"BABEȘ-BOLYAI" UNIVERSITY CLUJ-NAPOCA

Faculty of Biology and Geology

Doctoral School of Integrative Biology

Doctoral Thesis

Evaluating the antioxidant potential of several extracts from *Plantago*, *Galium* and *Hypericum*

- Summary -

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Abbreviation list

12-LOX–12-lipoxygenase;

3NT – 3-nitrotyrosin;

ABTS$\bullet^{+}$ – 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid);

ADN – Dezoxyribonucleic acid;

Af – final absorbance;

Ai – initial absorbance;

ALB – albumin;

ALT – alaninaminotransferase;

ANOVA – analysis of variance;

AST – aspartataminoransferase;

Casp3 – caspase 3;

CAT – catalase;

CD3 – T- cells co-receptors; Cluster of differentiation 3;

CIF – crystallographic information file;

CMF-BSS – calcium and magnesium buffer solution;

ConA – concavalin A;

COX-1– ciclooxygenase 1;

COX-2– ciclooxygenase 2;

CPA – cyclofosfamide;

Crea – creatinine;

CRP – C reactive protein;

CS – corticosterone;

CT – total cholesterol;

CYP450s – cytocromul 450;

D3 – dopamine receptor;

D4 – dopamine receptor;

Dcf – diclofenac;

DMEM – Dulbecco's modified eagle medium;

DMSO – dimethys ulfoxide;

DPPH – 2,2-diphenyl-1-picrylhydrazyl;

E.A.hy926 – endothelial human cells;

E2 – estradiol;

EC50 – Half maximal effective concentration;

ELISA – enzyme-linked immunosorbent assay;

EP – epinephrine;

EtOH – ethanol;

Fas-FasL – two molecules involved in the regulation of cell death;

FBS – Fetal bovine serum;

FC – Folin-Ciocâlteu;

FSH – Folicle-stimulanting hormone;

GABA – gamma-aminobutyric acid;

GAE – gallic acid equivalents;

GDF9 – growth and development factor 9;

GPX – glutation peroxidase;

GR – gluthation reductase;

GSH – glutathion;

H_2O_2 – oxygenated water;

HCT – hematocrit;

HGB – hemoglobin;

HHS – hypothalamic pituitary adrenal axis;

HIV-I – human immunodeficiency virus I;

HPLC-DAD – high performance liquid cromatography- diode array detector;

HPLC-MS – high performance liquid cromatography- diode array detector;

IC50 – The half maximal inhibitory concentration;

IFN-γ – gamma interferon;

IL - 1β – interleukin 1β;

IL- 6 – interleukin 6;

IL-4 – interleukin 4;

IOP – primary ovarian insuficiency;

JNK – Janus kinase;

LC-MS/MS – liquid cromatography coupled with with mass spectrometry; two analyzers coupled in one;

LDL – Low density lipoproteins;;

LOD – limit of detection;

LOQ – limit of cuantification;

LPS – lipopolysaccharides;

LYM – limfocytes;

MAO – monoamino oxidase;

MAP– mitogen activated kinases;

MCF-7 – breast cancer cell line;

MCH – mean corpuscular haemoglobin;

MCHC – mean corpuscular haemoglobin concentration;

MCV – mean corpuscular volume;

MDA – malondialdehyde;

metHB – methemoglobin;

MMP- 9 – matrix metallopeptidase 9;

MON – monocyte;

MPO – myeloperoxidase;

MPV – mean platelet volume;

MRC-5 – Medical Research Council cell strain 5, pulmonary fibroblasts;

$NADP^+$ – Nicotinamide adenine dinucleotide phosphate (oxy);

NADPH - Nicotinamide adenine dinucleotide phosphate (red);

NADPH oxidaza – Nicotinamide adenine dinucleotide phosphate oxidase;

NaOH – sodium hydroxide;

NAPQI – N-acetyl-p-benzoquinone imine;

NEU – neutrophils;

NO – nitric oxide;

NOx – nitric oxide species;

NRF2/Keap1 – nuclear factor associated with eritroid 2 factor/ Kelch-like ECH-associated protein 1

$O_2^{-}\bullet$ – superoxide radical;

OH^- – hydroxil radical;

OMS (WHO) –World health organisation;

OSI – oxidative stress index;

oxiHB – oxihemoglobin;

p38 – proteins belonging to MAPK family;

PCA – principal component analysis;

PCT – plateletcrit;

PDW – platelet distribution with;

PGD_2 – prostaglandin D2;

$PGF_{2\alpha}$ – prostaglandin F2-alfa;

PGI_2 – prostacyclin;

PHA – phytohaemagglutinin;

PLT – platelets;

PMN – polymorphonuclear cells;

PPAR γ – peroxisome proliferator-activated receptors;

PRO – progesterone;

RBC – red blood cells;

RDW – red blood cells distribution width;

RE – rutin equivalents;

RES – electroparamagnetic resonance spectroscopy;

SEM – scanning electron microscopy;

SERM – Selective estrogen receptor modulators;

SOD (1,2,3) – superoxid dismutase, 1, 2 or 3;

SRA (RNS) – reactive nitrogen species;

SRO (ROS) – reactive oxygen species;

SSRI – Selective serotonin reuptake inhibitors;

TAC – total antioxidant capacity;

TE – Trolox equivalents;

TEAC – Trolox equivalents antioxidant capacity;

TEM – transmission electron microscopy;

TLC – thin layer cromatography;

TNF-α –tumor necrosis factor;

TOS – total oxidative stress;

TXA – tromboxane;

Tyr – tyrosine;

UACC-62 – human melanom cell line;

UV-VIS – UV spectroscopy;

WBC – white blood cells;

Keywords: vegetal extracts, *Plantago, Galium, Hypericum*, antioxidants, inflammation, cells, oxidative stress, flavonoids, mimetism, estrogen;

Scope of the study

This doctoral thesis aims to obtain extracts, rich in compounds with antioxidant potential, from *Plantago, Galium* and *Hypericum* species, in order to investigate their potential on *in vivo* model of oxidative stress.

The objectives of the paper concern the following aspects:

1. Phytochemical evaluation, antioxidant activity and *in vitro* regenerative potential of *Plantago sempervirens* extract.

2. Investigating the adjuvant and anti-inflammatory potential of *P. sempervirens* extract in conditions of acute systemic inflammation in the laboratory rat.

3. Investigating the estrogen-mimetic effect of flavonoids from *Plantago* extracts on a biological model of oxidative stress on the female reproductive system.

4. Preparation of *Galium verum* extract and its chemical analysis for *in vivo* evaluation on a model of oxidative stress induced by neuropsychiatric stress.

5. Evaluating of rutin efficacy compared to rutin-rich *H. capitatum* extract in an *in vivo* model of oxidative stress associated with acute systemic inflammation.

6. Preparation of hydroalcoholic extracts of *P. cornuti, P. lanceolata, P. major* and *P. media* and their comparative analysis, both in terms of chemical composition and antioxidant activity.

I. Literature review

1.1. Biological characteristic of several *Plantago* species

1.1.1. Plant systematics and speciation

The genus *Plantago* belongs to the family Plantaginaceae, order Lamiales, subclass Asteridae, class Magnoliposida (Dicotyledonatae), branch Tracheophyta, kingdom Plantae. The Lamiales order includes herbaceous plants, with simple, unstipped leaves, grouped in basal rosettes, very rarely opposite. The flowers are actinomorphic, on type 4, gamosepalous and gamopetalous with persistent calyx, upper ovary and capsule fruit (Ciocârlan, 2009). The genus *Plantago* comprises about 250 species, with a widespread geographical distribution, especially in temperate areas, but also in tropical areas. (Hassemer et al., 2018). Species of this genus have been intensively studied, over time, both morphologically, anatomically and phytochemically, therapeutically etc. (Hetland et al., 2000; Rahn, 1996; Taskova et al., 1999).

Internationally, new species have been discovered and described, such as *Plantago nebularis* and *Plantago zoellneriana* in Latin America (Hassemer et al., 2018). In Romania, in 2003, the species *Plantago sempervirens* Crantz was reported for the first time, in the spontaneous flora, in Pusta village, Șincai locality from Mureș county (Puşcaş et al., 2003).

Among the *Plantago* species, in this doctoral thesis were researched *Plantago cornuti* Gouan, *Plantago major* L., *Plantago media* L., *Plantago lanceolata* L. and *Plantago sempervirens* Crantz.

Fig. 1. *P. major* L. (original image)

Fig. 2. *P. lanceolata* L. (original image)

Fig. 3. *P. media* L. (original image)

Fig. 4. *P. cornuti* Gouan (original image)

Fig. 5. *P. sempervirens* Crantz (original image)

1.1.3. Chemical composition

The genus Plantago is a promising source of new bioactive molecules, because only a part of the species of this genus have been closely studied in terms of their chemical composition. (Gonçalves and Romano, 2016). Most papers that describe the chemical composition of *Plantago* species comprise mostly *P. major*, *P. lanceolata* and *P. media* (Beara et al., 2009; Janković et al., 2012; Lukova et al., 2017).

The phytochemistry of the genus was investigated mainly by chromatographic methods (HPLC), coupled chromatographic (HPLC-MS, LC-MS / MS) methods for the determination of

flavonoid content (Janković et al., 2012), but also colorimetric (for the determination of total phenols, total iridoids or total phenylpropanoid glycosides) (Handjieva et al., 1993; Janković et al., 2012; Taskova et al., 1999). *Plantago* species contain many compounds with biological properties such as: polysaccharides, lipids, terpenes, benzoic compounds (vanillic acid), tannins, saponins, sterols, phenolic compounds, flavonoids (e.g. luteolin, apigenin, rutin), iridoid glycosides (e.g. aucubin , catalpol), caffeic acid (its main derivative is plantamajoside)(Samuelsen, 2000; Stanisavljevic et al., 2008).

The phenolic profile in *P. major* highlights several major compounds, namely ferulic acid, ellagic acid, but also luteolin, which according to the literature is the major compound in many species of *Plantago*, both leaves, flowers and seeds (Kawashty et al., 1994; Samuelsen, 2000).

P. media also belongs to the subgenus *Plantago*, so a phenolic profile similar to *P. major* can be considered. Other compounds identified in this species are: aucubin, melitosis, plantamajoroside, monomelitosis, 10-acetylmonomelitosis, acteoside, isoacteoside and chlorogenic acid (Olennikov et al., 2011; Saadi et al., 1988).

P. cornuti is another species belonging to the subgenus *Plantago* and from which a compound of the iridoid class, 10-hydroxyyridoid, was isolated for the first time, the second most important of this class (Handjieva et al., 1993). Aucubin and catalpol have also been identified in this species (Rønsted et al., 2000).

P. sempervirens belongs to subgenus *Psyllium* and it is less widespread geographically, which may be a reason why its medicinal potential is not yet known. Chromatographic studies, performed on this species, show only a few identified elements of the iridoid class, such as aucubin, plantarenaloside, karyoptoside, gardoside and 8-epilogonic acid. (Venditti et al., 2011).

1.1.4. Therapetical effects

Healing activity

During the healing process, a series of processes are activated in the skin of mammals, which involve inflammation, generation and remodelling of new tissue. Over a skin injury, the re-epithelialization process involves the migration of keratinocytes to the edges of the lesion, where they will begin to proliferate (Schäfer și Werner, 2007). Studies on cell proliferation stimulated by the presence of *P. major* extracts, at certain concentrations, have been associated with the healing effect of this species. Compounds responsible for the healing effect are not yet known for sure, but they may be associated with compounds responsible for anti-inflammatory and antioxidant activity, such as plantamajoside. (Mahmood și Phipps, 2006).

Antibacterial activity

In vitro, the antibacterial activity of *Plantago* extracts was investigated using the principle of antibiogram, in which the discs are impregnated with extract, and then applied to the seeded plate with the strains of interest. Extracts of *P. major* and *P. lanceolata* were experimentally tested on cultures of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and the reference antibiotics were penicillin and trimethoprim-sulfamethoxazole. Thus, *P. major* extract was effective in all three strains, and *P. lanceolata* extract had an effect only on *S. aureus* (Caceres et al., 1987; Mazzutti et al., 2017).

Antioxidant activity

Antioxidant activity is manifested in a wide variety of actions, including inhibition of oxidizing enzymes, chelation of transition metals, transfer of hydrogen or electrons unpaired to radicals, enzymatic detoxification of reactive oxygen species etc. (Bahadori et al., 2020; Beara et al., 2012). Stronger antioxidant activity is expressed by *P. lanceolata*, followed by *P.major*, then *P. arenaria* and *P. cornuti* (Nikolova et al., 2011). The phenolic compounds and flavonoids present in the extracts are mainly responsible for this activity; more specifically, the hydroxyl groups present in phenolic compounds act as free radical neutralizers (Beara et al., 2012).

Anti-inflammatory activity

P. major extracts have been shown to increase the production of tumour necrosis factor alpha (TNF-α) and nitric oxide (NO), which protect the host against infections and tumour growth (Nathan și Hibbs, 1991). The anti-inflammatory activity can be evaluated by several methods, including the evaluation of the enzymes cyclooxygenase-1 (COX-1) and lipooxygenase-12 (12-LOX), which are involved in the metabolism of arachidonic acid, and it usually derives from membrane phospholipids under the action of phospholipases. To establish the anti-inflammatory activity of the extracts, products of the enzymatic activity of COX-1 and 12-LOX were quantified, using liquid chromatography coupled with mass spectrometry. Both *P. major* and *P. lanceolata* extracts showed anti-inflammatory activity, inhibiting COX-1 and 12-LOX enzymes. The report was made against two standard compounds, which are known to be inhibitors of these enzymes (aspirin and quercetin). Thus, the studied extracts showed lower activity than aspirin, but higher than quercetin (Beara et al., 2010).

Cytotoxic activity

Some studies show that cytotoxic activity is associated with DNA cleavage, in which flavonoids intervene by activating a cleavage complex involving topoisomerase I and II (Gálvez et al., 2003). Treatment with *P. major*, *P. lanceolata* and *P. altissima* extracts demonstrated a dose-dependent inhibitory effect (Mello et al., 2015). Tests on healthy cells showed a weaker inhibitory effect than on tumour cells, suggesting that these extracts are more toxic to tumour

cells than to healthy ones (Beara et al., 2012).

Immuno-modulating activity

The presence of immunopotentiation agents in *P. major* could explain the prophylactic activity against the development of diseases such as cancer (Gomez-Flores et al., 2000). The compounds responsible for immunomodulatory activity are: aucubin, chlorogenic acid, ferulic acid, p-coumaric acid, vanillic acid, baicalein and baicalin. Oleanolic acid and ursolic acid did not stimulate lymphocyte proliferation, but showed strong activity in stimulating IFN-γ secretion (L. Chiang et al., 2003).

Gastroprotective activity

In Latin America and Turkey, *P. major* is used to prevent or combat digestive disorders such as gastric ulcer (Abud et al., 2017; Samuelsen, 2000). The gastroprotective effects of this species are associated with a number of active principles, such as polysaccharides, flavonoids and alkaloids. These classes of compounds are known for their antioxidant properties, neutralizing free radicals and inhibiting lipid lipoperoxidation (Beara et al., 2012; Samuelsen, 2000). Thus, these properties may be associated with the gastroprotective effect, given that the protective effects of flavonoids on the gastric mucosa have been reported in the literature, against the formation of lesions due to necrotic factors (Mahmood și Phipps, 2006).

Hepatoprotective activity

Returning to the secrets of traditional medicine, natural products with antioxidant properties are intensely appreciated for their protective effects on the liver. *P. major*, known for its many properties and beneficial effects, has also been investigated in conditions of liver toxicity, proving once again that the protective effects are due to a wide range of natural antioxidants. Experiments on model biosystems have shown that treatment with plantain extract not only prevents GSH depletion, but even reduces oxidative stress. Hepatic activity-specific enzymes (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)) are significantly reduced under *Plantago* treatment and due to drug poisoning (Beara et al., 2012; Samuelsen, 2000).

1.2. Biological characteristics of several species of *Galium*

1.2.1. Plant systematics and speciation

The genus *Galium* belongs to the family Rubiaceae, order Gentianales, subclass Asteridae, class Magnoliopsida (Dicotyledonatae), branch Tracheophyta, kingdom Plantae. The Rubiaceae family is the fourth largest family in terms of angiosperm size, with approximately 660 genera and 13526 species (De Toni și Mariath, 2015; Şik et al., 2016). *Galium* genus includes over 650 species, widely distributed in tropical and temperate areas, with annual and perennial species (Bradic et al., 2019; Friščić et al., 2018).

Fig. 6. *Galium verum* L. (original image)

1.2.3. Chemical composition

A number of bioactive compounds have been isolated from *Galium* species, which show, once again, the similarity between *G. verum* and *G. mollugo*, but there are some differences that are important chemotaxonomic indicators. The categories of chemical compounds identified in *G. verum* are as follows: glycosidic iridoids, phenolic compounds, anthraquinones, triterpenes, tannins, saponins, essential oils, pigments and vitamins (Bradic et al., 2016).

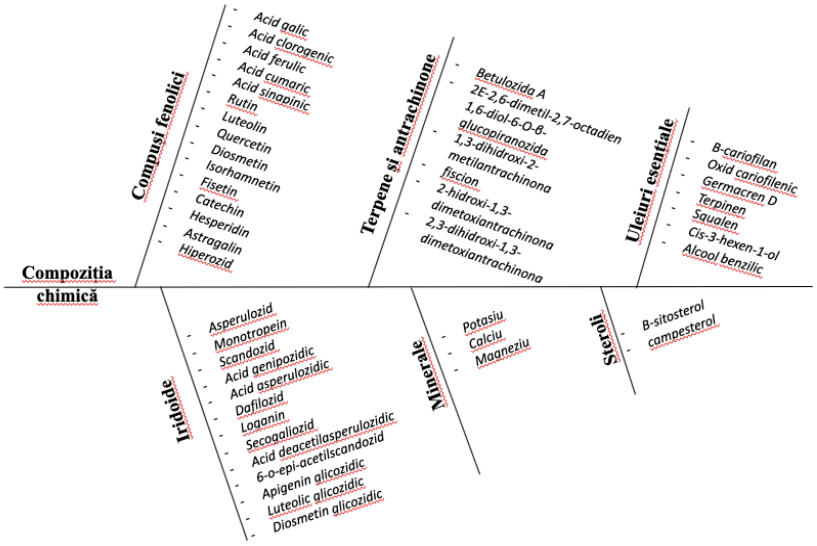


Fig. 9. Chemical profile of *G. verum*, reinterpreted after (Bradic et al., 2016; Mocan et al., 2019).

Figure 9 illustrates data from the literature, aimed at either identifying or quantifying chemical compounds of the species *G. verum*, which fall into the main classes of compounds present in plants.

1.2.4. Therapeuthical effects

Antioxidant activity

Recent papers prove the antioxidant activity of *Galium* extracts, obtained from different

species (*G. verum*, *G. mollugo*, *G. aparine*, *G. odoratum*) with solvents such as water, alcohol and methanol. The results obtained using the DPPH method show the 50% inhibitory concentration (IC50), which orders the four extracts of *Galium*, depending on the antioxidant potential. Thus *G. verum* ranks first (IC_{50}=105.43±0.15), they *G. mollugo* (IC_{50}=107.45±0.53), followed by *G. aparine* (IC_{50}=116.43±0.46) and *G. odoratum* (IC_{50}=264.42±0.74) (Vlase et al., 2014).

Antibacterial and antifungal activities

Experiments with gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes*) and gram-negative (*Salmonella typhimurium*, *Escherichia coli*) shows a higher sensitivity of gram-positive bacteria to the action of the *G. verum* extract (Bradic et al., 2016).

The antifungal activity of *Galium* extracts is very low. Studies of *C. albicans* colonies show an inefficient *G. verum*, but the complexes formed from it, together with *G. dasypodum, G. aparine* and *G. pseudomollugo* represents a very effective antifungal remedy (Vasilevna et al., 2016).

Anti-inflammatory activity

Against the background of acute, experimentally induced inflammation in the laboratory rat, oral administration of *G. verum* infusion reduced carrageenan edema by approximately 37%. (Fig. 10). Synthetic drugs used as an anti-inflammatory or positive control reduced edema by 47%, which shows a very small difference compared to the natural alternative (Mazko et al., 2017). The statistically significant result of reducing inflammation was recorded only two hours after the experimental induction of inflammation, which proves the rapid effect of *G. verum* extract.

Antitumoral activity

In vitro experiments have shown an increased sensitivity of laryngeal carcinoma cell lines, which shows the curative potential of *G. verum* extract, against throat and laryngeal cancers (Bradic et al., 2016). *G. verum* ethanolic extract has been shown to inhibit the development of breast cancer cells by activating apoptosis. Flavonoids in *G. verum* extract, especially diosmetin, are known to be an inhibitor of cervical and thymus tumours, by Fas-FasL-dependent signalling, with the activation of apoptosis-inducing caspases (Zhao et al., 2011).

1.3. Biological characteristic of several species of *Hypericum*

1.3.1. Plant systematics and speciation

The genus *Hypericum* belongs to the family Clusiaceae, order Malpighiales, class Magnoliopsida, branch Tracheophyta, kingdom Plantae[1]. In other databases, the genus *Hypericum*, belongs to the family Clusiaceae, order Theales, subclass Dilleniidae, class Magnoliopsida (Dicotyledonatae), branch Magnoliophyta, kingdom Plantae[2].

Fig. 11. *Hypericum capitatum* Choisy (https://www.turkiyebitkileri.com/en/photo-gallery/view-photo/3599/15974.html)

1.3.2. Morphology and ultrastructure

H. capitatum is a species native to Lebanon, Syria and Turkey. It is a perennial species, which can reach a height of up to 50 cm (Fig. 11). *H. capitatum* is characterized by the presence of small translucent secretory channels, specific to the genus, both on the stem and on the leaves and containing plant biomarkers (Esra și Afife, 2013). The flowers are colourful, but range from dark orange to deep red (Robson, 1987).

1.3.3. Chemical composition

The latest data on the chemical composition of *Hypericum* species indicate a rich content of bioactive secondary metabolites, such as essential oils, naphthodianthrones, flavonoids and others (Avato, 2005; Cirak et al., 2016). One specialized feature of *Hypericum* species is the presence of specialized glands, which have a special composition, having hydrocarbons, monoterpenes and sesquiterpenes, known for their antibacterial activity (Boga et al., 2016; Crockett și Robson, 2011; Ero și Mat, 2013).

From a pharmacological point of view, naphthodianthrones are one of the most interesting categories of chemical compounds in the genus *Hypericum*, due to the therapeutic effects that these structures determine. The most important naphthodianthrones are hypericin and pseudohypericin, which are located in leaves and flowers (Boga et al., 2016; Nahrstedt și Butterweck, 1997). Flavonoids and phenolic compounds are categories of chemical compounds present in high concentrations in species of the genus *Hypericum*, which are mainly responsible for antioxidant activity (Nahrstedt și Butterweck, 1997).

1.3.4. Therapeutical effects

Antibacterial, antifungal and antiviral activity

Several authors have studied the antibacterial effect of extracts *Hypericum,* such as *H.*

[1] https://eunis.eea.europa.eu/species/173352
[2] https://plants.sc.egov.usda.gov/core/profile?symbol=HYPER

heterophyllum, *H. hyssopifolium*, *H. scabrum* (Boga et al., 2016; Unal et al., 2008). These species have been shown to have remarkable antibacterial effects on colonies of gram-positive bacteria and less on gram-negative ones, and antifungal effects are absent. Thus, the chemicals responsible for this activity are considered to be flavonoids, phenolic compounds, naphthodiantrons and essential oils (Radulović et al., 2007). Moreover, the antiretroviral activity of the species against HIV-I virus has been proven, proving the inhibition of virus absorption (Sokmen et al., 1999).

Antioxidant and anti-inflammatory activity

Hypericum species are known for their antioxidant, anti-inflammatory and antidepressant effects, but the underlying mechanisms of these effects are not fully known (Du et al., 2014; Miller, 1998; Sánchez-Reus et al., 2007). What has been identified so far is the strong antioxidant activity of *H. performatum* extract, an activity that is based on the mechanism of action of flavonoids similar to that of reductones. These are chemical structures that react with certain peroxide precursors, thus preventing the formation of peroxides

DNA protection activity

The DNA protection activity was demonstrated using a plasmid DNA, oxidized with -OH radicals obtained from the photolysis of hydrogen peroxide and in the presence of methanolic extract of *H. capitatum* var. *capitatum*. The addition of the extract to the reaction mixture inhibited the formation of open circular and linear plasmid DNA in a dose-dependent manner. (Boga et al., 2016).

Antidepressant activity

Pharmacotherapy can reduce depression using antidepressant drugs, such as monoamine oxidase enzyme inhibitors (MAOs) and selective serotonin reuptake inhibitors (SSRIs). Although these substances are very effective in treating depressive disorders, side effects such as tachycardia, hypotension, dry mouth and liver toxicity have often been reported. *H. perforatum* has been introduced into depression therapy since the 1980s, is recommended for resolving depression and moderate (Wurglics și Schubert-Zsilavecz, 2006).

1.4. Physiology and pathology of oxidative stress

„ It is not stress that kills us, but our reaction to stress” Hans Selye

Daily exposure to various stressors, such as malnutrition, sedentary lifestyle, drugs, pollution, UV radiation, microorganisms, viruses, can lead to the development of pathologies associated with stress, such as depression, anxiety, gastrointestinal dysfunction, cardiovascular disease, etc. All of these conditions damage cell homeostasis by producing biochemical changes, even lesions that are known as oxidative stress. (Ochi et al., 2008; Sies et al., 2017).

1.4.1. Types of stress

Table 9. Classification of oxidative stress according to intensity, forms, terminology and types of associated responses

Criteria	Types of oxidative stress
Intensity	*basal* *Low* *Intermediate* *Raised / Vivid*
Specific types	*Physiological (eustres)* *Nutritional, postprandial* *Glyco-oxidative* *Proteotoxic, associated with endoplasmic reticulum (RE-stress) and / or disulfide bonds* *Photooxidative (UV-A, UV-B, visible, infrared)* *Radiation-induced stress* *nitrosative* *Nano-particle induced stress* *Environmental stress (SO_2, NO_2, O_3)*
Terminology	*Oxidant/pro-oxidant* *Redox, electrophilic stress* *Reducing, hypoxic stress* *Energy stress*
Associated responses	*Thermal shock* *Unpacked proteins* *Cell proliferation* *Autophagy, apoptosis, necrosis*

(Lushchak, 2014; Sies, 2015; Sies et al., 2017)

1.4.2. Cellular and molecular mechanisms of stress

Oxidative stress occurs in cells when the oxidant / antioxidant balance is in an imbalance, marked by an increase in the concentration of products known by the popular name of "free radicals". Thus, oxidative stress is a consequence of the excess of reactive species, such as these free radicals, which could not be neutralized by the cell's defensive systems. (Ahmadinejad et al., 2017; Crockett și Robson, 2011; Pan et al., 2019).

Data from the literature show that the largest number of reactive species comes from the electron transport chain, where at coenzyme Q there is a possibility that a large number of electrons are released, which then interact with molecular oxygen to form superoxide anions.

Polyphenols, although found in a fairly wide range, are considered to be a group of very strong antioxidants, sometimes even stronger than ascorbic acid (Prior și Cao, 2000). Among the mechanisms in which they engage are: direct purification of free radicals, activation of antioxidant enzymes (Table 10), chelation of metals, reduction of alpha-tocopheryl radicals, oxidase inhibition (Table 10), attenuation of nitro-oxidative stress, increased concentration of uric acid (Hirano et al., 2001; Lotito și Frei, 2006; Procházková et al., 2011a). A limiting stage of the antioxidant activity of polyphenols is the absorption in the cellular reaction microenvironment, which takes place with a previous microbial degradation in the small intestine. Thus, after that kind of degradation, phenolic compounds are either absorbed

individually or in condensed structures, called aglycones. The absorption phenomenon can be through two mechanisms:

a) *via* the sodium-dependent glucose carrier (Wurglics și Schubert-Zsilavecz, 2006);

b) passive diffusion of the molecule or aglycone into the bloodstream, preceded by deglycosylation of phenolic structures (e.g. rutin) using the enzyme floridzine hydrolase near the enterocyte membrane (Wurglics și Schubert-Zsilavecz, 2006).

However, exogenous antioxidants have a dual nature, in the sense that, in addition to the positive effects, they can also have a negative impact on the cellular microenvironment.

1.4.3. Oxidative stress and antioxidants in ovarian toxicity

Reactive oxygen species (SRO) affect several physiological processes, starting from oocyte maturation, fertilization, embryonic development and pregnancy. Being involved in ovarian function, these species contribute significantly to the development of diseases, which affect fertility (e.g. malformations, abnormal development of the embryo, puberty, sexual behaviour, etc.).

Plant flavonoids, in addition to the antioxidant effect offered by their chemical structure, activate cellular signalling pathways, which stimulate the internal fight of the cell against unstable radicals. Thus, by activating the signalling pathway of the transcription factors NRF2 (nuclear factor associated with erythroid factor 2) and PPARγ (peroxisome-activated gamma receptor), it activates antioxidant enzymes and blocks the production of ROS, inflammation and apoptosis. (Aladaileh et al., 2019; Sun et al., 2012). The side effects of CPA treatments are the production of reactive species ($^{\bullet}OH$, $O_2^{\bullet -}$, H_2O_2) in the ovary and follicles, especially during ovulation (Iorio et al., 2014). These radicals are known to be harmful to both follicles and oocytes, being associated with changes in proteins, fragments essential to maintaining the integrity of organs, cytoskeleton, so they can affect the activity, organization and distribution of cellular organs. (Devine et al., 2012; Goud et al., 2008). Thus, under pathological conditions, high levels of ROS /RNS and associated oxidative stress accelerate the aging of oocytes and cause qualitative deterioration of the oocyte. Increased levels of free radicals trigger lipoperoxidation in luteal cells, which can lead to regression of the corpus luteum. The intensification of the synthesis of unstable molecules is associated with the reduction of the activity of antioxidant enzymes, which contributes to apoptosis mediated *via* oxidative stress. Under pathological conditions, reactive species can contribute to the defective luteal phase, which can be a cause of infertility and miscarriage. (Devine et al., 2012; Zuo et al., 2016).

II. Personal contribution

2.1. Materials and methods

2.1.1. Vegetal extracts preparation

2.1.1.1. *Plantago* extracts

Species of *Plantago sempervirens*, *P. cornuti*, *P. lanceolata*, *P. major* and *P. media* were collected from "Alexandru Borza" Botanical Garden, Babeș-Bolyai University Cluj-Napoca, in September 2015. The hydroalcoholic extracts were obtained from the leaves by cold repercussion, using food alcohol 96% (Prodvinalco)(Pârvu et al., 2009).

2.1.1.2. *Galium verum* extract

The aerial parts of the species *Galium verum* L. were harvested during flowering, from Mihai Viteazu locality, Cluj county. The plants were dried and finely ground, of which 150 g of powder was macerated with 450 mL of a hydroalcoholic solution (70% ethanol) for 7 days at room temperature, and the extract obtained was filtered for further analysis. (Farcaș et al., 2018; Veličković et al., 2007).

2.1.1.3. *Hypericum capitatum* extract

Hypericum capitatum plants were collected in 2015 from Kilis area, Turkey, in different stages of development: before flowering, during flowering and after flowering. For comparative chemical analyses, an extract of *H. perforatum*, the most representative species of the genus *Hypericum*, was also prepared (Aidi Wannes et al., 2010; Farcaș et al., 2019).

2.1.2. Evaluating the chemical composition of the extracts

2.1.2.1. HPTLC analysis

Preliminary and qualitative chromatographic separation was performed using HPTLC thin layer chromatography plates (Cimpoiu et al., 2010; Hosu et al., 2016).

2.1.2.2. HPLC-DAD analysis

To perform a quantitative separation of the compounds of interest, high performance liquid chromatography coupled with diode array detector was used (HPLC-DAD) (Farcaș et al., 2019).

2.1.2.3. Electroparamagnetic resonance spectroscopy (EPR)

Antioxidant reactivity was also evaluated by the profile of semiquinone radicals generated by an alkaline treatment of the extracts and monitored by spin electromagnetic resonance (RES)(Moț et al., 2009, 2015).

2.1.3. Antioxidant activity of the extracts

2.1.3.1. DPPH method (2,2-difenil-1-picrylhydrazyl)

The DPPH method for evaluating the antioxidant activity of plant extracts is a method of neutralizing the DPPH radical, a stable radical, which can be monitored spectrophotometrically

for a determined period. of the radical, which can be translated into the antioxidant activity of the tested extract (Moț et al., 2011).

2.1.3.2. TEAC method (Trolox equivalent antioxidant capacity)

The TEAC method is based on the ability of the antioxidants present in the samples to neutralize the radical ABTS (2,2′-azinobis- (3-ethylbenzothiazoline-6-acidsulfonic)(Moț et al., 2009).

2.1.3.3. Folin-Ciocâlteu method

The Folin-Ciocâlteu (FC) method is used to quantify hydrophilic antioxidants and is based on the reduction of the FC reagent by compounds with antioxidant potential (Moț et al., 2009).

2.1.3.4. β-carotene method

β-carotene is a natural, yellow-orange compound, found in abundance in plant products. Due to its chemical structure, it performs antioxidant activities in interaction with biological membranes (Nimse și Pal, 2015). Evaluation of the antioxidant capacity of plant extracts, by the β-carotene method, involves the inhibition of the neutralization test induced by β-carotene (Moț et al., 2016).

2.1.4. Pro-oxidant activity evaluation of the extracts

Prooxidant activity was determined spectrophotometrically, using a method involving free radicals generated by an enzyme (lacase) and oxidation of haemoglobin (Moț et al., 2014).

2.1.5. Wound healing assay for *Plantago* extracts

The testing of the regeneration potential of the cells in the culture can be performed by damaging the cell cultures on solid culture medium. The ability of cells to recolonize the void indicates changes in cell dynamics (Jin et al., 2017).

2.1.6. Evaluating the antioxidant and antiinflammatory potential of the extracts using and *in vivo* experimental model

2.1.6.1. Biological models of stress using laboratory rats

2.1.6.1.1. Systemic acute inflammation model

To investigate the antioxidant and anti-inflammatory potential of *Plantago sempervirens* and *Hypericum capitatum* extracts, a model of acute systemic inflammation, induced with turpentine oil, was adopted in the laboratory rat. Turpentine oil functions as a non-antigenic stimulus and affects phagocytosis as part of the acute phase cellular response associated with inflammation. Oxidative lesions associated with the inflammatory response were quantified in terms of oxidative and nitro-oxidative stress parameters, such as total oxidative stress (TOS), MDA (malondialdehyde), OSI (oxidative stress index) and NO (nitric oxide). Antioxidant activity is determined by assessing glutathione level (GSH), enzymatic activities such as

superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (TAC). At the same time, using this model, the inflammatory response can be evaluated, by specific markers of inflammation, such as acute phase proteins (C-reactive protein - CRP), polymorphonuclear cell quantification (PMN), nitric oxide (NO), tyrosine nitration degree (3NT) (Andreicuț et al., 2018; Farcaș et al., 2019; Farcaș et al., 2019; Pârvu et al., 2014; Tiperciuc et al., 2013).

2.1.6.1.2. Neuropsychiatric stress model

The neuropsychic stress model was used to evaluate the antioxidant effects of *G. verum* extract *in vivo*. Neuropsychic stress was induced in laboratory rats by immobilization for 3 hours a day for 6 days. This model generates oxidative stress *in vivo* through another mechanism, unlike turpentine oil. Immobilization during 3 hours a day is a stimulus that induces many cellular changes and activates oxidative stress cascades with the production of an unusual state for the body (Liu et al., 2014; Rahal et al., 2014). The hypothalamic-pituitary-adrenal axis (HPA) is an essential component for stress response reactions. In the case of immobilization stress, the activity of the HPA axis increases and is reflected in the blood biochemical picture, in terms of stress hormone concentrations, such as corticosterone (CS) and epinephrine (EP) (Naila Sheikh et al., 2007). With hormonal changes, biochemical cascades are activated, and as a result, enzymatic antioxidant systems are altered in direct proportion to the level of oxidative stress (Selman et al., 2000). Thus, to evaluate the antioxidant activity of *Galium* extract, a set of specific stress markers were investigated from the serum of stressed, unstressed animals, but exposed to treatment with the extract. Parameters such as MDA, CS, EP, SOD, CAT, total cholesterol (CT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Crea) and total protein (PT) were evaluated from serum. Interpretation of results and statistical analyses help to demonstrate the antioxidant effect (Amin et al., 2017; Samarghandian et al., 2016; Thakare et al., 2016).

2.1.6.1.3. Cyclophosphamide (CPA)- induced ovarian toxicity

The model with CPA, in the laboratory rat, was induced against the background of a prophylactic treatment with *P. lanceolata* extract. To induce follicular depletion of approximately 50%, the CPA dose was set at 150 mg CPA / kg body weight, administered once, by intramuscular injection, according to the model proposed by (Horicks et al., 2015). The oxidative stress generated is also associated with an inflammatory response, which is also reflected in the sex glands. Thus, for the evaluation of oxidative stress and inflammatory response, parameters such as TOS, MDA, OSI, NO, 3NT were evaluated from animal serum, but also from ovarian tissue. In addition to these parameters, indicators of enzymatic (SOD, CAT, GPX-glutathione peroxidase) and non-enzymatic (GSH) antioxidant systems were determined. For a complete biochemical-endocrine picture, sex hormones such as follicle-stimulating

hormone (FSH), estrogen (E2), progesterone (PRO) were determined from blood serum in all animals in the experiment.

2.1.6.2. Animals and research ethics

All experiments were organized in accordance with Law 43/2014 on the protection of animals used for scientific purposes and European Directive 63/2010. The experiments obtained the approval of the Sanitary Veterinary and Food Safety Directorate of Cluj-Napoca (nr.22/13.12.2016).

2.1.6.3. Experimental design

A. Acute systemic inflammation and treatment with P. sempervirens

Table 11. Experimental groups and treatment with *P. sempervirens*

Groups/n=6	**C**	**EtOH**	**I**	**P25**	**P50**	**P100**	**I+P25**	**I+P50**	**I+P100**
Treatment	H_2O	Ethanol 30%	(0.6 mL/ kg b.w turpentine oil)	Extract 25%	Extract 50%	Extract 100%	I+ Extract 25%	I+ Extract 50%	I+ Extract 100%
Animals	Wistar rats, females, 160±20 g								
Time length	8 days								
Inflammatory agent administration	0.6 mL turpentine oil in day 7 of the experiment								
Treatment administration	Ethanol 30% and the extracts were intragastrical administered,1 mL, daily								
Conditions	Food and water *ad libitum*								

B. Gonadotoxicity associated with inflammatory response and treatment with luteolin and P. lanceolata

Table 12. Experimental groups and associated treatment

Groups/n=6	**C**	**CPA**	**CPA+E**	**CPA+L1**	**CPA+L2**	**CPA+L3**
Treatment	DMSO 3%	150 mg/kg corp, i.m., în ziua 15	CPA+ extract de P. lanceolata (0.4 mg luteolin în extract/ kg corp)	CPA+ 0.4 mg luteolin/ kg b.w	CPA+ 5 mg luteolin/ kg corp	CPA+ 10 mg luteolin/ kg corp
Animals	Wistar rats, females, 160±20 g					
Time length	21 days					
Toxic agent administration	CPA, 150 mg/ kg b.w., intramuscular injection in day 15					
Treatment administration	DMSO 3%, extract and luteolin solution were intragastrical administered, once at two days					
Conditions	Food and water *ad libitum*					

C. Acute systemic inflammation and treatment with H. capitatum

Table 13. Experimental groups and associated treatment with *H. capitatum*

Groups/n=5	C	I	I+Dcf	I+R	I+D1	I+D2	I+D3
Treatment	DMSO 4.7%	(0.6 mL/ kg b.w. turpentine oil)	I+ Diclofenac (10 mg/kg b.w.)	I+ 25 mg rutin/kg corp	I+ Extract cu 12.5 mg rutin in extract	I+ Extract cu 25 mg rutin in extract	I+ Extract cu 50 mg rutin in extract
Animals	Wistar rats, females, 160±20 g						
Time length	8 days						
Inflammation agent administration	0.6 mL turpentine oil in day 7 of the experiment						
Treatment administration	DMSO 4.7%, diclofenac (10 mg/kg b.w., rutin (25 mg kg b.w.) and extracts (D1, D2, D3) were intragastrical administered,1 mL, daily						
Conditions	Food and water *ad libitum*						

D. Neuropsychiatric stress induced by immobilization and treatment with G. verum

Table 14. Experimental groups and treatment with *G. verum*

Groups/n=6	C	S	SG1	SG2
Treatment	Normal diet	Restraint 3 h/day, in dark conditions	25 mg extract/ kg b.w.+ restraint 3 h/day in dark conditions	50 mg extract/kg b.w. restraint 3 h/day in dark conditions
Animals	Wistar rats, females, 160±20 g			
Time length	7 days			
Stressor	restraint in special containers, 3 h/day, for 7 days			
Treatment administration	extracts (G1 and G2) were intragastrical administered,1 mL, before restraint, daily			
Condition	Food and water *ad libitum*			

2.1.6.4. Markers of oxidative stress and inflammation

Oxidative and nitro-oxidative stress was investigated through several parameters that quantify, on the one hand, free radicals generated in the bloodstream (TOS, OSI, MDA, NO, 3NT) and on the other hand, endogenous antioxidant systems, enzymatic (CAT, SOD, GPX) and non-enzymatic (GSH), which contribute to the removal of oxidative species, as follows:

a. Total oxidative stress (TOS) (Erel, 2005);

b. Total antioxidant capacity (TAC) (Erel, 2004);

c. Oxidative stress index (OSI) represents TOS/TAC ratio (Harma et al., 2003; Miranda et al., 2001);

d. Nitric oxide (NO) (Miranda et al., 2001).

e. Malondialdehyde (MDA) (Mitev et al., 2010);

f. Thiol groups (SH) represents, overall, the non-enzymatic antioxidant system of cells (GSH) (Mitev et al., 2010);

g. Catalase (CAT) is an enzyme that belongs to the cell's own enzymatic antioxidant system and can be determined using a kinetic method (Aebi, 1984);

h. Superoxide dismutase (SOD) is an antioxidant enzyme that protects living cells against superoxide free radicals (Sun et al., 1988);

i. Glutathione peroxidase (GPX) is an oxidoreductase, which catalyses the reaction between glutathione and hydrogen peroxide;

j. C reactive protein (CRP) is one of the most important proteins of the acute phase, being involved in inflammation, trauma, myocardial infarction and other pathologies (Eckschlager et al., 2019);

k. Albumin (ALB) is a globular protein, predominant in human serum, with a role in maintaining osmotic pressure, antioxidant and support in the transport of drugs or other substances (Ueno et al., 2016);

l). Total proteins (PT) (Zheng et al., 2017);

2.1.6.5. Hematologic parameters

Complete blood count complete is performed by flow cytometry, using an automatic haematology analyser (Abacus Junior Vet, Diatron, Messtechnik, Budapest, Hungary).

2.1.6.6. Hormones

The main markers of follicular development are the hormones FSH and E2. FSH contributes to the stimulation and growth of follicles in post-pubertal mammals, and E2 has the role of forming the feedback loop of the pituitary-ovarian axis by inhibiting the concentration of FSH (Kaygusuzoglu et al., 2018).

2.1.6.7. Hepatic and renal markers

Liver enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), are markers of liver cell membrane integrity (Yang et al., 2010). Total cholesterol (CT) is a key parameter of lipid metabolism, and its serum level is determined by enzymatic colorimetric methods (Anwar și Meki, 2003). Creatinine (Crea) is a marker specific to renal function, which can be evaluated using the Jaffe method or the enzymatic method. (Schmidt et al., 2015).

2.1.6.8. Molecular markers

Growth and development factor 9 (GDF9) is a member of the TGF-superfamily and is responsible for the growth and development of granular cells during follicular development (Gürgen et al., 2013). Estrogen receptors (ERs) have a lot of physiological roles, due to their vulnerability to a lot of ligands (Imamov et al., 2005). Both ER and GDF9 were determined by ELISA using standard kits. Caspase 3 (Casp3) is an indicator of apoptosis (El-Readi et al., 2013).

2.1.6.9. Histological and ultrastructural analyses

For histological analysis, ovarian tissue was processed according to the described protocol (Toma et al., 2017). Ultrastructural analysis of granular and thecal cells in the ovary was performed using the transmission electron microscope (TEM) according to the protocol described by (Craciun și Barbu-Tudoran, 2013).

2.1.6.10. Molecular docking of flavonoids

Ligand-receptor simulations were performed using molecular docking, a computational method that, based on scaling functions, can predict the orientation of one molecule relative to another, as well as the stability of molecular complexes where they are formed. These data can be used to predict the binding power or binding affinity of these molecules (Ferreira et al., 2015).

2.1.7. Statistical analysis

All experimental data are expressed as mean ± SEM. The normal distribution of the data was verified with the Shapiro-Wilk test. The statistical test for investigating statistical significance was one-way ANOVA, followed by Bonferroni's multiple comparison. Statistical significance was set at p <0.05 (95% confidence interval). Statistical values were obtained with both Microsoft Excel and GraphPad Prism (5.0). Multivariate data analysis was performed with the PCA (Main Component Analysis) test, using the statistical program Statistics 12 (Statsoft, USA) (Farcaș et al., 2019).

2.2. Results and discussion

2.2.1. Biological characteristics of several *Plantago* extracts

2.2.1.1. Chemical composition

Phenolic profile of Plantago species studied (*P. cornuti, P. lanceolata, P. major, P. media* and *P. sempervirens*) follows a common profile, but each extract is distinguished by different concentrations of polyphenols (Table 15, Figure 20). The chemical profile of the species shows, on the one hand, the species *P. lanceolata* with an abundant quantitative polyphenolic content, and on the other hand, *P. sempervirens*, a species rich in numerous flavonoids and phenolic acids, with content above the quantification limit. (LOQ) (Table 15, Figure 20).

Table 15. Content in polyphenolic compounds of some *Plantago* extracts (ug/g plant material)(Farcaș et al., 2019).

Polyphenolic compounds	*P. cornuti* (ug/mL)	*P. lanceolata* (ug/mL)	*P. major* (ug/mL)	*P. media* (ug/mL)	*P. sempervirens* (ug/mL)
Gentisic acid	<0.2	<0.2	<0.2	<0.2	<0.2
Caffeic acid	<0.2	<0.2	<0.2	<0.2	<0.2
Chlorogenic acid	<0.2	8.253	<0.2	1.836	<0.2
***p*-coumaric acid**	<0.2	<0.2	<0.2	0.501	0.682
Ferulic acid	<0.2	<0.2	<0.2	<0.2	0.456
Rutin	<0.2	8.253	<0.2	<0.2	2.787
Luteolin	6.487	23.214	1.021	0.951	1.021
Apigenin	0.383	0.677	1.755	0.285	2.344
Quercitrin	<0.2	<0.2	<0.2	<0.2	NF

Note: Values are expressed as mean ± SD (n = 3).

Moreover, once the phenolic profile was established, the RES spectra for each extract could be simulated, depending on the fingerprint of the main compounds identified (apigenin, rutin, luteolin, chlorogenic acid, quercetin, kaempferol) (Figure 21). RES spectra were performed for each extract, using different dilutions (10x, 50x, 100x and 250x).

2.2.1.2. Antioxidant activity

Table 16. Antioxidant activity in the studied *Plantago* extracts (Farcaș et al., 2019).

Extracts / Method	*P. cornuti*	*P. lanceolata*	*P. major*	*P. media*	*P. sempervirens*
DPPH (mg QE/g plant)	0.95±0.15	1.25±0.15	0.90±0.15	2.20±0.15	6.03±0.48***
TEAC (µg TE/g plant)	132.40±9.42	194.20±1.41	188.70±16.77	272.10±9.18	459.50±35.78***
FC (mg GAE/g plant)	21.39±1.10	32.15±0.65	30.66±1.67	40.65±1.85##	59.14±4.34***

*** Significant at p <0.001 compared to *P. cornuti*, *P. lanceolata*, *P. major* and *P. media*

Significant at p <0.01 compared to *P. cornuti*

Note: Values are expressed as mean ± SD (n = 3)

Comparative analysis of the five extracts shows that the hydroalcoholic extract of *P. sempervirens* is by far 3-5x more antioxidant than its counterparts. (*P. cornuti*, *P. major*, *P. media* and *P. lanceolata*) (Table 16). Antioxidant activity results from reactions against DPPH and ABTS radicals, but also by reducing the Folin-Ciocâlteu reagent. These results are mainly associated with the concentrations in rutin and apigenin, but also with the whole spectrum of compounds identified over the LOQ, by the chromatographic method. *P. sempervirens* is a species vaguely described in the literature, with small exceptions, which also include the results present in this doctoral thesis. (Farcaș et al., 2019; Venditti et al., 2011).

2.2.1.3. Pro-oxidant activity

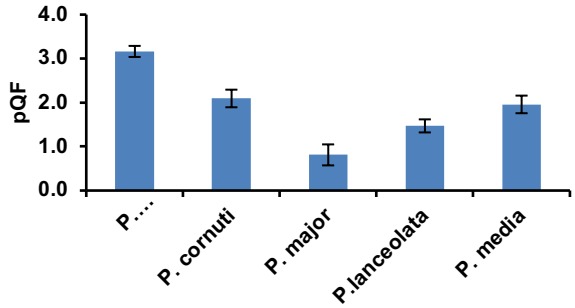


Figure 22. Prooxidant activity of *Plantago* extracts expressed as pro-oxidant quercetin (pQF)(Farcaș et al., 2019).

It is known that some polyphenolic compounds such as quercetin or rutin have the ability to generate radicals, which have prooxidant properties. (Moț et al., 2014).

Figure 22 shows the results of the prooxidant activity for each *Plantago* extract, but, although there are differences between the extracts, the results are not statistically significant. There are studies that show the possibility of luteolin, rutin and quercetin to generate free

radicals, which have prooxidant properties (Baldim et al., 2017; Cherrak et al., 2016; Eghbaliferiz și Iranshahi, 2016; Farcaș et al., 2018; Moț et al., 2014).

2.2.1.4. Wound healing assay

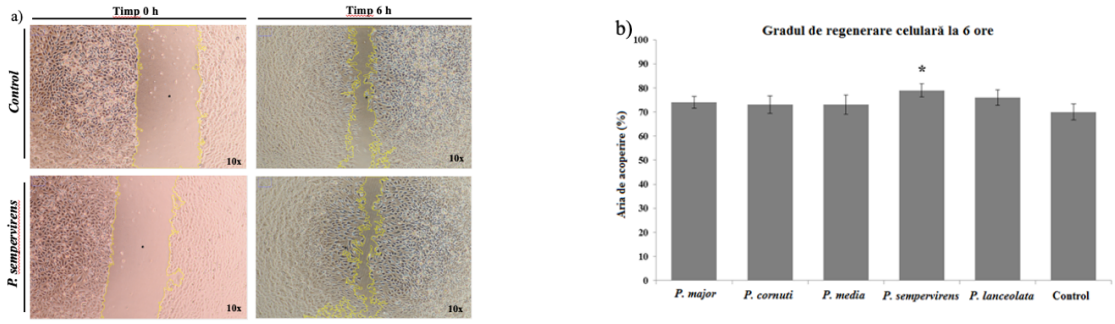


Figure 23. Degree of cell regeneration after exposure to *Plantago* extracts (b); Exposure of EA.hy926 cells to *P. sempervirens* induced constant cells migration even after 6 hours (Farcaș et al., 2019).

The results obtained from in vitro tests for cell regeneration (Figure 23 a, b) show the extract of *P. sempervirens* as a potential stimulant of the cell regeneration process, statistically significantly more efficient compared to its counterparts (Figure 23, b). Exposure of EA.hy926 cells to *P. sempervirens* induced constant cell migration even after 6 hours (Figure 23, a). At this point, statistical analyses show the regenerative process increased by 20% in *P. sempervirens* cells in the culture medium, as compared to the control group.

Therefore, the results obtained in this doctoral thesis demonstrate that *P. sempervirens* extract can modulate the cell migration process much more efficiently than homologous extracts. A rich content of flavonoids and phenylpropanoids, responsible for neutralizing radicals, can contribute to the process of cell migration (Zubair et al., 2012a).

2.2.1.5. Antioxidant and anti-inflammatory activity *in vivo*

Inflammation is associated with many chronic diseases, and the inflammatory response is closely linked to oxidative and nitro-oxidative stress (Akchurin și Kaskel, 2015; Verdile et al., 2015). To evaluate the antioxidant potential of *P. sempervirens* extract, an *in vivo* model of inflammation using turpentine oil was adopted.

Experimentally induced inflammation (Figure 24, ABCD, group I) stands out from all other experimental groups, due to the fact that the values of oxidative stress parameters are significantly modified, as follows: TOS (p <0.001); OSI (p <0.001) and MDA (p <0.01) significantly increased compared to control (C). As seen in Figure 24, the lowest dose (IP25) of *P. sempervirens* had the best inhibitory effect on TOS concentrations (p <0.05). A consequence of increased oxidative stress is the formation of lipoperoxides, a process that severely affects cell membranes. (Chen et al., 2013). As seen in Figure 24, the MDA level was strongly increased due to inflammatory processes.

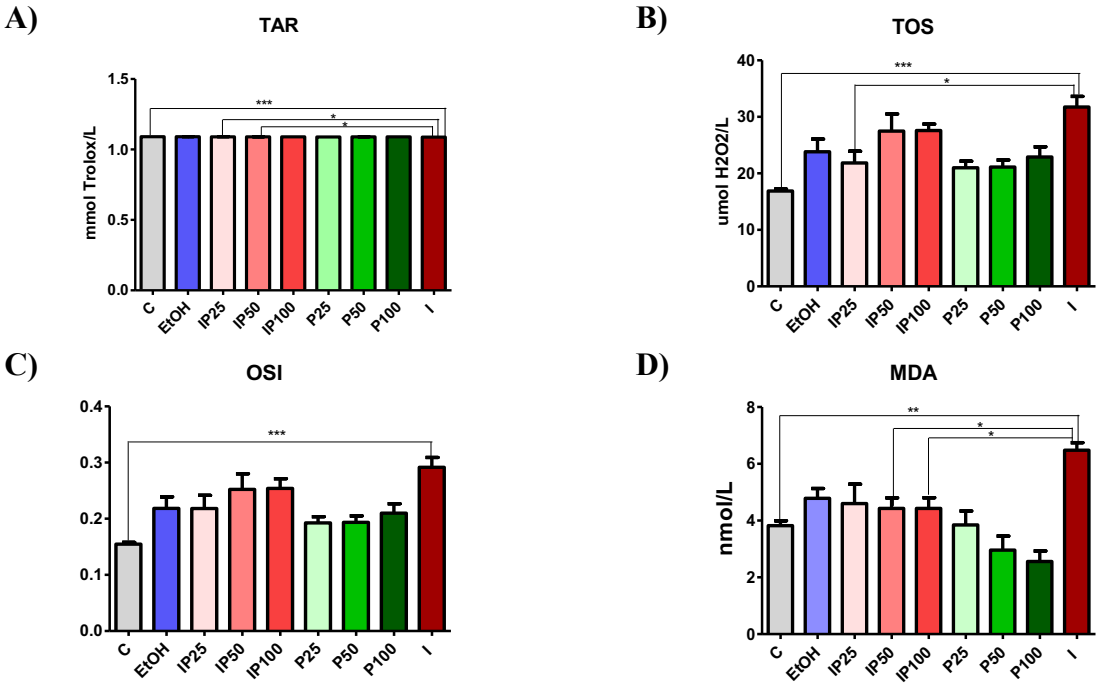


Figure 24. Effects of *P. sempervirens* extract on total antioxidant response (ART) (A), total oxidative stress (TOS) (B), oxidative stress index (OSI) (C) and malondialdehyde (MDA) (D) in rat serum . Data represent the mean ± SEM. The One-way ANOVA test was applied followed by Bonferroni's multiple comparison test; * Significant at $p < 0.05$; ** Significant at $p < 0.01$; *** Significant at $p < 0.00$ (Farcaș et al., 2019).

All three dilutions of *P. sempervirens* significantly reduced in vivo NO production, which could further reduce inflammation. Our results, related to NO levels, were consistent with TOS, OSI and MDA concentrations. The three doses of extract significantly increased SH concentrations in a dose-dependent manner. Moreover, oxidative-inflammatory reactions are correlated with the activity of antioxidant enzymes (Lee et al., 2012). SOD and CAT showed a decrease in inflammation activity, but *P. sempervirens* extract once again proved its beneficial potential by supporting its usefulness in increasing the activity of these two enzymes.

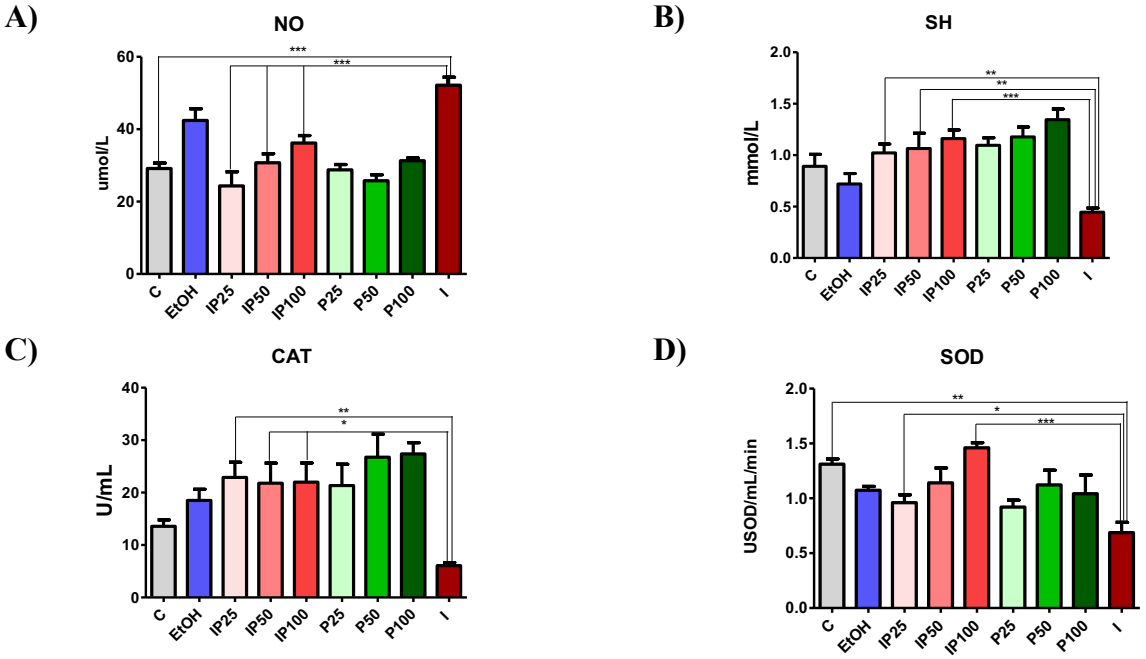


Figure 25. Effects of *P. sempervirens* extract on nitric oxide (NO) (A), thiol concentration (SH) (B), catalase activity (CAT) (C) and superoxide dismutase (SOD) activity (D) in rat serum. Data represent the mean ± SEM. The ANOVA

One-way test was applied followed by Bonferroni's multiple comparison test. * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00(Farcaș et al., 2019).

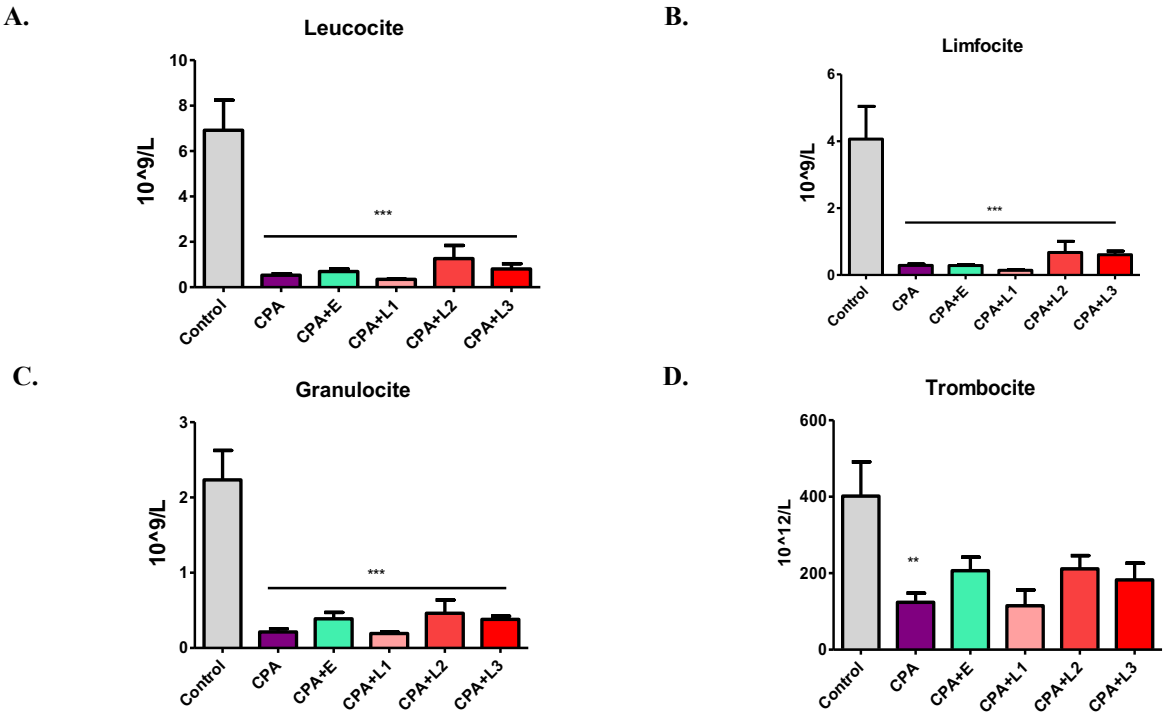
Biomarkers of inflammation, such as TP, Albumins (ALB), Globulins (Glob), ALB / Glob, and CRP, are shown in Table 17. The inflammatory effect of turpentine oil is associated with increased oxidative stress, as shown in previous sections, but also with increase in early biomarkers of inflammation protein metabolism (Table 17). Treatment with *P. sempervirens* resulted in decreased protein concentrations near or below the control level (CRP at IP100, p <0.05), (ALB at IP100, p <0.05), (TP at IP50, p <0.05, and at IP100, p <0.05).

Table 17. Concentration of C-reactive protein (CRP), albumin (ALB) and total protein (PT) in control and experimental animals. Values are expressed as mean ± SEM (Farcaș et al., 2019).

Param.	Control	EtOH	I	P25	P50	P100	IP25	IP50	IP100
CRP (mg/dL)	47.6±6.3	50.3±5.3	184.3±28.4*	110±25.2	79.6±251	162.7±29.3	72.3±27.5	64.3±11.5	40±4.3#
ALB (g/dL)	1.59±0.3	1.79±0.1	2.50±0.6**	1.31±0.9	1.70±0.5	2.01±0.8	1.67±0.3	1.46±0.4	1.16±0.0#
TP (g/dL)	6.5±0.6	9.6±0.6	11.3±1.0**	3.0±0.5	4.3±1.0	5.6±1.1	8.5±0.4	7.0±0.9#	6.8±0.6#
Glob (g/dL)	4.75±0.6	7.58±0.7	8.86±0.9*	1.69±0.52	2.6±0.9	3.67±0.9	6.82±0.4	5.55±0.9	5.22±0.4
ALB/Glob	0.36±0.05	0.31±0.08	0.29±0.02	0.44±1.08	-0.10±1.03	-0.07±0.50	0.25±0.04	0.34±0.10	0.22±0.06

* Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.001 (compared to Control); # Significant at p <0.05; ## Significant at p <0.01; ### Significant at p <0.001 (compared to I); Param = parameters.

2.2.1.6. Estrogen mimetism of flavonoids



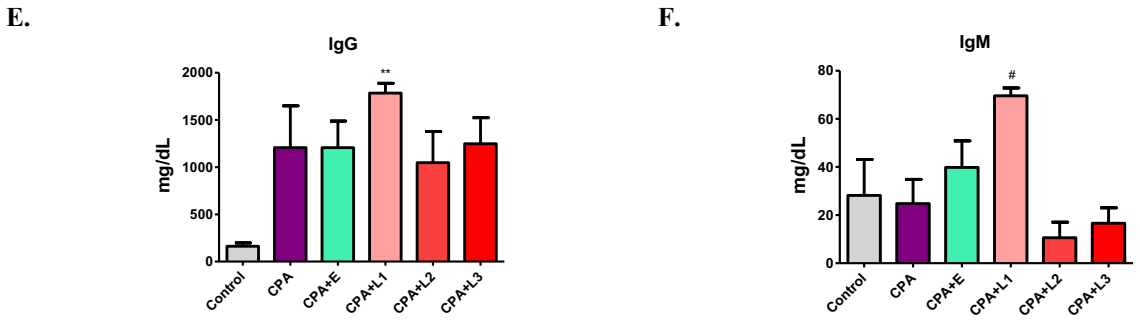


Figure 27. Effects of luteolin and *P. lanceolata* extract on the immune response in rats exposed to cyclophosphamide; Number of leukocytes (A); Number of lymphocytes (B); Number of granulocytes (C); Platelet count (D); IgG concentration (E); IgM concentration (F); Data represent the mean ± SEM. The One-way ANOVA test was applied followed by Bonferroni's multiple comparison test; * Significant at $p < 0.05$; ** Significant at $p < 0.01$; *** Significant at $p < 0.00$.

Haematological changes, in Figure 27 (A, B, C, D), show a massive immunosuppression, which involves a significant decrease in leukocytes ($p < 0.001$), lymphocytes ($p < 0.001$), granulocytes ($p < 0.001$) and platelets ($p < 0.01$), which demonstrates the immunosuppressive activity of cyclophosphamide. The extract and the three doses of luteolin do not counteract this effect, at least not at the cellular level. The concentration of immunoglobulins (IgG and IgM) (Figure 27, E and F) shows changes, but without statistical significance. The extract and the three doses of luteolin do not induce significant changes in these parameters, except for L1 which seems to significantly stimulate the production of antibodies, unlike the more concentrated doses (Figure 27, E and F).

Oxidative stress parameters (Figure 28, ABCD) varies in direct proportion to ovarian oxidative stress parameters (Figure 29, ABCD). CPA induces a significant increase in MDA values, from serum ($p < 0.05$) (Figure 28, A) and from tissue ($p < 0.001$) (Figure 29, A). Enzymatic activities and GSH values are considerably low ($p < 0.05$; $p < 0.01$), and *P. lanceolata* extract and the three doses of luteolin (L1, L2 and L3) contribute significantly to the restoration of homeostatic balance, at values close to the control group. C-reactive protein (CRP), although graphically visible, are not statistically significant. Massive GSH depletion can be attributed to the direct conjugation of acrolein, a secondary metabolite of CPA, thus reducing glutathione levels and generating oxidative stress (Nafees et al., 2015). Luteolin, as an individual treatment, but also luteolin in the extract, prevents glutathione depletion, and thus, antioxidant systems are stimulated to neutralize free radicals produced by the action of CPA. Cholesterol and total proteins determined from ovarian tissue show variations between the experimental groups, but without statistical significance (Figure 29, E, F).

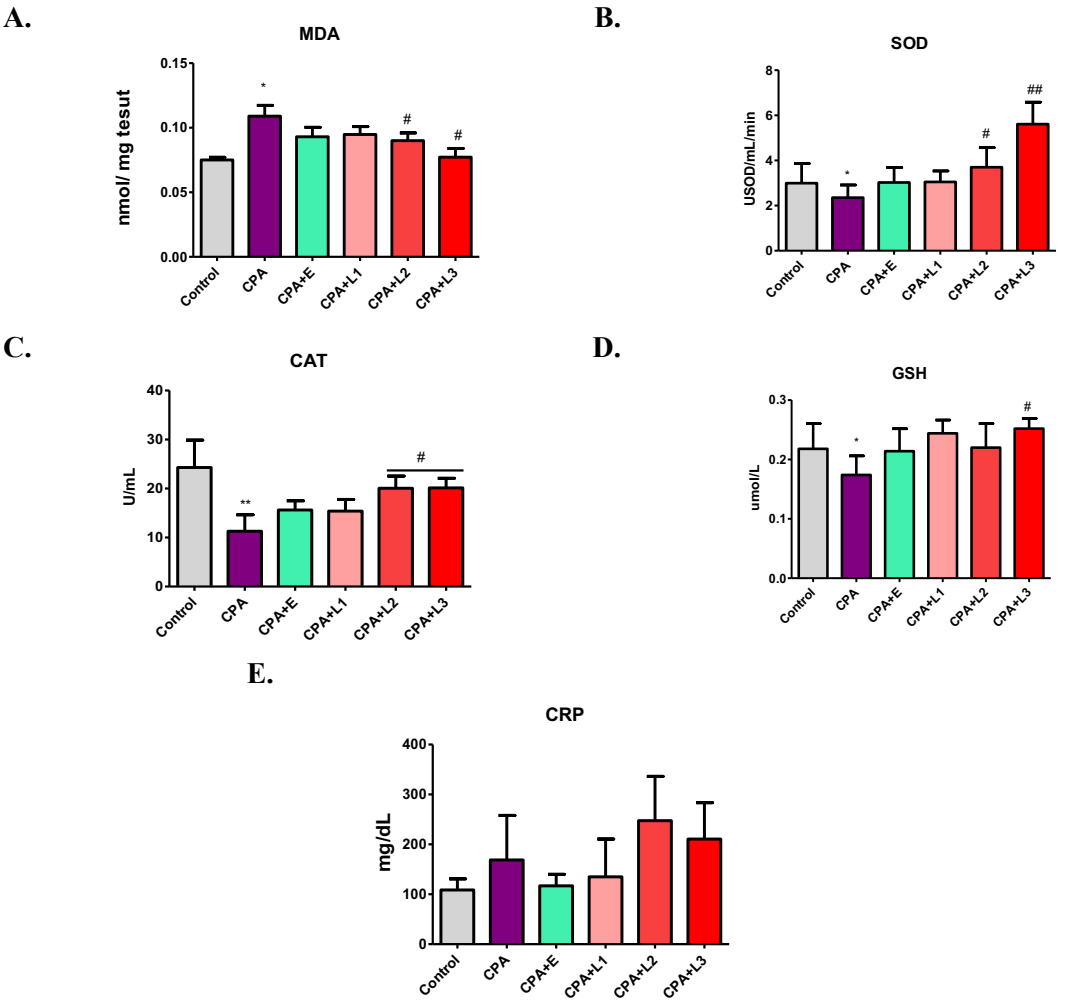


Figure 28. Effects of luteolin and *P. lanceolata* extract on oxidative stress in rat serum exposed to cyclophosphamide; concentration of malondialdehyde (MDA) (A); dismutase superoxide (SOD) activity (B); catalase activity (CAT) (C); glutathione concentration (D); C-reactive protein (CRP) concentration (E). Data represent the mean ± SEM. The One-way ANOVA test was applied followed by Bonferroni's multiple comparison test; * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00.

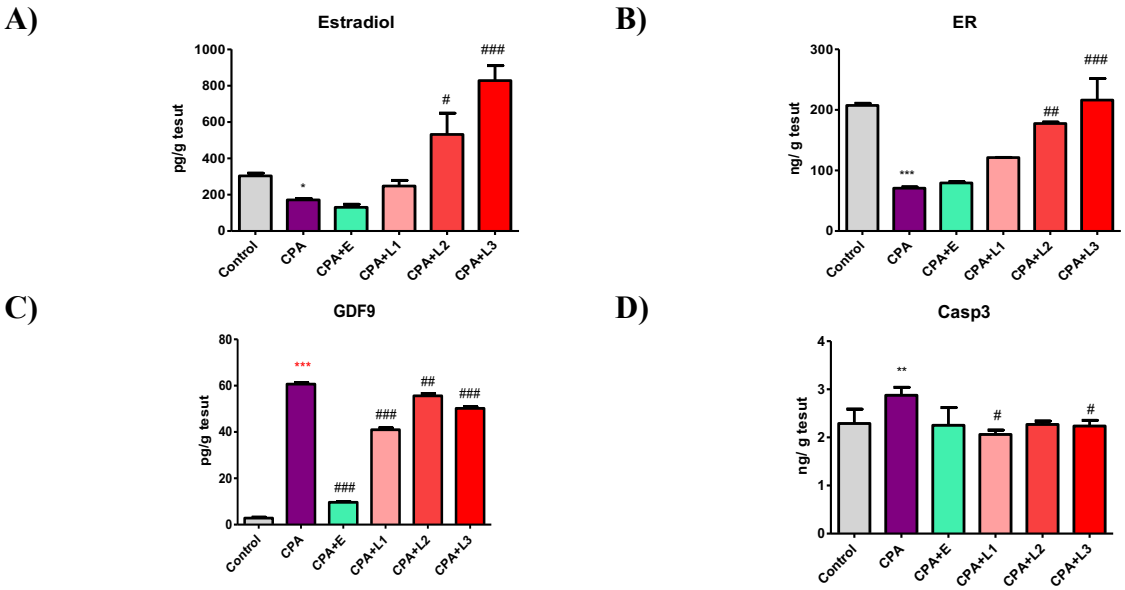


Figure 31. Effects of luteolin and *P. lanceolata* extract on reproductive function in ovarian tissue in rats exposed to cyclophosphamide; oestradiol concentration (A); estrogen receptor (ER) expression (B); growth and development

factor 9 (GDF9) (C); caspase 3 (Casp3) (D); Data represent the mean ± SEM. The One-way ANOVA test was applied followed by Bonferroni's multiple comparison test; * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00.

These hormones have the ability to maintain germ cells due to estrogen receptors (ER1 and ER2). Figure 31 shows the concentration of oestradiol (A) and its receptor expression (ER) (B), both of which are strongly affected by CPA. A key marker of folliculogenesis and ovulation regulation is growth and differentiation factor 9 (GDF9). Usually, the expression of this factor is associated with a normal development of oocytes and then cumulus cells, which surround the oocyte to form the first follicle, also called primordial (Sánchez și Smitz, 2012) (Figure 32). Figure 31 (C) shows an aberrant increase (p <0.001) in this growth indicator in the group exposed to cyclophosphamide, and the extract and the 3 doses of luteolin show significantly smaller changes (p <0.001) compared to CPA group. A potential therapeutic mechanism of luteolin, but also of the extract, could be based on the anti-apoptotic effect. Figure 31 (D) shows an increase in caspase 3, an indicator of apoptosis following the administration of cyclophosphamide, but the extract, but especially luteolin has an anti-apoptotic effect.

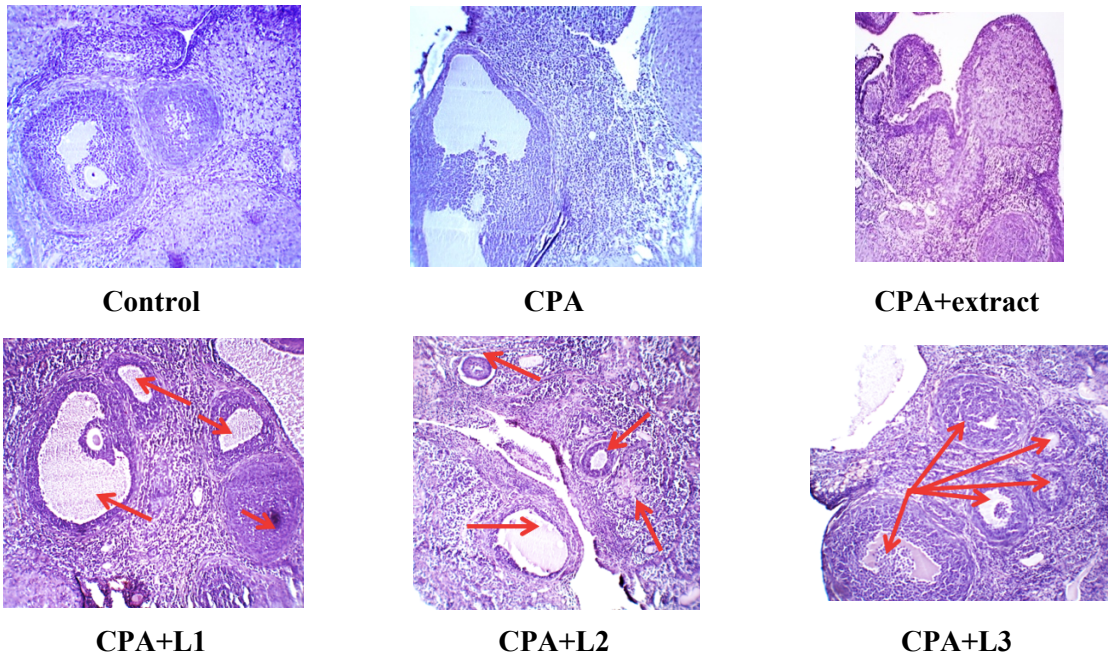


Figure 33. Representative microphotographs of follicular dynamics in rats (Control, CPA, CPA + extract, CPA + L1, CPA + L2, CPA + L3), haematoxylin-eosin staining, under an optical microscope, x200 magnification.

Histological analysis (Figure 33) shows follicular dynamics in all experimental groups (Control, CPA, CPA + extract, CPA + L1, CPA + L2, CPA + L3). In the images corresponding to the CPA group, a single antral follicle can be seen, which has accumulated a considerable amount of lipids inside it (Figure 33, CPA). The most spectacular follicular evolution is observed in the images corresponding to the CPA + L3 group, in which a series of primordial, primary, secondary, but also antral follicles can be easily identified (Figure 33, CPA + L3). The images in Figure 33 demonstrate the dose-dependent effect of luteolin, using mechanisms to reduce

oxidative stress and as a selective modulator of the estrogen receptor, but also of stimulating ovarian folliculogenesis.

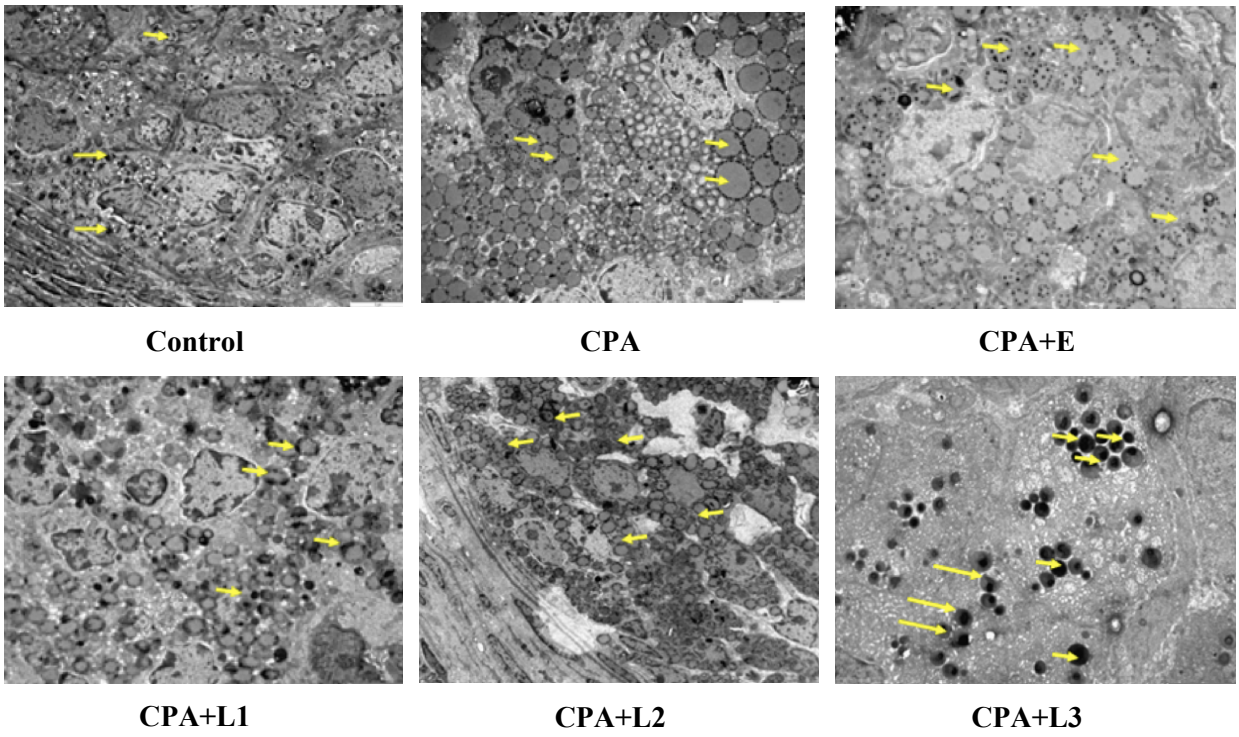


Figure 34. Ultrastructural details of granular cells and theca cells from rat ovaries (Control, CPA, CPA + extract, CPA + L1, CPA + L2, CPA + L3) at TEM, magnification x3000.

Evaluation by transmission electron microscopy (TEM) of granular cells, cumulus cells and theca cells show some surprising details of electron-dense corpuscles, which can be associated with hormone-secreting granules (Figure 34).

2.2.1.7. Conclusions

The investigated *Plantago* extracts (*P. cornuti, P. major. P. media, P. lanceolata* and *P. sempervirens*) were analysed by modern methods (HPLC) and differ phytochemically in terms of the number of phenolic compounds, especially apigenin , luteolin, rutin and chlorogenic acid;

The chemical profile of the species shows, on the one hand, the species *P. lanceolata* with an abundant polyphenolic content in luteolin, and on the other hand, the species *P. sempervirens*, a species rich in many compounds (flavonoids and phenolic acids), with content over quantification limit (Table 15, Figure 20) (Farcaș et al, 2019);

In vitro antioxidant activity was investigated by different methods (DPPH, TEAC, FC), which designate *P. sempervirens* as the most powerful antioxidant of all five investigated extracts (Table 16). (Farcaș et al, 2019);

P. sempervirens extract induced a constant cell migration of endothelial cells, even after 6 hours, which demonstrates a significantly higher proliferative effect compared to homologous extracts (Figure 23, a and b) (Farcaș et al, 2019);

The *in vivo* antioxidant activity was evaluated on a model of acute systemic inflammation in the laboratory rat, using turpentine oil. Preventive treatment with *P. sempervirens* extract, in three different doses, reduced the oxidative stress induced by inflammation, by decreasing the values of nitro-oxidative stress (MDA, TOS, OSI, NO) (Fig. 24 and Fig. 25) and by stimulation of endogenous non-enzymatic and enzymatic antioxidants (SOD, CAT, GSH, TAR) (Fig. 25) in a dose-dependent manner (Farcaș et al, 2019);

The estrogenic activity of luteolin was demonstrated by increasing the essential indicators of folliculogenesis such as: estrogen concentration, GDF9, follicular dynamics (Fig. 33), estrogen receptor expression, but also by the anti-apoptotic effect (Fig. 31).

2.2.2. Biological characteristics of *Galium verum* extract

2.2.2.1. Chemical composition

Phytochemical analysis of *G. verum* extract reveals a set of phenolic compounds, among which a large proportion of chlorogenic acid and rutin are noted (Table 21).

Table 21. Elution time, analytical parameters and concentrations of compounds determined in *Galium verum* extract samples (Farcaș et al., 2018).

Nr	Compounds	t_{elutie} (min)	R^2	LOD (µg/mL)	LOQ (µg/mL)	extract unhydrolyzed (µg/mL)	SD (µg/mL)	extract hydrolysed (µg/mL)	SD (µg/mL)
1	ac.chlorogenic	6.32	0.997	4.3	13.0	1748	42	568	59
2	ac. *p*-coumaric	8.43	0.998	1.4	4.1	82	1	80	3
3	ac. ferulic	9.28	0.999	1.5	4.7	24	0.1	39	7
4	rutin	12.28	0.999	1.9	5.8	1604	10	91	0.1
5	isoquercitrin	12.94	0.999	2.1	6.3	308	8	168	10
6	quercetin	19.27	0.999	6.0	18.1	36	1	659	139

LOD - limit of detection, LOQ - limit of quantification, R^2 - coefficient of determination for calibration curves, SD represents the standard deviation (n = 4).

In addition to its rich content of chlorogenic acid and rutin, *G. verum* extract contains significant amounts of p-coumaric acid, chlorogenic acid, isoquercitrin and quercetin. Data from the literature show a similar profile of phenolic compounds identified in the species *G. verum* (Chen et al., 2017; Kuhtinskaja și Vaher, 2018). The hydrolysed extract contains a much higher amount of quercetin that comes from quercetin-based glycosides, such as rutin and isoquercitrin.

On the one hand, after hydrolysis, the *G. verum* extract shows, first of all, a much higher amount of quercetin (from 36 µg / mL in the non-hydrolysed extract to 659 µg / mL in the hydrolysed extract; Table 21).

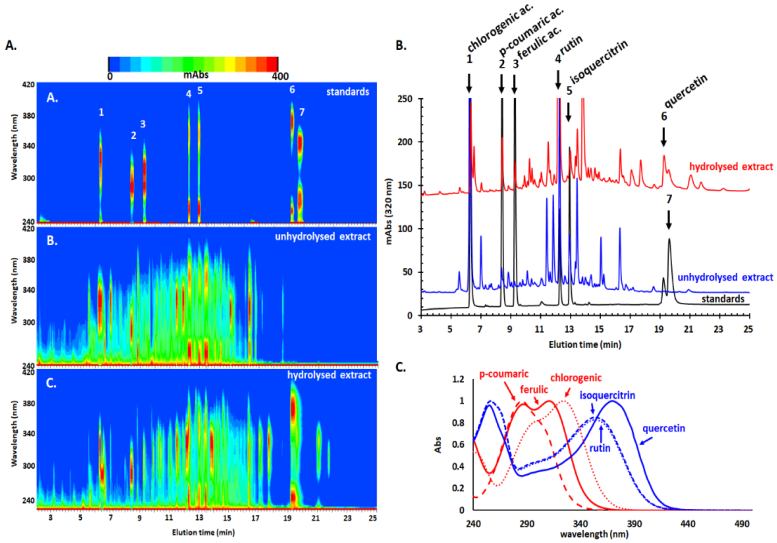


Figure 35. HPLC-UV-vis analysis of hydrolysed and non-hydrolysed *G. verum* extract **(A)** Heatmap graph of the chromatographic profile depending on the elution time; **(B)** Chromatograms of the two extracts (hydrolysed and non-hydrolysed) and of the standards monitored at 320 nm; **(C)** UV-vis spectrum of molecular absorption at six standards used (Farcaș et al., 2018).

2.2.2.2. Antioxidant and prooxidant activity

Compared to other plant extracts analysed, in the present doctoral thesis, *G. verum* appears to have a significantly higher activity by the DPPH test (4.6 mg equivalents of quercetin / g of plant material), compared to the TEAC method (0.082 mg equivalents quercetin / g plant). This means that most chemical constituents work better in a more hydrophobic environment or associated with hydrophobic cell compartments, such as membranes (Benedec et al., 2014b).

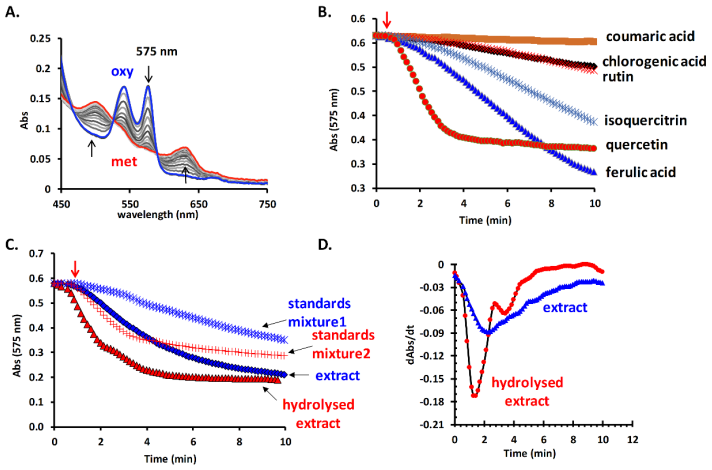


Figure 37. Evaluation of the prooxidant activity of *G. verum* extract. oxyHb (25 µM) is rapidly oxidized to metHb in the presence of the extract and laccase (100 nM) (A); Comparison between the prooxidant reactivity and the compounds identified in the extract at the same concentration (5 µM) (B); mixing compounds in identical proportions with those in the extract, before hydrolysis (Mixture 1) and after mixing (Mixture 2) (C); Comparison of the kinetic profile of oxiHb oxidation in the presence of the two extracts and the laccase (D)(Farcaș et al., 2018).

Previous experiments have shown that there is a direct relationship between the total phenolic content of plant extracts and the signal strength detected using the RES method, for samples treated in alkaline medium (Moț et al., 2009).

2.2.2.3. Antioxidant activity *in vivo*

Exposure to immobilization stress, combined with dark stress, increased plasma corticosterone (RS; p <0.05). The batches treated with *G. verum* (SG1 and SG2) showed a dose-dependent decrease in corticosterone levels (Figure 39A). On the other hand, plasma levels of epinephrine, under stress, changed similarly to corticosterone, more precisely, a significant increase (p <0.05) compared to the control group. Moreover, differences were found between the two distinct doses of extract (SG1 and SG2), as shown in Figure 39 (AB), in which the concentrated dose (SG2), as compared to SG1, decreased significantly and statistically significant both hormone levels. This indicator (cholesterol) changes dose-dependent, decreases to a higher concentration of extract (SG2), which may cause inhibition of the corticosterone synthesis pathway (Abobaker et al., 2019; Teixeira et al., 2017).

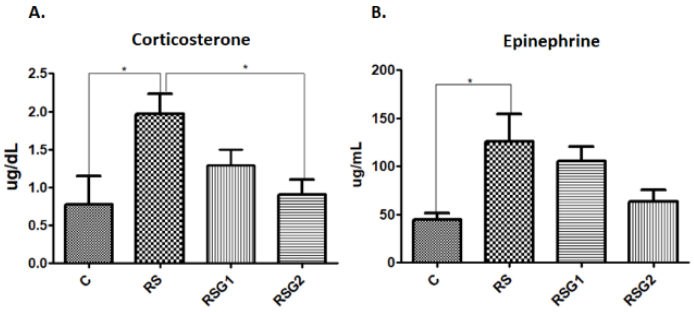


Figure 39. Determination of the concentration of stress hormones (corticosterone and epinephrine) in the control and experimental groups treated with *G. verum*; Histograms represent the mean ± SEM, n = 6, **(A)** C = 0.434 µg / dL; S = 1.96 µg / dL; SG1 = 1.29 µg / dL; SG2 = 0.90 µg / dL; **(B)** C = 44.4 µg / mL; S = 125.7 µg / mL; SG1 = 106 µg / mL; SG2 = 63.7 µg / mL; * Significant at p <0.05; (*t* test for corticosterone levels and ANOVA test followed by Bonferroni's post-hoc test for epinephrine concentrations; GraphPad software, San Diego, CA*)* (Farcaș et al., 2018).

The effects of repeated stress on the activities of antioxidant enzymes (CAT, SOD) along with TBARS (MDA), markers of oxidative stress, are described in Table 23. CAT and SOD activities were significantly (p <0.05) decreased in immobilization stress, in contrast of the values obtained at the control group. In addition, the TBARS level increased significantly (p <0.05) only under stress compared to control values. Treatment with *G. verum* extract resulted in a significant increase (p <0.05) in enzymatic activities, compared to the group exposed only to stress (S).

Table 23. Parameters of oxidative stress in control and experimental groups treated with *G. verum.* Values are expressed as mean ± SEM (Farcaș et al., 2018).

Parameters	Control	S	SG1	SG2
CAT(U/mL)	56.36±4.10	41.51±5.99*	56.95±3.14#	61.01±1.21#
SOD (USOD/mL/min)	0.80±0.00	0.68±0.01*	0.82±0.01	0.85±0.00#
TBARS (nmol/mL)	4.43±0.47	8.06±1.26*	4.74±0.67	3.73±0.69#

* Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.001 (compared to Control); # Significant at p <0.05; ## Significant at p <0.01; ### Significant at p <0.001 (compared to RS).

Similar changes were observed in the circulating levels of the biochemical markers of the control and treatment groups (Table 24). Cholesterol levels (p <0.05) and creatinine (p <0.001) were significantly increased only during stress, compared to control values, while exposure to *G. verum* reduced cholesterol levels. The most spectacular decrease was observed in the SG2 group (p <0.01) compared to group S. TP and ALT activity did not change after exposure to stress, but there is a tendency to increase AST activity. AST activity followed the same trend as cholesterol levels. Thus, the lowest activity was recorded in the SG2 group, significantly different (p <0.05) compared to group S. Compared to the control, ALT activity was significantly reduced (p <0.01) in both groups treated with extract (SG1 and SG2), but the results are significant only between SG1 and S (p <0.05).

Table 24. Serum control biochemistry and experimental groups treated with *G. verum*. Values are expressed as mean ± SEM (Farcaș et al., 2018).

Parameters	Control	S	SG1	SG2
TP (g/dL)	12.96±2.48	11.44±6.61	11.22±1.37	8.60±2.06$^{**,\#}$
Chol (mg/dL)	83.50±12.59	135.0±9.80*	118.0±14.39	74.29±3.79$^{\#\#}$
AST (U/L)	221.0±10.88	243.0±8.00	235.2±13.68	166.7±10.93$^{\#}$
ALT (U/L)	86.0±0.00	90.0±1.95	54.0±5.37$^{**,\#}$	59.8±3.73**
Crea (mg/dL)	0.80±0.00	1.17±0.02***	1.20±0.05***	1.26±0.04***

* Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.001 (compared to Control);

Significant at p <0.05; ## Significant at p <0.01; ### Significant at p <0.001 (compared to RS).

In our experiments, an immobilization stress model, associated with exposure to darkness, was used to disrupt homeostatic balance and to investigate and validate the protective and adaptogenic effects of *G. verum* extract. Animals exposed to stress showed significantly elevated corticosterone and epinephrine levels as evidence of an overactive adrenal gland. Thus, our research, in terms of oxidative stress markers (TBARS, SOD, CAT), liver parameters (ALT and AST, total protein concentration, cholesterol), renal parameters (creatinine), under conditions of oxidative stress, shows results similar to those reported in the literature. Both doses of *G. verum* extract, used in our experiments, improved the investigated clinical outcome, especially the concentrated dose (SG2). Our research shows that the extract contains polyphenols, especially chlorogenic acid and rutin, probably responsible for the improved outcome of clinical parameters. After exposure to stress, high activity of liver transaminases is a marker of liver damage (Bao et al., 2008). ALT and AST remain the most important indicators for the evaluation of liver cells, which, in our experiment, were strongly influenced by *G. verum* extract. The treatment proved to be extremely effective by reducing the levels of these enzymes, reaching values even lower than the control values. In addition, the decrease in liver enzymes occurs rarely and is not fully understood (Lum, 1995), but the decrease in these enzymes, after only 7 days of treatment with *G. verum*, shows a potential rapid regeneration of hepatocytes. SG2 also significantly decreased serum cholesterol levels, an effect that may be related to the

cholesterol-lowering effects of chlorogenic acid. There are also studies showing the effectiveness of chlorogenic acid in stimulating β-oxidation of fatty acids and inhibition of HMG-CoA reductase activities in the liver (Cho et al., 2010).

2.2.2.4. Conclusions

Based on the data from the literature and the results of the research carried out, several conclusions can be drawn on the *Galium verum* extract:

The chemical composition of *G. verum* extracts is represented by the following compounds: chlorogenic acid (predominantly quantitative), rutin, p-coumaric acid, ferulic acid, isoquercitrin and quercetin, identified in different amounts (Table 21), the hydrolysed *G. verum* extract contains a much higher amount of quercetin derived from quercetin-based glycosides, such as rutin and isoquercitrin (Table 21);

Evaluation of the *in vitro* antioxidant activity of *G. verum* extract was performed by different methods (DPPH and TEAC), which showed a significantly higher activity by the DPPH method, compared to the TEAC method (page 94);

G. verum extract decreases oxidative damage induced by neuropsychiatric stress having a modulating effect on the hypothalamic-pituitary-adrenocortical axis, by reducing plasma levels of corticosterone, epinephrine and oxidative stress parameters (Fig. 39, Table 23);

The biochemical profile was significantly improved, after treatment with the extract, under stress conditions (Table 24);

2.2.3. Biological characteristics of *Hypericum capitatum* extract

2.2.3.1. Chemical composition

High performance thin layer chromatographic analysis (HPTLC) was performed for a preliminary assessment of the overall chemical profile of each extract to be investigated. The compounds were identified, first, by the retention factor (Rf) and then by the colour emitted in fluorescence.

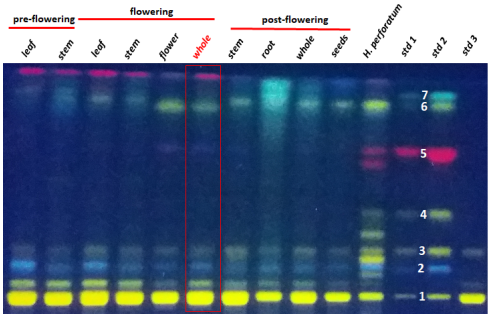


Figure 40. HPTLC analysis indicates the main secondary metabolites contained in *Hypericum capitatum* and *H. perforatum* extracts (each band contains 8 µL of 10 mg / mL); Standards: Standard 1 and 2 (std 1 - 2 µL and std 2 - 10 µL of 100 µg / mL stock solution containing a mixture of: rutin (1), chlorogenic acid (2), isoquercitrin (3), hyperoside (4), hypericin (5), quercetin (6) and kaempferol (7)). Std3 - routine standard - 2 µL from 3.5 mg / mL (Farcaș et al., 2019).

Table 25 shows that the most concentrated extract, from the routine point of view, is the one prepared from the stem in the flowering period (Strain (F)).

Table 25. Elution time (minutes), identified concentration (µg / mg, dry mass) for the standards tested in *H. capitatum* samples, prepared in different stages: prefloral (PreF), during flowering (F) and postfloral (PostF). The other standards (2, 3, 8, 10, Figure 40) are all below the detection limit (Farcaș et al., 2019).

No. standard	**1**	**4**	**5**	**6**	**7**	**9**	**11**	**12**
Compounds	chlorogenic	rutin	isoquercitrin	quercitrin	quercetin	kaempferol	hyperforin	hypericin
t_{elutie} (min)	***2.89***	***7.86***	***8.04***	***8.54***	***10.22***	***11.25***	***16.89***	***17.11***
Leaf (PreF)	42.1	638	8.4	1.4	<LOD	0.5	<LOD	<LOD
Strain (PreF)	3.3	521	5.8	3.3	<LOD	<LOD	<LOD	<LOD
Leaf (F)	22.2	707	9.8	8.6	<LOD	<LOD	<LOD	<LOD
Strain(F)	25.8	527	6.9	11	<LOD	<LOD	<LOD	<LOD
Flower(F)	6.5	332	2.4	47	16	<LOD	<LOD	**<LOQ**
Herba (F)	5	517	4.8	7.6	1.1	<LOD	<LOD	**<LOQ**
Strain (PostF)	3.9	609	7.6	9.0	<LOD	<LOD	<LOD	**<LOQ**
Roots (PostF)	10.1	163	5.5	5.6	<LOD	2.8	<LOD	<LOD
Herba (PostF)	3.4	363	5.2	5.0	<LOD	0.4	<LOD	<LOD
Seeds (PostF)	4.4	159	4.0	1.3	0.8	0.5	<LOD	<LOD
H. perforatum	36.8	49	23	4.2	6.1	0.9	0.9	0.31

In addition to rutin, chlorogenic acid, isoquercitrin and kaempferol were also identified, but in much lower concentrations compared to rutin. Isoquercitrin is found in high concentration in extracts prepared from leaves from the pre-flowering period (Leaf (PreF)) and quercetin in extracts prepared from flowers (Flower (F)).

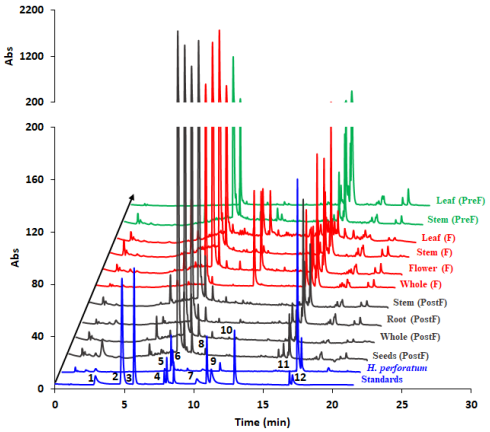


Figure 41. HPLC profiles of studied *Hypericum capitatum* extracts. Standards: 1 - chlorogenic acid, 2 - p-coumaric acid, 3 - caffeic acid, 4 - rutin, 5- isoquercitrin, 6 - quercitrin, 7 - quercetin, 8 - apigenin, 9 - kaempferol, 10 - chrysin, 11 - hyperforin , 11 - hypericin. The highest chromatographic peak belongs to the rutin, which represents more than 70% of the peak area, in all samples. (Farcaș et al., 2019).

The literature shows that the family Hypericaceae contains flavonol glycosides of quercetin in the aerial parts, especially rutin, and *H. capitatum* seems to have a much higher amount of rutin than the well-known *H. perforatum*. The concentrations of the secondary

metabolites of the plants change according to the periods of development, and the rutin is formed in higher concentrations in the flowering stage or in the postfloral stage (Avato, 2005).

Our research on *H. capitatum* extract indicates that only isoquercitrin was present in detectable amounts and at a much lower concentration compared to that determined for rutin. The literature highlights, when it comes to *Hypericum* species, the existence of a positive correlation between rutin concentration and environmental altitude, while there is a negative dependence if isoquercitrin levels are taken into account (Umekl et al., 1999).

A truly remarkable aspect, which seems to differentiate *H. capitatum* from other species, is the low level of naphthodianthrones, hypericin and pseudohypericin, detectable only in traces, during the flowering period. Naphthodiantrons are considered specific indicators of this family and are considered to be responsible for the therapeutic effects of the Hypericaceae family. (Crockett și Robson, 2011).

2.2.3.2. Antioxidant and prooxidant activity

The antioxidant activity of each *H. capitatum* extract was analysed by three very well-known methods (DPPH, TEAC, β-carotene) and, of course, by the total concentration of phenols. The whole plant extract, in the post-floral stage, has the lowest determined antioxidant activity. It should be noted that, by far, the antioxidant activities of the extracts prepared from flowers in the flowering stage and from leaves in the prefloral stage were the highest of all the samples analysed.

Table 26. Evaluation of the *in vitro* antioxidant activity of *Hypericum capitatum* extracts, by three different methods (DPPH, TEAC, β-carotene) and the method for total polyphenol content (TPC). The results are expressed as rutin equivalents (RE), Trolox equivalents (TE) and gallic acid equivalents (GE) in the dry extract (Farcaș et al., 2019).

No.	DPPH (mg RE/g)	TEAC (mg TE/g)	β-car. (mg RE/g)	TPC (mg GE/g)
Leaf (PreF)	107.6 ± 7.3	1253 ± 6	70.9 ± 3.9	38.4 ± 0.3
Strain (PreF)	57.5 ± 2.5	809 ± 27	44.9 ± 0.2	22.9 ± 0.9
Leaf (F)	31.0 ± 4.2	442 ± 2	48.9 ± 0.3	14.1 ± 0.1
Strain (F)	21.3 ± 0.6	29 ± 1	56.3 ± 0.5	8.5 ± 0.1
Flower (F)	90.5 ± 8.8	1260 ± 33	46.1 ± 0.1	37.8 ± 0.5
Herba (F)	36.4 ± 0.9	527 ± 9	56.5 ± 0.1	15.7 ± 0.1
Strain (PostF)	33.9 ± 1.6	401 ± 3	56.4 ± 0.7	12.3 ± 0.2
Roots (PostF)	22.4 ± 1.4	295 ± 3	76.5 ± 1.1	8.6 ± 0.1
Herba (PostF)	15.5 ± 2.5	207 ± 5	58.4 ± 0.5	6.3 ± 0.1
Seeds (PostF)	31.5 ± 0.7	430 ± 9	67.1 ± 7.1	15.1 ± 0.4

2.2.3.3. Anti-inflammatory and antioxidant activity *in vivo*

In the present research, oxidative stress was evaluated using TOS, TAC and OSI tests. The concentration of TOS was significantly increased (p <0.01) in group I. Preventive treatment with diclofenac, rutin and *H. capitatum* extract reduced TOS to almost normal values. In groups I + D2 (p <0.05) and I + D3 (p <0.01), *H. capitatum* had a more effective inhibitory effect on TOS

than rutin, which indicates the action of flavonoids in the extract.

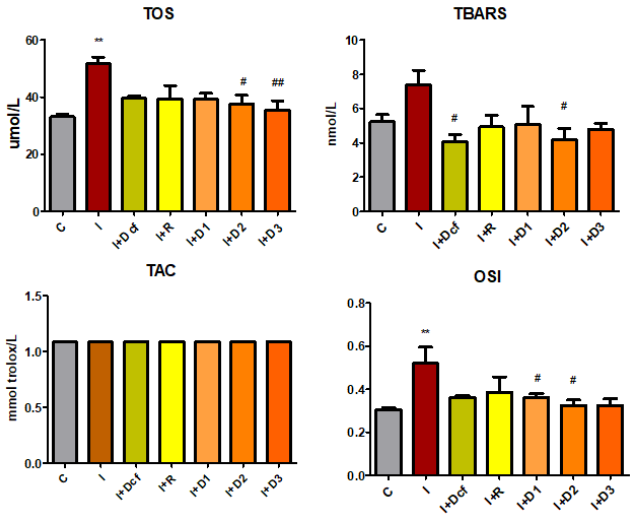


Figure 43. The *in vivo* antioxidant effects of *H. capitatum* (*herba*) extract on TOS, TBARS, TAC and OSI in rat serum. Data represent mean ± SEM; ANOVA One-way followed by Bonferroni's post-hoc test; * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00 - compared to group C; # Significant at p <0.05; ## Significant at p <0.01; Significant at p <0.00 - compared to group I; [C- Control; I- Inflammation; Dcf- Diclofenac; I + R- Inflammation + Rutin; I + D1 - Inflammation + Dose 1 of *H. capitatum* extract; I + D2 - Inflammation + Dose 2 of *H. capitatum* extract; I + D3 - Inflammation + Dose3 of *H. capitatum* extract] (Farcaș et al., 2019).

NO synthesis was determined by measuring serum NOx and 3-NT concentrations. As seen in Figure 44, the inflammation led to an increase in NOx, compared to the Control group (p <0.5). All treatments tested had an inhibitory effect on NOx (p <0.05), compared to group I. The three doses of *H. capitatum* extract reduced the NOx concentration almost to the control level and similar to diclofenac. Treatments with *H. capitatum* extract had an inverse inhibitory effect proportional to the dose administered on the 3-NT values, D1 having the strongest inhibitory effect and D3 the lowest.

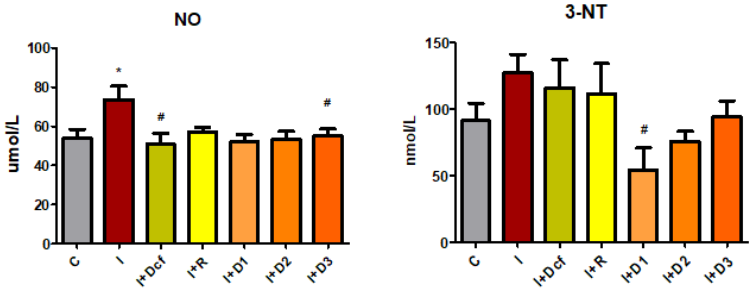


Figure 44. *In vivo* anti-inflammatory effects of *H. capitatum* extract on NO and 3-NT values in rat serum. Data represent mean ± SEM; ANOVA One-way followed by Bonferroni's post-hoc test; * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00 - compared to group C; # Significant at p <0.05; ## Significant at p <0.01; Significant at p <0.00 - compared to group I; [C- Control; I- Inflammation; Dcf- Diclofenac; I + R- Inflammation + Rutin; I + D1 - Inflammation + Dose 1 of *H. capitatum* extract; I + D2 - Inflammation + Dose 2 of *H. capitatum* extract; I + D3 - Inflammation + Dose3 of *H. capitatum* extract](Farcaș et al., 2019).

As shown in Figure 45, almost all experimental treatments contribute to the reduction of SOD activity, except D3, the highest concentration of extract, which managed to stimulate enzymatic activity above the control level (p <0.05).

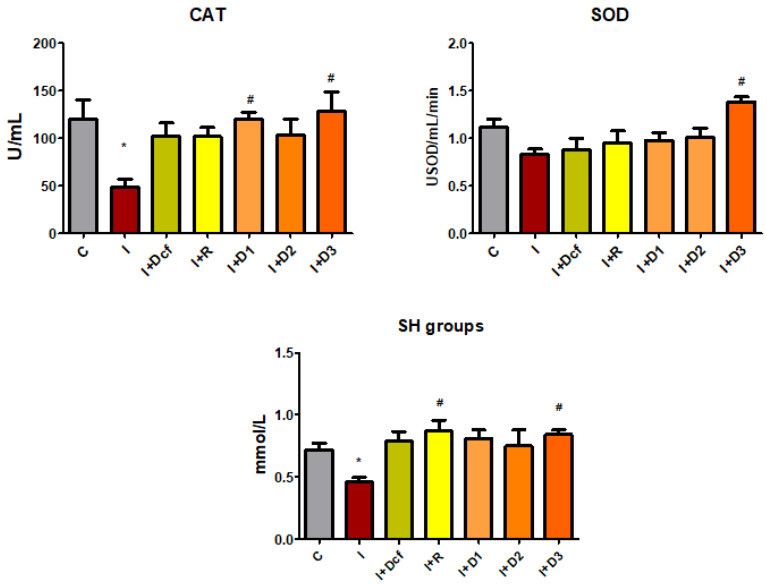


Figure 45. Effects of *H. capitatum* extract on CAT and SOD activities, as well as rat serum SH concentration. Data represent mean ± SEM; ANOVA One-way followed by Bonferroni's post-hoc test; * Significant at p <0.05; ** Significant at p <0.01; *** Significant at p <0.00 - compared to group C; # Significant at p <0.05; ## Significant at p <0.01; Significant at p <0.00 - compared to group I; [C- Control; I- Inflammation; Dcf- Diclofenac; I + R- Inflammation + Rutin; I + D1 - Inflammation + Dose 1 of *H. capitatum* extract; I + D2 - Inflammation + Dose 2 of *H. capitatum* extract; I + D3 - Inflammation + Dose3 of *H. capitatum* extract](Farcaș et al., 2019).

Management of intracellular oxidative stress is based on maintaining a balance in SOD and CAT activities. Thiol structures, such as glutathione, are involved in the plasma reactivity of antioxidants, as well as in signalling, defence and catalysis (Gilmore et al., 2008). Figure 45 reveals SH values in all experimental groups.

2.2.3.4. Conclusions

The chemical composition of *H. capitatum* extracts is represented by the compounds rutin (predominantly quantitative), chlorogenic acid, isoquercitrin, quercetin, kaempferol, hypericin and hyperforin, identified in different amounts (Table 25 and Figure 41), depending on the plant organ of which was obtained (extract);

The evaluation of the *in vitro* antioxidant activity of the 10 types of *H. capitatum* extracts was performed by three different methods (DPPH, TEAC and ß-carotene), and by the Folin-Ciocâlteu method the total polyphenol content (TPC) was determined (Table 26 and Fig. 42);

The *in vivo* antioxidant activity was determined on a model of acute systemic inflammation in the laboratory rat, using turpentine oil, as an inflammatory agent. Preventive treatment with *H. capitatum* extract reduced oxidative stress induced by inflammation, by decreasing the parameters of nitro-oxidative stress (TBARS, TOS, OSI, NO, 3-NT) (Fig. 43 and

Fig. 44) and by stimulating antioxidants non-enzymatic and endogenous enzymes (SOD, CAT, SH) (Fig. 45), in a dose-dependent manner;

General conclusions

Research on the chemical composition of plant extracts - obtained from the leaves of *Plantago cornuti, P. lanceolata, P. major, P. media* and *P. sempervirens*, from the herb of *Galium verum* and from various organs of the plant (root, stem, leaf, flower, seeds, herba) *Hypericum capitatum* and its various phenophases (prefloral, floral, postfloral) - it was made comparatively, using chromatographic methods (HPTLC, HPLC-DAD), methods for evaluating antioxidant activity (DPPH, TEAC, FC, ß-carotene, RES) and prooxidants (laccase and haemoglobin oxidation), methods for evaluating cell regeneration (*in vitro*) (measuring the distance of cell migration);

The chemical composition of *Plantago* extracts is represented by compounds: gentisic acid, caffeic acid, p-coumaric acid, chlorogenic acid, ferulic acid, rutin, apigenin, luteolin (predominantly quantitative), quercitrin, identified in different amounts, depending on the species;

The chemical composition of *G. verum* extracts is represented by compounds: chlorogenic acid (predominantly quantitative), rutin, p-coumaric acid, ferulic acid, isoquercitrin and quercetin, identified in different amounts;

The analysed *H. capitatum* extracts show very small amounts of hypericin (traces), and this happens only during the flowering period. The highest amount of rutin was determined in extracts of *H. capitatum* obtained from the leaf (from the floral and prefloral period, respectively), the stem (from the postfloral and prefloral period, respectively) and herba;

The evaluation of the *in vitro* antioxidant activity of *G. verum* extract was performed by different methods (DPPH and TEAC), which showed a significantly higher activity by the DPPH test, compared to the TEAC method. The prooxidant reactivity of *G. verum* extract is much higher in the hydrolysed extract, compared to the non-hydrolysed extract, due to its rich content of quercetin, which has the highest prooxidant reactivity;

H. capitatum extracts, prepared from flowers in the flowering stage and from leaves in the prefloral stage, showed the highest antioxidant activity, compared to all the analysed samples, and the extract prepared from the whole plant, in the post-floral stage, shows the highest low antioxidant activity;

In vivo, on a biological model of acute systemic inflammation, *P. sempervirens* reduces the markers of oxidative stress, neutrophilia and acute phase proteins, along with increasing the activities of antioxidant enzymes;

The most luteolin-rich extract was *P. lanceolata* and it was studied on a CPA-induced gonadotoxicity model. The *in vivo* effect of *P. lanceolata* extract and luteolin, as an individual treatment, was demonstrated by increasing specific indicators of folliculogenesis (estrogen concentration, GDF9, follicular dynamics, ER expression, by the anti-apoptotic effect and by the presence of electron-dense structures, in granulosa/cumulus/theca cells in the ovaries, which may be associated with hormone-secreting granules);

Treatment with *P. lanceolata* extract, in a model of cyclophosphamide-induced gonadotoxicity, proves the estrogen-mimetic effect of flavonoids, stimulating folliculogenesis and reducing oxidative stress;

G. verum extract diminishes *in vivo* oxidative damage induced by neuropsychiatric stress (immobilization stress combined with light/dark stress) through a modulating effect on the HPA axis. Both studies in the literature and the experiments in this doctoral thesis show that on a background of chronic pathologies, the administration of *G. verum* extract, rich in quercetin, produces an increase in renal vulnerability;

In vivo treatment with *H. capitatum* extract contributes, in a dose-dependent manner, to the reduction of nitro-oxidative stress associated with acute systemic inflammation. These results encourage future studies to develop herbal alternatives to conventional anti-inflammatory drugs, such as diclofenac;

The research results, described in this doctoral thesis, have been published in specialized journals with impact factor (IF), such as PlosOne, Oxidative Medicine and Cellular Longevity and BMC Complementary and Alternative Medicine. In addition to the 3 scientific articles in the field of doctoral thesis, published as first author, we mention: 2 scientific articles, with IF, published as main author; 3 scientific articles, with IF, published as a contributor; scientific articles, in BDI indexed journals, as first author and/or contributor; member in the research team for 5 projects; attending more than 10 national and international conferences.

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Personal contributions

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Studies

1. *Explorarea potenţialului adjuvant al unor fitocompuşi din genul Plantago în medicaţia bolilor degenerative prin interacţie cu biosisteme expuse unor metaboliţi toxici ai ciclofosfamidei* belonging to the project PN 16-30 02 03, from 2016, registered as a result of CDI at the National Research-Development Institute for Isotopic and Molecular Technologies (NRDIIMT) Cluj-Napoca.

Member of research projects

- **PN-III-P1.2-PCCDI-2017-0387** "Emerging technologies for the industrial capitalization of 2D structures (graphene and nongraphenic)".

- **Core project PN16-30 02 03** "Border reaserch for molecular technology development – New applications for isotopic and molecular processes for decontamination based on functionalized nanomaterials", 2017-2018, National Institute for Reseach and Development of Isotopic and Molecular Techologies Cluj-Napoca;

- **Young researcher teams** -PN-II-RU-TE-2014-4-2555 "Non-simbiotic Hemoglobins from plants and their reactions to stress", Babeș-Bolyai University;

- **Core Project PN09-440.213** „ Biochemical and biophysical evaluation of some proteins involved in oxidative metabolism, Phase I-2014/Phase II-IV-2015, National Institute for Reseach and Development of Isotopic and Molecular Techologies Cluj-Napoca.

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Attending conferences

Over 10 national and international conferences, of which the most representative are:

1. Evaluation of antioxidant properties and biological activities of several *Plantago* hidroalcoholic extracts, **The 15th International Symposium of the 3rd Millennium Agriculture, 29.09-1.10.2016, Cluj-Napoca, Romania**

Anca D. Farcaș, Augustin C. Moț, Vlad Al. Toma, Alina E. Pârvu, Ioana Roman, Silvia Neamțu, Marcel Pârvu

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