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Taraxacum officinale Leaves and Roots Suppress Glioma Cell Viability

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Abstract

Background: In spite of the progress that has been made towards development of many drugs, the accompanying toxicities and side effects sustain that further studies are required to reduce the incidence of tumor-related deaths. *Taraxacum officinale* (TO) known else as Dandelion, exerts many promising activities, like antioxidant, anti-inflammatory, antitumor etc. It demonstrated its effectiveness in case of many types of tumors, through different mechanisms of cytotoxicity. **The aim** of the present study was to determine the TO leaves and roots extracts influence on tumor glioblastoma cells line U-138 viability. **Material and Methods:** TO dry leaves and roots were extracted by DMSO, ethanol, and water. U-138 MG (Cell Lines Service) glioblastoma cells viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny-ltetrazolium bromide test. TO's influence was compared with Doxorubicin activity. **Results:** All TO extracts suppressed the tumor cells viability. The greatest inhibitory influence on glial cells viability was shown by TO roots extracts made in 80% ethanol (50,000 μg/L - 8.49 ± 1.04) and Doxorubicin (54350 μg/L - 8.64 ± 1.65). The lowest influence on astrocytes was determined in case of Doxorubicin at concentration of 1.28 μg/L (96.6 ± 2.76). **Conclusions:** *Taraxacum officinale* is a promising source of many substances with multiple benefits for humans. The exercised actions depend on type of extractant and plants' part. The greatest influence on glial tumors had the extract of roots made in ethanol of 80%. Further investigations are needed to explore the precise composition of the TO extracts, the importance of individual components and of their combination in neoplasms treatment and/or prevention.

Keywords

Taraxacum officinale, Glioblastoma, Extractant Type, MTT Test

1. Introduction

Glioblastomas are the leading primary tumors of the brain. The treatment of these kinds of neoplasms is very limited, because of their chemo-resistance, due to specific localization and the existence of natural barriers. These kinds of tumors develop different genetic subtypes, which respond differently to aggressive therapies, making treatment extremely difficult and challenging.

Taraxacum officinale (TO) commonly known as Dandelion, is considered nowadays as a garden weed, but it has been long used in traditional medicine as a remedy in case of pyrosis, liver, kidney and skin diseases. It contains more than 100 of bioactive components, which are under continuous research, due to antioxidant, anti-inflammatory, antiangiogenic, antinociceptive actions [1]. Different studies describe the TO functions in relation to specific substances from their composition. The antioxidant activity was attributed to luteolin and luteolin 7-glucoside [2]. Lupeol and taraxasterol can inhibit tumors initiation and promotion, that's why they are purposed as chemopreventive agents [3]. There are many literature evidences about TO effectiveness in case of different tumors, like melanoma, breast and colorectal cancers etc. [4] [5] [6] [7].

Regardless of the traditional usage of TO as an antitumor remedy, there are no biochemical studies of TO influence on astrocytes tumor cell lines. A single literature evidence of TO effectiveness in case of primary cultures of rat astrocytes was provided by Kim *et al.* (2000), whose results suggested that TO may inhibit TNF-α production, by inhibiting IL-1 production. This action was related to its anti-inflammatory activity [8].

In the present study we aimed to evaluate the antitumor effectiveness of different TO extracts by testing them on a human glioblastoma cell line. Our results describe TO efficiency in case of glial tumors, which depends on TO extract concentration, plant's part and type of extractant.

2. Material and Methods

2.1. Plant Material

The experiment was done with extracts prepared from leaves and roots of *Taraxacum officinale F. H. Wigg*. Plants were harvested in May 2017 from a natural habitat of Republic of Moldova (47˚4'8" North, 28˚40'47" East), just before flowering. The raw material (separated leaves and roots) was desiccated in the laboratory at room temperature for 2 weeks and pulverized using the mortar and pestle.

2.2. Preparation of the Extracts

A series of ethanolic (Luxfarmol) (20%, 50% and 80%) and DMSO (<0.1%, SigmaAldrich, nr.240060103) extracts were prepared. The water extracts were prepared as infusion with clean hot (100˚C) water (Adrona Cristal). The ratio biomass-to-solvent (mg/mL) was 10:1. The extraction was done in recipients of 100 ml for 24 hours. All extracts were filtered (Whatman nr.5, WHA1005090) and

stored at +4˚C. Aliquots of 1.5 ml of each extract were centrifuged (MPW 370, 5000 rpm, 5 min). The absence of stratification or sedimentation confirmed the samples purity.

For calculation of dry mass content, 200 µL of extracts were filtered again (Whatman nr.1, WHA10010155), then filters were dried and weighted (RADWAG PS210/C/2).

2.3. Cell Culture and MTT Test

Human glioblastoma cell line U-138 MG (Cell Lines Service) viability was evaluated by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. The experiment was performed according to method described by Mosmann, which is based on succinate dehydrogenase activity, from metabolically active mitochondria [9].

The cells were cultured in DMEM medium (Sigma) supplemented with 4.5 g/L glucose, 2 mM glutamine, 10% BSA (Invitrogen) and 5% CO_2 incubator conditions (Sanyo). Before the MTT test, cells viability was assessed with trypan blue 0.4% (Gibco^{TM}).

The cells in logarithmic growth phase were seeded in 96-well plates (5×10^3/100 µL per well). After 3 hours of incubation (37˚C, 5% CO_2), 90 µL of supernatant from every well was replaced with the same amount of tested compounds, procedures followed by 24 h incubation. 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) was added to each well. The plates were returned to incubator, periodically checking in microscope (OLYMPUS CK40) the presence of purple precipitate. When precipitate was clearly visible, 100 µL of detergent reagent (ATCC) was added to all wells and covered plates were left in dark conditions for 4 h at room temperature. The optical absorbance was measured at 540 nm (Synergy microplate reader, BioTek).

The TO action on glioblastoma cells viability was compared to Doxorubicin (the Food and Drug Administration approved antitumor drug) solutions of different concentrations.

The results were reported as percent of viable cells which survived after incubation with tested compounds. Taking into consideration Karakas *et al.* (2017) recommendation, we deduced the formula [10].

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

where,

$OD_{specimen}$—the optical density of tested specimens (cells + MTT + tested compounds);

$OD_{control}$—the optical density of control (cells + MTT + extractant);

OD_{Blank}—the optical density of Blank (extractant of the same concentration).

We did not use the term of cytotoxicity, because mitochondria of destroyed cells can preserve their viability for a while in the extracellular medium. All as-

says were done in triplicate.

2.4. Statistical Analysis

The half maximal inhibitory concentration (IC_{50}) of tested compounds on cells viability was calculated by GraphPad Prism 8 Software (San Diego, USA). Cells viability was described as mean and standard deviation (M ± SD). The Spearman assessment (r_s) was used to determine the existence of correlation between tested compounds concentration, ethanol percentage and cells viability. The most promising results were compared by Mann-Whitney U-test. The p-values equal or less than 0.05 were considered statistically significant.

This study was approved by the Research Ethics Committee of the *Nicolae Testemitanu* State University of Medicine and Pharmacy from Republic of Moldova.

3. Results

All tested substances could suppress cells viability (**Figures 1-4**).

The greatest inhibitory activity was determined in samples with highest Doxorubicin concentration. The dosage of 54,350 μg/L (8.64 ± 1.65) exhibited a better activity than 100,000 μg/L (15.32 ± 0.86) on U-138 MG cells (p = 0.01). The decrease of Doxo concentration led to increase of tumor cells survival, in case of Doxo100 as well as in case of Doxo54 (r_s = −0.98, p = 0.0001) (**Figure 1**). Leaves DMSO extracts inhibited cells viability better than roots DMSO extracts, even at lower concentration: LDMSO—110,000 μg/L (17.34 ± 8.02) *vs* RDMSO—145,000 μg/L (18.57 ± 4.54) (**Figure 1**, **Figure 4**), but without statistical significance (p = 0.74). The increase of TO concentration led to decrease of cells viability, but *p*-values did not reach *a* statistically significant level in case of both extracts.

The roots ethanolic extracts of 20% diminished cells viability to 60.96 ± 29.12 at TO concentration of 95,000 μg/L (**Figure 2**). In case of roots extracted with ethanol of 50% greatest inhibitory action was determined at 60,000 μg/L (64.74 ± 23.95). A much higher grade of inhibition of glioblastoma cells was determined in case roots extracts made on ethanol of 80% with lower concentration of TO (50,000 μg/L - 8.49 ± 1.04). In all tested groups the increase of TO concentration led to decrease of tumor cells viability, but in case of ethanolic extracts of 50% values did not reach the statistically significant level (r_s = −0.33, p = 0.21).

Leaves extracts on 20% of ethanol suppressed tumor cells and the greatest inhibitory values, which corresponds to 38.37 ± 21.05 where determined in case of highest TO concentration (140,000 μg/L) (**Figure 3**). The decrease of tumor viability was correlated with the increase of TO concentration (r_s = −0.88, p = 0.002). The same changes were determined in case of ethanolic extracts of 50% (150,000 μg/L - 13.65 ± 3.2), as well as of 80%. In the last case, the same antitumor activity (40,000 μg/L - 16.12 ± 9.03; p = 0.74) was noticed at lower TO concentration than in previous extracts.

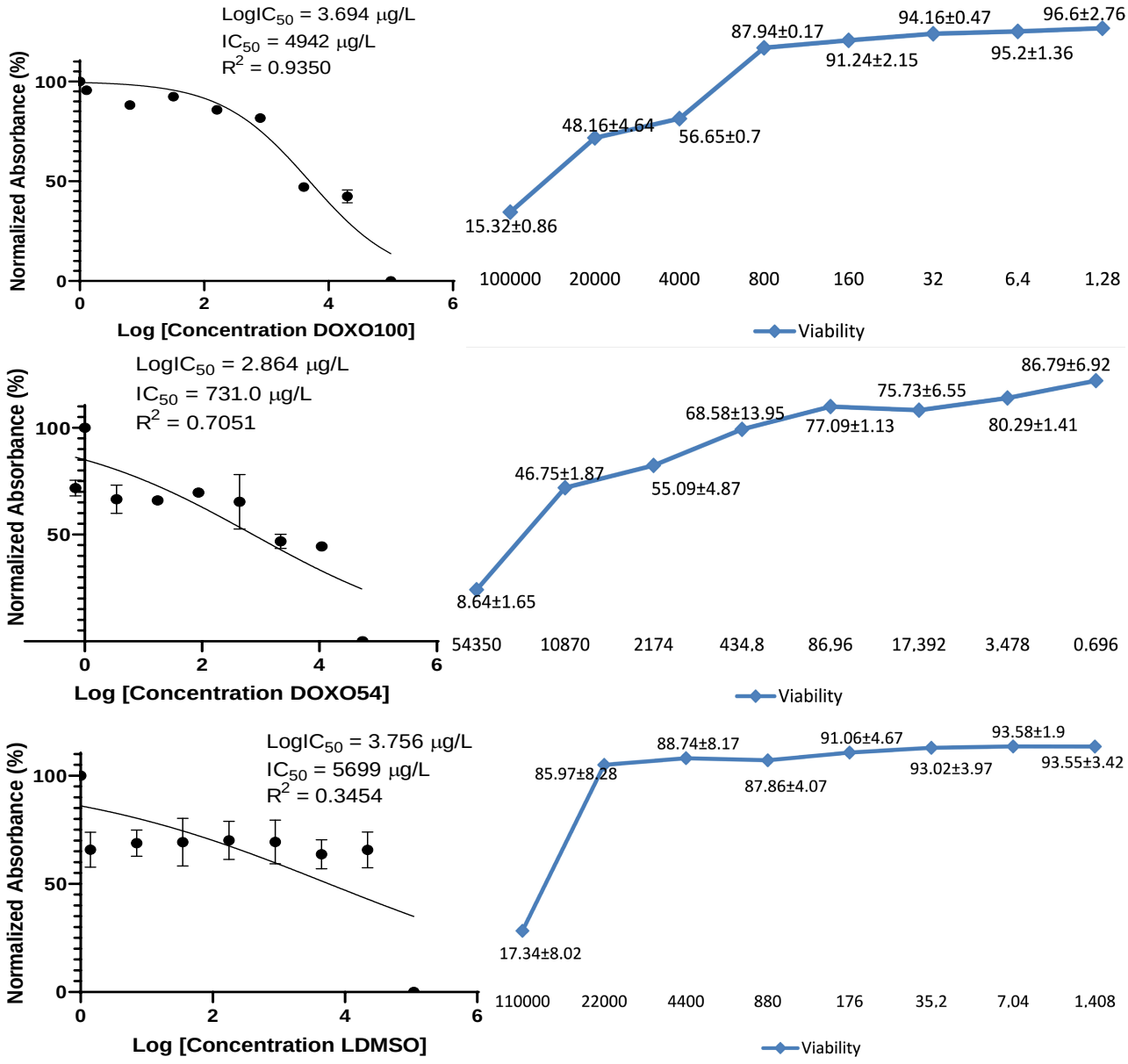
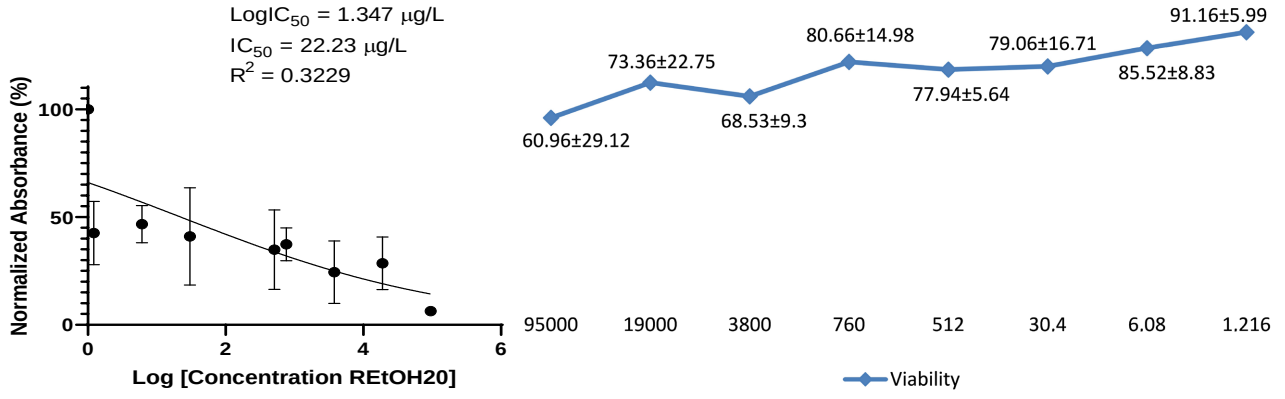


Figure 1. The effectiveness of Doxorubicin and leaves of TO extracted with DMSO (LDMSO) in inhibiting glioblastoma cells viability.



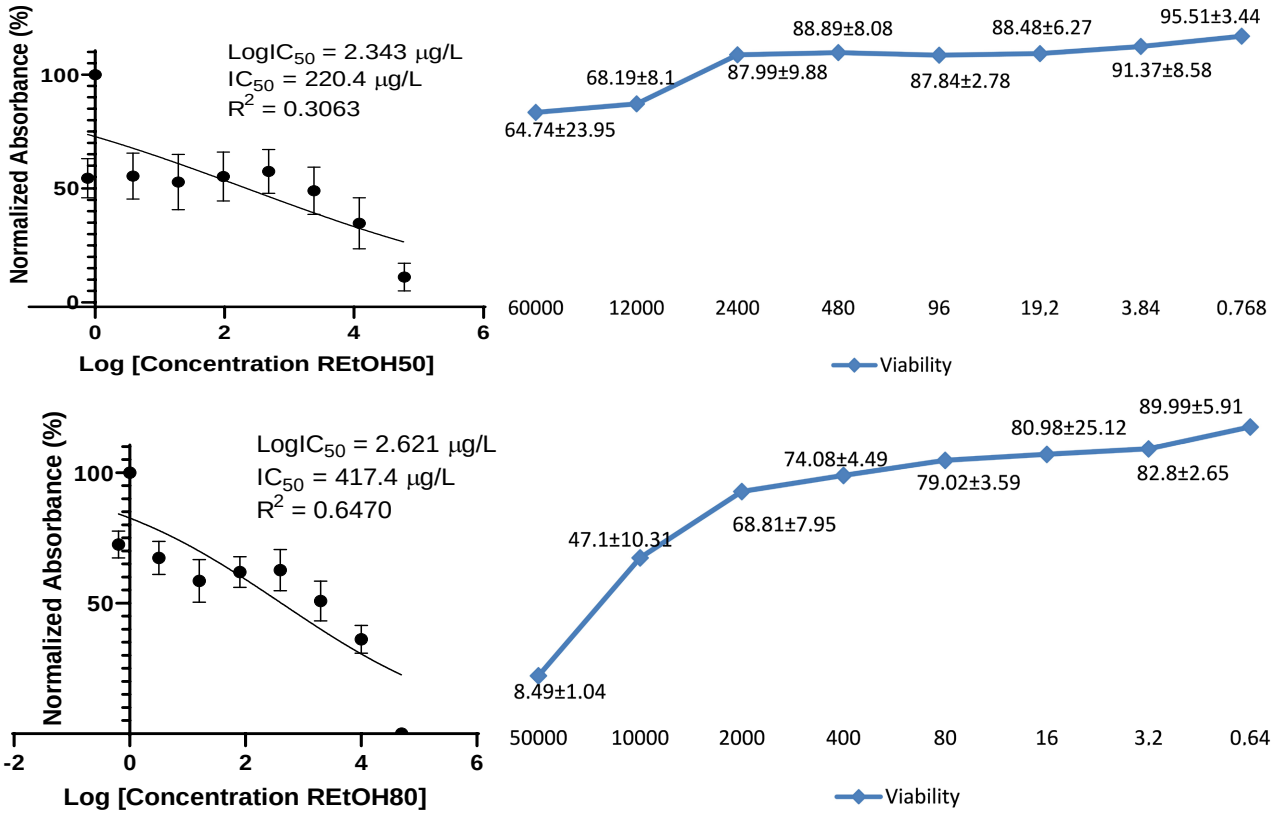
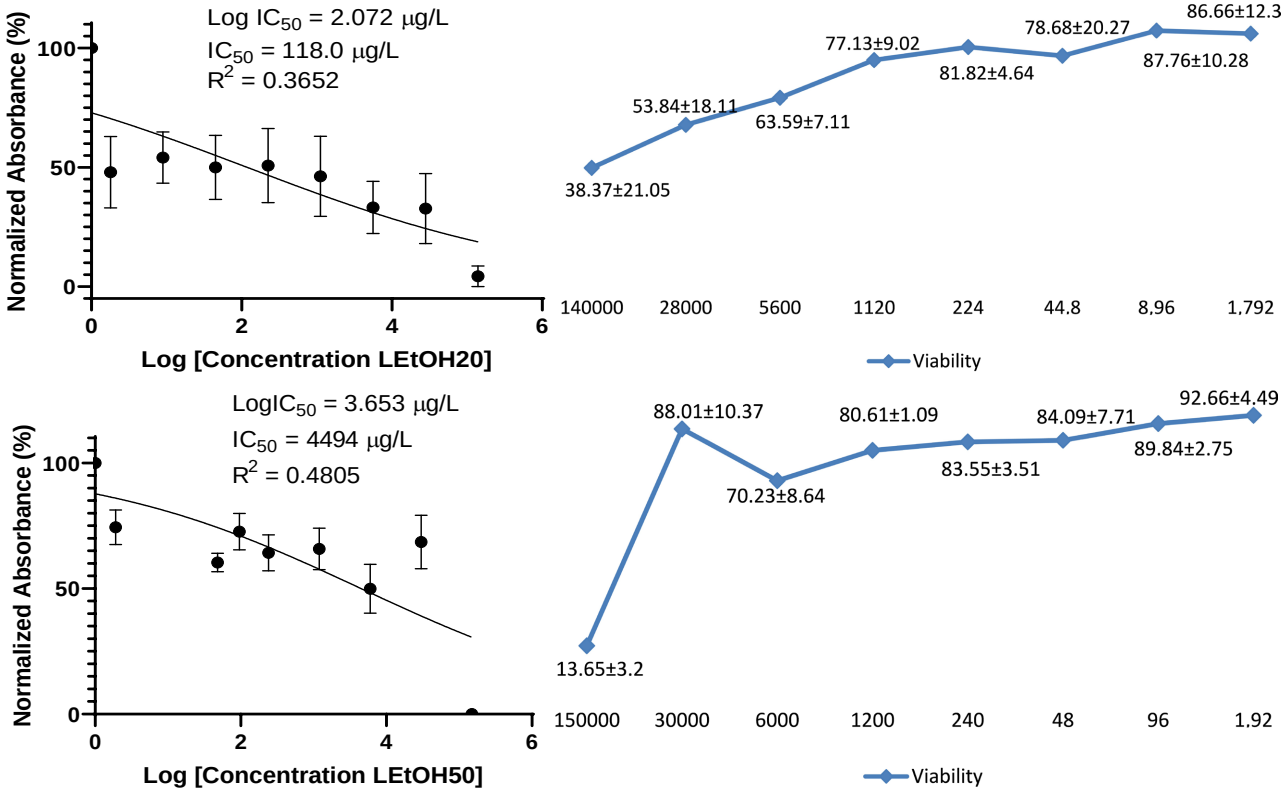


Figure 2. The effectiveness of roots of TO extracted with ethanol of 20% (REtOH20), 50% (REtOH50) and 80% (REtOH80) in inhibiting glioblastoma cells viability.



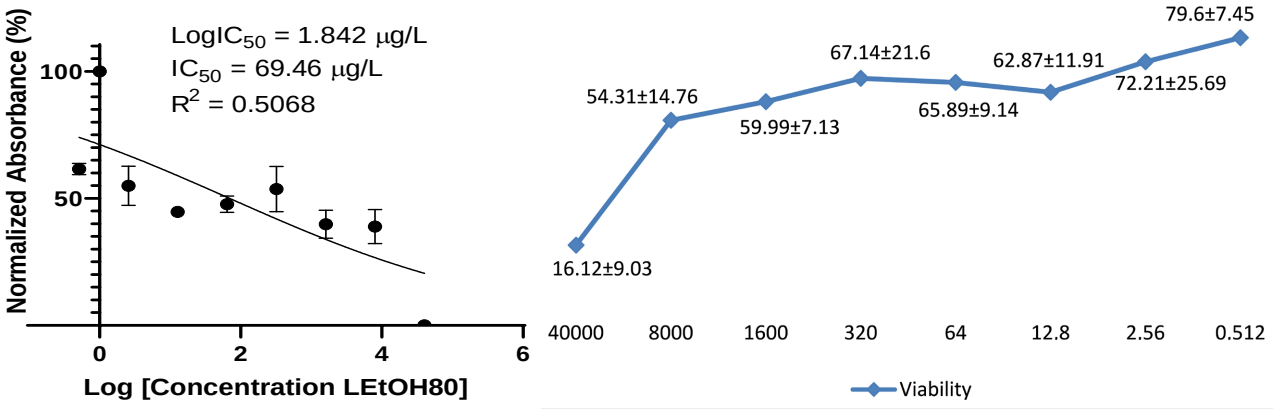


Figure 3. The inhibitory activity of leaves extracted with ethanol of 20% (LEtOH20), 50% (LEtOH50) and 80% (LEtOH80).

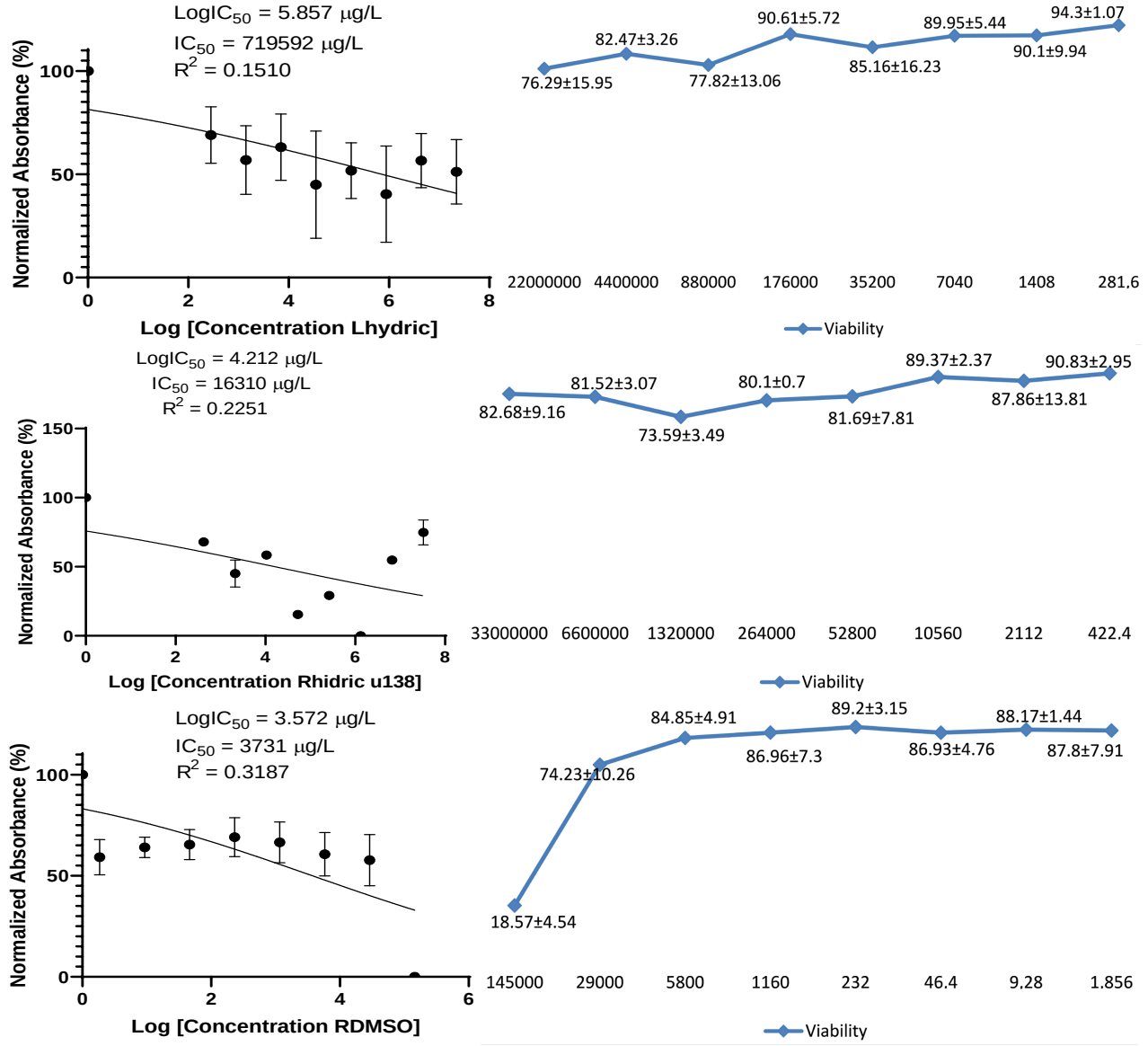


Figure 4. The aqueous extracts of leaves (Lhydric) and roots (Rhydric) of TO, as well roots extracts in DMSO (RDMSO) influence on glioblastoma cells viability.

A comparative analysis led to the conclusion that the use of root extracts prepared in 80% ethanol (50,000 μg/L - 8.49 ± 1.04) and Doxo54 (54,350 μg/L - 8.64 ± 1.65) had the greatest inhibitory effect on the viability of glioblastoma, results which were statistically different ($p = 0.003$). Doxo100 (1.28 μg/L - 96.6 ± 2.76) had the lowest inhibitory action on tumor cells. The lowest viability suppressing activity was determined in case of TO roots extracts made on ethanol of 50%, at 0.768 μg/L (95.51 ± 3.44). Last results compared by U-test showed the similarity between Doxorubicin of 1.28 μg/L and TO of 0.768 μg/L ($p = 0.64$).

For evaluation of the alcohol's concentration impact on glioblastoma cells viability the correlation test between ethanol concentration and cells viability results in vials with highest concentration of tested substances was done. A strong negative and statistically significant ($r_s = -0.87$, $p = 0.002$) correlation between alcohol concentration used for TO roots extraction and tumor cells viability was identified. The same test did not reach a statistically significant level ($r_s = -0.50$, $p = 0.07$) in assessing the relationship between ethanol concentration used in TO leaves extracts and cells viability, although the relation was a negative too.

4. Discussion

Glioblastoma is the most dangerous malignant brain tumor. Nowadays it requires a multidisciplinary treatment, including surgical resection in association with radiotherapy and temozolomide [11]. The addition of bevacizumab revealed no increase in overall survival. Regardless of advanced diagnostic and treatment modalities almost all patients experience tumor progression with high rate of mortality.

Nowadays, much attention is paid to the prevention and treatment of tumors using natural products. A significant amount of research has focused on the study of natural substances that have demonstrated the ability to selectively affect tumor cells. Species and subspecies of *Taraxacum* plants, widely distributed in the world, have been demonstrated different promising activities.

Takasaki *et al.* (1999) assigned anti-tumor activity of *Taraxacum* species to high content of triterpenes and sesquiterpenes [12]. Authors consider that several constituents of *Taraxacum japonicum* roots extracts have the ability to influence carcinogenesis at different stages (initiation and promotion), therefore can be used as valuable chemopreventive agents. Most promising inhibitory actions were established in case of two triterpenes, taraxasterol and taraxerol. Later, Ovesna *et al.* (2004) attributed the role of chemopreventive agents to taraxasterol and beta-sitosterol [13].

TO represents an exceptional source of multiple bioactive compounds that can act themselves or potentiate each other cytotoxic activity on cancer cells. Ovadje *et al.* (2016) experimental studies identified four pharmacologically active components: α-amirin, β-amirin, lupeol and taraxasterol, which showed weak antitumor activity as individual compounds compared to the effects of the entire extract.

Hata *et al.* (2006) isolated from TO the triterpene lupeol, which can block cell movement in B162F2 melanoma and neuroblastoma [4]. This compound selectively disrupted the assembly of actin fibers in neural crest-derived cells, which determined the inhibitory effects on cell migration. Moreover, luteol extracted from *Bombax ceiba* exhibited a significant *in vitro* antiangiogenic activity on tube formation of human umbilical venous endothelial cells [14]. The greatest inhibitory effect on endothelial cells was determined at 50 and 30 μg/mL, doses which did not affect the growth of tumor cell lines such as SK-MEL-2, A549, and B16-F10 melanoma.

Caffeic and chlorogenic acids, synthesized by TO roots, have been demonstrated to hamper the development of metastatic cancer [15]. Another compound of TO, apigenin, reduced prostate cancer stem cell survival and migration through downregulation of PI3K/Akt/NF-κB signaling, according to Erdogan *et al.* (2016) experiment results [16].

Taraxinic acid demonstrated its anti-proliferative efficacy on HL-60 cells [17]. In accordance with Koo HN *et al.* (2004) results, aqueous extract of TO reduced viability of HepG2 culture cells by 26% [18]. Authors determined that TO action depends of dosage and time: the maximum secretion of IL-1 (66 ± 1.7 pg/mL) and TNF-α (186 ± 2.0 pg/mL), followed by apoptosis was exhibited by 0.2 mg/ml of TO extract, after 48 hours of incubation.

We support the idea that dosage is important, but the highest concentration doesn't mean the best effect according to our data. The results obtained led to the conclusion that the type of extractant is more important than the concentration of TO extracts (**Figures 1-4**). Thus, the best antitumor activity (2.49 ± 1.08) was determined in case of roots extracts made on ethanol of 80% at 50,000 μg/L of TO, while roots extracted with alcohol of 50% had a much lower inhibitory activity (52.35 ± 21.70), even at higher concentration of TO (60,000 μg/L).

TO has the ability to influence various metabolisms. It seems to have a modulatory action, by expressing both antioxidant and pro-oxidant activities, depending on the cell state (normal or altered), as well as on the cell types. In case of normal cells, TO treatment led to suppression of both reactive oxygen species and nitric oxide, and prevention of lipids peroxidation, actions attributed to the presence of flavonoids [19].

In case of tumor cells (HT-29 and NCM460 cells) TO decreased the mitochondrial membrane potential and increased ROS levels in the isolated mitochondria [7]. Destabilization of mitochondrial membranes was associated with a rapid activation of caspase-8. The eloquent example of how TO activity is influenced by cell type was provided by Ovadje *et al.* (2016) who established that the same plant extract down-regulated the pro-survival genes, such as BCL2, GALNT5 (*Polypeptide N-Acetylgalactosaminyltransferase 5*) and PARP-2 in HT-29 cells, while it up-regulated them in NCM460 cells. At the same time, the pro-apoptotic genes: as CASP1, IFNG (*interferon gamma*) and TNF (ligands and receptors), were up-regulated by TO in HT-29 cells, and down-regulated in NCM460 cells.

Chatterjee *et al.* (2011) reported that TO root extract specifically and effectively induced apoptosis in human melanoma cells A375 without inducing toxicity in noncancerous cells [20]. Authors observed characteristic apoptotic morphology of nuclear condensation and phosphatidylserine flipping to the outer leaflet of the plasma membrane within 48 hours. They concluded, that TO can induce apoptosis by activation of caspase-8, thus demonstrating employment of extrinsic apoptotic pathway to kill A375 cells.

Interesting is that another tumor line, G361 initially resistant to TO, was blocked efficiently after combination with metformin, an anti-diabetes type II drug. Moreover, production of reactive oxygen species by TO treated mitochondria suggested that natural TO compounds can also directly target mitochondria.

The data presented by Ovadje *et al.* (2016) show that aqueous TO root extracts could trigger cell death in models of colon cancer HT-29 (p53−/−) and HCT116 (p53 WT)), *in vivo* and *in vitro* experiments [7]. Authors concluded that activation of caspase-8 is not the essential mechanism for the induction of apoptosis and TO is utilizing caspase-8 independent cell death pathway. They demonstrated TO cytotoxic action on tumor cells even after prior caspase-8 specific inhibition with IETD-fmk. It appears that TO may target metabolic defects and/or mitochondrial reactive oxygen species response only in cancer cells. Moreover, in HT-29 cells TO induced the up-regulation of pro-death genes—CASP1 (*caspase 1*), KCNIP1 (*encodes potassium voltage-gated channel interacting protein 1*), SNCA (*alpha synuclein*), TNF and some of its corresponding receptors like TNFRSF1A and TNFRSF11B (*tumor necrosis factor superfamily, member 1A and 11B*), as well the down-regulation of pro-survival, anti-apoptotic genes, BCL2 (*B-cell lymphoma 2*), BCL2A1 (*BCL2 Related Protein A1*) and PARP2 (*Poly* (*ADP-Ribose*) *Polymerase 2*).

Another described mechanism of action of TO is a decrease in nuclear GRB2 (*Growth Factor Receptor 2-Related Protein*), ERK1/2 (*Extracellular Signal-Regulated Protein Kinase*), and a corresponding increase in cytoplasmic ERK1/2 levels. TO selectively decreased the expression of COX-2 (*cyclooxygenase-2*) in colon tumor cells in a dose and time dependent manner. Thus, it can be concluded that TO is able to target several pathways of programmed cell death, as well as aberrant signaling pathways [7].

Other studies demonstrated that aqueous extracts of TO roots had the capacity to induce apoptosis of leukemia cells, in a dose and time dependent manner [21]. Extracts of low concentration were able to induce development of specific signs of apoptosis in leukemic T-cells line (Jurcat), without involvement of healthy cells. Authors considered that the most appropriate mechanism by which TO roots act is an early, even prophylactic activation of caspase-8, followed at the second stage by activation of caspase-3.

Hueber *et al.* (2000) reported that in Jurcat cells which express FADD (*Fas-associated death domain*) TO did not induce apoptosis, phenomena which

was explained by the fact that TO intervened in the extrinsic or receptor-mediated pathway of apoptosis [22]. Involvement of TO in mechanisms of cell mediated apoptosis was sustained by research results which identified that dominant-negative FADD cells that did not develope DISC (*death-inducing signaling complex*) complex were resistant to TO roots action. Non-tumor mononuclear blood cells were not affected by TO.

Jeon *et al.* (2008) reported, that leaves and flowers TO ethanolic extracts exhibit anti-angiogenic activity, which had an important impact in chemotherapy as well as in chemoprevention [23]. Such kind of activity was attributed to flavones, present in the extracts of TO.

TO can also influence the secretion of cytokines. Kim *et al.* (1999) reported that TO can induce, in peritoneal macrophages prestimulated with rIFN-γ, TNF-α secretion coupled with an increase in NO production [24].

Moreover, TO action seems to depend on the type of tumor cells and parts of the plant used in experiments. TO concentration of 100 and 1000 μg/mL inhibited significantly the production of IL-1 and TNF-α in astrocytes with or without preliminary LPS stimulation [8]. These data are in line with our results, which demonstrated the greatest TO roots influence on tumor cells even less concentration (50 μg/mL) (**Figure 2**). Stressing the plants part importance, Sigstedt *et al.* (2008) reported that roots extracts were more effective in case of MCF-7/AZ mammary cells cultures and leaves in case of LNCaP prostatic cancer [6].

According to Goldberg *et al.* (2007) results, aqueous extracts of TO were not able to modify metastatic activity of Lewis pulmonary cancer cells, but these extracts increased the activity of cyclophosphamide, that in consequence increased the rate of metastases inhibition from 4% to 77% [25]. According to the authors, TO carbohydrates, especially lentinan, exhibited such activity [26].

Sigstedt *et al.* (2008) compared the antitumor activity of different TO parts, by applying aqueous extracts of flowers, leaves and roots to MCF-7/AZ mammary cells cultures [6]. By these experiments authors established that only crud leaves extracts could diminish tumor cells growing by 40% after 96 hours of incubation. Authors consider that TO extracts exerted antitumor activity through ERK kinases, as well as by diminishing FAC (*focal adhesion kinase*) and SRC (*non-receptor tyrosine kinase*), and inhibition of MMP-2, MMP-9 (*matrix metalloproteinases*) activity.

Administration of TO ethanolic extracts for 10 days lead to a significant inhibition of ddY-Ehrlich tumors, inoculated intraperitoneally to ddY mice a weak earlier [27].

Antitumor activity was determined and in case of hot water TO extracts, which demonstrated effectiveness against Ehrlich tumors, as well syngeneic tumors derived of C3H/He-MM46 mice. These data correspond to our results, in which aqueous extracts, even of low concentration exhibited more powerful effect even than Doxo100 of 1.28 μg/L (**Figure 1**, **Figure 4**).

Recently, Yoon *et al.* (2016) selected TO of 500 plants as one of the most effective adjuvant TRAIL (*TNF-related apoptosis inducing ligand*) therapy [28]. The combined treatment, TRAIL and TO, induced apoptosis of Huh7 tumor cells, which initially were resistant to TRAIL. This effect of TO is mediated by inhibition of the MKK7-TIPRL interaction and activation of MKK7-JNK phosphorylation. The main component of these extracts was chicoric acid.

Another mechanism of cytotoxicity was described by Gerbino *et al.* (2018) based on results of HEK293 (*human embryonic kidney*) cells treatment with ethanolic roots extracts [29]. Authors related a great Ca^{2+} outflow from endoplasmic reticulum and its inflow in plasmalema.

TO, due to its molecular complexity, acts as an effective drug against tumors, as a selective trigger of programmed cell death pathways, as an antioxidant, prooxidant, or anti-inflammatory agent with additional antiangiogenic and antiproliferative activity. The substances extracted from TO, like taraxasterol, lupeol, sesquiterpene lactones and phenolic acids can lead to antioxidative, anti-inflammatory effects which assist in producing antitumor activity. In spite of all described functions above, Jedrejek *et al.* (2019) determined about 100 bioactive components in TO, but specific antitumor activity was not attributed to no one [1]. Their combinations seem to exert the greatest activity and not antitumor only.

This study has some limitations. We did not perform the tests which could describe the components of extracts of every dilution, as well as we did not appreciate the ratio of different chemicals in individual extracts. These are the goals of our future experiments, which will give additional information regarding the active compounds that can be responsible for specific action of the individual type of extract.

Nevertheless some strong point of this study should be stressed. Our research had demonstrated that selection of type of extractant, its concentration and plants part are requirements which should be taken into consideration in evaluation of TO activity, and may be not only TO. In addition, the two different types of solvents (protic and aprotic) that were compared are not only the most common solvents used by scientists, but also the solvents currently used by people in their lives when using the plant as a tea or tincture.

5. Conclusion

Taraxacum officinale is a promising source of many substances with multiple benefits for humans. The exercised actions depend on type of extractant and plants' part. The greatest influence on glial tumors had the extract of roots made in ethanol of 80%. Further investigations are needed to explore the precise composition of the TO extracts and the importance of individual components and of their combination in neoplasms treatment and/or prevention.

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Conflicts of Interest

Authors declare lack of any financial or non-financial conflict.

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