SUMMARY OF PHD THESIS

The therapeutic potential of *Craterellus cornucopioides L.*: A detailed analysis of its chemical composition and medicinal properties

PhD student **Mariana-Gabriela Bumbu**

Scientific coordinator **Prof. univ. dr. Ioan Marcus**

INTRODUCTION

Medicinal mushrooms have long been recognized as valuable sources of biologically active compounds, and their potential to contribute to modern therapies has attracted growing scientific interest. *Craterellus cornucopioides* L. (known as the chanterelle mushroom) is an edible mushroom of the *Cantharellaceae* family, highly prized in cooking, but its phytochemical richness and pharmacological potential remain incompletely characterized. Recent studies on various mushroom species indicate that mushroom-derived extracts may offer various bioactivities, including antioxidant, anti-inflammatory, antimicrobial, and hepatoprotective effects, making them promising candidates for drug discovery and nutraceutical development. However, with regard to *C. cornucopioides* specifically, systematic and rigorous investigations combining comprehensive chemical profiling with mechanistic bioassays are still limited.

This thesis addresses this gap by providing a study of this mushroom, combining biochemistry with *in vitro* and *in vivo* pharmacological evaluation. The paper primarily aims to elucidate the chemical composition of *C. cornucopioides* extracts using chromatographic and spectrophotometric techniques, thereby identifying and quantifying the major phenolic compounds, caffeic acid derivatives, and other relevant metabolites. In parallel with the composition analysis, the study evaluates antioxidant capacities (through tests such as DPPH, ABTS, ORAC, and CUPRAC), antimicrobial activity (microdilution in broth and related methods), and cytotoxic effects in cell models. Particular emphasis is placed on hepatoprotective potential, examined both through cell tests and experimental models that evaluate liver damage.

STRUCTURE OF THE DOCTORAL THESIS

The doctoral thesis entitled "*The therapeutic potential of Craterellus cornucopioides L.: A detailed analysis of the chemical composition and medicinal properties*" is structured in two main parts, namely: Part I - Current state of knowledge, and Part II - Personal contribution.

CURRENT STATE OF KNOWLEDGE

The first part consists of three chapters and provides a summary of current information on the chemical composition and biological properties of the mushroom *Craterellus cornucopioides*. This is followed by a presentation of the main experimental models used in *in vivo* studies on mushrooms, as well as an analysis of the role of phenolic compounds in the manifestation of biological activities.

PERSONAL CONTRIBUTION

Part II comprises three studies presenting the working hypothesis and main objectives of the research, the materials and methods used to achieve the proposed objectives, the results obtained, discussions, conclusions and recommendations, as well as the original elements of the thesis.

WORKING HYPOTHESIS AND MAIN OBJECTIVES OF THE THESIS

The research hypothesis is based on the fact that extracts from the wild mushroom *Craterellus cornucopioides* (L.) obtained from three distinct geographical regions in Romania (Cluj, Sălaj, and Suceava) contain biologically active compounds capable of exerting multiple pharmacological effects (hepatoprotective, antioxidant, antimicrobial, cytotoxic, and antiparasitic) whose intensity and specificity are influenced by environmental conditions, and the structural and quantitative variations of bioactive metabolites between regions determine differences in therapeutic potential. The purpose of this thesis is to quantify these effects through *in vitro* and *in vivo* tests and correlate them with the biochemical profile of each extract.

To achieve the set goal, the main objectives were structured as follows:

O1. Characterization of the profile of biochemical compounds obtained from extracts of *Craterellus cornucopioides* (L.) Pers. through quantitative and qualitative determinations, *in vitro* evaluation of antioxidant potential through complementary methods, and correlation with the analysis of soil parameters from the three harvesting locations.

O2. *In vitro* evaluation of the antimicrobial, cytotoxic, and antiparasitic effect of extracts obtained from *Cratrerellus cornucopioides L*..

O3. *In vivo* investigation of the hepatoprotective and antioxidant potential of extracts obtained from *Craterellus cornucopioides L.* in an experimental model of acute hepatotoxicity.

RESEARCH STUDIES

Study 1 - Integrated study on extracts from *Craterellus cornucopioides* L.: physicochemical characterization, antioxidant activity, and soil analysis in harvesting areas

Research activities of the study:

- Harvesting and obtaining ethanolic extracts from mushrooms;
- Sampling protocol and analytical methods for determining soil parameters;
- Nutritional profile and energy content of mushroom samples;
- Photometric dosage of bioactive compounds in extracts;
- HPLC-DAD-ESI$^+$ chromatographic analysis with identification of phenolic compounds;
- Evaluation of the antioxidant activity of *C. cornucopioides* extracts using the DPPH, ORAC, CUPRAC, ABTS, and FRAP methods.

Study results:

❖ **Analysis of fundamental edaphic indicators in soil samples**

Analysis of soils in Cluj, Suceava, and Sălaj revealed predominantly acidic conditions, with pH ranging from strongly acidic to moderately acidic depending on depth and location, which influences the growth and diversity of fungi. The humus and nitrogen content is highest in the litter layer, decreasing significantly at depths of 15 and 30 cm, with Suceava showing the best retention of organic matter and nitrogen. Mobile phosphorus and potassium are also concentrated in the surface layer, decreasing with depth, indicating reduced nutrient availability for roots and mycorrhiza in deeper layers.

❖ **Nutritional composition and energy value of mushroom samples**

Table 1

Nutritional composition and energy value of *Craterellus cornucopioides* L. samples harvested in Suceava County in 2018, 2022, and 2023, Sălaj County in 2022, and Cluj County in 2022.

C. cornucopioides samples	Humidity (mg/g ± SD)	Total fats (mg/g ± SD)	Protein (mg/g ± SD)	Ash (mg/g ± SD)	Carbohydrates (mg/g ± SD)	Energy (kcal/100 g)
Suceava 2018	118.4±0.2	34.8±0.5	465±1.0	127.9±0.4	253.9±0,7	318.88±0,05
Suceava 2022	106.3±1.5	41.4±0.1	473.2±0.8	98.7±0.1	280.4±1.0	338.7±0,09
Suceava 2023	129±0.2	25.8±0.7	462.1±0.5	91.5±0,2	291.6±0,4	324.7±0,03

Sălaj 2022	125.3±0.3	39.4±0.2	467.1±0.7	104±0.2	264.2±0,4	327.98±0,04
Cluj 2022	103.1±0.2	34.6±0.1	469.7±1.2	95.5±0.1	297.1±0,3	337.86±0,02

** Note: Each value represents the mean ± SD of three independent measurements.*

❖ Total content of polyphenols and carboxylic acids

Table 2

Total content of polyphenols and caffeic acid derivatives in extracts of *C. cornucopioides*

Analyzed extract	TPC (mg GAE/g+SD)	TPAC (mg CAE/g+SD)
C.C. Cluj 2022	23.21 ± 0.21	15.96 ± 0.14
C.C. Sălaj 2022	23.01 ± 0.02	16.05 ± 0.45
C.C. Suceava 2022	28.74 ± 0.25	19.74 ± 0.26
C.C. Suceava 2023	26.64 ± 0.36	18.64 ± 0.26

** Note: Each value represents the mean ± SD of three independent measurements.*

❖ Identification of phenolic compounds by HPLC-DAD-ESI+

Eight compounds belonging to two different subclasses were identified. Hydroxybenzoic acids were represented by gallic acid, gentisic acid, protocatechuic acid, and p-hydroxybenzoic acid. The hydroxycinnamic acid subclass was represented by chlorogenic acid, caffeic acid, ferulic acid, and p-coumaric acid.

In the 2022 Cluj extract, the highest concentration of phenolic compounds, expressed in GAE equivalent, was observed for gentisic acid (757.590 µg/mL), followed by protocatechuic acid (194.302 µg/mL), and the lowest concentration was for ferulic acid (29.545 µg/mL). In the extract from Sălaj in 2022, gentisic acid again had the highest amount of GAE (659.743 µg/mL), followed by p-hydroxybenzoic acid (324.410 µg/mL), while gallic acid had the lowest concentration (20.616 µg/mL). In the extracts from Suceava, for both 2022 and 2023, the highest amount of GAE was recorded for gentisic acid (842.946 µg/mL and 805.770 µg/mL, respectively), followed by protocatechuic acid (377.504 µg/mL and 439.365 µg/mL). The lowest GAE concentration was detected in gallic acid for Suceava 2022 (51.844 µg/mL) and in ferulic acid for Suceava 2023 (37.515 µg/mL).

❖ Antioxidant activity

Table 3

Antioxidant activity of the 4 extracts of *C. cornucopioides*

Analyzed extract	DPPH (µM TE/g ± SD)	FRAP (µM TE/g ± SD)	CUPRAC (µM TE/g ± SD)	ORAC (µM TE/g ±SD)	ABTS (µM TE/g ± SD)
C.C. Cluj 2022	13.01 ± 2.08	30.60 ± 0.25	157.61 ± 32.02	133.75 ± 0.49	34.78 ± 2.20
C.C. Sălaj 2022	8.31 ± 2.21	29.83 ± 1.98	83.13 ± 9.45	123.96 ± 2.93	32.28 ± 3.14
C.C. Suceava 2022	23.03 ± 1.34	24.47 ± 8.28	258.31 ± 12.89	147.06 ± 2.92	48.05 ± 1.26
C.C. Suecava 2023	12.98 ± 0.61	20.97 ± 3.38	185.62 ± 13.42	132.77 ± 2.72	38.68 ± 0.63

Study 2 - In vitro evaluation of the antimicrobial, cytoprotective, and anthelmintic effects of extracts from Craterellus cornucopioides L. on bacterial and fungal strains, LX1 cell cultures, and intestinal helminths Toxocara canis

Research activities of the study:

- Testing the antimicrobial potential of mushroom extracts;
- Assessment of cell viability using the CCK-8 test on the LX1 line;
- *In vitro* assessment of fungal extracts on *Toxocara canis* eggs.

Study results:

❖ **Antimicrobial and antifungal activity by the zone of inhibition method**

The extracts showed the strongest activity against Gram-positive bacteria such as *B. cereus*, MSSA, and MRSA, while Gram-negative bacteria, *E. coli* and *P. aeruginosa*, were more resistant. The complete lack of effect against P. aeruginosa highlights its resistance to fungal extracts, while the activity against MRSA suggests that the extracts may contain compounds with potential against antibiotic-resistant staphylococci (Table 4). The antifungal activity of the extracts against *C. albicans* showed moderate inhibition, with all four extracts exhibiting inhibition zones of 10.00 ± 0.00 mm. This indicates that the extracts have antifungal potential. Fluconazole produced a significantly larger inhibition zone of 21.00 ± 0.00 mm, confirming its superior antifungal efficacy (Table 5).

Table 5

Antimicrobial activity of the extracts under investigation by the zone of inhibition method (mm) and standard deviation

Extract	*MSSA*	*MRSA*	*Bacillus cereus*	*Enterococcus faecalis*	*Listeria monocytogenes*	*Escherichia coli*	*Pseudomonas aeruginosa*
Cluj 2022	15.75±0.43	15.00±0.00	16.00±0.00	11.75±0.43	11.00±0.00	10.00±0.00	0
Sălaj 2022	15.00±0.00	14.25±0.43	16.75±0.83	9.00±0.00	14.25±0.43	9.00±0.00	0
Suceava 2022	16.50±0.50	15.25±0.43	17.00±0.71	10.75±0.43	10.00±0.00	9.00±0.00	0

Suceava 2023	15.00±1.00	14.25±0.43	17.00±00	16.50±0.50	14.00±1.00	9.00±0.00	0
Gentamicine	19.00±0.00	17.00±0.25	20.00±0.00	10.00±0.00	22.00±0.50	19.00±0.00	18.00±0.00

Table 5

Antimycotic activity of extracts studied by the inhibition zone method (mm) and standard deviation

Extract	*Candida albicans*
Cluj 2022	10.00±0.00
Sălaj 2022	10.00±0.00
Suceava 2022	10.00±0.00
Suceava 2023	10.00±0.00
Fluconazole	21.00±0.00

❖ **Antimicrobial activity by the broth microdilution method**

The MIC and MBC/MFC values obtained for the tested extracts indicate variable antimicrobial activity depending on the origin of the sample and the target microorganism. The extract from Cluj 2022 showed the best overall antibacterial activity, with MIC values ranging from 0.23 to 0.93 mg/mL, showing effective inhibition of MSSA and MRSA strains, as well as Bacillus cereus. The extract from Sălaj 2022 showed comparable activity, maintaining similar MIC and MBC values, especially against MSSA, where the bactericidal effect occurs at the same concentration (0.23 mg/mL), suggesting a rapid and potent action. The extracts from Suceava 2022 had lower efficacy, requiring higher concentrations for bacterial inhibition (MIC 0.29–1.16 mg/mL), while Suceava 2023 showed improved activity, especially against *MSSA*, *MRSA*, and *Bacillus cereus* (MIC 0.27–0.54 mg/mL). In the case of *Candida albicans*, all extracts had MFC values >0.93–1.16 mg/mL, indicating weak antifungal activity compared to fluconazole (MIC 8 mg/L). Compared to gentamicin (MIC 3–4 mg/L), the extracts show lower activity but reveal significant natural antimicrobial potential, especially in the samples from Cluj and Sălaj.

❖ **Anti-biofilm antimicrobial activity**

The results highlight that the extracts demonstrated strain- and time-dependent antibiofilm activity. The strongest and most consistent effects were observed against MSSA, where all extracts remained active after 48 hours. The Cluj 2022 extract also showed promising late activity against MRSA. In contrast, E. coli showed the highest resistance, with weak inhibition observed at 48 hours. These results suggest that certain mushroom extracts, particularly in the Cluj and Suceava 2023 extracts, retain or even

enhance their antibiofilm potential as the biofilm matures, in some cases exhibiting activities comparable to the antibiotic control, gentamicin.

❖ **Evaluation of extract cytotoxicity by the CCK-8 method**

The results of the CCK-8 test showed a concentration-dependent antiproliferative effect of the *Craterellus cornucopioides* extract, manifested by a decrease in absorbance values with increasing concentration. The most pronounced cytotoxic effects were observed at concentrations of 2.5 mg/mL (for the 50 mg/mL extract) and 15 mg/mL (for the 200 mg/mL extract), but overall, all concentrations tested showed lower cytotoxicity than the control. The IC_{50} determination did not indicate a 50% inhibition of cell growth, suggesting low toxicity of the extract on LX1 liver cells. Therefore, it can be concluded that the extract of *C. cornucopioides* exerts a modest antiproliferative effect without being considered cytotoxic in the tested concentration range (Figure 1).

Figure 1

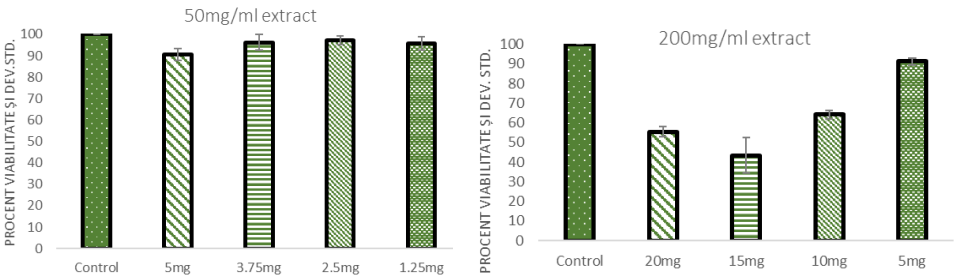

Fig 1. Cell viability of the LX1 cell line expressed as the mean OD450 ± SD after treatment with *C. cornucopioides* extract at concentrations of 50mg/ml and 200mg/ml

❖ **Evaluation of the antiparasitic effect**

When comparing the efficacy of extracts from Sălaj and Suceava, Cluj and Suceava, as well as Cluj and Sălaj, no statistically significant differences were recorded at the end of the evaluation period ($p > 0.05$). This result suggests that, although there were minor variations in ovicidal and larvicidal effects between extracts, the overall antiparasitic activity remained similar between the regions analyzed. Comparative analysis based on the time of evaluation showed a statistically significant difference between days 7 and 10 ($p < 0.05$), while the differences between days 3 and 7 to 10 were not significant ($p > 0.05$). Therefore, it can be concluded that the most pronounced variation in the efficacy of the extracts occurs between days 7 and 10 of exposure.

Study 3 - Determination *in vivo* of the hepatoprotective and antioxidant potential of extracts from *Craterellus cornucopioides L.* in carbon tetrachloride (CCl4)-induced hepatotoxicity in BALB/c mice

Research activities of the study:

- Obtaining effective and predictable experimental models of acute liver injury, *in vivo*, in BALB/c mice, by administering carbon tetrachloride;

Table 6. Organization of experimental groups

Experimental groups	Administration protocol
I Control - (*n=5*)	0.2 mL saline solution (NaCl) 0.9% p.o. for 6 weeks, 3 times a week
II Control + (*n=5*)	0.2 mL CCl4 oil p.o. for 6 weeks 3x/week
III Ac. Clor. (*n=5*)	0.2 mL CCl4 oil + 0.2 mL Chlorogenic acid p.o. for 6 weeks 3x/week
IV Crat 50(*n=5*)	0.2 mL CCl4 oil + 0.2 mL *Craterellus cornucopioides L.* extract 50 mg/kg p.o. for 6 weeks 3x/week
V Crat 200(*n=5*)	0.2 mL CCl4 oil + 0.2 mL *Craterellus cornucopioides L.* extract 200 mg/kg p.o. for 6 weeks 3x/week
VI Control - (*n=5*)	0.2 mL saline solution (NaCl) 0.9% p.o. single dose
VII Toxi (*n=5*)	0.2 mL *Craterellus cornucopioides L.* extract 2000 mg/kg p.o. single dose

- Evaluating the hepatoprotective and toxic effects of the extracts by determining biochemical parameters;
- Histopathological analysis of renal and hepatic tissue.

Study results:

- CCl_4 administration induced severe liver damage, evidenced by centrilobular necrosis, multifocal nodular inflammation, vacuolar degeneration, and micro- and macrovesicular steatosis, associated with congestion and vascular dilation, while the kidneys showed no significant pathological changes. Treatment with *Craterellus cornucopioides* extract demonstrated a dose-dependent hepatoprotective effect, progressively reducing the severity of the lesions. At a dose of 50 mg/kg, a decrease in inflammatory infiltrate and necrosis, maintenance of macrophage activity, and partial regeneration of the hepatic architecture were observed. The most obvious effects were recorded at a dose of 200 mg/kg, where the hepatic structure was almost completely restored, inflammation and cell degeneration were minimal, and steatosis and bile pigment accumulation were significantly reduced (Figure 2).
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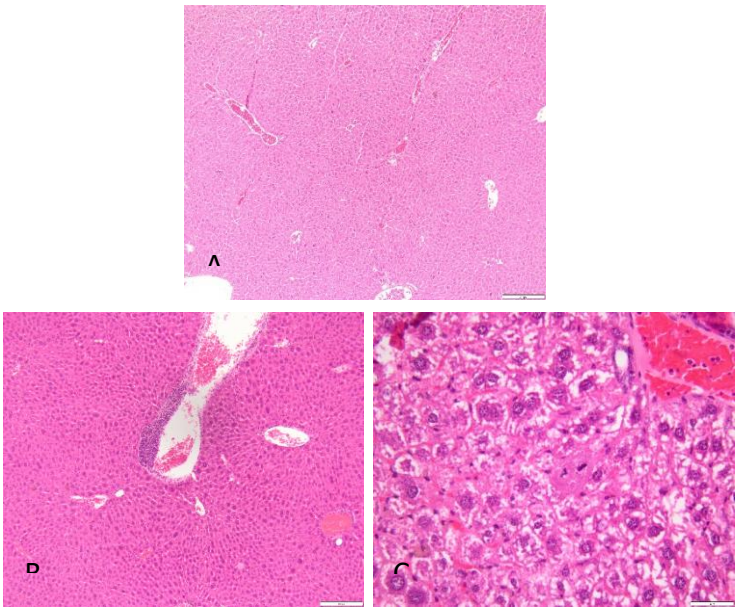
Figure 2



- Administration of the extract at the maximum tested dose of 2000 mg/kg did not produce any obvious toxic effects, indicating good tolerance and safety. The treated animals maintained their normal physiological state and even showed a slight but statistically significant increase in body weight compared to the control group ($p < 0.05$), suggesting the absence of any systemic toxicity and a favorable safety profile of the extract.

Table 7. Biochemical parameters of BALB/c mice after administration of *Craterellus cornucopioides* extract at 2000 mg/kg (acute toxicity)

	T1	T2	T3	T4	T5	Media	Dev. Std	ref	Unit
ALB	2.8	2.9	2.7	3	3	2.88	0.1304	2.5-4.8	g/dl
TP	4.8	4.9	5.5	5.8	5.6	5.32	0.4438	3.5-7.2	g/dl
GLOB	2	2.1	2.8	2.8	2.7	2.48	0.3962	0.6	g/dl
A/G	1.36	1.39	0.99	1.05	1.12	1.182	0.1824		
TB	0.2	0	0.9	0	0.3	0.28	0.3701	0.1-0.9	mg/dl
ALT	91	187	266	223	136	180.6	69.19	40-189	U/l
ALP	149	131	117	166	158	144.2	20.02	66-262	U/l
AMY	1897	1539	1920	1706	1815	1775	156.5	220-2000	U/l
Crea	0.2	0.2	0.1	0.1	0.2	0.16	0.05477	0.1-0.3	mg/dl
Urea	34.19	34.11	46.44	39.14	36.4	38.06	5.117	32.82-39.66	mg/dl
U/C	170.95	170.55	no	no	182,000	174.5	6.498		
GLU	307.96	176.5	189.94	179.72	184.54	207.7	56.26	196-278	mg/dl
Ca	9.76	9.3	9.23	9.38	9.32	9.398	0.2093	7.9-10.5	mg/dl
Phos	8.1	7.5	6.28	7.56	6.79	7.246	0.713	5.6-9.2	mg/dl
K	5.54	5.58	6.98	6.75	5.4	6.05	0.7514	5.7-11.1	mmol/dl
Na	149.4	149.3	147.8	149.4	148.7	148.9	0.6907	136-157	mmol/dl

- ❖ The treatment reduced serum levels of bilirubin, GGT, ALT, ALP, and TBA, suggesting recovery of hepatocellular integrity, improved bile flow, and normalization of excretory function.

RECOMMENDATIONS

1. Conducting *in vitro* and *in vivo* studies at multiple concentrations of the extract to determine the optimal therapeutic index and dose-effect ratio;
2. Conducting *in vitro* investigations on various types of biological samples to identify and fully characterize the pharmacological properties (antioxidant, anti-inflammatory, antimicrobial, and cytoprotective);
3. Conducting additional in vitro studies on standardized liver cell cultures to confirm and further investigate the cytoprotective and regenerative effects observed in in vivo tests;
4. Conducting in vivo investigations, both experimental and clinical, to highlight and consolidate the hepatoprotective effect and to support the inclusion of C. cornucopioides extracts as adjuvants in the treatment of liver disease;
5. Continuing research, both in vitro and in vivo, to accurately determine the molecular mechanisms and sites of action;
6. Evaluating the toxicological and safety profile of the extract at repeated and long-term doses to establish its potential clinical applicability;
7. Extending studies to other potential biological properties (antiparasitic, antitumor, immunomodulatory), which could support the use of this mushroom as a valuable source of natural bioactive compounds.

Original elements of the thesis

The present research offers innovative elements and represents the first study to evaluate the biological effects of the fungus *Craterellus cornucopioides* in liver pathology. Thus, it has been demonstrated that these mushrooms possess antioxidant and hepatoprotective properties in acute liver injury induced *in vitro* and *in vivo*.
These findings could encourage the use of medicinal plants as adjuvants in the treatment of liver diseases in both humans and pets.

At the same time, this paper highlights for the first time the antiparasitic effect against the larval forms of Toxocara canis, encouraging the use of phytochemical compounds as adjuvants to antiparasitic drugs, contributing to the reduction of antiparasitic resistance.