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Effects of *Taraxacum officinale* on Glioblastoma Cell Culture and Their Correlation with Hydroxycinnamic Acids Content †

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Abstract: Glioblastoma is an aggressive type of CNS tumors. The aim was to evaluate the antitumor activity of *Taraxacum officinale* (TO) extracts on U-138 MG glial cells and correlate it with the concentration of chicoric (ChA), chlorogenic (CGA), and caftaric (CA) acids (mg/mL) in the extract. TO dry leaves were extracted with DMSO and ethanol of different concentrations. The concentration of acids was determined by liquid chromatograph (Agilent 1260 with DAD). The viability of U-138 MG cells was assessed by MTT test (% of viable cells). The activity of TO was compared with that of doxorubicin. The best antitumor activity was shown by TO extracts prepared with DMSO (110,000 μg/L—17.3 ± 8%, that contained ChA—8976 × 10^{-6} mg/mL, CGA—316.8 × 10^{-6} mg/mL, CA—1628 × 10^{-6} mg/mL), with 50% ethanol (150,000 μg/L—13.7 ± 3.2%, that contained ChA—52,500 × 10^{-6} mg/mL, CGA—1746 × 10^{-6} mg/mL, CA—8460 × 10^{-6} mg/mL) and with 80% ethanol (40,000 μg/L—16.1 ± 9%, that contained ChA—904 × 10^{-6} mg/mL, CGA—114.4 × 10^{-6} mg/mL, CA—70.4 × 10^{-6} mg/mL). TO extracts activity was close to that of doxorubicin. In conclusion, the TO antitumor activity depends on the type of extractant and its concentration, as well as on the content of cinnamic acids.

Keywords: *Taraxacum officinale;* cinnamic acid derivatives; glioblastoma; U-138 MG; MTT test

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1. Introduction

Taraxacum officinale, known else as Dandelion have been found to be a rich source of triterpenes, flavonoids, polyphenols, including cinnamic acid derivatives [1]. The hydroxycinnamic acids have been reported to display a noteworthy number of valuable health properties, which are mostly related to antioxidant and antiradical activities [2]. Since elevated concentrations of reactive oxygen species are involved in carcinogenesis and promote tumor progression, compounds with phenolic groups realize their oncoprotective effect mainly due to their antioxidant activity, which, apparently, is realized mainly in the process of free radical scavenging. This and some additional mechanisms were described in case of mammary tumors, prostate cancer and melanoma [3,4]. Currently, there is no information about the effectiveness of TO in glioblastoma and the possible involvement of cinnamic acid derivatives in its effects. The aim of this study was to evaluate the antitumor activity of *Taraxacum officinale* extracts on U-138 MG glial cells and to identify a possible correlation of the effect with the concentration of hydroxycinnamic acids (cicoric, chlorogenic and cafftaric acids). We determined that TO components can

suppress glioblastoma cells viability and the activity depends on the type of extractant and its concentration, as well as on the content of cinnamic acids.

2. Materials and Methods

2.1. Plant Material

Taraxacum officinale F. H. Wigg (TO) leaves were harvested in May 2017 from a natural habitat (47°4′8″ North, 28°40′47″ East). The leaves, separated from roots, were dry at room temperature for 2 weeks and pulverized with the mortar and pestle.

2.2. Extracts Preparation

Extracts were prepared using ethanol (Luxfarmol, MD) of different concentrations (20%, 50% and 80%) and DMSO of 0.1% (Merck, DE). The extraction was done in dark conditions, in recipients of 100 mL for 24 h, where the ratio biomass/solvent (mg/mL) was 10:1. Extracts were filtered (Whatman no. 5, WHA1005090, Merck, DE) and stored at $+4^0$C. 1.5 mL of each extract was centrifuged 5 min at 5000 rpm (MPW 370, PL) to confirm the samples purity in the absence of stratification/sedimentation. The dry mass content of each extract was determined: 200 μL of the extracts were filtered (Whatman No. 1, WHA10010155, Merck, DE), then the filters were dried and weighed (RADWAG PS210/C/2, PL).

2.3. Cell Culture

The human glioblastoma cell line U-138 MG (Cell Lines Service, DE) were cultured in 5% CO_2 incubator conditions (Sanyo), in DMEM medium (Merck, DE) supplemented with 4.5 g/L glucose, 2 mM glutamine, 10% BSA (Invitrogen, USA). Cells viability was assessed (Olympus CK40, JP) with trypan blue 0.4% (Gibco™, USA).

2.4. MTT Test

The colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed according to method described by Mosmann [5]. The action of TO extracts was compared with that of Doxorubicin (Doxo) that was used in 2 stock concentrations—54,350 μg/L and 10^5 μg/L. The optical absorbance was measured at 540 nm (Synergy microplate reader, BioTek, USA). The percentage of viable cells which survived after incubation was determined by the Formula (1):

$$\text{Viability (\%)} = \frac{\text{OD}_{\text{specimen}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{Blank}}} \times 100 \quad (1)$$

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

All assays were done in triplicate.

2.5. TO Composition

The concentration of cinnamic acids was determined on an Agilent 1260 liquid chromatograph with DAD and an automatic injector. The following chromatographic conditions were used: Hyperpack Basic ODS column (5 mkm, 150 × 4.6 mm), with mobile phase (acetonitrile—0.05%, trifluoroacetic acid solution (32:68)), at 1.5 mL/min flow rate, with detection at 330 nm. The chlorogenic acid (HWI ANALYTIK GmbH), batch: HWI00352) was used as analytical reference substance.

2.6. Statistics

The GraphPad Prism 8 Software (San Diego, CA, USA) was used to calculate the mean of glioblastoma viability (%). Spearman analysis (r_s) was used to assess the existence of correlations between cinnamic acids concentrations and cell viability. The results were

compared by Mann-Whitney U-test. *p*-values equal or less than 0.05 were considered statistically significant.

3. Results

All TO extracts could suppress the viability of glioblastoma cells. The best antitumor activity (13.7 ± 3.2%) was determined in case of extracts made with ethanol of 50%, with highest TO concentration of 150,000 μg/L, that contained ChA—52,500 × 10^{-6} mg/mL, CGA—1746 × 10^{-6} mg/mL, CA—8460 × 10^{-6} mg/mL) (Table 1). The increase of cinnamic acids concentration led to a statistically significant decrease of tumor cells viability (r_s = −0.71, p = 0.02).

Table 1. Viability (Mean, %) of glioblastoma cells U-138 MG after exposure to TO ethanol extracts (20%, 50% and 80%) with different content of cinnamic acids.

										r_s	p
DMSO	**TO**	**110,000**	**22,000**	**4,400**	**880**	**176**	**35.2**	**7.04**	**1.408**		
	Viability	**17.3**	**86.0**	**88.7**	**87.9**	**91.1**	**93.0**	**93.6**	**93.6**		
	ChA	8976.0	1795.2	359.0	71.8	14.4	2.9	0.57	0.11	−0.95	0.0001
	CGA	316.8	63.4	12.7	2.5	0.5	0.1	0.02	0.004	−0.95	0.0001
	CA	1628.0	325.6	65.1	13.0	2.6	0.5	0.1	0.02	−0.95	0.0001
EtOH20	**TO**	**140,000**	**28,000**	**5,600**	**1120**	**224**	**44.8**	**8.96**	**1.792**		
	Viability	**38.4**	**53.8**	**63.6**	**77.1**	**81.8**	**78.7**	**87.8**	**86.7**		
	ChA	33320	6664	1332.8	266.6	53.3	10.7	2.1	0.4	−0.95	0.0001
	CGA	1232	246.4	49.3	9.9	2	0.4	0.08	0.02	−0.95	0.0001
	CA	7224	1444.8	289	57.8	11.6	2.3	0.5	0.09	−0.95	0.0001
EtOH50	**TO**	**150,000**	**30,000**	**6,000**	**1200**	**240**	**48**	**96**	**1.92**		
	Viability	**13.7**	**88.0**	**70.2**	**80.6**	**83.5**	**84.1**	**89.8**	**92.7**		
	ChA	52500	10500	2100	420	84	16.8	33.6	0.7	−0.71	0.02
	CGA	1746	349.2	69.8	14	2.8	0.6	1.1	0.02	−0.71	0.02
	CA	8460	1692	338.4	67.7	13.5	2.7	5.4	0.1	−0.71	0.02
EtOH80	**TO**	**40,000**	**8,000**	**1,600**	**320**	**64**	**12.8**	**2.56**	**0.512**		
	Viability	**16.1**	**54.3**	**60.0**	**67.1**	**65.9**	**62.9**	**72.2**	**79.6**		
	ChA	904	180.8	36.2	7.2	1.4	0.3	0.1	0.01	−0.90	0.001
	CGA	114.4	22.9	4.6	0.9	0.2	0.04	0.01	0.001	−0.90	0.001
	CA	70.4	14.1	2.8	0.6	0.1	0.02	0.005	0.001	−0.90	0.001

Note: TO—*Taraxacum officinale* concentration in extract (μg/L); Viability of tumor cells (%); Chicoric (ChA), chlorogenic (CGA) and caftaric (CA) acids concentration (×10^{-6} mg/mL); r_s—Spearman correlation (acids concentration to cells viability); p—statistical significance.

The second most effective antitumor activity (16.1 ± 9%) was shown by the alcoholic extract of 80%, with a TO concentration of 40,000 μg/L, which contained ChA—904 × 10^{-6} mg/mL, CGA—114.4 × 10^{-6} mg/mL ml, CA—70.4 × 10^{-6} mg/mL. The antitumor activity was dependent of cinnamic acids concentration (r_s = −0.90, p = 0.001).

DMSO 110,000 μg/L extracts, containing ChA—8976 × 10^{-6} mg/mL, CGA—316.8 × 10^{-6} mg/mL, CA—1628 × 10^{-6} mg/mL, were the third in the list of the most effective anti-tumor TO extracts (17.3 ± 8%). As in previous cases, the increase of cinnamic acids concentration was indirectly strong correlated with the glioblastoma viability (r_s = −0.95, p = 0.0001).

The stock concentration (140,000 μg/L) of 20% of ethanol TO extract exhibited a lower antitumor activity (38.4 ± 2.1%), where the content of cinnamic acids was ChA—33,320 × 10^{-6} mg/mL, CGA—1232 × 10^{-6} mg/mL, CA—7224 × 10^{-6} mg/mL. The increase of acids concentration decreased the tumor cells viability (r_s = −0.95, p = 0.0001).

The comparative assay determined statistical significant differences between the action realized by leaves extracted with ethanol of 50% and all other extracts, like follow: with 80% ethanolic extract p = 0.04, with DMSO p = 0.001 and with 20% p = 0.0001. The

20% ethanolic extract action was different of DMSO's (p = 0.0001). The action of 80% ethanolic extract was not different in comparison to DMSO effect (p = 0.16).

In case of Doxo, the best anti-tumor activity was determined at its highest concentration. The 54,350 μg/L of Doxo better (8.64 ± 1.65%) suppressed glioblastoma cells than 10^5 μg/L (15.32 ± 0.86%) and these results were statistically different (p = 0.01). The same test determined that TO leaf extract in ethanol of 50% action was different in comparison with Doxo of 54,350 μg/L (p = 0.05) and similar with Doxo of 10^5 μg/L (p = 0.17).

4. Discussion

A malignant neoplasm that still remains a therapeutic problem, mainly due to its heterogeneity and inaccessibility to therapeutic agents, is glioblastoma [6]. The identification of potential new drugs for the treatment of glioblastoma is of major importance. Natural sources are intensively studied as sources of antitumor compounds that can be developed to the drug stage.

Phenolic compounds containing aromatic ring and OH groups show anticancer action, the presence of these structural elements being considered the key characteristics necessary for their activity [7]. The increasing of hydroxyl groups' number enhances antitumor activity.

Cinnamic acid represents a natural aromatic fatty acid of low toxicity. The acid itself and its hydroxyl derivatives exert many beneficial activities, including anticancer one [2]. Liu et al. (1995) reported that cinnamic acid at a concentration of 1 to 4.5 mM caused a 50% reduction in cell proliferation in several tumor types such as melanoma, prostate cancer, lung carcinoma and glioblastoma. The authors concluded that this chemical induces cell differentiation and reduces the invasive capacity of tumors in many ways, such as modulating the expression of genes involved in tumor metastasis and immunogenicity by blocking mitogenic signal transmission [8]. Chkrabarti et al. (2018) results demonstrate, that cinnamic acid upregulate the expression of suppressor of cytokine signaling 3 molecule in glial cells via CREB (cAMP response element binding) pathway, involved in neuroinflammatory and neurodegenerative disorders [9]. Naumowicz et al. (2020) demonstrated that cinnamic acid could effectively suppress glioblastoma cell line LN-229, by changing the surface charge density of cell membranes [6]. Niero et al. (2013) determined that cinnamic acid, itself induces apoptotic cell death and cytoskeleton disruption in human melanoma cells with IC_{50} of 2.4 mM [10]. The novel antitumor mechanism of action of cinnamic acid is through TNFA-TNFR1 mediated extrinsic apoptotic pathway [4].

The list of cinnamic acid derivatives is enormous, where the hydroxy group is one of the most listed in the literature [11]. In the present study, we wanted to investigate the action of different TO extracts on glioblastoma U-138 MG cell line and correlate this activity with the content of hydroxy derivatives of cinnamic acid (chicoric, chlorogenic and caftaric acids) in the extract. As a result, we determined that increase of all cinnamic acids, involved in study, led to the decrease of tumor's viability.

Our data revealed that the extracts with the highest content of ChA were most effective in inhibiting U-138 MG glioblastoma cells viability (Table 1). There are several mechanisms of ChA action described until now that can underline the established effect. According to Sun et al. (2019) data, this acid can induce autophagy by promoting endoplasmic reticulum stress, process regulated through AMPK (AMP-activated protein kinase) [12]. Tsai et al. (2012) reported that ChA can inhibits cancer growth via inhibition cell proliferation, stimulation cell apoptosis, upregulation of DNA fragmentation with following deactivation of telomerase, cleavage of caspase-9 and poly (ADP-ribose) polymerase [13]. This action required a huge dosage, much higher than in our experiment, ranging from 105 to 315 μM.

Chlorogenic acid concentration also negatively correlated with glioblastoma viability in our study. This compound, which represents an ester of caffeic and quinic acids had the lowest concentration in all studied extracts (Table 1). Numerous functions of CGA,

such as regulation of glucose and lipid metabolism, protection of the nervous, cardiovascular, gastrointestinal systems, as well as kidney and liver, were noted in a comprehensive review by Lu et al. (2020) [14]. In case of glioma cells, CGA could increase the expression of specific biomarkers responsible for cell differentiation, like Tuj1 and GFAP [15]. These mechanisms suggest that CGA induces the differentiation of cancer cells rather than killing them. It was recently reported that CGA has been approved by the China Food and Drug Administration as a potential anti-cancer drug for Phase I (NCT02728349, April 2016) and Phase II (NCT03758014, November 2018) clinical trials in patients with glioma [15].

The caftaric acid (CA), is a tartaric ester of hydroxycinnamic acid (caffeic). CA has been reported to act as an inhibitor of protein-protein interactions mediated by Src family kinases, which is an antimutagenic activity and places the compound in the range of chemopreventive substances [16]. It was reported that CA, as a main component of juice of *Vitis coignetiae,* inhibited the clastogenicity and mutagenicity of heterocyclic amines (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) in the micronucleus assay and the Ames test. This acid was an effective inhibitor of the activities of detoxification phase I enzymes (cytochrome P450 1A1 and cytochrome P450 1A2) and an enhancer of the activities of phase II enzymes (uridine 5′-diphospho-glucuronosyltransferase and glutathione S-transferase). Until now, there are no data about its usage in tumors treatment [16].

Our study has several limitations. Although statistical analysis showed a correlation between the total amount of hydroxy derivatives of cinnamic acid and antitumor activity, TO extracts contain other biologically active components, which antitumor activity was described in the literature. This statement is proven by comparing the composition of the leaf extracts in our experiment: in the case of the 80% ethanolic extracts, which showed the 2nd most promising antitumor activity, the content of cinnamic acids was the lowest. (Table 1). Thus, it is necessary to continue studying the particularities of the action of some compounds from the chemical composition of TO extracts on metabolic processes, signal transduction mechanisms, apoptosis, etc. in glioblastoma cells as well as in normal cells to identify possible adverse effects of the extracts.

The results obtained allow us to conclude that *Taraxacum officinale* extracts significantly suppresses the viability of glioblastoma cells in vitro. This activity depends on the type of extractant and its concentration, as well as on the content of the hydroxyl derivatives of cinnamic acid.

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