SUMMARY OF PHD THESIS

The protective effect of natural antioxidants in diseases of the urinary system

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INTRODUCTION

Diseases of the urinary system represent impairments of kidney function and lower urinary tract. Among the most commonly diagnosed conditions are acute kidney injury (AKI) and urinary tract infection. Nephrotoxicity is one of the main causes of AKI. Oxidative stress is a contributing factor of nephrotoxicity. Strategies to combat drug-induced AKI are currently limited. For bacterial infection, numerous antibiotics are prescribed. The overuse of antibiotics promotes the growth of antimicrobial resistance, which is classified as a major public health problem.

Recently, nutrition is considered to play a crucial role in health. There is particular interest in living a healthy lifestyle by consuming foods and plants rich in phytonutrients. Recently, particular interest has been given to the fruits of *Cornus mas*, for which recent studies suggest to have great potential for application in alternative medicine. However, many fruits remain neglected due to the unpleasant taste sensed by consumers, despite their richness in phytonutrients. Among these fruits is Sorbus aucuparia, which has recently been shown to be a valuable source of phytochemical compounds with various biological properties. Therefore, research and integration of these fruits into both cultivation and consumption strategies could lead to considerable improvements in human and animal health.

PHD THESIS STRUCTURE

The doctoral thesis entitled "*The protective effect of natural antioxidants in diseases of the urinary system*" is structured in two main parts, namely **Part I - Current state of knowledge** and **Part II - Personal contribution**.

CURRENT STATE OF KNOWLEDGE

Part I consists of 7 chapters and summarizes current information on the pathologies of urinary system, in particular acute kidney injury and bacterial infection of the lower urinary tract, oxidative stress and its involvement in the development of urinary system diseases, the role of natural antioxidants in combating these diseases, and general aspects on *Cornus mas* and *Sorbus aucuparia*, two medicinal plants with potential beneficial effects in various pathologies.

PERSONAL CONTRIBUTION

Part II consists of 9 chapters presenting the working hypothesis and the main objectives of the research, the materials and methods used to achieve the proposed objectives, the results obtained, the discussions, conclusions and recommendations, as well as the originality of the thesis.

WORKING HYPOTHESIS AND MAIN OBJECTIVES

Effective treatments in AKI are still lacking, as inflammation, imbalances and oxidative stress are deeply involved in the pathophysiology of AKI. Medicinal plants could be an effective therapeutic alternative to combat oxidative stress involved in kidney damage.

Although considerable efforts have been made to develop new antibiotics to combat microbial infections, antimicrobial resistance is still being reported. Thus, there is a need to find alternative therapeutic strategies in order to avoid the development of new resistance that could no longer be controlled.

A natural remedy that could help treat urinary system disorders is *Cornus mas*. In traditional medicine, *Cornus mas* fruits have been used in the treatment of various conditions, including bacterial infections and kidney disease. Thus, these fruits have been the focus of several studies, which have shown that they possess multiple biological activities, the most important of which is their antioxidant effect.

Sorbus aucuparia is another medicinal plant, the fruit of which was often used in the past to cure a variety of diseases. The biological activities of *Sorbus aucuparia* have been little studied, but their antioxidant activity has been identified as a key component.

Thus, the main purpose of this thesis was to evaluate the bioactive compounds of *Cornus mas* and *Sorbus aucuparia* fruits, through *in vitro* and *in vivo* studies, in order to determine the antioxidant, nephroprotective and antimicrobial effects against urinary system pathologies.

In order to fulfil the set purpose, the main objectives have been structured as follows:

1. Phytochemical characterization of *Cornus mas* and *Sorbus aucuparia* fruit extracts and *in vitro* evaluation of their antioxidant effect;
2. *In vitro* evaluation of the antimicrobial effect of hydro-alcoholic extracts of *Cornus mas* and *Sorbus aucuparia* fruits against reference bacterial strains;
3. *In vitro* investigation of the antimicrobial effect of hydro-alcoholic extracts of *Cornus mas* and *Sorbus aucuparia* fruits against resistant clinical isolates from urine of companion animals in correlation with the prevalence of urinary tract infections in dogs and cats;
4. *In vitro* evaluation of the potential cytoprotective effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts on gentamicin-stressed primary renal cell lines;
5. *In vivo* investigation of the potential nephroprotective and antioxidant effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts in an experimental model of gentamicin-induced acute nephrotoxicity.

RESEARCH STUDIES

1. Determination of the profile of bioactive compounds and the *in vitro* evaluation of the antioxidant effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts

Study objectives:

- Obtaining hydro-alcoholic extracts from the fruits of *Cornus mas*, and hydro-alcoholic and carotenoid extracts from the fruits of *Sorbus aucuparia*;
- Determination of total polyphenols, total flavonoids and total carotenoids in fruit extracts by spectrophotometric methods;
- Separation and identification of phenolic compounds of fruit extracts by HPLC-DAD-ESI-MS chromatographic analysis;
- *In vitro* evaluation of the antioxidant activity of fruit extracts by radical inactivation (DPPH) and ferric ion reduction (FRAP) methods.

Study results:

- **Total content of polyphenols, flavonoids and carotenoids**

Table 1

Phytochemical composition of investigated fruit extracts

Analyzed extract	Total polyphenols mg GAE/mL	Total flavonoids µg QE/mL	Total carotenoids µg/g DM
Cornus mas	0.872 ± 0.003	139.14 ± 2.100	3.8 ± 0.0002
Sorbus aucuparia	1.393 ± 0.046	537.58 ± 3.255	95.68 ± 0.297

- **Identification of phenolic compounds by HPLC-DAD-ESI-MS**

In *Cornus mas* fruits, 13 compounds belonging to four different subclasses have been identified: **phenolic acids** - gallic acid, chlorogenic acid and caffeic acid; **anthocyanins** - Cy 3-O-coumaroyl glucoside, Cy 3-O-galactoside, Cy 3-O-galactoside, Cy 3-O-robinobiozide, Pg 3-O-galactoside and Pg 3-O-robinobiozide; **iridoids** - loganin and swerozide; and **flavonols** - rutin, K 3-O-galactoside and a procyanidin dimer.

The highest concentration of GAE was found in gallic acid (248.51 µg/mL), followed by procyanidin dimer (195.82 µg/mL) and loganin (111.47 µg/mL).

Ten compounds of three distinct subclasses were found in *Sorbus aucuparia* fruits: **phenolic acids** - gallic acid, chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid and ferulic acid; **flavonols** - Q 3,4′-O-diglucozide, Q 3-O-glucoside and Q 3-O-rutinoside; and **anthocyanins** - Cy 3-O-(caffeoyl-glucoside) and Cy 3-O-glucoside. The highest value of GAE was identified in chlorogenic acid (704.792 µg/mL), followed by neochlorogenic acid (376.610 µg/mL).

- **Antioxidant activity**

Table 2

Antioxidant activity of the tested extracts

Analyzed extracts	DPPH (IC_{50} mg/mL)	FRAP (µM TE/mL)
*Cornus mas**	0.466 ± 0.835	23.09 ± 0.610
*Sorbus aucuparia***	24.51 ± 0.577	0.016 ± 1.047

2. *In vitro* studies on the antimicrobial effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts on reference bacterial strains

Study objectives:

- Obtaining hydro-alcoholic extracts from Cornus mas and Sorbus aucuparia fruits;
- Determination of the *in vitro* antimicrobial effect of the investigated fruit extracts through agar-well diffusion and broth microdilution methods against six reference bacterial strains.

Study results:

Both extracts showed antimicrobial activity against all tested bacterial strains. In the case of *Cornus mas* fruits, the highest effect was recorded against *E. faecalis* and *Ps. aeruginosa* (22.33 ± 0.47 mm). *Sorbus aucuparia* showed significant efficacy against *E. coli* (25.67 ± 0.47 mm) and *E. faecalis* (25.67 ± 0.94 mm). In terms of MIC index, both extracts were found to exhibit bactericidal activity against all analyzed bacterial strains (MBC/MIC ≤ 4).

This is the first study to examine the antimicrobial activity of *Cornus mas* fruit extract against MRSA, which may be of particular interest for future antimicrobial resistance research. The zone of inhibition of the extract against *Ps. aeruginosa* in this study had the largest diameter of all published data, reinforcing the antimicrobial characteristics of these fruits.

However, *Sorbus aucuparia* extract showed a stronger antimicrobial effect against these strains compared to *Cornus mas* extract.

Tabel 3

Antimicrobial activity of investigated extracts by the broth microdilution method

	MIC Index MBC (µg GAE/µL) / MIC (µg GAE/µL)					
Bacterial Strains	*MSSA*	*MRSA*	*B. cereus*	*E. faecalis*	*E. coli*	*P. aeruginosa*
Cornus mas	**1** 0.02/0.02	**1** 0.02/0.02	**1** 0.02/0.02	**2** 0.04/0.02	**2** 0.04/0.02	**1** 0.32/0.32
Sorbus aucuparia	**1** 0.04/0.04	**1** 0.04/0.04	**1** 0.09/0.09	**1** 0.04/0.04	**1** 0.19/0.19	**1** 0.38/0.38

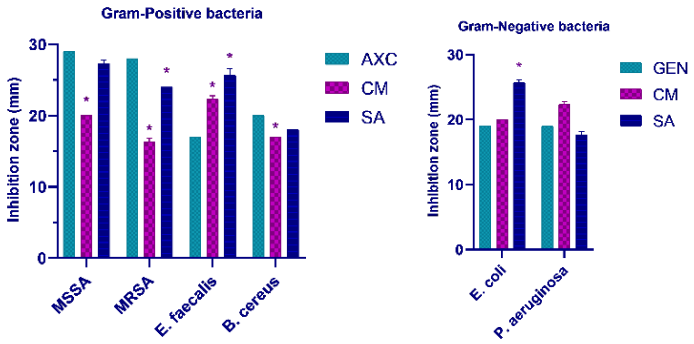

Fig. 1 Antimicrobial activity of investigated extracts by the agar-well diffusion method

3. *In vitro* studies on the antimicrobial effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts on clinical isolates from companion animals

Study objectives:

- Obtaining the hydro-alcoholic extracts from the investigated fruits;
- Urine sample collection from companion animals;
- Performing a microbiologic diagnosis on collected urine samples;
- Isolation, identification and selection of clinical isolates for antimicrobial testing;
- *In vitro* antimicrobial analysis of investigated fruit extracts by agar-well diffusion and broth microdilution methods;
- Characterizing the prevalence of urinary tract infections in dogs and cats.

Study results:

➢ **Prevalence of lower urinary tract infection in companion animals**

The species and sex distribution was relatively equal, with males in a slightly higher percentage. Adult dogs and young cats were more susceptible compared to other age categories; animals that had not undergone previous antibiotic treatment were more often diagnosed with urinary tract infections. Purebred dogs and European cats also tended to be more vulnerable in developing urinary tract infections.

➢ **Clinical isolates and antimicrobial resistance**

Escherichia coli was the most prevalent bacterium, being the most common pathogen in cats and the second most common in dogs. *Proteus mirabilis*, which was not isolated from cats, was the most common bacterium identified in dog urine. Another important pathogen present only in dogs was *Klebsiella pneumoniae*. *Enterococcus faecalis* was the most prevalent Gram-positive bacterium. Other bacteria identified were *Acinetobacter baumannii, Enterococcus faecium* and *Staphylococcus pseudintermedius*. Bacteria detected only in dogs were *E. cloacae, K. oxytoca, S. aureus,* and *Ps. luteola*, while *S. lentus, S. equorum, S. sciuri*, and *S. haemolyticus* were reported only in cats. A Gram-negative bacterium rarely documented in cases of urinary tract infection was *Leclercia adecarboxylata*. Also, two Gram-positive bacteria who have not previously been identified in urinary tract infections in pets were *Kocuria rhizophila* and *Kocuria rosea*.

The most effective antibiotic was doxycycline, while amoxicillin clavulanate showed the weakest antimicrobial activity against uropathogenic bacteria.

➢ **Antimicrobial activity of analyzed fruit extracts**

Cornus mas extract showed higher antimicrobial efficacy on Gram-negative bacteria, whereas *Sorbus aucuparia* extract showed higher antimicrobial activity against Gram-positive bacteria. Both extracts showed superior antimicrobial effect to amoxicillin. However, *Cornus mas* extract showed a stronger bactericidal effect, being manifested against 11 strains analyzed. By corroborating the results of both determinations, it was observed that *Cornus mas* extract showed the highest antimicrobial activity against *Pseudomonas luteola*, while *Sorbus aucuparia* extract showed maximum effects against *Staphylococcus lentus* and *Enterococcus faecalis*.

4. *In vitro* evaluation of the cytoprotective effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts on gentamicin-stressed renal cell cultures

Study objectives:

- Obtaining the primary renal epithelial cell cultures;
- Obtaining an efficient and predictable experimental model of acute kidney injury *in vitro* on primary renal cell cultures exposed to gentamicin;
- *In vitro* assessment of cell viability by MTT assay of gentamicin-stressed renal cells treated with the investigated fruit extracts at different concentrations;
- *In vitro* assessment of cell apoptotic rate by flow cytometry of gentamicin-stressed renal cells treated with the investigated fruit extracts at various concentrations.

Study results:

- **Cell viability**

In the MTT assay, gentamicin caused a drastic decrease in the percentage of renal cell viability (63.03%). Treatment of stressed cells with *Cornus mas* extract showed a significant increase in cell viability at all concentrations (71.63%, 74.73%, 71.25%, respectively). However, the lowest viability was observed at the highest concentration. Treatment of stressed cells with *Sorbus aucuparia* extract at low and moderate concentrations caused a significant increase in cell viability (74.49% and 67.63%, respectively). In contrast, the highest concentration of the extract caused a substantial reduction in the percentage of viable cells (58.66%).

- **Cell apoptotic rate**

In flow cytometry, gentamicin caused a drastic decrease in the percentage of cell viability (63.83%). There was an improvement in nephrotoxicity following cell exposure to *Cornus mas* extract at low and moderate concentrations (77.1% and 80.5%). In contrast, the highest concentration of extract showed cytotoxic activity (62.1%), probably due to the cumulative pro-oxidant effect of gentamicin and polyphenols at high concentrations. Also, the highest concentration of *Cornus mas* extract had the highest percentage of cells in early and late apoptosis (11.6% and 7.4%). Similarly, low and moderate concentrations of *Sorbus aucuparia* extract improved cell viability (72.8% and 73.77%, respectively). The highest concentration of extract caused a substantial reduction in the percentage of viable cells (60.2%). It also achieved the highest percentage of necrosis (40.3%).

Extracts of *Cornus mas* and *Sorbus aucuparia* show a cytoprotective effect on renal cells in small and moderate amounts, but with increasing concentration, they induce a gentamicin-like cytotoxic activity. Additionally, *Sorbus aucuparia* extract was found to be more cytotoxic at high concentrations compared to *Cornus mas* extract.

5. *In vivo* determination of the potential nephroprotective and antioxidant effect of *Cornus mas* and *Sorbus aucuparia* fruit extracts in gentamicin-induced nephrotoxicity on Wistar rats

Study objectives:

- Obtaining an effective and predictable experimental model of acute kidney injury *in vivo* in Wistar rats by administration of gentamicin;
- Evaluation of the nephroprotective effect of extracts by hematological analysis;
- Evaluation of the nephroprotective effect of the extracts by quantification of conventional biochemical markers as well as specific biochemical markers (Cystatin C, NAG Index and Renal Injury Molecule 1);
- Evaluation of the antioxidant capacity of the extracts by quantifying the markers of oxidative stress in both plasma and tissue;
- Evaluation of the protective effect of extracts by histopathological analysis of kidney and liver tissue.

Study results:

- In terms of the individuals' **general condition and body weight**, the oil-treated group showed the greatest increases in weight, while the gentamicin-treated group showed drastic decreases in body mass. The groups treated with extract and antibiotic did not show drastic decreases in body weight, highlighting their beneficial effect on the body.
- **The hematologic profile** reflected values within the reference limits, , thus requiring additional investigation of other parameters.
- **The biochemical profile** revealed some important changes. The antibiotic-treated group showed increases in PT, ALT, ALP, BUN and CREA. By administration of the tested extracts, the values of these markers were improved, both non-significantly (BUN) and significantly (PT, ALT, ALP, CREA), showing a protective effect on gentamicin-induced toxicity.
- By monitoring the activity of **Cystatin C**, which is a specific marker of glomerular function, it could be observed that the investigated extracts did not how a protective effect on renal glomerular function.
- **KIM-1**, which is a remarkable indicator of kidney damage in the rat, showed a substantial increase in the gentamicin-treated group, with both extracts lowering its values.
- After analyzing the **urinary NAG index**, which is a specific parameter of renal tubular function, it could be observed that extracts administered to the GCM and GSA groups showed a similar protective effect over time to that of the CM and SA groups.
- **Antioxidant enzyme analysis** reflected a decrease in CAT and GPx concentrations and an increase in SOD and TAC following *Cornus mas* and *Sorbus aucuparia* treatment. The increased levels of CAT and GPx in the stressed group could be due to a higher magnitude of oxidative stress, thus a greater release of antioxidant enzymes in the body to compensate the harmful effects caused by ROS, with the extracts ameliorating these aspects. The increase in SOD and TAC could also be attributed to the enhanced activity of antioxidant enzymes following natural antioxidant therapy. Thus, it can be concluded that both extracts show an antioxidant effect in gentamicin-induced oxidative stress.
- After analyzing the activity of **nitro-oxidative stress markers**, a decrease in MDA and NO values was observed in the extract-treated groups. This could be attributed to the ability of the fruits to reduce and neutralize ROS and RNS.
- Acute **histopathologic lesions** were identified following gentamicin administration in both the kidney and liver, with the fruit extracts ameliorating their severity.

RECOMMENDATIONS

- Conducting *in vitro* and *in vivo* studies on multiple extract concentrations in order to establish the therapeutic index for both extracts.
- Carrying out additional *in vitro* investigations on multiple biological samples in order to discover the therapeutic properties of these fruits.
- Performing additional *in vitro* studies on standardized renal cell cultures in order to strengthen the hypothesis regarding the cytoprotective effects of the two extracts.
- Conducting additional *in vivo* investigations, both experimental and clinical, in order to emphasize the nephroprotective effect of *Cornus mas* and *Sorbus aucuparia* fruits, and also, to encourage the use of these fruits as adjuvants in the treatment of renal pathology.
- Conducting further studies, both *in vitro* and *in vivo*, to establish the mechanisms and sites of action of these fruits.

ORIGINALITY OF THE THESIS

The present research provides innovative elements and represents the first study to evaluate the biological effects of *Sorbus aucuparia* fruits in renal pathology. Thus, it was demonstrated that these fruits possess antioxidant and nephroprotective properties in induced acute kidney injury, both *in vitro* and *in vivo*. Additionally, this is the first study that investigates the beneficial effect of *Cornus mas* fruits in gentamicin-induced nephrotoxicity, with the *in vitro* determination of the cell apoptotic score, and *in vivo* analysis of specific renal biomarkers. Furthermore, this research emphasizes for the first time the antimicrobial effect of *Cornus mas* and *Sorbus aucuparia* fruits against resistant uropathogenic bacteria in companion animals diagnosed with lower urinary tract infections, encouraging the use of phytochemical compounds as adjuvants or substitutes for antibiotics, and consequently, contributing to the reduction of antimicrobial resistance. Another original element is the identification for the first time bacteria from the *Kocuria* genus as pathogens in infected dog urine. In addition, a unique and advantageous element that contributed to these significant and authentic results was the implementation of the collaborative and interdisciplinary perspective, which aimed to investigate and prove the hypotheses of the present thesis.