https://doi.org/10.31925/farmacia.2021.1.2 **ORIGINAL ARTICLE**

CYTOTOXIC EFFECT OF CHLOROFORM EXTRACTS FROM *TANACETUM VULGARE, T. MACROPHYLLUM* AND *T. CORYMBOSUM* ON HELA, A375 AND V79 CELL LINES

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Abstract

The effect of *Tanacetum* extracts on cell viability was assessed by MTT method on HeLa (human cervical epithelioid carcinoma), A375 (human malignant melanoma) and V79 (Chinese hamster pulmonary fibroblasts). Apoptosis, cell cycle analysis, and genotoxicity tests were performed to identify the possible mechanism of action. Also, the antimicrobial activity was investigated, and LC-MS analysis of extracts was carried out. *Tanacetum* extracts substantially reduced the viability of all tested cells, normal cells being more sensitive than cancer cells. *T. vulgare* and *T. macrophyllum* extracts induced apoptosis in normal cells V79, while none of the extracts induced apoptosis on HeLa or A375 neoplastic cells. *T. vulgare* extract arrested cell cycle progression of V79 and A375 cells at the G_2/M phase and exhibited mild genotoxicity on melanoma cells A375. All extracts showed moderate antimicrobial activity against tested Gram-positive bacteria and fungi.

Rezumat

Efectul extractelor de *Tanacetum* asupra viabilității celulare a fost evaluat *in vitro* prin metoda MTT față de celulele HeLa (carcinom cervical uman epitelioid), A375 (melanom malign uman) și V79 (fibroblaste pulmonare de hamster chinezesc). Au fost efectuate teste de apoptoză, analiză a ciclului celular și genotoxicitate pentru a identifica mecanismul posibil de acțiune. De asemenea, s-a investigat activitatea antimicrobiană și s-a efectuat analiza LC-MS a extractelor. Extractele testate au redus substanțial viabilitatea tuturor celulelor testate, celulele normale fiind mai sensibile decât celulele canceroase. Extractele de *T. vulgare* și *T. macrophyllum* au indus apoptoza în celulele normale V79, în timp ce niciunul dintre extracte nu a indus apoptoza celulelor neoplazice HeLa sau A375. Extractul de *T. vulgare* a blocat progresia ciclului celular a celulelor V79 și A375 în faza G_2/M și a prezentat genotoxicitate ușoară față de celulele melanomului A375. Toate extractele au manifestat activitate antimicrobiană moderată împotriva bacteriilor și ciupercilor Gram-pozitive testate.

Keywords: anticancer activity, antimicrobial, toxicity

Introduction

One of the natural compounds that have attracted the attention of cancer researchers in recent decades is parthenolide, the main sesquiterpene lactone in *Tanacetum parthenium*, which is presently being tested in cancer clinical trials [9]. Sesquiterpene lactones are bioactive molecules primarily isolated from the *Asteraceae* family which exert anti-inflammatory, cytotoxic, antibacterial, antifungal and antiviral activity. The α-methylene-γ-lactone group present in most sesquiterpene lactones is a potent alkylating agent that reacts with sulfhydryl or amino groups in enzymes, transcription factors and other proteins causing steric and chemical changes which hinder their proper functioning [8]. This behaviour is considered to be the primary mechanism underlying the cytotoxic and

antimicrobial activity of sesquiterpene lactones, as well as the emergence of contact dermatitis in humans [1]. *Tanacetum* genus produces three skeletal types of lactones (germacranolides, guaianolides, eudesmanolides), all with a α-methylene-γ-lactone group [26], so it is possible that other *Tanacetum* species manifest cytotoxic activity. The present study aims to evaluate the cytotoxic effects of three species of *Tanacetum*, common in Romanian flora. A small number of studies investigated the activity of *T. vulgare* extracts and compounds against different types of tumour cells [10, 21, 27].

This paper is focused on the identification of the effects and possible action mechanisms of chloroform fractions from *T. vulgare* (T_1), *T. macrophyllum* (T_2) and *T. corymbosum* (T_3) against human cervical carcinoma HeLa, human malignant melanoma A375 and hamster pulmonary fibroblast V79 cells. The *in vitro* experimental approach was oriented to the cell viability, cell cycle analysis, apoptosis and genotoxicity studies to identify and assess the impact on neoplastic and normal cell lines. Another objective was the evaluation of antibacterial and antifungal activity of *Tanacetum* extracts.

Materials and Methods

Plant material and extraction procedure. The aerial parts of *T. vulgare* L. and *T. corymbosum* (L.) Sch. Bip. were harvested at the flowering stage from the spontaneous flora of Romania, near Iași and Valea Putnei, respectively. The leaves of *T. macrophyllum* (Waldst. et Kit.) Sch. Bip. were collected from the indigenous flora area within the Botanical Garden "A. Fătu" Iași, Romania. Voucher specimens were deposited in the Herbarium of Pharmaceutical Botany Department, Faculty of Pharmacy, "Gr T Popa" University of Medicine and Pharmacy, Iași, Romania. The plant material was air-dried and pulverized. 50 g of each plant were extracted three times with chloroform (1:5, 3 hours, 300 rpm) at room temperature. The extracts were filtered and evaporated to dryness. The resulting resins were dissolved in hot ethanol 60% (1:10), cooled to room temperature and extracted with chloroform [25]. The solvent was removed under reduced pressure to afford the dry extracts: 1.11 g *T. vulgare* (T_1), 1 g *T. macrophyllum* (T_2) and 0.1 g *T. corymbosum* (T_3). The extracts were appropriately diluted before injection in HPLC. Phytochemical analyses were also performed on crude chloroform extracts (1:10, 15 minutes ultrasound extraction) obtained from leaves, inflorescences and stems of plants.

Cellular assays

Cell lines. HeLa (human cervical epithelioid carcinoma, ECACC 93021013), A375 (human malignant melanoma, ECACC 88113005) and V79 (Chinese hamster pulmonary fibroblasts, ATCC CCL-93) cells were cultivated in a DMEM medium (Dulbeco's Modified Eagle's Medium, Biochrom AG, Germany), supplemented either with 10% (HeLa and V79) or with 15% (A375) foetal bovine serum (FBS, Biochrom AG, Germany) and also with 100 µg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany). The cell cultures were seeded at different densities according to the type of cell culture plates: 96 (viability assay, with an initial inoculum of 8000 cells/well) or 24 (apoptosis, cell cycle and COMET assays, with an initial inoculum of 20000 cells/well) well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and maintained in a CO_2 incubator (Binder CB 150, Tuttlingen, Germany), at 37°C.

Viability Assay. Cell cultures were seeded in 96 well plates and were allowed to grow overnight. After the medium was discarded and cells were washed, the renewal complete medium was supplemented with the tested extracts in doses of 25, 50, 100 and 200 µg/mL. Duration of treatment was of 24 hours and the cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. After treatment time has expired, the medium was discarded; cells were washed with PBS (phosphate-buffered saline) and covered with 100 µL fresh complete medium. 10 µL of MTT (5 mg/mL) were added in medium and cells were incubated for 3 hours. To solve the formed formazan DMSO (dimethyl sulfoxide, Merck) was used and the absorbance values were recorded at 540 nm [15]. Cellular viability (%) was calculated according to the formula: % cell viability = ($[\text{Absorbance}]_{\text{sample}}/[\text{Absorbance}]_{\text{control}}$) x100.

Apoptosis assay. The apoptosis was evaluated at 6 hours after treatment with extracts (25 µg/mL) by annexin V-FITC/propidium iodide assay [19]. Briefly, cells were detached by trypsinization, washed with cold PBS, resuspended in binding buffer and successively marked with Annexin V-FITC and propidium iodide (provided with eBioscience kit). The analysis of apoptosis was performed with a Beckman Coulter CellLab Quanta SC flow cytometer, equipped with a 488 nm laser and the fluorescence was collected for FITC on FL1 (525 nm bandpass filter) and for propidium iodide on FL3 (670 nm long-pass filter). All data were exported as LMD files and analysed by Flowing Software.

Cell cycle analysis. After 6 hours treatment with the extracts (25 µg/mL), cell suspension was transferred in cold 70% ethanol and incubated overnight at -20ºC. The ethanol permeabilizes the cell membranes, while low temperature preserves the cell integrity. After ethanol was discarded by centrifugation, the cell pellet was suspended in staining solution (propidium iodide, RNase A, Triton X-100), incubated for 40 minutes at 37ºC, pelleted and suspended in PBS for analysis on the flow cytometer (Beckman Coulter CellLab Quanta SC). The analysis used a blue laser for excitation with fluorescence collected on FL3 using a 670 nm long-pass filter [5].

COMET assay. The genotoxic potential was evaluated by the alkaline single-cell gel electrophoresis assay (Comet assay) as described by Olive *et al*. [17]. After incubation with the extracts (25 µg/mL) for 6 hours, cells were detached by trypsinization. The cell suspension (200 µL, about 40,000 cells) was mixed with 1% low melting agarose (1000 µL) at 37°C and quickly poured onto 1% normal melting agarose precoated slides. After agarose gelification, the slides were immersed in freshly prepared cold lysis solution and lysed overnight at 4°C under dark conditions. The slides were further washed with the electrophoresis buffer for three times at room temperature and placed in a horizontal gel electrophoresis tank very close to the anode. The electrophoresis was carried out at 0.6 V/cm for 25 minutes. The procedure was performed under dimmed light to prevent additional DNA damage. After electrophoresis, the slides were rinsed with distilled water. Comets were visualized by ethidium bromide staining (20 µg/mL, 30 seconds) using a fluorescence microscope (Nikon Eclipse 600, Japan). Comet scoring was performed by OpenComet plugin for ImageJ and classified according to Diem *et al*. [6]. The results were expressed as % tail DNA and % head DNA.

Antimicrobial susceptibility tests were performed on Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and pathogenic yeasts (*Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019). All strains were obtained from the Culture Collection of the Department of Microbiology, "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania.

Disc-diffusion method. Antimicrobial activity was evaluated by disc diffusion method according to described protocols [3, 4]. 0.1 mL of each extract was added into stainless steel cylinders (5 mm internal diameter; 10 mm height), applied on the agar surface in Petri plates. Commercial available discs containing Ciprofloxacin (5 µg/disc) and Nystatin (100 µg/disc) were used as positive controls. All assays were carried out in triplicate. Results are expressed as means ± standard deviation.

Broth microdilution method. The extracts were tested for the *Minimum Inhibitory Concentration (MIC)* and *Minimum Bactericidal Concentration (MBC)* against *S. aureus* ATCC 25923 according to described protocols [4, 18]. Serial double dilutions of each extract in Mueller Hinton broth (Oxoid) were inoculated with equal volumes of bacterial suspension (10^6 CFU/mL).

Phytochemical analysis

Methoxylated flavonoids were quantified through a LC-MS method described before [12]. Six standards were used: jaceosidin, eupatilin (ALB Technology, China), casticin, acacetin, eupatorin, hispidulin (Sigma, Germany). Calibration curves in the 0.02 - 6 µg/mL range with good linearity ($R^2 > 0.99$) were used to determine the concentration of methoxylated flavones.

Phytosterols analysis was performed by a previously reported LC-MS method [12] using five standards: β-sitosterol, stigmasterol, campesterol, brassicasterol and ergosterol, acquired from Sigma (Germany). Calibration curves of the sterols in the range of selected concentrations (0.06 - 6 µg/mL) showed a good linear correlation coefficient ($R^2 > 0.99$).

Parthenolide analysis was performed by LC-MS, using a Zorbax SB-C18 reversed-phase analytical column (100 × 3.0 mm i.d., 3.5 µm particles) operated at 45°C. The separation was achieved using a mobile phase consisting of 50:50 (v/v) methanol and ammonium formate 1 mM with a flow rate of 0.8 mL/min and an injection volume of 2 µL. Mass spectrometry analysis was performed on an Agilent Ion Trap1100 SL mass spectrometer with atmospheric pressure chemical ionization (APCI) interface. The instrument was operated in positive ion mode. The gas temperature (nitrogen) was 250°C at a flow rate of 7 L/min, nebulizer pressure 50 psi and capillary voltage 4000 V [11]. Selected reaction monitoring (SRM) was used for detection and quantification of parthenolide, by scanning *m/z* 231.2 from *m/z* 249.2. Calibration curve of parthenolide in the range of selected concentrations (1 - 100 µg/mL) showed an excellent linear correlation coefficient ($R^2 > 0.999$). Parthenolide standard and HPLC grade reagents were obtained from Sigma-Aldrich.

Statistical analysis

All of the experiments were carried out with at least three independent replicates, and all data were expressed as the mean value and standard error (SE) of the mean. The statistical analysis was performed using Student's "t" test, and the differences were expressed as significant at the level of $p < 0.05$.

Results and Discussion

Viability assay

Cell viability percentage variations, as determined by MTT assay, in HeLa, A375 and V79 cells cultures treated with the T_1 - T_3 extracts in doses ranging from 25 to 200 µg/mL are shown in the Figures 1, 2 and 3.

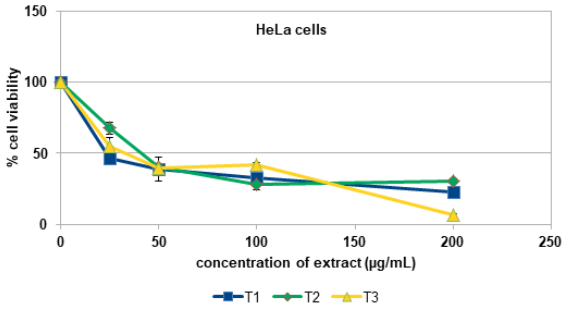

Figure 1.
The viability of HeLa cancer cells treated with *Tanacetum* extracts

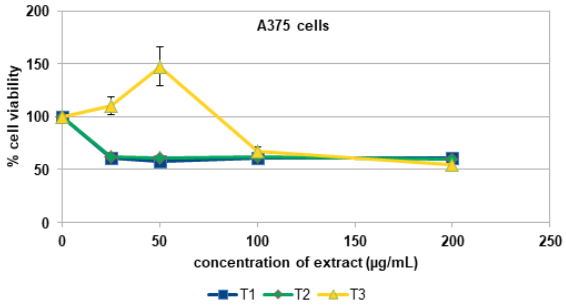

Figure 2.
The viability of A375 melanoma cells treated with *Tanacetum* extracts

As the control group was considered 100% viability for every experimental variant, the tested extracts determined a reduction of cell viability depending on the type of cell and concentration of extract, whit the exception of T_3 extract, which in case of A375 and V79 lines induced a proliferating effect (at 25 and 50 µg/mL). In the case of cancer cell cultures, the cytotoxic impact is more pronounced on HeLa cells, as compared to melanoma cells, the extracts reducing the cell viability in a concentration-dependent manner. All extracts had a pronounced cytotoxic effect on HeLa cells (between 69.87% and 93.71% at 200 µg/mL) while in the case of A375 melanoma cells the maximum cytotoxic effect did not exceed 45% at 200 µg/mL.

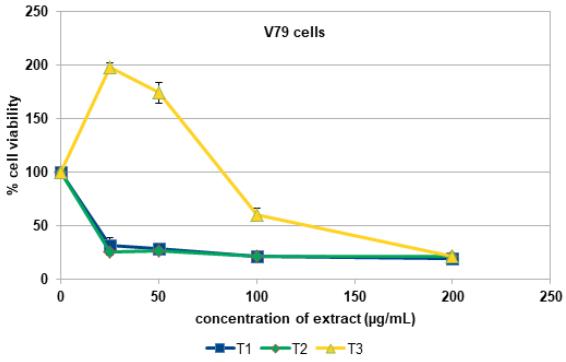

Figure 3.
The viability of V79 cells treated with *Tanacetum* extracts

The cytotoxic impact was higher in the case of normal V79 cells and was manifested even from the lowest concentrations of *T. vulgare* and *T. macrophyllum* extracts (68.78% and 74.60% at 25 µg/mL). We noticed that *T. corymbosum* extract initially induced an increase in cell proliferation on melanoma cells and normal pulmonary fibroblasts, at the first two doses, followed by a significant reduction of cell viability at higher doses. It appears that at low doses, *T. corymbosum* extract is acting as a growth factor on A375 and V79 cells.

In agreement with our results, previous studies reported the cytotoxic activity of *T. vulgare* extracts against J-45.01 human acute T leukaemia cell line [27], colon (WiDr), breast (MDA-MB-231) and lung (NCI-417) human cancer cell lines [20], human cervical adenocarcinoma HeLa, ovarian carcinoma A2780 and breast adenocarcinoma MCF7 [10]. Besides, five eudesmanolides from *T. vulgare* ssp. *siculum* were intensely cytotoxic against human lung carcinoma A549 and healthy hamster lung fibroblast V79379A cells [21].

More than 30 sesquiterpene lactones have been identified to date in *T. vulgare*, mostly of germacran and eudesman type, all possessing the α-methylene-γ-lactone moiety essential for the cytotoxic effect [26]. In the chloroform extracts of *T. macrophyllum* and *T. corymbosum*, similarly prepared as extracts T_1 - T_3, were identified nine and respectively, two sesquiterpene lactones with the α-methylene-γ-lactone group [24, 25]. It is probable that the cytotoxicity of T_1 - T_3 extracts is partially attributable to the sesquiterpene lactones, although other compounds may contribute to the final activity.

Apoptosis assay

Investigation of apoptosis by annexin V/propidium iodide resides in the strong affinity of annexin V for phosphatidylserine residues (normally hidden within the plasma membrane) on the surface of the cell. During apoptosis, phosphatidylserine is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Propidium iodide is used to discriminate between dead and living cells, also allowing the separation between preapoptotic and apoptotic cells in combination with Annexin V. The results of the apoptosis assay are shown in Table I.

Short-term treatment with T_1 - T_3 extracts in doses of 25 µg/mL did not show any significant changes in the distribution of viable, preapoptotic, apoptotic or dead cells in the treated groups as compared with the control in the case of HeLa and A375 cancer cells. A significant increase in the frequency of apoptotic cells was determined for *T. vulgare* and *T. macrophyllum* extracts in the case of normal V79 cells. This could be the mechanisms underlying the high cytotoxic impact of these extracts on normal cells, as observed on the MTT assay.

Table I

Percentage distribution of the viable, dead, apoptotic and preapoptotic cells after treatment with *Tanacetum* extracts

	Control	T_1		T_2		T_3	
HeLa	**Mean (%) ± SE**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**
Living cells	91,03 ± 1,97	92,67 ± 0,82	NS	92,94 ± 0,89	NS	91,09 ± 0,96	NS
Preapoptotic cells	0,00 ± 0,00	0,00 ± 0,00	NS	0,00 ± 0,00	NS	0,00 ± 0,00	NS
Apoptotic cells	8,83 ± 1,90	7,19 ± 0,80	NS	6,94 ± 0,88	NS	8,53 ± 0,84	NS
Dead cells	0,04 ± 0,00	0,15 ± 0,02	< 0.05	0,12 ± 0,01	NS	0,37 ± 0,18	NS
A375	**Mean (%) ± SE**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**
Living cells	86.70 ± 2.1	88.25 ± 1.23	NS	88.51 ± 2.8	NS	86.76 ± 0.89	NS
Preapoptotic cells	2.60 ± 0.05	3.00 ± 0.1	NS	1.60 ± 0.04	NS	2.56 ± 0.05	NS
Apoptotic cells	8.41 ± 0.95	6.84 ± 0.5	NS	6.61 ± 0.9	NS	8.13 ± 0.84	NS
Dead cells	2.29 ± 0.03	1.90 ± 0.01	NS	3.28 ± 0.06	NS	2.56 ± 0.21	NS
V79	**Mean (%) ± SE**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**	**Mean (%) ± SE**	**p**
Living cells	86.09 ± 2.98	23.21 ± 1.83	< 0.001	48.77 ± 1.33	< 0.001	76.54 ± 2.79	NS
Preapoptotic cells	0.01 ± 0.01	0.03 ± 0.01	NS	0.00 ± 0.00	NS	0.00 ± 0.00	NS
Apoptotic cells	13.41 ± 2.88	72.64 ± 1.56	< 0.001	49.45 ± 0.90	< 0.001	22.63 ± 2.84	NS
Dead cells	0.49 ± 0.13	4.12 ± 0.34	< 0.001	1.79 ± 0.68	NS	0.84 ± 0.05	NS

NS – not significant

Cell cycle analysis

Cell cycle checkpoints are used by the cell to monitor and regulate the progress of the cell cycle. The primary cell checkpoints are G_0/G_1 (ensures that everything is ready for DNA synthesis), S (synthesis, duplication of chromosomes), G_2/M (ensures that everything is ready to enter the mitosis phase and divide), and M (ensures that the cell is ready to complete cell division). If a cell does not meet quality standards to pass through any checkpoint, then the cell cycle is stopped, and the cell is directed to apoptosis. The analysis of the cell cycle distribution was performed 6 h after treatment with T_1 - T_3 extracts in a concentration of 25 µg/mL and the results are presented in Table II.

Table II

Percentage of cells in G_1, S and G_2 phases of the cell cycle after treatment with *Tanacetum* extracts

	G_0/G_1: %		S: %		G_2/M: %	
HeLa	**Mean ± SE**	**p**	**Mean ± SE**	**p**	**Mean ± SE**	**p**
Control	49.36 ± 1.20		27.79 ± 1.33		22.85 ± 1.13	
T_1	51.00 ± 1.19	NS	25.23 ± 2.04	NS	23.77 ± 1.21	NS
T_2	44.76 ± 0.89	< 0.05	27.50 ± 1.98	NS	27.74 ± 1.41	NS
T_3	50.22 ± 0.76	NS	28.52 ± 1.00	NS	21.26 ± 0.37	NS
A375	**Mean ± SE**	**p**	**Mean ± SE**	**p**	**Mean ± SE**	**p**
Control	61.96 ± 1.11		17.75 ± 0.49		4.41 ± 0.72	
T_1	58.56 ± 0.39	< 0.05	20.03 ± 0.56	NS	12.67 ± 0.45	< 0.001
T_2	60.82 ± 0.95	NS	20.49 ± 1.02	< 0.05	5.64 ± 0.61	NS
T_3	59.82 ± 1.77	NS	22.99 ± 0.63	< 0.01	10.89 ± 0.52	< 0.001
V79	**Mean ± SE**	**p**	**Mean ± SE**	**p**	**Mean ± SE**	**p**
Control	63.93 ± 0.94		17.53 ± 0.78		11.67 ± 0.35	
T_1	49.28 ± 1.52	< 0.002	16.09 ± 0.72	NS	23.21 ± 0.46	< 0.001
T_2	56.56 ± 2.45	NS	15.66 ± 0.89	NS	15.25 ± 0.62	< 0.01
T_3	60.03 ± 9.43	NS	19.96 ± 0.15	NS	10.25 ± 0.34	NS

NS – not significant

The results show that in the case of V79 cells the treatment with *T. vulgare* and *T. macrophyllum* extracts was responsible for the induction of a block in G_2/M stage and perturbation of cell cycle progression. The occurrence of G_2/M block, justified by a higher frequency of the cells in G_2/M stage compared to the reference, indicates the induction of DNA lesions by T_1 and T_2 extracts that have to be repaired before the cells pass to mitosis. The results on apoptosis suggested a fraction of cells severely damaged by T_1 and T_2 were eliminated while a fraction of cells was in G_0 phase (and reactivated for proliferation) and few other fractions of cells (with minor errors) followed reparatory mechanism. The unrepairable cells were directed to apoptosis and finally to death.

On the other hand, the impact of *Tanacetum* extracts on the progression of the cell cycle in HeLa carcinoma cells was negligible. As for A375 melanoma cells, only *T. vulgare* and *T. corymbosum* influenced the cell cycle progression, with an increase in frequency in the G_2/M stage.

COMET assay

Discrete lesions at DNA level could in time generate errors in the structure of genes or the correct copy

of the information to the daughter cells. Sometimes, those errors are responsible for the occurrence of different pathologies, one of the most prominent being cancer. In these conditions, it is essential to evaluate the genotoxic potential of different compounds used in humans or animals, the COMET assay being a sensitive method for genotoxicity assessment. The results achieved on the reactivity of the genetic material of normal and neoplastic cells to the treatment with T_1 - T_3 extracts, are included in Table III.

Table III

Percentages of DNA content in head and tail of the comets obtained by the treatment of cells with T_1 - T_3 in a dose of 25 µg/mL, for 24 hours

	HeLa			A375			V79		
	%DNA Head	%DNA Tail	p	%DNA Head	%DNA Tail	p	%DNA Head	%DNA Tail	p
Control	94.80 ± 0.69	15.20 ± 0.69		87.18 ± 2.10	12.82 ± 2.10		86,22 ± 1,11	13,78 ± 1,11	
T_1	74.57 ± 2.31	25.43 ± 2.31	NS	73.77 ± 3.13	26.23 ± 3.13	< 0.01	82,95 ± 1,43	17,05 ± 1,43	NS
T_2	65.03 ± 2.48	34.97 ± 2.48	NS	80.63 ± 2.69	19.34 ± 2.69	NS	82,46 ± 1,33	17,54 ± 1,33	NS
T_3	86.93 ± 1.27	13.07 ± 1.27	NS	81.25 ± 2.14	18.75 ± 2.14	NS	83,61 ± 1,02	16,39 ± 1,02	NS

NS – not significant

Data analysis after comet scoring has shown differences in response between cell lines. In the case of HeLa cells, *Tanacetum* extracts determined an increase in the proportion of DNA in the tail suggesting induction of a low damage for T_3 extract (5 - 20% DNA in tail) and a medium damage (20 - 40% DNA in tail) in the case of T_1 and T_2. On A375 cell line, T_2 and T_3 extracts shown a low damage effect on both A375 and V79 cell lines, while in the case of T_1 extract, a medium damage was registered in the case of A375 cells, but not on V79 (low damage). Overall, *Tanacetum* extracts did not exhibit a significant genotoxic impact on the tested cell lines.

Antimicrobial activity

The tested extracts inhibited the growth of *Staphylococcus aureus* and *Candida* sp. and did not affect *Escherichia coli* and *Pseudomonas aeruginosa*, as shown in Table IV. The minimum inhibitory concentrations against *S. aureus* were 0.625 mg/mL for all *Tanacetum* extracts, while minimum bactericidal concentrations were 5, 0.625 and 2.5 mg/mL for T_1, T_2 and T_3 extract, respectively. The results showed that *S. aureus* was more sensitive to the activity of *T. macrophyllum* extract, while the tested fungi were more sensitive to the action of *T. vulgare* extract.

Table IV

Antibacterial and antifungal activities of tested extracts

Extracts	The diameter of inhibition zones (mm)				
	S. aureus	***E. coli***	***P. aeruginosa***	***C. albicans***	***C. parapsilosis***
T_1	14.5 ± 0.50	0	0	15.0 ± 0.00	15.5 ± 0.50
T_2	15.5 ± 0.50	0	0	13.0 ± 0.00	14.5 ± 0.50
T_3	13.0 ± 0.00	0	0	12.5 ± 0.50	12.5 ± 0.50
Ciprofloxacin (5 µg/disc)	31.0 ± 0.00	32.5 ± 0.50	31.0 ± 0.00	NT	NT
Nystatin (100 µg/disc)	NT	NT	NT	22.5 ± 0.50	22.0 ± 0.00

NT – not tested

Studies carried out on the hydro-ethanolic extracts of *T. vulgare* are in accord with our findings: moderate activity on Gram-positive bacteria which the authors attributed to the flavonoids content of the extracts and weak or no activity against Gram-negative bacteria [2, 16]. Muresan *et al*. [16] also reported the lack of activity against fungi. In contrast, we noted significant antifungal activity of the chloroform extracts, probably due to more lipophilic compounds present in the extracts, such as sesquiterpene lactones and methoxylated flavonoids.

Phytochemical analysis

HPLC-MS analysis allowed the identification and quantification of five methoxylated flavonoids and three phytosterols in T_1 - T_3 extracts (Table V).

Table V

Concentration of methoxylated flavonoids and phytosterols in *Tanacetum* extracts (µg/g dry extract)

	T_1	T_2	T_3
Eupatilin	2.16	-	-
Eupatorin	934.86	16.81	-
Acacetin	-	54.86	30.04
Jaceosidin	3970.39	1.95	94.30
Hispidulin	76.29	3.67	60.04
Stigmasterol	196.52	168.26	231.90
β-Sitosterol	1468.24	866.76	3458.50
Campesterol	28.56	19.68	83.50

*Casticin, ergosterol and brassicasterol are absent from all samples

In all plant extracts, sterols concentrations varied in the order: β-sitosterol > stigmasterol > campesterol. *T. vulgare* extract had a remarkably high content of

eupatorin and jaceosidin which presumably contributed to the high cytotoxic effect of the T_1 extract on the tested cell lines.

Prior studies demonstrated eupatorin activity against different cancer cell lines through induction of apoptosis *via* mitochondrial pathway and cell cycle arrest at G_2/M phase, showing at the same time the lack of toxicity against healthy cells [7, 22]. Jaceosidin also promoted programmed cell death in human ovarian cancer cells through the mitochondrial pathway and stopped the proliferation of human bladder cancer cells through G_2/M phase arrest in the progression of cell cycle and apoptosis [13, 14]. Furthermore, the identified phytosterols added to the cytotoxic effect of the extracts, as they have been proven to exhibit direct actions against cancer cells, such as impeding the uncontrolled cell cycle progression, activating apoptosis and inhibiting tumour metastasis [23].

Parthenolide was not identified in any of the concentrated T_1 - T_3 extracts but was found in the brute chloroform extracts from *T. vulgare* flowers and leaves in a concentration of 808.93 µg/g and 4815.28 µg/g, respectively. Figures 4 and 5 present the chromatograms of these two extracts with the parthenolide peak.

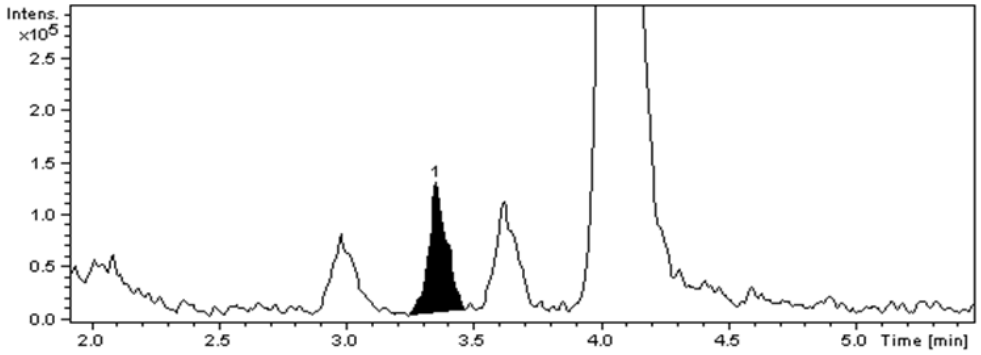

Figure 4.
Chromatogram of the brute chloroform extracts from *T. vulgare* flowers (1 - parthenolide)

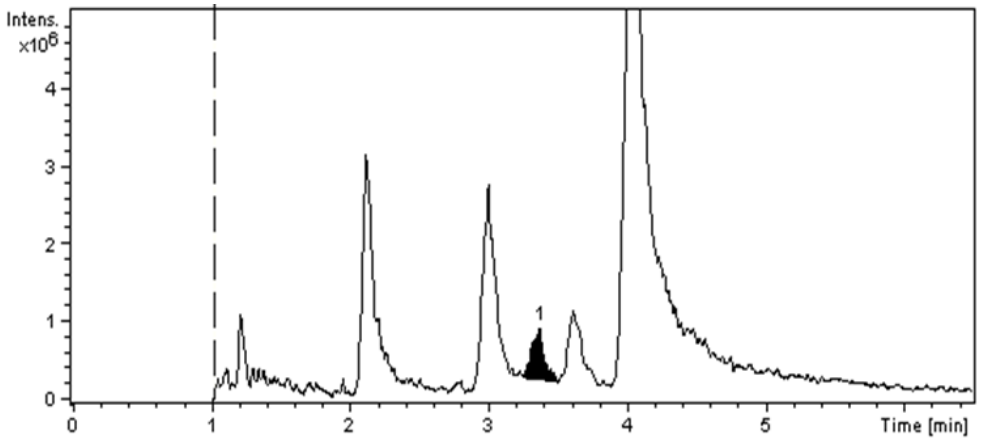

Figure 5.
Chromatogram of the brute chloroform extracts from *T. vulgare* leaves (1 - parthenolide)

Table VI
The concentration of methoxylated flavonoids and phytosterols in *Tanacetum* species (µg/g dw plant)

	***T. vulgare* flowers**	***T. vulgare* leaves**	***T. vulgare* stem**	***T. corymbosum* flowers**	***T. corymbosum* leaves**	***T. corymbosum* stem**	***T. macrophyllum* leaves**	***T. macrophyllum* stem**
Eupatilin	-	0.31	-	-	-	-	-	-
Eupatorin	3.83	41.92	9.37	-	-	-	-	-
Acacetin	-	-	-	-	-	-	2.39	-
Jaceosidin	62.12	750.26	39.94	1.42	1.39	0.90	0.56	0.51
Hispidulin	0.88	7.60	-	-	-	-	-	-
Stigmasterol	94.92	133.69	31.44	73.60	59.33	34.59	56.10	62.87
β-Sitosterol	892.78	1267.68	253.23	1061.15	1044.86	578.13	306.08	358.84
Campesterol	28.50	10.76	6.89	21.00	13.66	15.12	5.77	11.35
Ergosterol	-	-	-	2.35	3.38	-	0.92	-

*Casticin and brassicasterol are absent from all samples

Degradation of the compound during the preparation of the extract can explain the lack of parthenolide in the concentrated extract of *T. vulgare* (T_1). Aside parthenolide, in the crude chloroform extracts, were determined the same compounds as in the concentrated extracts, plus some traces of ergosterol (Table VI).

Conclusions

All tested *Tanacetum* extracts significantly reduced the viability of human cervical epithelioid carcinoma HeLa, human malignant melanoma A375, and Chinese hamster lung fibroblasts V79 cells. The apoptosis assay showed that none of the extracts had any effect on HeLa and A375 neoplastic cells, but *T. macrophyllum*, and especially *T. vulgare* chloroform fractions, induced programmed cell death in healthy cells V79. *T. vulgare* extract also disrupted the cell cycle progression of V79 and A375 cells through a block in G_2/M phase. A similar effect on the cell cycle of A375 melanoma cells was observed for *T. corymbosum* extract. The COMET assay highlighted a mild genotoxic impact of *T. vulgare* chloroform fraction on melanoma cells. In the antimicrobial test, all extracts inhibited the growth of *Staphylococcus aureus* and *Candida* sp. and did not affect tested Gram-negative bacteria. Phytochemical analysis of the extracts allowed the identification of active compounds with known anti-cancer properties which contribute to the overall cytotoxic effect, along with previously described sesquiterpene lactones.

Among the three species, *T. vulgare* appears to be more toxic against the tested cell lines and the healthy cells V79 are the most sensitive to the action of all *Tanacetum* extracts. Further studies are required to isolate and individually test the active principles of *Tanacetum* species.

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Conflict of interest

The authors declare no conflict of interest.

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