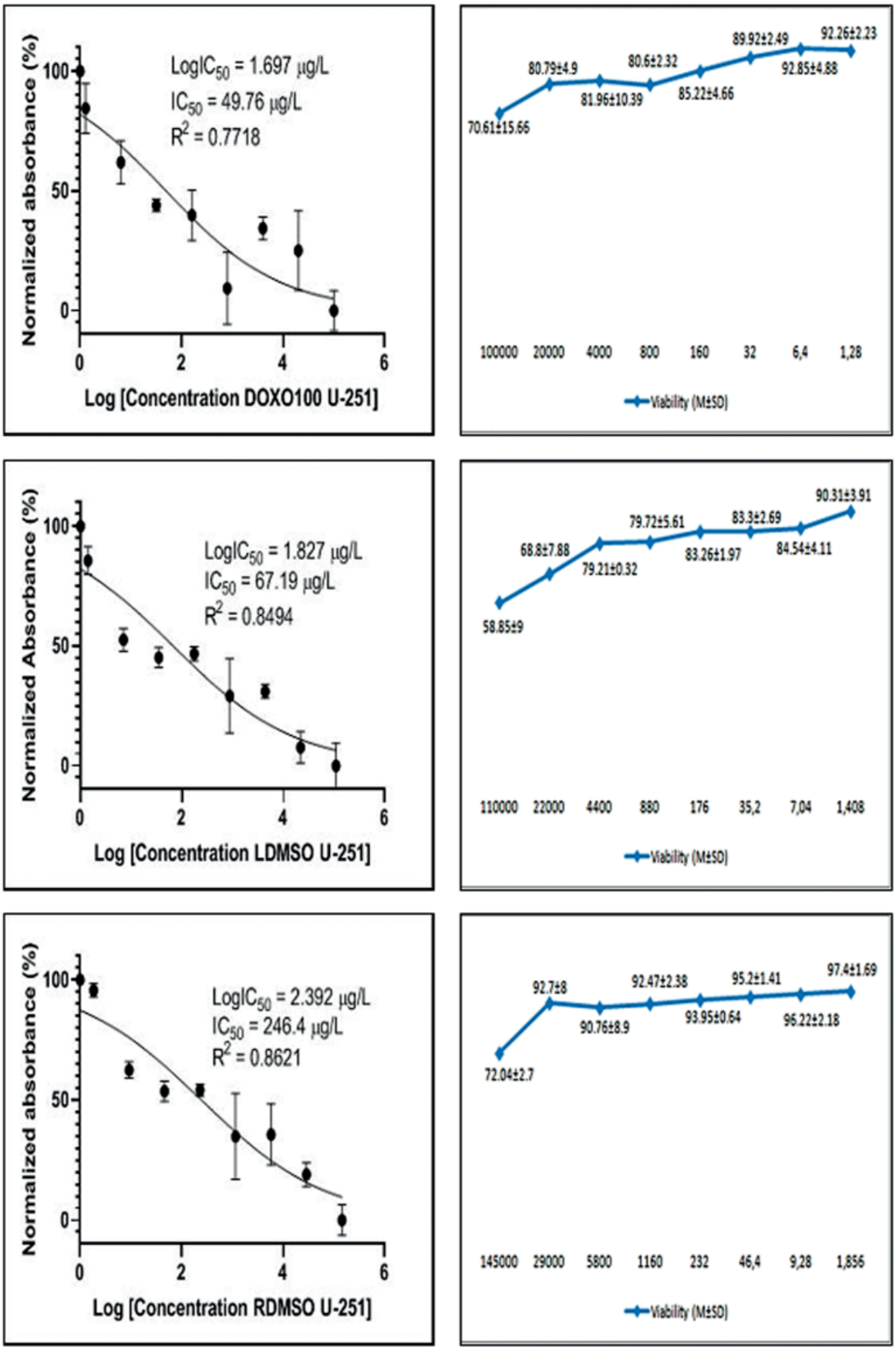


Figure 1. The effect of Doxo and DMSO extracts of TO leaves and roots on glioblastoma U-251 MG cells. Viability (M±SD) was represented in %.

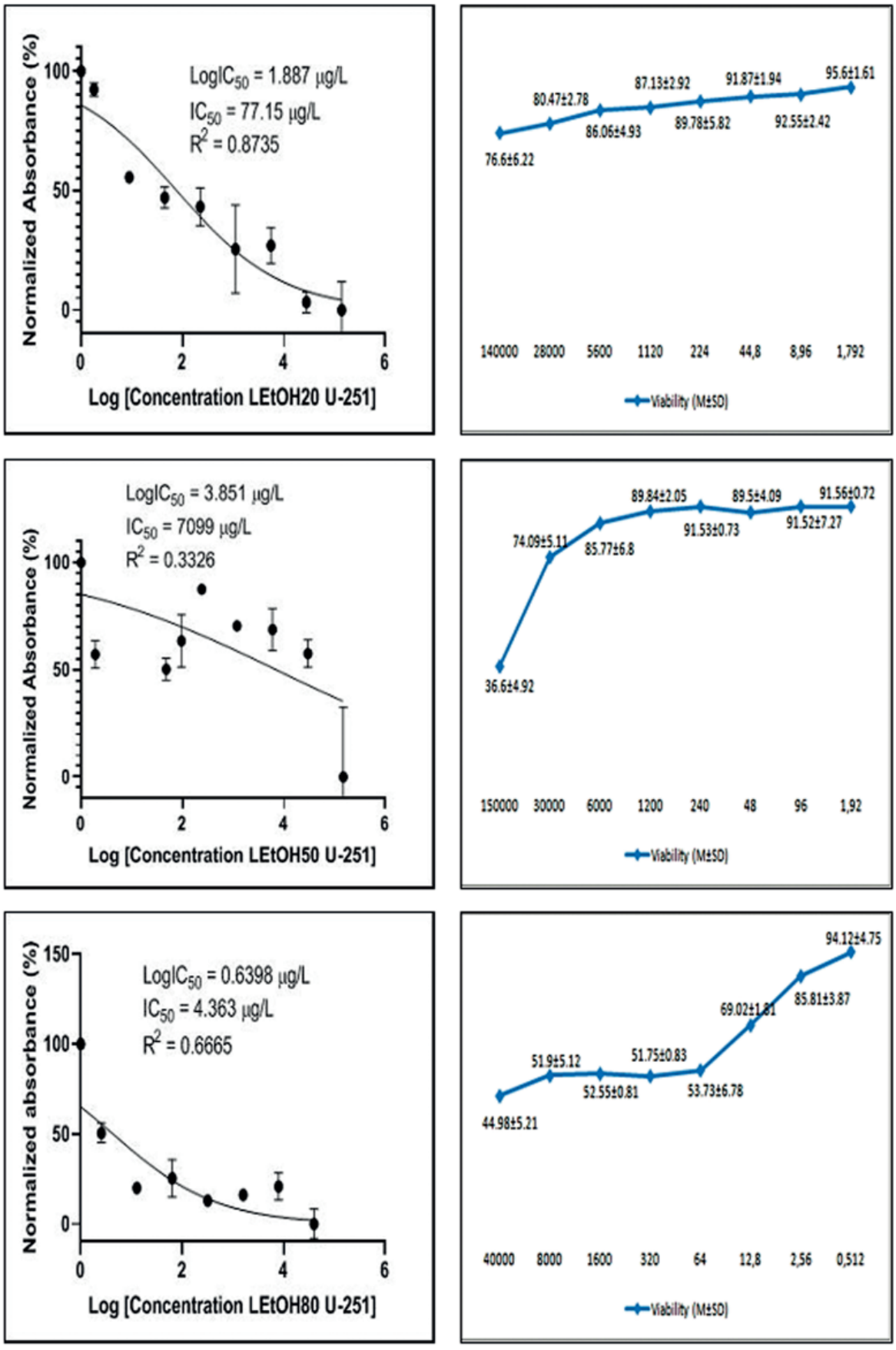


Figure 2. The effect of ethanolic extracts of TO leaves on glioblastoma U-251 MG cells. Viability (M±SD) was represented in %.

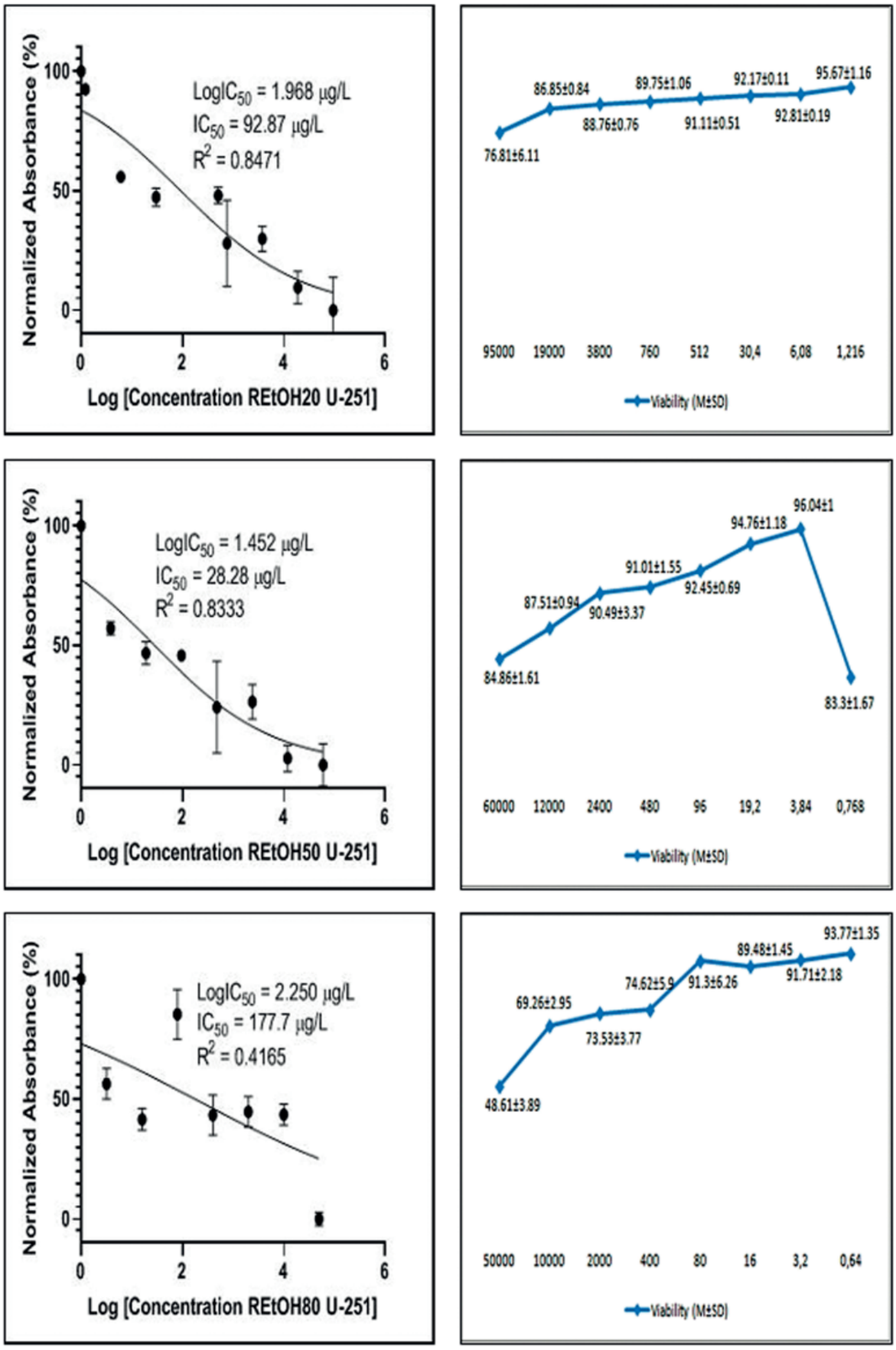


Figure 3. The effect of ethanolic extracts of TO roots on glioblastoma U-251 MG cells. Viability (M±SD) was represented in %.

curve was y = 3.9712x – 0.1725, where y is the absorbance and x – the concentration, mg TAE/mL. Phenols calibration curve showed a good linearity (R^2=0.9918).

Statistics. All assays were done in triplicate. The GraphPad Prism & Software (USA) was used to calculate the IC_{50} (the half maximal inhibitory concentration). The mean and standard deviation (M±SD) were used to describe cells viability. The correlation between tested compounds concentration, ethanol percentage, TPC content and cells viability was evaluated by Spearman (r_s) assessment. The non-parametric Mann-Whitney U-test was used to test the significance of the differences between the values in the compared groups. The p-values equal or less than 0.05 were considered statistically significant.

Results

All tested extracts inhibited the viability of U-251 MG cells (Figures 1 – 3).

In case of Doxo, the best suppression of tumour cells (70.61±15.66%) was determined in extracts with the highest concentration – 10^5 µg/L (Figure 1). The decrease of the concentration significantly correlated with the increase of cells viability (r_s=-0.67, p=0.001).

The same effect was identified in case of all TO extracts: more dilute solutions had lower inhibitory activity against glioblastoma cells. We used the term inhibitory activity rather than cytotoxicity, since the mitochondria of destroyed cells could retain their viability in the extracellular environment for some time. In addition, we did not count the number of cells at the end of the experiment.

The leaves DMSO (LDMSO) extracts inhibited cells viability better than roots extracts (RDMSO), even at lower concentration: LDMSO – 58.85±9% at 110,000 µg/L *vs* RDMSO – 72.04±2.7% at 145,000 µg/L (p=0.0001). The more powerful effect of leaves is sustained by 3.7 times lower IC_{50} value (p=0.0001) in case of LDMSO (67.19 µg/L) in comparison to RDMSO (246.4 µg/L).

In case of ethanolic leaves extracts (LEtOH), the best inhibitory effects were specific for LEtOH50 of 150,000 µg/L (36.6±4.92%) and LEtOH80 of 40,000 µg/L (44.98±5.21%) (Figure 2). The leaves extracted with 20% of ethanol, even at higher concentration of 140,000 µg/L, had a lower activity (76.6±6.22%). The increase of ethanol concentration in leaves extracts had a moderate, negative correlation with the cells viability (r_s=-0.51, p=0.001).

The best inhibitory activity was exhibited by roots ethanolic extracts (REtOH) with the highest concentration of alcohol, of 80% (50,000 µg/L – 48.61±3.89%) (Figure 3). However, in comparison to other roots extracts, the IC_{50} value was higher in case of REtOH50 (177.7 µg/L *vs* 28.3 µg/L) and of REtOH20 (92.9 µg/L *vs* 28.3 µg/L). In case of the roots, the extractant concentration had a very low impact on U-251 MG cells viability (r_s=-0.30, p=0.004).

A general comparative analysis of the effectiveness of all extracts led to the conclusion that the best antitumour activity was shown by leaf extracts in 50% ethanol of 150,000 µg/L (36.6±4.92%), which was statistically better (p=0.0004) than the one exhibited by the extracts of leaves of 40,000 µg/l (44.98±5.21%) and roots of 50,000 µg/l (48.61±3.89%) in alcohol 80%. The Doxo's best antitumour activity, attested at its highest concentration of 10^5 µg/L, was in the 12th place, behind a series of TO extracts. The lowest antitumour activity (97.4±1.69%) was determined in case of roots extracts made with DMSO (1.86 µg/L) (Figure 1).

According to our results, the highest values of TPC content were established in the leaves ethanolic extracts (Table 1).

In all cases, negative statistically significant, strong correlations were established between the content of TPC and the viability of tumour cells.

Discussion

Glioblastoma, also known as glioblastoma multiform, is an aggressive type of neoplasm of CNS, which because of its specific localization and the existence of natural barriers have limited possibilities of treatment. Therefore, studies of potential antiglioblastoma drugs, including compounds of natural origin (plant, animal, insect, etc.), are very important.

TO contains more than 100 bioactive components which exhibit antioxidant, anti-inflammatory, hepatoprotective, antidiabetic activities etc[4]. There are many evidences about its effectiveness in case of different type of tumours, like breast, prostate and colorectal cancers, leukaemia, melanoma etc[11,12]. Ovesna *et al.* reported that components of TO exhibit chemopreventive and chemoprotective effects[13].

The first evidence on the effectiveness of TO on glial cells were obtained by Kim et al (2000), who communicated that TO may inhibit TNF-α production, by inhibiting IL-1 production in astrocytes[14]. This effect was related to its anti-inflammatory activity. Until now, there are very few data about TO efficacy in case of astrocytes derived tumors.

Previous research aimed to establish the mechanisms of TO antitumour activity, identified that they are in the strong connection with plant's biochemical content. Lupeol disrupts the assembly of actin fibers, apigenin downregulates PI3K/Akt/NF-κB signaling[15], taraxinic acid has anti-proliferative activity[16], suppresses the reactive oxygen species, nitric oxide and prevents lipids peroxidation (attributed to the

Table 1. Correlation of the TPC content in the TO extracts with the tumour U-251 MG cells viability.

TO		*TPC*	*M*	r_s	*TO*		*TPC*	*M*	r_s
LDMSO	110,000	13.88	58.85	r_s=-1 p=0.000	RDMSO	145,000	4.04	72.04	r_s=-1 p=0.000
	22,000	6.94	68.80			29,000	2.02	92.70	
	4,400	3.47	79.21			5,800	1.01	90.76	
	880	1.74	79.72			1,160	0.51	92.47	
	176	0.87	83.26			232	0.25	93.95	
	35.2	0.43	83.30			46.4	0.13	95.20	
	7.04	0.22	84.54			9.28	0.06	96.22	
	1.408	0.11	90.31			1.856	0.03	97.40	
LEtOH20	140,000	31.4	76.60	r_s=-1 p=0.000	REtOH20	95,000	2	76.81	r_s=-1 p=0.000
	28,000	15.7	80.47			19,000	1	86.85	
	5,600	7.85	86.06			3,800	0.5	88.76	
	1120	3.93	87.13			760	0.25	89.75	
	224	1.96	89.78			512	0.125	91.11	
	44.8	0.98	91.87			30.4	0.06	92.17	
	8.96	0.49	92.55			6.08	0.03	92.81	
	1.792	0.25	95.60			1.216	0.015	95.67	
LEtOH50	150,000	38	36.60	r_s=-0.88 p=0.002	REtOH50	60,000	4.86	84.86	r_s=-0.88 p=0.002
	30,000	19	74.09			12,000	2.43	87.51	
	6,000	9.5	85.77			2,400	1.22	90.49	
	1,200	4.75	89.84			480	0.61	91.01	
	240	2.38	91.53			96	0.303	92.45	
	48	1.19	89.50			19.2	0.15	94.76	
	96	0.59	91.52			3.84	0.075	96.04	
	1.92	0.3	91.56			0.768	0.037	83.30	
LEtOH80	40,000	11.52	44.98	r_s=-0.93 p=0.000	REtOH80	50,000	8.3	48.61	r_s=-0.93 p=0.000
	8,000	5.76	51.90			10,000	4.15	69.26	
	1,600	2.88	52.55			2,000	2.08	73.53	
	320	1.44	51.75			400	1.04	74.62	
	64	0.72	53.73			80	0.52	91.30	
	12.8	0.36	69.02			16	0.26	89.48	
	2.56	0.18	85.81			3.2	0.13	91.71	
	0.512	0.09	94.12			0.64	0.065	93.77	

Note: TO – *Taraxacum officinale* content (μg/L) and type of extract; TPC – total phenolic content (mg TAE/mL); M – mean of cells viability (%); r_s –Spearman correlation coefficient; p – statistical significance.

presence of flavonoids)[17], anti-angiogenic activity was assigned to flavones[18(p20)], etc. However, there are effects of TO for which the compound responsible for this action has not been identified with certainty. Among them there are the decrease of the mitochondrial membrane potential, increase of reactive oxygen species (ROS) levels in the mitochondria, activation of caspase-8, decrease of nuclear levels of growth-factor receptor-bound protein-2 (GRB-2), extracellular signal-regulated kinase 1/2 (ERK1/2) and increase of cytoplasmic ERK1/2, down-regulation of pro-survival genes (BCL2, GALNT5) and up-regulation of CASP1, IFNG, TNF[11], apoptosis inducing[5], diminishing of focal adhesion kinase (FAC), proto-oncogene tyrosine-protein kinase (SRC) and inhibition of MMP-2, MMP-9 activity[19], Ca^{2+} outflow from endoplasmic reticulum and its inflow in plasmalema[20].

Polyphenols are organic compounds found abundantly in plants. They demonstrated effectiveness in the treatment of diabetes, neurodegenerative and cardiovascular diseases, as well as tumours. Schütz et al. (2004) described in TO about 43 compounds,

related to phenolic acids and flavonoids[21]. According to the data summarized by González et al. (2012), TO contains about thirteen benzenoids (hydroxycinnamic acid, dicaffeoyltartaric acid and their derivates, caffeoylquinic acid, chlorogenic acid, chicoric acid etc.) and various flavonoids such as luteolin, isorhamnetin, apigenin, and quercetin derivates[3]. Hagymási et al. (2000) reported a 3-fold higher content of polyphenols in the water extract from the leaves (9.9g%) than from the roots, which may underline the higher efficiency of TO leaves as a hydrogen donor (I_{50}= 160 µg), reducing agent (740 ASE mg^{-1}) and H_2O_2 scavenger (I_{50}=155 µg) compared to root extracts[22]. Our results confirm the higher content of polyphenols in the leaves, which was about 4.6 (p=0.007) times higher than in the roots.

The content of polyphenols seems to be dependent of plant's part. González et al. established that in case of roots the most abundant phenolic compound is chicoric acid and in case of leaves – hydroxycinnamic acid derivatives, particularly caffeic acid esters such as chlorogenic acid, dicaffeoyltartaric (chicoric) acid and monocaffeoyltartaric acid[3]. Moreover, the antitumour mechanisms of action of these compounds are different. Chlorogenic acid induces differentiation of cells through an increase of KHSRP, p53, p21, a decrease of poor differentiation-related genes c-Myc, CD44 and downregulation of oncogenic miRNA-17 family members in cancer cell lines[23], while chicoric acid increases deoxyribonucleic acid (DNA) fragmentation, cleavage of caspase-9 and poly (ADP-ribose) polymerases (PARP)[24]. Considering these differences in the content and mechanisms of action of individual polyphenols, we can assume that this factor determined the difference in the efficiency of the action on the viability of glioblastoma cells of the extracts of leaves and roots of TO studied by us.

Several previous studies have shown that the biomedical effects of plants depend on the form in which they were used – raw plants or different extracts. The selection of solvent depends on the specific nature of the bioactive compound being targeted[25]. The extraction of hydrophilic compounds requires polar solvents such as methanol, ethanol, while for the extraction of lipophilic compounds – solvents such as DMSO, dichloromethane or combinations such as dichloromethane and methanol. The role of solvent type in the quantity and quality of crude extracts, secondary metabolites, as well biological activities has been previously reported[26]. Thus, it can be expected that the content of the extracts, as well as the antitumour activity of DMSO and TO ethanol extracts, will differ. In the present study, it was shown that TPC content depends on the type of extractant, the best results in the extraction of phenols were obtained using ethanol. Moreover, an increase in the concentration of ethanol leads to an improvement in the extraction of TPC.

Data from our previous research on another glioma cell line (U-138 MG), revealed that TO roots extracts in 80% ethanol exhibited the greatest inhibitory action, which was higher than that of Doxo[27]. The extracts with higher concentration denoted stronger effects, as well as ethanol extracts were better inhibitors than DMSO ones. The present study data sustain the previous conclusions that TO tumour cell viability inhibition depends on extractant type and concentration, but also on the plant part. Moreover, the type of target cells seems to be important, too. In case of U-138 MG cells the IC_{50} of REtOH80 was 417.4 µg/L, while for the U-251 MG cells the half maximal inhibitory concentration of the same extract was lower, 177.7 µg/L.

The present study has some limitations. This research could have a bigger impact if every kind of extract could be sustained by its full, not only TPC, chemical composition, because the effects of TO are related not only to the nature of extractant and its concentration, but also to individual substances present in the extract and their ratio. Further investigations are required to explore the presence and quantity of individual compounds of the TO extracts and their correlation with the inhibitory effect on tumour cells. Another limitation is related to the assumption that alcohol itself, especially of high concentration, could block the cells viability. McDonald et al (2013) reported that alcohol has rather a pro-tumour than antitumour activity, at least in case of breast cancer[28]. In our study, ethanol solutions with the same concentrations were used in the control group to eliminate the effect of ethanol.

Conclusions

TO exhibits inhibitory action on viability of glioma cell line U-251 MG, which depends on the type of extractant, its concentration, plants' part and TPC content. The leaves and roots extracts made in ethanol of high concentration had the most promising antitumour activity. Further investigations are required to establish the composition of the TO extracts, as well as the most efficient chemicals combination in tumour cell viability inhibition.

Author Contributions:

Conceptualization, V.G., M.T. and O.T.; methodology, V.G. and A.F.; software, A.F.; validation, V.G. and O.T.; formal analysis, V.G. and A.F.; investigation, V.G. and A.F.; resources, V.G. and O.T.; data curation, V.G. and O.T.; writing–original draft preparation, A.F.; writing–review

and editing, V.G. and O.T.; visualization, A.F.; supervision, V.G., M.T. and O.T.; project administration, V.G., M.T. and O.T.; funding acquisition, V.G., M.T. and O.T. All authors have read and agreed to the published version of the manuscript.

Compliance with Ethics Requirements:

"The authors declare no conflict of interest regarding this article"

"The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law."

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