Study on the Phototoxicity and Antitumor Activity of Plant Extracts from *Tanacetum vulgare* L., *Epilobium parviflorum* Schreb., and *Geranium sanguineum* L.

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Abstract: *Our country is rich in medicinal plants with a thousand-year tradition of use for therapeutic and cosmetic purposes. World Health Organization estimates that around 70% of population is using traditional (folk) medicine to cure various ailments. In many cases, herbal preparations prove to be useful, but some of them can also exert toxic effects. Therefore, herbal extracts should be tested both as curatives and for safety application. The aim of the present work was to investigate extracts from Tanacetum vulgare (TVA), Epilobium parviflorum (EPE) and Geranium sanguineum (GSA), obtained by different organic solvents, for their phototoxicity and anticancer activity. The standard BALB/c 3T3 Neutral Red Uptake phototoxicity/cytotoxicity assay was used to evaluate the possible phototoxic properties of the extracts. The antitumor activity of the extracts was studied in vitro on a panel of human tumor cell lines in comparison to the non-tumorigenic MCF-10A cells. The selectivity indices and the photo-irritancy factors were determined. The results show that the studied extracts are not phototoxic at concentration range from 4 to 1000 μg/ml. Determined photo-irritancy factors (PIF) for the extracts was < 2 (PIF for EPE = 1.72, GSA = 1.04, TVA = 1.43), which assigns them to the category of non-phototoxic substances. In addition, at concentrations lower than < 60 μg/ml, no statistically significant cytotoxicity was observed. The selectivity index was calculated relative to the IC_{50} value for non-tumorigenic MCF-10A cells. The highest selective index obtained with respect to the HT-29 cells was shown by all testing extracts (selective index (SI) for EPE = 2.6, GSA = 2.4 and TVA = 2.5), to the HeLa cells by extract GSA (SI = 2.0) and to the PC3 cells*

by extracts GSA (SI = 2.6) and TVA (SI = 2.0). Presented data shows that the studied extracts have a high level of photosafety with a promising antitumor activity in certain cell lines.

Keywords: *Phototoxicity test, Plant extracts, Prolyl oligopeptidase*.

Introduction

Secondary metabolism of higher plants represents an inexhaustible source of potential therapeutic agents for treatment of many diseases. Different extracts of medicinal plants are used both in complementary alternative medicine and in the composition of modern medications. Current study is focusing our attention on three medical plants, including *Tanacetum vulgare*, *Epilobium parviflorum* and *Geranium sanguineum*, whose healing properties are world-renowned for years. *Tanacetum vulgare* is aromatic perennial herb from Asteraceae family, also known as tansy. This plant has broadly been used in traditional medicines for the treatment of a variety of medical disorders, such as intestinal worms, rheumatism, colds and fevers, digestive disorders, gout, kidney problems, and tuberculosis [4]. *Epilobium parviflorum*, also known as a willow herb, is the most common species belonging to the Onagraceae [33]. In the 20th century it became very popular to be used as a tea, especially in Austria, Germany and Poland, to treat benign prostatic hyperplasia, prostatitis, as well as bladder and kidney diseases [1]. It is also used to treat skin and mucosal infections [7]. The *Geranium sanguineum* (bloody geranium) is widely known in folk medicine for the treatment of eruptive skin disease and in malignant diseases of hematopoietic organs [11, 14, 26]. Despite the already existing modern information on the various properties of these traditional plants, the pharmacological data supporting their therapeutic application, there is a lack of research related to the photosafety of these plants. Phototoxic compounds have been found in many plant families [6]. Therefore, these investigations are necessary to predict possible phototoxic reactions that may occur from a combination of a photoactive compound of a medicinal plant and solar light. An exemplary mechanism of beneficial effects of the plant preparations is the inhibition of enzymes involved in pathogenesis of certain diseases. For instance, prolyl oligopeptidase (POP, EC 3.4.21.26) is a cytosolic serine-type endopeptidase associated with the pathogenesis of psychiatric and neurodegenerative diseases, connected with a memory loss and cognitive problems [30]. Selective POP inhibitors improve cognitive function in animal models [15] and have been tested even in clinical trials [29]. On the other hand, POP activity increases in a variety of solid tumors and its suppression leads to a restriction of tumor growth [10]. Our previous experiments showed that the ethyl acetate/water extract of *Tanacetum vulgare* (*TVA*) impedes the growth of MBA-MD-231 cells (human triple negative mammary gland carcinoma) by inhibiting the activity of POP [32]. More comprehensive studies proved that the main components of the extract were isomeric caffeoylquinic and dicaffeoylquinic acids which obviously are effective POP inhibitors [19, 31]. Similar studies were performed with the ethyl acetate extract of *Cotinus coggygria* leaves, which also proved to inhibit POP although the main constituents of this extract were galloyl glucoses or gallotannins (unpublished results). Previous data from other authors show that *Epilobium parviflorum* (*EPE*) and *Geranium sanguineum* (*GSA*) also contain substantial quantities of chlorogenic (cafeoylquinic) acids and gallotannins [7, 20] and so, they can be potential inhibitors of POP.

The aim of the present study was to evaluate the safety and anticancer activity of the organic solvents extracts from *Tanacetum vulgare*, *Epilobium parviflorum* and *Geranium sanguineum* from Bulgarian origin.

Materials and methods

Light source

The light source used is a light emitting diode (LED) matrix – an artificial solar light simulator Helios-iO, model LE-9ND55-H – 5500K (SERIC Ltd., Tokyo, Japan).

Chemicals

Dried *Tanacetum vulgare* flowers, *Epilobium parviflorum* (leaves and stems) and *Geranium sanguineum* (roots) were purchased from Vemo 99 Ltd (Sofia, Bulgaria) and were used after grinding (in a crushed state).

Cell culture reagents: Dulbecco's modified Eagle's medium (DMEM Sigma-Aldrich, Schnelldorf, Germany), fetal bovine serum (FBS Sigma-Aldrich, Schnelldorf, Germany), antibiotics (penicillin and streptomycin Sigma-Aldrich, Schnelldorf, Germany), the disposable consumables were supplied by Orange Scientific, Braine-l'Alleud, Belgium. Neutral Red and Thiazolyl Blue Tetrazolium Bromide were purchased from Sigma-Aldrich, Schnelldorf, Germany.

Cell lines

The BALB/3T3 clone A31, MCF-10A, MDA-MB-231, MCF-7, A549, H1299, HT-29, HeLa, HepG2 and PC3 cell lines were obtained from American Type Cultures Collection (ATCC, Manassas, Virginia, USA).

Extracts

The ethyl acetate/water (pH 3.0) extract of *Tanacetum vulgare* flowers was obtained exactly as described previously [31]. Ethanol extracts of *Epilobium parviflorum* and *Geranium sanguineum* were obtained by stirring of 5 g from the respective plant in 80% ethanol for 3 hours at room temperature. Combined filtrates were concentrated with rotary evaporator at 40 °C. Then, acetonitrile (5 ml) was added and later removed *in vacuo*. This procedure was repeated twice and the solid residue obtained was suspended in diisopropyl ether (10 ml), filtered and the residue was washed with diisopropyl ether and dried *in vacuo*.

Cytotoxicity and phototoxicity testing

BALB/3T3 cells were cultured in 75 cm^2 tissue culture flasks in DMEM, 10% FBS, 2 mM glutamine and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) at 37 °C, 5% CO_2 and 90% relative humidity. Cytotoxicity/phototoxicity was assessed by validated BALB/3T3 clone A31 Neutral Red Uptake Assay (3T3 NRU test) [5, 8]. Briefly, cells were plated in a 96-well microtiter plate at a density of 1×10^4 cells / 100 µl / well and were incubated for 24 h. Before treatment with test compounds, dry extracts dissolved in DMSO and further diluted in culture medium, so that the final concentration of DMSO was less than 1% (v/v). A wide concentration range of the test extracts was applied (from 4 to 1000 µg/ml). In phototoxicity tests, 96-well plates were irradiated with dose 2.4 J/cm^2 and the cells were incubated for additional 24 h. After treatment with Neutral Red medium, washing and treatment with the Ethanol/Acetic acid solution, the absorption was measured on a TECAN microplate reader (TECAN, Grödig, Austria) at wavelength 540 nm.

Cytotoxicity/phototoxicity were expressed as CC_{50}/PC_{50} values (concentrations required for 50% cytotoxicity/phototoxicity), calculated using non-linear regression analysis (GraphPad Software, San Diego, California, USA).

In vitro antiproliferative activity

The antiproliferative activity testing was performed on cell cultures from several human cell lines using the standard MTT dye-reduction assay, described by Mosmann [16]. The assay is based on the metabolism of the tetrazolium salt (MTT) to insoluble formazan by mitochondrial reductases. The formazan concentration can be determined spectrophotometrically. The measured absorption is an indicator of cell viability and metabolic activity. The following cell lines were used in the experiments: breast, non-tumorigenic epithelial cell line (MCF-10A), mammary gland type A adenocarcinoma (MCF-7); triple-negative breast cancer (MDA-MB-231); lung alveolar adenocarcinoma (A549), non-small cell lung carcinoma (H1299), colorectal adenocarcinoma (HT-29), cervical cancer (HeLa), hepatocellular carcinoma (HepG2) and prostate adenocarcinoma (PC3). The cell lines were routinely grown as monolayers in 75 cm^2 tissue culture flasks under standard conditions (described above). Cells were plated at a density of 1×10^3 cells in 100 µl in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with test extracts. A concentration range from 4 to 1000 µg/ml was applied for 72 h. Formazan absorption was registered using a microplate reader at λ = 540 nm. Antiproliferative activities were expressed as IC_{50} values (concentrations required for 50% inhibition of cell growth), calculated using nonlinear regression analysis (GraphPad Prism 4 Software).

The statistical analysis included application of One-way ANOVA followed by Bonferroni's post hoc test. $p < 0.05$ was accepted as the lowest level of statistical significance. The data obtained are on average from three independent experiments ± SD, $n = 6$.

Results and discussion

Safety testing

The extracts were studied for cytotoxicity/phototoxicity assessment by an *in vitro* 3T3 NRU test. The cells were incubated with the test substances at a concentration of 4 to 1000 µg/ml for 24 h at 37 °C, 5% CO_2 and 95% humidity. The cytotoxicity/ phototoxicity expressed in % relative to the negative control were determined. Dose-response dependence was observed for all extracts. The obtained results are shown in Fig. 1. At a concentration of 60 µg/ml, no cytotoxic effect was observed on the test substances. Based on the dose-response curves, CC_{50}/PC_{50} values (50% cytotoxic/phototoxic concentration) were calculated by nonlinear regression analysis (Table 1).

The CC_{50} values can be used to calculate the Photo-Irritancy Factor (PIF) for each test extracts, according to the following formula: $PIF = CC_{50}/PC_{50}$. The PIF shows us the probability that the test substance may cause a phototoxic effect (PIF < 2 not phototoxic, PIF ≥ 2 < 5 probable phototoxicity, PIF ≥ 5 phototoxic). For all tested extracts the calculated PIF < 2, which shows a high level of photo safety. These results show that extracts (EPE, GSA and TVA) are safe for use in pharmaceuticals and cosmetics.

Antiproliferative activity

The extracts were studied for antiproliferative activity by MTT dye-reduction assay. Cell cultures from panel of different cell lines were incubated with the test substances at a concentration of 4 to 1000 µg/ml for 72 h at 37 °C, 5% CO_2 and 95% humidity. The antiproliferative activity expressed in % relative to the negative control was determined. The obtained results are shown in Fig. 2. The IC_{50} (50% inhibitory concentration) values of the mean were calculated and presented in Table 2.

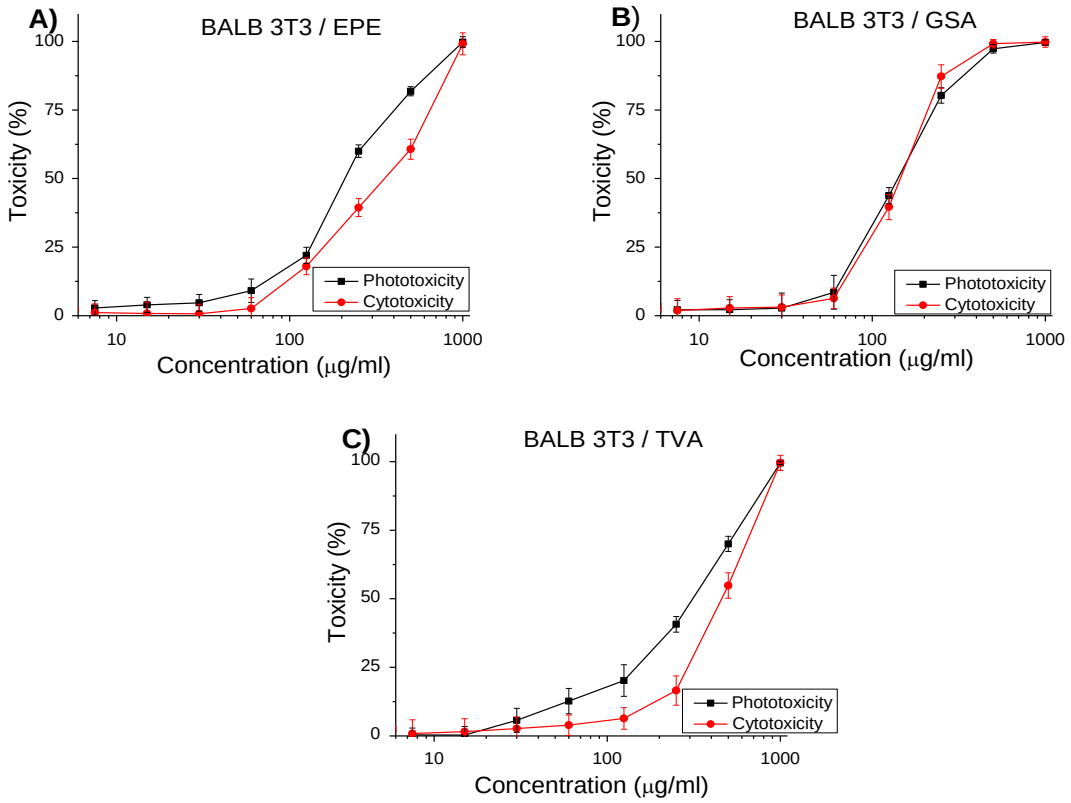
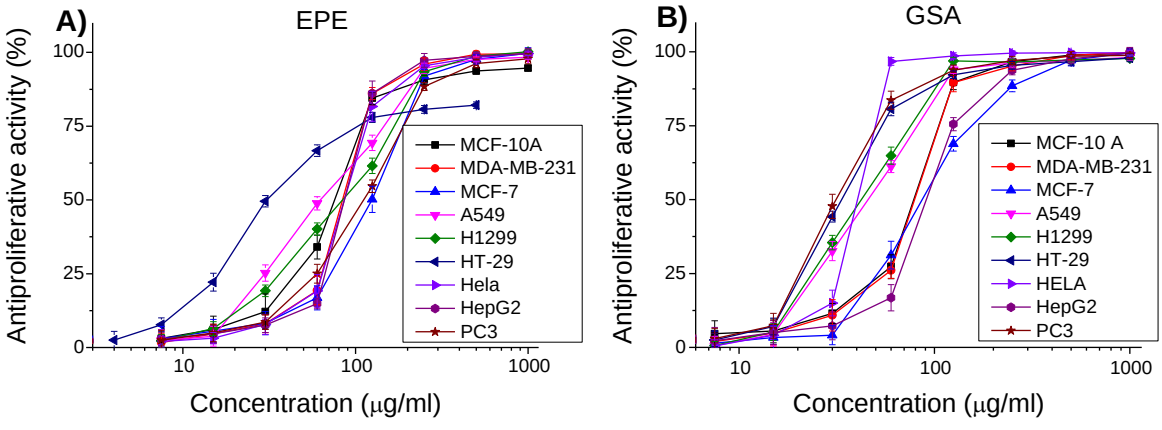


Fig. 1 Dose-response curves for cyto- and phototoxicity of extracts determined in BALB/3T3 cells: A) EPE; B) GSA; and C) TVA. Values are means ± SD from three independent experiments, $n = 6$.

Table 1. CC_{50}/PC_{50} values of mean and PIF

Cell line	Extracts	CC_{50} (PC_{50}) ± SD, (μg/ml)		PIF*
		Cytotoxicity	Phototoxicity	
BALB 3T3	EPE	375 ± 11.71	217.4 ± 2.4	1.72
	GSA	152.2 ± 4.88	146 ± 3.46	1.04
	TVA	470.1 ± 12.57	329.5 ± 9.4	1.43

* PIF < 2 not phototoxic, 2 ≥ PIF < 5 probable phototoxicity, PIF ≥ 5 phototoxic.



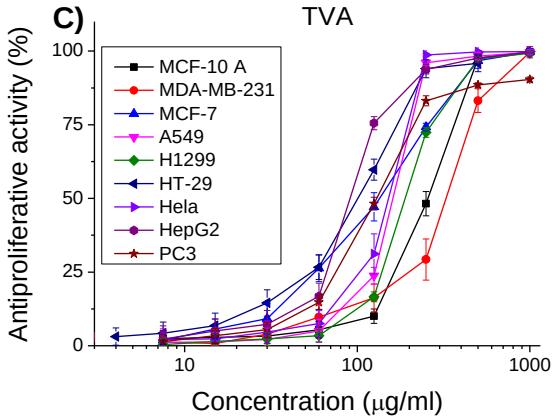


Fig. 2 Dose-response curves for antiproliferative activity of extracts determined in panel of cell lines: A) EPE; B) GSA; and C) TVA. Values are means ± SD from three independent experiments, n = 6.

Table 2. Antiproliferative activity of the studied extracts expressed by IC_{50} values of the mean ± SD and selectivity index

Cell lines	IC_{50} of mean ± SD, (μg/ml)			Selectivity index		
	EPE	GSA	TVA	EPE	GSA	TVA
MCF-10A	80.4 ± 1.4	83.6 ± 1.2	260.6 ± 6.8	-	-	-
MCF-7	126.2 ± 4.3	92.2 ± 1.9	137.2 ± 7.1	0.6	0.9	1.9
MDA-MB-231	89.7 ± 1.4	84.6 ± 0.8	344.8 ± 10.4	0.9	1.0	0.8
A549	64.6 ± 5.5	48.3 ± 1.8	170.3 ± 2.6	1.3	1.7	1.5
H1299	90.2 ± 6.5	**44.9 ± 1.4**	199.9 ± 3.4	0.9	1.9	1.3
HT-29	**31.2 ± 2.1**	**34.8 ± 1.5**	**106.1 ± 6.5**	**2.6**	**2.4**	**2.5**
Hela	92.1 ± 4.9	**42.8 ± 0.8**	159.4 ± 9.3	0.9	**2.0**	1.6
HepG2	92.1 ± 1.8	96.7 ± 3.0	312.7 ± 12.6	0.8	0.9	0.8
PC3	115.2 ± 1.1	**32.2 ± 2.6**	**131.4 ± 6.9**	0.7	**2.6**	**2.0**

The lowest values of IC_{50} and SI ≥ 2 in each column are marked in bold.

MCF-10A is a reliable model for normal human mammary epithelial cells, which serves as a control in experiments to determine antitumor activity [23]. In MCF-10A cells (Fig. 2), the least antiproliferative effect was caused by TVA (IC_{50} = 260.6 ± 6.8) while EPE and GSA show a significantly higher antiproliferative effect IC_{50} = 80.43 ± 1.43 and 83.56 ± 1.19, respectively (Table 2). In the tumor cell line HT-29 the strong effect is caused in all tested extracts (EPE with IC_{50} = 31.2 ± 2.1, GSA with IC_{50} = 34.8 ± 1.5, TVA with IC_{50} = 106.1 ± 6.5). In addition, PC3 cell line treated with GSA showed good antiproliferative activity at quite low IC_{50} values (32.2 ± 2.6), compared with the other two extracts with IC_{50} values 115.2 ± 1.1 for EPE and 131.4 ± 6.9 for TVA. Also, high antiproliferative activity was observed in H1299 and HeLa cell lines treated with GSA with IC_{50} values of about 40 μg/ml. The IC_{50} values, found in MCF-10A, are used to calculate a selective index (SI), which assesses the potential of a substance to be used as an antitumor agent with low side effects [7, 31]. We used the following formula to calculate the selective index SI = IC_{50} of MCF-10A/IC_{50} of tumor cells. A SI value > 10 was assumed to belong to a selected potential sample that can be further investigated [21]. Weerapreeyakul et al. [35] proposed a lower SI value (> 3) for classifying the prospective anti-cancer sample. Rashidi et al. [25] considered more than 2 SI values as high selectivity. A high selective index is indicative for a low level of side effects. The highest selective index with respect to HT-29 is shown by the all

tested extracts (SI for EPE = 2.6, GSA = 2.4 and TVA = 2.5). In PC3 cells the SI for GSA = 2.6 and TVA = 2. HeLa cells showed good selectivity by extract GSA (SI = 2.0). Regarding the MDA-MB-231 and HepG2 cell lines, SI < 1 were observed for each of the extracts tested.

Discussion

Medicinal plants are widely used, and extracts of them are included in many medicines and cosmetics [22]. At present, information on the chemical composition of many plants is limited. Their biological activity and level of safety (cytotoxicity and phototoxicity) have also been poorly studied. There are a large number of natural products with phototoxic action, such as some essential oils, plant extracts, pigments and others [3, 12, 28]. Plant extracts contain different types of molecules. Some of these molecules contain heterocyclic rings, such as phenolics and flavonoids, which is a prerequisite for photosensitivity and possibly for phototoxic action [10]. It is known from the literature that the plants of the Asteraceae family, to which the species *Tanacetum vulgare* refers, have two groups of photoactive compounds in their chemical composition, such as polyacetylenes and their thiophene derivatives. Also a flavonoid – 2,3-dehydrosilybin, which is present in the composition of various extracts obtained from natural products, is considered a compound with phototoxic potential [13]. However, the presence of substances with potential phototoxic effects does not always lead to a phototoxic effect of an extract. There are many other molecules in the extract that can neutralize highly reactive free radicals and reactive oxygen species. In addition, the absorbed photon energy can be transferred to nearby molecules by heat dissipation, thus reducing phototoxicity. Our study shows that no phototoxic effect was observed in the tested extracts (PIF < 2). Accordingly, they are safe for use in pharmaceuticals and cosmetics. The extracts EPE, TVA and GSA have the potential to be used as antitumor agents due to their high antitumor activity and selectivity. The observed anti-proliferative effect on the HT-29 cell line (colorectal carcinoma) is well expressed on three tested extracts and is consistent with the findings of other studies that link it to the flavonoids contained in the herbal extracts [2, 24, 36]. High antiproliferative activity was observed in hormone-dependent cancer cells MCF-7 and PC3 treated with TVA extract, which is explained by the presence of phytosterols (*β*-sitosterol) in its typical composition, which is able to specifically recognize and inhibit estrogen receptors [27]. According to the literature, *Epilobium parviflorum* extracts suppress the proliferation of prostate cancer cells. This effect is mainly associated with the presence of ellagitannins (oenothein A and B), which inhibit aromatase and 5-α-reductase enzymes involved in etiology of prostate cancer [37]. On the other hand, there are reports which indicate that this effect is not specific [9, 34]. In addition to this, our studies demonstrate the antiproliferative effect of HT-29 cells treated with EPE. Also, the obtained previous data in our laboratory and by other authors [19, 31, 32] shows that the above extracts contain substances that are potential inhibitors of POP. Since this peptidase is known to accelerate tumor cells division and vascularization of solid tumors [17, 18] its suppression may contribute to the antitumor activity of the whole extract. Additionally, POP inhibitors can be valuable tool for improving memory and cognitive functions in different psychiatric and neurodegenerative diseases [15]. In this respect, the possible applications of the studied medicinal plants extracts deserve to be considered in more details.

Conclusion

In conclusion, the studied extracts (TVA, EPE and GSA) from Bulgarian origin proved not to be phototoxic (PIF < 2) nor cytotoxic at a concentration lower than 60 μg/ml. The investigated extracts can be safety used in different preparations for medicinal, cosmetic and other applications. They suppress tumor cells growth/proliferation in concentration

dependent manner with promising selectivity indices (SI > 2) to cell lines HT-29, HeLa and PC3. All the extract contain components with potential inhibitory properties to POP – an enzyme, involved in tumor growth and in pathogenesis of psychiatric and neurodegenerative diseases. Thus, they can find a number of prospective applications.

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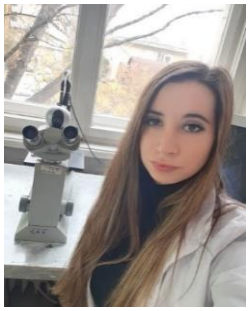

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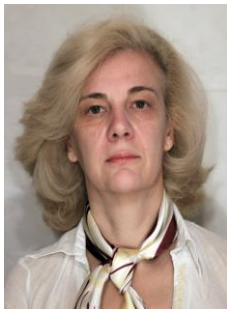

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