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ORIGINAL ARTICLE

ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *CHRYSANTHEMUM* AND *TAGETES* SELECTIVE EXTRACTS

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Abstract

The present study focuses on two ornamental species of the *Asteraceae* family and their biological properties, which can be correlated with the presence of different classes of natural compounds. Given the fact that secondary metabolites found in such plants can be used as valuable resources for the pharmaceutical industry, we chose to investigate the biological potential of *Chrysanthemum indicum* and *Tagetes erecta* inflorescences. Four types of extracts were obtained for each species, using organic solvents with different polarities. Bioactive compounds such as polyphenols were quantitatively analysed using a the Folin-Ciocâlteu method. The investigated compounds were present in all types of extracts in variable quantities, the highest amounts being found in the methanolic extracts. Positive dose-related values revealed that the investigated extracts exhibit good inhibitory activity against 15-lipoxygenase and a moderate metal-chelating activity. Moreover, all extracts showed promising antibacterial and antifungal activities on the tested strains.

Rezumat

Prezentul studiu se axează pe analiza unor specii ornamentale din familia *Asteraceae* și pe testarea acțiunilor lor biologice ce pot fi corelate cu prezența diferitelor clase de compuși naturali. Având în vedere faptul că metaboliții ai acestor plante pot fi utilizați ca resurse valoroase pentru industria farmaceutică, am ales să investigăm inflorescențele speciilor *Chrysanthemum indicum* și *Tagetes erecta*. Pentru fiecare specie au fost obținute patru tipuri de extracte, utilizând solvenți organici cu polarități diferite. Compușii bioactivi, precum polifenolii, au fost analizați cantitativ utilizând metoda spectrofotometrică. Compușii investigați s-au regăsit în toate tipurile de extracte în proporții variabile, cele mai mari cantități regăsindu-se în extractele metanolice. În ceea ce privește testarea acțiunilor biologice, valorile obținute au evidențiat faptul că extractele analizate prezintă o bună activitate inhibitorie asupra 15-lipooxigenazei și o activitate moderată de chelatare a ionului feros. Mai mult, toate extractele obținute din speciile investigate au prezentat activități antibacteriene și antifungice promițătoare asupra tulpinilor testate.

Keywords: *Asteraceae*, antimicrobial activity, 15-lipoxygenase, polyphenols

Introduction

Various secondary metabolites from plants have been chemically and biologically characterized so far, some of which have shown good antioxidant, antimicrobial or antiinflammatory actions [4, 8, 28]. The *Asteraceae* family is considered to be the vastest family of flowering plants [11]. Its species have been used for many centuries for their therapeutic, ornamental and nutritional properties. However, ornamental species have rarely been investigated regarding their potential therapeutic use [16].

The *Chrysanthemum* genus includes many species which are cultivated not only for ornamental purposes, but also for their use in cosmetic preparations [2, 5]. Chemical investigations have been carried out on different species, showing that they contain a variety of secondary metabolites such as flavonoids, phenolic acids, mono-, sesqui- and diterpenoids, steroids, carotenoids [12].

Several species belonging to the *Tagetes* genus are rich in secondary metabolites such as carotenoids, flavonoids, thiophenes and several classes of terpenoids [22, 24]. Different species of this genus, such as *Tagetes patula* and *Tagetes erecta* are used for ornamental purposes [2]. However, important active compounds such as lutein have been isolated from plants from this genus and are presently used as dietary supplements that could have a beneficial impact on macular degeneration [10, 19]. Therefore, given the large variety of secondary metabolites found in these ornamental plants, we investigated their potential implications in biochemical processes and inhibition of microorganisms in correlation with their chemical profile.

Materials and Methods

Plant material and extraction process.

The plant material was represented by the inflorescences of two ornamental species belonging to the *Asteraceae* family: *Chrysanthemum indicum* and *Tagetes erecta*. The plants were cultivated in ecological conditions in the North-Eastern part of Romania in the year of 2017. Voucher specimens are deposited at the Department of Pharmacognosy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania.

Four types of extracts were obtained for each sample, using as solvents chloroform, dichloromethane, hexane and methanol. 5 g of small fragments (0.5 - 1 cm) of dried inflorescences were extracted using 50 mL of either chloroform, dichloromethane and hexane, respectively, while 2.5 g of small fragments were extracted using 100 mL methanol. The first three extracts were obtained by maceration at room temperature for 7 days in a mixing chamber, while the methanolic extract was obtained through a three times extraction at 60ºC. Following filtration, the extracts were concentrated to residue.

Total phenolic content (TPC)

The quantity of polyphenols found in each extract was established with a well-known spectrophotometric method using Folin-Ciocâlteu reagent [26], with some modifications [13]. The dry extracts were dissolved in dimethyl sulfoxide for this determination. The absorbance was measured at 765 nm and gallic acid was used as standard. The results were expressed as g gallic acid equivalents (GAE)/100 g dry extract. The determination was carried out in triplicate.

Inhibition of 15-lipoxygenase (15-LOX)

The inhibition of 15-LOX was determined using the Malterud method [20] with some modifications [9]. 0.05 mL lipoxygenase buffer solution (pH 9) was treated with 0.05 mL extract dissolved in dimethyl sulfoxide in various concentrations and kept for 10 minutes at room temperature. Afterwards, 2 mL linoleic acid buffer solution 0,16 mM (pH 9) were added. The absorbance was measured at 234 nm for 120 seconds. The results were calculated using the following formula:

$$\% \text{ activity} = (A_{EFI} - A_{ECI}) \times 100/A_{EFI},$$

where A_{EFI} is the difference between the absorbance of the enzyme solution alone after 90 seconds and the absorbance of the same solution after 30 seconds and A_{ECI} is the difference between the absorbance of the enzyme solution mixed with the sample after 90 seconds and the absorbance of the same solution after 30 seconds. For each sample, the half maximal effective concentration (EC_{50}) was determined and expressed as µg sample/mL final solution. Quercetin was used as positive control.

Ferrous ion chelating activity

The capacity of the investigated extracts to chelate ferrous ions was determined according to the method described by Venditti *et al*. with slight modifications [29]. Briefly, the ferrous ion forms with ferrozine a complex with maximum absorbance at 562 nm. The presence of a chelating agent in the reaction medium decreases the absorbance of the formed complex. Over 0.2 mL sample solution, 0.74 mL of 0.1 M acetate buffer (pH 5.25) and 0.02 mL of 2 mM ferrous sulphate solution in 0.2 M hydrochloric acid were added. After mixing for 10 - 15 seconds, 0.04 mL of 5 mM ferrozine solution was also added. The absorbance of the solution was determined after being kept for 10 minutes in the dark, against a blank prepared under the same conditions. The metal chelating activity was determined using the following formula:

$$\text{Activity \%} = 100 \times (Ac - Ap)/(Ac),$$

where Ac is the absorbance of the control solution and Ap is the absorbance of the sample solution. For each extract, EC_{50} was determined and expressed as mg sample/mL final solution. Ethylenediamine-tetraacetic acid (EDTA) was used as positive control. The assay was carried out in triplicate.

Antimicrobial susceptibility tests

Microorganisms. The antimicrobial activity was studied using a Gram-positive bacterial strain (*Staphylococcus aureus* ATCC 25923), a Gram-negative bacterial strain (*Escherichia coli* ATCC 25922) and pathogenic yeasts (*Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019).

Disc-diffusion method. Antimicrobial tests for the selected microorganisms were carried out using a disc-diffusion method [6, 7]. A small amount of each microbial culture was diluted in sterile 0.9% sodium chloride solution until the turbidity was equivalent to McFarland standard no. 0.5. The suspensions were further diluted 1:10 in Mueller Hinton agar for bacteria (Oxoid) and Mueller-Hinton agar for yeasts (HiMedia) and then spread on sterile Petri plates. Sterile stainless-steel cylinders were applied on the agar surface in Petri plates. Afterwards, 0.1 mL of each sample was added into cylinders. Commercially available discs containing ciprofloxacin (5 µg/disc), fluconazole (25 µg/disc) and nystatin (100 µg/disc) were used as positive controls. The plates were incubated at 37°C for 24 h (bacteria) and at 24°C for 48 h (yeasts). After incubation, the diameters of the inhibition zones were measured in mm, including disc size.

Broth microdilution method. The samples were tested for the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. Serial double dilutions of each extract in Mueller Hinton broth were inoculated with equal volumes of bacterial suspension. MIC represents

the lowest concentration of extract where complete inhibition of visible growth was observed after 24 h incubation at 37°C. MBC values were determined by transferring 0.1 mL sample showing complete inhibition of visible growth on the surface of an agar plate. The subcultures were incubated 24 h at 37°C. MBC is the lowest concentration of extract required to kill more than 99.9% of microorganisms being tested. MIC of ciprofloxacin towards bacterial strains was also evaluated.

Results and Discussion

Total phenolic content

The comparison between the lipophilic extracts (in chloroform, dichloromethane and hexane) for *Tagetes erecta* showed a higher concentration of polyphenols in the chloroform extract, while for *Chrysanthemum indicum* the highest concentration was found in the dichloromethane extract (Table I). For *Tagetes erecta*, the high content of polyphenols in this type of extracts can be explained by the presence of complex molecules containing some hydroxyl groups [1].

Table I

Total amount of polyphenols found in the analysed extracts

Extract	Code	Concentration g GAE/100 g dry extract (mean ± SD*)
Chrysanthemum indicum chloroform extract	C1	1.654 ± 0.315
Chrysanthemum indicum dichloromethane extract	C2	3.144 ± 0.433
Chrysanthemum indicum hexane extract	C3	0.902 ± 0.553
Chrysanthemum indicum methanol extract	C4	11.856 ± 0.391
Tagetes erecta chloroform extract	T1	6.973 ± 0.376
Tagetes erecta dichloromethane extract	T2	4.185 ± 0.216
Tagetes erecta hexane extract	T3	5.378 ± 0.339
Tagetes erecta methanol extract	T4	15.676 ± 1.139

*SD – standard deviation

Regarding the concentrations of the polyphenolic compounds in the methanolic extracts, the comparison between the two species shows that *Tagetes erecta* contains the highest amount (15.676 ± 1.139 g GAE/ 100 g dry extract).

Inhibition of 15-lipoxygenase

Polyphenols as well as other compounds found in the extract have the capacity of blocking the action of lipoxygenase, which is responsible for catalysing the oxidation of linoleic acid, with the reduction of absorbance measured at 234 nm. The obtained values reveal that the methanolic extracts of both species show good inhibitory activity against 15-LOX (Figure 1, Figure 2). The EC_{50} values of the tested extracts can be found in Table II.

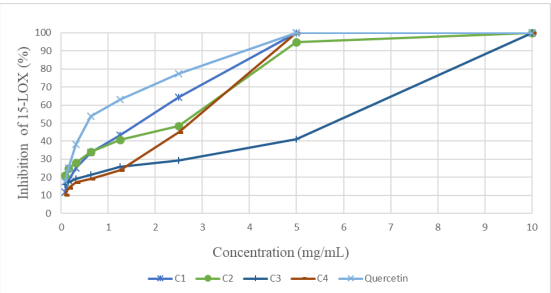

Figure 1.

15-LOX inhibition of *Chrysanthemum indicum* extracts and of quercetin

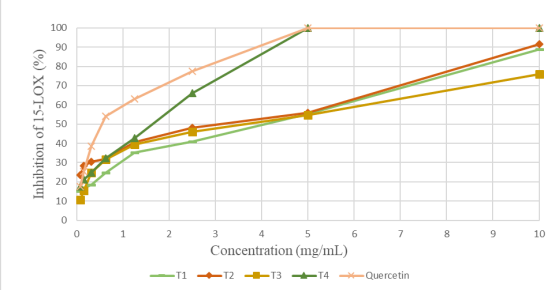

Figure 2.

15-LOX inhibition of *Tagetes erecta* extracts and of quercetin

Table II

EC_{50} values of tested samples and of quercetin

Sample	EC_{50} (µg/mL final solution)
C1	26.06 ± 0.34
C2	42.56 ± 0.45
C3	92.50 ± 0.61
C4	44.44 ± 0.31
T1	64.23 ± 2.41
T2	49.21 ± 3.24
T3	57.83 ± 3.29
T4	25.85 ± 0.67
Quercetin	17.45 ± 0.33

15-lipoxygenase is a non-heme iron enzyme. Its action can be modified by compounds that block the reversible oxidation of Fe^{2+} to Fe^{3+}. Consequently, the enzyme will no longer be able to catalyse the transformation of the substrate through the oxidation-reduction reaction. The substances that inhibit the enzyme through this mechanism have reducing capacity, releasing electrons and protons. Polyphenols and flavonoids found in the

extracts can reduce or even block the activity of the enzyme through the mentioned mechanism [17, 20]. The concentration in polyphenols for the investigated species can be correlated with the anti-inflammatory and antioxidant activities. Promising inhibition of the enzyme can be observed for the methanolic extracts of both species. However, this is also true for *Chrysanthemum indicum* chloroform and dichloromethane extracts, which indicates that lipophilic compounds such as carotenoids or terpenoids can also be responsible for the inhibitory activity, through a different mechanism of action [18, 25].

Ferrous ion chelating activity

Ferrous ion chelating capacity of all investigated extracts increased with concentration. According to the results, the most active extracts were the methanolic extracts of both species (C4, T4) and the *Chrysanthemum indicum* chloroform extract (C1), for which a 100% chelating activity was observed at a concentration of 5 mg/mL. All *Chrysanthemum indicum* extracts showed 100% activity at a concentration of 10 mg/mL, while only the methanolic extract of *Tagetes erecta* showed such result at the same concentration. *Chrysanthemum indicum* chloroform and methanol extracts (C1, C4) and *Tagetes erecta* hexane and methanol extracts (T3, T4) have the lowest EC_{50}, as it can be seen in Table III. However, the EC_{50} of EDTA was much lower than those of the tested samples (0.075 ± 0.001 mg/mL). It is generally believed that polyphenols are some of the most important compounds responsible for the metal chelating activity. While this can be proved by the correlation between the TPC of methanolic extracts and their EC_{50}, we believe that more classes of compounds can be responsible for the metal-chelating activity observed for other types of extracts [30], although different chemical assays are still ongoing.

Table III

EC_{50} of tested samples and of EDTA

Sample	EC_{50} (mg/mL final solution)
C1	0.282 ± 0.001
C2	0.382 ± 0.001
C3	0.346 ± 0.001
C4	0.307 ± 0.001
T1	0.492 ± 0.001
T2	1.605 ± 0.004
T3	0.437 ± 0.01
T4	0.441 ± 0.002
EDTA	0.075 ± 0.001

Antimicrobial susceptibility tests

The diameters of the inhibition zones corresponding to the tested samples are shown in Table IV. All assays were carried out in triplicate. Results are expressed as mean ± SD.

Table IV

Antibacterial and antifungal activities of the tested samples and of positive controls

Sample	Diameter of the inhibition zones (mm)			
	***S. aureus* ATCC 25923**	***E. coli* ATCC 25922**	***C. albicans* ATCC 90028**	***C. parapsilosis* ATCC 22019**
C1	16.7 ± 0.06	9.0 ± 0.00	15.5 ± 0.50	18.5 ± 0.50
C2	16.0 ± 0.00	11.5 ± 0.50	15.5 ± 0.50	20.5 ± 0.50
C3	15.5 ± 0.50	8.00 ± 0.00	20.5 ± 0.50	19.5 ± 0.50
C4	16.0 ± 0.00	7.0 ± 0.00	19.5 ± 0.50	15.5 ± 0.50
T1	17.0 ± 0.00	10.0 ± 0.00	20.5 ± 0.50	17.0 ± 0.00
T2	18.3 ± 0.57	7.0 ± 0.00	20.5 ± 0.50	15.5 ± 0.50
T3	15.3 ± 0.57	11.3 ± 0.57	14.0 ± 0.00	14.0 ± 0.00
T4	15.3 ± 0.57	11.0 ± 0.00	19.0 ± 0.00	17.0 ± 0.00
Ciprofloxacin	28.7 ± 0.06	36.5 ± 0.50	NT*	NT*
Fluconazole	NT*	NT*	30.5 ± 0.50	21.0 ± 0.00
Nystatin	NT*	NT*	23.5 ± 0.50	20.0 ± 0.00

*NT-not tested

All extracts have good antibacterial activity against *S. aureus* and medium activity against *E. coli*. Regarding the antifungal potential, all samples have shown good activity against the tested strains. Out of these samples, *Chrysanthemum indicum* hexane extract (C3) and *Tagetes erecta* chloroform and dichloromethane extracts (T1, T2) had the best antifungal activity against *C. albicans*. The *Chrysanthemum indicum* dichloromethane extract (C2) had a similar activity to that of nystatin and fluconazole against *C. parapsilosis*.

MIC and MBC values are shown in Table V. For most samples, the MBC values were 2 - 4 times greater than the MIC values. Out of the tested samples, both *Chrysanthemum indicum* and *Tagetes erecta* extracts showed good MIC values for *S. aureus*, while for *E. coli* the values were higher, which indicates that the studied extracts have a better activity on Gram-positive bacteria than on Gram-negative bacteria.

Table V

MIC and MBC values of the tested samples

Sample	*S. aureus* ATCC 25923		*E. coli* ATCC 25922	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
C1	0.25	0.5	0.5	> 0.5
C2	0.125	0.5	0.25	0.25
C3	0.125	0.5	0.5	> 0.5
C4	0.125	0.5	0.5	>0.5
T1	0.25	0.5	0.25	0.25
T2	0.125	0.25	0.5	> 0.5
T3	0.125	0.5	0.25	0.5
T4	0.125	0.5	0.25	0.5
Ciprofloxacin	1.0[a]	1.0[a]	1.0[a]	2.0[a]

[a] values are expressed in µg/mL

The results indicate that secondary metabolites found in these species possess good antimicrobial properties. Both polyphenol derivatives and sesquiterpenes can act as antimicrobials [8, 14]. It is worth mentioning that phenolic acids, especially caffeic and ferulic acids act against both Gram-positive and Gram-negative bacteria. More potent are flavan-3-ols, flavonols and hydrolysable tannins that present antibacterial and antifungal activity [8]. There is a definite difference in terms of antibacterial and antifungal activity, suggesting that certain compounds found in the investigated species act distinctively on various types of micro-organisms. This can be correlated with differences regarding the chemical structure and the position of several functional groups [8, 27]. Nevertheless, results indicate that lipophilic compounds are stronger anti-microbials and antifungals, fact supported by the possible direct mechanism on the bacterial membranes inducing alteration and transport deficit inside the bacteria [15, 24].

Our results complete some of the most recent studies on *Asteraceae* species, sustaining that such species are important resources, but care should be taken when handling the raw plant material which can induce contact dermatitis [23]. Previous studies sustain such claim by confirming the presence of alantolactone, a sesquiterpene lactone recognized for its allergenic properties [3, 21]. However, sesquiterpene lactones also possess important pharmacological benefits (anti-oxidant, anti-inflammatory, antimicrobial and anti-cancer activities) [4, 15].

Conclusions

Overall, the obtained results suggest that the choice of the extraction solvent is of extreme importance when searching for active compounds. Although most of the investigated extracts presented biological activity, the intensity and efficiency depend not only on the chemical variability of the plant material, but also on the extractability of certain groups of secondary metabolites. Both ornamental *Asteraceae* species present promising antioxidant, anti-inflammatory and antimicrobial potentials, but further studies regarding their chemical profile and toxicity still need to be done.

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