ORIGINAL ARTICLE

ANTIOXIDANT, ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF *TANACETUM VULGARE, TANACETUM CORYMBOSUM* AND *TANACETUM MACROPHYLLUM* EXTRACTS

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Abstract

The aim of this study was the evaluation of antioxidant, antimicrobial and cytotoxic activity of three *Tanacetum* species from Romanian flora and the identification of biologically active compounds in the analysed species. *T. vulgare* and *T. corymbosum* showed antioxidant activity in correlation with the total phenolic content of the extracts. All three species exhibited antimicrobial effect against the tested Gram-positive bacteria and fungi, and manifested strong cytotoxic activity against both cancer (HeLa) and healthy (Vero) cell lines. The chemical analyses allowed the identification of common phytosterols and many flavonoids, some reported for the first time: casticin in *T. vulgare*, eupatilin, casticin, acacetin, quercitrin and isoquercitrin in *T. macrophyllum*, eupatorin, apigenin and quercitrin in *T. corymbosum*. It is possible that the numerous flavonoids found in these species to contribute to the pharmacological actions of the extracts.

Rezumat

Studiul de față a avut drept scop evaluarea acțiunii antioxidante, antimicrobiene și citotoxice a extractelor obținute din trei specii de *Tanacetum* din flora României și identificarea de compuși biologic activi în speciile analizate. *T. vulgare* și *T. corymbosum* au prezentat o bună acțiune antioxidantă, corelată cu conținutul total fenolic al extractelor. Toate speciile analizate au exercitat un efect antimicrobian față de bacteriile Gram-pozitive și fungii testați și au manifestat o puternică activitate citotoxică, atât asupra celulelor canceroase (HeLa), cât și asupra celor normale (Vero). Analizele chimice au permis identificarea unor steroli și a numeroase flavonoide, unele raportate pentru prima dată la speciile respective: casticină în *T. vulgare*, eupatilină, casticină, acacetină, cvercitrină și izocvercitrină în *T. macrophyllum*, eupatorină, apigenină și cvercitrină în *T. corymbosum*. Este posibil ca numeroasele flavonoide identificate în aceste specii să contribuie la acțiunea farmacologică a extractelor testate.

Keywords: polyphenols, methoxylated flavones, phytosterols

Introduction

Tanacetum is a large genus with approximately 160 species distributed in the Northern Hemisphere. In the spontaneous Romanian flora, *Tanacetum* genus is represented by five native taxa (*T. achilleifolium, T. corymbosum, T. macrophyllum, T. millefolium, T. vulgare*) and one naturalized species, *T. parthenium* [20]. Different *Tanacetum* species are used as cosmetics, insecticides, balsams, dyes, food preservatives, flavouring agents and herbal remedies [2, 13].

In Romanian traditional medicine, flowers and aerial parts of *T. vulgare* (tansy) are used as anti-helminthic, stomachic, febrifuge and emmenagogue and *T. parthenium* (feverfew) for treating headache and earache [9]. Various herbal supplements with tansy flowers are marketed in Romania, in the form of hydro-alcoholic extracts, glycerinated macerate or dry packed plants for infusion, alone or in combination with other medicinal plants. In Mediterranean regions, infusions of *T. corymbosum* (corymbflower tansy) inflorescence are used to treat parasitic intestinal worms' infections and infusions of *T. vulgare* as relaxant in stomach pain [1].

Previous studies demonstrated antioxidant, anticancer, anti-inflammatory, anti-ulcer, anthelmintic, antiviral, antibacterial, vasorelaxant and insect-repellent activities of *Tanacetum vulgare* which are mostly attributable to compounds such as sesquiterpene lactones, volatile

oils, flavonoids and phenolic acids [13]. It is worth mentioning that *T. vulgare* shows an impressive intra-specific variability of chemical constituents, as seen in the volatile oil composition, but also in the non-volatile fraction: different chemo-types exist depending on the class of sesquiterpene lactones [23]. By comparison with common tansy, *T. corymbosum* and *T. macrophyllum* (rayed tansy) have been less studied: the antimicrobial activity of their essential oils and anticoagulant and anti-fibrinolytic activities of aqueous and chloroform extracts have been reported to date [13].

Literature data on the three species are mostly related to the volatile oils and sesquiterpene lactones, whereas information regarding other compounds is scarce, notably for *T. corymbosum* and *T. macrophyllum*. That is why, our research focused on polyphenols, especially flavonoids, and phytosterols as bioactive compounds. The present study aims to investigate the antimicrobial, antioxidant and cytotoxic effects of *T. vulgare, T. macrophyllum* and *T. corymbosum* in order to better characterize these medicinal plants and their health benefits.

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 (47□ 04′ 47″ N, 27□ 39′ 49″ E) and (47□ 29′ 27″ N, 25□ 21′ 19″ E), 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. All three species were gathered in July 2016 and a voucher specimen of each was deposited in the Herbarium of Pharmaceutical Botany Department, Faculty of Pharmacy, "Gr T Popa" University of Medicine and Pharmacy Iași, Romania. The plants were air-dried at room temperature and grounded to a fine powder. 10 g of plant material was extracted with 100 mL methanol for 15 minutes in an ultrasonic bath, at room temperature, and then filtered. The extracts were appropriately diluted before injection in the HPLC system.

Phytochemical analysis

Polyphenols were quantified using a HPLC-UV-MS method, previously described [12]. Eighteen polyphenolic standards were used: caffeic acid, chlorogenic acid, p-coumaric acid, kaempferol, apigenin, rutin, quercetin, quercitrin, isoquercitrin, fisetin, hyperoside, myricetin (Sigma, Germany), ferulic acid, gentisic acid, sinapic acid, patuletin, luteolin (Roth, Germany), caftaric acid (Dalton, USA). Calibration curves in the 0.5 - 50 µg/mL range with good linearity ($R^2 > 0.999$) were used to determine the concentration of polyphenols in plant samples.

Methoxylated flavonoids were quantified through a LC-MS method described before [15]. 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 [15] 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 ($R^2 > 0.99$).

Determination of total phenolic, flavonoid and phenolic acids contents

The concentration of total phenols in plant extracts was estimated by Folin-Ciocâlteu method [17] and the flavonoids content was estimated by the aluminium chloride colorimetric method [17] using an Able JascoV-550 UV-VIS spectrophotometer. Total phenolic content (TPC) was expressed as gallic acid equivalents (mg) in 1 g of dry material (mg GAE/g dw). Total flavonoids content (TFC) was expressed as mg quercetin equivalents per gram dry weight of sample (mg QE/g dw). Total phenolic acids content (TAC) was determined using the method described in the European Pharmacopoeia [7] and the results were expressed as mg chlorogenic acid equivalents *per* gram dry weight of sample (mg CGAE/g dw).

Antioxidant tests

The antioxidant activity of extracts was evaluated by DPPH radical-scavenging method and reducing power assay [17]. For this, the crude methanol extracts were evaporated to dryness and dissolved in DMSO at different concentrations ranging from 12.5 to 200 mg/mL. The antioxidant activities of extracts were expressed as the efficient concentration EC_{50} and quercetin was used as a positive control. All measurements were carried out in triplicate and results were expressed as mean value ± standard deviation.

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

The antimicrobial activity was evaluated by the disc diffusion method according to previously described protocols [5, 6]. 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 dishes. Commercial available discs containing nystatin

(100 µg/disc) and ciprofloxacin (5 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* according to described protocols [6]. Serial double dilutions of each extract in Mueller Hinton broth (Oxoid) were inoculated with equal volumes of bacterial suspension (10^6 CFU/mL).

Cytotoxicity assay

The cytotoxic activity was investigated against human HeLa cervical cancer cell line and normal African green monkey kidney epithelial cell line (Vero). The crude sesquiterpene lactones fraction, obtained as described by Todorova *et al*. [24], was used in this test. After 24 h incubation with the tested extracts in different doses (25 - 200 µg/mL) the cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test [16]. All results were expressed as mean ± standard deviation from triplicate determinations.

Results and Discussion

Phytochemical analysis

LC-MS analyses allowed the identification of the phenolic compounds listed in Table I. The results show the presence of previously identified flavonoid aglycones in *T. vulgare*, such as quercetin, luteolin, kaempferol, apigenin, eupatilin, acacetin and hispidulin [14, 17, 25] and of newly reported casticin. Methoxylated flavones jaceosidin and eupatorin described before in South-American *T. vulgare* and partially responsible for the anti-inflammatory effect of the plant [21] were not found in the extract, probably due to the existence of various chemotypes.

Table I

Concentration of phenolic compounds in *Tanacetum* species (µg/g dw)

	T. vulgare	*T. macrophyllum*	*T. corymbosum*
Chlorogenic acid	2576.08	63.66	937.87
Quercetin	8.89	-	-
Luteolin	30.95	-	*
Kaempferol	*	-	-
Apigenin	*	*	*
Quercitrin	-	*	205.56
Isoquercitrin	-	*	-
Eupatilin	0.19	144.09	-
Eupatorin	-	1.48	143.95
Acacetin	0.39	0.48	-
Jaceosidin	-	0.11	-
Casticin	42.91	0.29	1.60
Hispidulin	0.21	55.83	104.21

*compound present in sample (MS qualitative determination, UV signal < limit of quantification)

T. macrophyllum extract is characterized by a high number of methoxylated flavonoids, among which eupatilin (6-hydroxyluteolin 6,3',4'-trimethylether) and hispidulin (scutellarein 6-methylether) are in notable concentrations. Eupatilin, acacetin, casticin, quercitrin and isoquercitrin were identified for the first time in rayed tansy, while apigenin, eupatorin (6-hydroxyluteolin 6,7,4'-trimethylether), jaceosidin (6-hydroxyluteolin 6,3'-dimethylether) and hispidulin were reported before in *T. macrophyllum* [28]. Six flavonoids were found in *T. corymbosum* extract: three of them reported earlier in this species - luteolin, casticin, hispidulin [10, 28], and three newly identified in corymbflower tansy - apigenin, quercitrin and eupatorin.

The present results are in agreement with previous research published by Williams *et al*. who found that the lipophilic flavonoids of *T. vulgare*, *T. macrophyllum* and *T. corymbosum* are methyl ethers of scutellarein and 6-hydroxyluteolin. They also reported the anti-inflammatory activity of these 6-hydroxyflavones which act as inhibitors of cyclooxygenase and 5-lipoxygenase [28, 29].

It is well known that flavonoids exhibit a multitude of therapeutic actions, such as: anti-oxidative, anti-inflammatory, anti-cancer, hepato-protective, cardio-protective and antimicrobial. Their main disadvantage is the low bioavailability. But this is not the case for methoxylated flavonoids which have high oral bio-availability compared to other polyphenols due to their lipophilic nature and increased metabolic stability [26]. That is why methoxylated flavonoids, such as these found in *Tanacetum* species can be promising therapeutic candidates.

Among the phenolic acids, only chlorogenic acid was identified in all plants, in higher amount in *T. vulgare*. Muresan *et al*. [17] previously reported that chlorogenic acid is the major phenolic acid in tansy. On the other hand, Baczek *et al*. concluded that cichoric acid was the main hydroxycinnamic acid in

tansy, followed by chlorogenic acid (925.7 mg%), caffeic acid, ferulic acid and rosmarinic acid [2]. Caffeic acid was not found in any sample and for *T. vulgare* this result confirms the conclusion of another research conducted on Romanian plants [17].

Phytosterols analysis showed that all three *Tanacetum* species contain beta-sitosterol, stigmasterol and campesterol and traces of ergosterol, while brassicasterol is not present in any sample (as seen in Table II). Beta-sitosterol is the major sterol in all species, followed by stigmasterol and campesterol.

Table II

Concentration of phytosterols in *Tanacetum* species (µg/g dw)

	Stigmasterol	β-Sitosterol	Campesterol	Ergosterol
T. vulgare	89.90	696.32	20.88	0.76
T. macrophyllum	88.90	530.78	6.16	3.14
T. corymbosum	45.46	678.50	15.86	1.62

*Brassicasterol is absent from all samples

Regarding *T. vulgare*, our results confirm the findings of Chandler *et al*. who identified stigmasterol, campesterol and cholesterol, with beta-sitosterol as the major sterol in tansy [4]. The sterols of *T. macrophyllum* and *T. corymbosum* have not been previously reported. Plant sterols are considered to be functional food ingredients due to their health-promoting effects: they lower the circulating cholesterol level and offer protection against cardiovascular diseases; they also exhibit anti-inflammatory and anticancer properties and could be involved in the prevention of dementia [22].

Antioxidant activity

Both antioxidant tests showed a moderate activity for *T. vulgare* and *T. corymbosum* extracts, and a weak activity for *T. macrophyllum* extract. In all cases, the antioxidant effect was well correlated with the phenolic content of the plant material (Table III).

Table III

Antioxidant activity and content of phenolic compounds in *Tanacetum* extracts

	DPPH radical-scavenging assay EC_{50} (µg/mL)	Reducing power assay EC_{50} (µg/mL)	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAC (mg CGAE/g dw)
T. vulgare	242.8 ± 2.1	112.06 ± 1.1	26.37	1.38	0.55
T. macrophyllum	3269.56 ± 22.3	598.59 ± 1.39	0.64	0.30	0.09
T. corymbosum	344.8 ± 2.6	116.80 ± 0.94	5.90	3.76	0.40

T. corymbosum extract manifested a Fe^{3+}-reducing capacity comparable to that of common tansy, but a lower DPPH-scavenging ability. Also, it is important to note the high flavonoid content in *T. corymbosum* compared to the other species, in accord with the results of LC-MS analysis. We can assume that flavonoids are the principal compounds responsible for the antioxidant activity of *T. corymbosum*, especially since total phenol content is quite low in this extract. The low antioxidant activity of *T. macrophyllum* compared to the other two species and literature data [18] could be attributable to the different plant part used in this analysis: only leaves, as opposed to blooming aerial parts.

Tansy extract showed the highest activity in both antioxidant tests that was associated with the highest total phenol and phenolic acids content. Only the total flavonoid content was lower compared to that of *T. corymbosum*. The total polyphenol content of tansy was close to that previously reported for a 70% methanol extract from aerial parts (30.42 mg GAE/g), where it decreased in the order inflorescences > herbs > roots [27]. Baczek *et al*. concluded that the strong antioxidant activity of tansy is mainly due to the high amounts of phenolic acids and less to the flavonoid glycosides present in the hydro-ethanolic extract [2]. A stronger antioxidant effect was reported for aqueous and acetone extracts of *T. vulgare* and was attributed to mono and dicaffeoylquinic acids and flavonoid glycosides in the first case and to flavonoid aglycones in the second [3]. Furthermore, 3,5-O-dicaffeoylquinic acid, axillarin and luteolin were found to be the main antioxidant constituents of the crude methanol extract, responsible for its good DPPH radical-scavenging effect [11].

Antimicrobial activity

The diameters of the inhibition zones corresponding to the tested extracts are shown in Table IV. All extracts showed moderate activity against Gram-positive bacteria and fungi and had no effect on Gram-negative bacteria. *T. corymbosum* extract inhibited more intensely the growth of *Staphylococcus aureus* (MIC 3.12 mg/mL) compared with the other two extracts (MIC 6.25 mg/mL each). The MBC values of *Tanacetum* extracts were twice their concentrations inhibiting the growth of *Staphylococcus aureus*. The antibacterial activity of *T. corymbosum* could be related to the high concentration of flavonoids in the extract. The antifungal activity on *Candida* sp. varied in the order *T. vulgare* > *T. macrophyllum* > *T. corymbosum*.

Table IV

Antibacterial and antifungal activities of *Tanacetum* extracts

	Diameter of inhibition zones (mm)				
	S. aureus	*E. coli*	*Pseudomonas aeruginosa*	*C. albicans*	*C. parapsilosis*
T. vulgare	16.5 ± 0.50	0	0	12.0	12.0
T. macrophyllum	13.0	0	0	10.0	10.0
T. corymbosum	17.3 ± 0.57	0	0	9.0	9.0
Ciprofloxacin (5 µg/disc)	31.0	32.5 ± 0.50	31.0	*NT	*NT
Nystatin (100 µg/disc)	NT*	NT*	NT*	22.5 ± 0.50	22.0

*NT - not tested

Regarding *T. vulgare*, some studies undertaken on hydro-alcoholic extracts support our results, showing moderate activity on Gram-positive bacteria and weak activity on Gram-negative bacteria [2, 17].

Cytotoxic activity

The *in vitro* cytotoxic activity was investigated using the MTT assay, an efficient method for assessing mitochondrial impairment which correlates with the number of metabolically active cells, thus measuring the cell viability. After 24 hours of incubation with HeLa cells, all extracts strongly reduced the viability of cancer cells in a dose-dependent manner (Figure 1). At the highest applied concentration (200 µg/mL), cell growth inhibition ranged from 69.87% for *T. macrophyllum* extract, to 77.68% for *T. vulgare* extract and 93.71% for *T. corymbosum* extract.

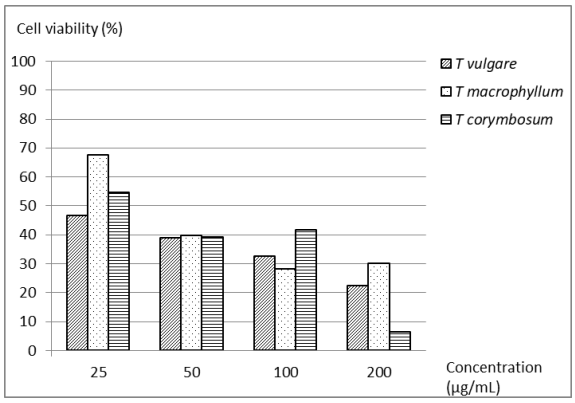

Figure 1.
Cytotoxic activity of *Tanacetum* extracts against HeLa cells

The cytotoxic effect of extracts on Vero cells (Figure 2) was even higher, varying between 95% and 96.98% at the greatest dose tested (200 µg/mL). The healthy cells were more sensitive to the action of *Tanacetum* extracts compared to cancer cells. Among the tested extracts, *T. corymbosum* extract proved to be the most efficient in killing both normal and cancer cells. In the case of *T. vulgare*, our results are in accordance with the findings of other researches who reported intense cytotoxic effect of tansy extracts against J-45.01 human acute T leukaemia cell line [27], human cervical adenocarcinoma HeLa, ovarian carcinoma A2780 and breast adenocarcinoma MCF7 cell lines [8]. Moreover, five eudesmanolides isolated from *T. vulgare* ssp. *siculum* manifested intense cytotoxic activity against human lung carcinoma A549 and healthy hamster lung fibroblast V79379A cells [19].

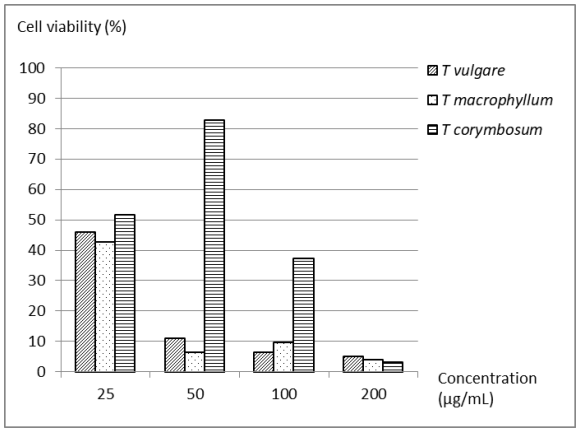

Figure 2.
Cytotoxic activity of *Tanacetum* extracts against Vero cells

Although sesquiterpene lactones are the main compounds in *Tanacetum* genus incriminated for the cytotoxic activity [19], it is probable that methoxylated flavones also play a part in this effect [26]. In addition, phytosterols present in *Tanacetum* extracts act as anticancer agents and can contribute to the overall cytotoxic effect [22]. Further studies are needed in order to characterize the extracts and elucidate the mechanism of action.

Conclusions

Tanacetum species have been used for centuries as medicinal plants and flavouring herbs, but in order to confirm their therapeutic value, detailed phyto-chemical and pharmacological analyses are required. Our research focused on three less studied species from Romanian flora. This is the first study documenting the cytotoxicity of *T. macrophyllum* and *T. corymbosum* and the antimicrobial and anti-oxidant activity of their methanolic extracts.

Phytochemical investigations permitted the identification of common phytosterols and numerous flavonoids, many of these biologically active compounds being reported for the first time in the studied plant

species. All extracts exhibited antimicrobial activity against *Staphylococcus aureus*, *Candida albicans* and *Candida parapsilosis* and had no effect on Gram-negative bacteria. *T. vulgare* and *T. corymbosum* extracts showed good antioxidant activity which was well correlated with the polyphenol content and, especially with the total flavonoid content in the case of *T. corymbosum* extract. Extracts from all three species manifested high cytotoxicity on HeLa cervical cancer and African green monkey kidney (Vero) cell lines. The impact on cell viability was dose dependent, the normal cells being more sensitive to the action of the tested extracts; an important cytotoxic effect had arisen at lower doses than in HeLa cells. The results show that the analysed species manifest health-promoting effects and could be promising sources of active ingredients in food and pharmaceutical industry.

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