EXPLORING THE ANTIOXIDANT, ANTITUMOR, AND IMMUNOMODULATORY POTENTIAL OF *TANACETUM VULGARE* L. EXTRACT: A BIOCHEMICAL AND PHARMACOLOGICAL PERSPECTIVE

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This study evaluated the antioxidant and antitumor potential of a 70% ethanolic tansy (Tanacetum vulgare L.) extract from Romania. The extract exhibited strong antioxidant activity, correlating with high phenolic content (23 g/100 g). In vitro tests on HEp-2 cells showed significant apoptosis induction (0.20% early, 0.99% late). LC-MS identified 64 bioactive compounds, including flavones, amino acids, and anthraquinones, suggesting broad therapeutic potential. These findings highlight Tansy's promise as an alternative or adjuvant therapy for oxidative stress-related diseases and malignancies.

Keywords: *Tanacetum vulgare*; Tansy extract; antioxidant activity; immunomodulatory potential; antitumoral activity

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1. Introduction

Reactive oxygen species represent the main trigger of infectious, cardiovascular, and cancer diseases [1,2] by suppressing both innate and adaptive immunity [3]. An important strategy in preventing diseases related to oxidative stress, including cancer, is the fight against free radicals [4,5]. In this regard, natural antioxidants, compounds recognized for their role in eliminating free radicals and implicitly reducing the risk of disease [6], have been increasingly tackled as treatment alternatives. The bioactive compounds resulting from the metabolism of medicinal plants represent an essential source of pharmacological products with antibacterial, antiviral, antifungal, and anticancer properties, supporting the immune system [7,8]. Phenolic compounds include flavonoids (e.g., anthocyanins, flavones) and phenolic acids (cinnamic and benzoic acid derivatives), representing the standard indicator in the quantification of antioxidant potential [8].

For this study, we have selected a plant with medicinal properties recognized since ancient times, a miraculous plant with multiple culinary and pharmaceutical uses, *Tanacetum vulgare* (Tansy), native to temperate Europe and Asia. According to a previously published report, tansy flowers are a source of essential oil [9,10], polysaccharides [11], phenolcarboxylic acids and flavones [12], and minerals like manganese, chromium, and magnesium [13]. Most studies have focused on the chemical composition of essential oil extracts, with very few examining hydroethanolic extracts. The concentration of bioactive compounds from the same plant differs greatly depending on climatic factors, specific abiotic and biotic factors, and processing, handling, and conservation techniques [14-16].

Tansy flowers are studied for their antioxidant, antibacterial [17], hepatoprotective [17], anti-parasitic [18], antifungal, antiviral, and cytotoxic properties [19]. The aim of our study is to assess the antioxidant and antitumor activities of a 70% ethanolic dry extract obtained from tansy flowers (*Tanacetum vulgare* L.), harvested from southwest Romania.

2. Materials and Methods

Tanacetum vulgare L. flowers were harvested during peak bloom (July-August 2023) from Sohodolului Valley, Gorj county, Romania. Flowers were naturally shade-dried and stored in laboratory conditions. Reagents and solvents used in the experiments (unless noted) were purchased from Roth, Germany.

2.1. Quality assessment of tansy flowers

The research involved several stages: 1) spectrophotometric quality assessment of tansy flowers using different solvents, 2) extraction and spectrophotometric/HPLC characterization of a selected tansy flowers dry extract (TFE), 3) *in vitro* antioxidant assessment of TFE, and 4) evaluation of TFE antiproliferative activity.

To select the best solvent, which extracts the highest amount of phenolic compounds, tansy flowers were heated for 30 min at 100 °C under a reflux condenser with several solvents (water, 50% ethanol, and 70% ethanol), using a drug: solvent ratio of 1:20 (2.5 g of flowers were heated with 50 mL of each solvent. Extracts were filtered and analyzed for total phenolic content (TPC), flavones ((FLs), and phenolcarboxylic acids (PCAs) using standard colorimetric assays. Absorbances were measured with a Jasco V-530 spectrophotometer (Jasco, Japan).

The total phenolic content (TPC) was determined based on polyphenols' ability to reduce molybdenic compounds (VI) using the Folin-Ciocalteu reagent [20-22]. Samples were left in the dark for 40 minutes. Subsequently, the absorbances were measured at λ = 765 nm and compared to a control sample (which contained all reagents except for tansy flower solutions). Based on a specific calibration curve, results were expressed as g gallic acid/100 g dry herbal product.

***Determination of phenol carboxylic acids (PCAs) content*:** Phenolcarboxylic acids (PCAs) were determined based on the formation of oxymes in the presence of sodium nitrite/ hydrochloric acid and sodium hydroxide [21-23]. Samples' absorbances were measured against a control sample (which contained all reagents except for Arnow) at λ = 525 nm.

Determination of flavones content (FLs): The flavones content was determined based on the chelating reaction with aluminum chloride [21,22,24,25]. The absorbance of the samples was measured against the corresponding control samples (containing all reagents except for aluminum chloride) at λ = 420 nm.

2.2. Obtaining and characterization of a selective dry extract

Based on our previously quantitative analysis of tansy flowers, 70% ethanol was selected for obtaining a selective dry extract (TFE), rich in phenolic compounds. For TFE preparation, 190–200 g of dried tansy flowers were heated twice with ethanol 70% (v/v) under a reflux condenser (100°C) for 30 min., using a 1:10 herbal product:solvent ratio (for the first extraction) and 1:5, for the second one. This is the usual literature protocol for two consecutive extractions of herbal drugs [26,27]. After cooling, the combined filtrates were concentrated, using a rotary evaporator (Buchi R 210-215) to remove the solvent, and then freeze- dried (Christ Alpha 1-2/B Braun freeze-dryer, Biotech International freeze-dryer) to yield 34 g dry extract.

2.2.1. Characterization of TFE dry extract using spectrophotometric methods

The content of active compounds (flavones, phenol carboxylic acids, and total phenolic contents) was determined by spectrophotometry. To evaluate active content, 0.3 g of TFE was dissolved in 100 mL of 70% ethanol by ultrasonication for 10-15 min (Digital Ultrasonic Bath, MRC Laboratories, UK) at 25°C. From this

stock solution, volumes ranging from 0.2 to 0.8 mL, 0.4 to 1 mL, and 0.2 to 0.8 mL were used for evaluating TPC, FLs, and PCAs contents, respectively.

2.2.2. HPLC analysis of TFE

LC-MS/MS analyzed TFE on an AB SCIEX TRIPLE TOF 5600+ mass spectrometer, with a NanoLC 425 system (Eksigent) and an analytical column Eksigent 5C18-CL-120, 300 μM ID, 150 mm length, as described in the literature [28]. Untargeted metabolite identification from SWATH data was performed using MS-DIAL ver.5.3.240719. Databases used for metabolite annotations were downloaded from the MSP spectral kit.

2.3. Evaluation of TFE antioxidant capacity

Antioxidant activity was assessed using two *in vitro* assays:

DPPH: 0.5 mL TFE (0.0238–0.298 mg/mL) was mixed with 3 mL 0.1 mM DPPH; absorbance was measured at 517 nm after 30 min. The mixture was kept in the dark, at room temperature, the absorbance was measured, before (A_{start}) and 30 min after adding the extractive solutions (A_{end}), and the inhibition (%) of DPPH radical activity was calculated according [21,22,29]. Results were expressed as % inhibition, EC_{50}, and Trolox equivalents.

ABTS•+: 0.5 mL TFE (0.01197–0.0597 mg/mL) was added to 3 mL ABTS•+ solution; absorbance was measured at 734 nm after 6 min in the dark [21,22,30]. The reduction in absorbance values represents the inhibition of the ABTS•+. Results were expressed as % inhibition, EC_{50}, and Trolox equivalents.

2.4. In vitro evaluation of antiproliferative activity of tansy hydroethanolic extracts

Cell viability was determined using the MTT assay and cell morphology using a microplate reader (Model EL 307C; BioTek, Shanghai, China) at 492 nm. The Hep2 (human epithelial cells derived from a laryngeal carcinoma) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM (high-glucose), supplemented with 10% fetal calf serum (Himedia, USA), at 37°C in a humidified atmosphere with 5% CO_2 in 96-well microplates (5000 cells/well/200 mL) and routinely cultured in a humidified incubator for 24 h [31]. Cell culture media was removed, TFE was added in serial concentrations (ranging from 0.05 to 10 mg/mL), and reincubated for 72 h. We also tested a control group (DMEM without TFE).

Cytometry using the annexin V staining method is used to determine the number of apoptotic cells. Propidium iodide (PI) stains necrotic cells with degraded DNA, thus allowing the differentiation between apoptotic and necrotic cells [32]. Annexin V-FITC/PI double staining kit (E-CK-A211) assay was used to evaluate the pro-apoptotic activity of TFE on the HEp-2 cell line that was treated with 0.1% TFE extract for 24 hours. The reading was done on the EPICS® XL™ Beckman-Coulter flow cytometer, and the FlowJo 7.2.5 software was used for the analysis.

Cells that were PI negative and Annexin V negative are considered healthy, cells that are PI negative and Annexin V positive are considered apoptotic, and cells that are positive to both PI and Annexin V are considered necrotic [32].

Caspase activity was evaluated using the Carboxyfluorescein Multi-Caspase Activity kit (Enzo Life Sciences, BML-AK117-001). At 24 hours after the treatment, the cells were recovered from the substrate and divided into two: one aliquot was kept as a control, and one aliquot was treated with 2.5 µl FAM-VAD-FMK/100 µl cell suspension, stirred, and incubated for one hour at 37°C, 5% CO_2, protected from light. The cells were washed twice, and 100 µL of each cell suspension was placed in duplicate in a black microtiter plate. The intensity of fluorescein fluorescence was quantified in the TtriStar S LB942 Multimode Reader (Berthold, GMBH), with results presented as the log of the ratio between the integrated value of the treated sample vs. the untreated control cells.

To highlight the activation of the apoptotic pathway, we used Bio-Plex Pro™ RBM Apoptosis Panel 3 #171WAR3CK (Biorad Inc., USA). The cells were collected after treatment, lysed in 750 µl lysate dilution buffer (LDB), and then the suspension was incubated on ice and homogenized by sonication. Detection was based on sandwich ELISA on magnetic microspheres, each with a distinct color code or spectral address to discriminate individual responses within a multiplex reaction. and quantified with Bio-Plex 200, and data analysis was performed using Bio-Plex Manager software.

Global DNA methylation was performed using the 5-mC DNA ELISA Kit (D5326, Zymo Research, USA). 24 hours after treatment, genomic DNA was extracted with the Wizard® purification kit, involving cell lysis, RNase digestion, protein precipitation, and DNA recovery via isopropanol precipitation. Each 100 ng DNA sample was denatured at 98°C for 5 min, cooled on ice for 10 min, and transferred to a 96-well plate for a 1-h incubation at 37°C. Then, anti-5-methylcytosine monoclonal antibody (anti-5-mC mAb) and HRP-conjugated secondary antibody prepared in 5-mC ELISA buffer were added, allowing the detection of 5-mC by measuring the absorbance at 405-450 nm (ELISA plate reader). The percentage of 5-mC in the DNA sample was quantified by using a standard curve and logarithmic regression.

All experiments were triplicated. The obtained data were calculated as the mean ± standard deviation and analyzed using IBM SPSS Statistics 29.0.2.0 for Windows (64–bit) or GraphPad version 7 for Windows (GraphPad, USA). One-way ANOVA with Duncan's/Tukey post-hoc tests was applied. Significant differences between means were determined according to Duncan's multiple range test (MRT)/Tukey post-hoc. Pearson correlation was used for antioxidant assays, with $p < 0.05$ being considered significant.

3. Results and Discussion

3.1. Quality assessment of tansy flowers

Our study showed that hydroalcoholic extracts of tansy flowers contain a high concentration of phenol carboxylic acids and flavones (Table 1). Significant differences were found between the total phenolic, flavones, and phenolcarboxylic acids contents of hydroalcoholic and aqueous solutions. Regarding 70% ethanolic and 50% ethanolic solutions, significant differences were only observed regarding the flavones content. The presence of phenolic compounds in the composition of flowers is also mentioned in the consulted scientific literature [17,21,24-26]. Several studies have shown the presence of flavones (hyperoside, rutin, isoquercitrin, quercetin, luteolin, apigenin), PCAs (chlorogenic, gentisic, caffeic, ferulic, rosmarinic, and chicoric acids) [17,22,26]. However, our results regarding the phenolic contents differ from those mentioned above, as pedoclimatic conditions are a significant factor influencing the active substance contents and biosynthesis.

As seen in Table 1, PCA content for all analyzed solutions was higher compared to the total phenolic contents. These discrepancies are the consequence of the Folin Ciocalteu reagent, which reacts not only with polyphenols but also with other compounds (ascorbic acid, polysaccharides, and amino acids) [33]. Our results are similar to those of other researchers, who found a higher concentration of flavones and total polyphenols in alcoholic extracts compared to aqueous or acetone extracts [25]. Based on our results, we chose 70% ethanol for obtaining tansy flowers' dry extract (TFE). The extraction yield was 15%. TFE is soluble in 70% ethanol, but also in water or phosphate buffer.

Table 1

Quality assessment of tansy flowers by means of spectrophotometric methods

Solvent	Active substances		
	TPC (g gallic acid / 100 g dry herbal product)	**FLs (g hyperoside / 100 g dry herbal product.)**	**PCAs (g chlorogenic acid /100 g dry herbal product)**
Ethanol 70%	4.9276 ± 0.5282^{ac}	2.4042 ± 0.4680^{abc}	6.2068 ± 0.3276^{ac}
Ethanol 50%	4.9382 ± 0.5477^{bc}	1.8396 ± 0.4697^{ac}	6.7912 ± 0.4601^{bc}
Water	2.9676 ± 0.3549	1.5156 ± 0.0952	5.3969 ± 0.089

Legend: Different letters in the same column mean statistical significance ($p<0.05$).

3.2. Characterization of tasy flowers dry extract (TFE)

Table 2 shows the results of spectrophotometric determinations for TFE.

Table 2

Results of spectrophotometric determinations for tansy flowers dry extract (TFE)

Active substance	g/100 g extract
TPC	23.0271 ± 1.7287
FLs	10.8830 ± 2.3963
PCAs	22.2016 ± 2.7845

It has been observed to be enriched in polyphenols, the content being superior to the herbal product 70% hydroalcoholic solution (Table 1).

The polyphenol content of TFE is superior to the results obtained by Devrnja et al. [24] for a methanolic extract from tansy aerial parts, which reported 11.26 g of total polyphenols (expressed in gallic acid)/100 g extract [20].

The chemical composition of *T. vulgare* is complex, with aglycones such as emodin, flavones like velutin, and casticin, as well as esters like colforsin, being recognized as the main antitumor, antibacterial, antiviral, antioxidant, or antifungal active compounds [34-40]. LC-MS/MS analysis identified 50 metabolites, of which 22 key metabolites are presented in Table 3, while Fig. 1 shows the absorption of the metabolite Eupafolin.

Table 3

The most representative metabolites isolated with the highest concentrations. The underlined metabolites are specific only to the inflorescence extract

No	Metabolite	Area
1	alpha-Cyperone	5709042
2	Diosmetin	2939277
3	Emodin	655758
4	3',4',7,8-Tetrahydroxyflavone	2536167
5	Jaceidin	769254
6	Casticin	265840
7	8-(2,3-dihydroxy-2-methylbutyl)-7-methoxychromen-2-one	394920
8	Tyrosine	15246
9	Luteolin	1971318
10	Apigenin	354490
11	Phenylalanine	1627743
12	Chrysosplenetin B	171280
13	Isorhamnetin	443954
14	Tetrahydroalstonine	98419
15	Flecainide	29215
16	Myricetin 3,7,3',5'-tetramethyl ether	200641
17	Eupafolin	110963
18	Resepine	81.260
19	(3*aR*,5*aS*,9*aS*,9*bR*)-5*a*,9-dimethyl-3-methylidene-4,5,6,7,9*a*,9*b*-hexahydro-3*aH*-benzo[g][1]benzofuran-2-one	1097663
20	5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)chroman-4-one	329379
21	1,3,8-trihydroxy-6-methylanthra-9,10-quinone	158000
22	Genipin 1-gentiobioside	8946

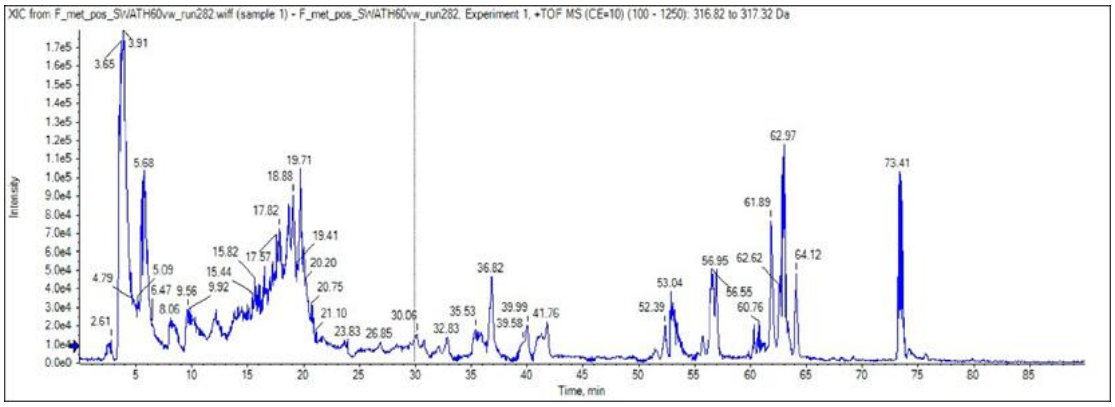

Fig. 1. The fractogram of the separation with the identification of Eupafolin.

Tyrosine is synthesized *de novo* only in plants and microorganisms, being a precursor of numerous specialized metabolites with physiological roles, as electron carriers, antioxidants, attractants, and defense [41]. Flecainide is a class IC antiarrhythmic that inhibits Nav1.5 sodium channels in heart muscle cells and modulates cardiac conduction [42]. Previous studies have reported that eupafolin, a flavonol obtained from *Salvia officinalis*, has antioxidant, anti-inflammatory, and antitumor effects, arresting cancer cell proliferation in the G0/G1 phase through the PI3K/Akt/mTOR signaling pathway [43]. Reserpine is an alkaloid initially identified in the roots of *R. serpentina* and *R. vomitoria*, recognized as an antihypertensive agent, while also being a first-generation antipsychotic [44]. Reserpine represented a significant clinical advance for its antipsychotic role in the treatment of schizophrenia, being one of the drugs that initiated the psychopharmacological era [45].

Alpha-Cyperone, which is most abundant in the inflorescence, is a hydrocarbon used in traditional Asian medicine for the treatment of inflammatory diseases. Its anti-inflammatory activity is linked to the downregulation of COX-2 and IL-6 through negative regulation of the NFκB pathway in LPS-stimulated RAW 264.7 cells [46]. 3',4',7,8-Tetrahydroxyflavone (fisetin) is an oxygenated flavonol whose main antioxidant activity was demonstrated by *in vitro* administration, with the effect of protecting hepatocytes by improving antioxidant competence in liver tissues [47]. Diosmetin, a natural flavonol, is the second most important metabolite in terms of concentration in inflorescences. The pharmacological activities are varied, including anti-inflammatory [48], anticancer, antioxidant, antibacterial, metabolic regulator, cardiovascular protector, and endocrine regulator [49]. Diosmetin also functions as a substrate of the cytochrome P450 enzyme CYP1 [50].

3.3. Evaluation of antioxidant activity of tansy flower dry extract (TFE)

TFE exhibited strong antioxidant activity, especially in the ABTS assay (EC50 = 0.0164 mg/mL) (Table 4). This activity is likely due to the presence of

phenolic compounds, as identified by spectrophotometric and HPLC assays. With the increase in concentration, there was an increase in the percentage of inhibition of ABTS free radical activity, which varies between 47.33% and 89% in the concentration range used. Regarding the DPPH assay, the inhibition varied between 23.20% (for 0.0238 mg/mL) and 63.81% (for 0.298 mg/mL). The obtaining of the highest antioxidant activity with ABTS assay is a consequence of its mechanism of action, as both lipophilic and hydrophilic compounds reduce the free radical [51].

Table 4

Results of antioxidant capacity evaluation

Antioxidant assay	EC50 (mg/mL)	Trolox equivalents (mM/g dry extract)	EDTA-Na equivalents (mM/g dry extract)
DPPH	0.1736 ±0.0044	0.8459 ± 0.2803	nd
ABTS	0.0164 ± 0.0014	4.2625 ± 1.1426	nd

Moreover, we also analyzed the interrelation between the antioxidant assays using Pearson correlation. The results showed a direct correlation, but statistically insignificant, between all the methods used, except for the reduction or chelation of iron, for which a negative correlation was found (Table 5).

Table 5

Correlation (Pearson coefficients) between the antioxidant methods used to test the antioxidant capacity of the dry hydroalcoholic extract.

Method	DPPH	ABTS
DPPH	-	0.1465 (p = 0.7539)
ABTS	-	-

3.4. Antiproliferative, pro-apoptotic, and cytotoxicity to cancer cell lines

The rate of annexin V-positive cells after treatment with TFE showed that the apoptosis was slightly increased. Early and late apoptosis rates in cells treated with 0.15 mg/mL TFE were 0.03% and 0.19%, compared to the control of 0.22% and 0.03%, respectively. Early and late apoptosis rates in cells treated with 0.3 mg/mL TFE were 0.20% and 0.99%. Also, necrosis increased from 1.96% (treatment with 0.15 mg/mL TFE) to 3.53% (treatment with 0.3 mg/mL of TFE) (Fig.2).

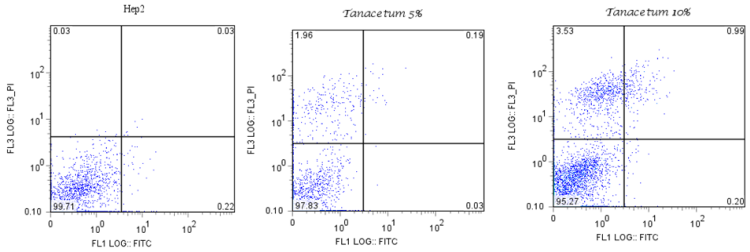

Fig.2. Flow cytometric analysis of Annexin V-FITC/PI staining. Dot plots show the percentage of viable (lower left quadrant), early apoptotic (lower right quadrant), late apoptotic (upper right quadrant), and necrotic (upper left quadrant) cells in untreated (control - first graph) and cells treated with 0.15 mg/mL and, corresponding, 0.3 mg/mL of TFE. The results are expressed in the histogram as total percentages of cells from four different groups.

The evaluation of pro-apoptotic activity revealed that *T. vulgare* extract induces a moderate activation of caspases, compared to that of quercetin or caffeic acid (Fig.3). This result is notable considering the low concentration of the extract and the fact that it contains a number of other components. Therefore, these results support the idea that *T. vulgare* possesses modest pro-apoptotic activity at the tested concentration (0.3 mg/mL), with biologically relevant potential.

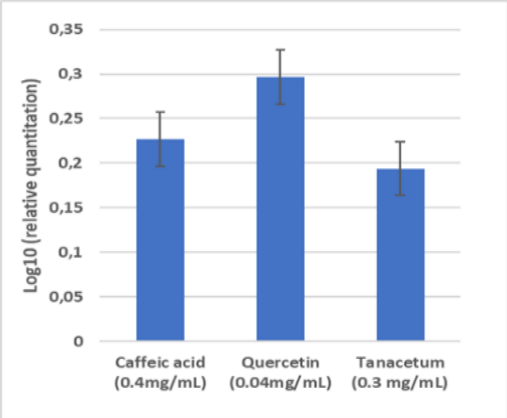

Fig.3. Apoptosis-inducing capacity of 0.3 mg/mL TFE compared to the effects of caffeic acid and quercetin. Results are presented as the mean ± SD (n = 3) of the logarithmic ratio between the integrated value of the treated sample and that of the untreated control cells.

At the protein level, a slight increase in activated caspase-3, Bcl-xL/Bak ratio, and surviving following treatment with TFE was observed, while the Mcl-1/Bak ratio showed a slight decrease (Fig.4). These results support the low percentage of mortality quantified by annexin-V and propidium iodide staining.

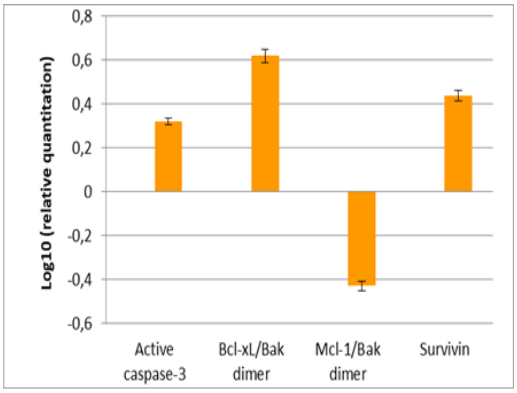

Fig.4. The protein expression levels of activated caspase-3, Bcl-xL, Mcl-1, and Survivin in HEp-2 cells following treatment with 0.3 mg/mL TFE. Results are expressed as mean ± SD (n = 3) of the logarithmic ratio between the integrated value of the treated sample and that of the untreated control cells.

In our experiments, treatment with TFE resulted in a slight reduction in global DNA methylation levels, as indicated by a decrease in the percentage of 5-

methylcytosine (%5-mC) relative to total DNA. In comparison, treatments with caffeic acid and quercetin had a more pronounced hypomethylating effect (Fig.5).

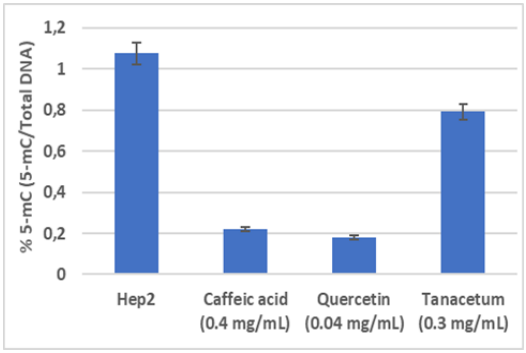

Fig.5. Global DNA methylation (% 5-mC) in HEp-2 cells treated with caffeic acid (0.4 mg/mL), quercetin (0.04 mg/mL), and TFE (0.3 mg/mL) for 72 hours. Negative control (HEp-2 cells). Results are expressed as mean ± SD (n = 3).

3.5. Discussion

Determining the antioxidant activity of phytochemicals is crucial in the preliminary analysis of medicinal plants, as it indicates their potential as antimicrobial and subsequently antitumor agents. Thus, our study proposes a thorough investigation of the relevant biological properties of Tansy extract to establish its therapeutic potential.

The lowest content of active ingredients was obtained for the aqueous solutions. The highest concentration of flavonoids was obtained from hydroalcoholic extracts at 70% concentration, and the highest concentrations of polyphenols and phenol carboxylic acids were in hydroalcoholic extracts at 50% concentration.

Comparing the results obtained by us with data from the specialized literature was difficult due to the different methods and solvents used for extracting the active substances. Thus, Baranauskiene et al. [29] determined a content of 14.23 g total polyphenols (expressed in gallic acid)/100 g extract, the value being lower than the one determined by us.

The antioxidant activity and concentration of bioactive compounds of tansy flower dry extract are corroborated by the therapeutic, antibacterial, and antitumor properties cited in the literature [52]. In our case, the antioxidant activity (determined by the ABTS method) of the Tansy extract was 4.2625 ± 1.1426 (equivalent to Trolox mM/g dry extract), with a degree of free radical inhibition of 89.91 ± 7.56%. The antioxidant activity of the extract was most likely imprinted by the phenolic constituents but also by the lipophilic active principles (sterols, sesquiterpenes), soluble in 70% ethanol, and cited in the consulted sources [21,53].

Other researchers also evaluated the antioxidant activity. For instance, Muresan et al. in 2015 [26] determined an antioxidant activity of 0.06-0.07 mg/mL

(by the DPPH method) for hydroalcoholic solutions (70% ethanol) obtained from aerial parts of *T. vulgare*, which indicates an antioxidant activity superior to that obtained by us for the dry extract (the lower the IC 50 value, the stronger the antioxidant effect). We believe the differences are likely due to the different harvesting areas and pedoclimatic conditions. Our results are closer to the values obtained by Ivănescu et al. [52], who found that for a methanolic extract, an antioxidant activity of IC50=0.248 mg/mL (DPPH method), respectively IC50=0.112 mg/mL (iron reduction capacity) [21]. Nonetheless, the different extraction methods and the solvent used did not allow us to make a fair comparison of the results obtained [21,54].

Concerning the identified metabolites, 50 were isolated from the flower extract. The most abundant metabolites identified in the inflorescence extracts were alpha-Cyperone, which showed the highest concentration expressed in the average area, 3',4',7,8-Tetrahydroxyflavone, followed by Diosmetin, Luteolin, Phenylalanine, Emodin, and Jaceidin, which are often described in the specialized literature concerning their antioxidant and antitumor activities.

We observed that the obtained TFE had pro-apoptotic effects. Apoptosis is a well-structured and orchestrated process with an important role in regulating cell number and tissue homeostasis by removing aged, damaged, and unwanted cells. This process is essential for organ development, tissue remodeling, and immune regulation [55]. Still, the essential feature of carcinogenesis is that dividing tumour cells fail to initiate apoptosis in response to DNA damage. In our study, treatment with TFE resulted in a significant increase in the percentage of cells undergoing both late apoptosis and necrosis, indicating that DNA fragmentation and cell membrane disintegration are the primary modes of cell death induced by the extract. This effect was more pronounced at a higher extract concentration.

Apoptosis mechanisms are carried out by two signaling pathways, the mitochondrial (intrinsic) and the membrane receptor death pathway (extrinsic). The death receptor-mediated pathway involves tumor necrosis factor, activating caspase-8, while the mitochondrial pathway, regulated by Bcl-2 family proteins, involves the release of the respiratory chain component cytochrome c into the cytoplasm [56]. Both these pathways lead to caspase 3 activation as an apoptosis effector [57]. Bcl-xl, Bak, and Mcl-1, members of the Bcl-2 family [58], as well as Survivin, a member of the inhibitor of apoptosis (IAP) protein family [59], is involved in the regulatory processes of apoptosis. The interaction between these components primarily consists of the regulation and execution of the apoptotic process: anti-apoptotic proteins (such as Bcl-xl, Mcl-1, and survivin) exert their roles to prevent caspase 3 activation and the initiation of apoptosis, while pro-apoptotic proteins (such as Bak) are upregulated and attempted to be suppressed to maintain cell survival [59,60].

In our study, TFE treatment induced an increase of Bcl-xL/Bak dimers, survivin, and activated caspase-3, and a decrease of Mcl-1/Bak dimers. Caspase-3 activation indicates the activation of apoptotic signaling pathways, particularly the execution phase. Additionally, the slight decrease in Mcl-1/Bak dimers suggests a reduced anti-apoptotic influence, thereby facilitating Bak activation and engagement of the mitochondrial pathway. Still, the increase in survivin expression, along with caspase-3 activation, may reflect a complex regulatory balance where cells attempt to counteract apoptosis. Moreover, Bcl-xL interaction with Bak may reflect an attempt by the cell to regulate mitochondrial outer membrane permeabilisation. Our data suggests that *T. vulgare* extract exerts a detectable pro-apoptotic effect, possibly through a partial activation of multiple caspases, indicating a selective cytotoxic potential or an indirect mechanism of action on the apoptotic pathway. Given the complexity of the extract, its effect may be modulated by the combination of phytochemicals and not exclusively manifested by direct activation of caspases. Also, compared to pure substances (quercetin, caffeic acid), Tanacetum extract may involve synergistic or additional mechanisms (anti-inflammatory, oxidative, or immunomodulatory).

DNA methylation, specifically at the 5-position of cytosine residues, is involved in controlling gene silencing, cellular differentiation, and maintaining genomic stability [61]. A decrease in 5-mC levels induced by TFE treatment may reflect an alteration in gene expression regulation, possibly promoting the activation of specific genes associated with cell cycle regulation, stress responses, or apoptosis. Integrated with apoptotic effects, the reduction in 5-mC could be a sign of DNA demethylation, which may activate genes involved in programmed cell death. DNA methylation is often linked to the suppression of pro-apoptotic genes, and demethylation can lead to the re-expression of these genes, thereby triggering apoptosis.

5. Conclusions

This study evaluated the biological activity of an extract obtained from Tanacetum vulgare flowers collected from a wild environment in Romania. The dry extract prepared using 70% ethanol showed a high content of biologically active substances. The extract showed strong antioxidant activity in multiple assays, demonstrating free radical scavenging and metal chelation capacities. In vitro, it induced apoptosis in HEp-2 cells, characterized by increased caspase-3 activation and changes in Bcl-xL/Bak and Mcl-1/Bak ratios, as well as a slight decrease in global DNA methylation. The results of our study demonstrate that Tanacetum vulgare extract exhibits promising antioxidant and antitumor effects, supporting its potential for therapeutic use.

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