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PhD THESIS

# **Evaluation of the therapeutic potential of some hydrogels enriched with Thieves and Oregano essential oils in induced periodontal disease in rat**

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PhD student **Ștefana Maria Cristina Mureșan**

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Scientific coordinator **Prof. Univ. Dr. Liviu Ioan Oana**

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# SUMMARY

## Introduction

Research in human dentistry aims to create new antimicrobial agents for use in various therapies related to dental hygiene and antisepticization, as well as the improvement of oral bacterial plaque. Currently, the same direction is being implemented in veterinary dental medicine, adapted to the ideologies and therapeutic methods created for human patients, with certain species differences being taken into account.

Natural phytochemical substances represent an efficient alternative for antibiotic treatment, which is considered increasingly harmful due to the unwanted effect of antimicrobial resistance. Consequently, the potency of essential oils is being extensively researched in both human and veterinary medical fields.

The synthesis of such materials involves the use of natural substances and their incorporation into composite materials tailored to the needs of targeted therapies. This includes adjusting the physicochemical properties to achieve gradual release, controlling absorption in the oral cavity, targeting specific affected areas, and avoiding negative systemic effects, local or generalized toxicity. Furthermore, these materials are more accessible to a wider public, thus improving the social level and life expectancy of both animal and human populations.

These studies aim to investigate the effects of hydrogels enriched with natural essential oils of Oregano® and Thieves®. The proposed research includes physicochemical and biochemical determinations for the characterization of biomaterials, as well as *in vitro* cytotoxicity tests and the antimicrobial effect of these compounds.

## Thesis Structure

The doctoral thesis entitled "Evaluation of the therapeutic potential of hydrogels enriched with essential oils in rat-induced periodontal disease" consists of 130 pages and is structured in two parts.

The first part provides an overview of the current state of knowledge, divided into three chapters and spanning 37 pages. It covers general notions about periodontal disease, its complex pathogenesis, as well as information about staging, symptoms, diagnosis, and therapeutic protocols with recent developments regarding the importance of antimicrobial potency. Additionally, it presents data on the beneficial properties of essential oils and their applications in the medical field. Moreover, the

thesis includes information on the medical approach to periodontal disease and experimental induction protocols for testing various parameters and biomaterials.

The second part comprises 93 pages and represents the personal contribution of the researcher. It is divided into eight chapters, with four of them dedicated to studies conducted between 2018 and 2022. Chapter 4 outlines the working hypothesis and research objectives, while chapters 5, 6, 7, and 8 focus on four distinct *in vitro* and *in vivo* studies. Each study includes objectives, materials, methods, results, and discussions, as well as the conclusions drawn from the research. Chapter 9 presents general conclusions and recommendations, while Chapter 10 highlights the originality and innovative contributions of the thesis.

## Research Objectives

The primary objective of the thesis is to create and test hydrogel products composed of polymers with additions of different essential oils, known for their antimicrobial, anti-inflammatory, immunomodulatory, and antioxidant properties in periodontal disease. The aim is to formulate alternative therapy recommendations applicable to rat-induced periodontal disease, which can improve, treat, and halt the periodontal process.

Among the multitude of composite biomaterials tested in oral pathologies, these hydrogels fall under the category of alternative and regenerative dental medicine. They are expected to have positive effects on antibacterial therapy, local regenerative therapy of the periodontal ligament and alveolar bone, as well as the improvement of clinical signs such as tooth mobility and bleeding, both in acute (periodontitis) and chronic processes (periodontosis).

Furthermore, in this study, we aim to evaluate the biocompatibility of hydrogels enriched with essential oils from several plants recognized for their beneficial effects in oral disease therapy. We hope that the results obtained in this study will lead to the possibility of proposing an effective therapy for periodontal diseases in animals.

The secondary objectives focus on physicochemical, microbiological, biocompatibility, and cellular toxicity studies, as well as the *in vivo* evaluation of hydrogels, which also aim to assess the biocompatibility and effects of hydrogels in experimentally induced periodontitis in rats. The study also investigates the potential regenerative capacity of the biomaterial in terms of soft tissue, namely the gingiva with periodontal ligaments, as well as the hard tissue of the alveolar bone. Thus, the secondary objectives are outlined as follows:

- *In vitro* testing of the hydrogel enriched with Oregano®, Frankincense® essential oil and the Thieves® blend using the MTT assay, which determines cellular cytotoxicity and biocompatibility. Additionally, the potential antimicrobial and bacteriostatic effects are evaluated by determining the minimum inhibitory

concentration (MIC) and minimum bactericidal concentration (MBC) indices on four reference bacterial cultures, including *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, and *Escherichia coli*.

- Induction of experimental periodontitis *in vivo* and monitoring of specific clinical signs of periodontal disease in Wistar rats.

- Therapeutic clinical evaluation of the materials used, by determining the bleeding score, laxity index, and body weight fluctuation.

- *In vivo* evaluation of the local anti-inflammatory and antioxidant effects of biomaterials and photodynamic therapy or combined therapy, by determining specific salivary biomarkers MMP8 and 8-OH-d-Guanosine, as well as systemic biomarkers for inflammation IL-1 $\beta$ , IL-6, and TNF- $\alpha$ .

- Post-mortem evaluation of induced lesions and the therapeutic potential of photodynamic therapy and hydrogels alone, as well as their combination, through imaging examination using micro-computed tomography and histopathological examination.

## Research Results

Chapter 5, titled "Physicochemical Evaluation of Hydrogels Enriched with Oregano<sup>®</sup>, Frankincense<sup>®</sup>, and Thieves<sup>®</sup> Essential Oils," presents a study that describes the synthesis method of hydrogels enriched with biologically active molecules and their physicochemical characterization. These include essential oils extracted from Oregano<sup>®</sup> and a mixture of several essential oils called Thieves<sup>®</sup>. The physicochemical determinations, such as GC-MS analysis identifying the active compounds of the essential oils in the studied hydrogels (eugenol, pinene, limonene, carvacrol, and cymene), represent the initial characterization. The second characterization includes UV-Vis analysis, which identifies the bioactive compounds from the essential oils incorporated into the polymeric matrix represented by the hydrogel. The inclusion of essential oils in the hydrogels did not affect their active compounds (eugenol, carvacrol, thymol, and other terpenes), providing good molecular stability.

Chapter 6, titled "Microbiological and Cytotoxic Analysis of Hydrogels Enriched with Essential Oils," conducted cytotoxicity and cellular biocompatibility testing for the synthesized hydrogels using the MTT assay on a specific cell line derived from human dental pulp. Additionally, the microbiological analysis was performed on reference strains, including *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*, and *Escherichia coli*, evaluating the minimum inhibitory and bactericidal effects.

Chapter 7, titled "Evaluation of the Effects of Oregano<sup>®</sup> and Thieves<sup>®</sup> Essential Oil-Based Hydrogels in Experimentally Induced Periodontal Disease in Rats," based on the obtained *in vitro* results, particularly regarding the antimicrobial effect, only two hydrogels based on Oregano<sup>®</sup> and Thieves<sup>®</sup> essential oils were selected for *in vivo*

testing. So the periodontitis was successfully induced through the applied experimental protocol, which involved placing a ligature at the base of the targeted molar. This resulted in the accumulation of a consistent bacterial plaque and the creation of a periodontal pocket that generated an acute inflammatory response. The induction of periodontal disease in rats was evaluated by monitoring body weight, post-ligation appetite, specific periodontal clinical examination including bleeding score and dental mobility/looseness index, as well as the evaluation of various specific biomarkers for local and systemic inflammation and salivary biomarkers associated with tissue destruction due to bacterial plaque accumulation, bleeding, suppurative processes, and local necrosis.

Chapter 8, titled "Imaging and Histopathological Evaluation of Therapeutic Effects in Experimentally Induced Periodontitis in Rats," aimed to evaluate the progression of bone and periodontal lesions in experimental periodontitis in rats using micro-computed tomography imaging. Additionally, histopathological evaluation was performed to assess the dynamics of the healing processes in the experimentally induced lesions. These evaluations and monitorings indicated that the therapeutic results are effective, and the lesions induced by acute periodontitis can be improved or even reversed.

## **General conclusions:**

The research findings lead to the following overarching conclusions:

1. The studied polymeric hydrogels uniformly incorporated Oregano® and Thieves® essential oils and proved suitable for the treatment of experimentally induced periodontitis in rats, as well as for various preclinical evaluations.
2. Considering the composition and demonstrated antibacterial, anti-inflammatory, and regenerative effects of the synthesized gels through *in vivo* and *in vitro* examinations, as well as the results of imaging evaluation, they recommend standalone periodontitis therapy using these hydrogels as a therapeutic alternative to currently used products primarily based on antibiotics and antiseptic substances.
3. The studied hydrogels demonstrated *in vitro* biocompatibility and effective antimicrobial properties, especially those enriched with Oregano® and Thives® essential oils
4. Both studied products exhibited efficient systemic biocompatibility, as evidenced by the improvement of acute inflammatory response, thus preventing complications in the periodontal process.
5. The therapies used in periodontitis treatment led to a reduction in specific salivary biomarkers associated with systemic and local inflammation, with a positive effect observed when combining hydrogels with photodynamic therapy.

6. Micro-CT provided staging of the induced periodontal process and highlighted bone resorption by quantifying the periodontal space.
7. Histopathological analysis confirmed the efficacy of the studied hydrogels in the treatment of periodontitis in rats, indicating good tissue regeneration activity.
8. The results of the histopathological examination revealed that the combination of photodynamic therapy with hydrogels enriched with essential oils had a synergistic therapeutic effect in experimentally induced periodontitis in rats.

The modification of the surgical method for inducing experimental periodontitis by placing a ligature at the base of the upper left first molar represents a valuable contribution. The technique involves passing the suture thread under the gingival mucosa and the hard palate for a length of 2-3mm after it has already been double-knotted at the base of the molar. This approach ensures better ligature retention, prevents slippage, and provides enhanced stability.

Another original aspect is the synthesis of composite biomaterials based on polymeric hydrogels and encapsulated natural extracts of Oregano® and Thieves® essential oils. These materials are intended for the treatment of periodontal pathology and other conditions due to their antimicrobial, anti-inflammatory, and regenerative effects.

The studied hydrogels in this study have demonstrated excellent stability of the active components derived from Oregano® and Thieves® essential oils, making them suitable for use in the therapy of periodontitis in rats. This represents a novel approach as the hydrogel used possesses a chemical structure in the form of a polymeric matrix, where the active ingredients are encapsulated within a thin film of polycaprolactone, a compound that facilitates the preservation of bioactive constituents without degradation.

The results obtained in this study open up new research opportunities, and our future goal is to develop hydrogel formulations with a consistency resembling "caramels," incorporating Oregano® and Thieves® essential oils. These formulations would be suitable for prolonged mastication, allowing the therapeutic components to be gradually released with efficacy in the prevention and treatment of oral cavity conditions in dogs.