GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

CHEMICAL AND BIOLOGICAL EVALUATION OF SOME EXTRACTIVE FRACTIONS FROM ORNAMENTAL *ASTERACEAE* SPECIES

DOCTORAL THESIS ABSTRACT

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The PhD thesis entitled ***Chemical and biological evaluation of some extractive fractions from ornamental Asteraceae species*** comprises of 164 pages, 20 tables, 106 figures and 355 references.

In this abstract, the numbering of figures, tables and references is the same as the one used in the doctoral thesis.

Keywords: *Asteraceae*, polyphenols, flavonoids, antioxidant, cytoprotective.

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PERSONAL PART

MOTIVATION, SCOPE AND AIMS

The scientific literature includes relatively little data regarding the chemical and biological potential of ornamental plant species. Medicinal plants have always been an important source of therapeutic agents, containing many active principles with different structures and biological activities. Today, active substances from plants and their derivatives represent approximately 50% of all existing medicinal products on the market. Taking into consideration the constant need for discovering new remedies for diseases such as cancer, inflammatory diseases, and, generally, those associated with oxidative stress, but also for infectious diseases, more and more researchers are now focusing on the plant kingdom with the aim of finding new substances and efficient treatments for various pathologies, or for the scientific confirmation of actions observed in traditional medicine (2, 257).

In the present doctoral research, we aim to evaluate a few ornamental species rich in various active principles, for which insufficient studies regarding chemical composition and possible therapeutic uses have been carried out, such as species from the *Rudbeckia*, *Tagetes* and *Zinnia* genera, all belonging to the *Asteraceae* family. Bearing in mind that species of these genera are cultivated especially for their ornamental role, a lack of phytochemical, biological and toxicological studies for their extractive fractions can be observed, especially for *Rudbeckia hirta* L. and *Zinnia elegans* Jacq. species. Only *Tagetes erecta* L. is currently cultivated for its inflorescences, which allow the extraction in appreciable quantities of carotenoids (66).

Therefore, we consider it would be of interest to carry out preliminary analyses in terms of active principles content, such as

polyphenolcarboxylic acids and flavonoids found in the three species, followed by the testing of biological actions for fractions rich in such polyphenolic compounds, as well as drawing certain correlations between the obtained results.

Consequently, the aim of the present doctoral thesis is represented by the chemical and biological evaluation of total extracts and selective fractions obtained from the inflorescences of the species taken into study, with the intent of confirming the presence of several polyphenolic representatives and of performing their quantitative analysis. Such compounds can be associated with several beneficial effects for the human body, which is why the present study focuses especially on the examination of the antioxidant, antimicrobial and cytoprotective actions.

The objectives of this doctoral thesis include the obtaining of certain types of plant extracts with therapeutic potential, which we will analyze taking into consideration the yield, the chemical composition and the biological potential. Therefore, in our research we have focused on three ornamental species originating in North America (*Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq.), but which are now widely cultivated in our country as garden plants or for landscaping. They also have a few uses in traditional medicine, as described in the first chapter of the general part.

It is worth mentioning that the complex research carried out in the present doctoral study on these species represents an element of novelty, given the fact that for *Rudbeckia hirta* L. and *Zinnia elegans* Jacq. there are very few studies regarding the chemical composition of the various organs of the plant or the bioactive potential of their extracts, that also include correlations between chemical composition and confirmed biological actions. In addition, the scientific literature includes few references for these plant species, which opens the possibility of publishing articles in an interesting and relatively unstudied field.

In this context, the specific objectives of the present research consisted of:

- highlighting morphological and histo-anatomical characters specific to the plant material harvested from each species included in the study (*Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq.), which can allow their differentiation;
- obtaining selective extracts and fractions, which could have important biological effects;
- carrying out the phytochemical study of classes of active principles that exist in a series of selective extracts and fractions, through hyphenated techniques, focusing on the determination of certain compounds;
- evaluating the *in vitro* biological potential of the total extracts and of some of their fractions, in terms of antioxidant, antimicrobial and cytoprotective activities, in correlation with the presence of certain classes of chemical compounds;
- *in vitro* pharmacotoxicological screening and determination of the cytotoxicity of total extracts.

Chapter III. Obtaining of the plant material and macro- and microscopic pharmacognostic analysis of the species considered in the study (*Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq.)

III.1. Cultivation of the species considered in the study

Due to the fertile soil and daily irrigation, a much larger amount of inflorescences than expected was obtained in the first two months after cultivation. Thus, 26,900 kg of inflorescences were harvested from *Rudbeckia hirta* L., of which, after drying, 7,520 kg remained, which represents a percentage of 27.95%, losing water up to 72.05%.

Tagetes erecta inflorescences lost about 80% water after drying, while the inflorescences of *Zinnia elegans* lost over 70% of the initial mass. For *Tagetes erecta*, 26,300 kg inflorescences were harvested and, after drying, 5,400 kg of plant material remained, which represents 20.53% of the initial floral mass.

For the last species included in the study, *Zinnia elegans*, 29,700 kg inflorescences were harvested, of which 8,500 kg remained after drying, which represents a percentage of 28.60% of the initial mass.

III.2. Macroscopic analysis of the cultivated species

For *Rudbeckia hirta*, the stem is semi-erect, covered with hairs, with a height between 50 and 100 cm, with pubescent leaves, opposite at the base and alternating at the top, ovate, with entire margins. The ligulate flowers are yellow or orange-yellow, and the tubular ones are brown, forming a central cone. The formed inflorescence is a capitula, which is specific to the *Asteraceae* family and has a diameter of 7 to 14 cm. The plant has no odor. The large presence of hairs found on the surface of the plant is easily notable.

For *Tagetes erecta*, the presence of an erect, glabrous stem with a height between 50 and 80 cm was noted. The alternating or opposite leaves have a dark green color, being feather-sectioned, with lanceolate, denticulate leaflets. The inflorescences are capitula with yellow or orange marginal flowers, while the tubular flowers are small, centrally located and have a yellow or orange color. The diameter of the inflorescence varies between 5 and 10 cm. The plant is aromatic, presenting a characteristic, pleasant smell.

Zinnia elegans (sin. *Zinnia violacea*) has a cylindrical, erect, simple or branched stem, which has a height ranging from 40 to 100 cm. Each branch has a single inflorescence. The leaves with entire margin are sessile and opposite and have a sharp tip. Their shape varies from triangular to ovate. The inflorescence is composed of marginal, ligulate, purple, red, yellow or white flowers and central, tubular flowers. The diameter of the inflorescence ranges from 7 to 12 cm. The plant has a very weak, particular odor.

III.3. Histo-anatomical analysis of the investigated species

Rudbeckia hirta

The anatomical structure of the leaf blade is bifacial equifacial, with palisade tissue under both epidermis: two layers on the adaxial surface and 1-2 layers on the abaxial surface. Both epidermises have isodiametric cells of different sizes, always larger at the upper side of the leaf blade.

At the epidermis level, two categories of trichomes can be noticed: long covering trichomes, with obtuse or sharp tip, with a vesicular basal cell and others, very short, which are glandular trichomes.

The scanning electron microscope shows that the multicellular hairs are larger on the edge of the leaf blade and have a particular structure, with a very large vesicular basal cell, a narrower transition cell and a very long terminal cell with sharp or obtuse tip. The glandular trichomes are very short in size.

The upper epidermis of the ligule corolla is composed of papilliform secretory cells, often conical in shape, with all walls slightly thickened. Sometimes, these secretory cells are separated by smaller, isodiametric cells (figure III.12).

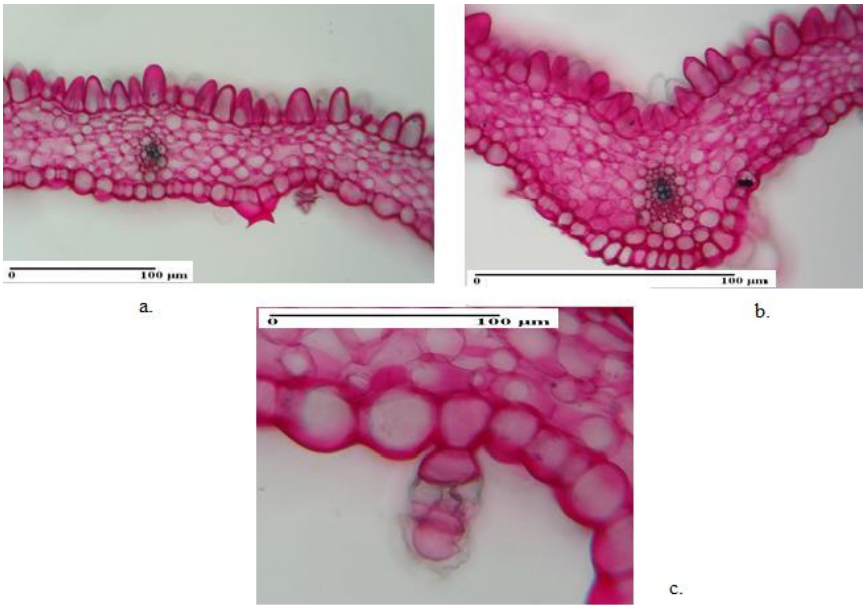
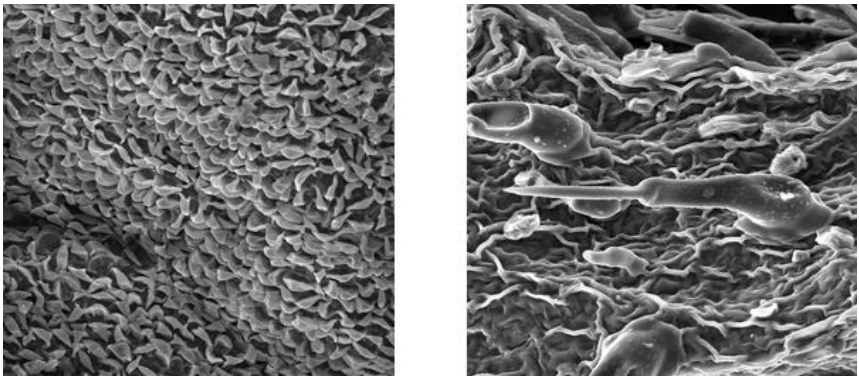


Figure III.12. Cross-section of *Rudbeckia hirta* ligule*:* a. overview, b. median vein, c. glandular trichome

At the lower epidermis level, unicellular or bicellular, relatively short covering trichomes, and short, multicellular, commonly biseriate secretory trichomes are visible, with the unicellular gland covered by a visibly distant cuticle.

Using the electron microscope, at the lower epidermis level, the same very long hairs, with the basal part much thicker than the terminal one, can be observed. The secretory trichomes are much shorter and are very rare, with a spherical gland (figure III.13).



a. b.

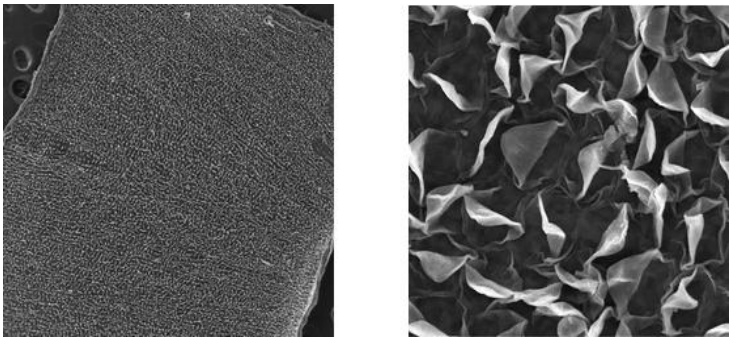
Figure III.13. *Rudbeckia hirta* ligule epidermis, front view: a. upper epidermis with papilliform secretory cells, b. lower epidermis with covering and glandular trichomes

Tagetes erecta

The anatomical structure of the leaf blade is bifacial heterofacial, with mono- or bilayered palisade tissue on the upper face, with long cells occupying 50% of the organ thickness. The lacunar tissue is multilayered, with parenchymal cells separated by relatively small spaces.

Both epidermises are made up of cells of different size, most of them being isodiametric, all covered by a thick outer wall. At this level, stomata are present and can be found in higher amounts in the lower epidermis. Covering trichomes are rare, long, single- or multi-cellular, uniseriate.

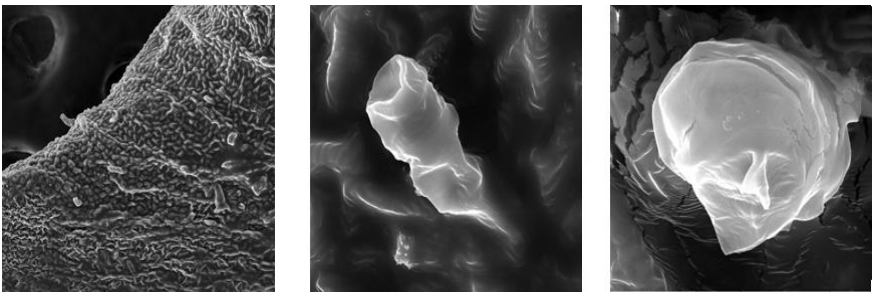
In the cross-section of the ligula of *Tagetes erecta* a strip of different thickness can be observed, with the median vein slightly protruding at the lower face. The upper epidermis is papilliform, with conical secretory cells that have a rounded tip and a conspicuous nucleus in the basal part (figure III.20). The lower epidermis has large, isodiametric or radially elongated cells, of different size, all with inner and especially outer thickened walls.



a. b.

Figure III.20. Upper epidermis of *Tagetes erecta* ligula, front view: a. overview, b. papilliform cells (detail)

At the lower epidermis level, relatively long covering trichomes and short, multicellular glandular trichomes can be observed using the electron microscope (figure III.21).



a. b. c.

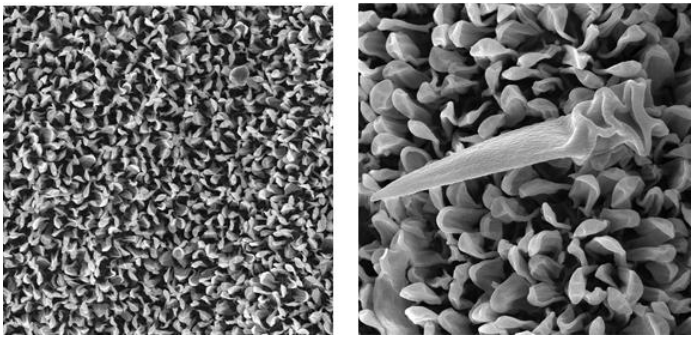
Figure III.21. Lower epidermis of *Tagetes erecta*, front view: a. overview, b. covering trichome, c. glandular trichome

Zinnia elegans

The median vein protrudes conspicuously at the lower surface of the leaf blade, and in the fundamental, parenchymatic, colorless tissue, a large vascular bundle of collateral type can be noticed. Stomata can be found on both epidermises. When using the scanning electron microscope, long, often bicellular, aculeiform,

long-pointed covering trichomes, and very short, multicellular, glandular trichomes can be observed on the surface of the blade.

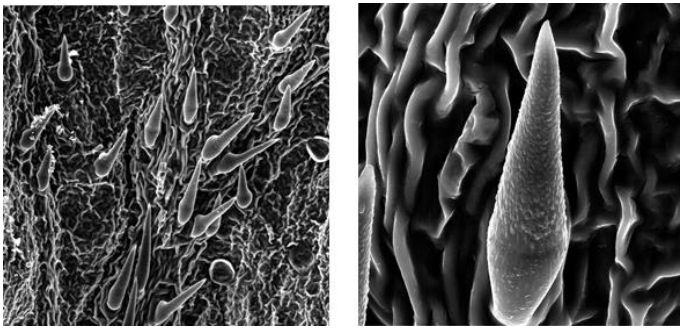
The ligule has a slightly protruding median area at the lower surface, where a collateral vascular bundle can be observed. The upper epidermis is papilliform, with conical secretory cells (figure III.28).



a. b.

Figure III.28. Upper epidermis of *Zinnia elegans* ligule, front view: a. papilliform cells, b. trichome

The lower epidermis has isodiametric cells, with the outer wall slightly thickened (figure III.29). From place to place, relatively long covering trichomes with vesicular base and pointed tip can be noticed, as well as very short, multicellular, glandular trichomes.



a. b.

Figure III.29. Lower epidermis of *Zinnia elegans* ligule, front view: a. overview, b. covering trichome

Chapter IV. Obtaining and phytochemical characterization of extractive fractions from *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq inflorescences

IV.1. Obtaining of methanolic extracts

In order to evaluate the extractability, the ratio of the mass of the initial dry plant material to the mass of the dry extract obtained after total evaporation of the solvent was calculated. This ratio is also known as drug-extract ratio (DER) (273, 274). The DER values calculated for the three dry methanolic extracts can be found in table IV.1.

Table IV.1. DER values for the obtained extracts

Code	Extract type	DER
4R	*Rudbeckia hirta* methanolic extract	10:0,9033
4Ta	*Tagetes erecta* methanolic extract	10:2,4922
4Z	*Zinnia elegans* methanolic extract	10:0,8649

The highest quantity of dry extract was obtained from *Tagetes erecta,* while the lowest yield was noticed for the dry methanolic extract obtained from *Zinnia elegans* inflorescences.

IV.2. Phytochemical analysis of extracts obtained from *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq. inflorescences

IV.2.1. Qualitative analysis of compounds found in the methanolic extracts using an UHPLC-MS method

For *Rudbeckia hirta,* over 25 compounds such as amino acids (phenylalanine, tryptophan), mono- and diacyl chlorogenic acids, as well as flavonoids, especially as glycosides of patuletin, eupalitin,

quercetin, quercetagetin, but also as aglycons (eupatolitin), were identified, only some of them being previously described in the scientific literature (32, 34).

The negative and positive mode chromatograms, as well as the CAD and UV-Vis chromatograms obtained for the total methanolic extract of *Rudbeckia hirta* inflorescences can be seen in figure IV.2.

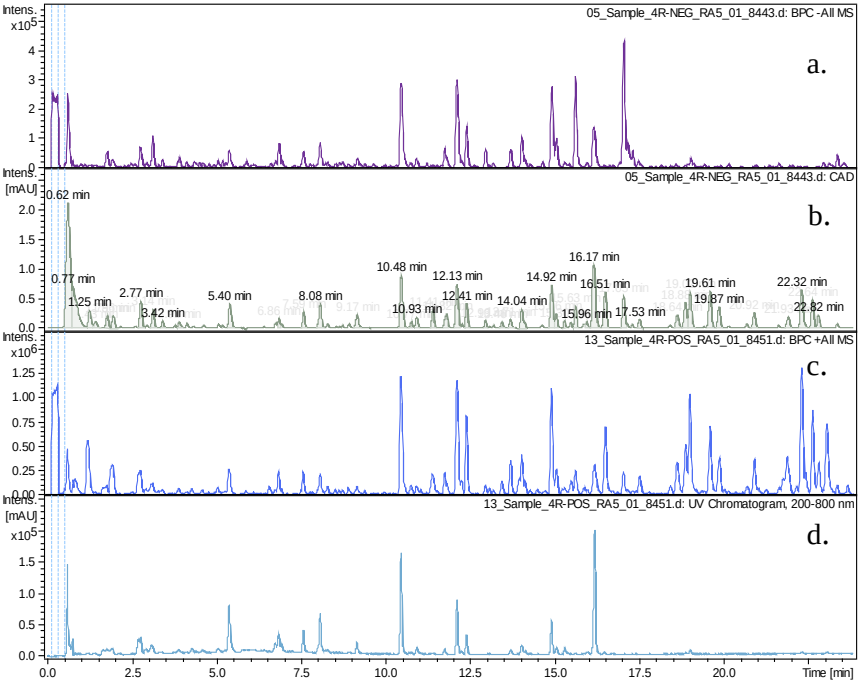


Figure IV.2. Chromatograms obtained for the methanolic extract of *Rudbeckia hirta* inflorescences (detection: a. negative mode, b. CAD, c. positive mode, d. UV-Vis)

For *Tagetes erecta*, over 50 compounds such as amino acids (phenylalanine, tryptophan), gallic, quinic, syringic and ellagic acid derivatives, mono- and diacyl chlorogenic acids, as well as flavonoids, commonly as glycosides of quercetagetin, quercetin,

kaempferol, patuletin, but also as aglycons (quercetin, kaempferol, patuletin, isorhamnetin and axillarin) could be identified.

The results are in accordance with the data found in literature, given the fact that compounds such as quercetagetin and its derivatives, syringic acid (85), quercetin, kaempferol (86), ellagic acid (291), gallic acid and its derivatives (83) have already been described for this species.

The negative and positive mode chromatograms, as well as the CAD and UV-Vis chromatograms obtained for the total methanolic extract of *Tagetes erecta* inflorescences can be seen in figure IV.3.

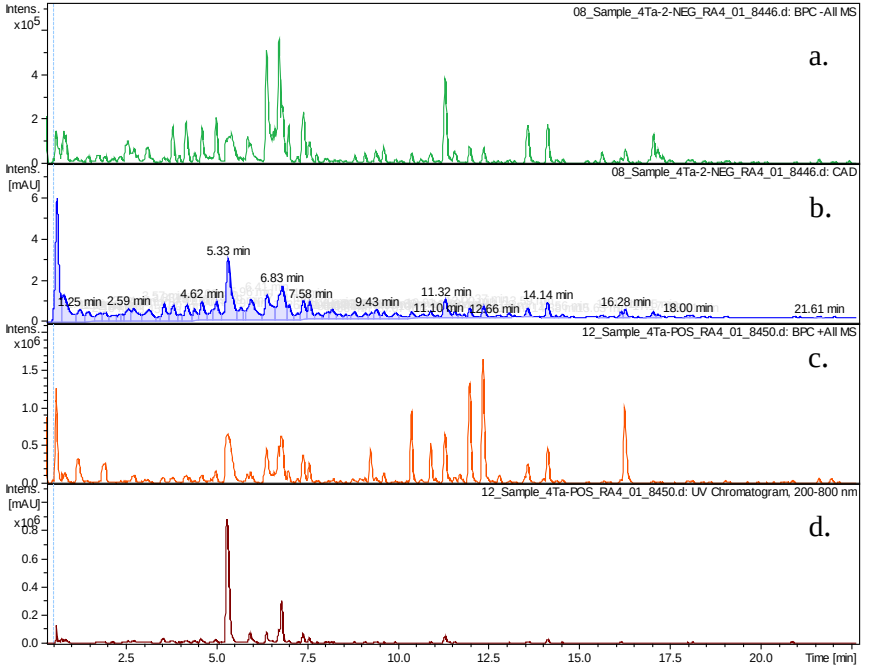


Figure IV.3. Chromatograms obtained for the methanolic extract of *Tagetes erecta* inflorescences (detection: a. negative mode, b. CAD, c. positive mode, d. UV-Vis)

Regarding *Zinnia elegans*, over 50 compounds were identified. Among these were some amino acids (phenylalanine, tryptophan, (iso)leucine), guanidine alkaloids (plantagoguanidinic acid, plumbagine B and their isomers), gallic acid derivatives, mono- and diacyl chlorogenic acids, as well as flavonoids, especially as glycosides of kaempferol, quercetin, apigenin and resokaempferol.

The results are in accordance with the data found in literature, compounds such as kaempferol, apigenin and their glycosides being previously described for other extracts obtained from the inflorescences of this species (153).

The chromatograms obtained for the total methanolic extract of *Zinnia elegans* inflorescences can be seen in figure IV.4.

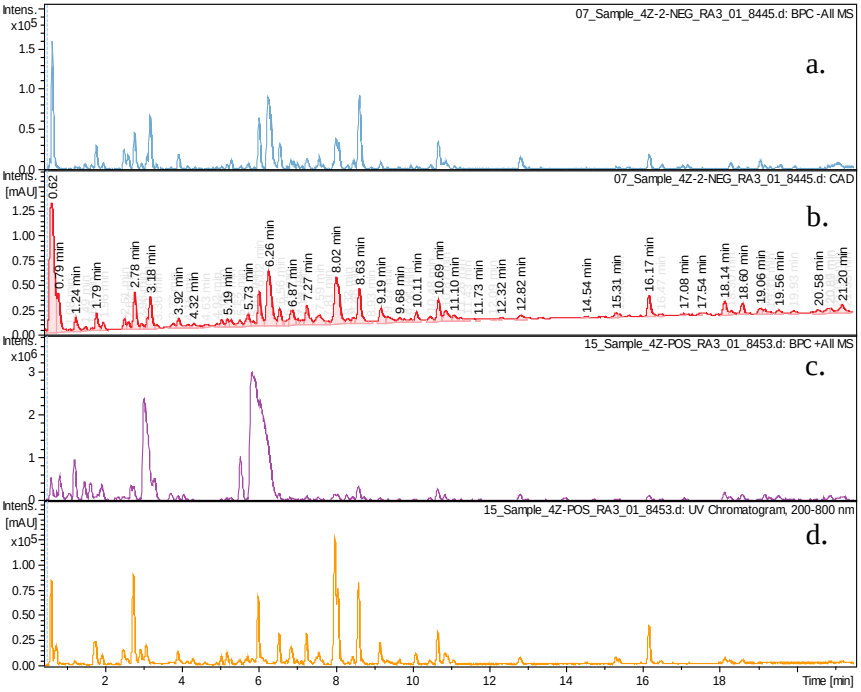


Figure IV.4. Chromatograms obtained for the methanolic extract of *Zinnia elegans* inflorescences (detection: a. negative mode, b. CAD, c. positive mode, d. UV-Vis)

IV.2.2. Quantitative determination of some classes of compounds present in the methanolic extracts

IV.2.2.1. Quantitative determination of total polyphenols

The obtained results indicate that the highest quantity of polyphenols is found in the methanolic extract of *Tagetes erecta* inflorescences (263,1667±1,7095 mg GAE/ g dry extract), while the lowest quantity is found in the extract obtained from *Rudbeckia hirta* inflorescences (table IV.7).

Table IV.7. Total polyphenols and total flavonoids content of the methanolic extracts obtained from the three species considered in the study (mean±standard deviation)

Sample	Total polyphenols (mg GAE/ g dry extract)	Total flavonoids (mg QE/ g dry extract)
4R	72,5433±2,1334	61,6333±1,5694
4Ta	263,1667±1,7095	218,2466±1,5723
4Z	136,1400±1,5878	269,978±1,2140

IV.2.2.2 Quantitative determination of total flavonoids

Regarding the total flavonoids content, it can be observed that the methanolic extract obtained from *Zinnia elegans* inflorescences contains the highest quantity (269,978±1,2140 mg QE/ g dry extract), followed by the extract obtained from *Tagetes erecta* and, finally, by the one obtained from *Rudbeckia hirta*.

IV.3. Obtaining of selective fractions by using solid phase extraction and LH-20 column chromatography

After applying solid-phase extraction, 5 fractions were obtained (coded A, B, C, D and E) for each methanolic extract. Fraction B (obtained using MeOH 85%), which is considered the

most valuable fraction regarding the content in polyphenolic compounds, was then subjected to Sephadex LH-20 column chromatography.

For *Rudbeckia hirta*, 8 fractions were obtained by mixing the eluates that correspond to certain signals found on the chromatogram: 4R 18-26, 4R 27-35, 4R 36-40, 4R 41-47, 4R 48-55, 4R 56-59, 4R 60-74 and 4R 75-87.

After analyzing the peaks, 4 representative fractions were obtained for *Tagetes erecta* by mixing the eluates that correspond to certain signals found on the chromatogram: 4Ta 16-33, 4Ta 34-49, 4Ta 63-75 and 4Ta 76-90.

For *Zinnia elegans*, 5 fractions were obtained by mixing the eluates that correspond to certain signals from the chromatogram: 4Z 15-35, 4Z 36-49, 4Z 50-54, 4Z 55-69 and 4Z 70-82.

IV.4. Identification of compounds present in the selective fractions using an HPLC-MS method

The first fraction eluted from the *Rudbeckia hirta* methanolic extract, coded 4R 18-26, contains two major compounds, one trihydroxy-octadecadienoic acid (9,10,11-trihydroxy-octadeca-12,15-dienoic acid or 9,12,13-trihydroxy-10,15-octadecadienoic acid) with *m/z* = 327.2 (measured in negative mode) and a trihydroxy-octadecenoic acid (*m/z* = 329.0). The structures established for these compounds are tentative identifications based on mass-to-charge ratios and fragmentation patterns. As expected, the 4R 27-35 fraction has a qualitative composition similar to the first fraction and contains as well as the first one a trihydroxy-octadecadienoic acid (9,10,11-trihydroxy-octadeca-12,15-dienoic acid or 9,12,13-trihydroxy-10,15-octadecadienoic acid) with *m/z* = 327.3 and a trihydroxy-octadecenoic acid (*m/z* = 329.6).

The 4R 36-40 fraction contains phenylalanine as major compound, which has a retention time of 1.96 min and *m/z* = 163.0, while the next eluted fraction, 4R 41-47, differs significantly regarding chemical composition and contains especially quinic acid

(*m/z* = 190.9), eupalitin-glucoside (*m/z* = 490.9) and acetylated eupatolin (*m/z* = 532.7).

The 4R 48-55 fraction contains three major compounds, easily detected on the negative mode chromatogram: eupatolitin-3-glucoside (*m/z* = 506.8), eupatolin (*m/z* = 490.7) and acetylated eupatolin (*m/z* = 532.9).

The 4R 56-69 fraction contains a series of secondary metabolites such as cyanidin-3-glucoside (*m/z* = 448.7), eupatolitin-3-glucoside (*m/z* = 507.1) and eupatolin (*m/z* = 490.7), its chemical composition being similar to the previously obtained fraction.

The major compounds identified in the 4R 60-74 fraction are, taking into consideration the order of their retention time, isoquercetin (*m/z* = 463.0), patuletin-glucoside (*m/z* = 492.9) and chlorogenic acid (*m/z* = 353.1).

The last eluted fraction, 4R 75-87, contains as major constituents quercetagitrin (*m/z* = 478.8) and helichrysoside (*m/z* = 609.0). The proposed structure for these compounds were established taking into consideration their mass-to-charge ratios and their fragmentation patterns. However, for a more accurate identification, other structure analyses are needed, including, for example, NMR or IR spectroscopy.

A series of metabolites found in the total methanolic extract obtained from *Tagetes erecta* inflorescences could be identified in the corresponding LH-20 fractions

The 4Ta 16-33 fraction contains compounds such as syringic acid (*m/z* = 197.1) and some if its derivatives as syringic acid-(dihydroxy-trimethoxy-benzoic acid)-hexoside with *m/z* = 568.7.

The 4Ta 34-49 fraction contains, as major constituents, syringic acid derivatives, such as di-syringic acid-hexoside (*m/z* = 539.1; *m/z* = 538.7), while the 4Ta 63-75 fraction contains di-galloyl-hexoside (*m/z* = 482.5), ellagic acid-hexoside (*m/z* = 462.9), a quercetagetin hexoside (*m/z* = 478.7) and quercetin (*m/z* = 300.9).

The last eluted fraction (4Ta 76-90) contains only one major compound, a quercetagetin hexoside, quercetagitrin, with *m/z* =

478.7. Given the high purity of the eluted fraction, the NMR spectroscopy analyses could be done without further purifications.

The metabolites identified in the total methanolic extract obtained from *Zinnia elegans* inflorescences could also be found in the LH-20 fractions. Therefore, the 4Z 15-35 fraction contains as major compounds two guanidine alkaloids (plumbagine B and plantagoguanidinic acid) with *m/z* = 222.1 and *m/z* = 224.0, respectively, values that were measured in negative mode.

The second fraction, 4Z 36-49, contains mostly mono-acyl chlorogenic acids (3-CQA, 5-CQA, 4-CQA, 5-*p*CoQA), but also kaempferol glycosides, such as kaempferol 3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranoside with *m/z* = 622.9.

The following major compounds are found in the 4Z 50-54 fraction: caffeic acid, clovamide and flavonoids as glycosides such as kaempferol 3-*O*-(pentosyl-hexoside) (*m/z* = 578.8), apigenin-7-*O*-dihexoside (*m/z* = 592.6) and resokaempferol 3-*O*-hexoside (*m/z* = 430.8).

The 4Z 55-69 fraction contains quercetin 3-*O*-hexoside (*m/z* = 462.8), diacyl chlorogenic acids, resokaempferol 3-*O*-hexoside (*m/z* = 430.9), while the last eluted fraction contains kaempferol-3-*O*-hexoside (*m/z* = 446.7), diacyl chlorogenic acids (*m/z* = 514.8) and apigenin (*m/z* = 268.9).

IV.5. Isolation of compounds from various selective fractions using preparative chromatography

Although two subfractions were collected for the 4Z 15-35 fraction (peak min. 13 and peak min. 20), given the obtained quantities, only one of them, which presents a peak around min. 20, was further subjected to NMR spectroscopy analyses. The shape of this peak indicates that the substance contains nitrogen in the molecule, given the tailing observed towards its end (figure IV.28).

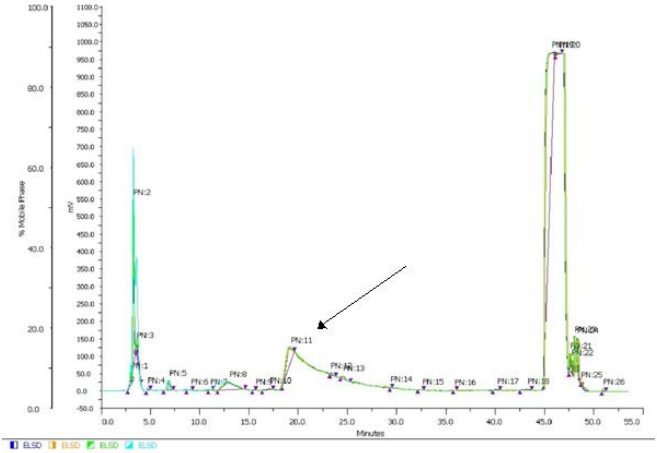


Figure IV.28. The chromatogram for the 4Z 15-35 fraction, peak of interest (min. 20) that was later subjected to NMR spectroscopy analyses

Although three subfractions were collected for the 4Z 36-49 fraction (peak min. 10, peak min. 17 and peak min. 35), for quantitative reasons, only one of them (peak min. 17) was further subjected to NMR spectroscopy analyses (figure IV.29).

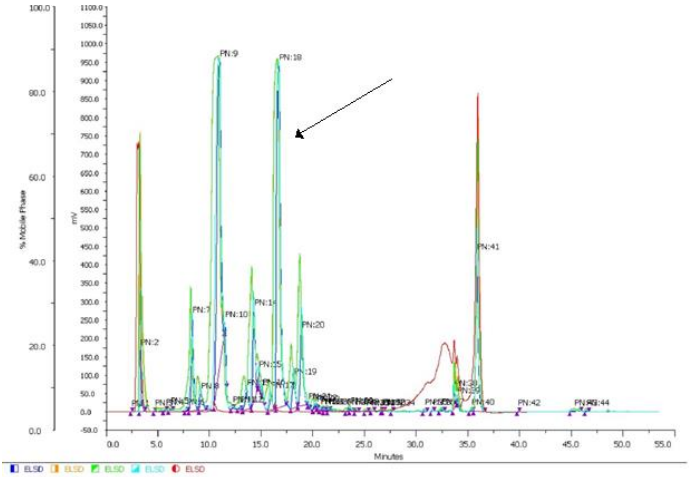


Figure IV.29. The chromatogram for the 4Z 36-49 fraction, peak of interest (min. 17) that was later subjected to NMR spectroscopy analyses

IV.6. Structural elucidation of compounds isolated from the plant extracts considered in the study by NMR spectroscopy

The FR85-P2 compound was purified through preparative chromatography techniques from the 4Z 36-49 fraction, which was previously obtained from the methanolic extract of *Zinnia elegans* inflorescences. Using the data provided by the one- and bidimensional NMR spectra, as well as the information obtained from the mass spectroscopy analyses, the following structure was proposed for the FR85-P2 compound, named kaempferol 3-*O*-[β-glucopyranosyl-(1→2)-β-glucuronopyranoside] (figure IV.30).

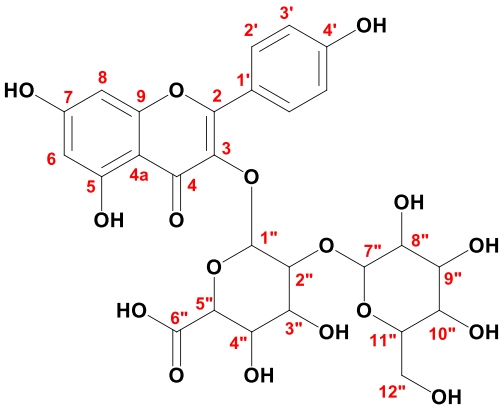


Figure IV.30. Proposed structure for the FR85-P2 compound

The ^{1}H-NMR spectrum for the FR85-P2 compound can be seen in figures IV.31 and IV.32. From this type of spectrum, information on the chemical shift, the multiplet structures, the coupling constants and the integration for all protons present in the sample can be obtained. All this information is essential for determining the chemical structure of individual compounds isolated from plants.

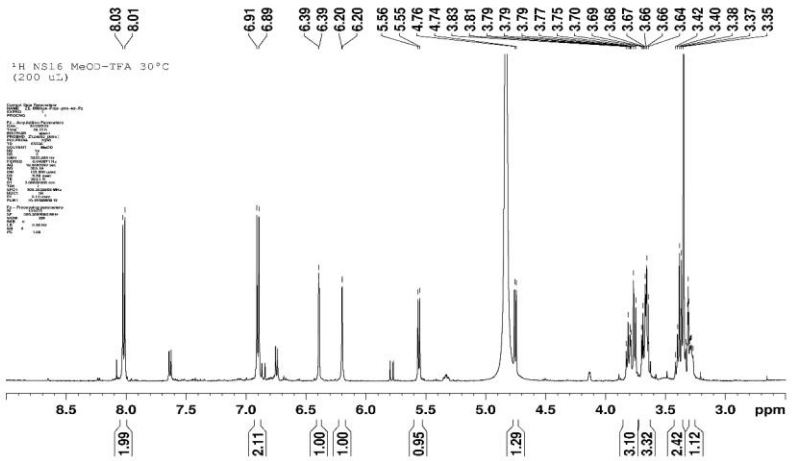


Figure IV.31. [1]H-NMR spectrum for the FR85-P2 compound, measured in MeOD with trifluoroacetic acid (TFA)

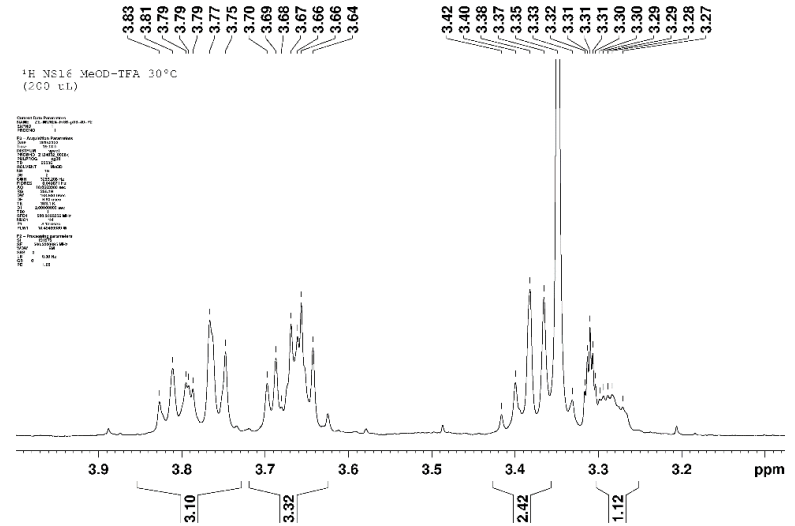


Figure IV.32. [1]H-NMR spectrum for the FR85-P2 compound, 3-4 ppm spectral window

The spectral profile indicates the presence of a *para*-substituted aromatic nucleus (characteristic signals in the 6.5-8.5 ppm interval), of a double bond or heteroaromatic (characteristic signals in the 5.5-6.0 ppm signals) and of a mono- or disaccharide unit. The doublets at 4.7 ppm and 5.5 ppm are characteristic to the anomeric protons found in saccharides. The H-1 proton found in β-glucose appears at 4.7 ppm, while the H-1 proton found in glucuronic acid appears at 5.5 ppm. The signals corresponding to the hydroxyl groups are no longer visible in the ^{1}H-NMR spectrum, being united with the trifluoroacetic acid signal. The attribution of the signals found in the ^{1}H-NMR spectrum can be found in table IV.8.

Table IV.8. Spectral characteristics of the FR85-P2 compound

Proton	**Chemical shift [ppm]**	**Signal shape**	**Coupling constant [Hz]**
H-11”	3.27-3.30	multiplet	-
H-8”, H-9” and H-10”	3.33-3.41	multiplet	-
H-3”, H-4’ and 1H from H-12”	3.64-3.70	multiplet	-
H-6” and 1H from H-12”	3.74-3.80	multiplet	
H-2”	3.81	triplet	8.0
H-7”	4.75	doublet	7.4
H-1”	5.56	doublet	7.4
H-6	6.19	doublet	1.6
H-8	6.39	doublet	1.7
H-3’	6.90	doublet	8.9
H-2’	8.02	doublet	8.8

The edited carbon spectrum, DEPTQ (Distorsionless enhancement by polarization transfer including the detection of quaternary nuclei) version for the FR85-P2 compound is shown in figure IV.33. In this type of experiment, the signals corresponding to carbon atoms are differentiated by the number of protons attached as follows: the CH_3 and CH groups usually have positive phase, while CH_2 and C have negative phase.

Unlike the ^{1}H- NMR spectrum, in the ^{13}C-NMR spectrum all signals are singlets, the couplings responsible for multiplets being removed during the recording of the experiment, by proton decoupling.

The exact attribution of the signals was obtained from bidemensional correlation spectra: 62.8 (CH_2-12"), 71.5 (CH-10"), 72.8 (CH-4"), 75.6 (CH-8"), 76.9 (CH-5"), 77.4 (CH-3"), 78.0 (CH-9"), 78.3 (CH-11"), 82.1 (CH-2"), 94.9 (CH-8), 100.1 (CH-6), 101.2 (CH-1"), 104.7 (CH-7"), 105.9 (C-4a), 116.4 (CH-3'), 122.8 (C-1'), 132.5 (CH-2'), 134.8 (C-3), 158.6 (C-9), 159.1 (C-2), 161.7 (C-4'), 163.2 (C-5), 166.0 (C-7), 179.6 (C-4).

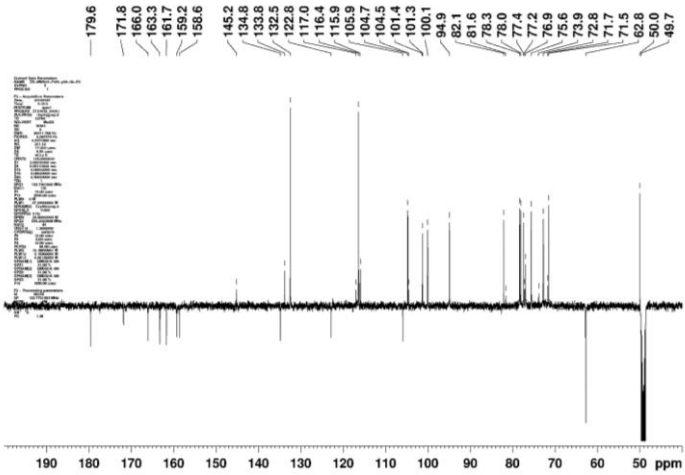


Figure IV.33. ^{13}C-DEPTQ-NMR spectrum for the FR85-P2 compound, measured in MeOD with TFA

The data obtained from bidimensional spectra was used for the exact attribution of the signals from one-dimensional spectra. One of the experiments used is COSY (Correlation spectroscopy). The homonuclear correlation spectrum allows gathering of information regarding vicinal and geminal proton coupling. The COSY spectrum for the FR85-P2 compound is shown in figure IV.36.

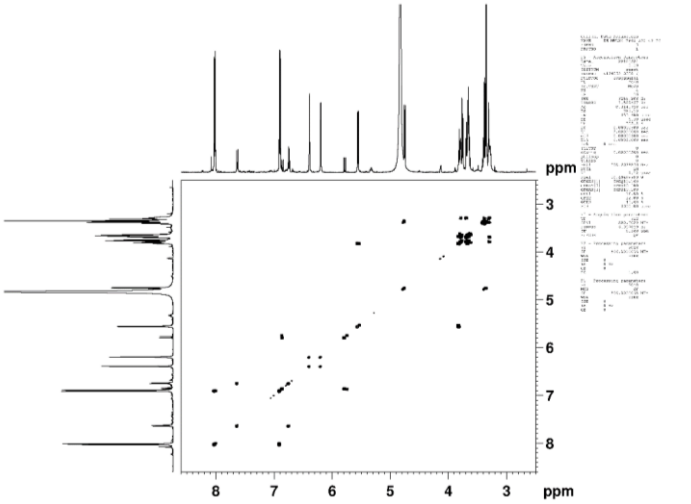


Figure IV.36. COSY spectrum for the FR85-P2 compound

The HSQC spectrum for the FR85-P2 compound is shown in figure IV.38. The one-dimensional ^{1}H-NMR and ^{13}C-DEPTQ-NMR spectra can be found on the axis of the bidimensional spectrum.

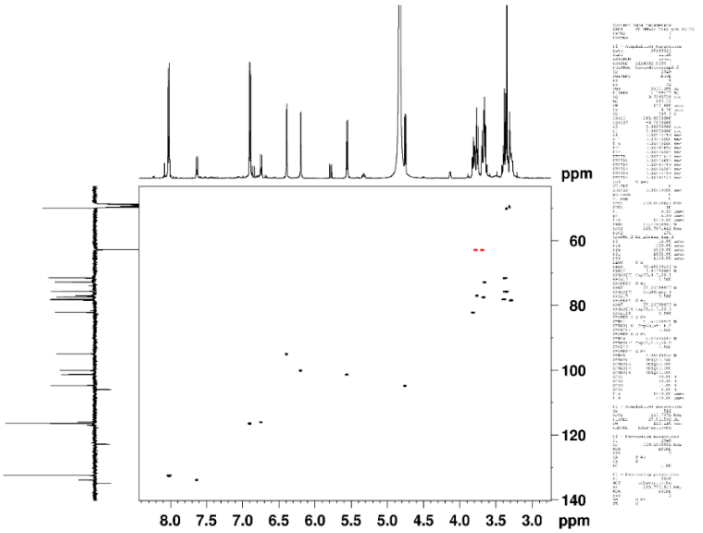


Figure IV.38. HSQC spectrum for the FR85-P2 compound

The signal attribution for quaternary carbon atoms was carried out using the data from the HMBC (Heteronuclear multiple bond correlation) spectrum. This experiment is similar to HSQC and the correlation signals obtained are due to the long-range proton-carbon scalar couplings, over 2, 3 or 4 chemical bonds.

The HMBC spectrum for the FR85-P2 compound can be seen in figure IV.40. The number of correlation signals is much higher compared to HSQC and it also gives the possibility of assigning signals for quaternary carbon atoms.

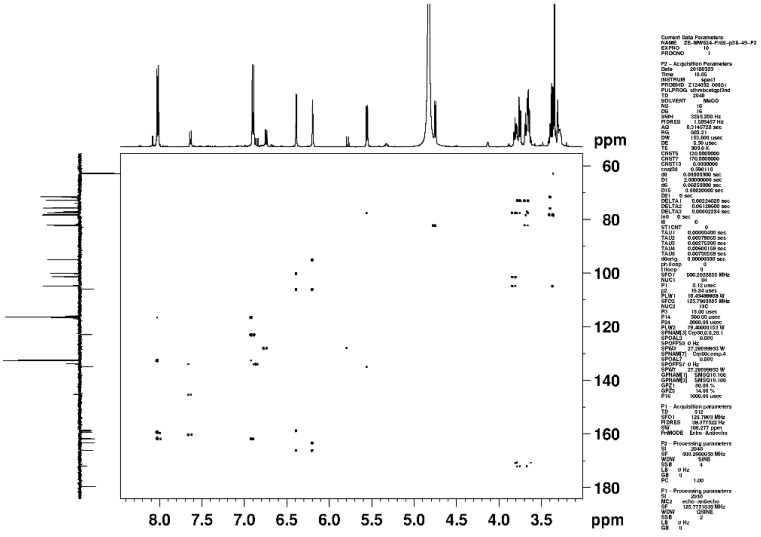


Figure IV.40. HMBC spectra for the FR85-P2 compound

The 4Ta 76-90 fraction obtained from the methanolic extract of *Tagetes erecta* inflorescences by using solid-phase extraction and LH-20 column chromatography was also subjected to NMR spectroscopy analyses. The sample did not require further purification through preparative chromatography techniques, having a purity of over 87%. The proton and carbon spectra indicated the presence of quercetagitrin.

As seen in the proton spectrum shown in figures IV.42-IV.44, this compound presents characteristic signals in the 3.8-6.5 ppm interval for the glycosidic ring and 7.5-10.5 ppm for the quercetagetin unit.

The signal attributions are: 4.01 (1H, t, H-4), 4.12-4.18 (2H, m, H-2 and H-3), 4.25-4.31 (2H, m, H-6 and H-5), 4.55 (1H, d, H-6), 5.43 (t, OH-6), 5.80 (1H, d, H-1), 5.86 (1H, d, OH-4), 5.89 (1H, bs, OH-3), 6.18 (1H, bs, OH-2), 7.70 (1H, d, H-3'), 7.72 (1H, s, H-8'), 8.33 (1H, dd, H-2'), 8.50 (1H, d, H-6'), 9.20 (1H, s, OH-6), 10.04 (1H, s, OH-5'), 10.10 (1H, s, OH-3), 10.38 (1H, s, OH-4'), 13.01 (1H, s, OH-5).

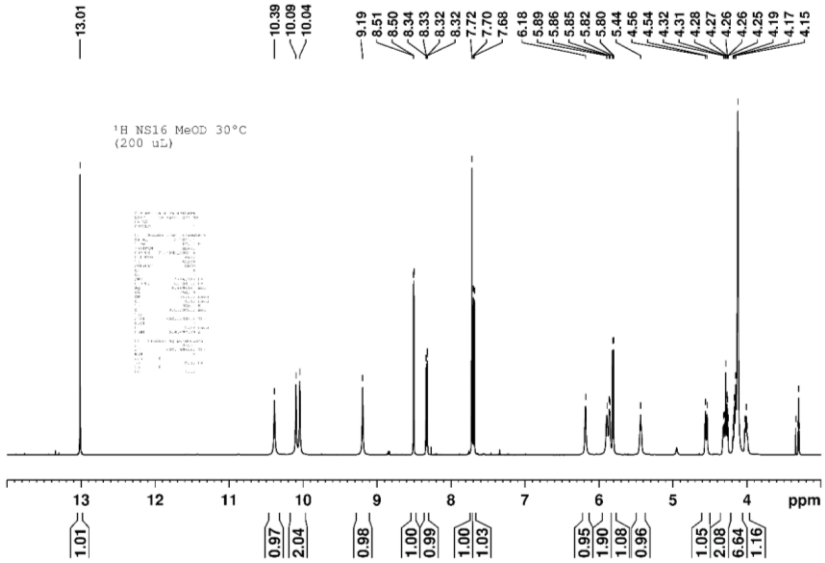


Figure IV.42. [1]H-NMR spectra for the compound found in the 4Ta 76-90 fraction, measured in MeOD with TFA

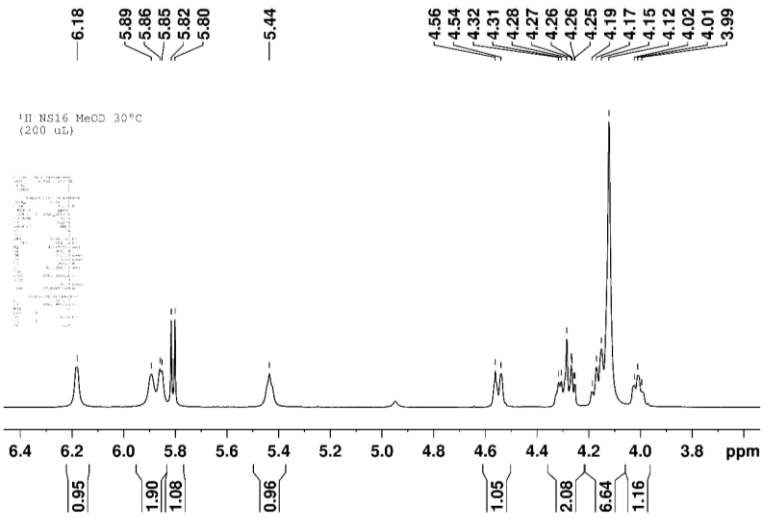


Figure IV.43. [1]H-NMR spectrum for the compound found in the 4Ta 76-90 fraction, measured in MeOD with TFA, 3.8-6.4 ppm spectral window

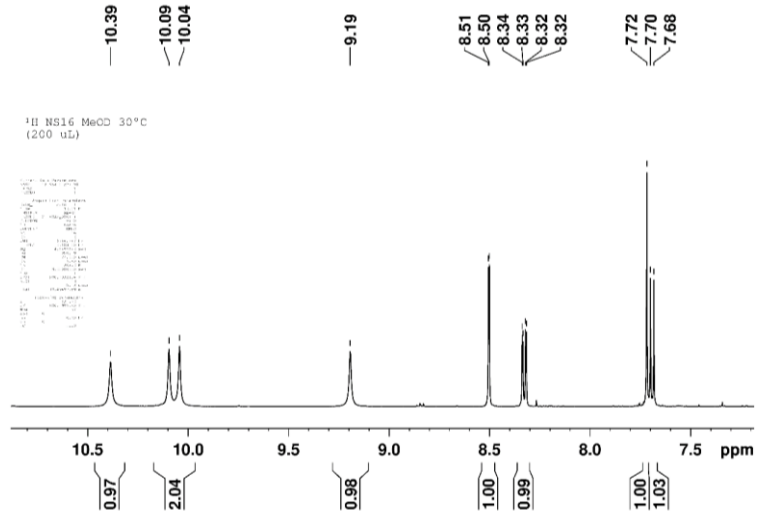


Figure IV.44. [1]H-NMR spectrum for the compound found in the 4Ta 76-90 fraction, measured in MeOD with TFA, 7.5-10.5 ppm spectral window

The number of signals and the chemical shift values from the DEPTQ spectrum (figure IV.45) confirm the presence of quercetagitrin. The signal attributions are: 70.3 (CH2-6), 79.3 (CH-4), 82.8 (CH-2), 85.4 (CH-3), 86.9 (CH-5), 103.2 (CH-8), 110.6 (CH-1), 114.7 (C-4a), 125.0 (CH-6'), 125.1 (CH-3'), 129.5 (CH-2'), 131.6 (C-1'), 139.3 (C-6), 145.2 (C-3), 154.6 (C-5'), 155.0 (C-5), 157.1 (C-2), 157.4 (C-4'), 157.7 (C-9), 161.2 (C-7), 186.7 (CO).

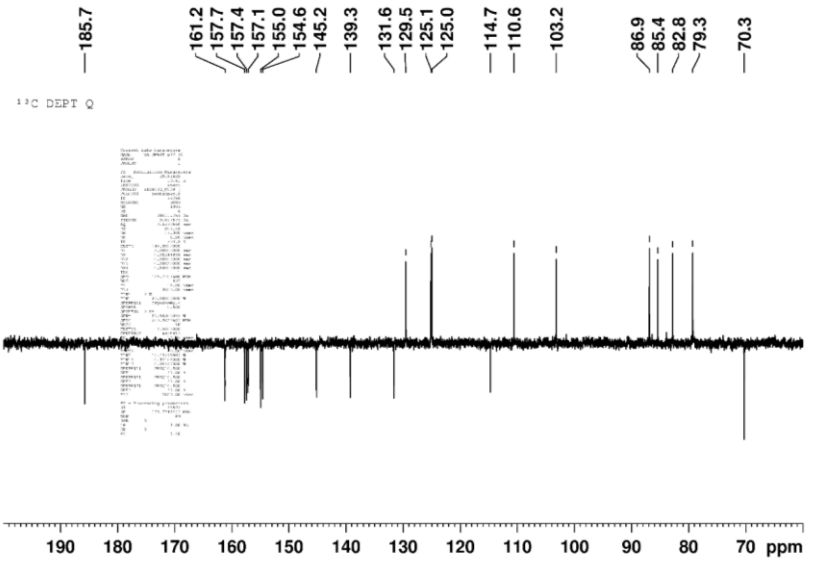


Figure IV.45. ^{13}C-DEPTQ-NMR spectrum for the compound found in the 4Ta 76-90 fraction, measured in MeOD with TFA

Another compound (MW-225) was subjected to NMR analyses, after being previously separated from the 4Z 15-35 fraction obtained from the methanolic extract of *Zinnia elegans* inflorescences and subjected to additional purification by solid-phase extraction and preparative chromatography. Consequently, the presence of plantagoguanidinic acid was revealed (305). The proton and carbon spectra are shown in figures IV.46-IV.48.

The signal attributions in the ^{1}H and ^{13}C-NMR spectra are the following : ^{1}H NMR: 1.61 (3H, s, H-7), 1.68 (3H, s, H-8), 1.60-1.67 (2H, m, H-3), 2.03 (1H, m, H-4b), 2.10 (1H, m, H-4a), 2.45 (1H, m, H-2), 3.54 (1H, m, H-5'b), 3.77 (1H, t, J = 9.5 Hz, H-5'a), 4.16 (1H, m, H-4'), 5.14 (1H, t, J = 6.9 Hz, H-5), 8.48 (OH). ^{13}C NMR: 18.0 (CH_3-7), 26.0 (CH_3-8), 27.0 (CH_2-4), 30.1 (CH_2-3), 48.2 (CH_2-5'), 53.1 (CH-2), 58.5 (CH-4'), 125.0 (CH-5), 133.3 (C-6), 161.5 (C-2'), 181.0 (C-1).

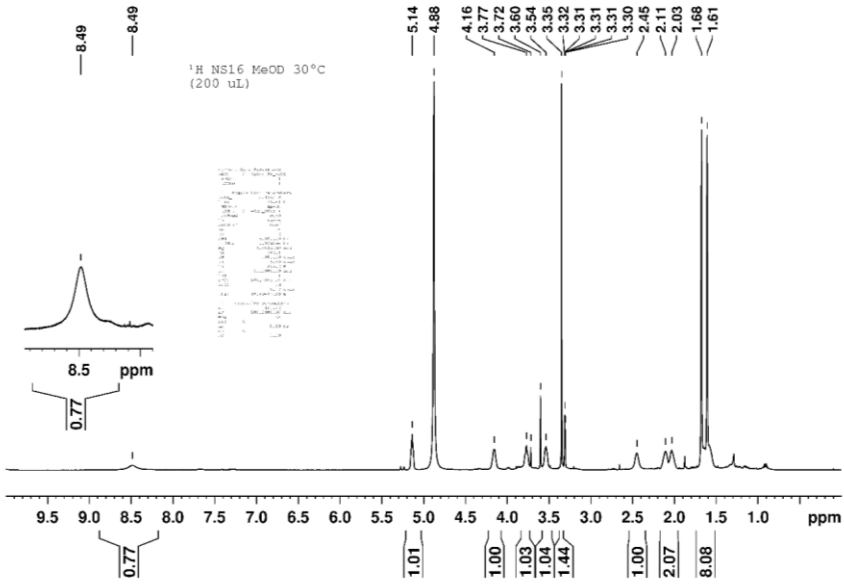


Figure IV.46. ^{1}H-NMR spectrum for compound MW-225, measured in MeOD with TFA, 3.8-6.4 ppm spectral window

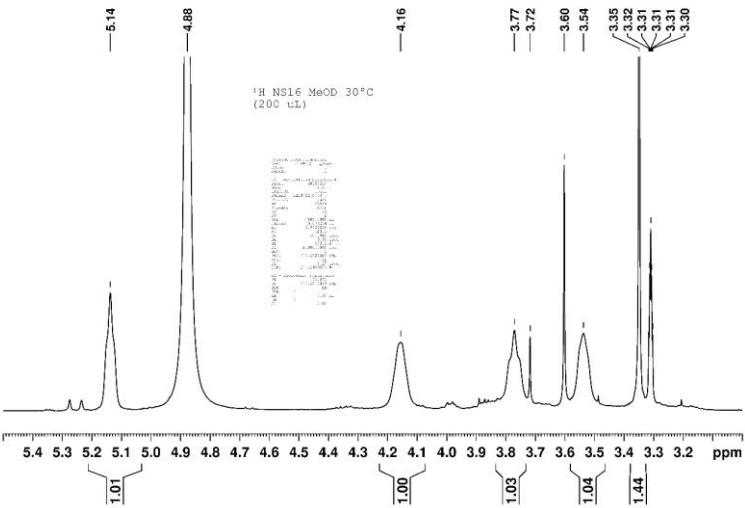


Figure IV.47. [1]H-NMR spectrum for compound MW-225, measured in MeOD with TFA, 3.0-5.5 ppm spectral window

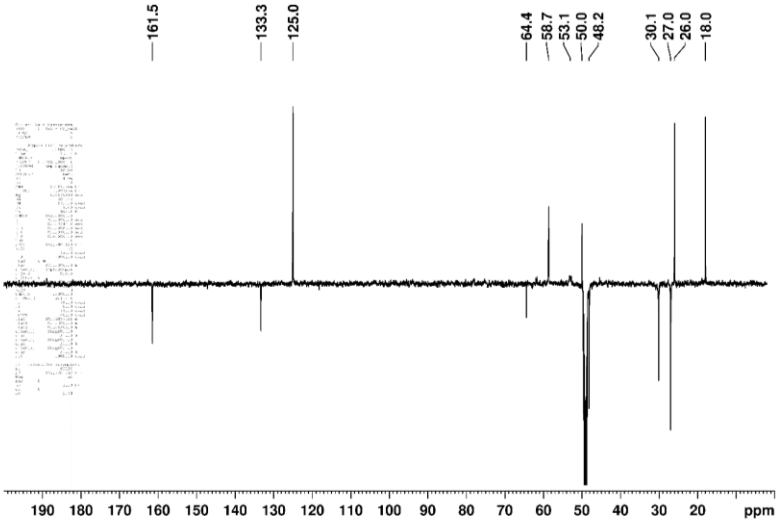


Figure IV.48. [13]C-DEPTQ-NMR spectrum for compound MW-225, measured in MeOD with TFA

Chapter V. Evaluation of the antioxidant action of extracts and fractions obtained from the species considered in the study (*Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq.)

V.1. Determination of DPPH radical scavenger activity

The results of the test indicate that all three extracts have DPPH radical scavenger activity, even at low concentrations (5 mg/mL).

Considering the data obtained in this test, it can be easily noticed that the antioxidant activity exhibited through this mechanism is directly proportional to the amount of extract/standard taken into consideration. Moreover, the methanolic extracts of the species with the highest amount of total polyphenols (*Tagetes erecta*) and total flavonoids (*Zinnia elegans*) presented good scavenging activity values.

In order to make an easy comparison between the results obtained for the samples and the standard, the intensity of the antioxidant action of the extracts in the 0.5 - 20 mg/mL concentration range, compared to that of the standard, is illustrated in figure V.1.

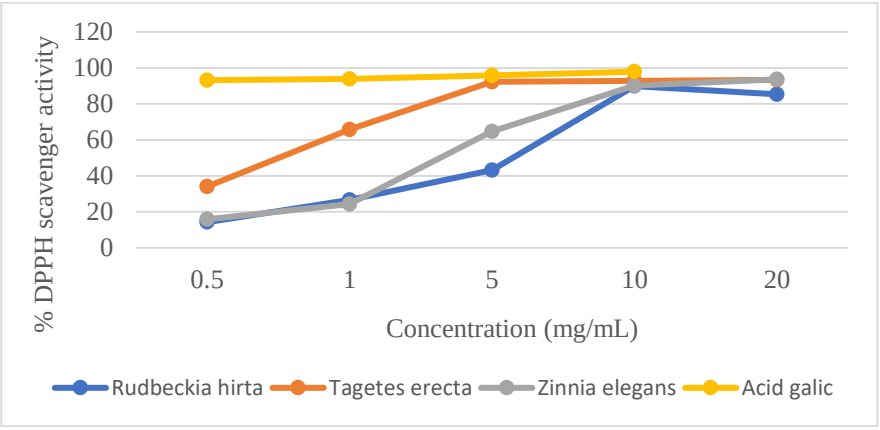


Figure V.1. The DPPH radical scavenging activity of the tested extracts and of gallic acid

In order to highlight the differences regarding the activity between the analyzed extracts and the standard, the effective concentration 50 (EC_{50}) was also calculated. A low EC_{50} value indicates a good antioxidant capacity. It is thus observed that the methanolic extract obtained from *Tagetes erecta* inflorescences has the most significant antioxidant activity through this mechanism, for which a DPPH scavenging activity greater than 50% can be noted starting with a concentration of 1 mg/mL and for which the EC_{50}=11.80±0.09 (table V.2). A promising DPPH scavenging capacity can also be observed for *Zinnia elegans*, while for *Rudbeckia hirta* a weaker activity can be noticed.

Table V.2. EC_{50} values obtained in the DPPH scavenging assay for the methanolic extracts and for the standard

	Sample			
	4R	**4Ta**	**4Z**	**Gallic acid**
EC_{50} (µg/mL final sol.)	92,25±0,44	11,80±0,09	46,38±1,26	1,584±0,02

V.2. Determination of iron (II) chelating activity

Generally, the obtained results indicate a better chelating activity for the three methanolic extracts compared to their fractions, except for the fraction obtained from the methanolic extract of *Rudbeckia hirta* inflorescences (4R 18-35), for which similar EC_{50} values were obtained. The results can be justified by the existence of several polyphenolic compounds such as tannins found in the total extract, which increase the number of free hydroxyl groups that can form complexes with ferrous ions. Depending on the potency of the three extracts, the species can be ordered in ascending order as follows: *Zinnia elegans*, *Rudbeckia hirta* and *Tagetes erecta.*

The total extract obtained from *Tagetes erecta* presented the lowest EC_{50} value, which is even lower compared to that of quercetin, which was used as a standard. This extract presented the highest total polyphenols content of the three tested extracts, which may justify the result. In addition, this species is known for being rich in carotenoids, compounds that can contribute to the antioxidant effect demonstrated by the ferrous ions' chelation mechanism (332). However, for the *Zinnia elegans* extract, in which the highest amount of flavonoids was detected, there is no direct correlation between the concentration in such substances and the activity exhibited in this test. This could be explained by the fact that a number of hydroxyl groups are linked to glycosides or other types of groups and, thus, cannot participate in the formation of the corresponding complexes with metal ions.

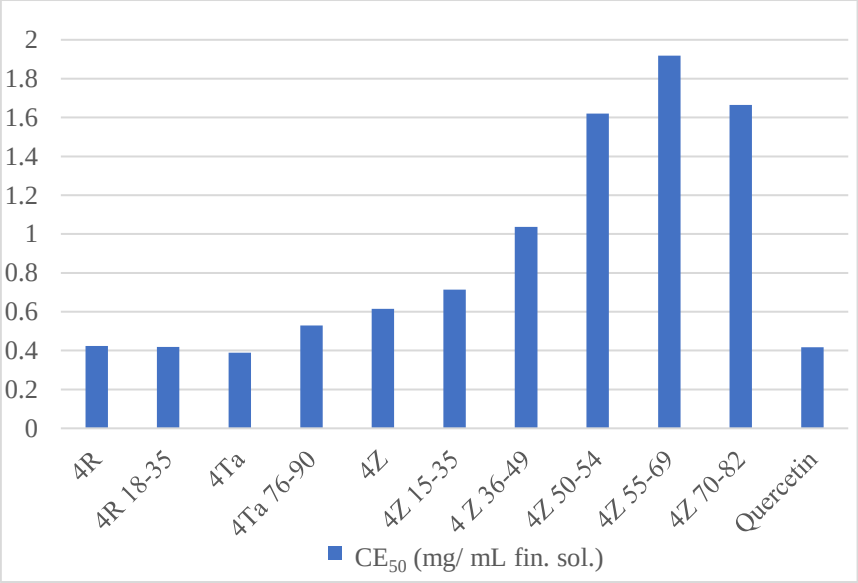


Figure V.2. Graphical representation of the EC_{50} values obtained for the initial extracts and for some fractions in the iron (II) chelating activity assay

V.3. Determination of 15-lipoxygenase inhibitory activity

Analyzing the results of the analysis, it can be easily noticed that the various separation methods applied to the total extracts are useful for obtaining fractions with high antioxidant activity, but not for *Rudbeckia hirta*. Comparing the obtained EC_{50} values for the three initial extracts, it can be observed that the intensity of the antioxidant action by this mechanism of action decreases in the following order: 4Ta> 4R> 4Z. Thus, once again, the correlation between the total polyphenols content and the antioxidant action demonstrated through this mechanism can be drawn, especially for *Tagetes erecta*.

Of the tested samples, the lowest EC_{50} values can be observed for the 4Ta 76-90 fraction, which contains mostly quercetagitrin, its value being lower than that obtained for the positive control, and also for the 4Z 36-49 fraction, which contains chlorogenic acids and kaempferol glycosides (figure V.3).

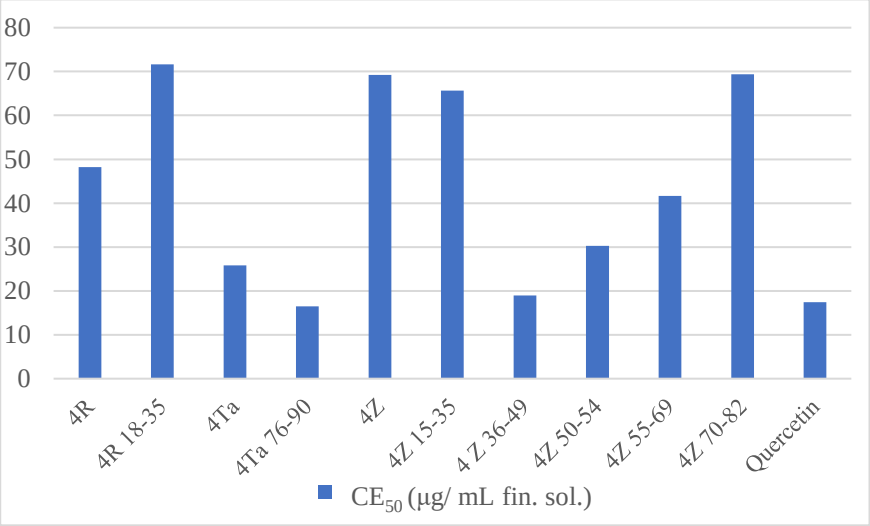


Figure V.3. Graphical representation of the EC_{50} values obtained for the initial extracts and for some fractions in the 15-LOX inhibitory activity assay

Chapter VI. Assessment of the antimicrobial activity of extracts obtained from *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq.

The inhibition zone diameters (in mm) for the three tested plant extracts and for the used standards are presented in table VI.1. The MIC and MBC values can be seen in table VI.2.

Table VI.1. Antibacterial and antifungal activity of the tested extracts

Plant extract/ Control/ Standard	**Inhibition zone diameters (mm)**				
	***Staphylococcus aureus* ATCC 25923**	***Escherichia coli* ATCC 25922**	***Pseudomonas aeruginosa* ATCC 27853**	***Candida albicans* ATCC 90028**	***Candida parapsilosis* ATCC 22019**
4R	16,0±0,00	10,0±0,00	10,0±0,00	15,0±0,00	17,0±0,00
4Ta	15,3±0,57	11,0±0,00	0	19,0±0,00	17,0±0,00
4Z	13,0±0,00	0	11,0±0,00	*NT	13,0±0,00
DMSO	0	0	0	0	0
Ciprofloxacin	28,7±0,06	36,5±0,50	31,5±0,50	*NT	*NT
Fluconazole	*NT	*NT	*NT	30,5±0,50	21,0±0,00
Nystatin	*NT	*NT	*NT	23,5±0,50	20,0±0,00

*NT – not tested

Table VI.2. MIC and MBC values for *Rudbeckia hirta* and *Tagetes erecta* extracts

Plant extract/ Standard	***Staphylococcus aureus* ATCC 25923**		***Escherichia coli* ATCC 25922**	
	MIC (mg/mL)	**MBC (mg/mL)**	**MIC (mg/mL)**	**MBC (mg/mL)**
4R	0,25	0,5	0,25	>0,5
4Ta	0,125	0,5	0,25	0,5
Ciprofloxacin	1*	1*	1*	2*

* values are expressed in µg/mL

Regarding the activity against the Gram-positive bacteria, the methanolic extract obtained from *Rudbeckia hirta* (4R) proved to be most active, having a good bacterial growth inhibitory capacity (16.0 mm).

The extract showed moderate growth inhibition activity against Gram-negative bacteria (10.0 mm). At the same time, the same extract showed good antifungal action, with a remarkable activity on *Candida parapsilosis* (17.0 mm), similar to that observed for the used standards (20.0 mm for nystatin and 21.0 mm for fluconazole, respectively). All extracts showed good antifungal action.

Regarding the evaluation of the *Tagetes erecta* extract (4Ta), it can be observed that it has the best antifungal activity of all the three tested extracts, having the potential to inhibit the growth of *Candida albicans* and *Candida parapsilosis* similar to that of nystatin. The extract shows inhibition of *Staphylococcus aureus* and *Escherichia coli*, but does not exhibit antibacterial activity on *Pseudomonas aeruginosa.*

The results obtained for *Zinnia elegans* indicate that the methanolic extract has the capacity to inhibit bacterial and fungal strains, but this action is weaker compared to the other two tested species. The antibacterial action seems to be more significant on Gram-positive bacteria than on Gram-negative bacteria.

Regarding MIC and MBC values determination, it is generally observed that MBC values are 2-4 times higher than the MIC values, but much higher than those of the used standard, ciprofloxacin, therefore indicating a relatively low potency compared to it.

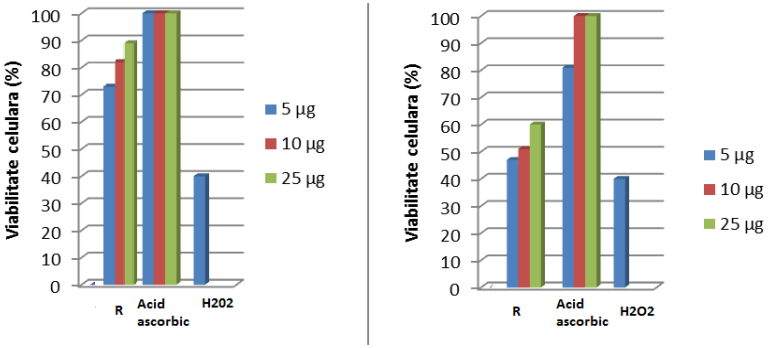
Chapter VII. The assessment of cytotoxicity and cytoprotective activity on cell cultures of extractive fractions obtained from *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq. inflorescences

VII.1. *In vitro* cytotoxicity testing of the studied total extracts

For this experiment, no significant cytotoxic effects were noticed on the healthy NIH3T3 fibroblast cell line for the tested samples. The IC_{50} values ranged from 64.71±3.22 to 91.91±2.92 µg/mL, concentrations that are difficult to obtain *in vivo.*

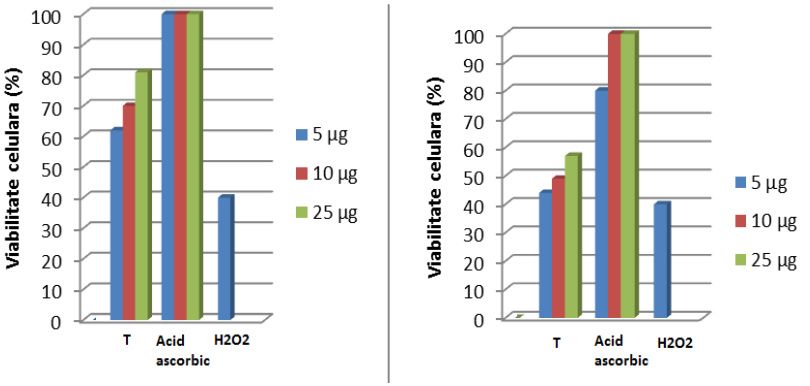
VII.2. Evaluation of the *in vitro* cytoprotective activity of the studied total extracts

The results for the cell viability under oxidative stress testing for the three total extracts can be seen in figures VII.5-VII.7.



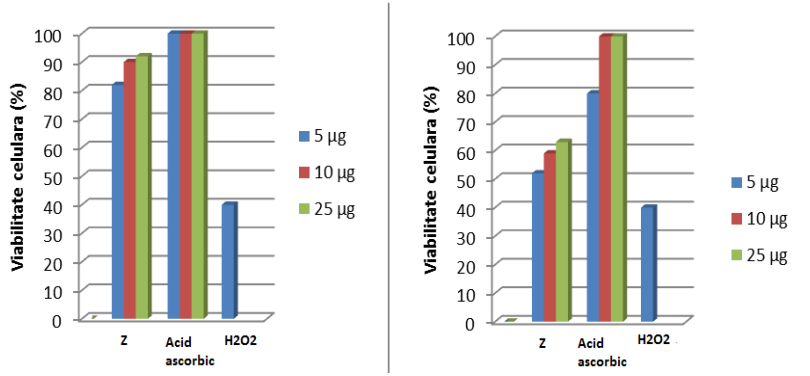
a. b.

Figure VII.5. Cytoprotective effect of the *Rudbeckia hirta* extract on the NIH3T3 cell line: a. 24h pretreatment before applying H_2O_2, b. 60 min pretreatment before applying H_2O_2



a. b.

Figure VII.6. Cytoprotective effect of the *Tagetes erecta* extract on the NIH3T3 cell line: a. 24h pretreatment before applying H_2O_2, b. 60 min pretreatment before applying H_2O_2



a. b.

Figure VII.7. Cytoprotective effect of the *Zinnia elegans* extract on the NIH3T3 cell line: a. 24h pretreatment before applying H_2O_2, b. 60 min pretreatment before applying H_2O_2

It can be noticed that in the case of a 24h pretreatment, the cells have developed protection mechanisms against oxidative stress, which could be seen through a high rate of cell survival. However, the cytoprotective effect was not significant for cells exposed for a short time (60 min) to the three plant extracts.

VII.3. Comparative evaluation of the cytotoxicity of the extract obtained from *Tagetes erecta* inflorescences and of one its fraction, 4Ta 76-90

The presence of viable cells found in the cell culture, which was previously incubated with the initial extract and the 4Ta 76-90 fraction, was evaluated using the MTT assay. The obtained results are presented in figures VII.10 and VII.11.

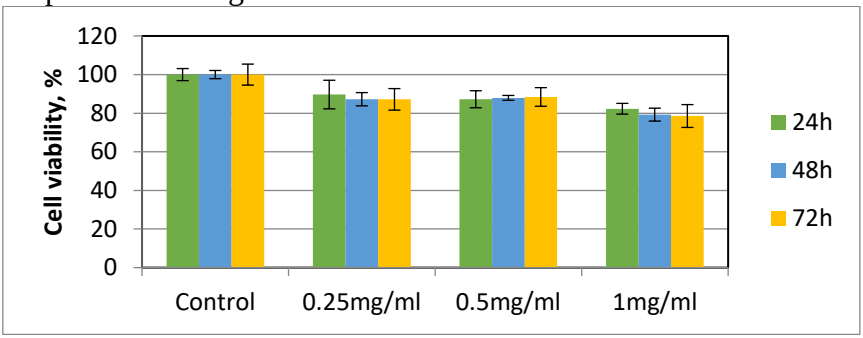


Figure VII.10. Cell viability observed during the MTT assay for the extract obtained from *Tagetes erecta* inflorescences

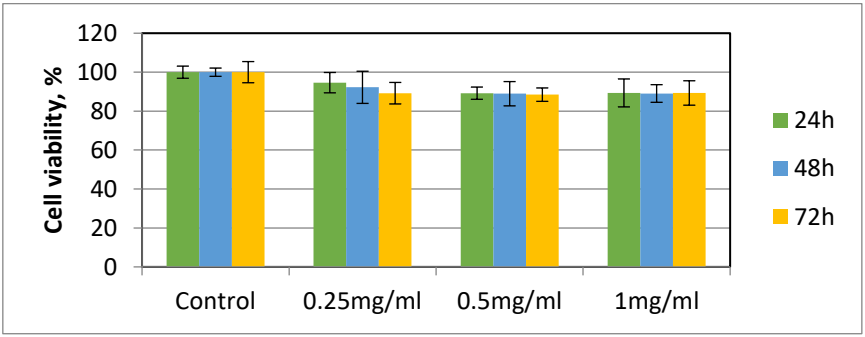


Figure VII.11. Cell viability observed during the MTT assay for fraction 4Ta 76-90

A slight tendency of diminished cell viability can be observed for the *Tagetes erecta* extract (4Ta) when increasing concentration and contact time, which indicates the existence of a cytotoxic potential at higher concentrations. However, the fraction did not present cytotoxicity in the studied concentration range.

GENERAL CONCLUSIONS. ORIGINAL CONTRIBUTIONS. RESEARCH PERSPECTIVES

GENERAL CONCLUSIONS

The main objective of the PhD thesis entitled **Chemical and biological evaluation of some extractive fractions from ornamental *Asteraceae species*** is the evaluation of the biological potential of total extracts obtained from three ornamental species: *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq. and of some of their fractions, in correlation with the presence of certain polyphenolic compounds. The chemical and biological analyses carried out on the initial extracts, as well as on some fractions obtained from these, represent elements of novelty regarding the investigation of the therapeutic potential of such plants, given the fact the scientific literature contains very little data in this regard.

The macroscopic evaluation allowed us to establish important differences between the three species of the *Asteraceae* family, especially regarding the size, smell, color, and also the structure of the leaves, but the inflorescences are all capitula.

Regarding the histo-anatomical analysis of the species, it has been shown that the leaf blade has an equifacial bifacial structure in *Rudbeckia hirta* and a heterofacial bifacial structure in *Tagetes erecta* and *Zinnia elegans.* All three species of the *Asteraceae* family have single or multicellular covering trichomes and pluricellular glandular trichomes. The latter are found in all the aerial parts of the plant, but especially in leaves and flowers. Generally, in tubular flowers, glandular trichomes, which are often biseriate, are present on both the corolla and the reproductive organs.

For the extraction of polyphenolic compounds from the plant matrix, we chose to obtain methanolic extracts from the inflorescences of the species taken into study. The largest amount of dry extract was obtained for *Tagetes erecta*.

The phytochemical evaluation of the inflorescences harvested in 2017 from the plants that were cultivated in ecological conditions included the qualitative and quantitative analysis of the methanolic extracts, as well as the qualitative analysis of the fractions obtained from these.

Regarding the qualitative analysis done on the methanolic extract obtained from *Rudbeckia hirta* L. inflorescences using UHPLC-MS techniques, it was discovered that it contains amino acids, chlorogenic acids, as well as flavonoids, especially glycosides of patuletin, eupalitin, quercetin and quercetagetin.

The methanolic extract obtained from *Tagetes erecta* L. inflorescences contains amino acids, chlorogenic acids, gallic, quinic, ellagic and syringic acids derivatives, as well as flavonoids, especially glycosides of quercetagetin, quercetin, kaempferol and patuletin.

For *Zinnia elegans*, some compounds such as guanidine alkaloids (plantagoguanidinic acid and plumbagine B), gallic acid derivatives, some mono- and diacyl chlorogenic acids, as well as glycosides of apigenin, quercetin, kaempferol and resokaempferol were identified in the methanolic extract.

Some compounds, especially from the polyphenols class, that were identified in the initial methanolic extract, can be selectively found in the fractions obtained after solid phase extraction and LH-20 column chromatographic separations, fact that was confirmed by LC-MS analyses.

Regarding the spectrophotometric quantitative determination of total flavonoids and total polyphenols from the methanolic extracts, it can be noticed that the *Tagetes erecta* extract has the highest content in polyphenols, while the highest content in flavonoids is found in the methanolic extract obtained from *Zinnia elegans* inflorescences.

The fractionations that were done through different extractive and chromatographic methods have led to the isolation of several compounds from *Zinnia elegans* and *Tagetes erecta.* Thus, in the present PhD thesis, the existence of kaempferol 3-*O*-[β-

glucopyranosyl-(1→2)-β-glucuronopyranoside] and its presence in *Zinnia elegans* were described for the first time, as well as the presence of plantagoguanidinic acid in the same species. The structure of these compounds was elucidated by corroborating the results obtained after spectroscopic analyses (mass spectrometry and NMR spectroscopy). Moreover, we have also succeeded in isolating quercetagitrin from *Tagetes erecta*, compound which has been previously described for this species.

The *in vitro* evaluation of the antioxidant activity of some extractive fractions was carried out through three different testing mechanisms. The DPPH scavenger activity represented a preliminary test which allowed the identification of the most active initial extracts and, afterwards, the inclusion of some of their fractions in the following antioxidant tests. The methanolic extracts obtained from *Tagetes erecta* and *Zinnia elegans* inflorescences had the best EC_{50} values.

The *Tagetes erecta* methanolic extract presented a better value than that obtained for quercetin in the iron chelating activity test. Generally speaking, for this test the results were more promising for the initial extracts than for their fractions.

For the 15-LOX inhibition assay, different results than those in the previous test were obtained. Therefore, the fractionations proved to be useful in obtaining fractions that were more active through this mechanism of action. Two of the fractions obtained from *Tagetes erecta* (4Ta 76-90) and *Zinnia elegans* (4Z 36-49), respectively, present similar values to that of quercetin, which was used as standard.

Considering the results of the antimicrobial evaluation of the methanolic extracts obtained from the inflorescences of the considered species, it can be stated that these have promising antifungal and antibacterial actions, especially on Gram-positive bacteria. Moreover, a certain growth inhibition on some Gram-negative bacteria could be noticed. The best antibacterial activity was observed for the methanolic extract obtained from *Rudbeckia hirta* inflorescences, while the most notable antifungal action was

seen for the *Tagetes erecta* extract, the obtained values being similar to those of the used standards (nystatin, fluconazole).

The *in vitro* evaluation of the cytotoxicity of the plant extracts on the NIH3T3 fibroblast cell line revealed some cytotoxic effects only at a concentration of over 50 µg/mL for all samples. Taking into consideration the used doses, we believe that the tested samples lack cytotoxicity.

Moreover, the cytoprotective activity testing on cell cultures emphasized a promising activity for all the extracts in the case of a 24-hour pretreatment, fact which was confirmed by high cell survival rates. The comparative evaluation of the cytotoxicity of the *Tagetes erecta* extract and of the 4Ta 76-90 fraction obtained from it, which contains mostly quercetagitrin, revealed the importance of applying various separation techniques in order to obtain compounds with enhanced biological actions and reduced cytotoxicity compared to the initial extract.

ORIGINAL CONTRIBUTIONS

The original contributions of the present study consist of the pharmacognostic and biological studies done for the first time in our country on the inflorescences of the ornamental species *Rudbeckia hirta* L., *Tagetes erecta* L. and *Zinnia elegans* Jacq., which were cultivated in ecological conditions. The morphological and histo-anatomical characterization of the inflorescences collected from the three species was performed, as well as the chemical evaluation of the total methanolic extracts and of the selective fractions obtained from them by various separation methods. Moreover, the presence of two compounds that have not previously been described in the literature for *Zinnia elegans* was demonstrated, their structure being established using mass spectrometry and NMR spectroscopy.

For the methanolic extracts and for some of their fractions the following analyses were done:

- *in vitro* antioxidant activity testing, using three different methods,

- ❖ antibacterial and antifungal testing,
- ❖ evaluation of the *in vitro* cytotoxicity of the methanolic extracts,
- ❖ evaluation of the cytoprotective activity of the methanolic extracts.

RESEARCH PERSPECTIVES

After correlating the promising results obtained in the present PhD study, the following research directions are viable:

- ❖ purification of the fractions already separated from the initial extracts, in order to isolate compounds and to evaluate their biological actions compared to those of the total initial extracts;
- ❖ the obtaining and investigation of volatile fractions from the studied species regarding qualitative and quantitative analysis of the extracted compounds, as well as observing their biological effects, compared to the fractions analyzed in this research;
- ❖ evaluating the antioxidant potential of all fractions obtained from the methanolic extracts, as well as applying other antioxidant tests;
- ❖ further evaluation of antimicrobial and antifungal potential by including other bacterial and fungal strains, as well as testing the obtained fractions;
- ❖ evaluation of several representative fractions separated from the three initial extracts through an additional number of tests on cell lines, in order to establish the mechanisms involved in the antioxidant and anti-inflammatory action.

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